2000-2001

Alternatives to the Use of Live Vertebrates in Biomedical Research and Testing

A Bibliography with Abstracts

TO ASSIST IN:

- REFINING EXISTING TEST METHODS
- REDUCING ANIMAL USAGE
- REPLACING ANIMALS AS TEST SYSTEMS

PREPARED BY
TOXICOLOGY AND ENVIRONMENTAL HEALTH INFORMATION PROGRAM
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The Scientific Community, concerned about animal welfare, is sensitive to concerns regarding how and why animals are used in biomedical research and testing to evaluate the toxicological potential of various substances. Although alternatives to methods based on the use of animals may not satisfy all requirements and needs of the biomedical research and toxicologic testing communities, alternatives to the use of vertebrates are being developed and evaluated. Research on such methodologies is aimed at refining procedures to reduce pain and discomfort; reduce the number of animals required to provide scientifically valuable results; and to replace live vertebrates when an alternative methodology can be verified and validated by the scientific community.

The purpose of these bibliographies on "animal alternatives" is to provide a survey of the literature in a format which facilitates easy scanning. This bibliography includes citations from published Articles, books, book chapters, and technical reports. Citations to items in non-English languages are indicated with [ ] around the title. The language is also indicated. Citations with abstracts or annotations relating to the method are organized under subject categories. This publication features citations which deal with methods, tests, assays or procedures which may prove useful in establishing alternatives to the use of intact vertebrates. Citations are selected and compiled through searching various computerized on-line bibliographic databases of the National Library of Medicine, National Institutes of Health.

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Suggestions and comments are welcome.

Table of Contents

GENERAL

CARCINOGENESIS

CYTOTOXICITY

DERMAL TOXICITY

ECOTOXICITY

GENOTOXICITY AND MUTAGENESIS

HEPATIC AND RENAL TOXICITY

IMMUNOTOXICITY

NEUROTOXICITY

OCULAR TOXICITY

PHARMACOKINETIC AND MECHANISTIC STUDIES

PULMONARY TOXICITY

QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIPS

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

MISCELLANEOUS

GENERAL

In vitro toxicology industrial platform (IVTIP) position paper, 9 February 2001: the role of an

industrial platform in the area of in vitro testing. Altern Lab Anim 2001;29(4):487-92.

Selected titles. Altern Lab Anim 2000;28(6):878-82.

Atterwill CK, Wing MG. **In vitro preclinical lead optimisation technologies (PLOTs) in pharmaceutical development.** Altern Lab Anim 2000;28(6):857-67.
Abstract: The explosion of genuine high throughput technologies has allowed large compound libraries to be screened with ever-increasing biological specificity, exacerbating the problem of lead candidate selection for subsequent drug development. To avoid creating a bottleneck, compounds identified from the high throughput screens undergo lead optimisation by employing medium-throughput screen which permits ranking in terms of their basic absorption, distribution, metabolism, excretion (ADME) and toxicological properties. The historical role of the CRO in the drug discovery/development continuum has been to perform efficacy and toxicology studies, simply to support the regulatory submission of lead candidates. This situation is, however, changing with the development of preclinical lead optimisation technologies facilitating the selection of leading candidates, thereby bridging the gap between high throughput efficacy screens and conventional safety assessment programmes.

Balls M. **Drunken monkeys and cruel humans.** Altern Lab Anim 2000;28(5):639-40.

Balls M, Sabbioni E. **Promotion of research on in vitro immunotoxicology.** Sci Total Environ 2001;270(1-3):21-5.
Abstract: ECVAM was established to play a leading role at the European level in the independent evaluation of the reliability and relevance of test methods and testing strategies for specific purposes through research on advanced methods and new test development and validation, so that chemicals and products of various kinds, including medicines, vaccines, medical devices, cosmetics, household products and agricultural products, can be manufactured, transported and used more economically and more safely, whilst the current relevance on animal test procedures is progressively reduced. Nowhere is this activity more necessary than in the field of immunotoxicology, where we know that chemicals and products of many kinds have the potential to stimulate, modulate or suppress the induction or expression of various types of immune responses. The problem is to effectively evaluate the potency of these effectors, and, since the available information is currently based on rather qualitative animal tests, to evaluate the true relevance of this knowledge and apply it intelligently in risk assessment processes which will protect human beings without unnecessarily limiting the development and use of materials which otherwise have economic, health and social benefits. The way forward must depend on the following: (a) a better understanding of immunotoxicological processes, based on a sounder understanding of the immune system itself (and of its network of control systems and interrelationships with other body systems); (b) The use of in vitro (not in vivo) systems based on human (not animal) cells and tissues; (c) integrated and tiered testing strategies, incorporating QSAR, as well as in vitro approaches; (d) taking advantage of the use of cells or factors from humans who have been exposed to potential immunotoxins, be this voluntarily, occupationally, environmentally or by accident; and (e) the recognition that virtually everything will effect one or more aspects of the immune system at some dose level and, in some circumstances, deciding when such effects are relevant, is the key to immunotoxicity testing. Some current ECVAM-sponsored work and activities at ECVAM are described.

Bee A, Theakston RD, Harrison RA, Carter SD. **Novel in vitro assays for assessing the haemorrhagic activity of snake venoms and for demonstration of venom metalloproteinase inhibitors.** Toxicon 2001;39(9):1429-34.
Abstract: Standard methods used for assessing the haemorrhagic toxicity of snake venoms and the effectiveness of antivenoms are laborious, expensive and involve the use of large numbers of laboratory animals. This paper examined the feasibility of using a gelatin degradation ELISA for preliminary screening of snake venom metalloproteinases (MPs). Potent gelatinolytic activity was observed in venoms from snakes of the family Viperidae and, as expected, little or no activity was evident in the venoms of snakes that induce neurotoxic pathology (most elapids). A reverse gelatin zymography assay was used on a variety of venoms to demonstrate a number of inhibitors of MP activity, the first such demonstration of its kind.

Booker S. **Alternative tests make the grade.** Environ Health Perspect 2000;108(3):A118-9.
Abstract: Toxicity testing is absolutely necessary for assessing the safety of substances in food, air, and water, in the workplace and at home. Although there are several tried-and-true toxicity assays, the search is always on for methods that can even better predict toxic effects. As scientific understanding of the effects of environmental toxicants grows, new tests are needed to evaluate previously unexamined end points and to take advantage of advances in biotechnology and the growing knowledge of how toxicants work at the molecular and cellular levels. Another issue is how to develop tests that can reliably and accurately assess toxicity using less time, money, and materials, and with greater regard for animal welfare. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 1997 to address these needs through the development, validation, acceptance, and harmonization of new and revised toxicological test methods throughout the federal government.

Broadhead CL, Betton G, Combes R, Damment S, Everett D, Garner C, Godsafe Z, Healing G, Heywood R, Jennings M, et al. **Prospects for reducing and refining the use of dogs in the regulatory toxicity testing of pharmaceuticals.** Hum Exp Toxicol 2000;19(8):440-7.
Abstract: A workshop was held to critically discuss the need for a nonrodent species and the role of the dog in regulatory toxicity testing of pharmaceuticals; to discuss opportunities to reduce and refine the use of dogs in preclinical toxicology; and to identify a number of specific recommendations which could be feasibly achieved to move the process forward. To facilitate a preliminary evaluation of the contribution of dog studies to the risk assessment process, anonymised, unpublished data were provided from fully evaluated, repeat-dose toxicity studies in the rat and dog. Results of the International Life Sciences Institute (ILSI) Human Toxicity Project were also presented and discussed. Analysis of the data demonstrated that the dog can provide additional toxicity information, which, in some cases, was shown to be predictive for humans. Discussions indicated that there is potential for achieving a reduction in dog use and several possible approaches were identified. To further the progress of this initiative, there is a need to collate the results of pharmacology, toxicology, and clinical studies to address some of the proposed approaches. One of the outcomes of the workshop will be the establishment of a steering group to co-ordinate data collation for further analysis.

Broadhead CL, Combes RD. **The current status of food additives toxicity testing and the potential**

for application of the three Rs. Altern Lab Anim 2001;29(4):471-85.

Croci L, Stacchini A, Cozzi L, Ciccaglioni G, Mazzei F, Botre F, Toti L. **Evaluation of rapid methods for the determination of okadaic acid in mussels.** J Appl Microbiol 2001;90(1):73-7.
Abstract: AIMS: Two different screening methods, a Buffalo Green Monkey cytotoxicity test and a biosensor test, have been considered to replace the official mouse bioassay in monitoring for okadaic acid (OA) levels in mussels. METHODS AND RESULTS: Diarrhoetic shellfish poison-contaminated mussels from the Adriatic Sea were assayed in parallel by means of the mouse bioassay and both alternative methods. Both the cytotoxicity test and the biosensor test showed high sensitivity (OA 0.01 mg g-1 hepatopancreas and 0.002 mg g-1 hepatopancreas, respectively) and a high correlation with the mouse bioassay (r=0.932, P Õ 0.001 and r=- 0.850, P < 0.001, respectively). CONCLUSION: Both methods are efficacious, quick, inexpensive and provide data on the amount of toxin present in mussels. SIGNIFICANCE AND IMPACT OF THE STUDY: Both methods, besides allowing the simultaneous assay of a great number of samples, comply with the ethical need to reduce the use of animals in the laboratory.

Cronin M. **Modeling environmental fate and toxicity.** Altern Lab Anim 2001;29(4):496-7.

De Bosschere H, Baert K, Ducatelle R, De Backer P. **The use of a Daphnia magna bioassay for rapid screening of acute intoxications with insecticides in dogs and cats.** Vet Res Commun 2001;25(5):421-7.
Abstract: Sudden death due to acute insecticide intoxications occurs frequently in dogs and cats. The absence of characteristic lesions at autopsy often renders post-mortem diagnosis dependent on the analysis of samples taken from the carcase at autopsy. In the present study, a bioassay utilizing Daphnia magna was proposed and tested as a rapid screening method for acute intoxications in dogs and cats. The bioassay was shown to be highly sensitive for detecting carbamate and organophosphate insecticides in the stomach contents. Generally. the mean survival time of the waterfleas in the control group was 5.17 h (SD = 1.24) and in the intoxicated group 1.32 h (SD = 1.49). during a 6 h observation period. If a cut-off is set at 4 h, this Daphnia bioassay gave 5.5% false negative results and 18.2% false positive results. using the results of toxicological analyses as a gold standard.

De Coen WM, Janssen CR, Segner H. **The use of biomarkers in Daphnia magna toxicity testing V. In vivo alterations in the carbohydrate metabolism of Daphnia magna exposed to sublethal concentrations of mercury and lindane.** Ecotoxicol Environ Saf 2001;48(3):223-34.
Abstract: Aspects of the carbohydrate metabolism of Daphnia magna exposed for 48 and 96 h to sublethal concentrations of mercury and lindane were investigated. General as well as toxicant-specific perturbations in the intermediary metabolism were observed. Both model toxicants caused an increase in glycolytic and hexose-monophosphate shunt activity. Mercury exposure increased lactate dehydrogenase and isocitrate activity (only after 96 h), while lindane exposure, on the contrary, inhibited the cellular lactate formation and increased the Krebs' cycle activity (only after 48 h). Daphnids exposed to sublethal mercury concentrations clearly exhibited increased glycogenolytic activity, while in lindane-exposed organisms mainly glycogen phosphorylase inhibition was detected. The short-term enzyme-based effect

levels (48--96 h LOEC and EC(10) values) were compared with the effects on the population dynamics. This evaluation for both model toxicants suggests that threshold levels (LOEC or EC(10) values) based on pyruvate kinase activity after 48 and 96 h of exposure could be potential early warning signals for long-term effects. A set of enzymatic endpoints, based on the intermediary metabolism, is suggested to characterize the metabolic state of the daphnids. Copyright 2001 Academic Press.

Evans SM, Casartelli A, Herreros E, Minnick DT, Day C, George E, Westmoreland C. **Development of a high throughput in vitro toxicity screen predictive of high acute in vivo toxic potential.** Toxicol In Vitro 2001;15(4-5):579-84.
Abstract: At an early stage of drug discovery high throughput screens are an invaluable tool to de-select compounds with undesirable properties. A high throughout in vitro toxicity screen has been developed and validated to identify compounds that have a high potential to be acutely toxic in vivo. This screen is based on treating Chinese hamster ovary (CHO) cells with test compounds for 24 h and then determining the degree of cytotoxicity by the reduction of Resazurin. Twenty-six structurally unrelated compounds were chosen that spanned a range of acute LD(50) values and mechanisms of toxicity. The acute LD(50) values (intraperitoneal and intravenous routes) from rat and mouse were taken from the RTECS database. Experimentally derived in vitro IC(35) results were compared to the 'most toxic' (lowest) LD(50) values for each compound. The resulting correlation was statistically significant (r=0.8475). However, due to the scatter of the data points, it was considered not appropriate to rank compounds according to their degree of in vivo toxicity on the basis of the in vitro result. However, by defining cut-off concentrations for both the in vivo (LD(50)) and the in vitro (IC(35)) values it was possible, using the in vitro result (IC(35) <10 &mgr;M), to identify compounds that had a high potential to be acutely toxic in vivo ('most toxic' LD(50) <25 &mgr;mol/kg). Further development led to a high throughput screen capable of giving a 'Yes', 'No' or 'Borderline' classification as to whether a compound has a high acute in vivo toxic potential. This screen is highly specific (no false positive classifications) and has a sensitivity of approximately 80%. This is deemed acceptable for a first tier toxicity screen at an early stage in the drug discovery process. Transfer of this screen from GlaxoSmithKline UK to sites in Italy, Spain and the USA resulted in very similar findings indicating the inter-laboratory robustness of this screen and therefore the ability to compare results across the GlaxoSmithKline sites.

Ford RA, Domeyer B, Easterday O, Maier K, Middleton J. **Criteria for development of a database for safety evaluation of fragrance ingredients.** Regul Toxicol Pharmacol 2000;31(2 Pt 1):166-81.
Abstract: Over 2000 different ingredients are used in the manufacture of fragrances. The majority of these ingredients have been used for many decades. Despite this long history of use, all of these ingredients need continued monitoring to ensure that each ingredient meets acceptable safety standards. As with other large databases of existing chemicals, fulfilling this need requires an organized approach to identify the most important potential hazards. One such approach, specifically considering the dermal route of exposure as the most relevant one for fragrance ingredients, has been developed. This approach provides a rational selection of materials for review and gives guidance for determining the test data that would normally be considered necessary for the elevation of safety under intended conditions of use. As a first step, the process takes into account the following criteria: quantity of use, consumer exposure, and chemical structure. These are then used for the orderly selection of materials for review with higher quantity, higher exposure, and the presence of defined structural alerts all contributing to a higher

priority for review. These structural alerts along with certain exposure and volume limits are then used to develop guidelines for determining the quality and quantity of data considered necessary to support an adequate safety evaluation of the chosen materials, taking into account existing data on the substance itself as well as on closely related analogs. This approach can be considered an alternative to testing; therefore, it is designed to be conservative but not so much so as to require excessive effort when not justified.

Garner RC. **Accelerator mass spectrometry in pharmaceutical research and development--a new ultrasensitive analytical method for isotope measurement.** Curr Drug Metab 2000;1(2):205-13.
Abstract: Accelerator mass spectrometry (AMS) permits the measurement of elemental isotopes at the individual atom level. The main application of AMS in drug discovery and development will be in the analysis of 14-carbon (14C). The principle behind AMS is the separation of individual positively charged atoms through mass, charge and momentum differences. In order to obtain the high-energy charge state required for separation, negative atoms are accelerated through a high voltage field (up to 10 million volts) generated by a tandem Van de Graaff accelerator. In the middle of the accelerator, the outer valency electrons are stripped from the atom and the resulting charged species are separated and counted. For 14C, AMS counts the number of individual atoms rather than measuring radioactive decays. The result is that AMS is up to one million times more sensitive than decay counting. Radioactivity levels as low 0.0001 dpm can be detected using AMS. The exquisite sensitivity of AMS analysis means that much lower amounts of 14C can be used than for conventional counting methods. This makes it easier to use 14C for in vitro, preclinical and clinical research programmes. As 14C poses both a biological and environmental hazard, AMS permits much lower doses to be used. Human drug mass balance studies have been conducted with doses of 50 nanoCuries and below. Radioactive HPLC metabolite profiles of plasma extracts from subjects given nanoCurie doses of 14C-labelled drug have been obtained by injecting as little as 0.25 dpm onto an HPLC column. In studies of biologics, biosynthetically 14C-labelled recombinant protein has been produced with a specific radioactivity sufficient to conduct human clinical studies with AMS analysis. For one human recombinant protein an increase in sensitivity of 2,000-fold over ELISA was obtained with AMS measurement. AMS is an enabling technology that should prove of value in increasing human and environmental safety as well as allowing new research directions to be followed.

Genschow E, Liebsch M, Halle W, Spielmann H. **[Report on the ICCVAM workshop on in vitro methods for assessing acute systemic toxicity].** ALTEX 2001;18(2):115-6 [Ger].
Abstract: It was suggested in the ICCVAM workshop that the Register of Cytotoxicity (RC), using in vitro cytotoxicity data to predict the in vivo starting doses, should be implemented into acute toxicity testing as soon as possible. The validity of the in vitro cytotoxicity data to establish appropriate starting doses for acute toxicity testing will be assessed experimentally. Secondly, in order to replace the use of animals in acute lethality testing a formal validation will be conducted in which the ability to predict rodent LD50 values and toxicity classes from cytotoxicity data will be evaluated.

Gerner I, Zinke S, Graetschel G, Schlede E. **Development of a decision support system for the introduction of alternative methods into local irritancy/corrosivity testing strategies. Creation of fundamental rules for a decision support system.** Altern Lab Anim 2000;28(5):665-98.

Abstract: The notification procedure of the European Union (EU) for new chemicals requires the application of protocols on physicochemical and toxicological tests for the evaluation of physicochemical properties and probable toxic effects of each notified substance. A computerised database was developed from data sets and toxicological test protocols relating to substance properties responsible for skin and eye irritation/corrosion. To develop specific structure-activity relationship (SAR) models and to find rules for a decision support system (DSS) to predict local irritation/corrosion, physical property data, chemical structure data and toxicological data for approximately 1300 chemicals, each having a purity of 95% or more, were evaluated. The evaluation demonstrated that the lipid solubility and aqueous solubility of a chemical are relevant to, or - in some cases - responsible for, the observed local effects of a substance on the skins and eyes of rabbits. The octanol/water partition coefficient and the measured value of the surface tension of a saturated aqueous solution of the substance give additional information that permits the definition of detailed SAR algorithms that use measured solubility values. Data on melting points and vapour pressure can be used to assess the intensity and duration of local contact with a chemical. Considerations relating to the reactivity of a pure chemical can be based on molecular weight and the nature of the heteroatoms present. With respect to local lesions produced following contact with the skin and eyes of rabbits, the data evaluation revealed that no general "local irritation/corrosion potential" of a chemical can be defined. A variety of mechanisms are responsible for the formation of local lesions on the skin or in the eyes: serious lesions are produced by mechanisms different from those that cause moderate irritation in these organs. In order to develop a DSS that uses the information extracted from the database, chemical main groups were categorised on the basis of their empirical formulae, and rules were defined of the type IF (physicochemical property) A, THEN not (toxic) effect B, based on correlations between specific local effects and measured physicochemical values. Other rules of the type IF substructure A, THEN effect B were developed based on correlations between specific local effects and the submitted structural formulae. Reactive chemical substructures relevant to the formation of local lesions and rules for the prediction of the absence of any skin irritation potential were identified. Proposals are made relating to the development of alternatives to eye irritation testing with rabbits.

Green S, Goldberg A, Zurlo J. **TestSmart-high production volume chemicals: an approach to implementing alternatives into regulatory toxicology.** Toxicol Sci 2001;63(1):6-14.
Abstract: This Article examines the status and application of alternatives defined as replacements, refinements, and reduction for screening high production volume (HPV) chemicals. It specifically focuses on the Screening Information Data Set (SIDS), a series of toxicological tests recommended by the Organization for Economic Cooperation and Development to screen such chemicals. Alternative tests associated with acute, repeat-dose, genetic, and reproductive and developmental toxicity were examined at 2 meetings of academic, industry, and regulatory scientists and their status determined. Tests were placed in 1 of 3 categories: ready for immediate use, in need of or currently undergoing validation, or needing research/developmental work. With respect to traditional acute toxicity testing, the basal cytotoxicity approach was placed in the category of research with the up-and-down, fixed-dose, limit test, and the acute toxic class categorized as available for immediate use and the neutral red assay under validation. Cell culture methods that could provide information on acute target organ toxicity were all categorized in the research stage. Studies of the Ah receptor were placed under validation. All alternative tests for repeat-dose toxicity were placed in the category of research. With

regard to genetic toxicity, the Ames, mouse lymphoma, and Chinese hamster ovary methods were considered ready for immediate use, while the in vitro micronucleus and Syrian hamster ovary assays were placed in the validation category. All alternatives for developmental toxicity, with the exception of gene chip technology, were placed in the category of validation. Gene chip technology is considered to be in the research stage. For reproductive toxicity, sperm motility and morphology were considered as ready for immediate use, with the other assays categorized as needing validation or in the research stage. Follow-up to these results is obvious. Work needs to be conducted to move those tests from the research stage to the validation and use stage. This is one approach to the development of alternatives to SIDS. Progress along these lines would apply not only to SIDS but also to toxicology in general.

Green S, Goldberg AM, Zurlo J. **The TestSmart-HPV Program--development of an integrated approach for testing high production volume chemicals.** Regul Toxicol Pharmacol 2001;33(2):105-9.
Abstract: The TestSmart program was developed in response to the High Production Volume Chemical Challenge, a voluntary initiative under which chemical producers provide basic toxicity data on chemicals produced in greater than one million pounds annually. Specifically, under the Challenge, chemical producers will generate data as needed to complete the Screening Information Data Set (SIDS), as defined by the Organization for Economic Cooperation and Development (OECD). The TestSmart program is a collaborative effort of the Johns Hopkins Center for Alternatives to Animal Testing, the Environmental Defense Fund, Carnegie-Mellon University, and the University of Pittsburgh. The goal of the TestSmart program is to provide a humane and efficient approach to collecting SIDS data. The program has two objectives, one immediate and the other more long term. The immediate objective has been to make recommendations to reduce the number of animals used in collecting SIDS data under the Challenge. This was accomplished, through a group process, by examining the current status of alternative methods for SIDS endpoints and by providing an assessment of the "state of readiness" of current and potential future alternatives. The long-term objective is to provide a model for other programs to follow the TestSmart concept of a more efficient and humane approach to obtain toxicological data of interest to regulators and the public. Copyright 2001 Academic Press.

Guilhermino L, Diamantino T, Silva MC, Soares AM. **Acute toxicity test with Daphnia magna: an alternative to mammals in the prescreening of chemical toxicity?** Ecotoxicol Environ Saf 2000;46 (3):357-62.
Abstract: In this study, the association between the acute toxicity of 15 compounds to Daphnia magna, expressed as 24- and 48-h LC(50) values, and the corresponding oral LD(50) values for the rat was tested. Since there was evidence of a strong relationship between the two species, the sample was extended to 54 cases by including the values for acute toxicity to D. magna and rat of more chemicals published by other authors. Thus, a total of 54 data points were further used to ascertain the relationship between the acute toxicity of chemical compounds to D. magna and that to the rat. To summarize its validity, the D. magna test is more specific than sensitive as an indicator of toxicity to the rat. When it is used with a chemical that has a high probability of being very toxic to D. magna (LC(50)< 0.22 mg/L), the test provides considerable information if it is positive, virtually giving evidence of toxicity to the rat (with a probability of 0.83). On the other hand, a negative test (D. magna LC(50)>0.22 mg/L) has a probability of correctly assigning nontoxicity to the rat equal to 0.74. This study and results published by other authors provide good evidence of the applicability of using invertebrate tests as prescreening

methods, thus considerably reducing the number of mammals required in toxicity testing.

Hastings KL. **Assessment of immunosuppressant drug carcinogenicity: standard and alternative animal models.** Hum Exp Toxicol 2000;19(4):261-5.
Abstract: Drugs intended for use in preventing allograft rejection in transplant patients are likely to be administered chronically; thus, it is normally expected that sponsors would conduct nonclinical studies to determine the carcinogenic potential of candidate compounds. For pharmaceuticals other than biologic agents, this would mean that rodent carcinogenicity bioassays would be performed under most circumstances. Immunosuppressant drugs have presented unique challenges with respect to the issue of carcinogenicity bioassays. The pharmacological activity of therapeutic immunosuppressants is thought to make them highly likely to act as promoters/cocarcinogens, even in the absence of genotoxic activity. Thus, it is assumed that this class of drug would represent a carcinogenic hazard in the absence of confirmatory standard rodent bioassay data. In addition, rodents typically have been sensitive to the pharmacological/toxicological effects of immunosuppressants. It has proven to be difficult, therefore, to conduct life-time bioassays at doses reasonably equivalent to those that would be used clinically. For this and other reasons, alternative models might be more appropriate for risk assessment with this class of drugs.

Jansson T, Loden M. **Strategy to decrease the risk of adverse effects of fragrance ingredients in cosmetic products.** Am J Contact Dermat 2001;12(3):166-9.
Abstract: In spite of extensive self-regulation of the fragrance industry, fragrance ingredients are still major causes of allergic contact dermatitis. There are indications that the problem is increasing in some countries, and that many nonregulated compounds are involved in the development of allergies. The use of essential oils in fragrance compounds might add both allergenic and carcinogenic compounds to a product and the exact composition of such ingredients is difficult to control. Herein, we propose a simple strategy to decrease the risk of adverse effects of fragrance ingredients in cosmetic products. This strategy consists of four major steps: (1) limit the concentration of fragrance compound in the products, (2) follow legislation and guidelines, (3) limit the concentration of a number of well-known sensitizing fragrance chemicals, and (4) limit the concentration of essential oils and materials with unknown composition. The strategy is discussed as an alternative to animal testing and in relation to other more resource-demanding approaches to the same problem.

Johnson BM, Bolton JL, van Breemen RB. **Screening botanical extracts for quinoid metabolites.** Chem Res Toxicol 2001;14(11):1546-51.
Abstract: Botanical dietary supplements represent a significant share of the growing market for alternative medicine in the USA, where current regulations do not require assessment of their safety. To help ensure the safety of such products, an in vitro assay using pulsed ultrafiltration and LC-MS-MS has been developed to screen botanical extracts for the formation of electrophilic and potentially toxic quinoid species upon bioactivation by hepatic cytochromes P450. Rat liver microsomes were trapped in a flow-through chamber by an ultrafiltration membrane, and samples containing botanical extracts, GSH and NADP(H), were flow-injected into the chamber. Botanical compounds that were metabolized to reactive intermediates formed stable GSH adducts mimicking a common in vivo detoxification pathway. If present in the ultrafiltrate, GSH conjugates were detected using LC-MS-MS with precursor ion

scanning followed by additional characterization using product ion scanning and comparison to standard compounds. As expected, no GSH adducts of reactive metabolites were found in extracts of Trifolium pratense L. (red clover), which are under investigation as botanical dietary supplements for the management of menopause. However, extracts of Sassafras albidum (Nutt.) Nees (sassafras), Symphytum officinale L. (comfrey), and Rosmarinus officinalis L. (rosemary), all of which are known to contain compounds that are either carcinogenic or toxic to mammals, produced GSH adducts during this screening assay. Several compounds that formed GSH conjugates including novel metabolites of rosmarinic acid were identified using database searching and additional LC-MS-MS studies. This assay should be useful as a preliminary toxicity screen during the development of botanical dietary supplements. A positive test suggests that additional toxicological studies are warranted before human consumption of a botanical product.

Johnson DE, Wolfgang GH. **Predicting human safety: screening and computational approaches.** Drug Discov Today 2000;5(10):445-54.
Abstract: Current preclinical safety evaluation programs use a combination of computational methods, mechanistic in vitro screening and - primarily - in vivo experimentation to predict human toxicity. The rapid transition of pharmaceutical R&D into electronic R&D (e-R&D) makes it imperative that predictive safety testing also develops into an information-rich, knowledge-based process in the near future. Accordingly, enhanced databases and computational tools are expected to change the way the pharmaceutical industry assesses drug toxicity during discovery and early development. Expert use of prediction tools should lead to lower failure rates in drug development and decrease the cost and time involved in successful drug approval.

Kaul S, Hoffmann A. **[Mediator release assay of rat basophil leukemia cells as alternative for passive cutaneous anaphylaxis testing (PCA) in laboratory animals].** ALTEX 2001;18(1):55-8 [Ger].
Abstract: Passive cutaneous anaphylaxis (PCA) is an animal model for inflammatory reactions in Type I allergy. An in vitro assay based on IgE dependent and allergen-induced mediator release of RBL-2H3 cells is presented as an alternative for PCA. The assay has been adopted to the special needs of examining allergen extracts and has been proven to be practicable and reliable. A high number of samples can be processed quickly in one assay, with intra-assay variations below 10%. The first successful applications are the measurement of biologic potencies in allergenic extracts and the determination of murine serum IgE antibodies. Further studies will show whether this assay is suited to evaluate the efficiency of anti-inflammatory and anti-allergic drugs.

Klemm M, Genschow E, Pohl I, Barrabas C, Liebsch M, Spielmann H. **[Permanent embryonic mouse germ cell-lines, an in vitro alternative to in vivo germ cell mutagenicity tests].** ALTEX 2001;18 (2):127-30 [Ger].
Abstract: Germ cell mutagenesis is required by the 7th amendment of the directive 67/548 EEC into the national regulations on existing chemicals. Officially accepted in vivo test systems for stage specific mutagenicity are the dominant lethal (DL) test and the specific locus test (SLT) in mice. An acceptable in vitro alternative designed to address germ cell mutagenesis and discriminate between male and female specific effects is not available at present. In order to offer a sensitive and predictive in vitro method to assess the genotoxic potential of chemical agents on male and female reproduction, we established

primordial germ (PG) cell-derived permanent embryonic germ (EG) cell lines of the mouse (strain BALB/cJ). The differences in developmental sensitivity of the EG(3) cell line and differentiated fibroblast cells 3T3 were comparatively tested with cytotoxicity assay (MTT test) and genotoxic studies (SCE-assay) under identical test conditions. The concentration-response curves reflected the female cell line EG(3) to be extremely sensitive concerning cytotoxic and genotoxic endpoints. Therefore this cell line was used to classify in vivo genotoxic and non-genotoxic test substances with different potential endpoints. Applying linear discriminant analysis three endpoints were identified for the correct classification (100%) of all test chemicals, namely the SCE(200) value (increase of 200% in the mean number of SCEs per metaphase spread) for EG(3) (3 hrs and 24 hrs assay) and the IC(5)0 value for EG (3) after 3 hrs of exposure to test chemicals.

Kobayashi K. **Methods of statistical analysis of quantitative data obtained by toxicological bioassays using rodents in Japan: historical transition of the decision tree.** J Environ Biol 2001;22 (1):1-9.
Abstract: Massive amounts of quantitative data are being obtained by toxicity tests with time. These data sometimes include the data with a different distribution pattern or with different numbers of animals in each group. The tree-type algorithm has long been used commonly to promptly analyze the difference between the control group and dosage groups. In Japan, the decision tree has been used since 1982 with several modifications of the initial procedure. Recently, the method has been revised to have a high power. The changes have been made in two directions; one is a sophisticated method and the other is a simplified method. In this paper, the historical transition of the decision tree is described and the future of the decision tree forecasted.

Kobayashi K, Kanamori M, Ohori K, Takeuchi H. **[A new decision tree method for statistical analysis of quantitative data obtained in toxicity studies on rodents].** Sangyo Eiseigaku Zasshi 2000;42 (4):125-9 [Jpn].
Abstract: Regarding the statistical analysis of the quantitative data obtained in control and dosage groups in toxicity studies, we tried to simplify the decision tree method. In a new decision tree presented in this Article, one-way analysis of variance and Kruskal-Wallis nonparametric analysis of variance are excluded from the traditional decision tree: (1) Bartlett's test is used as a test for the equality of k variances: (2) Then, if the k sampled populations have equal variances ($p > 0.05$ by the Bartlett's test), Dunnett's multiple comparison test is performed: otherwise, Steel's test is used. This new method, which increases the power in some conditions, may serve as an alternative to the traditional decision tree method.

Levenbook I, Dragunsky E, Pervikovc Y. **Development of a transgenic mouse neurovirulence test for oral poliovirus vaccine: international collaborative study 1993-1999.** Vaccine 2000;19(2-3):163-6.
Abstract: The neurovirulence safety of oral live poliovirus vaccine (OPV) has been tested in monkeys, because only primates are sensitive to all three types of poliovirus. The genetic engineering of transgenic mice susceptible to poliovirus led to studies on the suitability of these mice for a neurovirulence test of OPV. A WHO international collaborative study started with type-3 OPV in 1993 and was completed in 1999. The study produced a voluminous set of data proving that the TgPVR21 mice, inoculated with OPV samples into the lumbar cord, provided a test for neurovirulence of OPV as sensitive and

reproducible as the monkey test. A statistical decision model for acceptance/rejection of type-3 vaccines using the transgenic mouse test has been developed. The mouse neurovirulence test showed a number of essential advantages over the monkey test. This is the first example of a successful introduction of transgenic animals into control of biologicals. In October 1999, the WHO Expert Committee on Biological Standardization approved TgPVR21 mice as alternative to the monkey model for neurovirulence testing of OPV type 3. A final step of the collaborative study with OPV types 1 and 2 is in progress, and data obtained so far are promising. Two breeding stations for production of TgPVR21 mice are being established in Asia and Europe.

Libowitz L. **CAATSCAN: a review of alternatives. News and information.** In Vitr Mol Toxicol 2000;13(2):153-4.

Lovett RA. **Toxicogenomics. Toxicologists brace for genomics revolution.** Science 2000;289 (5479):536-7.

Luber-Narod J, Smith B, Grant W, Jimeno JM, Lopez-Lazaro L, Faircloth GT. **Evaluation of the use of in vitro methodologies as tools for screening new compounds for potential in vivo toxicity.** Toxicol In Vitro 2001;15(4-5):571-7.

Pennie WD, Tugwood JD, Oliver GJ, Kimber I. **The principles and practice of toxigenomics: applications and opportunities.** Toxicol Sci 2000;54(2):277-83.

Pfaller W, Balls M, Clothier R, Coecke S, Dierickx P, Ekwall B, Hanley BA, Hartung T, Prieto P, Ryan MP, et al. **Novel advanced in vitro methods for long-term toxicity testing: the report and recommendations of ECVAM workshop 45. European Centre for the Validation of Alternative Methods.** Altern Lab Anim 2001;29(4):393-426.

Prieto P. **ECVAM's in-house prevalidation/validation studies in the areas of haematotoxicity, reproductive toxicity, metabolism-mediated toxicity and epithelial barrier function.** Sci Total Environ 2000;247(2-3):349-54.
Abstract: The European Centre for the Validation of Alternative Methods (ECVAM) facilitates, co-ordinates and parcticipates in validation activities at the European Union level. Various experimental studies, e.g. in the areas of haematotoxicity, reproductive toxicity, nephrotoxicity and epithelial barrier function, and metabolism-mediated toxicity, are underway in ECVAM's laboratories. ECVAM itself is currently involved in the prevalidation/validation of two assays, the colony-forming unit granulocyte/ macrophage (CFU-GM) assays for predicting acute neutropenia and the embryonic stem cell test for predicting embryotoxicity. In the areas of metabolism-mediated toxicity and nephrotoxicity and epithelial barrier function, several assays are in the course of development. In many cases, the recommendations of various ECVAM workshops are being followed.

Ronai E, Danyi D. **Conventional and alternative acute oral toxicity tests in toxicological practice.** Cent Eur J Public Health 2000;8 Suppl:80

Sandbacka M, Christianson I, Isomaa B. **The acute toxicity of surfactants on fish cells, Daphnia magna and fish-a comparative study.** Toxicol In Vitro 2000;14(1):61-8.
Abstract: There is a need to replace acute toxicity tests on fish (LC(50)) with more cost effective assays. The main objective of this study was to explore whether gill epithelial cells, hepatocytes and Daphnia magna could be used to predict acute toxicity of surfactants on fish. The acute toxicity of 10 synthetic surfactants (anionic, cationic, nonionic and zwitterionic) and two resin acids were determined on hepatocytes and gill epithelial cells from rainbow trout (Oncorhynchus mykiss), on Daphnia magna and on fish. Cell viability was measured with the fluorescent viability probe calcein-AM, immobilization was determined for Daphnia and 24-hr LC(50) for rainbow trout. The EC(50) values for the cellular tests were clearly higher than the corresponding values for Daphnia and fish, indicating that the cellular tests with the endpoint used are less sensitive than whole organisms. A combination of the EC(50) values for Daphnia and freshly isolated gill epithelial cells in suspension showed, however, a good correlation with acute toxicity on fish (r(2)=0.91 and slope=1.09). The combination seems to be a promising in vitro alternative to acute toxicity tests on fish (LC(50)), but a more exhaustive comparison, including a broad spectrum of chemicals should be made before the predictive value of the combined in vitro test can be evaluated.

Sass N. **Humane endpoints and acute toxicity testing.** ILAR J 2000;41(2):114-23.

Schleger C, Krebsfaenger N, Kalkuhl A, Bader R, Singer T. **[Innovative cell culture methods in drug development].** ALTEX 2001;18(1):5-8 [Ger].
Abstract: The animal studies necessary for drug registration are time-consuming, costly, and often stressful for the animals. Toxicological screening of drug candidates early in development with in vitro cell culture systems is therefore of relevance. In contrast to animal studies, in vitro cell culture methods are characterized by a low compound requirement and a short duration. Additionally it is possible to include mechanistic studies or to test for toxicity specific to humans. Therefore, early toxicological screening can provide a useful support for selecting the most promising drug candidate. Primary hepatocytes can be used to measure the cytotoxicity of a test compound. These results can be used to estimate general toxicity. Measuring endpoints like apoptosis, redox status, or gene expression profiles can help to answer mechanistic questions. The use of primary human hepatocytes provides early predictivity for hepatotoxicity specific to humans. Since teratogenic findings in animal studies often lead to abandonment of development, it is reasonable to use an in vitro embryotoxicity assay for early determination of the teratogenic potential of a compound, e.g. the embryonic stem cell test (EST) which was recently developed by ZEBET. In the EST embryonic stem cells are investigated for their preserved capability to differentiate into cardiomyocytes following drug exposure. In comparison cytotoxicity of the test substance is analyzed in embryonic stem cells and in differentiated fibroblast cells. In a validation study initiated by ECVAM the EST shows a high correlation with in vivo data.

Schmuck G. **[The importance of standardised cell culture methods for the routine toxicology in pharmaceutical companies].** ALTEX 2001;18(1):79-80 [Ger].
Abstract: The establishment and the use of standardised cell cultures build the basis for toxicological investigations and guarantees the comparison over years. The requirement for these investigations in

vitro are mainly primary cell cultures from the target organs. The cell cultures should keep, also under long term culture conditions, the organ differentiation. This will be supported by new developments in the fields of cell culture media, supplements and coating material of the cell culture dishes. Routinely, cells from toxicologically important organs like liver, kidney and nervous system were used in vitro. However, mechanistic investigations of toxicological findings in vivo made the use of cell systems from other organs like cartilage, bone, skeletal and heart muscle cells, testis eg necessary. All cell culture systems were established and standardised to allow repeated tests under the same conditions. The determination of characteristic proteins or enzymes of the related organ will demonstrate the organ like quality of the cell cultures. The central question of toxicology is the risk assessment. Here, the in vitro toxicology will provide important information by a comparison between human and animal cell cultures under the same conditions.

Sells PG, Laing GD, Theakston RD. **An in vivo but insensate model for the evaluation of antivenoms (ED(50)) using fertile hens' eggs.** Toxicon 2001;39(5):665-8.
Abstract: The preclinical evaluation of the efficacy of new antivenoms (ED(50)) in animals is required by international regulatory authorities. In vitro testing alone does not provide the end point of lethality of a living system which is essential for an antivenom to prevent. Large numbers of mice are injected with venom/antivenom mixtures and the number of surviving mice is statistically analysed to give an ED(50) value reflecting the efficacy of that antivenom. Our objective was to develop a humane alternative to the conventional rodent ED(50) test using fertile hens' eggs. The egg test combines the advantages of an in vivo system, which gives a clear end point for haemorrhage and lethality, with the certain knowledge that no distress can be experienced by the embryo which is insensate at day 6 of development. Nine different, medically-important venoms and antivenoms were tested by both the rodent and egg ED(50) assays. A good correlation between the two tests was obtained ($p<0.01$) suggesting that the egg ED(50) method should be considered as an alternative to the rodent ED(50) test for non-neurotoxic venoms. Use of the egg ED(50) test would substantially reduce both the numbers of experimental animals required and also the considerable costs involved in delivering improved clinical treatment of snakebite.

Smith D, Trennery P, Farningham D, Klapwijk J. **The selection of marmoset monkeys (Callithrix jacchus) in pharmaceutical toxicology.** Lab Anim 2001;35(2):117-30.
Abstract: Prior to controlled clinical trials in human volunteers or patients it is required that novel pharmaceuticals are evaluated for pre-clinical safety in a rodent and a non-rodent ('second') species. In most cases the rodent species used has been the rat and the second species has been the dog or macaque (usually cynomolgus or rhesus) monkey. However, there is an increasing trend within the United Kingdom (UK) pharmaceutical industry to use the common marmoset (Callithrix jacchus) for pre-clinical toxicology programmes. This paper examines the practicality of using the common marmoset (henceforth referred to as 'the marmoset') in toxicological testing and reviews metabolic and pharmacodynamic similarities between this species and humans. It then discusses some of the advantages and disadvantages of the use of this species when compared with two other alternatives to the dog and macaque, namely the ferret and minipig. In parcticular, the marmoset has clear advantages over the macaque in terms of animal welfare and practicality. There is regulatory acceptance of this species for Investigational New Drug (IND), Clinical Trial Exemption (CTX), New Drug Application (NDA) and Marketing Authorization Application (MAA) registrations. Whilst the dog is likely to be

maintained as the primary non-rodent species in toxicology, the marmoset has been, and will likely continue to be, adopted as an additional non-rodent species in pre-clinical toxicology programmes where appropriate.

Spielmann H. **International co-operation: an essential requirement for replacing animal toxicity tests.** Altern Lab Anim 2001;29(6):633-4.
Abstract: The Three Rs concept, which was developed by Russell and Burch in 1959, was implemented into the legal framework in the European Union (EU) for the protection of vertebrate animals used for experimental and other scientific purposes, when Directive 86/609/EEC was adopted in 1986. One focus of activity under this Directive is the use of animals and alternative methods in regulatory testing. To reduce or replace animal testing for regulatory purposes, non-animal tests must be independently validated to prove that they can provide information that is relevant and reliable for hazard prediction in relation to specific types of toxicity in vivo. At the end of the 1980s, no scientific concept existed for the formal validation of in vitro toxicity tests, so a small group of European and American scientists met to develop a set of principles for experimental validation, which were first adopted by ECVAM in Europe in 1995, and, after harmonisation with experts from the USA and Japan, accepted internationally by the OECD in 1996. ECVAM has directly funded a number of validation studies, and a major breakthrough in the year 2000 was the acceptance for regulatory purposes in the EU of scientifically validated in vitro toxicity tests for phototoxic potential and for skin corrosivity. These, and other examples which are discussed, confirm that the internationally harmonised ECVAM/ICCVAM/OECD validation concept is a practical and effective way of making possible the replacement of regulatory testing in animals.

Spielmann H, Muller L, Averbeck D, Balls M, Brendler-Schwaab S, Castell JV, Curren R, de Silva O, Gibbs NK, Liebsch M, et al. **The second ECVAM workshop on phototoxicity testing. The report and recommendations of ECVAM workshop 42.** Altern Lab Anim 2000;28(6):777-814.

Stoklosowa S. **Three dimensional tissue and organ models in vitro: their application in basic and practical research.** Folia Histochem Cytobiol 2001;39(2):91-6.
Abstract: Good and reliable three dimensional biomodel in vitro should mimic the in vivo structure and function of investigated tissue or organ. This can be achieved through the interaction of various cell types and the environment (extracellular matrix, as well as spatial cell contacts). While designing such a model it is necessary to find best nutritive and adhesive factors, to allow access to components of optimal matrix and to enable cell contacts in three dimensions. The response and function of such models reminds physiological function of tissue of origin. Over several years there has been an increasing number of experiments and publications reporting research involving spatial cell models. This model and its structural and molecular analysis clearly showed, that in comparison with conventional cultures, mainly monocultures grown as monolayers, spatial cultures resemble the in vivo situation with regard to cell shape and biological behaviour. Spatial arrangement of cells and the environment can direct tissue differentiation. Interesting example of the importance of the environment for the latter, is the finding that the ectopic implantation of embryonic cells transforms them into malignant tissue while the same cells located in the uterus undergo normal embryogenesis. The present review describes several three-dimensional models in vitro and their application in basic and practical studies. It is especially interesting and important in the light of developments of tissue modeling to obtain in vitro substitutes of

damaged or impaired tissues and organs. Other demands come from animal protectionists who suggest replacement of experimental animals with cultures and tissue models in bio-, pharmacological, and toxicological assays. In response, European Union legislation introduces increasing restrictions on animal experiments. At last, researchers seek in vitro models which would enable avoiding discrepancies between in vitro and in vivo results.

Vanchieri C. **Move over, mouse: make way for the woodchucks, ferrets, and zebrafish.** J Natl Cancer Inst 2001;93(6):418-9.

Williams PL, Anderson GL, Johnstone JL, Nunn AD, Tweedle MF, Wedeking P. **Caenorhabditis elegans as an alternative animal species.** J Toxicol Environ Health A 2000;61(8):641-7.
Abstract: Caenorhabditis elegans has proven useful in toxicity testing of known toxicants, but its potential for assessing the toxicity of new pharmaceuticals is relatively unexplored. In this study the procedures used in aquatic testing of toxicants were modified to permit testing of small amounts (<40 mg) of gadolinium-based magnetic resonance imaging (MRI) compounds. Five blinded compounds were tested. The toxicity of these compounds determined using C. elegans was compared to existing mammalian test system data (minimum lethal dose [MLD] values for mice). Four of five compounds tested had the same relative sensitivity with C. elegans as with the mouse test system. Testing with C. elegans is efficient and could markedly reduce the cost of screening potentially useful compounds.

Zeilinger K, Auth S, Unger J, Grebe A, Mao L, Petrik M, Holland G, Appel K, Nussler A, Neuhaus P, et al. **[Liver cell culture in bioreactors for in vitro drug studies as an alternative to animal testing].** ALTEX 2000;17(1):3-10 [Ger].
Abstract: An important consideration for the utilisation of in vitro culture models for studies on drug metabolism as an alternative to animal testing is the maintenance of a defined degree of cell differentiation. Thus, in vitro conditions reflecting as near as possible the in vivo situation of the cells within the whole organ are required. A bioreactor was developed for the cultivation of liver cells which allows the reorganisation of hepatocytes and non-parenchymal cells of the liver in coculture to form three-dimensional, tissue-like structures including extracellular matrix components produced by the cells. In this study, the vitality and metabolic activity of isolated rat hepatocytes was investigated over a two week culture period in bioreactors. The results show that after a reorganisation phase, the cells preserve specific functions, such as protein and urea synthesis capacity and specific cytochrome P450 activities during the culture period, with maximal values during the first week. Possible applications of the model in pharmaceutical industry are studies on metabolite patterns, enzyme induction, drug-drug-interactions, first pass effects and long-term toxicity of drugs.

Zinke S, Gerner I. **Local irritation/corrosion testing strategies: extending a decision support system by applying self-learning classifiers.** Altern Lab Anim 2000;28(5):651-63.
Abstract: Procedures have been established and tested for the extension of a decision support system (DSS) for the prediction of the local irritation/corrosion potential of chemicals by using self-learning classifiers. The different approaches (decision trees, distances examinations in a multidimensional space, k-nearest-neighbour method) have been implemented, tested and evaluated independently. A combination of all of the established extension approaches was also developed and tested. Self-learning

classifiers are constructed "automatically" by a computer, i.e. they are not derived by a human expert, and thus they can be constructed with minimal effort. The classifiers presented here extend the existing DSS in a manner that increased significantly the predictive power of the extended system. However, automatically calculated results of self-learning classifiers are produced by a machine, and a machine is incapable of explaining the toxicological relevance of the results obtained. Thus, these results must be accepted, despite an inability to prove their reliability. Only the mathematical correctness of the method and the prediction rates for suitable test cases can lend some credibility to predictions produced by a computer calculating on a self-learning basis. This may not be adequate for regulatory hazard assessment purposes.

CARCINOGENESIS

Achanzar WE, Achanzar KB, Lewis JG, Webber MM, Waalkes MP. **Cadmium induces c-myc, p53, and c-jun expression in normal human prostate epithelial cells as a prelude to apoptosis.** Toxicol Appl Pharmacol 2000;164(3):291-300.
Abstract: Cadmium is a suspected human prostatic carcinogen shown to induce prostatic tumors and proliferative lesions in rats. The carcinogenic mechanism of cadmium is unknown, but its poor mutagenicity points toward an epigenetic mechanism. Here we studied the effect of cadmium on genes involved in growth regulation of prostate epithelial cell using the human prostate epithelial cell line RWPE-1, which is immortalized but not transformed and is androgen-responsive. Treatment with 10 &mgr;M cadmium resulted in transient increases in c-myc and p53 mRNA levels that peaked at 2-fold and 1.4-fold, respectively, compared to control after 2 h. In contrast, c-jun mRNA levels were increased >3-fold after 2, 4, and 6 h and 20-fold after 24 h. DNA synthesis decreased after 24 h of cadmium exposure. Further study revealed a significant increase in apoptosis after 48 h of cadmium exposure. However, approximately 35% of the cells were still viable and appeared normal, indicating this subpopulation was more resistant to cadmium. Furthermore, these resistant cells had 2.5-fold more metallothionein than untreated control cells. This suggests that cadmium could act to select for apoptotic-defective cells in vivo, thereby increasing the likelihood of tumor formation. This work represents the first description of cadmium affecting oncogene expression in a human cell model of a potential in vivo target site of cadmium carcinogenesis.

Ackland ML, Michalczyk A, Whitehead RH. **PMC42, a novel model for the differentiated human breast.** Exp Cell Res 2001;263(1):14-22.
Abstract: Cultured human breast carcinoma cell lines are important models for investigating the pathogenesis of breast cancer. Their use, however, is limited because of loss of expression of breast-specific markers and the development of a dedifferentiated phenotype after continuous culture. PMC42 is a unique human breast carcinoma line, previously shown to express secretory and myoepithelial markers. We have induced PMC42 cells to form hollow organoids in culture, similar to in vivo breast structures, using a combination of hormones including estrogen, progesterone, dexamethasone, insulin, and prolactin in combination with a permeable extracellular matrix. The organoids comprised polarized cells located around a central lumen. Expression of beta-casein was demonstrated in cells within organoids using reverse transcriptase-polymerase chain reaction, Western blot analysis, and confocal immunofluorescence. In this in vitro system, milk-specific gene expression was induced through

hormone and matrix interactions which may be similar to those operating in vivo. PMC42 is a novel model for investigations into the molecular mechanisms of carcinogenesis and differentiation in the human breast.

Andersen ME, Meek ME, Boorman GA, Brusick DJ, Cohen SM, Dragan YP, Frederick CB, Goodman JI, Hard GC, O'Flaherty EJ, et al. **Lessons learned in applying the U.S. EPA proposed cancer guidelines to specific compounds.** Toxicol Sci 2000;53(2):159-72.
Abstract: An expert panel was convened to evaluate the U.S. Environmental Protection Agency's "Proposed Guidelines for Carcinogen Risk Assessment" through their application to data sets for chloroform (CHCl3) and dichloroacetic acid (DCA). The panel also commented on perceived strengths and limitations encountered in applying the guidelines to these specific compounds. This latter aspect of the panel's activities is the focus of this perspective. The panel was very enthusiastic about the evolution of these proposed guidelines, which represent a major step forward from earlier EPA guidance on cancer-risk assessment. These new guidelines provide the latitude to consider diverse scientific data and allow considerable flexibility in dose-response assessments, depending on the chemical's mode of action. They serve as a very useful template for incorporating state-of-the-art science into carcinogen risk assessments. In addition, the new guidelines promote harmonization of methodologies for cancer- and noncancer-risk assessments. While new guidance on the qualitative decisions ensuing from the determination of mode of action is relatively straightforward, the description of the quantitative implementation of various risk-assessment options requires additional development. Specific areas needing clarification include: (1) the decision criteria for judging the adequacy of the weight of evidence for any parcticular mode of action; (2) the role of mode of action in guiding development of toxicokinetic, biologically based or case-specific models; (3) the manner in which mode of action and other technical considerations provide guidance on margin-of-exposure calculations; (4) the relative roles of the risk manager versus the risk assessor in evaluating the margin of exposure; and (5) the influence of mode of action in harmonizing cancer and noncancer risk assessment methodologies. These points are elaborated as recommendations for improvements to any revisions. In general, the incorporation of examples of quantitative assessments for specific chemicals would strengthen the guidelines. Clearly, any revisions should retain the emphasis present in these draft guidelines on flexibility in the use of scientific information with individual compounds, while simultaneously improving the description of the processes by which these mode-of-action data are organized and interpreted.

Anisimov VN. **Mutant and genetically modified mice as models for studying the relationship between aging and carcinogenesis.** Mech Ageing Dev 2001;122(12):1221-55.
Abstract: Increased interest is emerging in using mouse models to assess the genetics of aging and age-related diseases, including cancer. However, only limited information is available regarding the relationship between aging and spontaneous tumor development in genetically modified mice. Analysis of various transgenic and knockout rodent models with either a shortened or an extended life span, provides a unique opportunity to evaluate interactions of genes involved in the aging process and carcinogenesis. There are only a few models which show life span extension. Ames dwarf mutant mice, p66(-/-) knockout mice, alpha MUPA and MGMT transgenic mice live longer than wild-type strains. The incidence of spontaneous tumors in these mutant mice was usually similar to those in controls,

whereas the latent period of tumor development was increased. Practically all models of accelerated aging showed increased incidence and shorter latency of tumors. This phenomenon has been observed in animals which display a phenotype that more closely resembles natural aging, and in animals which manifest only some features of the normal aging process. These observations are in agreement with an earlier established positive correlation between tumor incidence and the rate of tumor incidence increase associated with aging and the aging rate in a population. Thus, genetically modified animals are a valuable tool in unravelling mechanisms underlying aging and cancer. Systemic evaluation of newly generated models should include onco-gerontological studies.

Ariyoshi N, Imaoka S, Nakayama K, Takahashi Y, Fujita K, Funae Y, Kamataki T. **Comparison of the levels of enzymes involved in drug metabolism between transgenic or gene-knockout and the parental mice.** Toxicol Pathol 2001;29 Suppl:161-72.
Abstract: Drug-metabolizing enzymes are involved in the metabolic activation or detoxification of carcinogens. To evaluate animals developed as models for alternative carcinogenicity testing, we investigated whether or not a gene manipulation including the transgene of ras and the knocking out of a tumor suppressor gene such as p53 or XPA could alter the expression of representative drug-metabolizing enzymes directly or indirectly. Expression of several isoforms of cytochrome P450 (CYP) in the liver of rasH2, p53 (+/-), Tg.AC, and XPA (-/-) mice with or without treatment of prototype inducer. phenobarbital or 3-methylcholanthrene, was analyzed by Western immunoblotting in comparison with their parental strains of mice. In addition, the activities of 3 major phase II enzymes, UDP-glucronosyltransferase, sulfotransferase, and glutathione S-transferase, were compared between the gene-manipulated and the corresponding parental strains of mice. Results demonstrate that XPA gene knockout appeared to increase constitutive expression of CYP2B and CYP3A isoforms. Overexpression of human c-Ha-ras gene or p53 gene knockout appeared to increase constitutive UGT activity toward 4-nitrophenol. The content or activities of almost all other enzymes examined in the present study do not appear to be affected by the gene manipulation.

Bai L, Kon K, Tatsumi M, Ito H, Hayashi S, Brautigam M. **A human B-cell CLL model established by transplantation of JOK-1 cells into SCID mice and an anti-leukemia efficacy of fludarabine phosphate.** Oncol Rep 2000;7(1):33-8.
Abstract: The present study was carried out to establish a human chronic lymphocytic leukemia (CLL) mouse model by transplantation of a JOK-1 human CLL cell line into SCID (severe combined immunodeficient) mice and to examine anti-leukemic effects of fludarabine phosphate, a prodrug of 9-beta-D-arabinofuranosyl-2-fluoroadenine (2F-ara-A). In vitro cytotoxic screening pattern of 2F-ara-A differed from those of other anticancer agents. Intraperitoneal inoculation with JOK-1 cells in SCID mice allowed the cells to infiltrate into a variety of organs including the liver and thymus, and resulted in the death of the mice with a median survival time of 29.5 days, associated with hepatomegaly, splenomegaly and enlarged lymph nodes. The ascitic cells expressing the human B-lymphocytic cell surface antigen CD19 actually grew after a latent period of 15 days. In this model, twice daily administration of fludarabine phosphate at a dose of 135 mg/kg for 5 days prolonged the survival time of the mice for considerably longer period than once-a-day treatment. Fludarabine phosphate in the doubled course of twice daily increased life span of 32.9%, which was in a similar range to that of doxorubicin. Thus, intraperitoneal inoculation of the human JOK-1 CLL cells into SCID mice seems to

serve as an animal model potentially for human leukemia, suggesting that transplantation and subsequent infiltration processes of human CLL cells is useful measures to explore mechanistic aspects for drug-induced modulation of the tumor progression.

Bar JK, Harlozinska A. **Morphological and phenotypic characterization of a new established ovarian carcinoma cell line (OvBH-1).** Anticancer Res 2000;20(5A):2975-80.
Abstract: BACKGROUND: A limited number of permanent ovarian carcinoma cell lines have been described so far and the majority of them have been derived from ovarian ascitic fluid cells taken from patients with serous ovarian carcinoma usually after chemotherapy treatment. MATERIALS AND METHODS: The cells for culture were obtained from ascitic fluid cells of a patient with ovarian clear cell carcinoma. Cytomorphological analysis of cultured cells at early and late passages was performed by hematoxylin-eosin staining. Immunophenotypic characterization of cells was performed using the following monoclonal antibodies against: intermediated cellular filaments (CK 6/18, CK 7, CK 1,5,6,8,10,14,18, V9) ovarian carcinoma-associated antigens (OC125, OV-TL3), carcinoembryonic antigen, p53 and c-erbB-2 oncoproteins. RESULTS: In the established ovarian carcinoma cell line (OvBH-1) two morphologically distinct cell types were recognized. Cytomorphologically the dominating type appears to frankly malignant features. The second cell subtype showed a lower degree of malignant features. The epithelial origin of both cell types was confirmed by immunohistochemical staining using antibodies against different cytokeratin epitopes. The expression of tumor-associated antigens (CA125, OV-TL3) was found in both cell subtypes reflecting their origin from ovarian carcinoma. The cell line was negative for CEA staining. The genetic defects of cultured cells were revealed by detection of p53 and c-erbB-2 overexpression. The level of both oncoproteins and especially c-erbB-2 was higher in the cell subtype with frankly malignant morphological features. CONCLUSIONS: A new established, well characterized ovarian clear cell carcinoma line OvBH-1 provides an experimental model for further investigation of the biological alterations responsible for carcinogenesis and chemoresistance of this uncommon subtype of epithelial ovarian carcinomas.

Barreto-Zuniga R, Kato Y, Bobadilla DJ, Okuyama M, Maruyama M, Ohta H, Takekoshi T, Shigematsu A. **[Importance of Helicobacter pylori in the pathogenesis of gastric cancer. Experimental models in rodents].** Rev Gastroenterol Mex 2000;65(4 Suppl 2):25-33 [Spa].
Abstract: We found that the seroprevalence in Cancer Institute of H. pylori infection was significantly more frequent in gastric cancer than in age- and gender-matched controls. This study suggested an epidemiological link between H. pylori infection and gastric cancer. H. pylori exhibits a complex system of enzymes which serve a range of functions. Toxic effects are produced by urease (UR), phospholipase (PL) and alcohol dehydrogenase (ADH). We embarked on an exploration of the enzyme activities of H. pylori infected patients using a TLC-autoradioluminography. This method has a wide dynamic range and could offer an analytical technique for studying a radioactive compound and its enzymes in H. pylori infected mucosa. Biopsies samples taken from 21 gastric cancer patients and 95 controls were studied. Although high activity of UR indicates well the presence of H. pylori impairment, activities of ADH and PL reflects more the chronicity of mucosal damage in both groups. Clearly, the enzyme profile showed in our study reflects the "physiological" adaptations behind chronic injured mucosal changes but its relation to gastric cancer and H. pylori needs further study. There is an urgent need to understand the carcinogenesis process using animal models. We performed previous study for to explore

the effect of H. pylori infection on N- methyl-N-nitrosourea-induced (MNU) gastric carcinogenesis in mice C57BL/6 mice were administered broth culture of H. pylori and given MNU in drinking water. In terms of the incidence of neoplasms development was increase in the MNU group pre-infected with H. pylori. That findings showed that C57BL/6 mice-infected model is well suited for investigating the bacteria promoter effect in the gastric carcinogenesis. Finally another rodent model study (still in process) showed rapid development of hyperplastic gastritis with gastric erosions in H. pylori-infected MTH1 knockout mice. We sought to further evaluate MTH1 knockout mice as potential test animal for carcinogenesis. CONCLUSION: It is suggested that H. pylori infection is an important risk factor for the development of gastric cancer. The possibility that this organism acts etiologically, exerting its effect over long period of time, is biologically plausible. However, the role of H. pylori per se in that process is still a matter of discussion. The various enzymes of H. pylori discussed in this paper support colonization, and are perhaps important for epithelial damage, they could contribute to the stimulation and modulation of the chronic inflammatory response, but its relation to gastric cancer and H. pylori needs further study. Finally H. pylori in C57BL/6 and knockout mice showed excellent colonization at two months and six months after infection there was adenomatous, hyperplastic and ulcerative changes. Those findings showed that both mice-infected models are well suited for investigating the bacteria promoter effect in the gastric carcinogenesis.

Beckwith LG, Moore JL, Tsao-Wu GS, Harshbarger JC, Cheng KC. **Ethylnitrosourea induces neoplasia in zebrafish (Danio rerio).** Lab Invest 2000;80(3):379-85.
Abstract: The zebrafish (Danio rerio) has been successfully used to discover hundreds of genes involved in development and organogenesis. To address the potential of zebrafish as a cancer model, it is important to determine the susceptibility of zebrafish to tumors. Germ line mutations are most commonly induced for zebrafish mutant screens by exposing adult male zebrafish to the alkylating agent, ethylnitrosourea (ENU). To determine whether ENU induces tumors, we compared the incidence of tumors in ENU-treated fish with untreated controls. Interestingly, 18 of 18 (100%) fish mutagenized with either 2.5 or 3.0 mM ENU developed epidermal papillomas, which numbered 1 to 22 per fish, within 1 year of treatment. The induced epidermal lesions included epidermal hyperplasia, flat papillomas (0.2 to 1.2 mm), and pedunculated papillomas (1.2 to 8 mm in greatest dimension), but no skin cancers. Angiogenesis was evident in papillomas larger than approximately 1 mm. All but two papillomas contained the three cell types (keratinocytes, club, and mucous cells) of normal zebrafish epidermis; histologic variants lacked either club cells or mucous cells. Two cavernous hemangiomas and a single malignant peripheral nerve sheath tumor were also found in the treated fish. None of five untreated controls developed tumors. These studies establish the feasibility of the zebrafish as an experimental model for the study of skin tumors.

Brennan LM, Boncavage-Hennessey EM, Wolfe MJ, Toussaint MW, Dennis WE, Rosencrance AB, Gardner HS Jr. **An in vivo method for using 5-bromo-2'-deoxyuridine (BrdU) as a marker of chemically-induced hepatocellular proliferation in the Japanese medaka (Oryzias latipes).** Toxicol Pathol 2001;29(3):387-97.
Abstract: Japanese medaka fish (Oryzias latipes) were used to develop an in vivo method to assess hepatocellular proliferation in a nonmammalian model. Proliferative responses were assessed in medaka at 7, 17, 24, and 94 days after a 48-hour exposure to 10 or 100 mg/L diethylnitrosamine (DEN).

Subgroups of medaka were exposed to 50 or 75 mg/L of 5-bromo-2'-deoxyuridine (BrdU) in water for 72 hours, sacrificed, and then processed for immunohistochemical staining. Proliferative indices of BrdU-labeled hepatocytes were quantified and compared using both count and area measurements. There was a significant increase ($p < 0.05$) in hepatocellular proliferation in the 100 mg/L DEN-treated fish as compared to controls and 10 mg/L DEN-treated fish for the first 3 time points. Hepatocarcinogenicity was evaluated 26 weeks post-DEN exposure. There was a significant increase (p X 0.0001) in hepatocellular neoplasms in 100 mg/L DEN-treated fish compared to other fish. Effective BrdU-labeling of S-phase hepatocytes in medaka was achieved by adding BrdU to the aquarium water, and an increase in hepatocellular proliferation using this method was detected 7 days after exposure to a carcinogenic concentration of DEN. Additionally, the new method of area measurement indices of proliferation were as precise as count indices (R2 > or = 0.92).

Briand P, Lykkesfeldt AE. **An in vitro model of human breast carcinogenesis: epigenetic aspects.** Breast Cancer Res Treat 2001;65(2):179-87.
Abstract: A review is given of 12 years of research on a human breast epithelial cell line, HMT-3522, which has undergone malignant transformation in vitro without being exposed to known carcinogenic agents. Epigenetic aspects of the malignant transformation have been considered and the results have been viewed in a clinical context. It has been concluded that the history and characteristics of the cell line resembles the comedocarcinoma of the human breast. It is hypothesized that progression from benign lesion to comedo in situ carcinoma and invasive carcinoma occurs if low levels of epidermal growth factor are prevailing in the microenvironment. Our data also suggest that breast cancer developed under high epidermal growth factor receptor activity is estrogen receptor negative, while suppression of epidermal growth factor receptor activity promotes estrogen responsive breast cancer.

Brown JJ, Qin M, William-Smith L, Coker JF, Zhou H, Nishitani J, Liu X. **Identification of differentially expressed genes in human papillomavirus type-16 infected oral cancer cells.** Otolaryngol Head Neck Surg 2001;124(6):663-8.
Abstract: OBJECTIVE: The goal of this study was to determine the genes required for head and neck cancer development. Study design and setting: Differential mRNA display analysis was performed using human papillomavirus Type-16 infected immortalized human oral keratinocytes (HOK-16B) and its benzo(a)pyrene-exposed tumorigenic derivative (HOK-16B-BaP-T). RESULTS: Twenty-one differentially expressed cDNA clones were identified between the 2 cell lines. Clone 4 with no known homology showed lower expression in tumorigenic cells compared with either normal or immortalized oral keratinocytes. Clone 6 expression was elevated in several head and neck cancer cells, in addition to Burkitt's lymphoma Raji harboring latent Epstein-Barr virus. CONCLUSION: These findings suggested that clone 6 may be involved in the oncogenic transformation whereas clone 4 may potentially function as a tumor suppressor gene. SIGNIFICANCE: Differential mRNA analysis using the in vitro oral carcinogenesis model may help to identify important genetic markers for the early detection and progression of head and neck cancer.

Budihardjo II, Boerner SA, Eckdahl S, Svingen PA, Rios R, Ames MM, Kaufmann SH. **Effect of 6-aminonicotinamide and other protein synthesis inhibitors on formation of platinum-DNA adducts and cisplatin sensitivity.** Mol Pharmacol 2000;57(3):529-38.

Abstract: The present study was undertaken to examine the mechanistic basis for the recent observation that the pyridine nucleotide derivative 6-aminonicotinamide (6AN, NSC 21206) enhances the accumulation and resulting cytotoxicity of cisplatin in a variety of tumor cell lines. When A549 lung cancer cells or K562 leukemia cells were treated with 62.5 &mgr;M 6AN for 21 h and then pulse-labeled with [(35)S]methionine for 1 h, increased labeling of five polypeptides, one of which corresponded to a M(r) approximately 78,000 glucose-regulated protein (GRP78), was observed. Two subsequent observations, however, suggested that up-regulation of these polypeptides was unlikely to explain the interaction between 6AN and cisplatin: 1) the concentration of 6AN required to induce GRP78 was 4-fold higher than the dose required to sensitize cells to cisplatin; and 2) simultaneous treatment of cells with 6AN and cycloheximide prevented the increase in GRP78 but not the sensitizing effect of 6AN. On the contrary, treatment with the protein synthesis inhibitors cycloheximide, anisomycin, or puromycin as well as prolonged exposure to the RNA synthesis inhibitor actinomycin D mimicked the biochemical modulating effects of 6AN on cisplatin action. Conversely, 6AN inhibited protein synthesis, whereas 18 6AN analogs that failed to enhance Pt-DNA adducts and cisplatin cytotoxicity failed to inhibit protein synthesis. These observations are consistent with a model in which 6AN and other inhibitors of protein synthesis act as modulating agents by increasing cisplatin accumulation, thereby enhancing the formation of Pt-DNA adducts and subsequent cisplatin-induced cell death.

Bunton TE. **Brown bullhead (Ameiurus nebulosus) skin carcinogenesis.** Exp Toxicol Pathol 2000;52 (3):209-20.

Abstract: Alternative models using fish species have been tested in liver toxicity and carcinogenesis bioassays. Similar models have not been developed for skin. The brown bullhead (Ameiurus nebulosus) has shown potential as a model for skin carcinogenesis studies due to its sensitivity to environmental chemical pollutants. The present study is an initial morphologic and biochemical characterization of the normal and neoplastic brown bullhead skin to assess its suitability as a model of skin carcinogenesis. Brown bullhead were removed from Back River in the Chesapeake Bay region, an area historically polluted with heavy metals and polycyclic aromatic hydrocarbons. Histology, histochemistry, and electron microscopy were used to stage the morphologic development and progression of neoplasia in skin. The distribution of keratin, a family of structural proteins with altered expression in mammalian tumorigenesis, was analyzed with one and two dimensional gel electrophoresis and nitrocellulose blots of extracts from normal skin. Keratin expression in skin and other organs was also assessed with immunohistochemistry using AE1, AE3, and PCK 26 antibodies, and the proliferation index in skin and neoplasms with PCNA antibody. Skin lesions appeared to progress from hyperplasia through carcinoma, and the proliferation index was increased in papilloma. Also in papilloma, intercellular interdigitations appeared increased and desmosomes decreased which may in future studies correlate with changes in expression of other molecular markers of neoplastic progression. Both Type I and Type II keratin subfamilies were detected in skin using gel electrophoresis with the complimentary keratin blot-binding assay. For further development of the brown bullhead model, future studies can compare and relate these baseline data to alterations in expression of keratin and other markers in fish neoplasms and to molecular events which occur in man.

Calaf G, Russo J, Alvarado ME. **Morphological phenotypes in neoplastic progression of benz(alpha)**

pyrene-treated breast epithelial cells. J Submicrosc Cytol Pathol 2000;32(4):535-45.
Abstract: The neoplastic conversion of a normal cell to a malignant one is a multistage process that occurs after a series of molecular alterations. Several chemical and physical agents can alter the morphology of different types of cells. Scanning and transmission electron microscopy have been valuable in evaluating changes that occur in the progression of transformation. MCF-10F, a spontaneously immortalized human breast epithelial cell line (Soule et al., 1990), was treated with benz (alpha)pyrene (BP) (Calaf and Russo, 1993) and then transfected with the c-Ha-ras oncogene (Calaf et al., 1995). The phenotypic changes of breast cancer progression were studied through the use of scanning and transmission electron microscopy. Activated oncogenes have been detected in a variety of malignant tumors and the altered expression of certain genes seems to play a role in the cancer process. Carcinogen-treated and transfected cells showed a progression of changes in the morphology, anchorage independent growth, invasiveness and capability of tumor formation in the SCID mice. This in vitro cancer model can parallel the progression of breast cancer seen through molecular changes that occur and have been observed during the natural development of this disease.

Calaf GM, Hei TK. **Establishment of a radiation- and estrogen-induced breast cancer model.** Carcinogenesis 2000;21(4):769-76.
Abstract: It is well accepted that cancer arises in a multistep fashion in which exposure to environmental carcinogens is a major etiological factor. The aim of this work was to establish an experimental breast cancer model in order to understand the mechanism of neoplastic transformation induced by high LET radiation in the presence of 17beta-estradiol (E). Immortalized human breast cells (MCF-10F) were exposed to low doses of high LET alpha pArticles (150 keV/microm) and subsequently cultured in the presence or absence of E for periods of up to 10 months post-irradiation. MCF-10F cells irradiated with either a single 60 cGy dose or 60/60 cGy doses of alpha pArticles showed gradual phenotypic changes including altered morphology, increase in cell proliferation relative to the control, anchorage-independent growth and invasive capability before becoming tumorigenic in nude mice. In alpha pArticle-irradiated cells and in those cells subsequently cultured in the presence of E, increased BRCA1, BRCA2 and RAD51 expression were detected by immunofluorescence staining and quantified by confocal microscopy. These studies showed that high LET radiation such as that emitted by radon progeny, in the presence of estrogen, induced a cascade of events indicative of cell transformation and tumorigenicity in human breast epithelial cells.

Carmichael NG, Debruyne EL, Bigot-Lasserre D. **The p53 heterozygous knockout mouse as a model for chemical carcinogenesis in vascular tissue.** Environ Health Perspect 2000;108(1):61-5.
Abstract: Heterozygous p53 knockout mice were investigated as a potential model for vascular tumor carcinogenesis. Groups of 20 male mice were exposed by gavage for 6 months to the vascular carcinogen urethane at 1, 10, or 100 mg/kg body weight/day. Wild-type and heterozygous p53 knockout control groups were exposed by gavage to the vehicle alone. Another group of 20 male mice received d-limonene by gavage (d-limonene is noncarcinogenic in mice). The high dose of urethane caused early mortality in the majority of mice associated with histopathologic evidence of toxicity and tumors, including a high incidence of benign and malignant vascular tumors, in all animals. At the intermediate dose, toxicity was less marked and 3 of 20 mice had tumors; mice that received the low dose did not have signs of toxicity or neoplasia. The two control groups had no tumors and the d-limonene group had

one tumor of the prostate, which was considered spontaneous. We conclude that the p53 knockout mouse is a useful tool for investigating vascular tumorogenesis.

Chen HC, Bhattacharyya N, Wang L, Recupero AJ, Klein EA, Harter ML, Banerjee S. **Defective DNA repair genes in a primary culture of human renal cell carcinoma.** J Cancer Res Clin Oncol 2000;126 (4):185-90.
Abstract: PURPOSE: Genomic stability is maintained by error-free DNA replication, repair, and recombination. To determine if repair genes contribute to genomic instability, we used a newly established cell line RCC-AJR (from clear-cell renal cell carcinoma) to examine hMSH2 (a mismatch-repair gene) and the gene encoding DNA beta polymerase (polbeta; a known contributor to base-excision repair). METHODS: Coding sequences of hMSH2 and polbeta were amplified by the polymerase chain reaction (PCR) using RNA from RCC-AJR cells and matched normal kidney (NK) cells from the same patient. Nucleotide sequences of the PCR products were determined by the dideoxy-DNA method and direct sequencing. Expressions of repair genes were assayed by Western blotting. Microsatellite stability in RCC-AJR cells was assayed by alteration in (CA)n repeats. RESULTS: In the RCC-AJR cells, we detected (a) a deletion of 1476 bp encoding 492 amino acids of hMSH2 cDNA, (b) an 87-bp deletion in the polbeta coding sequence, (c) truncated forms of hMSH2 and polbeta proteins, and (d) microsatellite instability. CONCLUSIONS: This study provides evidence of alterations in hMSH2 and polbeta in the homogeneous cell population of an RCC-AJR tumor culture. The data indicate that repair genes may help preserve genomic stability in this cell line. We believe that this new primary RCC-AJR cell line will prove a useful model for investigating the cascade of genetic events in renal cells that leads to renal carcinogenesis.

Clarke AR. **Manipulating the germline: its impact on the study of carcinogenesis.** Carcinogenesis 2000;21(3):435-41.
Abstract: Over the past two decades, the mouse has established itself as the primary organism in which to investigate the fundamental mechanisms of carcinogenesis and to model human neoplasia. The principal reason underlying such dominance almost certainly arises out of our ever increasing ability to manipulate the murine germline. Over the past 20 years we have moved from a position where animal models arose either spontaneously or were generated through exposure to carcinogen to a position in which it is possible to create and study precise mutations of choice. The most recent advances in inducible and conditional technologies now open the possibility for both temporal and tissue-specific gene manipulation. Each of these technological breakthroughs has facilitated significant steps forward in our understanding of the genetic basis of tumorigenesis. This review will highlight some of the major advances in the production and use of murine models of neoplasia over the last two decades.

Cohen SM. **Alternative models for carcinogenicity testing: weight of evidence evaluations across models.** Toxicol Pathol 2001;29 Suppl:183-90.
Abstract: Twenty-one chemicals were evaluated by standardized protocols in 6 mouse models that have been sugggested as alternatives to the 2-year mouse bioassay. Included were genotoxic and nongenotoxic chemicals, carcinogens and noncarcinogens, immunosuppressive and estrogenic agents, peroxisome proliferators, and chemicals producing cancer in rodents by other mechanisms. Mice were sacrificed at the end of 6 to 12 months, depending on the model. Standardized histopathology,

biostatistical analyses, and criteria for overall evaluation of the results were employed. The TgAC transgenic (dermal and oral administration), the Tg-rasH2 transgenic, the heterozygous p53 gene knockout, the homozygous XPA and homozygous XPA-heterozygous p53 gene knockout, and the neonatal mouse models were evaluated. The chemicals were also evaluated in the in vitro SHE assay. Comparison of the results between the various in vivo models suggest that they might have usefulness as screening bioassays for hazard identification for potential human carcinogens. They have the benefits of being quicker, less expensive, and involve fewer animals than the traditional 2-year mouse bioassay. They do not appear to be overly sensitive. However, they do not definitively distinguish between genotoxic and nongenotoxic carcinogens, and they do not have 100% specificity for identifying human carcinogens. Like the 2-year bioassay, the results from these models need to be evaluated in conjunction with other information on a chemical in an overall weight-of-evidence, integrated analytical approach to assess risk for human exposures.

Cohen SM, Robinson D, MacDonald J. **Alternative models for carcinogenicity testing.** Toxicol Sci 2001;64(1):14-9.
Abstract: The International Conference on Harmonisation Expert Working Group on Safety suggested that under certain circumstances, data from alternative assays could be used in safety evaluation in place of a second bioassay. Several alternatives were discussed. Six of these models were evaluated in a collaborative effort under the auspices of the Health and Environmental Sciences Institute (HESI) branch of the International Life Sciences Institute (ILSI). Standard protocols, pathology review, and statistical evaluations were developed. Twenty-one chemicals were evaluated, including genotoxic, nongenotoxic, carcinogenic, and noncarcinogenic chemicals. The models that were evaluated included the p53(+/-) heterozygous knockout mouse, the rasH2 transgenic mouse, the TgAC transgenic mouse (dermal and oral administration), the homozygous XPA knockout and the XPA/p53 knockout mouse models. Also evaluated were the neonatal mouse models and the Syrian Hamster Embryo (SHE) transformation assay. The results of this comprehensive study suggest that some of these models might be useful in hazard identification if used in conjunction with information from other sources in a weight of evidence, integrated analysis approach to risk assessment.

Corcoran C, Mehta C, Senchaudhuri P. **Power comparisons for tests of trend in dose-response studies.** Stat Med 2000;19(22):3037-50.
Abstract: The Cochran-Armitage test for trend is a popular statistical procedure for detecting increasing or decreasing probabilities of response when a categorical exposure is ordered. Such associations may arise in a variety of biomedical research settings, parcticularly in dose-response designs such as carcinogenicity experiments. Previously, computing limitations mandated the use of the asymptotic trend test, but with the availability of new algorithms, increased computing power, and appropriate software the exact trend test is now a practical option. Nevertheless, the exact test is sometimes criticized on the grounds that it is conservative. In this paper we investigate the implications of this conservatism by comparing the true type I error and power of three alternative tests of trend - the asymptotic test, the exact test and an admissible exact test proposed by Cohen and Sackrowitz. The computations are performed by an extension to the network algorithm of Mehta et al. This allows us to make precise power comparisons between the tests under any given design without resorting to simulation. We show how this tool can guide investigators in choosing the most appropriate test by

considering the design of two-year carcinogenicity studies carried out by the National Toxicology Program. We additionally compare the tests for various other combinations of sample sizes and number of groups or levels of exposure. We conclude that the asymptotic test, while more powerful where it is valid, generally does not preserve the type I error. This violation of the a priori testing level can be greatly affected by imbalance in the data or unequal spacing of dose levels. Copyright 2000 John Wiley & Sons, Ltd.

Croce MV, Colussi AG, Zambelli A, Price MR, Segal-Eiras A. **Establishment and characterization of a cell line (T201) derived from a human larynx squamous cell carcinoma.** Int J Oncol 2001;18 (4):729-35.
Abstract: The purpose of this report was the initiation and further maintenance of tumor cells from a primary larynx squamous cell carcinoma. A tumor fragment was mechanically dissociated, the cells were grown in RPMI medium, being the primary culture dependent on the presence of epidermal growth factor and insulin; during subsequent passages the adaptation to conventional growth conditions was obtained. Cells grew in monolayer with an epitheliod shape, showing a pavement-like arrangement; at confluence, cells piled up without contact inhibition maintaining the same morphology. Population doubling time was about 48 h with a colony-forming efficiency of 10%. Immunocytochemical characterization was performed with a panel of monoclonal antibodies reactive against tumor associated antigens, including mucin glycoproteins and related carbohydrate antigens, carcinoembryonic antigen (CEA), p53 as well as cytokeratins, vimentin and desmin. T201 expressed CEA, sialyl Lewis x, Lewis x, Lewis y, MUC1 mucin, Tn hapten, p53, vimentin and cytokeratins. On the other hand, a modal chromosome diploid number of 46 occurring in 74% of cells was detected. Present data confirmed that the methodology employed was adequate for the establishment and characterization of a new cell line which can provide a useful model to study biological and immunological aspects of larynx squamous cell carcinoma.

Custer L, Gibson DP, Aardema MJ, LeBoeuf RA. **A refined protocol for conducting the low pH 6.7 Syrian hamster embryo (SHE) cell transformation assay.** Mutat Res 2000;455(1-2):129-39.
Abstract: The Syrian hamster embryo (SHE) cell transformation assay evaluates the potential of chemicals to induce morphological transformation in karyotypically normal primary cells. Induction of transformation has been shown to correlate well with the carcinogenicity of many compounds in the rodent bioassay. Historically the assay has not received wide-spread use due to technical difficulty. An improved protocol for a low pH 6.7 assay was developed by LeBoeuf et al. [R.A. LeBoeuf, G.A. Kerckaert, M.J. Aardema, D.P. Gibson, R. Brauninger, R.J. Isfort, Mutat. Res., 356 (1996) 85-127], that greatly reduced many of the technical difficulties associated with the SHE assay. The purpose of this paper is to describe the most current execution of the pH 6.70 protocol including protocol refinements made since the publication of a comprehensive protocol for this assay in Kerckaert et al. [G.A. Kerckaert, R.J. Isfort, G.J. Carr, M.J. Aardema, Mutat. Res., 356 (1996) 65-84].

De Larco JE, Wuertz BR, Manivel JC, Furcht LT. **Progression and enhancement of metastatic potential after exposure of tumor cells to chemotherapeutic agents.** Cancer Res 2001;61(7):2857-61.
Abstract: Data presented in this report indicate short-term in vitro treatment of nonmetastatic MCF-7 breast carcinoma cells with the chemotherapeutic agents-, Adriamycin and/or 5-fluoro-2'-deoxyuridine

(FUdR), induced changes in the expressed phenotype. Cells treated sequentially with Adriamycin and FUdR expressed a metastatic phenotype. The results also show short-term exposure of MCF-7 cells to either Adriamycin or FUdR rapidly increases, in a dose-dependent manner, the release of the angiogenic cytokine, interleukin-8(IL-8), which is released at consistently higher levels in metastatic cell lines. Cell populations surviving a single treatment with either one or both of these chemotherapeutic agents continue to stably release IL-8. Survivors of sequential treatment with Adriamycin and FUdR (MCF-7 A/F) release the most IL-8 and express the greatest phenotypic variance from the parental, MCF-7 cells. Parental MCF-7 cells and MCF-7 A/F cells both form primary tumors when used in an orthotopic tumor model; however, the MCF-7 A/F tumors have a more rapid initial growth phase in situ and give rise to spontaneous lung metastases within 10 weeks. A cell line that is established from lung metastases releases more IL-8, has a higher cloning efficiency, and forms looser colonies in monolayer than do their parental cells. These experiments indicate the in vitro exposure of tumor cells to chemotherapeutic agents either selects more aggressive cells or enhances the metastatic potential of the surviving cells.

Elmore E, Luc TT, Li HR, Buckmeier JA, Steele VE, Kelloff GJ, Redpath JL. **Correlation of chemopreventive efficacy data from the human epidermal cell assay with in vivo data.** Anticancer Res 2000;20(1A):27-32.
Abstract: Continuous exposure to low doses of potentially mutagenic and carcinogenic chemicals over the human lifetime makes the identification of agents, which could reduce the ensuing risk of cancer, beneficial. The Human Epidermal Cell (HEC) Assay includes multiple exposures to low, non-toxic doses of propane sultone, which increases cellular growth and inhibits differentiation, and co-exposure to potential chemopreventive agents to determine their ability to inhibit the increased growth or increase differentiation. Original data are presented on the efficacy of twenty potential cancer chemopreventive agents were screened for efficacy in the HEC Assay. Efficacy was determined by the ability of agents, at nontoxic concentrations, to reverse either of the propane sultone-induced biomarkers, enhanced growth and reduced involucrin expression. Based on the number of positive concentrations and the lack of toxicity, 1,2-dithiol-3-thione, oltipraz, and a synthetic retinoid, Ro 16-9100, were the most active. Eleven of seventeen positive agents were active for both endpoints. S-Allylcysteine was only active for the growth inhibition endpoint, and DFMO, Iycopene, perillyl alcohol, ursodiol, and black tea polyphenols were only active for the involucrin endpoint. The three agents that have been shown to be negative in animal models, diphenhydramine, d-mannitol, and nordihydroguaiaretic acid, were correctly identified as negative by the assay. When the data from previous studies (Elmore et al, Anticancer Res, 19: 909-918, 1999) are included, a positive response in one or more endpoints of the HEC Assay correlates 100% (26/26) with a positive response in one or more of the animal cancer prevention models (8). The available data suggest that the HEC Assay response is highly predictive of efficacy in animals in vivo with an overall accuracy of 90%. Future studies will include data with additional negative agents. The correlation of the HEC Assay data with data from in vivo studies in animal models, which utilize multiple carcinogens and multiple target organs, would suggest that this in vitro assay has the ability to identify agents with the potential to prevent carcinogen-induced cancer. While our ultimate goal is to identify agents with potential efficacy for preventing human cancer, sufficient human data are not yet available to make this correlation.

Fang J, Shing Y, Wiederschain D, Yan L, Butterfield C, Jackson G, Harper J, Tamvakopoulos G, Moses

MA. **Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model.** Proc Natl Acad Sci U S A 2000;97(8):3884-9.
Abstract: Among the earliest and most important stages during tumorigenesis is the activation of the angiogenic process, an event that is termed the "switch to the angiogenic phenotype." We have developed an in vivo system that can reliably recapitulate the stages in tumor development that represent this transition. Using this model, we have harvested and studied tumor nodules that can be distinguished from each other on the basis of their degree of vascularization. Angiogenic tumor nodules were characterized by the presence of capillary vessels as determined by factor VIII immunohistochemistry, and both angiogenic and proteolytic activities in vitro. In contrast, preangiogenic nodules were devoid of microvessels and showed little angiogenic or proteolytic activity in vitro. Addition of a specific metalloproteinase inhibitor resulted in the abrogation of both angiogenic and proteolytic activities of the angiogenic nodules in vitro. Comparative substrate gel electrophoresis detected the presence of a prominent matrix metalloproteinase (MMP-2) in the angiogenic nodules when compared with the preangiogenic ones. Suppression of MMP-2 activity by antisense oligonucleotides in the vascular nodules resulted in the loss of angiogenic potential both in vitro and in vivo in the chick chorioallantoic membrane assay. Moreover, this suppression of MMP-2 activity in angiogenic nodules inhibited tumor growth in vivo by approximately 70%. These results strongly implicate the activity of MMP-2 as a requirement for the switch to the angiogenic phenotype and validate this model as a reliable and reproducible tool by which to study other cellular and biochemical factors involved in the acquisition of the angiogenic phenotype.

Fang MZ, Kim DY, Lee HW, Cho MH. **Improvement of in vitro two-stage transformation assay and determination of the promotional effect of cadmium.** Toxicol In Vitro 2001;15(3):225-31.
Abstract: The classical in vitro two-stage transformation method was modified for high transformation frequency, and the promotional effect of cadmium was evaluated. In this study, we reconfirmed the usefulness of the replating method and the optimal duration time between the initiator and promoter treatments for the optimal transformation of the Balb/3T3 cells. The results also showed that subsequent exposure to CdCl(2) for 2 weeks after initial exposure to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) markedly enhanced the transformation frequency. At the concentration of 360 ng/ml, the transformation frequency was increased by 35-fold that of the cultures treated only with MNNG, and was higher than that of the positive control group treated with 100 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA) after MNNG treatment. This transformation frequency was higher than that reported previously. Therefore, this in vitro two-stage transformation method can be used efficiently for the screening of genotoxic and non-genotoxic carcinogens and the study of multistage carcinogenesis. These results also indicate that cadmium has a strong potency as a promoter, and the promotional effect of cadmium is higher than that of TPA.

Farber E. **Risk assessment for possible carcinogens: a critical look.** Drug Metab Rev 2000;32(2):143-51.
Abstract: Our overall understanding of mechanisms of toxicology in relation to human disease, with prevention of disease as a major objective, depends in part on the development of an adequate number of ways to assess risks, both short term and long term. Despite the cost, the long duration of the test, and some pitfalls, the long-term animal tests remain, to date, the only reliable assay for possible carcinogens.

Recent work has concentrated increasingly on the development of short-term tests to replace the long-term tests. Such a development would be most welcome from several points of view. To date, a variety of approaches have been or are being used. These include (1) activation to an alkylating agent with DNA as the most important target, generating possible mutations in DNA and DNA damage with or without repair, (2) induction of cell proliferation, at least a few cycles, with DNA synthesis as the major target, again favoring mutations, and (3) decrease in cell-to-cell communication (gap-junctional intercellular communication) as a supposed test for promotion. None of these proposed assays are reliable indices for possible carcinogenic effects of chemicals or other agents; the scientific basis for this negative conclusion is discussed.

French J, Storer RD, Donehower LA. **The nature of the heterozygous Trp53 knockout model for identification of mutagenic carcinogens.** Toxicol Pathol 2001;29 Suppl:24-9.
Abstract: The heterozygous Trp53 null allele C57BL/6 (N5) mouse is susceptible to the rapid development of neoplasia by mutagenic carcinogens relative to control strains. This mouse model of chemical carcinogenesis demonstrates 1) dose-related rapid induction of tumors (26 wks), 2) multiple sites of carcinogen-specific tissue susceptibility, and 3) carcinogen-induced loss of heterozygosity involving the Trp53 wild-type allele or a p53 haploinsufficiency permitting mutation of other critical protooncogenes and/or inactivation of tumor suppressor genes driving tumorigenesis. Demonstration of mutation or loss of heterozygosity involving the Trp53 locus is consistent with a common finding in human cancers and supports extrapolation between rodents and humans. Using diverse experimental protocols, almost all mutagenic rodent carcinogens (including all mutagens that are carcinogenic to humans), but not nonmutagenic rodent carcinogens, induce tumors within 26 weeks of continuous exposure. These characteristics and results indicate that the mouse heterozygous for the Trp53 null allele may be of significant use for the prospective identification of mutagenic carcinogens of potential risk to human health.

Frowein J. **Hypothesis: chemical carcinogenesis mediated by a transiently active carcinogen receptor.** Cytogenet Cell Genet 2000;91(1-4):102-4.
Abstract: Biologically active small-molecular-weight compounds are actively transported into the cell nucleus by a specific receptor. This has been widely demonstrated for retinoids, polycyclic hydrocarbons (such as steroids), and dioxin. Thus, it is reasonable to assume that genotoxically active polycyclic hydrocarbons, and possibly all genotoxically active small-molecular-weight substances, exert their transformational effect in the cell nucleus via a specific receptor. I propose that the receptor is activated only at the end of the G(1) phase of the cell cycle and that the carcinogen receptor complex interferes directly with DNA synthesis, leading to mutations. This hypothesis may account for various characteristics of malignant growth, such as the organ specificity of carcinogens and the relationship between cell proliferation and malignant transformation. If so, it could form the basis for establishing a radioreceptor assay for carcinogens. Copyright 2001 S. Karger AG, Basel.

Fu PP, Von Tungeln LS, Hammons GJ, McMahon G, Wogan G, Flammang TJ, Kadlubar FF. **Metabolic activation capacity of neonatal mice in relation to the neonatal mouse tumorigenicity bioassay.** Drug Metab Rev 2000;32(2):241-66.
Abstract: The neonatal mouse tumorigenicity bioassay is a well-developed animal model that has

recently been recommended as an alternative tumorigenicity bioassay by the International Conference on Harmonization (ICH) for Technical Requirements for the Registration of Pharmaceuticals for Human Use. There are sufficient data to conclude that this animal model is highly sensitive to genotoxic chemical carcinogens that exert their tumorigenicity through mechanisms involving the formation of covalently bound exogenous DNA adducts that lead to mutation. On the other hand, it is not sensitive to chemical carcinogens that exert tumorigenicity through a secondary mechanism. The metabolizing enzymes present in the neonatal mouse, parcticularly the cytochromes P450, are critical factors in determining the tumorigenic potency of a chemical tested in this bioassay. However, compared to the metabolizing enzymes of the adult mouse and rat, the study of the metabolizing enzymes in neonatal mouse tissues has been relatively limited.

Ghanayem BI, Wang H, Sumner S. **Using cytochrome P-450 gene knock-out mice to study chemical metabolism, toxicity, and carcinogenicity.** Toxicol Pathol 2000;28(6):839-50.
Abstract: Cytochrome P-450 (CYP) enzymes are heme-containing proteins that carry out oxidative metabolism of a wide range of structurally diverse exogenous chemicals and therapeutic agents as well as endogenous compounds. For some of these xenobiotics, oxidative metabolism results in the formation of toxic, mutagenic, or carcinogenic metabolites. In the past, the role of CYP enzymes in metabolism and chemical-induced toxicity was studied indirectly through use of specific antibodies or inducers and inhibitors of these enzymes. Progress in molecular biology and the ability to bioengineer animal models that do not express CYP1A2, CYP1A1, CYP1B1, CYP2E1, or both CYP1A2 and CYP2E1 isozymes has allowed for direct investigations of the in vivo role of these enzymes in the metabolism, toxicity, and carcinogenicity of xenobiotics. This Article reviews research conducted to date that utilizes these genetically bioengineered mice in metabolism, toxicity, or carcinogenicity studies of chemicals. Some studies showed a positive correlation between in vivo results and in vitro predictions for the role of a specific CYP in chemical-induced effects, whereas other studies did not support in vitro predictions. Work reviewed herein demonstrates the importance of using animal models for investigating the role of specific CYP enzymes in metabolism and chemical-induced toxicity or carcinogenicity rather than relying solely on in vitro techniques. Eventually, studies of this nature will facilitate a more accurate assessment of human risks with regard to chemicals by helping us to understand the relationships between chemical metabolism, carcinogenicity, and polymorphisms in CYP enzymes.

Gonzalez FJ, Kimura S. **Understanding the role of xenobiotic-metabolism in chemical carcinogenesis using gene knockout mice.** Mutat Res 2001;477(1-2):79-87.
Abstract: Most chemical carcinogens require metabolic activation to electrophilic metabolites that are capable of binding to DNA and causing gene mutations. Carcinogen metabolism is carried out by large groups of xenobiotic-metabolizing enzymes that include the phase I cytochromes P450 (P450) and microsomal epoxide hydrolase, and various phase II transferase enzymes. It is extremely important to determine the role P450s play in the carcinogenesis and to establish if they are the rate limiting and critical interface between the chemical and its biological activities. The latter is essential in order to validate the use of rodent models to test safety of chemicals in humans. Since there are marked species differences in expressions and catalytic activities of the multiple P450 forms that activate carcinogens, this validation process becomes especially difficult. To address the role of P450s in whole animal carcinogenesis, mice were produced that lack the P450s known to catalyze carcinogen activation. Mouse

lines having disrupted genes encoding the P450s CYP1A2, CYP2E1, and CYP1B1 were developed. Mice lacking expression of microsomal epoxide hydrolase (mEH) and NADPH-quinone oxidoreductase (NQO1) were also made. All of these mice exhibit no gross abnormal phenotypes, suggesting that the xenobiotic-metabolizing enzymes have no critical roles in mammalian development and physiological homeostasis. This explains the occurrence of polymorphisms in xenobiotic-metabolizing enzymes among humans and other mammalian species. However, these null mice do show differences in sensitivities to acute chemical toxicities, thus establishing the importance of xenobiotic metabolism in activation pathways that lead to cell death. Rodent bioassays using null mice and known genotoxic carcinogens should establish whether these enzymes are required for carcinogenesis in an intact animal model. These studies will also provide a framework for the production of transgenic mice and carcinogen bioassay protocols that may be more predictive for identifying the human carcinogens and validate the molecular epidemiological studies ongoing in humans that seek to establish a role for polymorphisms in cancer risk.

Gupta A, Butts B, Kwei KA, Dvorakova K, Stratton SP, Briehl MM, Bowden GT. **Attenuation of catalase activity in the malignant phenotype plays a functional role in an in vitro model for tumor progression.** Cancer Lett 2001;173(2):115-25.
Abstract: We have developed an in vitro model to study the molecular mechanisms of tumor progression. Using repeated treatments with ionizing radiation or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), we caused malignant progression of a papilloma producing mouse keratinocyte cell line, 308 cells. In a previous study we have shown that the malignant variants of 308 cells have elevated reactive oxygen species (ROS) levels, and have established a functional role for the pro-oxidant state in the progressed phenotype (Carcinogenesis 20 (1999) 2063). In this study, we have evaluated the status of intracellular defense mechanisms for ROS scavenging in the progressed phenotype to identify sources that contribute to their pro-oxidant state. Our results demonstrate that a reduction in several anti-oxidant defense mechanisms, including catalase and glutathione S-transferase mu, correlates with the emergence of the malignant phenotype. We provide evidence that attenuation of catalase activity may play a functional role in the malignant progression of mouse keratinocytes.

Gupta S, Stanbridge EJ. **Paired human fibrosarcoma cell lines that possess or lack endogenous mutant N-ras alleles as experimental model for Ras signaling pathways.** Methods Enzymol 2001;333:290-306.
Abstract: We present here a human cell model for examination of mutant N-ras function. The HT1080 human fibrosarcoma cell line is pseudodiploid and contains a single endogenous mutant N-ras allele. MCH603c8 cells are a variant of HT1080 cells, in which the mutant allele has been deleted. The two cell lines differ dramatically in the constitutive levels of activation of downstream members of the Ras signaling pathways, and in biological features of transformation and tumorigenicity. Downregulation or activation of individual Ras-dependent pathways can be accomplished via transfection of dominant negatives or activated mutant cDNAs into HT1080 and MCH603c8 cells, respectively. The biochemical and biological consequences of expression of these mutant cDNAs can be assessed. There are dramatic effects on both the transformed and tumorigenic phenotype, depending on the cell line and mutant cDNA that is transfected.

Hanaoka T, Yamano Y, Hashimoto H, Kagawa J, Tsugane S. **A preliminary evaluation of intra- and interindividual variations of hOGG1 messenger RNA levels in peripheral blood cells as determined by a real-time polymerase chain reaction technique.** Cancer Epidemiol Biomarkers Prev 2000;9(11):1255-8.
Abstract: The hOGG1 (8-oxoguanine-DNA glycosylase) gene may contribute to further understanding of the relation between oxidative DNA damage and carcinogenesis. A real-time PCR technique was used to determine mRNA levels in peripheral blood cells to assess the possibility of using hOGG1 mRNA as a biomarker in epidemiological studies. To evaluate the intra- and interindividual variation of hOGG1 mRNA levels in peripheral blood cells, we measured them in five healthy nonsmokers three times over a 1-year period. The beta-actin mRNA level in each subject was set equal to 100, and the levels of hOGG1 mRNA were found to range from 1.6-17.6. The intraindividual variation range was 1.8-6.4. Although the difference in the mRNA levels between the sampling dates was not significant ($P = 0.73$), a significant difference in mRNA levels was found between the subjects ($P < 0.01$). The subjects seemed to fall into groups according to their individual levels. This preliminary study may provide initial information on the hOGG1 mRNA level of peripheral blood cells as a biomarker in epidemiological studies on oxygen radicals, oxygen radical-related agents, and cancer.

Hedberg JJ, Grafstrom RC, Vondracek M, Sarang Z, Warngard L, Hoog JO. **Micro-array chip analysis of carbonyl-metabolising enzymes in normal, immortalised and malignant human oral keratinocytes.** Cell Mol Life Sci 2001;58(11):1719-26.
Abstract: Enzymes involved in various protective and metabolic processes of carbonyl compounds were analysed utilising a micro-array method in a three-stage in vitro model for oral carcinogenesis involving cultured normal, immortalised and malignant human oral keratinocytes. A complete transcript profiling of identified carbonyl-metabolising enzymes belonging to the ADH, ALDH, SDR and AKR families is presented. Expression of 17 transcripts was detected in normal, 14 in immortalized and 19 in malignant keratinocytes of a total of 12,500 genes spotted on the micro-array chip. For the detected transcripts, about half were changed by cell transformation, and for the various enzyme families, differences in expression patterns were observed. The detected AKR transcripts displayed a conserved pattern of expression, indicating a requirement for the keratinocyte phenotype, while most of the detected SDRs displayed changed expression at the various stages of malignancy. The importance of multiple experiments in using a microarray technique for reliable results is underlined and, finally, the strength of the method in detecting co-expressed enzymes in metabolic pathways is exemplified by the detection of the formaldehyde-scavenging pathway enzymes and the polyol pathway enzymes.

Hoelting T, Goretzki PE, Duh QY. **Follicular thyroid cancer cells: a model of metastatic tumor in vitro (review).** Oncol Rep 2001;8(1):3-8.
Abstract: We used a thyroid metastatic tumor model to analyze some of the mechanisms of invasion and metastasis in culture. Chronic TSH stimulation (thyroid stimulating hormone) was associated with enhanced tumor proliferation and aggressiveness. We present a unique metastatic tumor model including three follicular thyroid cancer cell lines using a human primary tumor and two metastases of the same patient. They contain thyroglobulin, have intact thyroid functions and response to TSH. Investigating growth factor sensitivity we found that the amplitude of stimulation or inhibition of invasion was significantly smaller in both metastatic cell lines. Unstimulated cells of the lung metastasis had the

highest basal invasive potential, but were only minimally affected by the stimulation of growth factors. In contrast, the parental cell line had the lowest basal invasiveness, but was considerably stimulated by growth factors.

Hu Y, Lam KY, Wan TS, Fang W, Ma ES, Chan LC, Srivastava G. **Establishment and characterization of HKESC-1, a new cancer cell line from human esophageal squamous cell carcinoma.** Cancer Genet Cytogenet 2000;118(2):112-20.
Abstract: The establishment of an esophageal cancer cell line can facilitate the search for molecular mechanisms involved in esophageal carcinogenesis. A new human cancer cell line, HKESC-1, was established from a primary moderately-differentiated squamous cell carcinoma of the esophagus from a 47-year-old Hong Kong Chinese man. The pathological characteristics (morphology, immunohistochemical, and electron microscopic studies), the tumorigenecity in nude mice, the cytogenetic features, the DNA ploidy, and telomerase activity of the cell line were investigated. The HKESC-1 cells have been maintained continuously in vitro for more than 16 months and passaged over 96 times. HKESC-1 cells grow as a monolayer, with a doubling time of 46 hours. The HKESC-1 cells are of a squamous epithelial origin, as shown by their immunopositivity with the anti-cytokeratin antibodies and ultrastructural demonstration of tonofilaments and desmosomes. The HKESC-1 cells possess characteristics of malignancy because they are highly tumorigenic in nude mice and have strong telomerase activity. The HKESC-1 cells had an aneuploid DNA content, as demonstrated by flow cytometric analysis. Cytogenetic analysis revealed hyperdiploidy of greater than 50 in 80% of analyzable metaphases. Chromosome gains and losses were common, and loss of the Y chromosome was a consistent numerical aberration. Additionally, many structural chromosomal abnormalities were encountered, with frequent breakpoints at 1p32, 7p22, 7q34, and 20q13. This newly established cell line serves as a useful model for studying the molecular pathogenesis, and testing new therapeutic reagents for esophageal squamous cell carcinoma.

Johnson DG. **The paradox of E2F1: oncogene and tumor suppressor gene.** Mol Carcinog 2000;27(3):151-7.
Abstract: Cancer cells often contain mutations that lead to the loss of retinoblastoma tumor suppressor (Rb) function and the activation of E2F-dependent transcription. As a result, proliferation is deregulated, and sensitivity to apoptotic stimuli is increased. In cell culture studies, the transcription factor E2F1 has been shown to be equally adept at inducing proliferation and apoptosis. Several groups using mouse models have been examining how these E2F1-regulated processes impact the development of cancer. The conclusion from these studies is that E2F1 can function as both oncogene and tumor suppressor gene and that both positive and negative effects on tumorigenesis can be observed whether E2F1 is absent or overexpressed. These findings are discussed in the context of a model in which pathways controlling cell-cycle progression and apoptosis are intimately linked.

Jones B, Dale RG. **Radiobiological modeling and clinical trials.** Int J Radiat Oncol Biol Phys 2000;48(1):259-65.
Abstract: PURPOSE: Standard clinical trial designs can lead to restrictive conclusions: the "best recommended treatments" based on trial results, although generally applicable to patient populations, do not necessarily apply to individual patients. In theory, radiobiological modeling, coupled with reliable

predictive assays, can be used to rationalize the selection of patients for parcticular schedules in trials. MATERIALS AND METHODS: Linear-quadratic modeling of radiotherapy can be used to simulate a clinical trial. This is achieved by random sampling techniques where the key radiobiological parameters (alpha, beta, T(pot) and clonogen number) are selected from known or expected ranges. Clinical trial design in radiotherapy may be improved by formal radiobiological assessment designed to estimate the likely changes in tumor cure probability (TCP) and the likely normal tissue biologically effective dose (BED). Modeling may also be used to rationalize the allocation of patients to a test or standard schedule or for individual optimization of a treatment schedule. Such approaches depend on there being reliable predictive assays of the radiobiological parameters in individual patients. The influence of variations in predictive assay accuracy on the improved outcomes are assessed. RESULTS: Clinical trials, which have been preceded by modeling simulation, offer potentially substantial improvements in the results of cancer treatment by radiotherapy. These exceed the usual gains found in standard clinical trials. CONCLUSION: Future preclinical trial design should include modeling assessments that indicate how best to structure the trial.

Jonsson E, Friberg LE, Karlsson MO, Hassan SB, Freijs A, Hansen K, Larsson R. **Determination of drug effect on tumour cells, host animal toxicity and drug pharmacokinetics in a hollow-fibre model in rats.** Cancer Chemother Pharmacol 2000;46(6):493-500.
Abstract: PURPOSE: Based on the previously published hollow-fibre assay mainly used for early in vivo anticancer drug screening, we wanted to develop an extended hollow-fibre model in which antitumour activity, haematological toxicity and pharmacokinetics could be studied in the same animal. METHOD: The breast cancer cell lines MDA-MB-231 and MCF-7 were cultured in semipermeable hollow fibres. The fibres were implanted subcutaneously into immunocompetent male Sprague Dawley rats, and the rats were treated with 5-fluorouracil (5-FU, 125 mg/kg), epirubicin (EPI, 10 mg/kg) or cyclophosphamide (CP, 120 mg/kg) intraperitoneally, the new cyanoguanidine CHS 828 (375 mg/kg or 75 mg/kg x 5) orally, or vehicle only. After 6 days the fibres were retrieved and the cell density was evaluated. Haematological parameters were monitored and two to four samples per animal were drawn to determine the pharmacokinetic parameters in NONMEM. RESULTS: Drug treatment had generally low effects on the tumour cells. Of the standard drugs (5-FU, EPI and CP), only CP exerted a statistically significant antiproliferative effect. CHS 828 had only a minor effect as a single dose, but divided into five daily doses had a pronounced effect on both cell lines. 5-FU, EPI and CP all caused a marked decrease in leucocytes, platelets and haemoglobin, while CHS 828 did not seem to affect these parameters. The pharmacokinetics of 5-FU and EPI were in accordance with previously established pharmacokinetic models. The pharmacokinetics of CP and CHS 828 were both described by one-compartment models. CONCLUSIONS: This study illustrates the possibility of measuring antitumour effect, haematological toxicity and pharmacokinetics in the same animal using the hollow-fibre model.

Kallay E, Pietschmann P, Toyokuni S, Bajna E, Hahn P, Mazzucco K, Bieglmayer C, Kato S, Cross HS. **Characterization of a vitamin D receptor knockout mouse as a model of colorectal hyperproliferation and DNA damage.** Carcinogenesis 2001;22(9):1429-35.
Abstract: The vitamin D receptor knockout (VDR-KO) mouse presents with a skeletal phenotype typical for complete lack of genomic 1,25-dihydroxycholecalciferol effects. Our previous data from human colorectal tissue suggest that the steroid hormone and its receptor may have protective function against

tumour progression. In order to investigate the relevance of the vitamin D system for pre-malignant site-directed changes in the colon, we characterized the amount and site-specific distribution of the VDR along the large intestine in wild-type (WT), heterozygote (HT) and KO mice. We also evaluated expression of proliferating cell nuclear antigen (PCNA), of cyclin D1 and the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative stress. In colon ascendens, proliferative cells were dispersed all along the crypt and expression levels of all three markers were high in WT mice. A decrease of VDR expression did not affect expression significantly. In colon descendens, however, fewer proliferative cells were solely located in the lower third of the crypt, and an inverse relationship between VDR reduction, PCNA positivity and cyclin D1 expression was found in HT and KO mice. In parallel to enhanced proliferation a highly significant increase of 8-OHdG positivity occurred. Therefore, the sigmoid colon of VDR-KO mice, fed on an appropriate lactose/calcium-enriched diet to alleviate impaired calcium homeostasis-related phenotypic changes, is an excellent model for investigating induction and prevention of pre-malignant changes in one of the hotspots for human colorectal cancer incidence.

Kashiwagi H, Uchida K. **Genome-wide profiling of gene amplification and deletion in cancer.** Hum Cell 2000;13(3):135-41.
Abstract: Accumulations of genetic changes in somatic cells induce phenotypic transformations leading to cancer. Among these genetic changes, gene amplification and deletion are most frequently observed in several kinds of cancers. Amplification of oncogene and/or deletion of tumor suppressor gene, together with dysfunction of the gene by point mutation, are the main causes of cancer. Genome-wide analysis of amplification and deletion of genes in cancers is basic to resolving the mechanisms of carcinogenesis. Comparative genomic hybridization (CGH) developed in 1992 has been utilized to identify DNA copy number abnormalities in various kind of cancers and several reports have shown its usefulness in screening of the genes involved in carcinogenesis, and also in the identification of prognostic factors in cancer. We have shown that 1q23 gain is associated with neuroblastomas that are resistant to aggressive treatment, and have poor prognosis, and 1q and 13q gains are possibly related to drug resistance in ovarian cancers. Recently, the "rough draft" of the human genome was reported and we are ready to utilize the vast information on genomic sequences in cancer research. Moreover, microarray technology enables us to analyze more than ten thousand genes at a time and revealed genetic abnormalities in cancers at a genome-wide level. By combination of microarray and CGH, a powerful screening method for oncogenes and tumor suppressor genes in cancers, called array-CGH, has been developed by several groups. In this Article, we overview these genome-wide analytical methods, CGH and array-CGH, and discuss their potential in molecular characterization of cancers.

Katz AJ, Chiu A, Beaubier J, Shi X. **Combining Drosophila melanogaster somatic-mutation-recombination and electron-spin-resonance-spectroscopy data to interpret epidemiologic observations on chromium carcinogenicity.** Mol Cell Biochem 2001;222(1-2):61-8.
Abstract: Lung cancers are significantly increased among workers exposed to chromate (Cr6+, Cr3+), chromium pigments (Cr6+) and chromium plating (Cr6+). Chromium lung burdens and cancer risk increase proportionately with duration of employment at long latencies. However, this epidemiologic information alone is insufficient in determining whether Cr6+ or Cr3+ are equally important in causing cancer. We have attempted to combine epidemiologic data with data from the Drosophila melanogaster

somatic-mutation-recombination-test and from the in vitro electron-spin-resonance spectroscopy study to demonstrate that following somatic recombination plays a more important role than somatic mutation in chromium carcinogenesis. Cr4+ is more important than Cr5+ or Cr6+ in inducing somatic recombination while Cr6+ produces more and bigger clones than Cr4+ in somatic mutation. Cr3+ produces negative results in this fruit-fly wing-spot-assay. When the larvae and flies exposed to Cr6+ and Cr4+ are examined by ESR, only Cr5+ and Cr3+ are found. Thermodynamic parameters deltaE, deltaH, and deltaS are also estimated from these latter experiments to explain the relative importance of Cr6+, Cr4+, Cr3+ in chromium carcinogenesis among exposed industrial workers.

Kelloff GJ, Sigman CC, Johnson KM, Boone CW, Greenwald P, Crowell JA, Hawk ET, Doody LA.
Perspectives on surrogate end points in the development of drugs that reduce the risk of cancer.
Cancer Epidemiol Biomarkers Prev 2000;9(2):127-37.
Abstract: This paper proposes a scientific basis and possible strategy for applying surrogate end points in chemopreventive drug development. The potential surrogate end points for cancer incidence described are both phenotypic (at the tissue, cellular, and molecular levels) and genotypic biomarkers. To establish chemopreventive efficacy in randomized, placebo-controlled clinical trials, it is expected that in most cases it will be critical to ensure that virtually all of the biomarker lesions are prevented or that the lesions prevented are those with the potential to progress. This would require that both the phenotype and genotype of the target tissue in agent-treated subjects, especially in any new or remaining precancers, are equivalent to or show less progression than those of placebo-treated subjects. In the National Cancer Institute chemoprevention program, histological modulation of a precancer (intraepithelial neoplasia) has thus far been the primary phenotypic surrogate end point in chemoprevention trials. Additionally, we give high priority to biomarkers measuring specific and general genotypic changes correlating to the carcinogenesis progression model for the targeted cancer (e.g., progressive genomic instability as measured by loss of heterozygosity or amplification at a specific microsatellite loci). Other potential surrogate end points that may occur earlier in carcinogenesis are being analyzed in these precancers and in nearby normal appearing tissues. These biomarkers include proliferation and differentiation indices, specific gene and general chromosome damage, cell growth regulatory molecules, and biochemical activities (e.g., enzyme inhibition). Serum biomarkers also may be monitored (e.g., prostate-specific antigen) because of their accessibility. Potentially chemopreventive drug effects of the test agent also may be measured (e.g., tissue and serum estrogen levels in studies of steroid aromatase inhibitors). These initial studies are expected to expand the list of validated surrogate end points for future use. Continued discussion and research among the National Cancer Institute, the Food and Drug Administration, industry, and academia are needed to ensure that surrogate end point-based chemoprevention indications are feasible.

Khan SG, Metter EJ, Tarone RE, Bohr VA, Grossman L, Hedayati M, Bale SJ, Emmert S, Kraemer KH.
A new xeroderma pigmentosum group C poly(AT) insertion/deletion polymorphism.
Carcinogenesis 2000;21(10):1821-5.
Abstract: We found a common biallelic polymorphism (PAT) in the xeroderma pigmentosum complementation group C (XPC) DNA repair gene consisting of an insertion of 83 bases of A and T [poly(AT)] and a 5 base deletion within intron 9. We developed a PCR assay to resolve the XPC PAT+ and PAT- alleles and found that the PAT+ allele frequency was 0.44 in 156 cancer-free donors from the

Johns Hopkins School of Public Health, 0.41 in 263 cancer-free donors from the Baltimore Longitudinal Study of Aging and 0.36 in samples from 216 unselected donors from NIH. We also found a single nucleotide polymorphism in exon 15 of the XPC gene (A2920C, Lys939-->Gln) that creates a new enzyme restriction site. This XPC exon 15 single nucleotide polymorphism occurred at a frequency of 0.38 in 98 NIH donors and is in linkage disequilibrium with the PAT locus. We developed an allele-specific complementation assay utilizing post-UV host cell reactivation to assess DNA repair capacity of polymorphic alleles. We found similar DNA repair with XPC 2920A and XPC 2920C. These common polymorphisms in the XPC DNA repair gene may be useful for molecular epidemiological studies of cancer susceptibility.

King RD, Srinivasan A, Dehaspe L. **Warmr: a data mining tool for chemical data.** J Comput Aided Mol Des 2001;15(2):173-81.
Abstract: Data mining techniques are becoming increasingly important in chemistry as databases become too large to examine manually. Data mining methods from the field of Inductive Logic Programming (ILP) have potential advantages for structural chemical data. In this paper we present Warmr, the first ILP data mining algorithm to be applied to chemoinformatic data. We illustrate the value of Warmr by applying it to a well studied database of chemical compounds tested for carcinogenicity in rodents. Data mining was used to find all frequent substructures in the database, and knowledge of these frequent substructures is shown to add value to the database. One use of the frequent substructures was to convert them into probabilistic prediction rules relating compound description to carcinogenesis. These rules were found to be accurate on test data, and to give some insight into the relationship between structure and activity in carcinogenesis. The substructures were also used to prove that there existed no accurate rule, based purely on atom-bond substructure with less than seven conditions, that could predict carcinogenicity. This results put a lower bound on the complexity of the relationship between chemical structure and carcinogenicity. Only by using a data mining algorithm, and by doing a complete search, is it possible to prove such a result. Finally the frequent substructures were shown to add value by increasing the accuracy of statistical and machine learning programs that were trained to predict chemical carcinogenicity. We conclude that Warmr, and ILP data mining methods generally, are an important new tool for analysing chemical databases.

Kodell RL, Lin KK, Thorn BT, Chen JJ. **Bioassays of shortened duration for drugs: statistical implications.** Toxicol Sci 2000;55(2):415-32.
Abstract: Declining survival rates in rodent carcinogenesis bioassays have raised a concern that continuing the practice of terminating such studies at 24 months could result in too few live animals at termination for adequate pathological evaluation. One option for ensuring sufficient numbers of animals at the terminal sacrifice is to shorten the duration of the bioassay, but this approach is accompanied by a reduction in statistical power for detecting carcinogenic potential. The present study was conducted to evaluate the loss of power associated with early termination. Data from drug studies in rats were used to formulate biologically based dose-response models of carcinogenesis using the 2-stage clonal expansion model as a context. These dose-response models, which were chosen to represent 6 variations of the initiation-promotion-completion cancer model, were employed to generate a large number of representative bioassay data sets using Monte Carlo simulation techniques. For a variety of tumor dose-response trends, tumor lethality, and competing risk-survival rates, the power of age-adjusted statistical

tests to assess the significance of carcinogenic potential was evaluated at 18 and 21 months, and compared to the power at the normal 24-month stopping time. The results showed that stopping at 18 months would reduce power to an unacceptable level for all 6 submodels of the 2-stage clonal expansion model, with the pure-promoter and pure-completer models being most adversely affected. For the 21-month stopping time, the results showed that, unless pure promotion can be ruled out a priori as a potential carcinogenic mode of action, the loss of power is too great to warrant early stopping.

Kowalczyk DW, Wlazlo AP, Blaszczyk-Thurin M, Xiang ZQ, Giles-Davis W, Ertl HC. **A method that allows easy characterization of tumor-infiltrating lymphocytes.** J Immunol Methods 2001;253(1-2):163-75.
Abstract: A method was developed to compare the lymphocytic infiltrates in regressing vs. progressing experimental mouse tumors using a model for human papillomavirus-16 (HPV-16) oncoprotein-linked cancer. Tumor cells mixed with matrigel, composed of natural matrix substances that provide a basement membrane structure for adherent cells, were inoculated into mice vaccinated with an efficacious vaccine to the E7 oncoprotein or a vaccine to a control antigen. The tumor cells remained within the solidified gel and recruited a cellular infiltrate that could readily be analyzed upon removal of the gelatinous mass containing progressing or regressing tumors. The results show that tumors recruit activated CD8(+) T cells regardless of their antigen specificity. In regressing tumors expressing an appropriate target antigen for the vaccine-induced CD8(+) T cells, a strong increase of the tumor antigen-specific T cell population was observed over time. Progressing tumors that lacked the target antigen for the activated CD8(+) T cell population did not show this selective enrichment.

Kowalski LA, Assi KP, Wee RK, Madden Z. **In vitro prediction of carcinogenicity using a bovine papillomavirus DNA--carrying C3H/10T 1/2 cell line (T1). II: Results from the testing of 100 chemicals.** Environ Mol Mutagen 2001;37(3):231-40.
Abstract: A new in vitro test for identifying carcinogens is evaluated against a testing database of 100 chemicals including the following groups: steroids, antineoplastics, PCBs, dioxins, alkyl halides, aromatic amines, nitrogen heterocycles, polyaromatic hydrocarbons, mustards, and benzodioxoles. The assay uses focus formation in a stable, BPV-1-DNA-carrying C3H/10T 1/2 mouse embryo fibroblast cell line (T1), which does not require transfection, infection with virus, or isolation of primary cells from animals. For this group of chemicals, the T1 assay correctly predicted the rodent carcinogenicity or noncarcinogenicity of 77% of the chemicals for which carcinogenicity is reported. Based on published data the bacterial mutagenicity assay would have correctly predicted carcinogenicity or noncarcinogenicity of 53% of the chemicals. The Syrian hamster embryo test would have correctly predicted carcinogenicity or noncarcinogenicity of 61% of the chemicals. We also demonstrate dose--response relationships for two of the chemicals. We report the responses of T1 cells to the group of chemicals used in the International Life Sciences Institute's program for screening of alternative methods of predicting carcinogenicity. Copyright 2001 Wiley-Liss, Inc.

Kozusko F, Chen P, Grant SG, Day BW, Panetta JC. **A mathematical model of in vitro cancer cell growth and treatment with the antimitotic agent curacin A.** Math Biosci 2001;170(1):1-16.
Abstract: A mathematical model of cancer cell growth and response to treatment with the experimental antimitotic agent curacin A is presented. Rate parameters for the untreated growth of MCF-7/LY2 breast

cancer and A2780 ovarian cell lines are determined from in vitro growth studies. Subsequent growth studies following treatments with 2.5, 25 and 50 nanomolar (nM), concentrations of curacin A are used to determine effects on the cell cycle and cell viability. The model's system of ordinary differential equations yields an approximate analytical solution which predicts the minimum concentration necessary to prevent growth. The model shows that cell growth is arrested when the apoptotic rate is greater than the mitotic rate and that the S-phase transition rate acts to amplify this effect. Analysis of the data suggests that curacin A is rapidly absorbed into both cell lines causing an increase in the S-phase transition and a decrease in the M-phase transition. The model also indicates that the rate of apoptosis remains virtually constant for MCF-7/LY2 while that of A2780 increases 38% at 2.5 nM and 59% at 50 nM as compared to the untreated apoptotic rate.

Krupp G, Klapper W, Parwaresch R. **Cell proliferation, carcinogenesis and diverse mechanisms of telomerase regulation.** Cell Mol Life Sci 2000;57(3):464-86.
Abstract: Replication of linear genomes is incomplete and leaves terminal gaps. Solutions to this 'end replication' problem can be traced back to the prebiotic RNA world: 'fossils' of the presumptive archetypes of telomere structure and of the telomerase enzyme are retained in the terminal structures of some RNA viruses. Telomerase expression in mammals is ubiquitous in embryonic tissues but downregulated in somatic tissues of adults. Exceptions are regenerative tissues and, notably, tumor cells. Telomerase activation is controlled by cellular proliferation, and it is an early step in the development of many tumors. In contrast to mammals, indeterminately growing multicellular organisms, such as fish and crustaceae, maintain telomerase competence in all somatic tissues. In human tumor diagnostics, detection of proliferation markers with monoclonal antibodies is well established, and in this review, the significance of additional telomerase assays is evaluated. Telomerase inhibitors are attractive goals for application in tumor therapy, and telomerase knockout mice have proven that telomere erosion limits the lifespan of cells in vivo. In contrast, telomerase stimulation can be used to expand the potential of cellular proliferation in vitro, with possible applications for transplantation of in vitro expanded human cells, for immortalizing primary human cells as improved tissue models and for the isolation of otherwise intractable products, such as genuine human monoclonal antibodies.

Kunzi-Rapp K, Genze F, Kufer R, Reich E, Hautmann RE, Gschwend JE. **Chorioallantoic membrane assay: vascularized 3-dimensional cell culture system for human prostate cancer cells as an animal substitute model.** J Urol 2001;166(4):1502-7.
Abstract: PURPOSE: Chorioallantoic membranes have been used as a reliable biomedical assay system for many years. Chicken eggs in the early phase of breeding are between in vitro and in vivo systems but may provide an immunodeficient, vascularized test environment. We tested this model as an in vivo system for prostate cancer research. MATERIALS AND METHODS: Single cell suspensions of LNCaP, PC-3 and Tsu-Pr1 human prostatic cancer cell lines as well as 2 immortalized normal human prostate epithelial cell lines were inoculated on the chorioallantoic membrane of fertilized chicken eggs on day 5 or 6 of breeding. Tumor growth and viability of the embryo was evaluated by stereo microscopy. At day 10 the membranes were removed and embedded in paraffin. Cell morphology was assessed after hematoxylin and eosin staining. Cellular expression of cytokeratin, prostate specific antigen and androgen receptor as well as apoptosis induction was confirmed by immunohistochemistry. RESULTS: Three days after tumor cell inoculation on the extraembryonic vascular system of the

chorioallantoic membrane cell growth and formation of 3-dimensional tumors became apparent in 100% of inoculated membranes. Strong neo-angiogenesis was detected next to the established tumors and tumor cells invading the stroma of the chorioallantoic membrane. Cytokeratin expression as well as prostate specific antigen and androgen receptor in LNCaP cells confirmed the human prostate tumor origin. Assessment of quantitative in vivo apoptosis induction in LNCaP cells after intravenous injection of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate confirmed the model as a versatile in vivo system. CONCLUSIONS: The well vascularized chorioallantoic membrane of bred chicken eggs is a suitable system for early in vivo cancer research. Reliable growth of prostate cancer cell lines is feasible and allows the evaluation of proliferation and apoptosis induction after intravascular or topic application of anticancer drugs. Exploitation of this assay enables a substantial reduction in or substitution for subsequent animal experiments.

Kwack SJ, Lee BM. **Correlation between DNA or protein adducts and benzo[a]pyrene diol epoxide I-triglyceride adduct detected in vitro and in vivo.** Carcinogenesis 2000;21(4):629-32.
Abstract: In this study, we demonstrated the in vitro and in vivo formation of carcinogen-lipid adduct and its correlation with DNA or protein adducts. The lipids from serum or hepatocyte membranes of Sprague-Dawley rats, human serum and standard major lipids were in vitro reacted with benzo[a]pyrene (B[a]P) and B[a]P metabolites. 7, 8-Dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene(BPDE-I), an ultimate carcinogenic form of B[a]P, was covalently bound to triglyceride (TG). BPDE-I-TG adducts isolated by thin-layer chromatography (TLC) were further detected by high-performance liquid chromatography. TGs, including triolein, tripalmitin and tristearin, showed positive reactions with BPDE-I. However, cholesterol, phospholipids (phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidyl-inositol and sphingomyelin) and non-esterified fatty acids (palmitic acid, oleic acid, linoleic acid and stearic acid) did not react with BPDE-I. In addition, other B[a]P metabolites (B[a]P-phenols and -diols) did not react with TG. TG appeared to be the most reactive lipid yet studied with respect to its ability to form an adduct with BPDE-I. There was a clear-cut dose-related formation of [1,3-(3)H]BPDE-I-lipid adducts in vitro between TG and [1,3-(3)H]BPDE-I. In an animal study, BPDE-I-TG was also formed in the serum of rats orally treated with B[a]P (25 mg/rat). Also, obvious correlations between [(3)H]B[a]P related-biomolecule adducts (DNA or protein) or lipid damage and the BPDE-I-TG adducts were obtained in various tissues of mice i.p. treated with [(3)H]B[a]P. These data suggest that TG can form an adduct with BPDE-I, as do other macromolecules (DNA, RNA and protein). Therefore, a carcinogen-lipid adduct would be a useful biomarker for chemical carcinogenesis research and cancer risk assessment.

Laconi S, Pani P, Pillai S, Pasciu D, Sarma DS, Laconi E. **A growth-constrained environment drives tumor progression invivo.** Proc Natl Acad Sci U S A 2001;98(14):7806-11.
Abstract: We recently have shown that selective growth of transplanted normal hepatocytes can be achieved in a setting of cell cycle block of endogenous parenchymal cells. Thus, massive proliferation of donor-derived normal hepatocytes was observed in the liver of rats previously given retrorsine (RS), a naturally occurring alkaloid that blocks proliferation of resident liver cells. In the present study, the fate of nodular hepatocytes transplanted into RS-treated or normal syngeneic recipients was followed. The dipeptidyl peptidase type IV-deficient (DPPIV(-)) rat model for hepatocyte transplantation was used to distinguish donor-derived cells from recipient cells. Hepatocyte nodules were chemically induced in

Fischer 344, DPPIV(+) rats; livers were then perfused and larger (>5 mm) nodules were separated from surrounding tissue. Cells isolated from either tissue were then injected into normal or RS-treated DPPIV (-) recipients. One month after transplantation, grossly visible nodules (2--3 mm) were seen in RS-treated recipients transplanted with nodular cells. They grew rapidly, occupying 80--90% of the host liver at 2 months, and progressed to hepatocellular carcinoma within 4 months. By contrast, no liver nodules developed within 6 months when nodular hepatocytes were injected into the liver of recipients not exposed to RS, although small clusters of donor-derived cells were present in these animals. Taken together, these results directly point to a fundamental role played by the host environment in modulating the growth and the progression rate of altered cells during carcinogenesis. In parcticular, they indicate that conditions associated with growth constraint of the host tissue can drive tumor progression in vivo.

Lanari C, Luthy I, Lamb CA, Fabris V, Pagano E, Helguero LA, Sanjuan N, Merani S, Molinolo AA. **Five novel hormone-responsive cell lines derived from murine mammary ductal carcinomas: in vivo and in vitro effects of estrogens and progestins.** Cancer Res 2001;61(1):293-302.
Abstract: We have developed an experimental model of mammary carcinogenesis in which the administration of medroxyprogesterone acetate (MPA) to female BALB/c mice induces progestin-dependent ductal metastatic mammary tumors with high levels of estrogen receptor (ER) and progesterone receptor (PR). Through selective transplants in untreated mice, we have obtained progestin-independent variants, still expressing high levels of ER and PR. Primary cultures of the MPA-induced carcinomas C4-HD and C7-HI were set up, and after 3-4 months, several different cell lines were obtained. Four of these, MC4-L1, MC4-L2, MC4-L3, and MC4-L5 were established from C4-HD and a fifth, MC7-L1, from C7-HI. All cells were of epithelial origin, as demonstrated by electron microscopy and by immunocytochemical identification of cytokeratin and cadherin. In vitro MC4-L1, MC4-L3, and MC4-L5 showed a typical epithelial morphology; when transplanted in vivo, they originated metastatic carcinomas with different degrees of differentiation. MC4-L2 and MC7-L1 deviated from the standard epithelial picture; they disclosed a spindle-shaped morphology in vitro and in vivo gave rise to a biphasic spindle cell/tubular carcinoma and an anaplastic carcinoma, respectively; both lines gave rise to metastases. This differential morphology correlated with a higher degree of aggressiveness, as compared with MC4-L1, MC4-L3, and MC4-L5. ERs and PRs were detected by binding, immunocytochemistry, and Western blot. In vitro, MC4-L2 and MC7-L1 were stimulated by MPA (nM to microM) and 17beta-estradiol (nM and 10 nM); no significant stimulation was observed in MC4-L1, MC4-L3, and MC4-L5 under the same experimental conditions. In vivo, MPA significantly stimulated tumor growth in all epithelioid lines but not in MC4-L2 and MC7-L1. A progestin-dependent growth pattern was confirmed for MC4-L1, MC4-L3, and MC4-L5 in successive transplants, whereas MC4-L2 and MC7-L1 behaved as progestin independent. This is the first description of mouse mammary carcinoma cell lines expressing ER and PR. The different in vitro hormone responses as compared with in vivo and the differential effects of 17beta-estradiol in the parental tumors and in cell lines render these lines useful tools for the in vitro and in vivo study of hormone regulation of tumor growth and metastases.

Langa F, Kress C, Colucci-Guyon E, Khun H, Vandormael-Pournin S, Huerre M, Babinet C. **Teratocarcinomas induced by embryonic stem (ES) cells lacking vimentin: an approach to study the role of vimentin in tumorigenesis.** J Cell Sci 2000;113 Pt 19:3463-72.
Abstract: Vimentin is a class III intermediate filament protein widely expressed in the developing

embryo and in cells of mesenchymal origin in the adult. Vimentin knock-out mice develop and reproduce without any obvious defect. This is an unexpected finding in view of the high degree of conservation of the vimentin gene among vertebrates. However, it does not exclude the possibility of a role for vimentin in pathological conditions, like tumorigenesis. To address this question directly, we have used a teratocarcinoma model involving the injection of ES cells into syngeneic mice. ES cells lacking vimentin were generated from 129/Sv Vim-/- mice with high efficiency. The absence of vimentin did not affect ES cell morphology, viability or growth rate in vitro. Tumours were induced by subcutaneous injection of either Vim-/- or Vim+/+ ES cells into Vim+/+ and Vim-/- mice, in order to analyse the effect of the absence of vimentin in either the tumorigenic cells or the host mice. No significant differences were found in either tumour incidence, size or vascularization of teratocarcinomas obtained with all possible combinations. Vim-/- ES-derived tumours showed the same cellular composition typical of teratocarcinomas induced by wild-type ES cells together with a very similar apoptotic pattern. Taken together, these results demonstrate that in this model vimentin is not essential for efficient tumour growth and differentiation in vivo.

Law JM. **Mechanistic considerations in small fish carcinogenicity testing.** ILAR J 2001;42(4):274-84.
Abstract: Historically, small fish species have proven useful both as environmental sentinels and as versatile test animals in toxicity and carcinogenicity bioassays. They can be bred in large numbers, have low maintenance and bioassay costs, and have a low background incidence of tumors. However, more mechanistic information is needed to help validate the information garnered from these models and to keep pace with other more fully developed animal models. This paper focuses on mechanistic considerations when using small fish models for carcinogenicity testing. Several small aquarium fish species have proven useful. The Japanese medaka is perhaps the best characterized small fish model for carcinogenicity testing; however, the zebrafish is emerging as an important model because it is well characterized genetically. Both route and methodology of exposure may affect the outcome of the study. Most studies have been conducted by introducing the test compound into the ambient water, but dietary exposures and embryo microinjection have also been used. Other considerations in study design include use of an initiating carcinogen, such as diethlynitrosamine, and differences in xenobiotic metabolism, such as the fact that fish CYP2B is refractory to phenobarbital induction. The small size of these models has perhaps limited some types of mechanistic studies, such as formation and repair of DNA adducts in response to carcinogen exposure. However, improved analytical methods are allowing greater resolution and should be applied to small fish species. Slide-based methods such as immunohistochemistry are an important adjunct to routine histopathology and should be included in study design. However, there is a need for development of more species-specific antibodies for fish research. There is also a need for more fish-specific data on cytokines, serum biochemistry, and oncogenes to strengthen the use of these important test models.

Lin SC, Chang KW, Chang CS, Yu SY, Chao SY, Wong YK. **Establishment and characterization of a cell line (HCDB-1) derived from a hamster buccal pouch carcinoma induced by DMBA and Taiwanese betel quid extract.** Proc Natl Sci Counc Repub China B 2000;24(3):129-35.
Abstract: This study identified that the carcinogenesis of hamster buccal pouch (HBP) induced by 7,12-dimethylbenz[a]anthracene (DMBA) was greatly enhanced (18 folds) by a combination treatment with Taiwanese betel quid (BQ) extract. A new cell line, HCDB-1, has been established from induced

carcinomas. The cultured monolayer cells were epithelioid in shape with irregular nuclei. They demonstrated abundant cytokeratin and tonofilaments; however, ultrastructural well-organized desmosomes were lacking. The HCDB-1 cell exhibited population doubling in 19 h and was highly tumorigenic in nude mice. A C-->T transition at codon 141 (Ala to Val) of the p53 gene was detected in this cell. This mutation is equivalent to a specific temperature-sensitive mouse p53Ala135Val mutant that causes transformation by shifting to 37.5 degrees C. HCDB-1 is the first cell line established from the HBP model of oral carcinogenesis induced by DMBA/Taiwanese BQ extract. It might be valuable for exploring the molecular pathogenesis of oral cancer.

Makinen K, Loimas S, Nuutinen P, Eskelinen M, Alhava E. **The growth pattern and microvasculature of pancreatic tumours induced with cultured carcinoma cells.** Br J Cancer 2000;82(4):900-4.
Abstract: Pancreatic cancer is one of the most frustrating problems in gastroenterological surgery, since there is little we can do to improve the survival of patients with current treatment strategies. If one is to elucidate factors related to carcinogenesis, tumour biology, diagnostics and new treatment modalities of this malignant disease, then it is essential to develop a suitable animal model. In the present study we investigated rat pancreatic tumour growth after intrapancreatic injection of cultured pancreatic carcinoma cells (DSL-6A/C1), originally derived from an azaserine-induced tumour, as well as the features of tumour microcirculation using the microangiography technique. After intrapancreatic inoculation, tumours were detected in 64% of animals. A 1 cm3 tumour volume was reached within 20 weeks after inoculation. The tumours were ductal adenocarcinomas. Larger tumours showed invasive growth and spreading into the surrounding tissues, mainly into spleen and peritoneum. Microangiography revealed that the pancreatic tumours had an irregular and scanty vessel network and there were avascular areas in the center of the tumour. The area between normal pancreas and the induced tumour had dense vascularization. Intrapancreatic tumour induction with cultured pancreatic carcinoma cells produced a solid and uniformly growing tumour in Lewis rats and it thus provides a possible model for pancreatic cancer studies.

Manickan E, Satoi J, Wang TC, Liang TJ. **Conditional liver-specific expression of simian virus 40 T antigen leads to regulatable development of hepatic neoplasm in transgenic mice.** J Biol Chem 2001;276(17):13989-94.
Abstract: Adaptive epigenetic changes and toxicity often accompany constitutive expression of a transgene or knockout of an endogenous gene in mice. These considerations potentially limit the usefulness of transgenic technology in studying the in vivo functions of a gene. Using conditional gene expression technology, it is possible to override such restrictions to achieve temporal and tissue-specific manipulation of gene expression in vivo. Based on the tetracycline regulatory system, we established a binary transgenic model in which the conditional expression of two transgenes, SV40 T antigen (TAg) and lacZ, can be tightly regulated in the liver by administration of tetracycline. The mouse albumin or mouse major urinary protein promoter was used to achieve liver-specific expression of the tetracycline-responsive transcriptional activator (tTA) in one set of transgenic mice. These mice were crossed with transgenic mice carrying either TAg or lacZ under the control of the tTA-regulated promoter. Analyses of mice transgenic for both tTA and TAg (or lacZ) revealed that the liver-specific expression of the transgenes could be suppressed to undetectable levels and regulated in a reversible fashion by

tetracycline administration and withdrawal. Mice with tTA and TAg transgenes developed hepatocellular adenomas and hyperplasia that could be prevented by continuous tetracycline administration. Our report demonstrates the value of this binary transgenic model in studying the physiological functions of any potential genes of interest in a liver-specific manner.

Maronpot RR. **The use of genetically modified animals in carcinogenicity bioassays.** Toxicol Pathol 2000;28(3):450-3.

Matsuo A, Watanabe A, Takahashi T, Futamura M, Mori S, Sugiyama Y, Takahashi Y, Saji S. **A simple method for classification of cell death by use of thin layer collagen gel for the detection of apoptosis and/or necrosis after cancer chemotherapy.** Jpn J Cancer Res 2001;92(7):813-9.
Abstract: To assess the efficacy of cancer chemotherapy, an important index is apoptosis of the target cells, which can usually be confirmed by electron microscopy (EM). We established a new experimental technique, whereby cancer cells (MKN45) were distributed in thin collagen gel as one or two cell layers, and cultured with anti-cancer drugs (5-FU and CDDP). The cells were stained with fluorescent Hoechst 33258 (Ho) and photographed, then with hematoxylin and eosin (H&E) and again photographed, and processed for EM. This approach allowed us to characterize the patterns of death of single cells in detail. There were six patterns of cell damage: two patterns of apoptosis, early peripheral condensation of chromatin and late apoptotic bodies, two patterns of necrosis, cytoplasmic swelling and washed-out images, and two further patterns, with morphological features of both apoptosis and necrosis, neither classified into necrosis nor apoptosis. The results show that cell death patterns can be mostly determined by combining observations of Ho and H&E-stained cells without the necessity for EM observation.

Mauthe RJ, Gibson DP, Bunch RT, Custer L. **The syrian hamster embryo (SHE) cell transformation assay: review of the methods and results.** Toxicol Pathol 2001;29 Suppl:138-46.
Abstract: The Syrian hamster embryo (SHE) cell-transformation assay represents a short-term in vitro assay capable of predicting rodent carcinogenicity of chemicals with a high degree of concordance (LeBoeuf et al [1996]. Mutat Res 356: 85-127). The SHE assay models the earliest identifiable stage in carcinogenicity, morphological cell transformation. In contrast to other short-term in vitro assays, both genotoxic and epigenetic carcinogens are detected. The SHE assay, originally developed by Berwald and Sachs (J Natl Cancer Inst 35: 641-661) and modified as described by LeBoeuf and Kerckaert (Carcinogenesis 7: 1431-1440), was included in the International Life Sciences Institute, Health and Environmental Sciences Institute (ILSI/HESI). Alternative Carcinogenicity Testing (ACT) collaboration to provide additional information on the use of short-term in vitro tests in predicting carcinogenic potential. A total of 19 ILSI compounds have been tested in the SHE assay: 15 were tested for this project, whereas clofibrate, methapyrilene, reserpine, and Di(2-ethylhexyl)phalate (DEHP) were tested previously. Of the 3 noncarcinogenic compounds tested, 2 were negative in the SHE assay, whereas ampicillin was tested positive. The remaining 16 compounds tested were either known rodent carcinogens and/or human carcinogens. From this group, 15 tested positive in the SHE assay whereas phenacetin, a genotoxic carcinogen, was tested negative. Therefore, overall concordance between the SHE assay and rodent bioassay was 89% (17/19), whereas concordance with known or predicted human carcinogens was 37% (7/19). Based on these data, it is concluded that the SHE cell-transformation assay has utility for predicting the results of the rodent carcinogenesis bioassay but lacks the selectivity to

distinguish between rodent and human carcinogens.

Oda H. **[p53 transgenic and knockout mice].** Nippon Rinsho 2000;58(6):1250-4 [Jpn].
Abstract: Li-Fraumeni syndrome(LFS) and its relation to the p53 gene and p53 gene transgenic and targeting mice are reviewed. LFS is a hereditary cancer-prone syndrome mainly with germ line mutation of p53 gene. p53 deficient mice, especially heterozygous mice, may serve as a model for human LFS, although there are several discrepancies between them. Carcinogenesis experiment using p53 deficient mice may be important to clarify the role of p53 in tumorigenesis in vivo.

Ogawa K, Nakanishi H, Takeshita F, Futakuchi M, Asamoto M, Imaida K, Tatematsu M, Shirai T. **Establishment of rat hepatocellular carcinoma cell lines with differing metastatic potential in nude mice.** Int J Cancer 2001;91(6):797-802.
Abstract: For better understanding of cancer metastasis, we have established an in vivo model for induction of highly metastatic hepatocellular carcinomas (HCC) in male F344 rats. From 1 tumor, 4 cell lines with differing metastatic potential (C1, C2, C6, C5F) were established by subcloning using the limited-dilution cloning technique. Two other lines, N1 and L2, arose from another primary HCC and a lung metastatic lesion, respectively. Although cell adhesion of each cell line in culture medium was different, tumors developing in the subcutis of nude mice after transplantation were all moderately differentiated HCC with a trabecular pattern. On subcutaneous injection into nude mice, all 6 cell lines proved to be tumorigenic in the injection site and C5F was highly metastatic to the lung. With injection into the tail vein, N1 and L2 formed frequent metastases in the lung as well as in lymph nodes. Using intraperitoneal injection, C1, C6, N1 and L2 showed marked disseminated growth in the abdominal cavity with bloody ascitis. Northern blot analysis revealed expression of known metastasis-related genes, KAI1 and heparanase, to be decreased in C5F, but no differences in expression of nm23-H1 were evident. A point mutation in the GSK-3beta phosphorylation site of the beta-catenin gene was found in L2. These transplantable HCC cell lines that have different metastatic ability should be useful for elucidation of mechanisms of metastasis. Copyright 2001 Wiley-Liss, Inc.

Omenn GS. **Assessment of human cancer risk: challenges for alternative approaches.** Toxicol Pathol 2001;29 Suppl:5-12.
Abstract: The ILSI/HESI Workshop on Alternatives to Carcinogenicity Testing aims to develop and apply new methods for assessment of potential carcinogenic risk to humans from various chemicals. The Workshop represents a major cooperative scientific effort. The long-term goals should be to greatly enhance the efficiency and reliability of such testing and to supplant, not just supplement, lifetime rodent bioassays. There are now well-established frameworks for risk assessment and risk management, putting risks into public health context and engaging stakeholders. The Lave-Omenn value-of-information model provides a useful way to assess the social costs and benefits of different strategies for testing large numbers of chemicals.

Popp S, Waltering S, Holtgreve-Grez H, Jauch A, Proby C, Leigh IM, Boukamp P. **Genetic characterization of a human skin carcinoma progression model: from primary tumor to metastasis.** J Invest Dermatol 2000;115(6):1095-103.
Abstract: The type and number of genetic aberrations required for a fully malignant tumor are still

unclear. This study describes the genetic analysis of a series of skin squamous cell carcinomas, representing the primary tumor, two recurrences, and a metastatic lesion from a single patient and cell lines established therefrom (MET-1 to MET-4). Comparative genomic hybridization demonstrated that: (i) most of the gains and losses were common for tumors and cell lines and affected chromosomes 3 (3p loss, 3q gain), 5 (5p gain, 5q loss), 7 (7p gain), 8 (8p loss, 8q gain), 11 (11q gain), and 17 (17p loss), and (ii) only one aberration was present in a tumor but not in the cell line (10 loss in tumor 4); and only few aberrations were cell line specific. From these, 10p loss and 17q gain were shared by all lines and tumor 4, suggesting that they were already present in all tumors, although in only a subpopulation of cells, whereas 20q gain (shared by all lines), 4q loss (MET-2), and 18p gain/18q loss (MET-3) seem to be culture derived. In agreement, multiplex fluorescence in situ hybridization demonstrated a set of common translocations for all lines thereby further confirming their common origin. In addition, each cell line, exhibited one or more individual translocation chromosomes, which suggested that MET-1 was a precursor of MET-4, whereas MET-2 and MET-3 developed in parallel. Whereas MET-1 to MET-3 were hypodiploid or hyperdiploid, MET-4 was characterized by polyploidization, a set of specific aberrations (t(3;7), t(X;2), i(10q)), and increased heterogeneity (varying translocations in individual metaphases). Using sequencing and expression studies, cells from all lines were wild type for p53, did not exhibit mutations in any of the ras genes (Harvey, Kirsten, or N-ras), and expressed wild-type fragile histidine triad gene (FHIT; mapped to 3p14.2, a locus underrepresented in all cells) transcripts. Thus, with the MET cell lines we present an in vivo skin carcinoma progression model that was genetically well defined, and which, despite originating from a sun-exposed site, is wild type for p53.

Qin X, Shibata D, Gerson SL. **Heterozygous DNA mismatch repair gene PMS2-knockout mice are susceptible to intestinal tumor induction with N-methyl-N-nitrosourea.** Carcinogenesis 2000;21 (4):833-8.

Abstract: PMS2-deficient (PMS2(-/-)) mice are hypersensitive to N-methyl-N-nitrosourea (MNU)-induced thymic lymphomas based on the failure to initiate mismatch repair (MMR) at O(6)-methylguanine:T mismatches formed after MNU exposure. However, heterozygous PMS2 knockout (PMS2(+/-)) mice do not develop spontaneous tumors, suggesting that they have sufficient MMR function to prevent genomic instability. We hypothesized that in PMS2(+/-) mice, exogenous carcinogens may either mutationally knockout the remaining normal allele leading cells to develop tumors or introduce sufficient DNA adducts and mismatches to overload the lower capacity for MMR, leading in either case to an increased rate of tumor production. In the present study, PMS2(+/-) mice and their littermate PMS2(+/+) mice were monitored for tumor incidence following MNU treatment. Mice were given 50 mg MNU/kg i.p. when 5 weeks old. They demonstrated a similar incidence of thymic lymphomas, suggesting that expression of the single normal PMS2 allele is sufficient to protect the thymus and implying that a single dose of MNU may not efficiently knock out the remaining PMS2 allele in the thymus. Surprisingly, PMS2(+/-) mice were significantly more likely to develop intestinal tumors-both adenomas and adenocarcinomas-after MNU than were PMS2(+/+) mice (2.34 +/- 0.34 tumors per mouse versus 1.34 +/- 0.25 tumors per mouse; $P < 0.05$). The intestinal tumors were located mainly in the small intestine. However, these tumors in both the PMS2(+/-) mice and PMS2(+/+) mice did not show microsatellite instability characteristic of loss of MMR. These results suggest that a single normal PMS2 allele can protect thymus but not intestine from MNU carcinogenesis. Organ-specific factors might influence MMR- mediated resistance to methylating agents. Heterozygous PMS2

knockout mice may be used as a promising animal model for intestinal tumorigenesis studies involving environmental carcinogens.

Quader ST, Bello-DeOcampo D, Williams DE, Kleinman HK, Webber MM. **Evaluation of the chemopreventive potential of retinoids using a novel in vitro human prostate carcinogenesis model.** Mutat Res 2001;496(1-2):153-61.
Abstract: The prevalence of prostatic intraepithelial neoplasia (PIN) and latent prostatic carcinoma, representing multiple steps in carcinogenesis and progression to invasive carcinoma, makes them relevant targets for prevention. A unique family of human prostate epithelial cell lines, which mimic steps in prostate carcinogenesis and progression, were used to evaluate the chemopreventive potential of all-trans-retinoic acid (RA) and N-(4-hydroxyphenyl)retinamide (4-HPR). The effects of RA and 4-HPR on anchorage-dependent growth of an immortalized, non-tumorigenic cell line RWPE-1 and two tumorigenic cell lines, WPE1-NB14 and WPE1-NB11, derived from RWPE-1 by exposure to N-methyl-N-nitrosourea (MNU), were examined. Both tumorigenic cell lines grow more rapidly than the parent RWPE-1 cell line in monolayer culture. Further, while RWPE-1 cells do not form colonies in agar, both tumorigenic cell lines do, with a colony forming efficiency (CFE) of 1.85 and 2.04% for WPE1-NB14 and WPE1-NB11 cells, respectively. Both RA and 4-HPR inhibited anchorage-dependent growth of all cell lines and anchorage-independent growth of WPE1-NB14 and WPE1-NB11 cells, in a dose-dependent manner, however, 10 times more RA than 4-HPR was required to produce the same effect. RWPE-1 cells are not invasive but WPE1-NB11 cells are significantly more invasive than WPE1-NB14 cells. Both RA and 4-HPR inhibited invasion in vitro by WPE1-NB11 and WPE1-NB14 cells where the more malignant WPE1-NB11 cells showed greater inhibition of invasion by 4-HPR than by RA. Overall, 4-HPR was more effective than RA in inhibiting growth and invasion but the response varied amongst the cell lines. These three cell lines mimic progressive steps in carcinogenesis and progression, from immortalized, non-tumorigenic RWPE-1 cells, to the less malignant WPE1-NB14 to the more malignant WPE1-NB11 cells, and provide powerful models for studies on secondary and tertiary prevention, i.e. promotion and progression stages, respectively, of prostate cancer.

Resor L, Bowen TJ, Wynshaw-Boris A. **Unraveling human cancer in the mouse: recent refinements to modeling and analysis.** Hum Mol Genet 2001;10(7):669-75.
Abstract: The ability to manipulate the mouse genome has made the mouse the primary mammalian genetic model organism. It has been possible to model human cancer in the mouse by overexpressing oncogenes or inactivating tumor suppressor genes, and these experiments have provided much of our in vivo understanding of cancer. However, these transgenic approaches do not always completely and accurately model human carcinogenesis. Recent developments in transgenic and knockout approaches have improved the accuracy of modeling somatic cancer in the mouse and analyzing the genomic instability that occurs in murine tumors. It is possible to use retroviral gene delivery, chromosome engineering and inducible transgenes to selectively manipulate the genome in a more precise spatial and temporal pattern. In addition, the development of powerful cytogenetic tools such as spectral karyotyping, fluorescence in situ hybridization and comparative genome hybridization have improved our ability to detect chromosomal rearrangements. Finally, global patterns of gene expression can be determined by microarray analysis to decipher complex gene patterns which occur in cancers. Several of these advances in mouse modeling of human cancer are discussed in this review.

Rhim JS. **Development of human cell lines from multiple organs.** Ann N Y Acad Sci 2000;919:16-25.
Abstract: While the majority of carcinogenesis studies have relied on the use of rodent cells in culture, experimental models to define the role of carcinogenic agents in the development of cancers must be established by using a variety of human cells. Unlike rodent cells, normal human cells in culture rarely undergo spontaneous transformation and have generally proven to be resistant to neoplastic transformation by carcinogens. Remarkable progress has been made during the past decade in human cell transformation systems. Malignant transformation of human cells in culture has been achieved by a stepwise process: immortalization and conversion of the immortalized cells to tumorigenic cells. One of the critical initial events in the progression of normal human cells to tumor cells is the escape from cellular senescence, with few exceptions; normal human cells require immortalization to provide a practical system for carcinogenesis studies. Different cell types require different conditions and transforming agents to achieve a useful cell line. The current state of the art in immortalization of human cells will be presented.

Rivedal E, Mikalsen SO, Sanner T. **Morphological transformation and effect on gap junction intercellular communication in Syrian hamster embryo cells as screening tests for carcinogens devoid of mutagenic activity.** Toxicol In Vitro 2000;14(2):185-92.
Abstract: A large fraction of chemicals observed to cause cancer in experimental animals is devoid of mutagenic activity. It is therefore of importance to develop methods that can be used to detect and study environmental carcinogenic agents that do not interact directly with DNA. Previous studies have indicated that induction of in vitro cell transformation and inhibition of gap junction intercellular communication are endpoints that could be useful for the detection of non-genotoxic carcinogens. In the present work, 13 compounds [chlordane, Arochlor 1260, di(2-ethylhexyl)phthalate, 1,1,1-trichloro-2, 2-bis(4-chlorophenyl)ethane, limonene, sodium fluoride, ethionine, o-anisidine, benzoyl peroxide, o-vanadate, phenobarbital, 12-O-tetradecanoylphorbol 13-acetate and clofibrate] have been tested for their ability to induce morphological transformation and affect intercellular communication in Syrian hamster embryo cells. The substances were selected on the basis of being proven or suspected non-genotoxic carcinogens, and thus difficult to detect in short-term tests. The data show that nine of the 13 compounds induced morphological transformation, and seven of the 13 inhibited intercellular communication in hamster embryo cells. Taken together, 12 of the 13 substances either induced transformation or caused inhibition of communication. The data suggest that the combined use of morphological transformation and gap junction intercellular communication in Syrian hamster embryo cells may be beneficial when screening for non-genotoxic carcinogens.

Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, Persons DL, Smith PG, Terranova PF. **Development of a syngeneic mouse model for events related to ovarian cancer.** Carcinogenesis 2000;21(4):585-91.
Abstract: Mouse ovarian surface epithelial cells (MOSEC) were obtained from virgin, mature mice by mild trypsinization and were repeatedly passaged in vitro. Early passage cells (Z20 passages) exhibited a cobblestone morphology and contact inhibition of growth. After approximately 20 passages in vitro, cobblestone morphology and contact inhibition of growth was lost. Tumor forming potential was determined by s.c. and i.p. injection of early and late passage cells into athymic and syngeneic C57BL6

mice. Subcutaneous tumors formed in approximately 4 months and were present only at the injection site. Intraperitoneal injection of late passage MOSEC into athymic and syngeneic mice resulted in growth of tumor implants throughout the abdominal cavity, and production of hemorrhagic ascitic fluid. Early passage MOSEC did not form tumors in vivo. Histopathologic analysis of tumors revealed a highly malignant neoplasm containing both carcinomatous and sarcomatous components. Late passage MOSEC expressed cytokeratin and did not produce ovarian steroids in response to gonadotropin stimulation in vitro. Ten clonal lines were established from late passage MOSEC. Each clone formed multiple peritoneal tumors and ascitic fluid after i.p. injection into C57BL6 mice. Three cell lines examined cytogenetically were polyploid with near-tetraploid modal chromosome numbers. Common clonal chromosome gains and losses included +5, +15, +19 and -X, -3, -4. One cell line had a clonal translocation between chromosomes 15 and 18 and another had a small marker chromosome; common structural abnormalities were not observed. These data describe the development of a mouse model for the study of events related to ovarian cancer in humans. The ability of the MOSEC to form extensive tumors within the peritoneal cavity, similar to those seen in women with Stage III and IV cancer, and the ability of the MOSEC to produce tumors in mice with intact immune systems, makes this model unique for investigations of molecular and immune interactions in ovarian cancer development.

Salter ER, Tichansky D, Furth EE, Herlyn AM. **Tumor-associated antigen expression and growth requirements predict tumorigenesis in squamous cell carcinoma.** In Vitro Cell Dev Biol Anim 2001;37(8):530-5.

Abstract: Squamous cell carcinomas (SCCs) are the most common malignancies in man. While clinical specimens are theoretically ideal to study tumor development and progression, practical difficulties such as normal cell contamination, the presence of different cell types. and limited material make preclinical studies of model systems involving a homogeneous population of normal or transformed cells preferable. Tumor-associated antigens (TAAs) found on the cell surface, including integrins, mucins, cadherins, growth factor receptors, membrane bound antigens, and glycoproteins are known to play an important role in squamous carcinogenesis. We hypothesized that (1) alterations in TAA expression in vitro predict in vivo alterations, (2) analysis of a group of TAAs would provide a better indication of SCC tumorigenesis than any single marker, and (3) SCCs with independence from exogenous growth factors in vitro would demonstrate the most aggressive growth in vivo. The cell line which grew best in vitro without serum or other supplements demonstrated the most rapid tumor growth. whereas cell lines which grew only with supplements rarely formed tumors. Normal keratinocytes. eight SCC and two immortal keratinocyte cell lines were evaluated by flow cytometry for the expression of 10 cell surface markers, including alpha and beta integrins, minor blood group-related carbohydrate determinants. carcinoembryonic antigen-related proteins, E-cadherin, and GA733 (epithelial glycoprotein. epithelial cell adhesion molecule). None of the cell lines with abnormal expression of < or = 2 markers formed tumors, whereas all lines with altered expression of > or = 3 markers formed tumors. Using GA733 expression as an example, we found that altered TAA expression in vitro predicted the presence of TAA alterations in clinical specimens. In summary, in vitro independence from supplements for optimal growth and altered expression of > or = 3 cell surface markers were good predictors of SCC tumorigenesis. These findings may be useful in decreasing the need for whole animal tumorigenicity experiments.

Sasaguri K, Ganss B, Sodek J, Chen JK. **Expression of bone sialoprotein in mineralized tissues of tooth and bone and in buccal-pouch carcinomas of Syrian golden hamsters.** Arch Oral Biol 2000;45 (7):551-62.
Abstract: The expression of bone sialoprotein (BSP) is normally restricted to mineralized connective tissues of bones and teeth where it has been associated with mineral crystal formation. However, recent studies have revealed ectopic expression of BSP in various lesions, including oral and extraoral carcinomas, in which it has been associated with the formation of microcrystalline deposits and the metastasis of cancer cells to bone. To develop a model to study the induction of BSP in carcinoma development, BSP expression in squamous-cell carcinomas induced by chemical carcinogen in the hamster cheek-pouch epithelium was investigated. Hamster BSP cDNA was first isolated and characterized, then used to prepare probes for Northern and in situ hybridization. The protein sequence of hamster BSP displayed 86% amino acid identity with a consensus mammalian BSP sequence and retained polyglutamate sequences, the RGD sequence and sites of phosphorylation, glycosylation and sulphation. The tissue-specific expression of hamster BSP mRNA and protein was confirmed by in situ hybridization and immunolocalization in developing tissues. Squamous-cell carcinomas induced in the buccal pouches of 5-week-old male Syrian golden hamsters treated with chemical carcinogen had BSP mRNA and BSP in the proliferating neoplastic epithelium. In contrast, neither BSP mRNA nor the protein could be detected in the stroma within which islands of the transformed tissue had formed. Thus, the hamster cheek pouch is a well-characterized model that can be used to study the induced expression of BSP in association with the development of squamous-cell carcinomas.

Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ, Hall EJ. **The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells.** Radiat Res 2001;155(3):397-401.
Abstract: It has long been accepted that radiation-induced genetic effects require that DNA be hit and damaged directly by the radiation. Recently, evidence has accumulated that in cell populations exposed to low doses of alpha pArticles, biological effects occur in a larger proportion of cells than are estimated to have been traversed by alpha pArticles. The end points observed include chromosome aberrations, mutations and gene expression. The development of a fast single-cell microbeam now makes it possible to expose a precisely known proportion of cells in a population to exactly defined numbers of alpha pArticles, and to assay for oncogenic transformation. The single-cell microbeam delivered no, one, two, four or eight alpha pArticles through the nuclei of all or just 10% of C3H 10T1/2 cells. We show that (a) more cells can be inactivated than were actually traversed by alpha pArticles and (b) when 10% of the cells on a dish are exposed to alpha pArticles, the resulting frequency of induced transformation is not less than that observed when every cell on the dish is exposed to the same number of alpha pArticles. These observations constitute evidence suggesting a bystander effect, i.e., that unirradiated cells are responding to damage induced in irradiated cells. This bystander effect in a biological system of relevance to carcinogenesis could have significant implications for risk estimation for low-dose radiation.

Schuldiner O, Benvenisty N. **A DNA microarray screen for genes involved in c-MYC and N-MYC oncogenesis in human tumors.** Oncogene 2001;20(36):4984-94.
Abstract: MYC proto-oncogenes play a major role in various types of human tumors. The products of these genes are transcription factors that bind to specific sequences and activate the expression of target

genes. Identifying these target genes and their downstream effectors is a crucial step in understanding and preventing MYC induced oncogenesis. Until now, most of the efforts to identify such genes were performed by analysing in vitro systems whose relevance to the malignant process in vivo remains unclear. We aimed at identifying genes that play a major role in the malignant process of MYC induced carcinogenesis. Thus, we analysed the expression profiles of human MYC induced tumors and compared them to similar, non-MYC tumors. Moreover, we looked for the common characteristics of different types of MYC induced tumors. We identified several genes, most of them involved in cell cycle regulation, that are over expressed in MYC induced lymphomas as well as MYC induced neuronal-like tumors. In order to determine whether MYC induced oncogenesis is similar in human and in the mouse model system, we analysed the expression of the identified genes in cells derived from transgenic mice tumors. We also present the distribution of MYC putative binding sites in the regulatory sequences of the genes identified in our analysis. This analysis pointed to two genes (E2F1 and TSC2) as candidates to be targets of Myc activity. We thus further analysed the expression of these genes in the tumor cell lines, and examined the plausibility that elements in their promoter bind the Myc protein. Our data points to several genes that may be involved in c-MYC and N-MYC induced tumors and to two genes that may be targets for MYC activity.

Shattuck-Brandt RL, Varilek GW, Radhika A, Yang F, Washington MK, DuBois RN. **Cyclooxygenase 2 expression is increased in the stroma of colon carcinomas from IL-10(-/-) mice.** Gastroenterology 2000;118(2):337-45.
Abstract: BACKGROUND & AIMS: The pathological and molecular changes associated with colitis-associated colorectal cancer and sporadic colorectal cancer are considered to be distinct. Therefore, we have used a mouse model of ulcerative colitis to determine if expression of the enzyme cyclooxygenase (COX)-2 is increased in colitis-associated tumors. METHODS: Reverse-transcription polymerase chain reaction and Western analysis were used to determine if COX-2 expression is increased in these tumors; in situ hybridization and immunohistochemistry were used to determine the localization of COX-2. RESULTS: Increased levels of COX-2 messenger RNA and protein were detected in interleukin (IL)-10 (-/-) tumors and in an inflamed region of the colon that contained no macroscopically detected tumors. This expression was localized to the inflammatory cells associated with ulcerated regions of the tumor by in situ hybridization and immunohistochemistry. Increased COX-2 expression was also associated with the areas of the tumor expressing alpha-smooth muscle actin, which is a molecular marker for subepithelial myofibroblasts. The association between COX-2 expression and subepithelial myofibroblasts was also noted in tumors derived from the multiple intestinal neoplasia mice (Min/+) and from carcinogen-induced tumors. CONCLUSIONS: These results indicate that COX-2 is expressed very early in the pathogenesis of colitis-associated tumors, and that the expression pattern is similar to that seen in tumors from azoxymethane-treated and Min/+ mice.

Shen Z, Cen S, Shen J, Cai W, Xu J, Teng Z, Hu Z, Zeng Y. **Study of immortalization and malignant transformation of human embryonic esophageal epithelial cells induced by HPV18 E6E7.** J Cancer Res Clin Oncol 2000;126(10):589-94.
Abstract: In order to study the effect of viruses and tumor promoters on the tumorigenicity of the esophagus, human embryonic esophageal epithelial cells were infected with human papilloma virus HPV18 E6E7-AAV in synergy with 12-O-tetradecanoylphorbol 13-acetate (TPA) to observe their

malignant transformation. The cultured esophageal epithelial cells incubated with HPV18 E6E7-AAV were divided into two groups: the SHEEC1 group was exposed to TPA (5 ng/ml) for 4 weeks at the 5th passage of the cells; the SHEE group served as the control and was cultured in the same medium without TPA. The morphological phenotype, the DNA content during the cell cycle and the chromosomes were analyzed. The tumorigenicity was assessed by colony formation after cultivation in soft agar and transplanting the cells into nude mice. HPV18 E6E7 DNA was assayed by fluorescent in situ hybridization (FISH) and the polymerase chain reaction (PCR). The SHEE group, at its 20th passage, grew as a monolayer with the cells showing anchorage dependence and contact inhibition. The chromosome analysis showed diploidy, and soft-agar cultivation and injection into nude mice showed the cells to be non-tumorigenic. They were therefore immortalized cells. In contrast, the SHEEC1 group (TPA group) showed increased DNA synthesis and a proliferative index that was higher (45%) than that of the SHEE group (34%). The number of large colonies of dense multilayer cells (positively transformed foci) in soft agar was high in SHEEC1 group (4.0%) but low in the SHEE group (0.1%). Tumors resulting from transplantation were observed in all six nude mice injected subcutaneously with cells of the SHEEC1 group but no tumor developed in mice receiving cells of the SHEE group. In both groups of cells, HPV18 E6E7 DNA was positively detected by FISH and PCR. The malignant transformation of human embryonic epithelial cells was induced in vitro by HPV18 E6E7 in synergy with TPA. This is a good evidence for the close relationship between HPV and the etiology and pathogenicity of esophageal carcinoma. It is also a reliable model for studying the cellular and molecular mechanisms of carcinogenesis of esophageal carcinoma.

Smith WA, Freeman JW, Gupta RC. **Effect of chemopreventive agents on DNA adduction induced by the potent mammary carcinogen dibenzo[a,l]pyrene in the human breast cells MCF-7.** Mutat Res 2001;480-481:97-108.
Abstract: Over 1500 structurally diverse chemicals have been identified which have potential cancer chemopreventive properties. The efficacy and mechanisms of this growing list of chemoprotective agents may be studied using short-term bioassays that employ relevant end-points of the carcinogenic process. In this study, we have examined the effects of eight potential chemopreventive agents, N-acetylcysteine (NAC), benzylisocyanate (BIC), chlorophyllin, curcumin, 1,2-dithiole-3-thione (D3T), ellagic acid, genistein, and oltipraz, on DNA adduction of the potent mammary carcinogen dibenzo[a,l]pyrene (DBP) using the human breast cell line MCF-7. Bioactivation of DBP by MCF-7 cells resulted in the formation of one predominant (55%) dA-derived and several other dA- or dG-derived DNA adducts. Three test agents, oltipraz, D3T, and chlorophyllin substantially (>65%) inhibited DBP-DNA adduction at the highest dose tested (30 microM). These agents also significantly inhibited DBP adduct levels at a lower dose of 15 microM, while oltipraz was effective even at the lowest dose of 5 microM. Two other agents, genistein and ellagic acid were moderate (45%) DBP-DNA adduct inhibitors at the highest dose tested, while NAC, curcumin, and BIC were ineffective. These studies indicate that the MCF-7 cell line is an applicable model to study the efficacy of cancer chemopreventive agents in a human setting. Moreover, this model may also provide information regarding the effect of the test agents on carcinogen bioactivation and detoxification enzymes.

Spalding JW, French JE, Stasiewicz S, Furedi-Machacek M, Conner F, Tice RR, Tennant RW. **Responses of transgenic mouse lines p53(+/-) and Tg.AC to agents tested in conventional**

carcinogenicity bioassays. Toxicol Sci 2000;53(2):213-23.
Abstract: The haplo-insufficient p53 knockout (p53+/-) and zetaglobin v-Ha-ras (Tg.AC) transgenic mouse models were compared to the conventional two rodent species carcinogen bioassay by prospectively testing nine chemicals. Seven of the chemicals classified as carcinogens in the conventional bioassay induced tumors in the liver or kidneys of B6C3F1 mice, and one (pentachlorophenol) also induced tumors in other tissues. Only three chemicals, furfuryl alcohol, pyridine, and pentachlorophenol, induced tumors in rats. The tumorigenic effect of pyridine was seen in F344 rats but not in Wistar strain rats. None of the chemicals induced tumors in the p53+/- transgenic mice, which is consistent with the absence of genotoxicity of these chemicals. Only two of the seven nongenotoxic carcinogens were positive in the Tg.AC model (lauric acid diethanolamine and pentachlorophenol). These results show that these transgenic models do not respond to many chemicals that show strain- or species-specific responses in conventional bioassays.

Storer RD, French JE, Haseman J, Hajian G, LeGrand EK, Long GG, Mixson LA, Ochoa R, Sagartz JE, Soper KA. **P53+/- hemizygous knockout mouse: overview of available data.** Toxicol Pathol 2001;29 Suppl:30-50.
Abstract: The performance of the p53-/- transgenic (knockout) mouse model was evaluated through review of the data from 31 short-term carcinogenicity studies with 21 compounds tested as part of the International Life Sciences Institute's (ILSI) Alternatives to Carcinogenicity Testing (ACT) project, together with data from other studies which used comparable protocols. As expected based on the hypothesis for the model, a significant number (12/16 or 75%) of the genotoxic human and/or rodent carcinogens tested were positive and the positive control, p-cresidine, gave reproducible responses across laboratories (18/19 studies positive in bladder). An immunosuppressive human carcinogen, cyclosporin A, was positive for lymphomas but produced a similar response in wild type mice. Two hormones that are human tumorigens, diethylstilbestrol and 17beta-estradiol, gave positive and equivocal results, respectively, in the pituitary with p53-deficient mice showing a greater incidence of proliferative lesions than wild type. None of the 22 nongenotoxic rodent carcinogens that have been tested produced a positive response but 2 compounds in this category, chloroform and diethylhexylphthalate, were judged equivocal based on effects in liver and kidney respectively. Four genotoxic noncarcinogens and 6 nongenotoxic, noncarcinogens were also negative. In total (excluding compounds with equivocal results), 42 of 48 compounds or 88% gave results that were concordant with expectations. The technical lessons learned from the ILSI ACT-sponsored testing in the p53+/- model are discussed.

Tagliaferri F, Teodori L, Valente MG, Stipa F, Cucina A, Gohde W, Colettii D, Alo P, Stipa S. **In vitro proliferation and in vivo malignancy of cell lines simultaneously derived from a chemically-induced heterogeneous rat mammary tumor.** In Vitro Cell Dev Biol Anim 2000;36(3):163-6.
Abstract: Identification of clones in primary tumors responsible for proliferation, invasion, and metastasis was carried out. Four different aneuploid established cell lines derived from a ductal infiltrating mammary rat tumor induced by 7,12-dimethylbenz[a]anthracene were studied for proliferative and growth features in vitro and for tumorigenic and metastatic potential in vivo in nude mice. Clones, named RM1, RM2, RM3, and RM4, were characterized by different proliferative activity. Clone RM1 showed the highest proliferative activity by both tritiated thymidine incorporation and S-

phase flow cytometry, followed by clone RM4. Conversely, clones RM2 and RM3 showed a lower proliferation rate. Growth-promoting activity, tested on 3T3 Swiss cells, was high in all clones, although RM1 showed significantly lower growth factors-releasing activity. Nude mice tumorigenesis demonstrated a strong tumor induction of line RM1 (100% of the mice after 47 +/- 7 d) and a slightly lower tumor induction of line RM4 (70% of the mice after 69 +/- 9 d). Line RM3 showed tumor induction in 40% of the mice after 186 +/- 16 d. Lines RM2 showed no tumor induction. Metastasis occurred in mice treated with line RM1 only. Therefore, tumorigenesis and metastasis correlate with proliferation but not with the release of growth factors. In conclusion, flow cytometry monitoring of clones from heterogeneous primary tumors proved to be a suitable model for the study of in vivo malignancy and in vitro proliferation.

Thielecke H, Mack A, Robitzki A. **Biohybrid microarrays--impedimetric biosensors with 3D in vitro tissues for toxicological and biomedical screening.** Fresenius J Anal Chem 2001;369(1):23-9.
Abstract: To investigate the effectiveness of potential anticancer therapeutics or therapies, efficient screening methods are required. On the one hand, multicellular 3D aggregates (spheroids) are a powerful in vitro model for simulating the in vivo situation and on the other hand, planar electrode structures are generally highly suitable for automation and parallel testing. Here, the detection of the effect of active substances on spheroids positioned on electrodes of substrate integrated electrode arrays is exemplarily investigated. As a 3D tissue model a reaggregation system of T47D clone 11 tumor cells is used. The effect of cytotoxins (DMSO, Triton X-100) on spheroids can be detected by recording the effective impedance of planar electrodes covered by spheroids. The equivalent circuit model parameter of electrodes covered by cytotoxin treated spheroids are determined from recorded impedance spectra and compared to the parameter of electrodes covered by control spheroids as well as not covered electrodes. Spheroids on electrodes mainly influence the electrode impedance in the frequency range of 10 kHz to 1 MHz. The results are discussed in view of an optimal electrode/spheroid-interface for sensing the effects of therapeutics with high sensitivity.

Thompson KL, Rosenzweig BA, Tsong Y, Sistare FD. **Evaluation of in vitro reporter gene induction assays for use in a rapid prescreen for compound selection to test specificity in the Tg.AC mouse short-term carcinogenicity assay.** Toxicol Sci 2000;57(1):43-53.
Abstract: Under ICH guidelines, short-term carcinogenicity assays such as the Tg.AC assay are allowed alternatives for one species in the 2-year rodent bioassay. The Tg.AC transgenic mouse, which carries the v-Ha-ras oncogene under control of the zeta-globin promoter, develops skin papillomas in response to dermal application of carcinogens and tumor promoters. The appropriate specificity of the Tg.AC model for testing pharmaceuticals has not been systematically evaluated. The selection of candidate test compounds among noncarcinogenic pharmaceuticals would be aided by a high-throughput in vitro prescreen correlative of activity in the in vivo Tg.AC assay. Here we describe the development of a prescreen based on correct response to 24 compounds tested previously in Tg.AC mice. The in vitro prescreens, chosen to reflect molecular pathways possibly involved in Tg.AC papilloma formation, consisted of a zeta-globin promoter-luciferase construct stably expressed in K562 cells (Zeta-Luc) and three of the stress-response element-chloramphenicol acetyltransferase (CAT) fusion constructs stably expressed in HepG2 cells that are part of the CAT-Tox (L)iver assay. The stress response elements chosen were the c-fos promoter, the gadd153 promoter, and p53 response element repeats. Of the four

assays, the gadd153-CAT assay showed the strongest concordance with activity in the Tg.AC assay, correctly classifying 78% of Tg.AC positive and 83% of Tg.AC negative compounds. The correlation was further improved by adding the Zeta-Luc assay as a second-stage screen. These cell-based assays will be used in a novel approach to selecting candidate compounds that challenge the specificity of the Tg.AC assay toward pharmaceuticals.

Torrance CJ, Agrawal V, Vogelstein B, Kinzler KW. **Use of isogenic human cancer cells for high-throughput screening and drug discovery.** Nat Biotechnol 2001;19(10):940-5.
Abstract: Cell-based screening for novel tumor-specific drugs has been compromised by the lack of appropriate control cells. We describe a strategy for drug screening based on isogenic human cancer cell lines in which key tumorigenic genes have been deleted by targeted homologous recombination. As a test case, a yellow fluorescent protein (YFP) expression vector was introduced into the colon cancer cell line DLD-1, and a blue fluorescent protein (BFP) expression vector was introduced into an isogenic derivative in which the mutant K-Ras allele had been deleted. Co-culture of both cell lines allowed facile screening for compounds with selective toxicity toward the mutant Ras genotype. Among 30,000 compounds screened, a novel cytidine nucleoside analog was identified that displayed selective activity in vitro and inhibited tumor xenografts containing mutant Ras. The present data demonstrate a broadly applicable approach for mining therapeutic agents targeted to the specific genetic alterations responsible for cancer development.

Trombino AF, Near RI, Matulka RA, Yang S, Hafer LJ, Toselli PA, Kim DW, Rogers AE, Sonenshein GE, Sherr DH. **Expression of the aryl hydrocarbon receptor/transcription factor (AhR) and AhR-regulated CYP1 gene transcripts in a rat model of mammary tumorigenesis.** Breast Cancer Res Treat 2000;63(2):117-31.
Abstract: Exposure to ubiquitous environmental chemicals, such as polycyclic aromatic hydrocarbons (PAH), may contribute to human breast cancer. In animals, PAH induce tumors in part by activating the aryl hydrocarbon receptor (AhR)/transcription factor. Historically, investigations into AhR-regulated carcinogenesis have focused on AhR-dependent transcriptional regulation of cytochrome P450 (CYP) enzymes which oxidize PAH to mutagenic intermediates. However, recent studies suggest that the AhR directly regulates cell growth. Given the postulated role of the AhR in carcinogenesis, we predicted that: (1) tissue predisposed to PAH tumorigenesis would express the AhR and (2) aberrant AhR and/or AhR-regulated gene expression would accompany malignant transformation. To test these hypotheses, AhR and CYP1 protein and/or mRNA levels were evaluated in rat mammary tumors induced with 7, 12-dimethylbenz[a]anthracene (DMBA), a prototypic PAH and AhR ligand. Results indicate modest AhR expression in normal mammary myoepithelial and ductal epithelial cells. In contrast, high AhR levels were detected in DMBA-induced tumors. Nuclear AhR localization in tumors suggested constitutive AhR activation. In situ hybridization and quantitative RT-PCR assays indicated high AhR mRNA levels in neoplastic epithelial cells. While both AhR-regulated CYP1A1 and CYP1B1 mRNAs were induced in breast tissue within 6 h of DMBA gavage, only CYP1B1 mRNA remained elevated in tumors. These results: (1) help explain targeting of breast tissue by carcinogenic PAH, (2) imply that AhR and CYP1B1 hyper-expression represent molecular biomarkers for, at least, PAH-induced mammary cell transformation, and (3) suggest mechanisms through which the AhR may contribute to carcinogenesis well after exogenous AhR ligands have been eliminated.

Tsutsumi S, Kuwano H, Morinaga N, Shimura T, Asao T. **Animal model of para-aortic lymph node metastasis.** Cancer Lett 2001;169(1):77-85.
Abstract: The purpose of this study was to establish a model of experimental lymph node metastasis by intra-rectal implantation of human cancer cells in nude mice. Four types of human cancer cell lines (TE-1, MKN-45, HT-29, and MIAPaca-2) were investigated. Tumor cells suspended in Matrigel were injected into the submucosal layer of the rectum. All cancer cell lines produced locally aggressive rectal tumors and, subsequently, para-aortic lymph node metastasis. We were unable to produce other distant metastases in the dying state in such locations as the liver, spleen, lung, and peritoneum. However, using this method, we were able to evaluate the effect of the anti-cancer agent uracil/tegafur (UFT) on primary tumor growth and lymph node metastasis. Oral intake of UFT significantly suppressed implanted tumor volume and inhibited lymph node metastasis. We expect that the process of lymph node metastasis shown in this model will be studied as an experimental model of lymph node metastasis simulating human cancers.

van Kreijl CF, McAnulty PA, Beems RB, Vynckier A, van Steeg H, Fransson-Steen R, Alden CL, Forster R, van der Laan JW, Vandenberghe J. **Xpa and Xpa/p53+/- knockout mice: overview of available data.** Toxicol Pathol 2001;29 Suppl:117-27.
Abstract: DNA repair deficient Xpa-/- and Xpa-/-/p53+/- knock-out mice in a C57BL/6 genetic background, referred to as respectively the XPA and XPA/p53 model, were investigated in the international collaborative research program coordinated by International Life Sciences Institute (ILSI)/ Health and Environmental Science Institute. From the selected list of 21 ILSI compounds, 13 were tested in the XPA model, and 10 in the XPA/p53 model. With one exception, all studies had a duration of 9 months (39 weeks). The observed spontaneous tumor incidence for the XPA model after 9 months was comparable to that of wild-type mice (total 6%). For the XPA/p53 model, this was somewhat higher (9%/13% for males/females). The 3 positive control compounds used, B[a]P, p-cresidine, and 2-AAF, gave positive and consistent tumor responses in both the XPA and XPA/p53 model, but no or lower responses in wild-type mice. From the 13 ILSI compounds tested, the single genotoxic carcinogen (phenacetin) was negative in both the XPA and XPA/p53 model. Positive tumor responses were observed for 4 compounds, the immunosuppressant cyclosporin A, the hormone carcinogens DES and estradiol, and the peroxisome proliferator WY-14,643. Negative results were obtained with 5 other nongenotoxic rodent carcinogens, and 2 noncarcinogens tested. As expected, both DNA repair deficient models respond to genotoxic carcinogens. Combined with previous results, 6 out of 7 (86%) of the genotoxic human and/or rodent carcinogens tested are positive in the XPA model. The positive results obtained with the 4 mentioned nongenotoxic ILSI compounds may point to other carcinogenic mechanisms involved, or may raise some doubts about their true nongenotoxic nature. In general. the XPA/p53 model appears to be more sensitive to carcinogens than the XPA model.

van Steeg H, de Vries A, van Oostrom CTh, van Benthem J, Beems RB, van Kreijl CF. **DNA repair-deficient Xpa and Xpa/p53+/- knock-out mice: nature of the models.** Toxicol Pathol 2001;29 Suppl:109-16.
Abstract: Xeroderma pigmentosum (XP) is a rare autosomal recessive disease in which repair of ultraviolet (UV)-induced DNA damage is impaired or is totally absent due to mutations in genes

controlling the DNA repair pathway known as nucleotide excision repair (NER). XP is characterized, in part, by extreme sensitivity of the skin to sunlight, and XP patients have a more than 1000-fold increased risk of developing cancer at sun-exposed areas of the skin. To study the role of NER in chemical-induced tumorigenesis in more detail, the authors developed Xpa-/- homozygous knockout mice with a complete defect in NER (designated as Xpa mice or XPA model). Xpa mice develop skin tumors at high frequency when exposed to UV light, and as such, they mimic the phenotype of human XP. Moreover, the Xpa mice also appear to be susceptible to genotoxic carcinogens given orally. Based on these phenotypic characteristics, the Xpa mice were considered to be an attractive candidate mouse model for use in identifying human carcinogens. In an attempt to further increase both the sensitivity and specificity of the XPA model in carcinogenicity testing, the authors crossed Xpa mice with mice having a heterozygous defect in the tumor suppressor gene p53. Xpa/p53+/- double knockout mice develop tumors earlier and with higher incidences upon exposure to carcinogens as compared to their single knockout counterparts. Here the authors describe the development and features of the Xpa mouse and present some examples of the Xpa and Xpa/p53+/- mouse models' sensitivity towards genotoxic carcinogens. It appeared that the Xpa/p53+/- double knockout mouse model is favorable over both the Xpa and p53+/- single knockout models in short-term carcinogenicity testing. In addition to the fact that the double knockout mice respond more robustly to carcinogens, they also appear to respond in a very discriminative way. All compounds identified thus far are true (human) carcinogens, and, therefore, the authors believe that the Xpa/p53+/- mouse model is an excellent candidate for a future replacement of the chronic mouse bioassay, at least for certain classes of chemicals.

Wang Y, Sudilovsky D, Zhang B, Haughney PC, Rosen MA, Wu DS, Cunha TJ, Dahiya R, Cunha GR, Hayward SW. **A human prostatic epithelial model of hormonal carcinogenesis.** Cancer Res 2001;61 (16):6064-72.

Abstract: The effects of stromal and hormonal environment on the immortalized but nontumorigenic human prostatic epithelial cell line BPH-1 were investigated in an in vivo model. BPH-1 cells were recombined with rat urogenital sinus mesenchyme (UGM), and the tissue recombinants were grafted to the renal capsule of adult male athymic mouse hosts. BPH-1 + UGM recombinants formed solid branching epithelial cords with a well-defined basement membrane. The cords canalized to form ductal structures. The mesenchymal cells formed thick sheets of well-differentiated smooth muscle surrounding the epithelium, reinforcing the idea that the epithelium dictates the patterning of prostatic stromal cells. When hosts carrying BPH-1 + UGM tissue recombinants were exposed to testosterone propionate and 17-beta-estradiol (T + E2), the tissue recombinants responded by forming invasive carcinomas, demonstrating mixed, predominantly squamous as well as adenocarcinomatous (small acinar and mucinous) differentiation. When either untreated or T + E2-treated hosts were castrated, epithelial apoptosis was observed in the grafts. When tumors were removed and regrafted to fresh hosts they grew rapidly. Tumors were serially regrafted through six generations. Histologically these tumors consisted largely of focally keratinizing squamous cell carcinoma with high-grade malignant cytological features. BPH-1 cells grown in the absence of UGM survived at the graft site but did not form tumors or organized structures. This behavior was not influenced by the presence or absence of T + E2 stimulation. These data show that an immortalized, nontumorigenic human prostatic epithelial cell line can undergo hormonal carcinogenesis in response to T + E2 stimulation. In addition, the data demonstrate that the stromal environment plays an important role in mediating hormonal carcinogenesis.

Warbrick EV, Dearman RJ, Ashby J, Schmezer P, Kimber I. **Preliminary assessment of the skin sensitizing activity of selected rodent carcinogens using the local lymph node assay.** Toxicology 2001;163(1):63-9.

Abstract: It has been demonstrated previously that there exists an incomplete correlation between the skin sensitizing potential of chemicals and their mutagenic properties as judged by activity in the Salmonella mutation assay. More recently, it has been proposed that there may exist a broader association between carcinogenicity in rodents (including non-genotoxic carcinogenesis) and skin sensitizing activity. To explore further these putative relationships we have here examined the skin sensitizing potential of two non-genotoxic rodent carcinogens which are generally considered not to represent a carcinogenic hazard in humans (limonene and saccharin) and of three genotoxic rodent carcinogens (vinylidene dichloride, ethyl acrylate and bisphenol A diglycidyl ether). For this purpose we have used the local lymph node assay (LLNA), a method for the identification and characterization of skin sensitizing chemicals that has recently been recognized as a stand-alone method for hazard identification purposes. Activity in the LLNA was compared with the results of Salmonella tests conducted previously. This small series of investigations reveals that there exists no general relationship between skin sensitizing potential and rodent carcinogenicity. Furthermore, although a general correlation does exist between mutagenic activity and skin sensitization, this association is not universal and activity in the Salmonella mutation assay does not necessarily imply skin sensitizing potential. Collectively these data suggest that it is inappropriate currently to recommend the use of skin sensitization tests as an adjunct to conventional approaches to the evaluation of potential carcinogenicity.

Webber MM, Quader ST, Kleinman HK, Bello-DeOcampo D, Storto PD, Bice G, DeMendonca-Calaca W, Williams DE. **Human cell lines as an in vitro/in vivo model for prostate carcinogenesis and progression.** Prostate 2001;47(1):1-13.

Abstract: BACKGROUND: The study of prostate carcinogenesis and tumor progression is made difficult by the lack of appropriate in vitro and in vivo models. High prevalence of prostatic intra-epithelial neoplasia and latent prostatic carcinoma, representing multiple steps in carcinogenesis to invasive carcinoma, are relevant targets for cancer prevention. From the RWPE-1, immortalized, non-tumorigenic, human prostate epithelial cell line, we have derived four tumorigenic cell lines with progressive malignant characteristics. METHODS: Cell lines were derived by exposure of RWPE-1 to N-methyl-N-nitrosourea (MNU), selected and cloned in vivo and in vitro, and characterized by prostatic epithelial and differentiation markers, karyotype analysis, anchorage-independent growth, invasiveness, tumorigenicity, and pathology of the derived tumors. RESULTS: Cytokeratins 8 and 18, androgen receptor, and prostate-specific antigen expression in response to androgen, confirm prostatic epithelial origin. RWPE-1 cells do not grow in agar and are not tumorigenic in mice, but the growth, tumorigenicity, and tumor pathology of the MNU cell lines correlate with their invasive ability. The WPE1-NA22 (least malignant) form small, well-differentiated, and WPE1-NB26 cells (most malignant) form large, poorly differentiated, invasive tumors. Overall, loss of heterozygosity for chromosomes 7q, 13q, 18q, and 22, and gain of 5, 9q, 11q, and 20, was observed. The MNU cell lines, in order of increasing malignancy are; WPE1-NA22, WPE1-NB14, WPE1-NB11, and WPE1-NB26. CONCLUSIONS: This family of cell lines with a common lineage represents a unique and relevant model which mimics stages in prostatic intra-epithelial neoplasia (PIN) and progression to invasive

cancer, and can be used to study carcinogenesis, progression, intervention, and chemoprevention.

Wiese FW, Thompson PA, Kadlubar FF. **Carcinogen substrate specificity of human COX-1 and COX-2.** Carcinogenesis 2001;22(1):5-10.
Abstract: The activation of carcinogenic aromatic and heterocyclic amines and benzo[a]pyrene-7,8-diol to intracellular electrophiles by prostaglandin H synthase (COX) is well documented for ovine sources of this enzyme. Here, the arachidonic acid-dependent activation of substrates by human (h)COX-1 and-2 is examined, utilizing recombinant enzymes. The COX-dependent activation of benzidine (BZ), 4-aminobiphenyl, (+)benzo[a]pyrene-7,8-diol, (+)benzo[a]pyrene-7,8-diol, 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx), 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), and 4,4'-methylenebis(2-chloroaniline) (MOCA) is assessed by means of COX-catalyzed, covalent DNA binding. The hCOX isozymes activated all substrates tested, activation varied from barely detectable for IQ (0.76 and 1.52 pmol bound/mg DNA for COX-1 and -2, respectively) to a high of 65 and 117 pmol bound/mg DNA for COX-1 and -2, respectively, for the activation of MOCA. BZ, which is an excellent peroxidase substrate, did not exhibit high DNA binding levels in hCOX assays and this phenomenon was found to be due to high levels of binding to protein, which effectively competed with the DNA for binding in the assay. The demonstrated ability of the COX enzymes to activate a variety of environmental and dietary carcinogens indicates a potential role for COX in the activation pathway of aromatic and heterocyclic amines and polycyclic hydrocarbons at extra-hepatic sites during early or late stages of carcinogenesis.

Ashby J. **Expectations for transgenic rodent cancer bioassay models.** Toxicol Pathol 2001;29 Suppl:177-82.
Abstract: The results of the present study have advanced dramatically the database on transgenic mouse abbreviated carcinogenicity bioassay models. As such, it will provide a secure foundation for future evaluations of these assays and for their eventual validation as models for the prediction of possible human carcinogens. Based upon the results derived from the present study, it is suggested that 5 areas require discussion as a prelude to the further evaluation of existing models and the future evaluation of new models. First, there is the need to agree a standard list of calibration chemicals to be studied and to derive agreement on optimal bioassay group sizes, statistical methods, and exposure periods. Second, general agreement must be reached regarding the classes/types of known rodent carcinogens so that it is acceptable for the new models to find negative, by implication, those rodent carcinogens considered not to pose a carcinogenic hazard to humans. Third, current understanding of mechanisms of carcinogenesis should be integrated into the evaluation of new bioassay models. Fourth, any changes made to the standard rodent carcinogenicity bioassay protocol will require compromises being made, and these should be commonly owned between interested parties in order to reduce the number of regional/agency-specific carcinogenicity testing schemes. Fifth, a mechanism needs to be developed by which assays can be adopted or rejected for use in the routine bioassay of chemicals. In the absence of such initiatives the increasing number of new bioassay models will come to exist along side of the standard 2-species bioassay, and this may potentially lead to confusion regarding the true future role of these assays.

Bahler D, Stone B, Wellington C, Bristol DW. **Symbolic, neural, and Bayesian machine learning models for predicting carcinogenicity of chemical compounds.** J Chem Inf Comput Sci 2000;40

(4):906-14.
Abstract: Experimental programs have been underway for several years to determine the environmental effects of chemical compounds, mixtures, and the like. Among these programs is the National Toxicology Program (NTP) on rodent carcinogenicity. Because these experiments are costly and time-consuming, the rate at which test Articles (i.e., chemicals) can be tested is limited. The ability to predict the outcome of the analysis at various points in the process would facilitate informed decisions about the allocation of testing resources. To assist human experts in organizing an empirical testing regime, and to try to shed light on mechanisms of toxicity, we constructed toxicity models using various machine learning and data mining methods, both existing and those of our own devising. These models took the form of decision trees, rule sets, neural networks, rules extracted from trained neural networks, and Bayesian classifiers. As a training set, we used recent results from rodent carcinogenicity bioassays conducted by the NTP on 226 test Articles. We performed 10-way cross-validation on each of our models to approximate their expected error rates on unseen data. The data set consists of physical-chemical parameters of test Articles, alerting chemical substructures, salmonella mutagenicity assay results, subchronic histopathology data, and information on route, strain, and sex/species for 744 individual experiments. These results contribute to the ongoing process of evaluating and interpreting the data collected from chemical toxicity studies.

Bannasch P, Nehrbass D, Kopp-Schneider A. **Significance of hepatic preneoplasia for cancer chemoprevention.** IARC Sci Publ 2001;154:223-40.
Abstract: Hepatic preneoplasia represents an early stage in neoplastic development, preceding both benign and malignant neoplasia. This applies parcticularly to foci of altered hepatocytes (FAH), that precede the manifestation of hepatocellular adenomas and carcinomas in all species investigated. Morphological, microbiochemical and molecular biological approaches in situ have provided evidence for striking similarities in specific changes of the cellular phenotype of preneoplastic FAH emerging in experimental and human hepatocarcinogenesis, irrespective of whether this was elicited by chemicals, hormones, radiation, viruses or, in animal models, by transgenic oncogenes or Helicobacter hepaticus. Different types of FAH have been distinguished and related to three main preneoplastic hepatocellular lineages: (1) the glycogenotic-basophilic cell lineage, (2) its xenomorphic-tigroid cell variant, and (3) the amphophilic-basophilic cell lineage. The predominant glycogenotic-basophilic and tigroid cell lineages develop especially after exposure to DNA-reactive chemicals, radiation, hepadnaviridae, transgenic oncogenes and local hyperinsulinism, their phenotype indicating initiation by insulin or insulinomimetic effects of the oncogenic agents. In contrast, the amphophilic cell lineage of hepatocarcinogenesis has been observed mainly after exposure of rodents to peroxisome proliferators that are not directly DNA-reactive or to hepadnaviridae, the biochemical pattern mimicking an effect of thyroid hormone, including mitochondrial proliferation and activation of mitochondrial enzymes. Hepatic preneoplastic lesions are increasingly used as end-points in carcinogenicity testing, parcticularly in medium-term carcinogenesis bioassays. This has been complemented more recently by the use of FAH as indicators of chemoprevention, although possible pitfalls of this approach have to be considered carefully. Our ever-increasing knowledge on the metabolic and molecular changes that characterize preneoplastic lesions and their progression to neoplasia provides a new basis for rational approaches to chemoprevention by drugs, hormones or components of the diet.

Brooks N, McHugh PJ, Lee M, Hartley JA. **Alteration in the choice of DNA repair pathway with increasing sequence selective DNA alkylation in the minor groove.** Chem Biol 2000;7(9):659-68.
Abstract: BACKGROUND: Many conventional DNA alkylating anticancer drugs form adducts in the major groove of DNA. These are known to be chiefly repaired by both nucleotide (NER) and base (BER) excision repair in eukaryotic cells. Much less is known about the repair pathways acting on sequence specific minor groove purine adducts, which result from a promising new class of anti-tumour agents. RESULTS: Benzoic acid mustards (BAMs) tethering 1-3 pyrrole units (compounds 1, 2 and 3) show increasing DNA sequence selectivity for alkylation from BAM and 1, alkylating primarily at guanine-N7 in the major groove, to 3 which is selective for alkylation in the minor groove at purine-N3 in the sequence 5'-TTTTGPu (Pu=guanine or adenine). This increasing sequence selectivity is reflected in increased toxicity in human cells. In the yeast Saccharomyces cerevisiae, the repair of untargeted DNA adducts produced by BAM, 1 and 2 depends upon both the NER and BER pathways. In contrast, the repair of the sequence specific minor groove adducts of 3 does not involve known BER or NER activities. In addition, neither recombination nor mismatch repair are involved. Two disruptants from the RAD6 mutagenesis defective epistasis group (rad6 and rad18), however, showed increased sensitivity to 3. In parcticular, the rad18 mutant was over three orders of magnitude more sensitive to 3 compared to its isogenic parent, and 3 was highly mutagenic in the absence of RAD18. Elimination of the sequence specific DNA adducts formed by 3 was observed in the wild type strain, but these lesions persisted in the rad18 mutant. CONCLUSIONS: We have demonstrated that the repair of DNA adducts produced by the highly sequence specific minor groove alkylating agent 3 involves an error free adduct elimination pathway dependent on the Rad18 protein. This represents the first systematic analysis of the cellular pathways which modulate sensitivity to this new class of DNA sequence specific drugs, and indicates that the enhanced cytotoxicity of certain sequence specific minor groove adducts in DNA is the result of evasion of the common excision repair pathways.

Bucher JR. **Doses in rodent cancer studies: sorting fact from fiction.** Drug Metab Rev 2000;32 (2):153-63.
Abstract: The belief that rodent cancer bioassays predict for human cancers is a fundamental public health precept based on sound biological principles. Nonetheless, it is appropriate to periodically debate this point as scientific understanding of cancer causation advances. This presentation addresses one of the many factors that determines the predictive value of rodent tumor bioassay results for human health. This is the issue of dose. Examination of several recent National Toxicology Program (NTP) studies demonstrates that the applied dose often far overestimates the actual effective dose, or maximum blood concentration attained in a rodent, when compared with similar relationships in humans. Further examination of the NTP database on rodent toxicity and carcinogenicity studies revealed summary information on factors that were pivotal in prechronic studies for selecting doses for chronic studies. Contrary to popular belief, target organ toxicity was a determining factor in only about half of the studies. The typically minimal nature of the lesions which limit doses for chronic studies is described for several common target sites. Taken together, these facts paint a far different picture than the common public perception of the "massive" doses used in chronic rodent studies and suggest that, in some cases, dose limitations are actually so severe as to limit the sensitivity of a chronic bioassay to detect a carcinogenic effect.

de Boer JG. **Protection by dietary compounds against mutation in a transgenic rodent.** J Nutr 2001;131(11):3082S-6S.
Abstract: One of the most relevant biomarkers of genotoxicity and, potentially, carcinogenesis is the occurrence of mutations. Data indicate that carcinogens are highly specific with regard to their target tissue in inducing both tumors and mutations. This specificity may reflect the dependence on tissue-specific metabolic activation, the organ-specific environment or both. Ideally, therefore, mutation should be determined in a real animal rather than in a cell culture system. The lacI transgenic rodent model provides such a system. We have used this model to investigate tissue, species and sex specificity of mutation induced by selected dietary carcinogens and to examine how some compounds may alter the induction of mutation. We have studied mutation using several chemicals, including the dietary heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), the environmentally important aromatic hydrocarbon benzo[a]pyrene and the food contaminant aflatoxin B(1). We have shown that the mutagenic potency of these chemicals can be modulated by other dietary compounds, including green tea and conjugated linoleic acid, and the dioxin 2,3,7,8-tetrachlorodibenzo[b,e][1,4] dioxin (TCDD). These results demonstrate that the lacI transgenic rodent is a useful model for the study of chemoprevention in vivo.

Di Tomaso E, Pang JC, Lam HK, Tian XX, Suen KW, Hui AB, Hjelm NM. **Establishment and characterization of a human cell line from paediatric cerebellar glioblastoma multiforme.** Neuropathol Appl Neurobiol 2000;26(1):22-30.
Abstract: Permanent glioma cell lines are invaluable tools in understanding the biology of glioblastomas. The present study reports the establishment of a clonal human cell line, GBM6840, derived from a biopsy of paediatric cerebellar glioblastoma multiforme. GBM6840 had a doubling time of 32 h and grew as a monolayer of large round cells that retained immunopositivity for glial fibrillary acidic protein and vimentin. Karyotypic analysis revealed a modal chromosome number of 68 and polysomies of chromosomes 3, 5 and 20, as well as the presence of 3-4 marker chromosomes. GBM6840 also showed anchorage-independent growth in soft agar and tumour formation in nude mice. The p16(CDKN2A) gene was transcriptionally silenced by hypermethylation, consistent with the lack of protein expression observed in the original tumour and cultured cells. Western blot analysis revealed normal protein expression of pRb and CDK4. It appears that p16 is the major component altered in the cell cycle pathway and may confer these cells unrestrained proliferation potential. Neither EGFR gene amplification nor over-expression of the protein was detected in the cultured cells. Over-expression of the p53 protein was observed in the majority of cells, despite undetectable mutation (exons 5-8) in the gene. One allele of the PTEN gene was found to be mutated during in vitro cultivation. Telomerase activity was demonstrated in the cultured cells but not in the original tumour, supporting the hypothesis that telomerase is required for the in vitro immortalization process.

Dunson DB, Haseman JK, van Birgelen AP, Stasiewicz S, Tennant RW. **Statistical analysis of skin tumor data from Tg.AC mouse bioassays.** Toxicol Sci 2000;55(2):293-302.
Abstract: New strategies for identifying chemical carcinogens and assessing risk have been proposed based on the Tg.AC (zetaglobin promoted v-Ha-ras) transgenic mouse. Preliminary studies suggest that the Tg. AC mouse bioassay may be an effective means of quickly evaluating the carcinogenic potential of a test agent. The skin of the Tg.AC mouse is genetically initiated, and the induction of epidermal

papillomas in response to dermal or oral exposure to a chemical agent acts as a reporter phenotype of the activity of the test chemical. In Tg.AC mouse bioassays, the test agent is typically applied topically for up to 26 weeks, and the number of papillomas in the treated area is counted weekly. Statistical analyses are complicated by within-animal and serial dependency in the papilloma counts, survival differences between animals, and missing data. In this paper, we describe a statistical model for the analysis of skin tumor data from a Tg.AC mouse bioassay. The model separates effects on papilloma latency and multiplicity and accommodates important features of the data, including variability in expression of the transgene and dependency in the tumor counts. Methods are described for carcinogenicity testing and risk assessment. We illustrate our approach using data from a study of the effect of 2,3,7, 8-tetrachlorodibenzo-p-dioxin (TCDD) exposure on tumorigenesis.

Eastin WC, Mennear JH, Tennant RW, Stoll RE, Branstetter DG, Bucher JR, McCullough B, Binder RL, Spalding JW, Mahler JF. **Tg.AC genetically altered mouse: assay working group overview of available data.** Toxicol Pathol 2001;29 Suppl:60-80.
Abstract: In a Government/Industry/Academic partnership to evaluate alternative approaches to carcinogenicity testing, 21 pharmaceutical agents representing a variety of chemical and pharmacological classes and possessing known human and or rodent carcinogenic potential were selected for study in several rodent models. The studies from this partnership project, coordinated by the International Life Sciences Institute, provide additional data to better understand the models' limitations and sensitivity in identifying carcinogens. The results of these alternative model studies were reviewed by members of Assay Working Groups (AWG) composed of scientists from government and industry with expertise in toxicology, genetics, statistics, and pathology. The Tg.AC genetically manipulated mouse was one of the models selected for this project based on previous studies indicating that Tg.AC mice seem to respond to topical application of either mutagenic or nonmutagenic carcinogens with papilloma formation at the site of application. This communication describes the results and AWG interpretations of studies conducted on 14 chemicals administered by the topical and oral (gavage and/or diet) routes to Tg.AC genetically manipulated mice. Cyclosporin A, an immunosuppresant human carcinogen, ethinyl estradiol and diethylstilbestrol (human hormone carcinogens) and clofibrate, an hepatocarcinogenic peroxisome proliferator in rodents, were considered clearly positive in the topical studies. In the oral studies, ethinyl estradiol and diethylstilbestrol were negative, cyclosporin was considered equivocal, and results were not available for the clofibrate study. Of the 3 genotoxic human carcinogens (phenacetin, melphalan, and cyclophosphamide), phenacetin was negative by both the topical and oral routes. Melphalan and cyclophosphamide are, respectively, direct and indirect DNA alkylating agents and topical administration of both caused equivocal responses. With the exception of clofibrate, Tg.AC mice did not exhibit tumor responses to the rodent carcinogens that were putative human noncarcinogens, (di(2-ethylhexyl) phthalate, methapyraline HCl, phenobarbital Na, reserpine, sulfamethoxazole or WY-14643, or the nongenotoxic, noncarcinogen, sulfisoxazole) regardless of route of administration. Based on the observed responses in these studies, it was concluded by the AWG that the Tg.AC model was not overly sensitive and possesses utility as an adjunct to the battery of toxicity studies used to establish human carcinogenic risk.

French JE, Lacks GD, Trempus C, Dunnick JK, Foley J, Mahler J, Tice RR, Tennant RW. **Loss of heterozygosity frequency at the Trp53 locus in p53-deficient (+/-) mouse tumors is carcinogen-and**

tissue-dependent. Carcinogenesis 2001;22(1):99-106.
Abstract: Mutagenic carcinogens rapidly induced tumors in the p53 haploinsufficient mouse. Heterozygous p53-deficient (+/-) mice were exposed to different mutagenic carcinogens to determine whether p53 loss of heterozygosity (LOH) was carcinogen-and tissue-dependent. For 26 weeks, C57BL/6 (N5) p53-deficient (+/-) male or female mice were exposed to p-cresidine, benzene or phenolphthalein. Tumors were examined first for loss of the wild-type p53 allele. p-cresidine induced p53 LOH in three of 13 bladder tumors, whereas hepatocellular tumors showed p53 LOH in carcinomas (2/2), but not in adenomas (0/3). Benzene induced p53 LOH in 13 of 16 tumors examined. Finally, phenolphthalein induced p53 LOH in all tumors analyzed (21/21). Analysis of the p-cresidine-induced bladder tumors by cold single-strand conformation polymorphism (SSCP) analysis of exon 4-9 amplicons failed to demonstrate polymorphisms associated with mutations in tumors that retained the p53 wild-type allele. p-cresidine induced a dose-related increase in lacI mutations in bladder DNA. In summary, these data demonstrate that loss of the wild-type allele occurred frequently in thymic lymphomas and sarcomas, but less frequently in carcinomas of the urinary bladder. In the bladder carcinomas other mechanisms may be operational. These might include (i) other mechanisms of p53 inactivation, (ii) inactivating mutations occurring outside exons 4-9 or (iii) p53 haploinsufficiency creating a condition that favors other critical genetic events which drive bladder carcinogenesis, as evidenced by the significant decrease in tumor latency. Understanding the mechanisms of p53 LOH and chemical carcinogenesis in this genetically altered model could lead to better models for prospective identification and understanding of potential human carcinogens and the role of the p53 tumor suppressor gene in different pathways of chemical carcinogenesis.

French JL, Williams PL. **Characterizing dose-response relationships in multiple cancer bioassays.** Risk Anal 2001;21(1):91-102.
Abstract: In the evaluation of chemical compounds for carcinogenic risk, regulatory agencies such as the U.S. Environmental Protection Agency and National Toxicology Program (NTP) have traditionally fit a dose-response model to data from rodent bioassays, and then used the fitted model to estimate a Virtually Safe Dose or the dose corresponding to a very small increase (usually 10(-6)) in risk over background. Much recent interest has been directed at incorporating additional scientific information regarding the properties of the specific chemical under investigation into the risk assessment process, including biological mechanisms of cancer induction, metabolic pathways, and chemical structure and activity. Despite the fact that regulatory agencies are currently poised to allow use of nonlinear dose-response models based on the concept of an underlying threshold for nongenotoxic chemicals, there have been few attempts to investigate the overall relationship between the shape of dose-response curves and mutagenicity. Using data from an historical database of NTP cancer bioassays, the authors conducted a repeated-measures Analysis of the estimated shape from fitting extended Weibull dose-response curves. It was concluded that genotoxic chemicals have dose-response curves that are closer to linear than those for nongenotoxic chemicals, though on average, both types of compounds have dose-response curves that are convex and the effect of genotoxicity is small.

Goodman JI. **A perspective on current and future uses of alternative models for carcinogenicity testing.** Toxicol Pathol 2001;29 Suppl:173-6.
Abstract: This perspective is based upon the data presented at the International Life Sciences Institute

(ILSI), Health and Environmental Sciences Institute Workshop on the Evaluation of Alternative Methods for Carcinogenicity Testing (ILSI Workshop). It is important to understand that all models discussed at the Workshop have limitations and that they are not designed to be employed as stand-alone assays. Although they may have other, appropriate applications. I do not recommend use of the SHE cell assay and the Tg.AC model for the regulatory purposes of a safety assessment. In my view, the neonatal mouse, p53+/-, XPA-/-, XPA-/- and p53+/-, and the rasH2 models can, as a component of an overall assessment, provide information on potential carcinogenicity of a chemical that is appropriate for consideration in a regulatory context. Generally, these models exhibit the ability to detect genotoxic compounds. In most cases these compounds would be detected in a standard battery of genotoxicity tests and, therefore, quite often the use of an alternative is not necessary. Actually, I believe that a bioassay in rats will suffice most of the time, that is, in my view, a routine bioassay in mice is not necessary. Specific circumstances where data obtained from one of the "recommended" alternative models might be helpful are discussed. With regard to lessons for the future, there is a parcticular need for models that are responsive to chemicals that exhibit a nongenotoxic mode of action. Additionally, new models will continue to be developed and their half-life will likely be substantially shorter than the time required for traditional validation. The development of enhanced paradigms for validation should be a priority so that improved safety assessment decisions can be made more quickly. However, while evaluating and validating such models, it is important to consider the fundamental issues, for example, rational dose selection, evaluation of mode of action in the context of dose-response relationships including the existence of thresholds and secondary mechanisms, and species-to-species extrapolation. The alternatives to carcinogenicity testing project was a very major undertaking. In addition to the valuable information provided, it serves to illustrate the value of cooperation between academia, government, and industry. Furthermore, the involvement of the International Life Sciences Institute as the overall organizing, facilitating umbrella was crucial for the success of the project.

Gulezian D, Jacobson-Ram D, McCullough CB, Olson H, Recio L, Robinson D, Storer R, Tennant R, Ward JM, Neumann DA. **Use of transgenic animals for carcinogenicity testing: considerations and implications for risk assessment.** Toxicol Pathol 2000;28(3):482-99.
Abstract: Advances in genetic engineering have created opportunities for improved understanding of the molecular basis of carcinogenesis. Through selective introduction, activation, and inactivation of specific genes, investigators can produce mice of unique genotypes and phenotypes that afford insights into the events and mechanisms responsible for tumor formation. It has been suggested that such animals might be used for routine testing of chemicals to determine their carcinogenic potential because the animals may be mechanistically relevant for understanding and predicting the human response to exposure to the chemical being tested. Before transgenic and knockout mice can be used as an adjunct or alternative to the conventional 2-year rodent bioassay, information related to the animal line to be used, study design, and data analysis and interpretation must be carefully considered. Here, we identify and review such information relative to Tg.AC and rasH2 transgenic mice and p53+/- and XPA-/- knockout mice, all of which have been proposed for use in chemical carcinogenicity testing. In addition, the implications of findings of tumors in transgenic and knockout animals when exposed to chemicals is discussed in the context of human health risk assessment.

Hartwig A, Kasper P, Madle S, Speit G, Staedtler F, Sengstag C. **The potential use of mutation**

spectra in cancer related genes in genetic toxicology: a statement of a GUM working group. Mutat Res 2001;473(2):263-7.
Abstract: In recent years, there has been widespread interest in the relationship between carcinogenic exposure and mutation spectra in cancer-related genes. To evaluate potential benefits and/or limitations in the use of mutation spectra in genetic toxicology, a GUM working group has been established to discuss this subject. Based on methodological possibilities and limitations, the impact of mutation spectra in the interpretation of animal experiments and in the identification of etiological agents in human cancer has been considered. With respect to experimental animals, the analyses of mutation spectra within long-term rodent carcinogenicity studies may provide some additional information on the mode of action of the respective carcinogen, however, the interpretation of results should be done carefully and only in context with other toxicological data available. Regarding human exposure, the analysis of mutation spectra in p53 or ras genes supplies information on the genotoxic properties of the respective agent. Nevertheless, on the individual level, the presence or absence of defined mutations in cancer-related genes in human tumors does not permit a definite conclusion about the causative agent.

Haseman J, Melnick R, Tomatis L, Huff J. **Carcinogenesis bioassays: study duration and biological relevance**. Food Chem Toxicol 2001;39(7):739-44.
Abstract: Criticisms of the scientific value of rodent carcinogenicity bioassays have focused on the arguments that the studies are too long and that most organ-specific carcinogenic effects observed in experimental animals have little or no relevance to humans. For example, Davies et al. (Davies, T.S., Lynch, B.S., Monro, A.M., Munro, I.C., Nestmann, E.R., 2000. Rodent carcinogenicity tests need be no longer than 18 months: an analysis based on 210 chemicals in the IARC Monographs. Food and Chemical Toxicology 38, 219-235) concluded that the duration of rodent bioassays should be no more than 18 months, based on their analysis of 210 International Agency for Research on Cancer (IARC) rodent carcinogens in which they report that most chemicals showed "tumorigenic effects" at or before 12 months. However, many of these "tumorigenic effects" reflect the occurrence of a single neoplasm, with most tumors occurring much later in the study. Reliance on a single tumor at an early time point as providing definitive evidence of rodent carcinogenicity is a dangerous practice that could produce both false positive and false negative outcomes. An extensive evaluation of the NTP database reveals that many rodent carcinogens produce later-appearing tumors that would not be detected as statistically significant in a 12-18 month study. Such a shortened duration study would be roughly equivalent to evaluating human cancer in subjects 30-50 years of age, which would result in markedly reduced study sensitivity. In fact, many investigators recommend extending the duration of rodent studies to 30 months or to a true lifetime to increase study sensitivity. We also do not agree with the second conclusion of Davies et al. (2000) that the mode of action of rodent carcinogenesis is sufficiently well understood to justify discounting the majority of organ-specific carcinogenic effects found in these studies. The consequences of performing rodent carcinogenicity studies with inadequate sensitivity, and then discounting most of the carcinogenic effects that are observed will be that potential human carcinogens will not be detected, thus forcing near total reliance on human studies for this purpose. This is not prudent public health policy.

Hofmanova J, Machala M, Kozubik A. **Epigenetic mechanisms of the carcinogenic effects of xenobiotics and in vitro methods of their detection.** Folia Biol (Praha) 2000;46(5):165-73.

Abstract: Carcinogenesis is associated with various epigenetic mechanisms, which can alter intra- and intercellular communication and gene expression and thus affect cytokinetics, i.e. regulation of cell proliferation, differentiation, and apoptosis. These processes lead to a loss of homeostatic control. In addition to "classical" epigenetic events such as DNA methylation and histone acetylation, the major mechanisms include changes in concentrations of signal molecules (hormones, growth factors, fatty acids, etc.), modulation of cell receptors and drug-, hormone- and fatty acid-metabolizing enzymes, oxidative stress, and interference with intracellular signal transduction pathways. Multidisciplinary and multibiomarker approach is necessary for setting up a battery of specific biochemical, molecular, and cellular in vitro methods detecting the epigenetic carcinogenic potential of individual chemicals or their environmental mixtures. This approach is based on studies of modes of action of xenobiotics at various levels, including the molecular mechanisms and modulations of cytokinetics, each of them having its specific predictive value.

Ito N, Imaida K, Asamoto M, Shirai T. **Early detection of carcinogenic substances and modifiers in rats.** Mutat Res 2000;462(2-3):209-17.
Abstract: Over the past 20 years, we have been developing in vivo medium-term bioassay systems in rats for detecting carcinogenic and modifying effects of test compounds. The systems are based on the two-step hypothesis of carcinogenesis. In a liver model, male F344 rats are initially given a single dose of diethylnitrosamine (DEN, 200 mg/kg, i.p.) and starting 2 weeks later are treated with test compounds for 6 weeks and then killed, all rats being subjected to two-thirds partial hepatectomy at week 3. Carcinogenic potential is scored by comparing the numbers and areas per cm(2) of induced glutathione S-transferase placental form (GST-P) positive foci in the livers of groups of about 15 rats with those of corresponding control groups given DEN alone. A positive response is defined as a significant increase in the quantitative values of GST-P-positive foci, such a negative response as no change or a decrease. The results obtained have been compared with reported Salmonella/microsome and long-term carcinogenicity test findings for the same compounds. Of the liver carcinogens, 30 out of 31 (97%) mutagenic and 29 out of 33 (88%) non-mutagenic compounds gave positive results. Carcinogens other than hepatocarcinogens gave a lower proportion of positive results (9 out of 42, 21%). This bioassay also provides information concerning inhibitory potential. The practical utility and benefits of a multi-organ medium-term experimental protocol for early detection of carcinogenic agents and modifiers acting at sites other than the liver are also discussed.

Johnson FM. **The "rodent carcinogen" dilemma: formidable challenge for the technologies of the new millennium.** Ann N Y Acad Sci 2000;919:288-99.

Kazianis S, Gimenez-Conti I, Setlow RB, Woodhead AD, Harshbarger JC, Trono D, Ledesma M, Nairn RS, Walter RB. **MNU induction of neoplasia in a platyfish model.** Lab Invest 2001;81(9):1191-8.
Abstract: Interspecific hybrid crosses between members of the fish genus Xiphophorus have been used for over 70 years to study the genetic aspects of melanoma formation. In the well-established "Gordon-Kosswig" cross, the platyfish X. maculatus is outcrossed to the swordtail X. helleri, and the resulting backcross segregants spontaneously develop melanoma. We recently produced a distinct cross between X. maculatus and another platyfish species, X. couchianus. X. maculatus strain Jp 163 A is homozygous for several X-linked pigment pattern genes, including the Spotted dorsal (Sd), Dorsal red (Dr), and Anal

fin spot (Af). Af is a sex-limited trait, coding exclusively for melanophores distributed on the modified anal fin or "gonopodium" in the adult male fish. Within F1 and BC1 hybrids (to X. couchianus), the Sd pigment pattern is phenotypically suppressed, whereas Dr and Af are enhanced. We exposed BC1 hybrids to the direct-acting carcinogen N-methyl-N-nitrosourea (MNU). Treatment led to the development of schwannomas, fibrosarcomas, and retinoblastomas. In addition, numerous MNU-treated males that inherited Af developed a pronounced melanotic phenotype, with melanin-containing cells oftentimes totally covering the gonopodium and extending further to grow within the ventral regions of the fish. Genetic linkage analysis of the BC1 hybrids revealed a significant ($p < 0.01$) association between CDKN2X genotype and the phenotypic degree of melanization. Such an association is consistent with a locus within linkage group V playing a role in the development of melanosis and delineates three genetic preconditions and a carcinogenic scheme resulting in melanosis of the ventral regions of hybrid fish. The overall study further alludes to the potential of using Xiphophorus fish to study carcinogenic mechanisms for tumors other than melanoma (schwannoma, fibrosarcoma, and retinoblastoma) and should enable extensive pathologic and molecular genetic studies of derived neoplastic abnormalities.

Kowalski LA. **In vitro carcinogenicity testing: present and future perspectives in pharmaceutical development.** Curr Opin Drug Discov Devel 2001;4(1):29-35.
Abstract: Almost all new drugs must be tested for carcinogenicity at some point during their development, and ultimately, a lifetime in vivo assay, usually in rodents, must be performed. Many in vitro assays of carcinogenicity have been developed for use before short- or long-term in vivo testing in order to remove from the development stream those drugs that are likely to produce tumors in vivo. This review discusses in vitro assays that are required by the International Conference on Harmonization, followed by a discussion of in vitro carcinogenicity assays, which are currently in use, but are not specifically required. The concluding section is devoted to a discussion of high-throughput compatible carcinogenicity screens and potential human cell-based high-throughput compatible screens with reference to future methods in silico.

Kowalski LA, Laitinen AM, Mortazavi-Asl B, Wee RK, Erb HE, Assi KP, Madden Z. **In vitro determination of carcinogenicity of sixty-four compounds using a bovine papillomavirus DNA-carrying C3H/10T(1/2) cell line.** Environ Mol Mutagen 2000;35(4):300-11.
Abstract: A new in vitro test for predicting rodent carcinogenicity is evaluated against a testing database of 64 chemicals including both genotoxic and nongenotoxic carcinogens and carcinogens that normally require addition of an S-9 microsomal fraction for detection in the bacterial mutagenicity assay. The assay uses focus formation in a stable, bovine papillomavirus type 1 (BPV-1) DNA carrying C3H/10T (1/2) mouse embryo fibroblast cell line (T1) that does not require transfection, infection with virus, isolation of primary cells from animals, or addition of a microsomal fraction. Of a total database of 64 compounds, 92% of the carcinogens, promoters, or noncarcinogens were correctly predicted. Based on previously reported results, the test of bacterial mutagenicity would have correctly predicted 58% of carcinogens, promoters or noncarcinogens and the Syrian hamster embryo test would have correctly predicted 87% of carcinogens, promoters, or noncarcinogens of this database. Of carcinogens that normally require addition of an S-9 fraction, T1 cells correctly predicted rodent carcinogenicity of polyaromatic hydrocarbons, aflatoxins, azo-compounds, nitrosamines, and hydrazine without the

addition of an S-9 fraction. Of nongenotoxic carcinogens, T1 cells correctly predicted diethylstilbestroel, diethylhexylphthalate, acetamides, alkyl halides, ethyl carbamate, and phorbol ester tumour promoters.

Kreklau EL, Limp-Foster M, Liu N, Xu Y, Kelley MR, Erickson LC. **A novel fluorometric oligonucleotide assay to measure O(6)-methylguanine DNA methyltransferase, methylpurine DNA glycosylase, 8-oxoguanine DNA glycosylase and abasic endonuclease activities: DNA repair status in human breast carcinoma cells overexpressing methylpurine DNA glycosylase.** Nucleic Acids Res 2001;29(12):2558-66.
Abstract: DNA repair status plays a major role in mutagenesis, carcinogenesis and resistance to genotoxic agents. Because DNA repair processes involve multiple enzymatic steps, understanding cellular DNA repair status has required several assay procedures. We have developed a novel in vitro assay that allows quantitative measurement of alkylation repair via O(6)-methylguanine DNA methyltransferase (MGMT) and base excision repair (BER) involving methylpurine DNA glycosylase (MPG), human 8-oxoguanine DNA glycosylase (hOGG1) and yeast and human abasic endonuclease (APN1 and APE/ref-1, respectively) from a single cell extract. This approach involves preparation of cell extracts in a common buffer in which all of the DNA repair proteins are active and the use of fluorometrically labeled oligonucleotide substrates containing DNA lesions specific to each repair protein. This method enables methylation and BER capacities to be determined rapidly from a small amount of starting sample. In addition, the stability of the fluorometric oligonucleotides precludes the substrate variability caused by continual radiolabeling. In this report this technique was applied to human breast carcinoma MDA-MB231 cells overexpressing human MPG in order to assess whether up-regulation of the initial step in BER alters the activity of selected other BER (hOGG1 and APE/ref-1) or direct reversal (MGMT) repair activities.

Krinke GJ, Kaufmann W, Mahrous AT, Schaetti P. **Morphologic characterization of spontaneous nervous system tumors in mice and rats.** Toxicol Pathol 2000;28(1):178-92.
Abstract: Spontaneous rodent nervous system tumors, in comparison to those of man, are less well differentiated. Among the central nervous system (CNS) tumors, the "embryonic" forms (medulloblastoma, pineoblastoma) occur both in rodents and humans, whereas the human "adult" forms (gliomas, ependymomas, meningiomas) have fewer counterparts in rodents. In general, the incidence of spontaneous CNS tumors is higher in rats (>1%) than in mice (>0.001%). A characteristic rat CNS tumor is the granular cell tumor. Usually it is associated with the meninges, and most meningeal tumors in rats seem to be totally or at least partly composed of granular cells, which have eosinophilic granular cytoplasm, are periodic acid-Schiff reaction (PAS)-positive, and contain lysosomes. Such tumors are frequently found on the cerebellar surface or at the brain basis. Rat astrocytomas are diffuse, frequently multifocal, and they invade perivascular spaces and meninges. The neoplastic cells with round to oval nuclei and indistinct cytoplasm grow around preexisting neurons, producing satellitosis. In large tumors, there are necrotic areas surrounded by palisading cells. Extensive damage of brain tissue is associated with the presence of scavenger cells that react positively with histiocytic/macrophage markers. The neoplastic astrocytes do not stain positively for glial fibrillary acidic protein; they probably represent an immature phenotype. In contrast to neoplastic oligodendroglia, they bind the lectin RCA-1. Astrocytomas are frequently located in the brain stem, especially the basal ganglia. Rat oligodendroglial tumors are well circumscribed and frequently grow in the walls of brain ventricles. Their cells have

water-clear cytoplasm and round, dark-staining nuclei. Atypical vascular endothelial proliferation occurs, especially at the tumor periphery. Occasionally in the oligodendrogliomas, primitive glial elements with large nuclei occur in the form of cell groups that form rows and circles. Primitive neuroectodermal tumors of rats, such as pineal tumors or medulloblastomas, appear to have features similar to those found in man. In mice, the meningeal tumors are mostly devoid of granular cells and the astrocytomas are similar to those occurring in rats, whereas spontaneous oligodendrogliomas are observed extremely rarely. Tumorlike lesions, such as lipomatous hamartomas or epidermoid cysts, are occasionally encountered in the mouse CNS. It is suggested that we classify rodent CNS lesions as "low grade" and "high grade" rather than as "benign" and "malignant." The size of CNS tumors is generally related to their malignancy. Tumors of the peripheral nervous system are schwannomas and neurofibromas or neurofibrosarcomas consisting of Schwann cells, fibroblasts, and perineural cells. Well-differentiated schwannomas are characterized by S-100 positivity and the presence of basement membrane. They show either Antoni A pattern with fusiform palisading cells or Antoni B pattern, which is sparsely cellular and has a clear matrix. The rat develops specific forms of schwannomas in the areas of the submandibular salivary gland, the external ear, the orbit, and the endocardium. Spontaneous ganglioneuromas occur in the rat adrenal medulla or thyroid gland. Compared to experimentally induced neoplasms, the spontaneous tumors of the rodent nervous system are poor and impractical models of human disease, although they may serve as general indicators of the carcinogenic potential of tested chemicals.

Lersch RA, Fung J, Hsieh HB, Smida J, Weier HU. **Monitoring signal transduction in cancer: from chips to fish.** J Histochem Cytochem 2001;49(7):925-6.
Abstract: The microarray format of RNA transcript analysis should provide new clues to carcinogenic processes. Because of the complex and heterogeneous nature of most tumor samples, histochemical techniques, parcticularly RNA fluorescent in situ hybridization (FISH), are required to test the predictions from microarray expression experiments. Here we describe our approach to verify new microarray data by examining RNA expression levels of five to seven different transcripts in a very few cells via FISH. (J Histochem Cytochem 49:925-926, 2001).

Low-Baselli A, Hufnagl K, Parzefall W, Schulte-Hermann R, Grasl-Kraupp B. **Initiated rat hepatocytes in primary culture: a novel tool to study alterations in growth control during the first stage of carcinogenesis.** Carcinogenesis 2000;21(1):79-86.
Abstract: To study growth regulation in the beginning of carcinogenesis, we established a novel ex vivo model for co-cultivation of normal and putatively initiated hepatocytes. Rats received the genotoxic hepatocarcinogen N-nitrosomorpholine (NNM). This led to the appearance of hepatocytes expressing placental glutathione S-transferase (G(+) cells). These cells exhibited elevated rates of cell replication and apoptosis, as known from further advanced preneoplasia; G(+) cells were considered initiated. At days 20-22 post-NNM treatment their frequency was maximal (1-2%); approximately 40% were still single and 60% were arranged in mini foci. At this time-point liver cells were isolated by collagenase perfusion and cultivated. G(+) cells, identified by immunostaining of the culture-plates, were present at the same percentage as in vivo, excluding selective loss, enrichment or spontaneous expression of the G (+) phenotype. In untreated cultures G(+) hepatocytes showed significantly higher rates of replicative DNA synthesis than normal G(-) cells. Application of the hepatomitogen cyproterone acetate (CPA)

elevated DNA replication preferentially in G(+) cells. Transforming growth factor beta1 (TGF-beta1) suppressed replicative DNA synthesis which was more pronounced in G(+) than in G(-) hepatocytes. Combined treatment with CPA and TGF-beta1 had no effect on G- cells, but considerably inhibited DNA replication in G(+) cells. This suggests that the effects of TGF-beta1 predominated in G(+) hepatocytes. We conclude that putatively initiated G(+) hepatocytes, both in vivo and in culture, exhibit higher basal rates of DNA replication than normal G(-) hepatocytes and an over-response to mitogens and growth inhibitors. Therefore, G(+) cells show (i) nearly identical behaviour in intact liver and in primary culture and (ii) inherent defects in growth control that are principally similar although somewhat less pronounced than in later stages of carcinogenesis. The present ex vivo system thus provides a novel and useful tool to elucidate biological and molecular changes during initiation of carcinogenesis.

Mancuso JY, Ahn H, Chen JJ. **Order-restricted dose-related trend tests.** Stat Med 2001;20(15):2305-18.
Abstract: Methods of isotonic regression are proposed to increase the power of common trend tests in situations where a monotonicity constraint is imposed upon the dose-response function. Isotonic versions of Cochran-Armitage type trend tests for binary response data are developed, and the bootstrap method is used in finding the empirical distributions of the test statistics and their critical values. The isotonic likelihood ratio test with a survival adjustment is also proposed. This survival adjustment can be applied to the likelihood ratio test for either the order-restricted or unrestricted parameter cases. To achieve the isotonic modifications, an amalgamation algorithm is applied when the observed dose-response is non-monotonic. A Monte Carlo simulation study comparing these trend tests shows the advantages of the isotonic modifications and survival adjustment. By applying the proposed methods to data from a toxicology and carcinogenesis study conducted as part of the National Toxicology Program, the effect of CI Pigment Red 23 is investigated. Copyright 2001 John Wiley & Sons, Ltd.

Maronpot RR, Mitsumori K, Mann P, Takaoka M, Yamamoto S, Usui T, Okamiya H, Nishikawa S, Nomura T. **Interlaboratory comparison of the CB6F1-Tg rasH2 rapid carcinogenicity testing model.** Toxicology 2000;146(2-3):149-59.
Abstract: Several genetically engineered mouse models are currently being examined for potential use in cancer hazard identification. We have undertaken an interlaboratory comparison of the performance of the CB6F1-Tg rasH2 transgenic mouse in cancer bioassays concurrently conducted in the United States and Japan. Chemicals selected for study included known human carcinogens (melphalan and cyclosporin A) and known rodent carcinogens (p-cresidine and vinyl carbamate) tested at carcinogenic doses, and non-carcinogens (p-anisidine and resorcinol) tested at appropriate high doses. Because of abdominal adhesions caused by the intraperitoneal dosing vehicle, melphalan was excluded from the study results. The remaining five studies showed similar results between the two laboratories conducting each study. Vinyl carbamate gave the strongest positive response inducing lung adenomas and carcinomas and splenic hemangiosarcomas. p-Cresidine was considered positive for urinary bladder transitional neoplasia. Cyclosporin A, p-anisidine, and resorcinol were negative in all studies. Although only five chemicals were successfully tested in this interlaboratory comparison, there was good concordance in outcome for the strong carcinogens and for the non-carcinogens. Successful testing of chemicals with less carcinogenic potential may require modifications in study design to include more animals and

longer study duration.

McClain RM, Keller D, Casciano D, Fu P, MacDonald J, Popp J, Sagartz J. **Neonatal mouse model: review of methods and results.** Toxicol Pathol 2001;29 Suppl:128-37.
Abstract: The neonatal mouse model, in various forms, has been used experimentally since 1959 and a large number of chemicals have been tested. The neonatal model is known to be very sensitive for the detection of carcinogens that operate via a genotoxic mode of action. In contrast, it is known not to respond to chemicals that act via epigenetic mechanisms, commonly observed in the two-year carcinogenicity studies. As such, the model has a high sensitivity and specificity in its response. Dose selection for the neonatal model is based on the maximum tolerated or feasible dose. Traditionally, compounds have been tested via the IP route of administration in this model. In some cases, this has limited the amount of material that can be administered because of the low dosing volumes (10 to 20 microL) that can be administered IP. For the ILSI project, the neonatal model was adapted for oral administration, which has the advantages of being the same route for which most pharmaceuticals are administered. In addition, a 10-fold increase in the volume of administration (100 to 200 microL) and the ability to dose drugs in suspension, permits much higher doses to be used as compared to the IP route of administration. The spontaneous tumors in the neonatal model occurred mainly in the liver of male mice and lung of male and female mice with a few tumors observed in the Harderian gland. The positive control, DEN produced a robust, uniform, and reproducible tumor response with the target organs essentially limited to liver and lung. A total of 13 compounds out of the 21 ILSI ACT compounds were evaluated in the neonatal model involving 18 studies with duplicate studies for some compounds. The genotoxic carcinogens including those used as positive controls were clearly positive (cyclophosphamide, diethylnitrosamine, 6-nitrochrysene). The non-genotoxic rodent carcinogens were clearly negative (chlorpromazine, sulfisoxazole, sulfamethoxazole, clofibrate, DEHP, haloperidol, metaproteranol, and phenobarbital). The non-genotoxic human carcinogen (cyclosporin) was clearly negative. The two other human carcinogens phenacetin and DES were negative and interestingly estradiol was negative in one of the two oral studies, but was clearly positive in the other. Considering the mode of action for three of the human carcinogens (DES, cyclosporin and phenacetin), which were negative in this model, the mode of action in humans is likely to be epigenetic. Overall, for the 3 clearly genotoxic chemicals, all were positive. For the 9 clearly non-genotoxic chemicals, all 9 were negative. The two human carcinogens for which genotoxicity may or may not play a role (DES and phenacetin) were negative and estradiol was positive in I of the two oral studies. Overall, the extensive database for compounds tested in the neonatal mouse model would support its use as an alternative model for the assessment of the carcinogenic potential of a chemical. The model responds to chemicals that act via a genotoxic mode of action that represent a greater concern for human cancer risk.

Moolgavkar SH, Turim J, Brown RC. **The power of the European Union protocol to test for carcinogenicity of inhaled fibers.** Regul Toxicol Pharmacol 2001;33(3):350-5.
Abstract: We evaluate the power of a recent protocol proposed by the European Union (EU) for testing the carcinogenicity of inhaled fibers. We assume that every fiber has oncogenic potential determined by its biopersistence. We use a recently estimated potency for the oncogenic potential of fibers together with experimentally determined "weighted" half-lives (WHL) of a dozen fibers to generate simulations of long-term bioassays conducted according to the EU protocol. We analyze these experiments using

standard statistical techniques and determine the number of tests that would have yielded significant results. We conclude that the EU protocol will readily detect the carcinogenic effect of long-lived fibers, such as amosite with a WHL of more than 450 days, and usually detect the effect of fibers, such as RCF1a, with WHL of about 40-50 days. However, the EU protocol has very low power to detect effects of short-lived fibers, such as X607, with WHL of about 10 days. Copyright 2001 Academic Press.

Nishikawa A, Suzuki T, Masumura K, Furukawa F, Miyauchi M, Nakamura H, Son HY, Nohmi T, Hayashi M, Hirose M. **Reporter gene transgenic mice as a tool for analyzing the molecular mechanisms underlying experimental carcinogenesis.** J Exp Clin Cancer Res 2001;20(1):111-5.
Abstract: Carcinogenic compounds are classified into 2 categories, genotoxic and non-genotoxic, which are basically judged from in vitro genotoxicity data. However, it is well documented that genotoxicants do not necessarily exert in vivo carcinogenicity in rodents, partly because of a discrepancy between in vitro and in vivo mutagenicities. Recently, transgenic animal models with reporter genes such as lacI, lacZ and gpt have been developed as a tool for assessing in vivo mutagenicity as well as carcinogenicity. In this Article, data using lacI transgenic mice and gpt delta mice are presented and their application is discussed. In lacI transgenic mice, dimethylnitrosamine (DMN) treatment significantly increased lacI mutant frequency (MF) in the liver, kidenys and lungs, but not in other non-target organs. Repeated dose ip administration of DMN was more effective than single dose treatment in the induction of lacI MF. The spectrum of mutant plaques induced by DMN was characterized by deletions as well as GC to AT base transitions. The remaining mice receiving DMN proved to have liver adenomas at a high frequency after 78 weeks. Meanwhile, dietary 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MelQx) significantly increased lacI and gpt MFs in the liver and colon. The characteristic spectrum of mutant plaques induced by MeIQx was a GC to TA base transversion in both the lacI and gpt mutations. Our results thus strongly suggest that these reporter gene transgenic animal models could offer a useful tool for analyzing molecular mechanisms underlying experimental carcinogenesis and for assessing the carcinogenic risk of environmental chemicals.

Osborn LV, Kurek JT, Kriech AJ, Fehsenfeld FM. **Luminescence spectroscopy as a screening tool for the potential carcinogenicity of asphalt fumes.** J Environ Monit 2001;3(2):185-90.
Abstract: A subset of polycyclic aromatic compounds (PACs), which contain 4-6 annulated rings, has been documented as the source of carcinogenicity in animal skin painting studies of petroleum products and asphalt fumes (M. L. Machado, P. W. Beatty, J. C. Fetzer, A. H. Glickman and E. L. McGinnis, Fundam. Appl. Toxicol., 1993, 21, 492; T. A. Roy, S. W. Johnson, G. R. Blackburn and C. R. Mackerer, Fundam. Appl. Toxicol., 1988, 10, 466). Because of the chemical complexity of these materials, it has been difficult to identify the specific compounds within this broad range of PACs responsible for their carcinogenicity. An alternative approach using luminescence spectroscopy was taken in this study to quantify, without identification, a subset of these compounds that appears to cause cancer. The fluorescence response at a specific wavelength pair was obtained for 39 laboratory asphalt fume condensates from animal skin painting studies, yielding a linear correlation coefficient of R2 = 0.96 between the fluorescence response in these materials and the carcinogenicity found in animal studies. In the absence of other asphalt fume condensates from animal studies, 17 petroleum oils were also evaluated using this method and compared with the available animal skin painting data. The details of the method include a clean-up step that removes the highly polar compounds and spectral subtraction of

two- and three-ring PAC interference, both of which add to the fluorescence response, yet were not found to contribute to a carcinogenic response from skin painting studies. Full scan fluorescence plots also produce a fingerprint which can be used to assess contamination, such as coal tar products or mixtures of materials, that are not defined as asphalt, yet may be present in the working environment.

Pylev LN, Vasil'eva LA, Pliss GB. **[Methodological approaches to the study of the carcinogenic properties of substances].** Vopr Onkol 2000;46(3):320-6 [Rus].
Abstract: Since the methods of investigation of carcinogenicity of different agents have changed drastically, relevant manuals need to be revised and supplemented. A new concept of carcinogenic agents evaluation, criteria for their selection and study are discussed. Working out of criteria for establishing priorities of hazardous substance selection is of vital importance.

Robinson DE, MacDonald JS. **Background and framework for ILSI's collaborative evaluation program on alternative models for carcinogenicity assessment. International Life Sciences Institute.** Toxicol Pathol 2001;29 Suppl:13-9.
Abstract: The willingness of the agencies involved in the regulation of pharmaceuticals to accept data from newly proposed models for carcinogenicity testing (eg, transgenic animals, neonatal rodent models, initiation-promotion models) has stimulated international interest in gaining experience and a greater understanding of the strengths and limitations of the specific models. Over a 4-year period, the International Life Sciences Institute (ILSI) Health and Environmental Science Institute (HESI) has coordinated a large-scale collaborative research program to help to better characterize the responsiveness of several models proposed for use in carcinogenicity assessment. The overall objective of this partnership among industry, government, and academic scientists was to evaluate the ability of these new models to provide useful information for human cancer risk assessment. This research program reflected a commitment of nearly US$35 million by over 50 industrial, govemment, and academic laboratories from the United States, Europe, and Japan. Evaluation of the models required the development of standardized protocols to allow reproducibility and comparability of data obtained across multiple laboratories. Test compounds were selected on the basis of mechanistically meaningful carcinogenic activity or noncarcinogenicity in the rodent bioassay as well as humans. Criteria were established for dose selection, pathology review, quality control, and for evaluation of study outcome. The database from these studies represents an important contribution to the future application of new models for human cancer risk assessment. Beyond the data, the collaborative process by which the models were evaluated may also represent a prototype for assessing new methods in the future.

Rockett JC, Swales KE, Esdaile DJ, Gibson GG. **Use of suppression-PCR subtractive hybridisation to identify genes that demonstrate altered expression in male rat and guinea pig livers following exposure to Wy-14,643, a peroxisome proliferator and non-genotoxic hepatocarcinogen.** Toxicology 2000;144(1-3):13-29.
Abstract: Understanding the genetic profile of a cell at all stages of normal and carcinogenic development should provide an essential aid to developing new strategies for the prevention, early detection, diagnosis and treatment of cancers. We have attempted to identify some of the genes that may be involved in peroxisome-proliferator (PP)-induced non-genotoxic hepatocarcinogenesis using suppression-PCR subtractive hybridisation (SSH). Wistar rats (male) were chosen as a representative

susceptible species and Duncan-Hartley guinea pigs (male) as a resistant species to the hepatocarcinogenic effects of the PP, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14,643). In each case, groups of four test animals were administered a single dose of Wy-14,643 (250 mg/kg per day in corn oil) by gastric intubation for 3 consecutive days. The control animals received corn oil only. On the fourth day the animals were killed and liver mRNA extracted. SSH was carried out using mRNA extracted from the rat and guinea pig livers, and used to isolate genes that were up and downregulated following Wy-14,643 treatment. These genes included some predictable (and hence positive control) species such as CYP4A1 and CYP2C11 (upregulated and downregulated in rat liver, respectively). Several genes that may be implicated in hepatocarcinogenesis have also been identified, as have some unidentified species. This work thus provides a starting point for developing a molecular profile of the early effects of a non-genotoxic carcinogen in sensitive and resistant species that could ultimately lead to a short-term assay for this type of toxicity.

Sanner T, Dybing E, Willems MI, Kroese ED. **A simple method for quantitative risk assessment of non-threshold carcinogens based on the dose descriptor T25.** Pharmacol Toxicol 2001;88(6):331-41.
Abstract: This report provides guidance for using the dose-descriptor T25 from animal studies as a basis for quantitative risk characterisation of non-threshold carcinogens. T25 is presently used within the European Union for setting specific concentration limits for carcinogens in relation to labelling of preparations (formulations). The T25 is defined as the chronic dose rate which will give 25% of the animals tumours at a specific tissue site, after correction for spontaneous incidence, within the standard life-time of that species. The T25 is converted to the corresponding human dose descriptor, HT25, by dividing it with the appropriate scaling factor for interspecies dose scaling based on comparative metabolic rates. Subsequently, the human dose (expressed in mg per kg body-weight per day) is calculated from the available exposure data. The corresponding human life-time cancer risk is then obtained by using linear extrapolation by dividing the exposure dose with the coefficient (HT25/0.25). The results with this new method, which can easily be calculated without computer programmes, are in excellent agreement with results from computer-based extrapolation methods such as the linearised multistage model and the benchmark method using LED10, even though the present method only takes into consideration one single dose-response point. To overcome possible shortcomings of the present method, the estimated life-time risks are proposed to be accompanied by a commentary statement giving an overall evaluation of data that may have bearing on the carcinogenic risk and that may indicate whether the real human risk is likely to be higher or lower than the calculated life-time risk. By using the present guidance and a harmonized set of criteria and default values, the calculation of life-time cancer risk should be transparent and easy to comprehend.

Shen Z, Bosbach D, Hochella MF Jr, Bish DL, Williams MG Jr, Dodson RF, Aust AE. **Using in vitro iron deposition on asbestos to model asbestos bodies formed in human lung.** Chem Res Toxicol 2000;13(9):913-21.
Abstract: Recent studies have shown that iron is an important factor in the chemical activity of asbestos and may play a key role in its biological effects. The most carcinogenic forms of asbestos, crocidolite and amosite, contain up to 27% iron by weight as part of their crystal structure. These minerals can acquire more iron after being inhaled, thereby forming asbestos bodies. Reported here is a method for depositing iron on asbestos fibers in vitro which produced iron deposits of the same form as observed on

asbestos bodies removed from human lungs. Crocidolite and amosite were incubated in either FeCl(2) or FeCl(3) solutions for 2 h. To assess the effect of longer-term binding, crocidolite was incubated in FeCl (2) or FeCl(3) and amosite in FeCl(3) for 14 days. The amount of iron bound by the fibers was determined by measuring the amount remaining in the incubation solution using an iron assay with the chelator ferrozine. After iron loading had been carried out, the fibers were also examined for the presence of an increased amount of surface iron using X-ray photoelectron spectroscopy (XPS). XPS analysis showed an increased amount of surface iron on both Fe(II)- and Fe(III)-loaded crocidolite and only on Fe(III)-loaded amosite. In addition, atomic force microscopy revealed that the topography of amosite, incubated in 1 mM FeCl(3) solutions for 2 h, was very rough compared with that of the untreated fibers, further evidence of Fe(III) accumulation on the fiber surfaces. Analysis of long-term Fe (III)-loaded crocidolite and amosite using X-ray diffraction (XRD) suggested that ferrihydrite, a poorly crystallized hydrous ferric iron oxide, had formed. XRD also showed that ferrihydrite was present in amosite-core asbestos bodies taken from human lung. Auger electron spectroscopy (AES) confirmed that Fe and O were the only constituent elements present on the surface of the asbestos bodies, although H cannot be detected by AES and is presumably also present. Taken together for all samples, the data reported here suggest that Fe(II) binding may result from ion exchange, possibly with Na, on the fiber surfaces, whereas Fe(III) binding forms ferrihydrite on the fibers under the conditions used in this study. Therefore, fibers carefully loaded with Fe(III) in vitro may be a parcticularly appropriate and useful model for the study of chemical characteristics associated with asbestos bodies and their potential for interactions in a biosystem.

Sills RC, French JE, Cunningham ML. **New models for assessing carcinogenesis: an ongoing process.** Toxicol Lett 2001;120(1-3):187-98.
Abstract: Traditionally, the use of rodent models in assessing the carcinogenic potential of chemicals has been expensive and lengthy, and the relevance of the carcinogenic effect to humans is often not fully understood. Today, however, with the rapid advances in molecular biology, genetically altered mice containing genes relevant to humans (e.g. oncogenes, tumor suppressor genes) and reporter genes (e.g. lacI) provide powerful tools for examining specific chemical-gene interactions thereby allowing a better understanding of the mechanisms of carcinogenesis in a shorter period of time. This paper will cover an overview of ongoing validation efforts, followed by examples of studies using several genetically engineered models including the p53def mouse model and the Big Blue transgenic mouse model. Specifically, examples where transgenic models were integrated into the testing program based on specific hypotheses dealing with genetic alterations in cancer genes and reporter genes will be discussed. The examples will highlight possible ways genetically altered mice may be integrated into a comprehensive research and testing strategy and thereby provide an improved estimation of human health risks.

Storer RD. **Current status and use of short/medium term models for carcinogenicity testing of pharmaceuticals--scientific perspective.** Toxicol Lett 2000;112-113:557-66.
Abstract: Short- and medium-term rodent bioassays have been proposed under ICH guidelines for use in testing for the carcinogenic potential of pharmaceuticals. Further evaluation of these models is needed urgently and coordinated efforts are in progress worldwide to expand the available database. Models currently being investigated include transgenic mice (Tg-rasH2, Tg.AC, p53(+/-), XPA(-/-)) and

neonatal mice. As more data become available on the performance of these assays, regulatory and industry scientists will be faced with the difficult challenge of determining how the performance (accuracy) of each assay will be measured and deciding which assays have value in the risk assessment process.

Sueoka E, Sueoka N, Goto Y, Matsuyama S, Nishimura H, Sato M, Fujimura S, Chiba H, Fujiki H. **Heterogeneous nuclear ribonucleoprotein B1 as early cancer biomarker for occult cancer of human lungs and bronchial dysplasia.** Cancer Res 2001;61(5):1896-902.
Abstract: Heterogeneous nuclear ribonucleoprotein (hnRNP) B1 is a RNA-binding protein of Mr 37,000. We previously reported that hnRNP B1 was specifically overexpressed in the nuclei of human lung cancer cells, parcticularly in squamous cell carcinoma (E. Sueoka et al., Cancer Res., 59: 1404-1407, 1999). We extended this study to determine whether hnRNP BL was overexpressed in roentgenographically occult cancers of the lungs and premalignant lesions of squamous cell carcinomas, such as bronchial dysplasia. The additional object of our study was to examine the usefulness of hnRNP B1 as a potential diagnostic marker for squamous cell carcinoma of various organs, such as the oral cavity and esophagus in humans. Surgically resected specimens of bronchial dysplasia, lung cancers, and various human squamous cell carcinomas, collected at two hospitals in Japan, were subjected to immunohistochemical staining with anti-hnRNP B1 antibody. Overexpression of hnRNP B1 protein was observed in 100% of stage I lung cancer tissues, but it was not found in normal bronchial epithelium. Squamous cell carcinoma of the lungs showed stronger staining than other histological types, and elevation of hnRNP B1 was found in both roentgenographically occult lung cancers and bronchial dysplasia. Furthermore, cytological examination with anti-hnRNP B1 antibody detected cancer cells in sputum, suggesting the potential of hnRNP B1 protein as a new biomarker for the very early stage of lung cancer in humans. Because strong staining of hnRNP B1 was also observed in various squamous cell carcinomas of oral and esophageal tissues as shown in our recent reports, overexpression of hnRNP B1 seems to be a common event in the carcinogenic processes of squamous cell carcinoma. These results suggest that hnRNP B1 protein could be a useful diagnostic biomarker for both the very early stages of lung cancer and various squamous cell carcinomas in humans.

Thorgeirsson SS, Factor VM, Snyderwine EG. **Transgenic mouse models in carcinogenesis research and testing.** Toxicol Lett 2000;112-113:553-5.
Abstract: Double transgenic mice bearing fusion genes consisting of mouse albumin enhancer/promoter-mouse c-myc cDNA and mouse metallothionein 1 promoter-human TGF-alpha cDNA were generated to investigate the interaction of these genes in hepatic oncogenesis and to provide a general paradigm for characterizing both the interaction of nuclear oncogenes and growth factors in tumorigenesis. In addition, these mice provide an experimental model to test how environmental chemicals might interact with the c-myc and TGF-alpha transgenes during the neoplastic process. We show experimental evidence that co-expression of TGF-alpha and c-myc transgenes in mouse liver promotes overproduction of ROS and thus creates an oxidative stress environment. This phenomenon may account for the massive DNA damage and acceleration of hepatocarcinogenesis observed in the TGF-alpha/c-myc mouse model. Also, the role of mutagenesis in hepatocarcinogenesis induced by 2-amino-3,8-dimethylimidazo(4,5-f)-quinoxaline (MeIQx) was demonstrated in C57BL/lacZ (Muta Mice) and double transgenic c-myc/lacZ mice that carry the lacZ mutation reporter gene. The MeLQx

hepatocarcinogenicity was associated with an increase in in vivo mutagenicity as scored by mutations in the lacZ reporter gene. These results suggest that transgenic mouse models may provide important tools for testing both the carcinogenic potential of environmental chemicals and the interaction/cooperation of these compounds with specific genes during the neoplastic process.

van der Laan JW. **Current status and use of short/medium-term models for assessment of carcinogenicity of human pharmaceuticals: regulatory perspectives.** Toxicol Lett 2000;112-113:567-72.
Abstract: In the summer of 1997 international governmental organizations and industry partners agreed upon a new document on 'Testing for Carcinogenicity of Pharmaceuticals'. The most important element in the new guidance was the acceptability of only one life-time carcinogenicity study in a rodent species (preferably the rat). In addition a choice could be made to test the pharmaceutical in one of the newly developed models, i.e. the newborn mouse assay or one of the various transgenic mouse assays. In the present paper the strengths and weaknesses of various models are discussed from a regulatory point of view. The aim of the new animal models would eventually be replacing animal life-span studies without compromizing human safety. Such studies should supplement the life-span studies and provide additional information not readily available from the long-term assay. At present there is insufficient information to predict or offer guidance on which of the models may be the most suitable. New models are not useful to test the carcinogenic potential of biotechnological products.

Wiltse JA, Dellarco VL. U.S. **Environmental Protection Agency's revised guidelines for carcinogen risk assessment: evaluating a postulated mode of carcinogenic action in guiding dose-response extrapolation.** Mutat Res 2000;464(1):105-15.
Abstract: There are new opportunities to using data from molecular and cellular studies in order to bring together a fuller biological understanding of how chemicals induce neoplasia. In 1996, the Environmental Protection Agency (EPA) published a proposal to replace its 1986 Guidelines for Carcinogen Risk Assessment to take advantage of these new scientific advances in cancer biology. The analytical framework within the new guidelines focuses on an understanding of the mode of carcinogenic action. Mode of action data come into play in a couple of ways in these new guidelines. For example, such information can inform the dose-response relationship below the experimental observable range of tumours. Thus, mode of action data can be useful in establishing more appropriate guidance levels for environmental contaminants. It is the understanding of the biological processes that lead to tumour development along with the response data derived from experimental studies that can help discern the shape of the dose-response at low doses (linear vs. nonlinear). Because it is experimentally difficult to establish "true thresholds" from others with a nonlinear dose-response relationship, the proposed guidelines take a practical approach to depart from low-dose linear extrapolation procedures when there is sufficient experimental support for a mode of action consistent with nonlinear biological processes (e.g., tumours resulting from the disruption of normal physiological processes).

Zhang DL, Li LJ, Xia GT, He XY, Gao BX, Bai XH, Huang GS, Liu SG, Yan LF, Fang FD, et al. **[Analyses of chromosomal karyotypes and cytogenetic variations of animal cell lines].** Yi Chuan Xue Bao 2001;28(4):327-44 [Chi].

Abstract: After the master cell stock(mcs) and working cell bank of more than 30 different strains of 7 animal kidney cell lines (F-81 or CRFK cell line, MDCK cell line, Vero or Vero-2 cell line, MA-104 cell line and BHK-21 cell line) were established in China, the chromosomal number variations and structural aberrations of the above lines, primary feline or canine kidney cell (FKC or CKC) and HeLa cell line were investigated and their karyotypes of routine or Giemsa chromosomal bands were analyzed. The carcinogenesis or tumorigenicity testing of these cells in about 700 nude mice and for colony formation in soft agar (SA) and for agglutination under different concentration of plant lectins was carried out. Both tumorigenicity-negative strains of F-81, CRFK, Vero or Vero-2 lines and very-low-tumorigenicity strains of MDCK line were successfully selected and evaluated for the production of canine or feline combination viral vaccines, which are free of infectious agents, and described with respect to cytogenetic characteristics and tumorigenicity. Rate of modal chromosome number represents the ratio of cell number having modal chromosome number to all the split cell number analyzed at random. Rate of difference represents the ratio of difference of the rate of modal chromosome number between mcs (master cell stock) + n and mcs passages. The chromosomal analysis results showed that the ratio of difference of the rate of modal chromosome number between mcs + n and mcs passages was not more than 5%-15% and the structure aberrations was generally 0%-3%, not more than 5%-10%, thus the hereditary character of cell lines is comparatively stable without significant difference between different passages. The genetic characteristics of chromosomal number of cell lines determines their tumorigenicity, but it is species specific. Experimental models were established for the researches on the prevention and prophylaxis of malignant tumors or cancers and their genetically biological characteristics. Tests showed that there was correlation among cell line chromosome number variations, anchorage independence in soft agar, agglutination under plant lectins and tumor-forming ability in nude mice. Since testing in vitro is more economic, simpler, faster, and is thought to be reliable, we recommend plant lectins followed by SA or analysis of karyotypes as the initial means for monitoring tumorigenicity of animal cell line in nude mice.

CYTOTOXICITY

Apasov SG, Chen JF, Smith PT, Schwarzschild MA, Fink JS, Sitkovsky MV. **Study of A(2A) adenosine receptor gene deficient mice reveals that adenosine analogue CGS 21680 possesses no A (2A) receptor-unrelated lymphotoxicity.** Br J Pharmacol 2000;131(1):43-50.

Abstract: Cell surface A(2A) adenosine receptor (A(2A)R) mediated signalling affects a variety of important processes and adenosine analogues possess promising pharmacological properties. Demonstrating the receptor specificity of potentially lymphotoxic adenosine-based drugs facilitates their development for clinical applications. To distinguish between the receptor-dependent and -independent lymphotoxicity and apoptotic activity of adenosine and its analogues we used lymphocytes from A(2A) R-deficient mice. Comparison of A(2A)R-expressing (+/+) and A(2A)R-deficient (-/-) cells in cyclic AMP accumulation assays confirmed that the A(2A)R agonist CGS 21680 is indeed selective for A(2A) receptors in T-lymphocytes. Incubation of A(2A)R-expressing thymocytes with extracellular adenosine or CGS 21680 in vitro results in the death of about 7-15% of thymocytes. In contrast, no death was induced in parallel assays in cells from A(2A)R-deficient mice, providing genetic evidence that CGS 21680 does not display adenosine receptor-independent intracellular cytotoxicity. The A(2A) receptor-specific lymphotoxicity of CGS 21680 is also demonstrated in a long-term (6-day) in vitro model of

thymocyte positive selection where addition of A(2A)R antagonist ZM 241,385 did block the effects of CGS 21680, allowing the survival of T cells. The use of cells from adenosine receptor-deficient animals is proposed as a part of the screening process for potential adenosine-based drugs for their receptor-independent cytotoxicity and lymphotoxicity.

Babich H, Sinensky MC. **Indirect cytotoxicity of dental materials: a study with Transwell inserts and the neutral red uptake assay.** Altern Lab Anim 2001;29(1):9-13.
Abstract: A modification of the Transwell insert methodology was evaluated by using the neutral red uptake (NRU) assay in a cytotoxicity test. The Transwell insert methodology was developed to assess the biocompatibility of solid materials used in dentistry and, when initially designed, used the release of radiochromium ((51)Cr) in the cytotoxicity assay. Another aim of this study was to evaluate different exposure regimes with which to assess cytotoxicity. The exposure regimes included: a 1-hour exposure in buffer followed by a 24-hour incubation in growth medium; a 2-hour exposure in buffer followed by a 24-hour incubation in growth medium; a 24-hour exposure in serum-limited medium; and a 24-hour exposure in a serum-sufficient medium. The bioindicator target was the Smulow-Glickman (S-G) human gingival cell line and the biomaterials were dental restoratives. The Transwell insert methodology with the NRU cytotoxicity assay as the cytotoxicity endpoint was effective in differentiating the potencies of the dental restoratives; a 2-hour exposure in buffer and a 24-hour exposure in serum-limited medium were the exposure regimes that most clearly differentiated the test agents according to their potencies. The sequence of cytotoxicity of the dental restoratives to the S-G cells was Vitremer > Ketac-Molar Aplicap > Flow-It.

Barrett KL, Willingham JM, Garvin AJ, Willingham MC. **Advances in cytochemical methods for detection of apoptosis.** J Histochem Cytochem 2001;49(7):821-32.
Abstract: In an earlier Article from this laboratory, the current methods developed to detect apoptosis in cells and tissues were highlighted, along with the challenges in their interpretation. Recent discoveries concerning the underlying biochemical mechanisms of apoptotic effector pathways have made possible further assays that allow a more direct measure of the activation of the apoptotic machinery in cells. This Article summarizes some of these newer methods and extends the interpretation of the more classical assays of apoptosis in a defined cell system. We present data in KB and PC3 cell model culture systems induced to undergo apoptosis by the plant toxin ricin. Using a modified in situ nick translation assay (ISNT) with either Bodipy or BUdR labeling, we confirm that most cells showing altered nuclear morphology do not show reactivity with this assay until very late in the apoptotic process. We also show that only a minority of cells label with fluorescent annexin V during apoptosis but that apoptotic cells continue to internalize material from the cell surface through endocytosis after becoming reactive with annexin V. In addition, we describe the utility of a prototype of new assays for caspase substrate cleavage products, the detection of cleaved cytokeratin 18. It is these newer cleavage product assays that perhaps hold the greatest promise for specific detection of apoptosis in cells either in cell culture or in intact tissues. (J Histochem Cytochem 49:821-832, 2001).

Bogen KT, Enns L, Hall LC, Keating GA, Weinfeld M, Murphy G, Wu RW, Panteleakos FN. **Gel microdrop flow cytometry assay for low-dose studies of chemical and radiation cytotoxicity.** Toxicology 2001;160(1-3):5-10.

Abstract: Low-level cytotoxicity may affect low-dose dose-response relations for cancer and other endpoints. Conventional colony-forming assays are rarely sensitive enough to examine small changes in cell survival and growth. Automated image-analysis techniques are limited to ca. 10(4) cells/plate. An alternative method involves encapsulation of single proliferating cells into ca. 35-75-microm-diameter agarose gel microdrops (GMDs) that are randomly grouped, differential exposure of these groups, culture at 37 degrees C for 3-5 days, and finally GMD analysis by flow cytometry (FC) to determine the ratio of GMDs containing multiple versus single cells as a measure of clonogenic survival. This GMD/FC assay was used to examine low-dose cell killing induced by a cooked-meat mutagen/rodent-carcinogen (MeIQx) in DNA-repair-deficient/metabolically-sensitive CHO cells. Results of conventional colony-forming assays using up to 30 replicate plates indicate a shouldered, threshold-like dose-response; in contrast, those obtained using the GMD/FC assay suggest "hypersensitivity"-like nonlinearity in dose-response. The GMD/FC assay was also applied to human A549 lung cells after GMD-encapsulation and gamma radiation followed by culture for a total of 4 days, to examine survival after exposure to > or =100 cGy delivered at a relatively low dose rate (0.18 cGy/min). Dose-response for clonogenic growth was again observed to be reduced with apparent nonlinear suggesting hypersensitivity between 0 and 50 cGy, insofar as doses of 5 and 10 cGy appear to be ca. fivefold more effective per unit dose than the 50- or 100-cGy doses used. The GMD/FC assay may thus reveal low-dose dose-response relations for chemical and radiation effects on cell proliferation/killing with implications for low-dose risk assessment.

Bugelski PJ, Atif U, Molton S, Toeg I, Lord PG, Morgan DG. **A strategy for primary high throughput cytotoxicity screening in pharmaceutical toxicology.** Pharm Res 2000;17(10):1265-72.
Abstract: PURPOSE: Recent advances in combinatorial chemistry and high throughput screens for pharmacologic activity have created an increasing demand for in vitro high throughput screens for toxicological evaluation in the early phases of drug discovery. METHODS: To develop a strategy for such a screen, we have conducted a data mining study of the National Cancer Institute's Developmental Therapeutics Program (DTP) cytotoxicity database. RESULTS: Using hierarchical cluster analysis, we confirmed that the different tissues of origin and individual cell lines showed differential sensitivity to compounds in the DTP Standard Agents database. Surprisingly, however, approaching the data globally, linear regression analysis showed that the differences were relatively minor. Comparison with the literature on acute toxicity in mice showed that the predictive power of growth inhibition was marginally superior to that of cell death. CONCLUSIONS: This datamining study suggests that in designing a strategy for high throughput cytotoxicity screening: a single cell line, the choice of which may not be critical, can be used as a primary screen; a single end point may be an adequate measure and a cut off value for 50% growth inhibition between 10(-6) and 10(-8) M may be a reasonable starting point for accepting a cytotoxic compound for scale up and further study.

Byth HA, Mchunu BI, Dubery IA, Bornman L. **Assessment of a simple, non-toxic Alamar blue cell survival assay to monitor tomato cell viability.** Phytochem Anal 2001;12(5):340-6.
Abstract: The Alamar Blue (AB) assay, which incorporates a medox indicator that changes colour or fluorescence in response to metabolic activity, is commonly used to assess quantitatively the viability and/or proliferation of mammalian cells and micro-organisms. In this study the AB assay was adapted for the determination of the viability of plant cells. Cell suspension cultures of tomato, Lycopersicon

esculentum, L., with differing viabilities, served as the experimental model for a comparison of the AB assay with the conventional 2,3,5-triphenyltetrazolium chloride (TTC) viability assay. The AB assay showed a sigmoidal relationship between cell viability and AB reduction (as quantified by spectrofluorometry or spectrophotometry), which was similar to that obtained using the TTC assay. Both assays detected a significant reduction in cell viability after 48 h exposure to virulent Ralstonia solanacearum (biovar III), while the TTC assay, in addition, revealed cell proliferation in control cells from 24 to 72 h. The TTC assay detected cell proliferation over a wider range of cell densities, while the AB assay was more rapid and versatile whilst being non-toxic and thus allowing subsequent cell analysis.

Cook RS, Meyer GD, Miller TE, Curran MA, Cmar CB, Miller GL, Carmichael L. **Assessing the feasibility of an in vitro cytotoxicity method to detect harmful ubiquitous chemicals (detection of non-warfare hazardous chemicals in the operational theater).** Drug Chem Toxicol 2000;23(1):95-111.
Abstract: The objective of this work was to assess the feasibility of accomplishing aqueous extracts of soil samples and determining if the extracted solution induced adverse effects in the human myelomonocytic cell line, HL60. Dosing of HL60 cells was accomplished over a 24-hour period using 100% of extracted media from standard soil samples containing known contaminants. Assessments of viability, apoptosis, reduced thiols, and mitochondrial membrane integrity were accomplished by argon-ion laser flow cytometric analysis, using chemical labels specific for each end-point. The in vitro cytotoxicity data was compared with the results of Microtox and Mutatox tests as well as earthworm and plant toxicity tests. In vitro cytotoxicity tests' results exhibited good correlation with other tests' results.

Fenoglio I, Croce A, Di Renzo F, Tiozzo R, Fubini B. **Pure-silica zeolites (Porosils) as model solids for the evaluation of the physicochemical features determining silica toxicity to macrophages.** Chem Res Toxicol 2000;13(6):489-500.
Abstract: The interaction between inhaled pArticles and alveolar macrophages plays a key role in silica-related diseases. It has been previously shown [Fubini, B., et al. (1999) Chem. Res. Toxicol. 12, 737-745] that a monocyte-macrophage cell line (J774) may be employed in the evaluation of the degree of cytotoxicity to alveolar macrophages of various silica dusts. In this paper, pure-silica zeolites (porosils) in microcrystalline form have been employed as "model solids" in an effort to show which physicochemical properties of the silica pArticle are playing a major role in the toxicity to macrophages. The samples employed covered four different porosil crystal structures (MFI, FAU, TON, and MTT) and also include a synthetic rodlike cristobalite (CRIS-rd). When compared at equal weight, the samples cover a wide range of cytotoxicity from inert to toxic as unheated mineral cristobalite [Fubini, B., et al. (1999) Chem. Res. Toxicol. 12, 737-745]. Mild grinding did not affect cytotoxicity. Calcined (open pores) and uncalcined (pore filled with template) TON exhibited the same cytotoxicity, indicating that only the outer surface is implied. The hydrophobic and/or hydrophilic character of TON, evaluated by adsorption calorimetry, is close to what has been previously found for silicalite and is consistent with a hydrophilic outer surface and hydrophobic pore walls. The potential for generating hydroxyl radicals from hydrogen peroxide varies among the various porosils that have been studied. A model is proposed for the correlation between inhibition of growth on proliferating cells and physicochemical properties varying from one to the other sample. The extent of external surface and the aspect ratio were related to the intensity of the cytotoxic effect, while the level of radical release was not. This suggests, on one

hand, that comparison of toxicity among various dusts should be made at equal pArticle surface and, on the other, that in the model studied, free radical release does not play a crucial role in the primary event of toxicity to alveolar macrophages.

Flanagan AF, Callanan KR, Donlon J, Palmer R, Forde A, Kane M. **A cytotoxicity assay for the detection and differentiation of two families of shellfish toxins.** Toxicon 2001;39(7):1021-7.
Abstract: There is an urgent need for an alternative to the mouse bioassay for the detection of algal toxins in shellfish on both analytical and animal welfare grounds. Several alternative methodologies have been described, but have not gained widespread acceptance to date, because each assay measures only one or a small number of related phycotoxins out of the increasing range that needs to be detected. A simple cytotoxicity assay using either the HepG2 or ECV-304 cell lines is described with two end-point measurements, which can detect and distinguish between two unrelated classes of phycotoxins. Morphological examination following 3h exposure to the sample enables the detection of the diarrhetic shellfish poisons, including okadaic acid and related toxins. Viability testing using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), following 24h exposure of the same cells to the sample, reveals a second class of toxin, which is most probably the newly-described toxin, azaspiracid. This assay should play an important role in shellfish monitoring in the future.

Gandhi VM, Cherian KM. **Red cell haemolysis test as an in vitro approach for the assessment of toxicity of karanja oil.** Toxicol In Vitro 2000;14(6):513-6.
Abstract: The karanja tree grows in parts of India and Australia. The oil from seed kernels was found to be toxic to animals. The annual potential availability of the oil is around 135,000 tons in India. In order to use it for beneficial purposes, it is necessary to detoxify the oil. In the present study, the oil was assessed for toxicity by the red cell haemolysis test and estimating the LDH in the supernatant. The non-lipid constituents were isolated from raw oil by aqueous methanol extraction. The raw oil and the non-lipid fraction were found to haemolyse the red cells with release of LDH, whereas the extracted oil did not show such a manifestation. There was a good correlation between haemolytic activity and LDH released from cells. These findings were further confirmed with in vivo studies where the raw and extracted karanja oils showed 100% and nil mortality in rats dosed orally at 10 and 20 ml/kg body weight, respectively. This haemolysis test can be used as an in vitro method to predict toxicity and to monitor the detoxification of the oils prior to use in in vivo studies for toxicological evaluation. The fatty acid composition of the raw and extracted karanja oils showed no difference.

Hall LA, Krauthauser CM, Wexler RS, Hollingshead MG, Slee AM, Kerr JS. **The hollow fiber assay: continued characterization with novel approaches.** Anticancer Res 2000;20(2A):903-11.
Abstract: The hollow fiber assay, a unique in vivo model, permits the simultaneous evaluation of compound efficacy against multiple cell lines in two physiological compartments. This assay has been used to characterize in vivo activity of cytotoxic compounds. The purpose of the present study was to characterize and optimize this assay for compounds with a defined mechanism of action, specifically cell cycle inhibition. Two human tumor cell lines and one normal human cell line were loaded into polyvinylidene fluoride hollow fibers at two or more cell concentrations and grown in mice for 3-10 days. The data demonstrate the importance of characterizing the initial loading density of various cell lines in the evaluation of compounds. All studies were performed with cells in the linear part of the cell

growth curves. Initial loading densities of 1-2 x 10(4) cells/fiber gave the greatest opportunity for growth in the three human cell lines tested (HCT116 colon carcinoma, NCI-H460 non-small cell carcinoma, and AG 1523 normal fibroblast). Utilizing the MTT assay, standard curves were constructed to correlate the final number of cells with optical density (OD) readings at 540 nm in order to calculate cell numbers in the fibers. Insights into the mechanism of action of cisplatin have been gained using Western blot analysis of the cell cycle markers PCNA (a protein present throughout the cell cycle) and Rb (a protein that acts as a tumor suppressor gene product) from the hollow fiber cells. In cisplatin-treated NCI-H460 cells both PCNA and Rb phosphorylation decreased, suggesting the arrest of the cells prior to the S phase. Standard therapeutic agents, cisplatin, racemic flavopiridol, cyclophosphamide and mitomycin C, were evaluated independently in the hollow fiber assay and the xenograft model. The data demonstrate that compounds active in the hollow fiber assay are also active in the xenograft.

Jean F, Roudot AC, Parent-Massin D. **An automatic method for the evaluation of xenobiotic toxicity on haematopoietic progenitors.** Comput Methods Programs Biomed 2000;63(1):1-8.
Abstract: Culture of haematopoietic progenitors is used in toxicology to evaluate the effects of xenobiotic contaminants on humans. One of the most important parts of this analysis is the quantification of cell aggregates in the culture. These aggregates can have different sizes and shapes, which reflect the category of cells (granulocyte or monocyte), and the growth percentage reflects the toxic effect of the contaminant. A proposal for an automatic method based on image analysis is described in this work. Algorithms able to solve the basic problems are proposed and tested, and the problem concerning a tridimensional analysis is exposed and discussed.

Kayser O, Kiderlen AF, Laatsch H, Croft SL. **In vitro leishmanicidal activity of monomeric and dimeric naphthoquinones.** Acta Trop 2000;76(2):131-8.
Abstract: A series of monomeric and dimeric naphthoquinones with potential for treatment of Leishmania infections was identified in vitro using both a direct cytotoxicity assay against extracellular promastigotes of Leishmania donovani, Leishmania infanturn, Leishmania enriettii, and Leishmania major and a test against intracellular amastigote L. donovani residing within murine macrophages. Several naphthoquinones proved to be active at concentrations in the microgram range (EC(50) 0.9-17.0 microg/ml). When tested against a panel of human cancer cell lines (KB, SKMel, A549, MDA) and murine bone marrow culture-derived macrophages (BMMPhi) as mammalian host cell controls, compounds with anti-Leishmania-activity showed moderate (EC(50)>25 microg/ml) to pronounced (EC (50)<10 microg/ml) toxic effects.

Kim S, Stair EL, Lochmiller RL, Lish JW, Qualls CW Jr. **Evaluation of myelotoxicity in cotton rats (Sigmodon hispidus) exposed to environmental contaminants. I. In vitro bone-marrow progenitor culture.** J Toxicol Environ Health A 2001;62(2):83-96.
Abstract: Bone marrow is extremely sensitive to toxicants, and in vitro culture of bone-marrow progenitor cells has been shown to be a sensitive indicator of bone-marrow injury in laboratory rodents. The ability of a bone-marrow progenitor cell assay to detect myelotoxicity in a wild rodent model (cotton rat, Sigmodon hispidus) that inhabits many contaminated ecosystems in the southern United States was examined. Responsiveness of progenitor cells to recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and cotton rat lung-conditioned medium (LCM) was

determined to optimize culture conditions for cotton rats. Myelotoxicity was induced in cotton rats by treating animals with either cyclophosphamide (8 or 80 mg/kg) or dexamethasone (500 microg/kg) over a 5-d period. Administration of a high dose of cyclophosphamide caused nearly total suppression of colony formation of granulocyte-macrophage progenitor cells (CFU-GM). Marked histological changes in both the bone marrow and spleen were also observed in cotton rats treated with a high dose of cyclophosphamide. Although histological lesions were not apparent, the number of CFU-GM in the bone marrow of low-dose cyclophosphamide- and dexamethasone-treated cotton rats was significantly suppressed compared to controls. The number of CFU-GM was consistently higher using LCM than recombinant murine GM-CSF. This reproducible, quantitative, in vitro bone-marrow progenitor cell culture system was a sensitive indicator of myelotoxicity in wild cotton rats and should be useful for monitoring chronic exposures to low levels of environmental toxicants in wild rodent populations.

Niu Q, Zhao C, Jing Z. **An evaluation of the colorimetric assays based on enzymatic reactions used in the measurement of human natural cytotoxicity.** J Immunol Methods 2001;251(1-2):11-9.
Abstract: In recent years colorimetric assays based on an enzymatic reaction such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay have been used in an attempt to replace the conventional isotopic assay for cell-mediated cytotoxicity. To clarify the problems in the colorimetric assays for natural cytotoxicity, K562 cells were employed as target cells and peripheral mononuclear cells (PBMCs) from cancer patients were used as effector cells. No correlation was found between the 51Cr assay and the MTT assay (P>0.05) or the N-acetyl-beta-D-glycosaminidase (NAG) release assay (P>0.05) in 16 cancer patients. Labeling effector cells showed that the 51Cr release levels of such cells in 19 chemotherapy patients were significantly higher than the levels from target cells in this group (P<0.01) and from effector cells in the control group (PZ0.01). There was no correlation between the positive and negative 51Cr assays (P>0.05). The sensitivity of the MTT assay was greatly decreased by washing K562 cells prior to loading MTT solution. Enzyme release occurs as a result of cell metabolism and elevated enzyme release is associated with freezing. These findings indicate that the colorimetric assays based on an enzymatic reaction are not suitable for the detection of natural cytotoxicity in all populations, and are especially not suitable for the assay of natural cytotoxicity in chemotherapy patients.

Ruppova K, Wsolova L, Urbancikova M, Slamenova D. **Comparison of three in vitro assays at evaluation of IC50 of acetylsalicylic acid, ferrous sulfate, amitriptyline, methanol, isopropanol and ethylene glycol in human cancer cells HeLa.** Neoplasma 2000;47(3):172-6.
Abstract: Evaluation of the 50% inhibitory concentration (IC50) of acetylsalicylic acid, ferrous sulfate, amitriptyline, methanol, isopropanol and ethylene glycol was done on human cancer cells cultured in in vitro conditions. Three different in vitro assays were used in this study: the plating efficiency test, the microprotein test and the neutral red uptake test. Obtained results were evaluated by statistical methods. All used methods seem to be useful for screening a cytotoxic potential of the tested chemicals. The knowledge of cytotoxic effects of frequently used chemicals on mammalian cells is important not only for necessary in vitro genotoxicity and carcinogenicity studies but also for assessing the toxicity of chemicals to find out possible hazards to the human health. Results presented in this paper underline the usefulness of the wider methodological approach for the comparison of the different endpoints as well as a necessity for selection of a battery of in vitro cytotoxicity tests allowing to estimate the possible

harmful effects of xenobiotics.

Schuster U, Schmalz G, Thonemann B, Mendel N, Metzl C. **Cytotoxicity testing with three-dimensional cultures of transfected pulp-derived cells.** J Endod 2001;27(4):259-65.
Abstract: SV40 large T-antigen-transfected bovine pulp-derived cells were grown three-dimensionally on polyamide meshes. For optimal cell growth, various cell numbers and mesh coatings were tested. Next the three-dimensional cultures were used in a dentin barrier test device, and the system was evaluated by testing a set of dental filling materials. After 24 hr exposure with or without perfusion of the pulpal part of the test device, cell survival was evaluated using an MTT assay. In all experiments pulp-derived cells transfected with SV40 large T-antigen grew three-dimensionally on polyamide meshes and showed growth kinetics similar to those on cell culture plates with lag, log, and plateau phases (reached after about 14 days of incubation). Cross-sections of the three-dimensional cell cultures revealed about 15 to 20 cell layers. In vitro cytotoxicity tests resulted in cell survival rates which are in good agreement with in vivo data and with results obtained from cytotoxicity tests with three-dimensional cultures of human foreskin fibroblasts.

Snyder EL, O'Donnell L, Dengler TJ, Pomper GJ, Velleca MA, Dincecco DM, Baril LL, Min K, Gudino MD, Bender JR. **Ex vivo evaluation of PBMNCs collected with a new cell separator.** Transfusion 2001;41(7):940-9.
Abstract: BACKGROUND: This study reports on an evaluation of the ability of a cell separator (Amicus, Baxter Healthcare) and the integral MNC computer software program to collect a variety of MNC subsets. The collection efficiency (CE) of the Amicus for these MNC subsets was compared to that of another cell separator (CS-3000 Plus, Baxter). The collected MNCs were also assayed ex vivo to determine if these cells remained functional. STUDY DESIGN AND METHODS: Healthy volunteer blood donors were recruited to provide PBMNCs for the isolation of CD3+, CD4+, CD8+, CD19+, NK, and gammadelta+ cells and monocytes. Cells were collected with an Amicus (test arm; n = 16) or a CS-3000 Plus (control arm; n = 11) cell separator. Cells were counted on a flow cytometer and CEs were calculated. For functional studies, the Amicus-collected MNC data were compared to CS-3000 Plus historical data. Functional studies performed included surface antigen expression assays (CD8+), proliferation assays (CD4+ and CD8+ cells), NK cytotoxicity assays for K562 and HUVE cells, and E-selectin induction on endothelial cells through NK+ contact dependency. Dendritic cells (DCs) were generated from CD34+ cells collected on the Amicus, positively selected by the use of antibody-bound, magnetic bead technology, and then cultured ex vivo with a combination of growth factors to generate the DCs. RESULTS: CEs were higher on the Amicus than on the CS-3000 Plus for CD3+ (68 vs. 54%), CD4+ (70 vs. 56%), CD8+ (68 vs. 52%), and CD19+ (60 vs. 48%) cells ($p<0.05$). For the two separators, CEs were equivalent for monocytes, NK+, and gammadelta+ cells. The Amicus separator collected significantly fewer platelets than did the CS-3000 Plus ($p<0.00001$). CD4+, CD8+, and NK cells proliferated normally. NK cells appropriately stimulated E-selectin expression on endothelial cells. Culture-generated DCs obtained by using Amicus-collected CD34+ cells expressed appropriate cell surface markers. CONCLUSION: The Amicus separator is acceptable for the collection of PBMNC subsets. The device collects CD3+, CD4+, CD8+, and CD19+ T- and B-cell subsets with greater efficiency and collects MNCs with significantly fewer contaminating platelets than does the CS-3000 Plus. Cells collected on the Amicus are suitable for use in a variety of research and clinical

immunobiologic studies.

Soos K, Valkusz Z, Vetro O, Juhasz A, Petri A, Molnar G, Molnar J, Galfi M. **Primary mono-layer cell cultures as model system for studying of environmental toxic agents: organochlorine compounds.** Cent Eur J Public Health 2000;8 Suppl:35-6.
Abstract: Organic pollution of water and soil has various harmful effects on biological systems (1). Chlorine substituted benzol compounds are one these xenobiotic substances, which are toxic to the environment (2). They can also accumulate in plant and animal tissues (3), which provides ample reason to study the effects of sublethal doses of chloro-benzols on various cell cultures. In this study the toxic effects of chloro-benzols were investigated on avian fibroblast and mammalian hepatocyte cultures. The fibroblast cultures were prepared from eggs preadapted to chloro-benzol during a fourteen-day-long incubation period. The Wistar rat hepatocyte monolayer cultures were exposed to a direct treatment of 1,2,4-tri-chloro-benzol (0.01 microgram/ml-1 microgram/ml) for 3 hours. Following the treatment with chloro-benzol, the viability of the cells was measured, together with lactic dehydrogenase activity, in both kinds of cultures. The effect of tri-chloro-benzol treatment on chicken eggs was not significant. The cells of chicken embryos were not damaged after the 1,2,4-tri-chloro-benzol treatment. The hepatocyte cultures showed the toxic effects of 1,2,4-tri-chloro-benzol after the direct and acute treatment. The cell viability decreased and the LDH activity increased significantly. These results show that the primary cell cultures are suitable for studying the effects of organochlorine compounds.

Turco L, De Angelis I, Stammati A, Zucco F. **Apoptosis evaluation in epithelial cells exposed to different chemicals: relevance of floating cells.** Cell Biol Toxicol 2000;16(1):53-62.
Abstract: The recent increase in understanding of cell death has promoted new approaches in toxicological studies, mainly those dealing with in vitro systems where the evaluation of cell death has been the most widely adopted end-point in measuring the effects of chemical toxicants. The aim of this study was to investigate the possibility of improving the traditional cytotoxicity test protocols in order to produce more specific information on the type of cell death induced by exposure to toxicants. In parcticular, we characterized the mode of cell death in an established epithelial cell line, HEp-2 cells, which is frequently used in cytotoxicity testing owing to its easy handling and standardization of culture conditions. Reference chemicals for apoptosis and necrosis were selected as controls, together with other molecules that have been shown, in preliminary studies, to induce various morphological and structural modifications in relation to cell death. The results obtained show that: (a) the floating fraction of treated cells gives the clearest picture of the necrotic/apoptotic distribution; (b) morphological analysis is crucial for characterization of apoptosis; (c) more than one cytotoxic end-point is necessary to clearly identify the type of cell death.

Ulrichova J, Dvorak Z, Vicar J, Lata J, Smrzova J, Sedo A, Simanek V. **Cytotoxicity of natural compounds in hepatocyte cell culture models.** The case of quaternary benzo[c]phenanthridine alkaloids. Toxicol Lett 2001;125(1-3):125-32.
Abstract: The quaternary benzo[c]phenanthridine alkaloids (QBA) produce a plethora of species- and tissue-specific effects but the molecular basis of their biological activities remain mysterious. The objective of the present study was to investigate the cytotoxicity of QBA alkaloids, sanguinarine (SA), chelerythrine (CHE), fagaronine (FA), and the extract from Macleaya cordata in primary cultures of

human and porcine hepatocytes. The cellular damage was assessed by the MTT assay, lactate dehydrogenase (LDH) leakage and the determination of intracellular glutathione (GSH) levels. The results are summarised as follows: (i) The alkaloids tested in doses 0.1 and 10 &mgr;M did not display statistically significant cytotoxicity for 0-3 h incubation; (ii) SA and CHE showed the dose- and time-dependent toxicity within the range 25-100 &mgr;M whereas FA was not toxic; (iii) the LDH leakage into the medium was higher for SA than for CHE, thus revealing a potent potential of SA to disturb cell-membrane integrity; (iv) after 3 h incubation with 100 &mgr;M SA/CHE, mitochondrial dehydrogenase activity (MTT assay) and the cellular GSH levels decreased to residual values of about 40% suggesting that mitochondria are unlikely to be a primary target for SA/CHE in the cell; (v) no differences were found in the response to QBA application in human vs porcine hepatocyte.

Van Den Heuvel RL, Leppens H, Schoeters GE. **Use of in vitro assays to assess hematotoxic effects of environmental compounds.** Cell Biol Toxicol 2001;17(2):107-16.
Abstract: The number of chemicals being introduced into the environment increases and many of these substances may pose a health risk to exposed individuals. Many environmental toxicants with a potential toxicity to the hematopoietic system have been identified by animal experiments. Owing to the risks of severe chronic hematopoietic disorders, it is important to screen chemicals for their hematotoxicity. The aim of this work was to identify, through the use of in vitro techniques, targets for hematotoxic effects. Our study focused on myeloid and erythroid hematopoietic progenitors and stromal stem cells as possible targets. The in vitro assays showed that various hematotoxic compounds exert different effects on these cell populations. In vitro exposure of murine bone marrow cells to various inorganic (cadmium, lead) and organic (benzene metabolites, lindane. benzo-[a]-pyrene (BaP), PCB (polychlorinated biphenyl) congeners) environmental chemicals indicated that hematopoietic or stromal bone marrow cells were targets for most of the chemicals. Stromal cells were more affected by lead, cadmium, and BaP compared to myeloid cells. Benzene and phenol gave no response, but the metabolites catechol and hydroquinone were equally toxic to the stromal and the myeloid progenitor cells. Among the PCBs tested, PCB126 was most toxic. Human progenitor cells derived from cord blood were exposed in vitro to catechol, hydroquinone, lead nitrate, and PCBs. Human hematopoietic cells were sensitive to the tested compounds. Human erythroid progenitors are more susceptible to lead exposure than are myeloid progenitors. Based on the in vitro tests, humans are more sensitive to lead, catechol, and PCB126 than are mice. In contrast to the murine data, humans responded with individual differences to lead and PCB126.

van Wyk CW, Olivier A, Maritz JS. **Cultured pulp fibroblasts: are they suitable for in vitro cytotoxicity testing?** J Oral Pathol Med 2001;30(3):168-77.
Abstract: The use of cell cultures to test the biocompatibility of dental materials is gaining in importance. Any cytotoxic effects that restorative materials may have will be on the dental pulp and for that reason cultured pulp cells should be the model of choice for biocompatibility testing. The aim of this investigation was to study the growth and morphologic characteristics and toxic response of human pulp lines and to compare these parameters to those of human buccal mucosa fibroblasts. Twenty-one specimens of pulp tissue and six from buccal mucosa were cultured using standard techniques. Six pulp cell lines were cultured successfully as were all six from the buccal mucosa specimens. From these specimens, 12 growth curves were computed. To study the morphology of the cultured cells, they were

observed microscopically and classified into three morphological types: slender elongated cells (type I), epithelioid shaped cells (type II) and large stellate cells (type III). Their numbers and proportions were determined for each cell line and compared statistically. To gauge sensitivity to toxic materials, cells were exposed to concentrations of arecoline. An analysis of the growth curves showed no statistical difference between pulp cells and buccal mucosa cells; the slopes of the curves, however, differed significantly between individual cell lines, and these individual differences were greater among pulp cell lines. The morphology of the pulp and mucosa fibroblasts was similar microscopically. There was no significant difference between the number and proportion of the cell types in the two groups, but there were significant differences between the individual cell lines. Pulp cells showed a greater inhibition of growth when exposed to arecoline. Because pulp fibroblasts are difficult to culture, their reported survival rate is poor; due to the differences that exist between individual cell lines, we conclude that pulp cells when used as single cell lines or even pooled may not be ideal for testing biocompatibility, especially if reproducibility is a prerequisite. Any evaluation will require tests on not one, but several cell lines in order to minimize the effect of inter-cell-line differences. Their greater sensitivity to toxic substances, on the other hand, may show that pulp cells could be more sensitive indicators of cytotoxicity.

Wilson MR, Stone V, Cullen RT, Searl A, Maynard RL, Donaldson K. **In vitro toxicology of respirable Montserrat volcanic ash.** Occup Environ Med 2000;57(11):727-33.
Abstract: OBJECTIVES: In July 1995 the Soufriere Hills volcano on the island of Montserrat began to erupt. Preliminary reports showed that the ash contained a substantial respirable component and a large percentage of the toxic silica polymorph, cristobalite. In this study the cytotoxicity of three respirable Montserrat volcanic ash (MVA) samples was investigated: M1 from a single explosive event, M2 accumulated ash predominantly derived from pyroclastic flows, and M3 from a single pyroclastic flow. These were compared with the relatively inert dust TiO(2) and the known toxic quartz dust, DQ12. METHODS: Surface area of the pArticles was measured with the Brunauer, Emmet, and Teller (BET) adsorption method and cristobalite content of MVA was determined by x ray diffraction (XRD). After exposure to pArticles, the metabolic competence of the epithelial cell line A549 was assessed to determine cytotoxic effects. The ability of the pArticles to induce sheep blood erythrocyte haemolysis was used to assess surface reactivity. RESULTS: Treatment with either MVA, quartz, or titanium dioxide decreased A549 epithelial cell metabolic competence as measured by ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). On addition of mannitol, the cytotoxic effect was significantly less with M1, quartz, and TiO(2). All MVA samples induced a dose dependent increase in haemolysis, which, although less than the haemolysis induced by quartz, was significantly greater than that induced by TiO(2). Addition of mannitol and superoxide dismutase (SOD) significantly reduced the haemolytic activity only of M1, but not M2 or M3, the samples derived from predominantly pyroclastic flow events. CONCLUSIONS: Neither the cristobalite content nor the surface area of the MVA samples correlated with observed in vitro reactivity. A role for reactive oxygen species could only be shown in the cytotoxicity of M1, which was the only sample derived from a purely explosive event. These results suggest that in general the bioreactivity of MVA samples in vitro is low compared with pure quartz, but that the bioreactivity and mechanisms of biological interaction may vary according to the ash source.

Yamamoto O, Hamada T, Tokui N, Sasaguri Y. **Comparison of three in vitro assay systems used for assessing cytotoxic effect of heavy metals on cultured human keratinocytes.** J UOEH 2001;23(1):35-44.
Abstract: The cell viability assay using cultured cells is of great advantage to elucidate the biological effect of potentially toxic substances. Recently, a novel assay system, Tetracolor One cell proliferation assay (Seikagaku Co., Tokyo, Japan), has been developed. In this report, we compare the results of the Tetracolor One assay regarding the cytotoxic effect of three heavy metal salts on cultured adult keratinocytes to those of the neutral red dye uptake assay and the MTT eluted stain assay. In this study, these three methods showed almost similar results. Compared to the other two methods, however, the Tetracolor One assay, which requires only one-step procedure before spectrophotometric measurement, is easier to use, and errors in measurement, which may be produced through the multistep procedure, are much less in this assay. Therefore, we believe that the Tetracolor One assay system is useful for assessing the cytotoxic effect of heavy metals on cultured human keratinocytes.

Zuang V. **The neutral red release assay: a review.** Altern Lab Anim 2001;29(5):575-99.
Abstract: The neutral red release (NRR) assay is a cytotoxicity test that can be used to measure the immediate toxic effects of test substances on the cell membrane, resulting in the leaking of intracellular contents. The assay has already been used for several years to evaluate the cytotoxicities of various kinds of products, such as cosmetics, pharmaceuticals, industrial chemicals and household products. It has undergone in-house validation by many companies, and has been found to be parcticularly useful for identifying substances that are potentially capable of causing adverse reactions on coming into brief contact with the eye or the skin at relatively high concentrations, such as might occur in an adventitious splash into the eye or onto the skin, followed by a quick rinse. Because of the relatively long existence of the NRR assay, its practicality and its proven usefulness for parcticular purposes, ECVAM decided to review the status of the method, in order to decide whether prevalidation and formal validation studies on the test might be profitable. The review of the status of the test was carried out by performing a comprehensive review of the literature, and by conducting a survey involving companies and institutes with experience in using the test. Both the review and the survey revealed that the assay could provide extremely valuable information when it was used for parcticular purposes, such as for the evaluation and comparison of immediate toxic effects on the eye or the skin caused by certain products or chemicals such as surfactants. Most of those who responded in the survey favoured a prevalidation/validation study.

DERMAL TOXICITY

Arrighi JF, Rebsamen M, Rousset F, Kindler V, Hauser C. **A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers.** J Immunol 2001;166(6):3837-45.
Abstract: We investigated the involvement of mitogen-activated protein kinases (MAPKs) in the maturation of CD83(-) dendritic cells (DC) derived from human blood monocytes. Maturating agents such as LPS and TNF-alpha induced the phosphorylation of members of the three families of MAPK (extracellular signal-regulated kinase l/2, p46/54 c-Jun N-terminal kinase, and p38 MAPK). SB203580, an inhibitor of the p38 MAPK, but not the extracellular signal-regulated kinase l/2 pathway blocker PD98059, inhibited the up-regulation of CD1a, CD40, CD80, CD86, HLA-DR, and the DC maturation

marker CD83 induced by LPS and TNF-alpha. In addition, SB203580 inhibited the enhancement of the allostimulatory capacity and partially prevented the down-regulation of FITC-dextran uptake induced by LPS and TNF-alpha. Likewise, SB203580 partially prevented the up-regulation of IL-1alpha, IL-1beta, IL-lRa, and TNF-alpha mRNA upon stimulation with LPS and TNF-alpha, as well as the release of bioactive TNF-alpha induced by LPS. DC maturation induced by the contact sensitizers 2,4-dinitrofluorobenzene and NiSO(4), as seen by the up-regulation of CD80, CD86, and CD83, was also coupled to the phosphorylation of p38 MAPK, and was inhibited by SB203580. The irritants SDS and benzalkonium chloride that do not induce DC maturation did not trigger p38 MAPK phosphorylation. Together, these data indicate that phosphorylation of p38 MAPK is critical for the maturation of immature DC. These results also suggest that p38 MAPK phosphorylation in DC may become useful for the identification of potential skin contact sensitizers.

Asbill C, Kim N, El-Kattan A, Creek K, Wertz P, Michniak B. **Evaluation of a human bio-engineered skin equivalent for drug permeation studies.** Pharm Res 2000;17(9):1092-7.
Abstract: PURPOSE: To test the barrier function of a bio-engineered human skin (BHS) using three model drugs (caffeine, hydrocortisone, and tamoxifen) in vitro. To investigate the lipid composition and microscopic structure of the BHS. METHODS: The human skin substitute was composed of both epidermal and dermal layers, the latter having a bovine collagen matrix. The permeability of the BHS to three model drugs was compared to that obtained in other percutaneous testing models (human cadaver skin, hairless mouse skin, and EpiDerm). Lipid analysis of the BHS was performed by high performance thin layered chromatography. Histological evaluation of the BHS was performed using routine H&E staining. RESULTS: The BHS mimicked human skin in terms of lipid composition, gross ultrastructure, and the formation of a stratum corneum. However, the permeability of the BHS to caffeine, hydrocortisone, and tamoxifen was 3-4 fold higher than that of human cadaver skin. CONCLUSIONS: In summary, the results indicate that the BHS may be an acceptable in vitro model for drug permeability testing.

Barany E, Lindberg M, Loden M. **Unexpected skin barrier influence from nonionic emulsifiers.** Int J Pharm 2000;195(1-2):189-95.
Abstract: Skin disorders are often treated with creams containing various active substances. The creams also contain emulsifiers, which are surface-active ingredients used to stabilize the emulsion. Emulsifiers are potential irritants and in the present study the influence of stearic acid, glyceryl stearate, PEG-2, -9, -40, and -100 stearate, steareth-2, -10 and -21 on normal as well as on irritated skin have been evaluated with non-invasive measurements. Test emulsions were created by incorporating 5% emulsifiers in a water/mineral oil mixture (50:50). The emulsions and their vehicle were then applied to normal skin for 48 h and to sodium lauryl sulfate (SLS) damaged skin for 17 h in aluminum chambers. Twenty-four hours after removal of the chambers the test sites were evaluated for degree of irritation. In normal skin, the emulsifiers induced significant differences in TEWL but not in skin blood flow. Five of the emulsifiers increased TEWL. In SLS-damaged skin an aggravation of the irritation was expected. However, no differences regarding skin blood flow was noted from the emulsifiers. Furthermore, three emulsifiers unexpectedly decreased TEWL. These results highlight the possibility of absorption of these emulsifiers into the lipid bilayer, which increase TEWL in normal skin and decrease TEWL in damaged skin.

Bauerova K, Kassai Z, Koprda V, Harangozo M. **Contribution to the penetration of radionuclides across the skin.** Concentration dependence of strontium through the skin in vitro. J Appl Toxicol 2001;21(3):241-3.
Abstract: Toxicological studies of radionuclide passage across the skin, which represents a crucial barrier of radiation, are important for ensuring the quality of the environment. Both (137)Cs and (90)Sr are most frequently involved in radionuclide contamination of the human body. In our study, we selected (90)Sr because this radionuclide is chemically very close to the bio-element calcium. The permeation of (90)Sr from donor solution across the intact skin of 5- or 9-day-old rats (5DR, 9DR) and across stripped and split skin of the 5DR was studied. The experiments in vitro were carried out using vertical diffusion cells. Strontium chloride (SrCl(2)) was used as carrier in the donor solution in different concentrations. Liquid scintillation spectrometry was applied for radiation detection. The experiments showed that: the permeated fraction of (90)Sr(2+) was indirectly proportional to the carrier concentration in the donor solution; the stratum corneum was found to be the principal penetration barrier of strontium; and in the case of the 9DR the dominant route of strontium penetration was along the follicles. Copyright 2001 John Wiley & Sons, Ltd.

Blaha M, Bowers W Jr, Kohl J, DuBose D, Walker J, Alkhyyat A, Wong G. **Effects of CEES on inflammatory mediators, heat shock protein 70A, histology and ultrastructure in two skin models.** J Appl Toxicol 2000;20 Suppl 1:S101-8.
Abstract: Chemical warfare threats require the development of diverse models for the assessment of countermeasures. Human skin products, Skin2 (differentiating keratinocytes on a fibroblast-collagen matrix) and EpiDerm (differentiating keratinocytes) were exposed (2 h) to the sulfur mustard 2-chloroethyl ethyl sulfide (CEES, 1-2 mg l(-1) min(-1)) in humidified air or to humidified air alone. Tissues were evaluated histologically, ultrastructurally and for viability 22 h later; media and tissues were also analyzed for inflammatory mediators. Histology showed that CEES induced the separation of dermal and epidermal regions in Skin2 with severe damage to basal keratinocytes. Histology and electron microscopy of both products revealed condensation of nuclear chromatin, retraction of spinous processes, collapse of the tonofibrillar network and cytoplasmic vacuolization and blebbing in those cells with loss of pseudobasement membrane integrity. Exposure of Skin2 to CEES increased extracellular interleukin-1alpha (IL-1alpha), prostaglandin-E2 (PGE2) and especially IL-1 receptor antagonist (IL-1Ra) release (56,334 vs 84,614 pg ml(-1)), but decreased interleukin-6 (IL-6, 4,755 vs 351 pg ml(-1)). Exposure of EpiDerm to CEES led to unaffected extracellular and reduced intracelluar IL-1alpha (371 vs 92 pg ml(-1)). Extracellular IL-1Ra greatly increased (2,375 vs 24,875 pg ml(-1)), whereas cellular levels decreased (16,5425 vs 96,625 pg ml(-1)). Extracellular (224 vs 68 pg ml(-1)) and intracellular (485 vs 233 pg ml(-1)) soluble interleukin-1 receptor H (sIL-1RII) decreased. Prostanglandin E2 increased (1,835 vs 2,582 pg ml(-1)), whereas heat shock protein 70A (Hsp70A) remained statistically unchanged (57,000 vs 96,000 pg ml(-1)). Failure to obtain a heat shock response to CEES may contribute to the susceptibility of tissue to the alkylating agent. Consistent and marked responses of cellular and extracellular IL-1Ra to CEES suggest a potential for use as a tissue status marker and primary antiinflammatory regulator in skin.

Blotz A, Michel L, Moysan A, Blumel J, Dubertret L, Ahr HJ, Vohr HW. **Analyses of cutaneous**

fluoroquinolones photoreactivity using the integrated model for the differentiation of skin reactions. J Photochem Photobiol B 2000;58(1):46-53.
Abstract: Currently available test models for the differentiation of photoallergic and photoirritant reactions are extremely time consuming and the protocols are very heterogeneous. In vitro tests are of proven value in predicting irritant or toxic effects, but these tests fail to predict chemical-induced allergic side effects. We developed test systems for this endpoint which is not easily detected by existing assays. In a previous publication we were able to discriminate between a contact sensitizer and a skin irritant with a combination of primary ear swelling analysis and cell counting of the ear-draining lymph nodes [Toxicol. Appl. Pharm. 153 (1998) 83; Arch. Toxicol. 73 (2000) 501]. This combination of tests was called the Integrated Model for the Differentiation of chemical-induced allergic and irritant Skin reactions (IMDS). In addition, it had been shown before that inclusion of UV irradiation in the local lymph node assay enables discrimination of photoallergic from photoirritant reactions after dermal application [Photodermatol. Photoimmunol. Photomed. 10 (1994) 57]. Because of the fact that fluoroquinolones are known to induce photoreactions after oral but not dermal treatment, the aim of the present study was to apply the IMDS for the fast and reliable differentiation of photoreactions due to fluoroquinolones after oral treatment. Enoxacin, lomefloxacin, ofloxacin, sparfloxacin and BAY y 3118 were tested in this system. We found a good correlation between the results of UV light-irradiated IMDS and a guinea pig model with the quinolones as far as photoirritancy was concerned. This holds true also for the photoallergic standard olaquindox and the photoirritant standard 8-methoxypsoralen. However, in contrast to the guinea pig assays the IMDS is fast and extremely predictive for the risk of both photosensitization and photoirritancy depending on the route of exposure. Thus, the UV light-irradiated IMDS turned out to be a good tool for the preclinical risk assessment procedure in terms of discriminating photoreactions. In addition, flow cytometric analyses were used to underline the fact that antigen-independent activation occurred after the induction of photoirritant reactions.

Bodin A, Shao LP, Nilsson JL, Karlberg AT. **Identification and allergenic activity of hydroxyaldehydes - a new type of oxidation product from an ethoxylated non-ionic surfactant.** Contact Dermatitis 2001;44(4):207-12.
Abstract: Ethoxylated alcohols, which are widely used as surfactants, are susceptible to oxidation on air exposure. A complex mixture of oxidation products is formed, among which alkylated aldehydes, alkylated formates, formaldehyde and peroxides have previously been identified by our group. In the present study, we have identified a new class of oxidation product from the nonionic ethoxylated surfactant C12E5. These oxidation products are highly water-soluble hydroxyaldehydes with the general formula HO(CH2CH2O)nCH2CHO, n=1-4. To facilitate the identification of the hydroxyaldehydes in oxidized C12E5, reference compounds were synthesized. The sensitizing potential of 1 of the identified hydroxyaldehydes, HO(CH2CH2O)3CH2CHO, was studied in guinea pigs and was found to be weak. A significant cross-reactivity between this aldehyde and the next shorter homologue, HO(CH2CH2O)2CH2CHO, was observed. Irritant components, present in the oxidation mixture, facilitate the skin penetration of allergens, which further accentuates the importance of controlling the conditions of storage and handling of ethoxylated surfactants, to reduce the formation of allergenic and irritant oxidation products.

Boelsma E, Gibbs S, Faller C, Ponec M. **Characterization and comparison of reconstructed skin**

models: morphological and immunohistochemical evaluation. Acta Derm Venereol 2000;80(2):82-8.
Abstract: Reconstructed human skin equivalents are currently being investigated as in vitro models for the prediction of human skin toxicity and irritation responses. Three different industrial reconstructed skin models (EpiDerm, Episkin and SkinEthic) and one in-house equivalent were characterized and compared using light microscopy, immunohistochemistry and reduction of (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT). Their inter- and intra-batch variation was evaluated. Histological examination showed a completely stratified epithelium in all skin models, which closely resembled normal human epidermis. Low intra-batch variation in tissue architecture was observed in all skin models, but moderate to considerable inter-batch variation was noticed. Evaluation of the expression and localization of a number of differentiation-specific protein markers revealed that all skin models showed an aberrant expression of keratin 6, skin-derived antileukoproteinase, small proline rich proteins, involucrin and transglutaminase. Although variation within batches was low, in parcticular keratin 6, involucrin and skin-derived antileukoproteinase expression demonstrated some inter-batch variation. Reduction of MTT in vehicle-treated cultures showed high similarities between skin models, but marked differences were observed when 1.0% sodium lauryl sulfate was applied topically for 3 or 16 h. Most pronounced effects were noticed in SkinEthic cultures. Intra-batch variations were low and moderate variations were observed between batches. All skin models tested reproduced many of the characteristics of normal human epidermis and therefore provide a morphologically relevant in vitro means to assess skin irritation and other skin-related studies.

Cotovio J, Onno L, Justine P, Lamure S, Catroux P. **Generation of oxidative stress in human cutaneous models following in vitro ozone exposure.** Toxicol In Vitro 2001;15(4-5):357-62.
Abstract: Ozone, one of the main components of photochemical smog, represents an important source of environmental oxidative stress. The skin, being the outermost barrier of the body, is directly exposed to environmental oxidant toxicants. Skin sebum and cellular plasma membrane lipids contain polyunsaturated fatty acids which are primary targets for ozone and free radical attack induced lipid peroxides. These ozonation processes in skin can also generate aldehydes, hydroxyhydroperoxides and specific Criegee's ozonides. In order to evaluate in vitro human skin susceptibility to ozone, we have exposed cultured immortalized human keratinocytes (DK7-NR) and the reconstructed human epidermis Episkin(R) to 10 ppm of ozone in a specific incubator. We measured the formation of protein carbonyls by an ELISA method and monitored the oxidative stress using the fluorogenic probe 2',7'-dichlorofluorescin-diacetate (DCFH-DA). Results showed a time-dependent increase of fluorescence levels (linked to oxidative stress) in both models exposed to ozone. Using this protocol, we investigated the protective potential of different products including vitamin C, a thiol derivative and a plant extract. All products dramatically reduced oxidative responses during ozone exposure. Decreases observed in fluorescence levels were between 60 and 90% as compared to non-protected controls. These results demonstrate: (a) cutaneous in vitro models are remarkably susceptible to oxidative stress generated by an environmental air pollutant as ozone, and (b) raw antioxidants, thiols and vitamin C were efficient products to prevent ozone induced cellular oxidative damage.

Dahl JE, Polyzois GL. **Irritation test of tissue adhesives for facial prostheses.** J Prosthet Dent 2000;84 (4):453-7.
Abstract: STATEMENT OF PROBLEM: Adhesives are commonly used to improve the retention of a

facial prosthesis to the skin. Although no requirement exists for facial prosthetic adhesives, an adhesive should be nonirritating and nontoxic. PURPOSE: This study assessed the irritative potential of facial prosthetic adhesives by using an in vitro technique for detection of eye-irritating chemicals. MATERIAL AND METHODS: Ten adhesives were evaluated by the hens egg test chorioallantoic membrane method. Adhesives were applied to the chorioallantoic membrane in fertilized hen eggs, and the membrane examined by a photomacroscope for injury to the blood vessels. The average irritation score was calculated from the recorded times for the debut of hemorrhage, lysis, and coagulation, and the products were classified as being non, slight, moderate, or strong irritants, based on the irritation score. RESULTS: The predominant injury to the membrane was coagulation of blood vessels, and the exposure time needed to initiate the reaction was dependent on the composition of the product. Four products were classified as strong irritants, 1 as moderate, and the remaining 5 as slight or nonirritant. CONCLUSION: On the basis of a test for eye irritation, the irritant potential of tissue adhesives varied from non to severe. The most severe reactions were mainly seen in products containing the solvent ethyl acetate.

Fentem JH, Briggs D, Chesne C, Elliott GR, Harbell JW, Heylings JR, Portes P, Roguet R, van de Sandt JJ, Botham PA. **A prevalidation study on in vitro tests for acute skin irritation. results and evaluation by the Management Team.** Toxicol In Vitro 2001;15(1):57-93.
Abstract: A prevalidation study on in vitro tests for acute skin irritation was conducted during 1999 and 2000. The overall objective of validation in this area, of which this prevalidation study is an initial stage, is to identify tests capable of discriminating irritants (I) from non-irritants (NI), as defined according to European Union (EU) risk phrases ("R38"; no classification) and the harmonised OECD criteria ("Irritant"; no label). This prevalidation study specifically addressed aspects of: protocol refinement (phase I), protocol transfer (phase II), and protocol performance (phase III), in accordance with the prevalidation scheme defined by the European Centre for the Validation of Alternative Methods (ECVAM). The tests evaluated were: EpiDerm (phases I, II and III), EPISKIN (phases I, II and III), PREDISKIN (phases I and II, and additional protocol refinement), the non-perfused pig ear method (phases I and II, and additional protocol refinement), and the mouse skin integrity function test (SIFT; phases I and II). Modified, standardised test protocols and well-defined prediction models were available for each of the tests at the end of phase I. The results of phase I (intralaboratory reproducibility) were sufficiently promising for all of the tests to progress to phase II. Protocol transfer between the Lead Laboratory and Laboratory 2 was undertaken for all five tests during phase II, and additional refinements were made to the test protocols. For EpiDerm, EPISKIN and the SIFT, the intralaboratory and interlaboratory reproducibilities were acceptable; however, better standardisation of certain aspects of the test protocols was needed prior to commencing phase III. Neither PREDISKIN nor the pig ear test performed sufficiently well in phase II to progress to phase III. The PREDISKIN protocol was overly sensitive, resulting in the prediction of all the NI chemicals as I. The variability in the pig ear test results was too great, indicating that the test would show limited predictive ability. In additional studies (a repeat of phase I), further modification of the PREDISKIN protocol and a change in the prediction model considerably improved the ability of the test to distinguish I from NI chemicals. However, attempts to improve the intralaboratory reproducibility of the pig ear test were unsuccessful. In phase III an initial assessment of the reproducibility and predictive ability, in three independent laboratories per test, was undertaken for the EpiDerm and EPISKIN tests (the SIFT was a late inclusion in the

prevalidation study, and is being evaluated in a separate phase III study). A set of 20 coded chemicals (10 I, 10 NI) were tested with the final, refined, test protocols. The intralaboratory reproducibility was acceptable for both EpiDerm and EPISKIN. The interlaboratory reproducibility was considered to be acceptable for EPISKIN; however, for EpiDerm, analysis of variance (ANOVA) indicated that there was a statistically significant laboratory effect on the overall variability, suggesting that the interlaboratory transferability of the test needs to be improved. The EpiDerm test had an overall accuracy of 58%, with an over-prediction rate of 37% and an under-prediction rate of 47%. The EPISKIN test had an overall accuracy of 58%, showing an under-prediction rate of 23% and an over-prediction rate of 60%. It is concluded that, as yet, none of the tests evaluated in this prevalidation study are ready for inclusion in a formal validation study on in vitro tests for acute skin irritation. Overall protocol performance of the SIFT is currently being evaluated in a phase III study. Further studies are also in progress to improve the test protocols and prediction models for EpiDerm and EPISKIN.

Fuchs J, Groth N, Herrling T. **In vitro and in vivo assessment of the irritation potential of different spin traps in human skin.** Toxicology 2000;151(1-3):55-63.
Abstract: No clinical data are available on the acute cutaneous toxicity of spin traps which are frequently used in combination with the electron paramagnetic resonance (EPR) technique for detection of free radicals and reactive oxygen/nitrogen species. The purpose of this study was to evaluate the acute dermatotoxicity of the following spin traps in human skin: C-phenyl-N-tert.-butyl nitrone (PBN), C-(4-pyridinyl-N-oxide)-N-tert.-butylnitrone (POBN), 5, 5-dimethyl-l-pyrroline-N-oxide(DMPO), 5 diethoxyphosphoryl-5-methyl-l-pyrroline-N-oxide (DEPMPO), diethyldithiocarbamate (DDC) and N-methyl-D-glucamine dithiocarbamate (MGD). The corrosivity of the test substances was first assessed in human skin in vitro by measurement of transcutaneous electrical resistance (TER). In this assay all spin traps were non-corrosive at 500 mM concentration. Subsequently cutaneous irritation of the spin traps was determined at different concentrations (50, 250 and 500 mM) in human skin according to a routine four h human patch test in comparison to the standardized irritant sodium laurylsulfate (SLS, 20%). The response was evaluated clinically as well as by a biophysical method analyzing transepidermal water loss (TEWL). PBN and DEPMPO caused a transient and weak inflammatory reaction at 500 mM in four of 17 and in two of 17 volunteers, respectively. DMPO, POBN, DDC, MGID, and the iron complexes of DDC and MGD were clinically non-irritant at all concentrations tested and no delayed-acute inflammatory reactions were observed. However, the TEWL values were significantly increased by all spin traps except DMPO at 500 mM, indicating disturbed epidermal barrier function. We conclude that the spin traps investigated have a low potential to cause acute skin toxicity and may be used safely for in vivo EPR studies in human skin.

Gabard B, Chatelain E, Bieli E, Haas S. **Surfactant irritation: in vitro corneosurfametry and in vivo bioengineering.** Skin Res Technol 2001;7(1):49-55.
Abstract: BACKGROUND/AIMS: Irritant reactions to surfactants, cleansing products, soaps and detergents are common in clinical and occupational dermatology. Mildness has become a major benefit claimed, and testing for mildness now ranks among the first concerns of the manufacturing industry. A wealth of publications deals with this problem, trying to improve the methodology, reduce the costs of testing and facilitate decision-making. Differences in vivo can be measured clinically and/or instrumentally. This is difficult, as commercially available products are generally safe to use and none

are harsh in the absolute sense. METHODS: Nineteen different products (syndets, shampoos, personal cleansers), all claiming to be mild, were tested in vitro by a newly introduced method, corneosurfametry. For evaluating the aggressiveness of the products, the calculation of an index of irritation (IOI) was proposed. A concentration-effect curve of sodium lauryl sulfate (SLS) as standard and model surfactant was obtained. Some of the products were further tested in vivo with a flex wash test and with a soap chamber test and compared to SLS. Bioengineering methods (transepidermal water loss TEWL, skin color) were used to evaluate the results. RESULTS AND CONCLUSIONS: The results of the corneosurfametry allowed us to classify the products in three categories, with increasing aggressiveness towards the stratum corneum, according to their IOIs. The in vivo tests were not able to discriminate between the products, but ranks from the results of the bioengineering measurements showed a good correlation between TEWL changes, but not between colour changes, and IOIs from corneosurfametry. Corneosurfametry emerged as a simple, low-cost and fast method for ranking commercial products according to their mildness. However, the skin bioengineering techniques showed that some products could lead to skin reactions, such as erythema, that could not be detected by the in vitro technique.

Gerberick GF, Robinson MK. **A skin sensitization risk assessment approach for evaluation of new ingredients and products.** Am J Contact Dermat 2000;11(2):65-73.
Abstract: Skin sensitization risk assessment of new ingredients or products is critical before their introduction into the marketplace. The risk assessment process described in this Article involves evaluation of skin sensitization hazard, consideration of all potential human exposures, comparative ingredient/product benchmarking, and, when appropriate, the management of the risk. In this Article, a risk assessment process is reviewed along with a description of the risk assessment tools that are employed for evaluating a new ingredient or product. The basic process we use for evaluating the skin sensitization risk of a new product or ingredient is considered a no effect/safety factor approach. The tools used for conducting a risk assessment include structure activity relationship analysis, exposure assessment, preclinical testing (e.g., local lymph node assay [LNNA]) and clinical testing (e.g., human repeat insult patch testing [HRIPT]). The skin sensitization risk assessment process described in this paper has been used successfully for many years for the safe introduction of new products into the marketplace. This process is dynamic--it can be applied to a diversity of product categories (e.g., shampoo, transdermal drug). In summary, the skin sensitization risk assessment process described in this Article allows one to carefully assess the skin sensitization potential of a new ingredient or product so that it can be safely introduced into the marketplace.

Goffin V, Pierard GE. **Microwave corneosurfametry and the short-duration dansyl chloride extraction test for rating concentrated irritant surfactants.** Dermatology 2001;202(1):46-8.
Abstract: BACKGROUND: There are ethical concerns to conduct in vivo tests in the animal and human to provide evidence that cosmetics and other topical products are safe. OBJECTIVE: To compare two methods, namely the short-duration dansyl chloride extraction test and the microwave corneosurfametry bioassay, to test the irritation potential of concentrated surfactant systems. METHODS: Five surfactants and water were tested using the in vivo test and the bioassay. RESULTS: A significant linear correlation was found between data yielded by the two procedures. CONCLUSION: The short-duration dansyl chloride extraction test and the microwave corneosurfametry bioassay provide similar information. The latter has the advantage of being safe.

Green BG. **Measurement of sensory irritation of the skin.** Am J Contact Dermat 2000;11(3):170-80.
Abstract: The sensory reaction to contact with chemical irritants has not been extensively studied. This neglect has been caused partly by a lack of understanding of the physiological basis of subjective irritation, and partly by inadequate methods of perceptual measurement. This Article begins with a brief overview of the neurophysiology of cutaneous chemoreception (chemesthesis), and continues with a discussion of numerous sensory, physical, and perceptual factors that can affect the sensitivity to irritants. Then, guidelines for conducting perceptual measurements of subjective irritation that take these factors into account are offered. An alternative approach to direct chemosensory measurement also is proposed that relies on the fact that many of the cutaneous receptors that are sensitive to chemicals also are sensitive to temperature and/or mechanical stimulation (pain or itch). It is suggested that thermal and mechanical sensitivity might prove useful as measures of the severity of acute sensory irritation and as indicators of subclinical sensitization by environmental irritants.

Lee JK, Kim DB, Kim JI, Kim PY. **In vitro cytotoxicity tests on cultured human skin fibroblasts to predict skin irritation potential of surfactants.** Toxicol In Vitro 2000;14(4):345-9.
Abstract: Cultured human skin cells are a potentially useful model for skin irritancy testing. We have investigated the use of human skin fibroblasts for in vitro screening for skin toxicity. To assess the cytotoxic effects of surfactants, cell viability was measured by the NRU (neutral red uptake) assay and AB (Alamar blue) assay as in vitro methods. The skin irritation potential of surfactants by human skin patch test was assessed as in vivo methods. The close relationship was found between AB assay with human skin fibroblasts and human patch test (r=0.867). There was a relatively good agreement between the NRU and in vivo patch test (r=0.648). These results suggest that AB and NRU assay using cultured human fibroblast could be predictable methods for the irritancy of various surfactants in human.

Loffler H, Freyschmidt-Paul P, Effendy I, Maibach HI. **Pitfalls of irritant patch testing using different test chamber sizes.** Am J Contact Dermat 2001;12(1):28-32.
Abstract: BACKGROUND: Test chambers for irritant patch testing are usually larger than those used in allergic patch testing. In general, larger areas show stronger skin reactions than smaller areas. OBJECTIVE: This study investigated whether this difference is of practical relevance, when a model irritant is applied in small and large Finn chambers and evaluated by measurement of transepidermal water loss (TEWL). METHODS: Patch testing was performed with 2 concentrations of sodium lauryl sulfate (SLS) (0.25% and 0.5%) on forearms of healthy volunteers. Large (inner diameter, 12 mm) and small (inner diameter, 8 mm) chambers were used. RESULTS: A variance analysis (3 factors, 2-tailed) showed that the test outcome, as assessed by TEWL, was strongly dependent on SLS test concentration and test chamber size. The larger chambers gave approximately 30% to 50% higher values than the smaller. CONCLUSIONS: This may be explained by the fact that with the small chambers, the adjacent small area of nontreated skin was also assessed by the evaporimeter, biasing the results. A formula estimating TEWL value of the large chamber from values of the small chambers has been proposed. Copyright 2001 by W.B. Saunders Company.

Lovell WW, Jones PA. **Evaluation of mechanistic in vitro tests for the discrimination of photoallergic and photoirritant potential.** Altern Lab Anim 2000;28(5):707-24.

Abstract: Photochemical tests were used to discriminate photoallergens and photoirritants. UV absorption spectrometry was employed to identify chemicals which absorbed sunlight wavelengths and which required further testing. Photoallergic potential was assessed by studying photobinding of the test chemicals to human serum albumin. Photobinding was determined by increased UV absorbance of the protein fraction after gel filtration chromatography. Photooxidation of histidine was used to screen for a mechanism of photoirritancy. Efficient photooxidisers may be considered photoirritant rather than photoallergic. The substances selected for the EU/COLIPA phototoxicity project were tested. There were 14 photoirritants (three tested as both free acid/base and salts, i.e., 17 samples in total), four photoallergens, three which were photoirritant and photoallergenic (i.e., 17 photoirritants and seven photoallergens) and six "negatives" (four clearly non-phototoxic and two unclear). UV spectrometry showed that 28 of the 30 substances absorbed sunlight significantly and had the potential for adverse photoreaction. Six of seven photoallergens were identified as such by the photobinding assay. Most photoirritants also caused photomodification of protein, but eleven of these photooxidised histidine efficiently and so were classified as photoirritants. Four photoirritants remained falsely predicted as photoallergens. Two photoirritants were negative for both photomodification of protein and for histidine photooxidation. Four chemicals negative in vivo were negative in vitro. The remaining two chemicals could not be classified because of unclear data both in vivo and in vitro. The in vitro test battery, therefore, was useful for the discrimination of photoallergic and photoirritant potential.

Medina J, de Brugerolle de Fraissinette, Chibout SD, Kolopp M, Kammermann R, Burtin P, Ebelin ME, Cordier A. **Use of human skin equivalent Apligraf for in vitro assessment of cumulative skin irritation potential of topical products.** Toxicol Appl Pharmacol 2000;164(1):38-45.
Abstract: The main goal of the present study was to investigate the response of the human skin equivalent Apligraf in vitro to the application of irritant substances and its predictivity as a screening tool for cumulative skin irritant potential in humans. Vaseline, calcipotriol, trans-retinoic acid, and sodium lauryl sulfate were applied to Apligraf in vitro for 24 h. Cell viability (lactate dehydrogenase leakage), release and mRNA expression of the proinflammatory cytokines IL-1alpha and IL-8, and morphological changes were assessed. The same products were applied to 30 healthy volunteers in a double-blind, randomized, vehicle-controlled within-subject study. The skin reactions after repeated 24-h applications over 3 weeks under Finn chamber patches were monitored by visual scoring and biophysical methods (trans-epidermal water loss, chromametry, and blood flow). Sodium lauryl sulfate was cytotoxic to Apligraf, and increased the release and expression of cytokines at low (0.2%, 0. 4%), but not at high (0.8%, 1%) concentrations. It induced severe irritancy in vivo. Trans-retinoic acid increased the expression and release of cytokines with no detectable cytotoxicity and showed moderate irritancy in humans. Although calcipotriol did neither affect cell viability nor the production of cytokines, it induced morphological signs of irritation and was mildly irritant for healthy volunteers. Vaseline was innocuous in vivo and induced no changes in Apligraf. In conclusion, the cumulative skin irritation potential of the tested products could be predicted with Apligraf in a sensitive and specific manner, by monitoring cytotoxicity, proinflammatory cytokines, and morphological changes.

Muller L, Brendler-Schwaab S, Kasper P, Kersten B. **[In vitro methods for phototoxicity and photocarcinogenicity testing of drugs]**. ALTEX 2001;18(2):117-21 [Ger].
Abstract: Phototoxicity is an acknowledged property of some UV and/or visible light absorbing

substances some of which are used as pharmaceuticals or in cosmetic preparations. In recent years attention has been called upon the fact that toxic intermediates that are generated upon photoactivation of a substance can also lead to DNA damage. Such damage may lead to mutated/initiated skin cells which in turn can contribute to an elevated skin cancer risk. The method of choice to test for photo-related skin carcinogenesis is a 1-year study in genetically hairless mice in which the formation of skin papilloma and their latency time are assessed. Here, in vitro test approaches to test for photogenotoxicity can be used in a tiered assessment approach asking the use of in vitro genotoxicity tests for prediction of rodent/human carcinogenicity. In the past few years some effort has been put into the evaluation for such systems, in parcticular standard test protocols have been generated for the in vitro photo-micronucleus test and the in vitro photo-comet assay with Chinese hamster V79 cells. The data that have been produced so far show promising results regarding the implementation of these systems in a tiered approach for photocarcinogenicity assessment of UV- and/or visible light absorbing substances but the systems will have to be validated in further collaborative studies.

Nylander-French LA. **A tape-stripping method for measuring dermal exposure to multifunctional acrylates.** Ann Occup Hyg 2000;44(8):645-51.
Abstract: Current methods for measuring dermal exposure to skin irritants and allergens, such as acrylates, have significant drawbacks for exposure assessment. A noninvasive sampling method has been developed and tested for measuring dermal exposure to a multifunctional acrylate employing a tape stripping of the nonviable epidermis (stratum corneum). Samples were subsequently extracted and a gas chromatographic method was employed for quantitative analysis of tripropylene glycol diacrylate (TPGDA). This method was tested in 10 human volunteers exposed to an a priori determined amount of TPGDA or a UV-radiation curable acrylate coating containing TPGDA (UV-resin) at different sites on hands and arms. On the average, the first tape stripping removed 94% (coefficient of variation 16%) of the theoretical quantity of deposited TPGDA and 89% (coefficient of variation 15%) of the theoretical quantity of deposited TPGDA in UV-resin 30min after exposure. Quantities of TPGDA recovered from two consecutive tape strippings accounted for all of the test agent, demonstrating both the efficiency of the method to measure dermal exposure and the potential to determine the rate of absorption with successive samples over time. In general, the amount removed by the first stripping was greater for TPGDA than for UV-resin while the second stripping removed approximately 6 and 21% of TPGDA and UV-resin, respectively. However, when the amounts removed with the first tape stripping for TPGDA or UV-resin from the five different individual sites were compared, no significant differences were observed (P=0.111 and 0.893, respectively). No significant difference was observed in recovery between TPGDA and UV-resin for the first tape stripping when calculated as a percentage of the theoretical amount (P=0.262). The results indicate that this tape-stripping technique can be used to quantify dermal exposure to multifunctional acrylates.

Qvist MH, Hoeck U, Kreilgaard B, Madsen F, Frokjaer S. **Evaluation of Gottingen minipig skin for transdermal in vitro permeation studies.** Eur J Pharm Sci 2000;11(1):59-68.
Abstract: The optimal skin type for in vitro permeability studies depends on the purpose of the specific transdermal study. In a number of cases, it may be advantageous to use animal skin as an alternative to human skin although they have different characteristics. Recently, Gottingen minipigs have been reported as good models in toxicological and pharmacokinetic studies of drug substances. In this paper,

the potential use of skin from the Gottingen minipig is evaluated by studying three model drug substances (nicotine, salicylic acid and testosterone) through skin from humans, domestic pigs and three ages of the Gottingen minipig. An analysis of variance and a Student's t-test showed that both the skin from the Gottingen minipig and the domestic pig possessed transdermal permeabilities, which correlated with human skin and exhibited a lower intra- and intervariation. Furthermore, it was shown that permeability and variation of fluxes through skin from Gottingen minipigs were dependent on the age of the minipig and of the drug substance. It is concluded that the Gottingen minipig, like the domestic pig, is a good skin model for in vitro permeation through human skin.

Robinson MK, McFadden JP, Basketter DA. **Validity and ethics of the human 4-h patch test as an alternative method to assess acute skin irritation potential.** Contact Dermatitis 2001;45(1):1-12.
Abstract: For more than 50 years, the Draize rabbit skin irritation test has reigned supreme as the regulatory method of choice for the identification of skin irritant chemicals. To date no in vitro alternative test has been validated as an adequate replacement. However, one potential option, to test the endpoint of concern (skin irritation) in the species of concern (man) has been overlooked. The advent of predictive in vitro tools for the identification of substances corrosive to the skin has opened up the practical possibility of carrying out safe and ethical studies on small panels of humans. The human 4-h patch test has been developed to meet the needs of identifying chemical skin irritation potential, providing data which is inherently superior to that given by a surrogate model, such as the rabbit. This paper reviews in detail the present state of the human 4-h patch test, highlighting its advantages and noting its utility as the 'gold standard' on which to build future in vitro models.

Robinson MK, Osborne R, Perkins MA. **In vitro and human testing strategies for skin irritation.** Ann N Y Acad Sci 2000;919:192-204.
Abstract: Prior to the manufacture, transport, and marketing of chemicals or products, it is critical to assess their potential for skin toxicity (corrosion or irritation), thereby protecting the worker and consumer from adverse skin effects due to intended or accidental skin exposure. Traditionally, animal testing procedures have provided the data needed to assess the more severe forms of skin toxicity, and current regulations may require animal test data before permission can be obtained to manufacture, transport, or market chemicals or the products that contain them. In recent years, the use of animals to assess skin safety has been opposed by some as inhumane and unnecessary. The conflicting needs of the industrial toxicologist to (1) protect human safety, (2) comply with regulations, and (3) reduce animal testing have led to major efforts to develop alternative, yet predictive, test methods. A variety of in vitro skin corrosion test methods have been developed and several have successfully passed initial international validation. These have included skin or epidermal equivalent assays that have been shown to distinguish corrosive from noncorrosive chemicals. These skin/epidermal equivalent assays have also been modified and used to assess skin irritation potential relative to existing human exposure test data. The data show a good correlation between in vitro assay data and different types of human skin irritation data for both chemicals and consumer products. The effort to eliminate animal tests has also led to the development of a novel human patch test for assessment of acute skin irritation potential. A case study shows the benefits of in vitro and human skin irritation tests compared to the animal tests they seek to replace, and strategies now exist to adequately assess human skin irritation potential without the need to rely on animal test methods.

Ross JH, Driver JH, Cochran RC, Thongsinthusak T, Krieger RI. **Could pesticide toxicology studies be more relevant to occupational risk assessment?** Ann Occup Hyg 2001;45 Suppl 1:S5-S17
Abstract: Pesticide toxicology study design has evolved from concern for oral exposure via food residues. The emphasis on the oral route does not generally apply to workers that are exposed primarily via the dermal route either handling pesticides or re-entering treated fields. As a result numerous assumptions about how oral toxicology results relate to dermal exposure must be made when conducting worker risk assessments. These assumptions introduce a high degree of uncertainty. Alternative toxicology study designs are suggested to reduce uncertainty when assessing risk. Because the dermal route is so important to characterizing occupational risk, methods to improve the accuracy of dermal absorption estimates are suggested, including the use of human subjects to study dermal absorption. Additional suggestions include tailoring dermal, oral and inhalation kinetic study designs to reflect worker exposure dosages. Suggestions are made to routinely conduct a single dose toxicity study patterned after the neurotoxicity study design to distinguish single dose effects and NOAELs from those resulting from multiple doses. Finally, interspecies pharmacokinetics studies are proposed to determine which toxicology study regimen of dosing best reflects intermittent worker exposure.

Ryan CA, Gerberick GF, Cruse LW, Basketter DA, Lea L, Blaikie L, Dearman RJ, Warbrick EV, Kimber I. **Activity of human contact allergens in the murine local lymph node assay.** Contact Dermatitis 2000;43(2):95-102.
Abstract: The murine local lymph node assay (LLNA) is a predictive test for the identification of chemicals that have the potential to cause skin sensitization. Since its original development, the assay has been the subject of national and international evaluation studies and extensive comparisons with guinea pig tests and human data. On the basis of these investigations, the LLNA has recently been endorsed by ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) as a stand-alone method for skin sensitization hazard identification. At the same time, ICCVAM confirmed that, although the LLNA is not an in vitro method, it does represent a refinement in the way animals are used and can provide a means for reducing the number of animals used in sensitization hazard assessment. The investigations described here were designed to explore further the ability of the LLNA to identify accurately those chemicals that cause allergic contact dermatitis in humans. To that end we have measured, amongst 3 independent laboratories, LLNA responses induced by a total of 18 test chemicals, 11 of which are known to cause skin sensitization and 7 of which are believed not to be associated with any significant evidence of allergic contact dermatitis in humans. The LLNA correctly classified 16 of the 18 materials. The 11 chemicals tested which are associated with allergic contact dermatitis in humans were found to be positive in the LLNA. Of the 7 materials believed to be non-sensitizers, 5 were negative in the LLNA and 2 produced positive results. Collectively, these data provide additional evidence that the LLNA is able to discriminate skin sensitizers from those chemicals which do not possess a significant skin sensitization potential and thus provides a method for hazard identification that offers important animal welfare benefits.

Singh RP, Das M, Khanna R, Khanna SK. **Evaluation of dermal irritancy potential of benzanthrone-derived dye analogs: structure activity relationship.** Skin Pharmacol Appl Skin Physiol 2000;13(3-4):165-73.

Abstract: The twelve structural analogs of benzanthrone-derived dyes of commercial use were screened for their dermal irritation potential response using the Draize occlusive patch test. The test dyes, dissolved in DMSO as vehicle, were topically applied on the skin of the male Druckery rats (160 +/- 10 g) according to the OECD protocol. The potential dermal hazard was assessed in terms of the primary cutaneous irritation (PCI) index and irritancy. Irritancy was evaluated according to the AFNOR scale. In terms of irritancy, the twelve benzanthrone dyes qualified as moderately irritant (3.0-5.0) according to the above scale. In decreasing order, PCI index of the various dyes was: Navy Blue R (4.5); Jade Green XBN (4.25); 16, 17-dihydroxydibenzanthrone (3.84); Black NB (3.75), Jade Green 2G (3. 75); 3-bromobenzanthrone (3.58); Brilliant Purple 4R (3.58); Olive D (3.50); Dark Blue 2R (3.41); Olive Green B (3.33); isodibenzanthrone (3.33), and benzanthrone (3.16). These results indicate that benzanthrone-derived dyes/dye intermediates caused dermal toxicity which appears to be influenced by the number of carbonyl and amino-anthraquinone groups as well as by the presence of functional groups like halogen, nitro, hydroxy and methoxy in the parent molecule, benzanthrone.

Skowronski GA, Turkall RM, Abdel-Rahman MS. **In vitro penetration of soil-aged mercury through pig skin.** J Toxicol Environ Health A 2000;61(3):189-200.
Abstract: The dermal bioavailability of mercury "aged" in soil for 3 mo was compared to that of pure mercury (without soil) and to mercury in brief contact with soil (16 h). Studies were conducted in vitro with [203Hg]mercuric chloride on dermatomed male pig skin by flow-through diffusion cell methodology. Less than 0.5% of the initial mercury dose penetrated through skin into receptor fluid after each treatment. The majority of pure mercury became covalently bound to skin. However, a short contact time with either an Atsion (sandy) or Keyport (clay) soil significantly decreased the total penetration of mercury (sum of receptor fluid and skin) by 40%. After aging, a 95% reduction in total penetration was observed for the compound relative to chemical without soil. Both soils bind mercury more strongly with time, as evidenced by larger quantities of radioactivity in soil and smaller amounts in skin decontaminate after aging than in soil for 16 h. Decreased mercury bioavailability with aging indicates lower health risk and reduced need for soil cleanup.

Steiling W, Kreutz J, Hofer H. **Percutaneous penetration/dermal absorption of hair dyes in vitro.** Toxicol In Vitro 2001;15(4-5):565-70.
Abstract: This paper presents an in vitro technique to analyse percutaneous penetration and dermal absorption of hair dyes, topically applied to excised pig skin. Representative examples are given by the radio-labelled hair dyes p-phenylenediamine and bis-(5-amino-1-hydroxyphenyl)-methane. Both compounds were assessed under simulated use conditions and were analysed in representative formulations including the specific conditions for oxidation hair dyes. To be able to differentiate between topically adsorbed and systemically available amounts, the bioavailability of the hair dyes is defined as the amount penetrated and/or remaining in the exposed skin after removing the stratum corneum. Less than 1% of the assessed topically applied dyes was found to be bioavailable in the presence of hydrogen peroxide, typically added to oxidation hair dyes prior to applications. Compared with published results and unpublished in-house in vivo data, the level of confidence was high. Owing to in-house experience over about 5 years in using excised pig skin for measurements of percutaneous penetration and dermal absorption of hair dyes, the technique was found to be successful and appropriate to reduce the number of test animals normally used for toxicological assessments. The essentials of this

technique are actually recommended by the SCCNFP (The Scientific Committee on Cosmetic Products and Non Food Products intended for Consumers) for the safety evaluation of cosmetic ingredients, parcticularly for hair dyes. The corresponding OECD guideline as well as the guidance document has been drafted and is currently in discussion on the level of the national co-ordinators.

Traynor NJ, Barratt MD, Lovell WW, Ferguson J, Gibbs NK. **Comparison of an in vitro cellular phototoxicity model against controlled clinical trials of fluoroquinolone skin phototoxicity.** Toxicol In Vitro 2000;14(3):275-83.
Abstract: Many therapeutic drugs induce phototoxic skin responses following exposure to solar or artificial ultraviolet radiation sources. Several in vitro model systems have been developed to predict drug phototoxicity but none have been conducted in parallel with controlled clinical phototoxicity studies on systemically administered pharmaceuticals. The in vitro phototoxicity of eight fluoroquinolone (FQ) antibiotics (ciprofloxacin, grepafloxacin, lomefloxacin, norfloxacin, ofloxacin, trovafloxacin, BAYy3118, moxifloxacin) was determined by exposing Chinese hamster fibroblasts to UVA radiation. Cell damage was quantified with standard MTT or neutral red assays and an in vitro phototoxic index calculated (PI(vit)=% cell viability with UVA alone /% cell viability with UVA+FQ) for each endpoint. Clinical photosensitizing ability of the eight systemically administered FQ was investigated using double-blind, placebo and positive controlled, clinical skin phototesting of normal subjects. Minimal erythema doses at 365+/-30nm were determined before and after 6-7 days of FQ ingestion and PI(clin) (minimal erythema dose without FQ/minimal erythema dose with FQ) calculated. Linear regression analysis of PI(vit) vs PI(clin) gave correlations of up to 0.893. Principal components analysis of PI(vit), daily dose, plasma levels and photophysical (absorption) properties of the eight FQ showed that phototoxic (arbitrarily defined as PI(clin)> or =2) and non-phototoxic (PI(clin)<2) FQ could be completely discriminated using these parameters, and that the in vitro models were able to rank the relative phototoxic potential of the eight FQ.

Tuschl H, Kovac R, Weber E. **The expression of surface markers on dendritic cells as indicators for the sensitizing potential of chemicals.** Toxicol In Vitro 2000;14(6):541-9.
Abstract: Novel approaches to testing of skin sensitizing chemicals have made use of immature dendritic cells (DCs) cultured from different hematopoietic progenitors. These cells resemble Langerhans cells (LCs), which are the most potent antigen presenting cells in the skin. Former research has focused on the phenotypic and functional changes of LCs after application of skin sensitizers. But it has proven difficult to isolate sufficient numbers of LCs from skin. This disadvantage is overcome by cultures of immature DCs providing high numbers of reactive cells. The aim of the present investigation was to test the response of DC cultures established from different blood donors to known sensitizers, an irritant and a vehicle. The sensitizers NiSO(4), dinitrochlorobenzene (DNCB), 2,4,6 trinitrobenzene sulfonic acid (TNBS), alpha-hexylcinnamaldehyde (Cinn) and eugenol (Eu) induced the up-regulation of the co-stimulatory molecule CD86, of intercellular adhesion molecule CD54 and of the HLA-DR antigen. The irritant sodium dodecyl sulfate (SDS) and the vehicle dimethyl sulfoxide (DMSO) had no effect. A high rate of responders within blood donors was found for NiSO(4), TNBS, Cinn and Eu, while DNCB was less effective. The augmentation of surface marker expression in dendritic cells obtained from peripheral human blood seems to be a promising readout in prescreening for strong and moderate sensitizers. This test could thus help to reduce animal numbers for in vivo testing.

Vohr HW, Blumel J, Blotz A, Homey B, Ahr HJ. **An intra-laboratory validation of the Integrated Model for the Differentiation of Skin Reactions (IMDS): discrimination between (photo)allergic and (photo)irritant skin reactions in mice.** Arch Toxicol 2000;73(10-11):501-9.
Abstract: We recently presented a modified local lymph node test which made it possible to quickly and reliably differentiate between irritative and allergic skin reactions with extremely simple parameters. The Integrated Model for the Differentiation of Skin Reactions (IMDS) test combines measurement of cell proliferation in draining lymph nodes with measurement of primary ear swelling after topical application of the test substance on three consecutive days. In contrast to the 'classic' skin sensitisation test in guinea-pigs the IMDS test is considerably faster and is based on objective measured data, not subjective skin evaluations. Like the Local Lymph Node Assay (LLNA), measurement of allergic potential in the IMDS test is based on the underlying immunological mechanisms, but also considers the behaviour of immune competent cells following non-specific activation by irritants. In addition, the IMDS test can employ UV radiation after application of the substance and, therefore, make differentiation possible between different types of skin photoreaction (photoallergy and photoirritation) after both topical and systemic administration. Attempts to achieve this kind of discrimination with the LLNA necessitate considerably greater expenditure, as proliferation in the draining lymph nodes can also be induced by moderate to extreme (photo)irritants. In a previous paper in which we presented the IMDS test, we examined each type of reaction in reference to one single standard; the next logical step was therefore a broad-based intra-laboratory validation. An important factor in the validation was the use of standards that had been thoroughly examined in both guinea pig and mouse systems and were also relevant with regard to estimation of the risk for humans. The data presented here show that the IMDS is a simple and reliable tool for obtaining fast and reproducible assessments of potential (photo)allergic and (photo)irritant skin reactions to substances.

Wilson TD, Steck WF. **A modified HET-CAM assay approach to the assessment of anti-irritant properties of plant extracts.** Food Chem Toxicol 2000;38(10):867-72.
Abstract: Hen's egg--chorioallantoic membranes were used to screen for and assess anti-irritant properties among aqueous extracts of plants (HET-CAM tests), in connection with searches for plant-derived substances with topical anti-irritant action. The main question to be answered was whether CAM-assay screening of plant extracts could provide a useful route to identifying promising anti-irritant extracts for follow-up clinical testing. To be useful, the method would have to flag materials with strong anti-irritant properties, and would have to avoid registering false negatives. The tests conducted provided positive indications. We measured the delays in onset of three manifestations of membrane irritation-vascular hemorrhaging, membrane lysis and membrane coagulation-observed with test substances relative to positive controls. Aqueous 15% lactic acid, a commonly used irritant in direct tests on human skin, was employed as the test irritant in this study. The ratio [irritation onset times after test substance pre-treatment]:[onset times without test substance pretreatment] was used to measure the anti-irritant power of test substances. A scoring notation was devised for this which treats the delay parameters as independent effects. Most tested plant extracts showed no significant irritant or anti-irritant effects. Among the apparently anti-irritant plant extracts (approx. 10% of all those tested), most showed their greatest effect against hemorrhaging. Lesser but still readily measurable effects against membrane lysis and coagulation were also observed in nearly all the apparently anti-irritant extracts.

Two of the tested extracts proved to be membrane irritants. Some key CAM assay results were compared with results obtained in direct tests on human skin using the same test irritant (15% lactic acid). In these comparative tests on skin, an essentially similar pattern of efficacy was obtained, with the plant extract deemed best in the CAM screenings, outperforming the benchmark anti-irritant hydrocortisone. From these initial results it appears that physiological CAM assays may prove useful in screening natural materials for anti-irritant properties, as alternatives to mechanism-dependent biochemical assays, or expensive direct screening tests on human subjects. Further work remains to extend the CAM screening approach to irritants other than lactic acid, and to assess its quantitative powers of prediction of topical anti-irritancy.

Worth AP, Cronin MT. **The use of bootstrap resampling to assess the uncertainty of cooper statistics.** Altern Lab Anim 2001;29(4):447-59.
Abstract: The predictive abilities of two-group classification models (CMs) are often expressed in terms of their Cooper statistics. These statistics are often reported without any indication of their uncertainty, making it impossible to judge whether the predicted classifications are significantly better than the predictions made by a different CM, or whether the predictive performance of the CM exceeds predefined performance criteria in a statistically significant way. Bootstrap resampling routines are reported that provide a means of expressing the uncertainty associated with Cooper statistics. The usefulness of the bootstrapping routines is illustrated by constructing 95% confidence intervals for the Cooper statistics of four alternative skin-corrosivity tests (the rat skin transcutaneous electrical resistance assay, EPISKIN, Skin(2) and CORROSITEX), and four two-step sequences in which each in vitro test is used in combination with a physicochemical test for skin corrosion based on pH measurements.

Yang EK, Yoon HH, Lee DH, Park JK. **Assessment of toxic potential of industrial chemicals using a cultured human bioartificial skin model: production of interleukin 1alpha and hydroxyeicosatetraenoic acids.** Skin Pharmacol Appl Skin Physiol 2000;13(5):246-57.
Abstract: Cytotoxicity assays using artificial skin are proposed as alternative methods for in vitro tests to minimize animals used in ocular and dermal irritation testing. The responses of the artificial skins were studied to a well-characterized chemical irritant, such as toluene, glutaraldehyde and sodium lauryl sulfate (SLS), and a nonirritant, such as polyethylene glycol. The evaluation of irritating and nonirritating test chemicals was also compared with responses seen in human dermal fibroblasts and human epidermal keratinocytes grown in monolayer culture. The responses monitored included the MTT mitochondrial functionality assay. In order to better understand the local mechanisms involved in skin damage and repair, the productions of several mitogenic proinflammatory mediators such as interleukin-1alpha (IL-1alpha), 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-HETE were investigated. Dose-dependent increases in the levels of IL-1alpha and HETEs were observed in the underlying medium of the skin systems exposed to two skin irritants, glutaraldehyde and SLS. The results of the present study show that both human artificial skins can be used as efficient testing models for the evaluation of skin toxicity in vitro and for screening the contact skin irritancy in vitro.

Zuang V, Rona C, Archer G, Berardesca E. **Detection of skin irritation potential of cosmetics by non-invasive measurements.** Skin Pharmacol Appl Skin Physiol 2000;13(6):358-71.

Abstract: This study analyses the ability of four non-invasive techniques (laser Doppler velocimetry, evaporimetry, chromametry and corneometry) to discriminate between irritant and non-irritant products, when compared to appropriate controls, and to detect subliminal changes in barrier function and erythema. These changes, which remain undetectable in the traditional visual and palpable clinical assessment, can be used as early reactions that are followed by the development of overt skin irritation. Laser Doppler velocimetry and evaporimetry were good discriminators between irritant and non-irritant substances, whereas corneometry and chromametry did not clearly distinguish between them. Laser Doppler velocimetry and evaporimetry detected early stages in the development of an irritant reaction before it became visible, but chromametry was not able to detect an early irritant response. It was concluded that non-invasive measurements could improve the quality and relevance of data obtained from human irritation testing, since the data they provide are objective, quantitative and sometimes subclinical, which also allows the concentration of a positive control to be reduced, resulting in the induction of less skin damage in human volunteers and reducing the ethical concerns related to the deliberate induction of an irritant response in a 'healthy' volunteer.

ECOTOXICITY

Amanuma K, Aoki Y. **[Application of transgenic fish for detecting hazardous chemicals in water environment].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2973-81 [Jpn}.

Amanuma K, Takeda H, Amanuma H, Aoki Y. **Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments.** Nat Biotechnol 2000;18(1):62-5.
Abstract: We have established a transgenic zebrafish line carrying a shuttle vector plasmid (pML4) for detecting mutagens in aquatic environments. The plasmid contains the rpsL gene of Escherichia coli as a mutational target gene, and the kanamycin-resistance gene for recovering the plasmid from the chromosomal DNA. To evaluate the system, we treated embryos of the transgenic fish with N-ethyl-N-nitrosourea (ENU), which induces a dose-dependent increase in the mutation frequency of the target gene. The mutation spectrum was consistent with the proposed mechanism of ENU mutagenesis. Similarly, treating the embryos with benzo[a]pyrene or 2-amino-3, 8-dimethylimidazo[4,5- f] quinoxaline, which are found in naturally polluted water, significantly increased the frequency of mutations in the target gene.

Andersson PL, Berg AH, Bjerselius R, Norrgren L, Olsen H, Olsson PE, Orn S, Tysklind M. **Bioaccumulation of selected PCBs in zebrafish, three-spined stickleback, and arctic char after three different routes of exposure.** Arch Environ Contam Toxicol 2001;40(4):519-30.
Abstract: The uptake and elimination of 20 structurally diverse tetra- to heptachlorinated biphenyls were studied in zebrafish (Danio rerio), three-spined stickleback (Gasterosteus aculeatus), and arctic char (Salvelinus alpinus). The polychlorinated biphenyls (PCBs) were administered to the fish through food, intraperitoneal injection of peanut oil, or intraperitoneal implantation of silicone capsules. The retention of the PCBs in fish exposed through their diet was related with the substitution patterns of the compounds. Ortho-substituted congeners with no unsubstituted meta-para positions had high biomagnification potential. PCBs with low biomagnification all had adjacent vicinal hydrogens, indicating that congeners with this feature may have been metabolically eliminated. The retention

characteristics of the PCBs in the diet-exposed and the injected zebrafish were similar. The pattern of congeners in arctic char indicates that they have a lower capacity to metabolize PCBs compared to three-spined sticklebacks and zebrafish. The levels in the fish exposed to the PCBs through a silastic implant were negatively correlated with the hydrophobicity of the congeners. Most probably congener-specific release rates of the PCBs from the implants mask their retention characteristics. It is suggested that food, mimicking the natural intake route, should be used in PCB exposure studies to validate extrapolations to natural situations.

Araujo CS, Marques SA, Carrondo MJ, Goncalves LM. **In vitro response of the brown bullhead catfish (BB) and rainbow trout (RTG-2) cell lines to benzo[a]pyrene.** Sci Total Environ 2000;247(2-3):127-35.
Abstract: Established cell lines from rainbow trout (RTG-2) and brown bullhead catfish (BB) were evaluated as bioindicators of benzo[a]pyrene (B[a]P) toxicity with 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction and neutral red (NR) uptake assays. Cytochrome P450 1A1 (CYP1A1) enzymatic activity was also evaluated, and taken as a biological indicator of the B[a]P induction power by ethoxyresorufin O-deethylase (EROD) and ethoxycoumarin O-deethylase (ECOD) assays. The BB and RTG-2 cells were compared after 1 and 6 days of exposure to B[a]P. The photoactivation of the compound (B[a]PUV) was another parameter taken into consideration. Cytotoxicity was not observed after 1 day of incubation with B[a]P in both cell lines, although the enzymatic activities of ECOD and EROD presented an induction. Apparently, after 1 day, cells did not metabolise sufficient amounts of B[a]P to cytotoxic metabolites. After 6 days of exposure to this compound a significant reduction in cell viability was observed, this reduction being superior to 50% at the highest B[a]P concentrations for the RTG-2 cell line. These results are in agreement with the values observed for the ECOD and EROD induction. The B[a]P cytotoxicity determined in both cell lines could be ascribed to the significant increase of EROD activity by 6 days of exposure. The photoactivation of B [a]P showed marked differences in both cytotoxic assays and CYP1A1 enzymatic activities, for both cell lines. After 1 day of exposure there was a significant reduction in cell viability, superior to 50% for the RTG-2 cell line. However, it was observed that no induction occurred but rather a decrease in ECOD and EROD activities. Six days of incubation with B[a]PUV showed a decrease in cell viability at the highest concentrations for the BB cells and at the lowest concentrations for the RTG-2 cell line, and the CYP1A1 enzymatic activity presented a significant induction. These results and those observed after 1 day of exposure suggest that B[a]PUV acts as a direct-acting toxicant as well as a metabolism-mediated toxicant-like B[a]P. The RTG-2 cells were more sensitive to B[a]P and its toxic metabolites as well as to the photoactivation of the compound, in both exposure times tested. The finding that the cell lines responded to the CYP1A1 induction in a very efficient way gives proof of the applicability of this system to environmental biomonitoring and toxicology.

Bello SM, Franks DG, Stegeman JJ, Hahn ME. **Acquired resistance to Ah receptor agonists in a population of Atlantic killifish (Fundulus heteroclitus) inhabiting a marine superfund site: in vivo and in vitro studies on the inducibility of xenobiotic metabolizing enzymes.** Toxicol Sci 2001;60 (1):77-91.
Abstract: New Bedford Harbor (NBH), MA, is a federal Superfund site that is heavily contaminated with polychlorinated biphenyls (PCBs) and other halogenated aromatic hydrocarbons (HAHs), including

some potent aryl hydrocarbon receptor (AhR) agonists. A population of Atlantic killifish (Fundulus heteroclitus) continues to inhabit this site, despite accumulating extraordinarily high concentrations of PCBs (272 microg/g dry weight). To determine if NBH killifish have developed resistance to HAHs that act through the AhR, we examined the inducibility of cytochrome P4501A1 (CYP1A1), UDP glucuronosyl transferase (UGT), and glutathione S-transferase (GST) in fish from NBH and a reference site, Scorton Creek (SC, Cape Cod, MA; PCB concentrations 0.177 microg/g dry weight). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) induced CYP1A1 mRNA, protein, and activity in SC fish in all tissues examined (liver, heart, gut, gill, kidney, spleen, and gonad). In contrast, NBH fish expressed low levels of CYP1A1 and showed no induction of CYP1A1 mRNA, protein, or activity by TCDF, or induction that was lower in magnitude or required higher doses of inducer. p-Nitrophenol UGT activity was not induced by TCDF in either population, while GST activity with 1-chloro-2,4-dinitrobenzene as substrate was induced only in NBH fish in one experiment. Inducibility of CYP1A1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or beta-naphthoflavone (BNF) was measured in primary hepatocyte cultures prepared from SC and NBH fish. TCDD induced CYP1A1 activity (ethoxyresorufin O-deethylase) to the same degree in hepatocytes from both populations, demonstrating the functionality of the AhR signaling pathway in NBH fish. However, hepatocytes from NBH fish were 14-fold less sensitive to TCDD than were those from SC fish. The nonhalogenated AhR agonist BNF also induced CYP1A1 in cells from both populations, although with only a 3-fold difference in sensitivity (NBH < SC). These results indicate that chronic exposure to high levels of HAHs has led to a reduction in the sensitivity of NBH killifish to AhR agonists. The resistance is systemic and pretranslational, and exhibits compound-specific differences in magnitude. These findings suggest an alteration in the AhR signal transduction pathway in NBH fish.

Bi S, An S, Tang W, Xue R, Wen L, Liu F. **Computer simulation of the distribution of aluminum speciation in soil solutions in equilibrium with the mineral phase imogolite.** J Inorg Biochem 2001;87(1-2):97-104.
Abstract: The speciation of aluminum (Al) is a critical issue when evaluating the environmental and biological significance of elevated Al concentrations in soil solutions caused by acidic precipitation. Numerous studies have revealed that, with increased concentrations of silica acid in soil, the activity of Al species in soil solutions is greatly modified by SiO(4)(2-). However, thus far there has been little thorough theoretical modeling of this subject. This paper reports a computer simulation of the distribution of Al speciation in soil solutions in equilibrium with the mineral phase imogolite based on a chemical equilibrium calculation. The unique characteristic associated with imogolite reported by previous researchers can be explained theoretically by the proposed model. The dissolved silica has a remarkable influence on Al speciation: increasing concentrations of silica acid may effectively inhibit the formation of polymeric alumino-hydroxo species, and, furthermore, detoxify Al toxicity to plants.

Boese BL, Ozretich RJ, Lamberson JO, Cole FA, Swartz RC, Ferraro SP. **Phototoxic evaluation of marine sediments collected from a PAH-contaminated site.** Arch Environ Contam Toxicol 2000;38 (3):274-82.
Abstract: The phototoxicity potential of PAH-contaminated field sediment was evaluated and compared to standard sediment toxicity test results. Marine sediments were collected from 30 sites along a presumed PAH sediment pollution gradient in Elliot Bay, WA. Standard 10-day acute and 28-day

chronic sediment toxicity tests were conducted with the infaunal amphipods Rhepoxynius abronius and Leptocheirus plumulosus using mortality and the ability to rebury as endpoints. The survivors of these tests were then subjected to 1-h exposures to UV radiation with mortality and reburial again determined. The most highly toxic sediments identified in these experiments were evaluated further for toxicity and phototoxicity by serially diluting them with uncontaminated sediment and repeating the toxicity tests. Standard 10-day toxicity test results indicated that over 70% of the sites sampled in Elliot Bay exhibited measurable toxicity with nine sites being highly toxic to both species of amphipods. Results of standard 28-day chronic sediment toxicity tests were similar. In contrast, almost all of the sites were found to be highly phototoxic. Results indicated that exposure to UV increased toxicity five- to eightfold. This suggests that standard toxicity tests underestimate the potential ecological risk of PAH-contaminated sediments in animals exposed to sunlight. However, only when PAH contamination was between 0.05 and 1.0 toxic units would conducting a phototoxicity evaluation add information to that gained from conducting a standard sediment toxicity test alone.

Boischio AA. **Re: evaluating mercury exposure through fish consumption in the Amazon--the roles of biomarkers and predictive models.** Environ Res 2000;82(1):91-2; discussion 93.

Bombardier M, Bermingham N, Legault R, Fouquet A. **Evaluation of an SOS-Chromotest-based approach for the isolation and detection of sediment-associated genotoxins.** Chemosphere 2001;42 (8):931-44.

Abstract: A series of experiments was conducted to evaluate an approach advanced by the St. Lawrence Centre (SLC) of Environment Canada for assessing the genotoxic potential of sediments. The SLC method entails the extraction, isolation and solvent exchange of the organic constituents in sediment, and the testing of these solubilized extracts with the SOS Chromotest (Escherichia coli PQ37). A total of five sediments, three variously contaminated by organic compounds and two reference materials certified for persistent organic chemicals, were Soxhlet-extracted. Each of the five extracts was then split, with a portion remaining in crude form and another portion fractionated into two molecular-weight classes of organic contaminants, thus yielding a total of 15 extract samples. The ability of the SOS Chromotest to detect genotoxins in the various organic extracts was evaluated and compared with that of the Ames Fluctuation Assay (Salmonella typhimurium, strain TA100). The intra-laboratory variance associated with the SOS Chromotest was also assessed. Procedural details are presented and results are discussed. The SOS Chromotest results were in good agreement with those of the Ames Fluctuation Assay, especially after metabolic activation. However, the E. coli PQ37 system was slightly more sensitive than the Salmonella assay for detecting genotoxins in the sediment extracts. The SOS Chromotest was also the most discriminating of the two assays, generating SOS-induction factors that were consistent with the organic contamination gradient reported in the sediment samples. The removal of macromolecules from the dichloromethane extracts by size-exclusion chromatography prior to testing enhanced the sensitivity of both test systems. The intra-laboratory variance of the SOS Chromotest ranged from 0.24% to 23.82%, depending on the extract sample. As applied in this study, the SOS Chromotest can serve as a sensitive test for screening the genotoxic potential of uncharacterized sediment extracts. A more sensitive assay would be appropriate, however, as a confirmation for definitive investigations, especially for the detection of direct-acting genotoxins.

Brousseau P, Pellerin J, Morin Y, Cyr D, Blakley B, Boermans H, Fournier M. **Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam Mya arenaria hemocytes following in vitro exposure to heavy metals.** Toxicology 2000;142(2):145-56.
Abstract: The effectiveness of toxicology biomonitoring programs could be improved by the addition of sensitive biomarkers. In this study the cell viability and sensitivity of phagocytic function of phagocytes from bivalves (Mya arenaria) to selected heavy metals were measured by flow cytometry, a novel approach. Hemocytes (phagocytes) collected from bivalves by puncture of the posterior adductor muscle were incubated in vitro for 18 h in hemolymph containing 10(-9)-10(-3)M of cadmium chloride, zinc chloride, mercuric chloride, methylmercury chloride or silver nitrate, before determining their capacity to phagocytose fluorescent latex beads by flow cytometry. Heterogeneity of the hemocyte cell population was determined by forward scatter (FSC) and side scatter (SSC) cytometric profile which showed two distinct cell populations. At low doses (10(-9), 10(-8) M), all the metal compounds studied stimulated phagocytic activity except silver nitrate. At higher levels of exposure (10(-6), 10(7) M), all metals caused a significant concentration-related decrease in hemocyte phagocytosis activity. From the concentration of each metal inducing 50% suppression (IC50) of the phagocytic activity, the immunotoxic potential of metals with respect to phagocytic function can be ranked in the following increasing order: $ZnCl_2 < CdCl_2 < AgNO_3 < HgCl_2 < CH_3HgCl$. Parallel analysis of hemocyte viability showed that suppression of phagocytosis by heavy metals was not solely related to a decreased cell viability. These results reveal the high but different degree of sensitivity of the phagocytosis activity of bivalves with respect to heavy metals, as measured by flow cytometry, and demonstrate that flow cytometry is a potentially useful tool in ecotoxicological monitoring.

Burkholder JM, Marshall HG, Glasgow HB, Seaborn DW, Deamer-Melia NJ. **The standardized fish bioassay procedure for detecting and culturing actively toxic (italic)pfiesteria(/italic), used by two reference laboratories for Atlantic and Gulf Coast states.** Environ Health Perspect 2001;109 Suppl 5:745-56.
Abstract: In the absence of purified standards of toxins from (italic)Pfiesteria(/italic) species, appropriately conducted fish bioassays are the "gold standard" that must be used to detect toxic strains of (italic)Pfiesteria(/italic) spp. from natural estuarine water or sediment samples and to culture actively toxic (italic)Pfiesteria(/italic). In this Article, we describe the standardized steps of our fish bioassay as an abbreviated term for a procedure that includes two sets of trials with fish, following the Henle-Koch postulates modified for toxic rather than infectious agents. This procedure was developed in 1991, and has been refined over more than 12 years of experience in research with toxic (italic)Pfiesteria(/italic). The steps involve isolating toxic strains of (italic)Pfiesteria(/italic) (or other potentially, as-yet-undetected, toxic (italic)Pfiesteria(/italic) or (italic)Pfiesteria(/italic)-like species) from fish-killing bioassays with natural samples; growing the clones with axenic algal prey; and retesting the isolates in a second set of fish bioassays. The specific environmental conditions used (e.g., temperature, salinity, light, other factors) must remain flexible, given the wide range of conditions from which natural estuarine samples are derived. We present a comparison of information provided for fish culture conditions, reported in international science journals in which such research is routinely published, and we provide information from more than 2,000 fish bioassays with toxic (italic)Pfiesteria,(/italic) along with recommendations for suitable ranges and frequency of monitoring of environmental variables. We present data demonstrating that algal assays, unlike these standardized fish bioassays, should not be used

to detect toxic strains of (italic)Pfiesteria(/italic) spp. Finally, we recommend how quality control/ assurance can be most rapidly advanced among laboratories engaged in studies that require research-quality isolates of toxic (italic)Pfiesteria(/italic) spp.

Carvan MJ 3rd, Dalton TP, Stuart GW, Nebert DW. **Transgenic zebrafish as sentinels for aquatic pollution.** Ann N Y Acad Sci 2000;919:133-47.
Abstract: Using the golden mutant zebrafish having a decrease in interfering pigmentation, we are developing transgenic lines in which DNA motifs that respond to selected environmental pollutants are capable of activating a reporter gene that can be easily assayed. We have begun with three response elements that recognize three important classes of foreign chemicals. Aromatic hydrocarbon response elements (AHREs) respond to numerous polycyclic hydrocarbons and halogenated coplanar molecules such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin) and polychlorinated biphenyls. Electrophile response elements (EPREs) respond to quinones and numerous other potent electrophilic oxidants. Metal response elements (MREs) respond to heavy metal cations such as mercury, copper, nickel, cadmium, and zinc. Soon, we will include estrogen response elements (EREs) to detect the effects of environmental endocrine disruptors, and retinoic acid response elements (RARE, RXRE) to detect the effects of retinoids in the environment. Each of these substances is known to be bioconcentrated in fish to varying degrees; for example, 10(-17) M TCDD in a body of water becomes concentrated to approximately 10(-12) M TCDD in a fish, where it would act upon the AHRE motif and turn on the luciferase (LUC) reporter gene. The living fish as a sentinel will not only be assayed intact in the luminometer, but--upon several days or weeks of depuration--would be usable again. To date, we have established that zebrafish transcription factors are able to recognize both mammalian and trout AHRE, EPRE, and MRE sequences in a dose-dependent and chemical-class-specific manner, and that expression of both the LUC and jellyfish green fluorescent protein (GFP) reporter genes is easily detected in zebrafish cell cultures and in the intact live zebrafish. Variations in sensitivity of this model system can be achieved by increasing the copy number of response elements and perhaps by altering the sequence of each core consensus response element and flanking regions. This transgenic technology should allow for a simple, exquisitely sensitive, and inexpensive assay for monitoring aquatic pollution. We have already initiated studies using sentinel zebrafish to monitor a public drinking water source.

Carvan MJ 3rd, Solis WA, Gedamu L, Nebert DW. **Activation of transcription factors in zebrafish cell cultures by environmental pollutants.** Arch Biochem Biophys 2000;376(2):320-7.
Abstract: Many classes of environmental pollutants are found at significant levels in the aquatic environment. We are designing a fish model as an inexpensive and efficient system for the assessment of aquatic pollution. Three classes of environmental pollutants-halogenated and nonhalogenated aromatic hydrocarbons, heavy metals, and potent electrophiles-are known to upregulate parcticular mammalian genes via the activation of specific DNA motifs called aromatic hydrocarbon (AHREs), heavy metal (MREs), and electrophile (EPREs) response elements, respectively. We have made plasmid constructs, using these mammalian or trout response elements to drive the luciferase reporter gene. Here we show that transient transfection of the zebrafish ZEM2S cell line with these reporter constructs imparts dose-dependent gene induction upon exposure to a variety of chemicals within each of these three classes of inducers: [a] (AHRE-mediated) 2,3,7,8-tetrachlorodibenzo-p-dioxin, 3-methylcholanthrene, 3,4,5,3',4',5'-hexabromobiphenyl, Aroclor 1254, and benzo[a]pyrene; [b] (MRE-mediated) Cd(2+), Zn(2+), Hg(2+),

and Al(3+); and [c] (EPRE-mediated) tert-butylhydroquinone, Hg(2+), Pb(2+), As(3+), Cu(2+), and Cd (2+). As expected, some agents gave a response to only one of the three classes, whereas others gave a mixed (AHRE- plus EPRE-mediated or MRE- plus EPRE-mediated) response. In response to several environmental agents, we found that differences in the electrophoretic mobility shift assay, using the AHRE or MRE as probe, were consistent with the degree of transcriptional activation seen with the reporter constructs. Our data suggest that these reporter constructs might be valuable for the generation of transgenic zebrafish in order to carry out mechanistic and developmental studies of transcriptional activation by environmental contaminants; moreover, such transgenic zebrafish lines might be useful as a sentinel for assessing aquatic pollution.

Cassells NP, Lane CS, Depala M, Saeed M, Craston DH. **Microtox testing of pentachlorophenol in soil extracts and quantification by capillary electrochromatography (CEC)--a rapid screening approach for contaminated land.** Chemosphere 2000;40(6):609-18.
Abstract: An approach to rapid soil testing which involved the use of simple solvent extraction methods was developed. The analytes of interest were priority pollutants of low water solubility which could not be readily removed from the soil using water. Direct toxicity testing of the soil samples by Microtox showed a high background toxicity which prevented realistic toxicity data from being obtained for the contaminants present. A range of different extraction solutions was used in an attempt to extract the contaminants while eliminating the matrix effects of the soil. It was necessary that the solvents selected for extraction of the soil samples were not of significant toxicity, as this could potentially mask the toxic effects of any compounds extracted from the soil. The extraction efficiencies of solvent systems were evaluated using pentachlorophenol (PCP) as a model compound of known toxicity in the Microtox assay. A rapid and cost-effective method was developed in order to determine the amount of PCP recovered from the soil by the extraction solvents employed. This method consisted of a solid phase extraction (SPE) step followed by quantification using capillary electrochromatography (CEC). Recoveries were greater when a higher proportion of organic solvent (methanol) was used in the extraction process, and lowest when water was used. An extraction based on water could provide information on the potential for leaching of contaminants from the soil into nearby water bodies in an environmental setting. An organic solvent extraction method could indicate how much toxicity soil-dependent organisms might be exposed to through ingestion. Extraction based on 50% (v/v) methanol in water was considered to be the most suitable overall extraction solution for soil screening, given that this permitted extraction of the water-insoluble compound PCP at a level which was clearly toxic in the Microtox assay while also retaining the capability to extract water-soluble contaminants.

Castano A, Sanchez P, Llorente MT, Carballo M, de la Torre A, Munoz MJ. **The use of alternative systems for the ecotoxicological screening of complex mixtures on fish populations.** Sci Total Environ 2000;247(2-3):337-48.
Abstract: This paper presents the results of the use of alternative systems in a screening study of four complex mixtures. The following tests were performed: in vitro induction of micronuclei in a rainbow trout-derived cell line by flow cytometry, and hatching percentage, time of hatching and teratogenic alterations on the embryolarval development on medaka fish eggs. The results obtained with the proposed tests in this study allows an increase in the information level in a short period of time (2 weeks), using very low sample volumes (< 100 ml). Inclusion of chronic and specific effects

(genotoxicity and teratogenicity) allows the selection of the most sensitive endpoint to increase security factors in the ecotoxicological assessment of complex mixtures, so that detailed studies can be focused only on those samples which require further research.

Chandler GT, Green AS. **Developmental stage-specific life-cycle bioassay for assessment of sediment-associated toxicant effects on benthic copepod production.** Environ Toxicol Chem 2001;20 (1):171-8.
Abstract: In chronic bioassays of sediment organic compounds, toxicant exposures often decline through time, such that the beginning of a test yields disproportionately higher exposures than the end. Thus, those life stages initiating a test often are exposed to the highest concentrations, and for rapidly maturing test fauna, this may lead to varying conclusions regarding compound toxicities depending on the initial life stage chosen. This problem can be addressed by comparative full life-cycle tests initiated with different test-organism life stages. Thus, a full life stage-to-life stage toxicity test was developed for the rapidly maturing meiobenthic copepod Amphiascus tenuiremis to assess the importance of developmental stage at the onset of sediment toxicant exposure relative to reproduction, net population growth, and sex and age structure. Tests were conducted with a model spiked-sediment insecticide, chlorpyrifos, for each of the major life stages (P1) of A. tenuiremis (nauplius, copepodite, and adult). Each P1 stage was allowed to mature and reproduce in low chlorpyrifos concentrations (6-33% of stage-specific 96-h LC50s; 4-22 ng chlorpyrifos/g dry sediment) for 26 d. Test endpoints were numbers of surviving adult females, males, eggs per female (clutch), first generation (F1) nauplii, F1 copepodites, F1 total production, and realized F1 production per surviving female. Only the copepodite P1 test showed a significant decline in survival of an adult age class: females declined by 28% at 22 ng/g. Reductions in total production ranged from 33-96% of controls from nauplius to adult. The P1 naupliar stage was most sensitive, with F1 production being 33-47% of that in controls. However, on a realized production per female basis, both the copepodite and naupliar P1 yielded significantly reduced F1s of 23 and 40% of controls at 11 and 22 ng/g.

Cheng SH, Chan PK, Wu RS. **The use of microangiography in detecting aberrant vasculature in zebrafish embryos exposed to cadmium.** Aquat Toxicol 2001;52(1):61-71.
Abstract: Embryonic vascular patterns in zebrafish (Danio rerio) could be visualised by confocal microscopy coupled with microinjected fluorescent microbeads. This microangiographic technique was adopted here, for the first time, to study the effects of cadmium on cardiovascular development in zebrafish embryos. Zebrafish embryos were incubated in culture medium containing 100 microM cadmium from 5 h post fertilisation (hpf) to 48 hpf. At 48 hpf, embryos were examined for viability and occurrence of malformations. The 100 microM cadmium caused 32.21 +/- 3.65% mortality and 20.33 +/- 4.04% visible malformations in surviving embryos. In the remaining embryos with no visible signs of malformations, further assessments for less obvious abnormalities were performed. Assessments on craniofacial development were made by digital measurements on areas of brains and eyes. Cardiac development was assessed by immunostaining the heart with the antibody MF20 specific for myosin heavy chain. Body lengths of the embryos were also measured. Embryonic development of brains, eyes, hearts and body lengths of visibly healthy embryos in the cadmium treatment group showed no significant difference from the controls. Embryonic vasculature of these visibly healthy embryos was then studied by microinjecting fluorescent microbeads of diameter 0.02 microm into the circulation. All

the cadmium treated embryos showed localised vascular defects in the dorsal aortae, segmental and cranial vessels while none of the control embryos showed any aberrant patterns in the networking of the vasculature. Improved image analyses on the anterior regions revealed that cadmium treated embryos had markedly less complex networks of cranial vessels with fewer vessels perfusing the craniofacial regions. The number of branch points in the vascular network was counted. In untreated embryos, there were 135.6 +/- 51 branches in the vasculature in entire body. In the cadmium treated embryos, there were 64.5+/-31 branches. The difference was significant when assessed with Student's t-test. It appeared that although cadmium did not cause any signs of external malformations in these visibly healthy embryos, nonetheless induced impaired branching and anastomsis of the cranial vessels. This study revealed, for the first time, that vital vascular structures in fish embryos could be affected by exposure to cadmium. This technique allowed visualisation of vascular anomalies in embryos showing no external signs of malformations. The impairment of anatomical features during embryonic development might serve as meaningful health endpoints in ecotoxicological studies and in risk assessment.

Fargasova A. **Winter third- to fourth-instar larvae of Chironomus plumosus as bioassay tools for assessment of acute toxicity of metals and their binary combinations.** Ecotoxicol Environ Saf 2001;48(1):1-5.
Abstract: The ecotoxicological effect expressed as mortality of four metal ions (Cd, Cu, Zn, Al) and their associations on winter third- to fourth-instar larvae of Chironomus plumosus was determined. The effect of individual metals was introduced as acute toxicological effect and expressed as LC(50) and LC95 values with 95% intervals of confidence. On the basis of the LC50 values the toxicity of metals after 96 h treatment was ranked Cu>>Cd>Zn>Al. Copper was at least 100 times more toxic than other metals tested. When the acute toxic effect of metal pairs was observed, in general, deleterious effects were directly proportional to metal concentrations. The toxicity of metals in combinations was different from that of individual metals, because of either antagonism or synergism. From the results obtained it can be concluded that when in metal pairs in which the original metal (the metal for which the interaction was determined) is at the lower concentration (Cd 10 mg x L(-1), Zn 25 mg x L(-1), Al 25 mg x L(-1), Cu 0.1 mg x L(-1)), the prevailing interaction is synergism (mortality was higher for metal combinations than for individual metals). Except for an overadditive effect (synergism), additivity was also confirmed in some cases (Al25+Cd10; Al25+Cd25; Al25+Cu0.1; Cu0.1+Cd10; Cu0.1+Cd25; Cu0.1 +Al50). Synergism, in combinations in which the original metal is at the higher concentration (Cd 25 mg x L(-1), Zn 50 mg x L(-1), Al 50 mg x L(-1), Cu 1.0 mg x L(-1)), was observed only for the pairs Zn50 +Al25 and Zn50+Cu1.0. Reciprocal additivity was observed after 96 h of treatment only for the combination Zn50+Al50. For all other binary combinations in which the original metal was at the higher concentration, an antagonistic effect was confirmed.

Fent K. **Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples.** Toxicol In Vitro 2001;15(4-5):477-88.
Abstract: In vitro systems such as primary cells and cell lines are of growing importance in ecotoxicology. Cells from different tissues and species of fish are used for the assessment of toxic action of chemicals and evaluation of environmental samples. For organotins and substituted phenols, we have found that the in vitro cytotoxicity is positively correlated with the acute toxicity in vivo, and therefore

cytotoxicity assays may serve as an alternative for acute fish toxicity testing. We have been using the hepatocellular carcinoma (PLHC-1) cell line for the assessment of the cytochrome P4501A (CYP1A) induction potential of polyaromatic hydrocarbons (PAHs), nitro-PAHs and azaarenes. For these compounds, the CYP1A induction potential is found to be related to the molecular structure and lipophilicity. In mixtures, CYP1A induction of individual compounds is additive. Based on the comparative investigation of the induction potential we derived an induction equivalency (IEQ) concept that can be applied for the evaluation of environmental samples such as landfill leachates, sediments and motorway runoffs. Fish cell lines are also valuable, rapid and cost-effective tools for the assessment of estrogenic activity of chemicals and environmental samples. We have developed an estrogen-responsive reporter gene system using the rainbow trout gonad cell line RTG-2, in which an estrogen receptor beta form is expressed at very low levels, but is not inducible. As the estrogenic activity is dependent on the cellular level of estrogen receptor (ER), ER has to be co-transfected in transient transfections in addition to an estrogen-responsive reporter gene. Using a dual luciferase system, the estrogenic activity of 12 compounds including alkylphenols, DDT-isomers and its metabolites have been assessed. Our system shows a high sensitivity with a detection limit of 0.05 nM estradiol and is therefore more sensitive than many other mammalian or yeast systems. The relative estrogenic activity (e.g. o,p'-DDT) and other toxicological effects may differ from those in mammalian systems, indicating that a risk evaluation for fish could only be meaningfully assessed in fish-specific systems. This paper illustrates the versatility and high potential of fish cell lines in ecotoxicology.

Fredrickson HL, Perkins EJ, Bridges TS, Tonucci RJ, Fleming JK, Nagel A, Diedrich K, Mendez-Tenorio A, Doktycz MJ, Beattie KL. **Towards environmental toxicogenomics -- development of a flow-through, high-density DNA hybridization array and its application to ecotoxicity assessment.** Sci Total Environ 2001;274(1-3):137-49.
Abstract: Assessment of the environmental hazard posed by soils/sediments containing low to moderate levels of contaminants using standard analytical chemical methods is uncertain due (in part) to a lack of information on contaminant bioavailability, the unknown interactive effects of contaminant mixtures, our inability to determine the species of a metal in an environmental matrix, and the relative sensitivity of bioassay species. Regulatory agencies compensate for this uncertainty by lowering cleanup goals, but in this process they effectively exclude otherwise attractive cleanup options (i.e. bioremediation). Direct evaluations of soil and sediment toxicity preclude uncertainty from most of these sources. However, the time and cost of chronic toxicity tests limits their general application to higher levels of tiered toxicity assessments. Transcriptional level (mRNA) toxicity assessments offer great advantages in terms of speed, cost and sample throughput. These advantages are currently offset by questions about the environmental relevance of molecular level responses. To this end a flow-through, high-density DNA hybridization array (genosensor) system specifically designed for environmental risk assessment was developed. The genosensor is based on highly regular microchannel glass wafers to which gene probes are covalently bound at discrete (200-microm diameter spot) and addressable (250-microm spot pitch) locations. The flow-through design enables hybridization and washing times to be reduced from approximately 18 h to 20 min. The genosensor was configured so that DNA from 28 environmental samples can be simultaneously hybridized with up to 64 different gene probes. The standard microscopic slide format facilitates data capture with most automated array readers and, thus high sample throughput (> 350 sample/h). In conclusion, hardware development for molecular analysis is enabling very tractable

means for analyzing RNA and DNA. These developments have underscored the need for further developmental work in probe design software, and the need to relate transcriptional level data to whole-organism toxicity indicators.

Garay V, Roman G, Isnard P. **Evaluation of PNEC values: extrapolation from Microtox, algae, daphnid, and fish data to HC5.** Chemosphere 2000;40(3):267-73.
Abstract: In order to evaluate the risk to the environment from long term exposure of any discharged substance, toxicity thresholds are estimated, and parcticularly the Predicted No Effect Concentration (PNEC). This concentration can be estimated by the classic assessment factor approach or by statistical methods. These are more scientifically sound but they require several (at least 5-6) chronic ecotoxicity data, implying greater cost and time. New extrapolation methods derived from the statistical concept but requiring less data have been studied. Results show that methods based on chronic data are more reliable than methods based on acute data but the improvement is quite small. Considering the costs of chronic tests compared to acute tests, approaches based on acute data are an attractive alternative. A simple regression on the mean of the acute data gives the best results.

Geffard O, Budzinski H, Augagneur S, Seaman MN, His E. **Assessment of sediment contamination by spermiotoxicity and embryotoxicity bioassays with sea urchins (Paracentrotus lividus) and oysters (Crassostrea gigas).** Environ Toxicol Chem 2001;20(7):1605-11.
Abstract: Gametes (sperm) and fertilized eggs (embryos) of the Mediterranean sea urchin, Paracentrotus lividus, and the Japanese oyster, Crassostrea gigas, were used to investigate the toxicity of two marine sediments, one polluted by polycyclic aromatic hydrocarbons (PAH) and the other by heavy metals. The sediment samples were freeze-dried for storage, and three different treatments were used for analysis: whole sediment, unfiltered elutriate, and filtered elutriate. The two sediments were toxic to sea urchin spermatozoa but not to oyster spermatozoa, and embryotoxicity was almost always the more sensitive endpoint for toxicity assessment. As a rule, whole sediment was more toxic than the elutriates by nearly two orders of magnitude. With respect to embryotoxicity, the whole sediments and the elutriates of the PAH-contaminated sediment were more toxic to oyster embryos, whereas the elutriates of the sediment polluted by heavy metals had stronger effects on sea urchin embryos. The results confirm that bioassays with Japanese oyster embryos provide a more sensitive appraisal of toxicity in the marine environment than bioassays with other developmental stages. As a whole, Mediterranean sea urchins and Japanese oysters were similar in overall sensitivity and are therefore both equally suited as bioassay organisms, but tests with oysters are more reproducible because of the better performance of the controls.

Grant RJ. **A bioassay for the measurement of insecticide concentration.** Arch Environ Contam Toxicol 2001;41(3):319-24.
Abstract: A bioassay was developed to measure insecticide residues using fruit flies (Drosophila melongaster). After adding a known volume of sampling solution, the time at which 50% of the flies were dead (LT(50)) was recorded and cross-referenced to the appropriate calibration curve. Using known standards, comparable results were obtained using the bioassay and GC-MS. The bioassay allows concentrations of synthetic pyrethroids as low as 1 pg L(-1) to be measured with a variance of < 5%. The bioassay can be used reliably over a wide range of temperatures and it is tolerant to a range of pH and surface tensions of the test solution. The whole bioassay is compact, physically robust, and simple to

use; hence, it could be of use in the field as a quick preliminary assessment of water contamination.

Jha AN, Cheung VV, Foulkes ME, Hill SJ, Depledge MH. **Detection of genotoxins in the marine environment: adoption and evaluation of an integrated approach using the embryo-larval stages of the marine mussel, Mytilus edulis.** Mutat Res 2000;464(2):213-28.
Abstract: In genetic ecotoxicology or eco-genotoxicology, there is lack of well-validated systems which could demonstrate the utility of multiple endpoints in environmental quality assessment. For an evaluation of genotoxic potential of heterogeneous marine sediment samples collected from a small fishing harbour in the UK, an in vivo test system using embryo-larval stages of the common mussel, Mytilus edulis was validated against direct and indirect acting reference mutagens. The system appeared to be sensitive and reproducible for cytogenetic endpoints analysed (sister chromatid exchanges (SCEs) and chromosomal aberrations (CAbs)). Following validation and chemical characterisation of the environmental samples, multiple endpoints were measured. Determination of the maximum tolerated dose (MTD) was carried out as a measure to determine cytotoxic effects as a confounding factor for genotoxicity, based on developmental and cytotoxic (in terms of proliferative rate index or PRI) effects. Evaluation of the genotoxic potential of the samples gave a positive response for all the endpoints tested, linking different levels of biological organisation (i.e., chromosomal, cellular and organismal) for the observed effects. The study also emphasises the need for the assessment of the short and long-term impacts of dredge disposal on marine biota by including laboratory-based bioassays and incorporating an integrated approach which could yield as much useful information as possible in overall hazard and risk assessment for aquatic genotoxicity.

Jonczyk E, Gilron G, Zajdlik B. **Sea urchin fertilization assay: an evaluation of assumptions related to sample salinity adjustment and use of natural and synthetic marine waters for testing.** Environ Toxicol Chem 2001;20(4):804-9.
Abstract: Most industrial effluents discharged into the marine coastal environment are freshwater in nature and therefore require manipulation prior to testing with marine organisms. The sea urchin fertilization test is a common marine bioassay used for routine environmental monitoring, investigative evaluations, and/or regulatory testing of effluents and sediment pore waters. The existing Canadian and U.S. Environmental Protection Agencies test procedures using sea urchin (and sand dollar) gametes allow for sample salinity adjustment using either brine or dry salts. Moreover, these procedures also allow for the use of either natural or synthetic marine water for culturing/holding test organisms and for full-scale testing. At present, it is unclear to what extent these variables affect test results for whole effluents. The test methods simply state that there are no data available and that the use of artificial dry sea salts should be considered provisional. We conducted a series of concurrent experiments aimed at comparing the two different treatments of sample salinity adjustment and the use of natural versus synthetic seawater in order to test these assumptions and evaluate effects on the estimated end points generated by the sea urchin fertilization sublethal toxicity test. Results from these experiments indicated that there is no significant difference in test end points when dry salts or brine are used for sample salinity adjustment. Similarly, results obtained from parallel (split-sample) industrial effluent tests with natural and artificial seawater suggest that both dilution waters produce similar test results. However, data obtained from concurrent tests with the reference toxicant, copper sulfate, showed higher variability and greater sensitivity when using natural seawater as control/dilution water.

Kammann U, Riggers JC, Theobald N, Steinhart H. **Genotoxic potential of marine sediments from the North Sea.** Mutat Res 2000;467(2):161-8.
Abstract: The alkaline comet assay is a method for detecting DNA strand breaks and alkali labile sites in individual cells. An in vitro system was used to investigate the genotoxic potential of complex mixtures such as organic extracts of marine sediments. DNA damage was induced in leukocytes isolated from carp (Cyprius carpio) by exposure to organic sediment extracts from the North Sea or hydrogen peroxide as positive control, respectively. The minimum concentration for significant effects ranged from 1 to 40 mg sediment dry weight per milliliter assay volume. The sensitivity of the method was enhanced by using the DNA repair inhibitor, 1-beta-D-arabinofuranosylcytosine (ara C). From the results, it can be suggested that total organic carbon (TOC) as well as the different compositions of contaminants present in the sediment extracts may contribute to the genotoxic effects observed. The comet assay can be applied successfully as an in vitro bioassay for investigations on genotoxicity of marine sediment extracts.

Kater BJ, Postma JF, Dubbeldam M, Prins JT. **Comparison of laboratory and in situ sediment bioassays using Corophium volutator.** Environ Toxicol Chem 2001;20(6):1291-5.
Abstract: Bioassays with the marine amphipod Corophium volutator were performed simultaneously in situ and in the laboratory using sediments sampled from the in situ locations. In most cases, the in situ response was significantly higher compared to the laboratory response. This difference was not caused by direct influence of the use of the field chamber on Corophium sp., nor was the difference caused by the overlying water used. Experiments showed homogenization can affect the toxicity of a sediment, but not in such a way that it can completely explain the difference between the response in situ and in the laboratory. Possible explanatory factors are harbor activity, storms, and temperature. To reduce the influence of some of these factors, the best period of the year to perform in situ bioassays with C. volutator is May, June, or September.

Koh CH, Kim GB, Maruya KA, Anderson JW, Jones JM, Kang SG. **Induction of the P450 reporter gene system bioassay by polycyclic aromatic hydrocarbons in Ulsan Bay (South Korea) sediments.** Environ Pollut 2001;111(3):437-45.
Abstract: Polycyclic aromatic hydrocarbons (PAHs) and induction of the P450 reporter gene system (RGS) for 6- and 16-h exposure periods were determined in organic extracts of Ulsan Bay (South Korea) sediments to assess the utility of this bioassay as a screening tool for PAH contamination. The sum of the concentrations of 23 individual PAHs in 30 sediment samples (sigma PAH) based on GC-MS analysis ranged from 0.05 to 6.1 micrograms/g dry wt. P450 RGS fold induction ranged from 4.0 to 320 micrograms/g based on benzo[a]pyrene toxic equivalents (BaPEq). P450 RGS BaPEq and the 'chemical BaPEq', defined as the sum of the products of individual PAH concentrations and pre-determined toxic equivalency factors, exhibited very strong positive correlations with sigma PAH ($r2 > 0.90$; $P < 0.001$). Fold induction did not increase (and in some cases decreased) after the optimal incubation period (6 h) for PAHs, indicating that other compounds known to induce the P450 RGS (e.g. chlorinated organics) were not present at levels effecting significant induction. This was supported by GC-ECD analysis where non-ortho and mono-ortho polychlorinated biphenyls (PCBs) known to be strong P450 RGS inducers were found to be at very low or non-detectable levels in samples with the highest P450 RGS

responses. The profound difference in PAH profiles for the two most contaminated sites suggested that this assay is especially sensitive for selected PAHs with greater than four rings. Combined with previous results, the P450 RGS shows promise as a useful screening tool for predicting deleterious biological effects resulting from CYP1A1-inducing, sediment-associated chemicals, parcticularly high molecular weight PAHs.

Lyytikainen M, Sormunen A, Ristola T, Juvonen R, Kukkonen JV. **Toxicity of freshwater sediments in the vicinity of an old sawmill: application of three bioassays.** Arch Environ Contam Toxicol 2001;40 (3):318-26.
Abstract: Toxicity of contaminated sediments collected from an old sawmill area and the downstream river-lake system was assessed with three different bioassays. Survival and growth were used as endpoints in subchronic (10-day) test with Chironomus riparius and growth and reproduction in long-term (28-day) test with Lumbriculus variegatus. A microbial bioluminescent direct contact assay, the Flash test, was also included in the test set to measure acute toxicity. In every bioassay, sediment from a pool of the sawmill was found to be toxic, and some adverse effects were found in other sediments as well. The bioassays were then compared to chemical analysis results, which showed the presence of several toxicants. These results could not, however, be directly connected to any individual toxicant, nor did they show any obvious trend downstream from the mill.

Martinez-Tabche L, Ortega MD, Ramirez Mora B, German Faz C, Lopez Lopez E, Galar Martinez M. **Hemoglobin concentration and acetylcholinesterase activity of oligochaetes in relation to lead concentration in spiked sediments from Ignacio Ramirez reservoir.** Ecotoxicol Environ Saf 2001;49 (1):76-83.
Abstract: This paper presents toxicity and uptake data of Limnodrilus hoffmeisteri generated by lead in systems using natural sediments from Ignacio Ramirez reservoir. Lead uptake, hemoglobin concentration, and acetylcholinesterase activity were studied in L. hoffmeisteri exposed to spiked sediments. All assays of lead uptake were conducted using whole sediments. Sediment texture was also considered. Acetylcholinesterase activity and hemoglobin concentration decreased after treatment with lead. Acetylcholinesterase activity and hemoglobin concentration tests indicated a response to the concentration of lead. These results suggest the usefulness of a diversity of bioassays to evaluate the toxicity of sediments polluted with heavy metals. Copyright 2001 Academic Press.

Marvin CH, McCarry BE, Villella J, Allan LM, Bryant DW. **Chemical and biological profiles of sediments as indicators of sources of contamination in Hamilton Harbour. Part II: bioassay-directed fractionation using the Ames Salmonella/microsome assay.** Chemosphere 2000;41(7):989-99.
Abstract: Bottom sediment and suspended sediment samples from Hamilton Harbour (western Lake Ontario) and from a major tributary were profiled using a bioassay-directed fractionation approach. Sample extracts were fractionated using an alumina/Sephadex gel clean-up procedure to afford non-polar aromatic fractions which were characterized using chemical analyses and the Ames/microsome bacterial assay in Salmonella typhimurium strains YG1025 with the addition of oxidative metabolism (S9), and YG1024 without S9. Non-polar aromatic fractions of selected samples were separated by normal phase HPLC into 1-min fractions which were subjected to bioassay analyses. The bioassays

using strain YG1025+S9, a TA100-type strain, were performed to assess genotoxicity arising from the presence of polycyclic aromatic hydrocarbons (PAH). Fractions which exhibited mutagenic activity contained PAH with molecular masses of 252, 276 and 278 amu; these fractions contained over 80% of the genotoxicity attributable to PAH. Individual compounds identified using Gas Chromatography-Mass Spectrometry analyses in these active fractions included benzo[a]pyrene, indeno[cd]pyrene and dibenz[a, h]anthracene. The YG1025+S9 mutagenic activity profiles were similar for all samples. Mutagenic activity profiles generated using strain YG1024-S9, a TA98-type strain sensitive to compounds characteristic of mobile source emissions, were very different. The mutagenic activities in strain YG1024-S9 were greatest for harbour-suspended sediment samples collected from sites impacted by a major tributary. Suspended sediments collected near areas known to contain high levels of coal tar-contamination in the bottom sediments contained higher levels of genotoxic PAH than suspended sediments collected from other areas of the harbour.

Meregalli G, Vermeulen AC, Ollevier F. **The use of chironomid deformation in an in situ test for sediment toxicity.** Ecotoxicol Environ Saf 2000;47(3):231-8.
Abstract: An in situ bioassay using mouthpart deformities in Chironomus riparius larvae was developed to monitor sediment toxicity. Second-instar larvae, along with a standardized amount of food and sediment taken from the study locations, were enclosed in cages that were placed on the sediment surface of rivers. Mouthpart deformities were screened after larval molting to the fourth instar (exposure time: 7-10 days). Mouthpart deformities of caged and field larvae (when present) were related to the estimated sediment toxicity. By summing toxicant concentrations and normalizing them to the organic matter and clay contents, a significant relationship between toxicity levels and mouthpart deformities in the mentum was revealed. Results suggest that the pattern of observed deformities was indicative of site toxicity rather than a characteristic of the laboratory larval population used. The main advantage of the proposed in situ bioassay is the possibility to assess the incidence of deformities at sites where C. riparius does not occur naturally.

Nadeau D, Corneau S, Plante I, Morrow G, Tanguay RM. **Evaluation for Hsp70 as a biomarker of effect of pollutants on the earthworm Lumbricus terrestris**. Cell Stress Chaperones 2001;6(2):153-63.
Abstract: Induction of heat shock proteins (Hsps) is often associated with a cellular response to a harmful stress or to adverse life conditions. The main aims of the present study were (1) to assess if stress-induced Hsp70 could be used to monitor exposure of the earthworm species Lumbricus terrestris to various soil pollutants, (2) to assess the specificity of pollutants in their tissue targeting and in Hsp70 induction, and (3) to evaluate if dose-response relationships could be established and if the stress-response observed was specific. The midgut/intestinal tissues of L. terrestris are shown to express an inducible member of the Hsp70 family after heat shock treatment in vitro and exposures to different soil toxicants in vivo (re: artificial soil). Short-term (24-72 hours) and long-term (14-16 days) exposures to the chemical standards chloroacetamide and pentachlorophenol and to heavy metals (Pb++, Cd++, Cu++, and Hg++) also affected the earthworms, and Hsp70 was induced in their midgut/intestinal tissues. After a 3-day exposure to heavy metals, the level of Hsp70 induction in the midgut/intestinal tissues appears to correlate well with the reported in vivo and in vitro toxicity data. Comparatively, in proximal and midbody wall muscle tissues of animals exposed to the heavy metals, a decrease in expression of

Hsp70 was sometimes detected. Thus Hsp analysis by Western blot in L. terrestris tissues and parcticularly in the midgut/intestine proved to be a suitable and sensitive assay for adverse effects in earthworms and showed a good level of reproducibility despite some individual variations. The use of pristine/nonexposed animals transposed into contaminated environments as in the present study should therefore be of high ecological relevance. Induction of Hsp70 in earthworms should represent not only a good wide-spectrum biomarker of exposure but also a biomarker of effect since known toxicants altered gene expression in tissues of these animals, as contrasted with a simple accumulation of Hsp. Hence, the detection of Hsp70 in earthworms can constitute an early-warning marker for the presence of potentially deleterious agents in soils, with L. terrestris in parcticular and earthworms in general acting as potential sentinel animal species.

Radix P, Leonard M, Papantoniou C, Roman G, Saouter E, Gallotti-Schmitt S, Thiebaud H, Vasseur P. **Comparison of four chronic toxicity tests using algae, bacteria, and invertebrates assessed with sixteen chemicals.** Ecotoxicol Environ Saf 2000;47(2):186-94.
Abstract: The performances of four chronic toxicity tests, comprising the Daphnia magna 21-day (d) (crustacean), Brachionus calyciflorus 2-d (rotifer), Pseudokirchneriella subcapitata 72-h (green algae), and the Microtox chronic 22-h (bacteria) tests, were compared. Sixteen chemicals with toxicity covering 6 orders of magnitude were studied. Very high correlations were found between the NOEC/EC(10) Pseudokirchneriella 72-h, NOEC/EC(10) Brachionus 2-d, and the NOEC Daphnia 21-d tests. The toxicological response of rotifers and microalgae were within the same order of magnitude as the response of Daphnia in 80% of cases (13/16 chemicals). The Microtox chronic test also anticipated the overall results of the Daphnia 21-d test, but the prediction was rather imprecise, compared with microalgae and rotifers. The test measuring the algal growth inhibition of P. subcapitata after 72h was the most sensitive bioassay. Toxicity on microalgae after 72h could be estimated after 5h by measuring either the direct fluorescence of either photosynthetic pigments or fluorescein diacetate in 56 and 43% of cases, respectively. The median value of the ratio between EC(10) and EC(50) was 3.75, 2, and 1.5 with the algae, the rotifers, and the bacteria, respectively.

Repetto G, Jos A, Hazen MJ, Molero ML, del Peso A, Salguero M, Castillo PD, Rodriguez-Vicente MC, Repetto M. **A test battery for the ecotoxicological evaluation of pentachlorophenol.** Toxicol In Vitro 2001;15(4-5):503-9.
Abstract: Experimental bioassays are currently used in ecotoxicology and environmental toxicology to provide information for risk assessment evaluation of new chemicals and to investigate their effects and mechanisms of action; in addition, ecotoxicological models are used for the detection, control and monitoring of the presence of pollutants in the environment. As a single bioassay will never provide a full picture of the quality of the environment, a representative, cost-effective and quantitative test battery should be developed. The effects of pentachlorophenol were studied using a battery of ecotoxicological model systems, including immobilization of Daphnia magna, bioluminiscence inhibition in the bacterium Vibrio fischeri, growth inhibition of the alga Chlorella vulgaris, and micronuclei induction in the plant Allium cepa. The inhibition of cell proliferation and MTT reduction were investigated in Vero cells. Neutral red uptake, cell growth, MTT reduction, lactate dehydrogenase leakage and activity were studied in the salmonid fish cell line RTG-2, derived from the gonad of rainbow trout. Pentachlorophenol was very toxic for all biota and cells. The system most sensitive to

pentachlorophenol, was micronuclei induction in A. cepa, followed by D. magna immobilization, bioluminescence inhibition in V. fischeri bacteria at 60 min and cell proliferation inhibition of RTG-2 cells at 72 h. Inhibition of cell proliferation and MTT reduction on Vero monkey cells showed intermediate sensitivity.

Rice CA, Myers MS, Willis ML, French BL, Casillas E. **From sediment bioassay to fish biomarker--connecting the dots using simple trophic relationships.** Mar Environ Res 2000;50(1-5):527-33.
Abstract: Two common problems in applying and interpreting invertebrate bioassays and fish biomarkers in sediment toxicology are the wide gap between significant effects concentrations determined by these two approaches, and a general lack of ecological context. We have devised an exposure system that is able to reconcile much of the disparity between invertebrate bioassay and fish biomarker results by incorporating realistic ecological processes based on deposit feeding and predator-prey interactions. This system relates the disturbance of interest (sediment contamination) to biologically meaningful effects in a resource of interest (marine flatfish) via a realistic contaminant vector (a deposit-feeding polychaete worm). In this pilot study, polychaetes (Armandia brevis) were exposed for 28 days to clean sediments supplemented with benzo(a)pyrene (BaP), para-para dichlorodiphenyldichloroethylene (pp'DDE), Aroclor 1254, or field sediments collected from two sites in Puget Sound, Washington, contaminated predominantly with polcyclic aromatic hydrocarbons (PAHs) or chlorinated compounds. Exposed worms were then fed live to juvenile English sole (Pleuronectes vetulus) for 10 or 12 days. At the end of the exposure period, fish were measured for length and weight, sacrificed, and preserved for either routine histopathology and immunohistochemical analysis of cytochrome P450 1A induction, or 32P post-labeling determination of hepatic PAH-DNA adducts. Growth of predatory flatfish was lower than reference in all but one of eight groups fed contaminant-exposed polychaetes; however, statistically significant reductions in growth were only observed in three of these eight groups, at least in part due to low statistical power. Juvenile sole from all contaminant-exposed groups showed increased expression of CYP1A, and fish exposed to BaP-exposed worms showed clear evidence of hepatic PAH-DNA adducts. This method allows the concurrent evaluation of sediment contamination at multiple biological and ecological levels. These results indicate that sediments determined to be nontoxic by common invertebrate bioassays may have the potential to cause adverse effects at higher trophic levels.

Robidoux PY, Choucri A, Bastien C, Sunahara GI, Lopez-Gastey J. **Interlaboratory study for the validation of an ecotoxicological procedure to monitor the quality of septic sludge received at a wastewater treatment plant.** Environ Toxicol 2001;16(2):158-71.
Abstract: Septic tank sludge is regularly hauled to the Montreal Urban Community (MUC) wastewater treatment plant. It is then discharged and mixed with the wastewater inflow before entering the primary chemical treatment process. An ecotoxicological procedure integrating chemical and toxicological analyses has been recently developed and applied to screen for the illicit discharge of toxic substances in septic sludge. The toxicity tests used were the Microtox, the bacterial-respiration, and the lettuce (Lactuca sativa) root elongation tests. In order to validate the applicability of the proposed procedure, a two-year interlaboratory study was carried out. In general, the results obtained by two independent laboratories (MUC and the Centre d'expertise en analyse environnementale du Quebec) were comparable and reproducible. Some differences were found using the Microtox test. Organic (e.g.,

phenol and formaldehyde) and inorganic (e.g., nickel and cyanide) spiked septic sludge were detected with good reliability and high efficiency. The relative efficiency to detect spiked substances was > 70% and confirms the results of previous studies. In addition, the respiration test was the most efficient toxicological tool to detect spiked substances, whereas the Microtox was the least efficient (< 15%). Efficiencies to detect spiked contaminants were also similar for both laboratories. These results support previous data presented earlier and contribute to the validation of the ecotoxicological procedure used by the MUC to screen toxicity in septic sludge.

Schnurstein A, Braunbeck T. **Tail moment versus tail length--application of an in vitro version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish (Danio rerio).** Ecotoxicol Environ Saf 2001;49(2):187-96.
Abstract: In order to investigate the suitability of an in vitro version of the comet assay with primary hepatocytes and gill cells from zebrafish (Danio rerio), cells were isolated by immersion in trypsin/ EDTA solution after whole-body perfusion with phosphate-buffered saline. Within the scope of an 18-month biomonitoring study, primary cells were used to identify the genotoxic potential of native water samples from different sites along the major German rivers, Rhine and Elbe, and to evaluate the sensitivity and practicability of the chosen assay. Depending on the endpoint measured, considerable differences were detected with respect to the number of genotoxic surface water samples: Whereas no differences could be recorded for tail moment and relative DNA contents of head and tail, the number of positively tested native surface water samples significantly increased with tail length as endpoint. Copyright 2001 Academic Press.

Schramm KW, Klimm C, Hofmaier A, Kettrup A. **Comparison of dioxin-like-response in vitro and chemical analysis of emissions and materials.** Chemosphere 2001;42(5-7):551-7.
Abstract: Samples from industrial and domestic emissions and materials were investigated by using in vitro assays for dioxin-like-response (DLR) and chemical analysis. The results show a small part of persistent bioaccumulative toxicants (PBT) to be responsive in vitro. Clean-up procedures directed to persistence decrease the amount of inducing toxicants substantially. The comparison of biological in vitro (B) and chemical (A) analysis show ratios between B and A below 10 for DLR of persistent inductors. The consideration of PAH improved the knowledge about unknown less persistent agonists.

Shaw JP, Large AT, Chipman JK, Livingstone DR, Peters LD. **Seasonal variation in mussel Mytilus edulis digestive gland cytochrome P4501A- and 2E-immunoidentified protein levels and DNA strand breaks (Comet assay).** Mar Environ Res 2000;50(1-5):405-9.
Abstract: Mytilus edulis digestive gland microsomes were prepared from indigenous populations sampled from a clean reference site (Port Quin) and an urban-industrial contaminated site (Blackpool) in the UK. Samples were collected in March/April, May, August and December 1998. Western blot analysis was performed using polyclonal antibodies to fish CYP1A and rat CYP2E using partially purified M. edulis CYP as a positive control, to aid identification. CYP1A- and CYP2E-immunopositive protein levels showed different site-specific seasonal variation with higher levels of CYP2E determined in May ($P < 0.05$). At both sites, lower levels of CYP1A-immunopositive protein but not CYP2E-immunopositive protein were observed in the samples collected in December ($P < 0.05$). This correlated with lower levels of nuclear DNA damage (Comet assay expressed as per cent tail DNA) observed in

December compared to August ($P < 0.05$).

Simms W, Ross PS. **Vitamin A physiology and its application as a biomarker of contaminant-related toxicity in marine mammals: a review.** Toxicol Ind Health 2001;16(7-8):291-302.
Abstract: In recent decades, marine mammal populations living in highly polluted areas have experienced incidences of low reproductive success, developmental abnormalities and disease outbreaks. In many of these cases, environmental contaminants were suspected as causal or contributing factors. However, demonstrating a mechanistic link between contaminant exposure and effect in marine mammal populations has proven challenging. Consequently, the development and application of relatively noninvasive biomarkers represents a potentially valuable means of monitoring wildlife populations exposed to elevated levels of contaminants. One touted biomarker is vitamin A (retinol), a "dietary hormone" whose metabolites are required for reproduction, growth, development, immune function, vision and epithelial maintenance. Laboratory studies have shown that many contaminants, including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), can disrupt vitamin A physiology and alter the distribution of its essential metabolites. Field studies suggest that complex environmental mixtures of these chemicals can also interfere with vitamin A dynamics in free-ranging marine mammals and other fish-eating wildlife. However, circulatory retinol, which is the least invasive measurement of vitamin A status, appears to have variable responses to contaminant exposure. In addition, "normal" circulatory retinol levels have not yet been described for most wildlife species, and not enough is known about the natural physiological events that can alter these concentrations. Confounding factors must therefore be characterized before retinoids can be used as an effective indicator of adverse health effects in marine mammals exposed to elevated levels of environmental contaminants.

Sisinno CL, Oliveira-Filho EC, Dufrayer MC, Moreira JC, Paumgartten FJ. **Toxicity evaluation of a municipal dump leachate using zebrafish acute tests.** Bull Environ Contam Toxicol 2000;64(1):107-13.

Szamosi-Hernadi D, Olah B, Gaty S, Pap L. **Ecotoxicological experiences on Lemna minor test system.** Cent Eur J Public Health 2000;8 Suppl:96-7.
Abstract: Are plants generally less sensitive to toxicity than aquatic animals as indicated by some authors (1)? The use of phytotoxicity (higher plant) tests as a part of ecotoxicology is relatively underdeveloped. The objective of this study was to compare the results of the OECD Lemna Growth Inhibition Test with other mainly used test systems. Three toxicants were tested applying the Alga Growth Inhibition Test (Seleneastrum capricornutum), the Daphnia Immobilization Test (Daphnia magna) and the MICROTOX-test (Photobacterium phosphoreum). Duckweed plants were found to be sensitive to the chemicals tested, with nearly 100% inhibition of frond production occurring in the highest concentrations. The plants in some samples showed lesions (break up of the colony structure) and loss of green pigments (chlorosis), others showed localized dead tissues (necrosis). In addition to the morphological deviations (appearance) there is a wide range of characteristics to be observed on the test plants: number of fronds, number of plants, biomass, dry weight, mortality, CO_2 and O_2 exchange, concentration of chlorophyll a and chlorophyll b. It is consistent that the number of fronds is slightly more sensitive than or the same as the number of plants and that the biomass tend to be somewhat more

tolerant than those for the number of plants (2). Applying the simplest way of observation, changes in the frond number were recorded. As Lemna minor show a characteristical sensitivity to the different kind of chemicals, it is a promising indicator of aquatic toxicity.

Takanashi H, Urano K, Hirata M, Hano T, Ohgaki S. **Method for measuring mutagen formation potential (MFP) on chlorination as a new water quality index.** Water Res 2001;35(7):1627-34.
Abstract: A novel water quality index, the mutagen formation potential (MFP) is proposed for use in evaluation of the quality of drinking water which may contain pollutants capable of forming mutagens when chlorinated under the conditions used in water purification processes. A method for measuring MFP was established as follows. The water sample to be tested is diluted until the TOC reaches 3-4 mg l-1, the pH is adjusted to 7.0 +/- 0.2, sodium hypochlorite is added to obtain conditions where Cl/TOC = 3-4 mg Cl (mg C)-1, and the water sample is left standing for 24 +/- 2 h at room temperature. Thereafter, 21 of the chlorinated water sample at pH 2.0 +/- 0.1 is passed through a Sep-Pak Plus CSP-800 cartridge to adsorb any mutagens formed, and DMSO is applied to the cartridge to desorb the mutagens. Then, a 2 ml sample of the eluate is collected after the DMSO had begun to flow out of the cartridge and evaluated by the Ames Salmonella mutagenicity assay (preincubation method).

Verhaar HJ, Solbe J, Speksnijder J, van Leeuwen CJ, Hermens JL. **Classifying environmental pollutants: Part 3. External validation of the classification system.** Chemosphere 2000;40(8):875-83.
Abstract: In order to validate a classification system for the prediction of the toxic effect concentrations of organic environmental pollutants to fish, all available fish acute toxicity data were retrieved from the ECETOC database, a database of quality-evaluated aquatic toxicity measurements created and maintained by the European Centre for the Ecotoxicology and Toxicology of Chemicals. The individual chemicals for which these data were available were classified according to the rulebase under consideration and predictions of effect concentrations or ranges of possible effect concentrations were generated. These predictions were compared to the actual toxicity data retrieved from the database. The results of this comparison show that generally, the classification system provides adequate predictions of either the aquatic toxicity (class 1) or the possible range of toxicity (other classes) of organic compounds. A slight underestimation of effect concentrations occurs for some highly water soluble, reactive chemicals with low log K(ow) values. On the other end of the scale, some compounds that are classified as belonging to a relatively toxic class appear to belong to the so-called baseline toxicity compounds. For some of these, additional classification rules are proposed. Furthermore, some groups of compounds cannot be classified, although they should be amenable to predictions. For these compounds additional research as to class membership and associated prediction rules is proposed.

Vighi M, Gramatica P, Consolaro F, Todeschini R. **QSAR and chemometric approaches for setting water quality objectives for dangerous chemicals.** Ecotoxicol Environ Saf 2001;49(3):206-20.
Abstract: In order to evaluate environmentally safe levels of dangerous chemicals, there is the need for a set of toxicological data on organisms representative of the ecosystems, which is often unavailable or inadequate. In this Article, a predictive approach was applied to a set of 125 chemicals (derived from the European priority list in compliance with Directive 76/464/EEC), for which water quality objectives were available. Toxicological data on organisms representative of the aquatic environment (algae, Daphnia, and fish) were taken from the literature or predicted by means of quantitative structure--

activity relationships. This provided toxicological data on all three organisms for 97 of 125 chemicals and on at least two organisms (Daphnia and fish) for the whole data set. Principal Component Analysis was applied in order to perform an a priori classification of chemicals based on toxicity data. Then several classification models, based on traditional and nontraditional molecular descriptors, were applied. Classification models gave results in agreement with the a priori classification as well as with the original water quality objectives classification. The behavior of some outliers was explained. The approach described appears to be a useful tool for the preliminary classification of chemicals that are dangerous to the aquatic environment for which toxicological data are inadequate. Copyright 2001 Academic Press.

Wells PG, Depledge MH, Butler JN, Manock JJ, Knap AH. **Rapid toxicity assessment and biomonitoring of marine contaminants--exploiting the potential of rapid biomarker assays and microscale toxicity tests.** Mar Pollut Bull 2001;42(10):799-804.
Abstract: There is a great need for an integrated international effort in research and training using rapid, easy to use, biomarker and microscale ecotoxicity techniques. These techniques must be directed, coordinated and formulated into protocols that contribute to the prevention and reduction of marine pollution world-wide and the improvement of ocean and human health. This need should be considered as urgent by marine environmental scientists, managers and policy makers throughout the world. Our paper discusses such techniques and suggests a four-point framework for advancing work towards their wider use, parcticularly in developing coastal nations.

Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE. **Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure.** Crit Rev Toxicol 2000;30(4):347-570.
Abstract: This review compiles and evaluates existing scientific information on the use, limitations, and procedural considerations for EROD activity (a catalytic measurement of cytochrome P4501A induction) as a biomarker in fish. A multitude of chemicals induce EROD activity in a variety of fish species, the most potent inducers being structural analogs of 2,3,7,8-tetracholordibenzo-p-dioxin. Although certain chemicals may inhibit EROD induction/activity, this interference is generally not a drawback to the use of EROD induction as a biomarker. The various methods of EROD analysis currently in use yield comparable results, parcticularly when data are expressed as relative rates of EROD activity. EROD induction in fish is well characterized, the most important modifying factors being fish species, reproductive status and age, all of which can be controlled through proper study design. Good candidate species for biomonitoring should have a wide range between basal and induced EROD activity (e.g., common carp, channel catfish, and mummichog). EROD activity has proven value as a biomarker in a number of field investigations of bleached kraft mill and industrial effluents, contaminated sediments, and chemical spills. Research on mechanisms of CYP1A-induced toxicity suggests that EROD activity may not only indicate chemical exposure, but also may also precede effects at various levels of biological organization. A current research need is the development of chemical exposure-response relationships for EROD activity in fish. In addition, routine reporting in the literature of EROD activity in standard positive and negative control material will enhance confidence in comparing results from different studies using this biomarker.

Atienzar FA, Cordi B, Donkin ME, Evenden AJ, Jha AN, Depledge MH. **Comparison of ultraviolet-**

induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, Palmaria palmata. Aquatic Toxicol 2000;50(1-2):1-12.
Abstract: The random amplified polymorphic DNA (RAPD) technique was used to detect DNA damage in the sublittoral macroalgae Palmaria palmata (Rhodophyta) exposed to both ambient and elevated irradiances of UV-B (280-315 nm). To investigate the potential of this method in ecotoxicological assessments, the qualitative and quantitative modifications in RAPD profiles were compared with changes in a number of physiological and fitness parameters. RAPD detectable modifications in DNA profiles were observed in all UV exposed individuals compared with controls. Changes in chlorophyll fluorescence (F(v)/F(m) ratio), in vivo pigment absorptance, thallus growth and RAPD profiles, examined simultaneously, provided a sensitive measure of UV-induced toxicity. In conclusion, the application of the RAPD method in conjunction with other suitable physiological and fitness measurements, may prove to be a valuable tool for investigating the specific effects of genotoxic agents upon marine algal populations. Ultimately, this methodology may allow the ecotoxicological examination of the link between molecular alterations and measurable adverse effects at higher levels of biological organisation.

Bobeldijk I, Brandt A, Wullings B, Noij T. **High-performance liquid chromatography--ToxPrint: chromatographic analysis with a novel (geno)toxicity detection.** J Chromatogr A 2001;918(2):277-91.
Abstract: In order to aid the monitoring of the overall quality of (surface) waters a new analytical approach has been developed, combining on-line solid-phase extraction, HPLC separation and effect-related detection. Compounds present in surface water or wastewater samples are extracted on-line with Oasis [poly(divinylbenzene-co-N-vinylpyrrolidone)] material and directly fractionated by reversed-phase HPLC. The eluent of the total chromatogram is collected on a microtitre plate in fractions of 1 min each. After evaporation and re-dissolvation in a suitable solvent, the (geno)toxicity of the individual fractions before and after enzymatic activation with S9, is determined with the umu test. In this way, harmful compounds can be detected and localized in the HPLC-diode array detection trace even without their identity and exact concentration being known at that moment. The method was developed using two test compounds, 4-nitroquinoline-N-oxide and 2-aminoanthracene. Compounds with mutagenic properties comparable to those of the test compounds can be detected from 0.1 microg/l, which is a concentration relevant for surface waters. The new analytical approach was successfully applied to various types of model samples, as well as real wastewater.

Czyz A, Jasiecki J, Bogdan A, Szpilewska H, Wegrzyn G. **Genetically modified Vibrio harveyi strains as potential bioindicators of mutagenic pollution of marine environments.** Appl Environ Microbiol 2000;66(2):599-605.
Abstract: For biodetection of mutagenic pollution of marine environments, an organism naturally occurring in these habitats should be used. We found that marine bacterium Vibrio harveyi may be an appropriate bioindicator of mutagenic pollution. For positive selection of mutants, we developed a simple method for isolation of V. harveyi mutants resistant to neomycin. We constructed genetically modified V. harveyi strains that produce significantly more neomycin-resistant mutants upon treatment with low concentrations of mutagens than the wild-type counterpart. The sensitivity of the mutagenicity test with the V. harveyi strains is at least comparable to (if not higher than) that of the commonly used

Ames test, which uses Salmonella enterica serovar Typhimurium strains. Therefore, we consider that the V. harveyi strains described in this report could be used as potential bioindicators of mutagenic pollution of marine environments.

Le Pennec G, Le Pennec M. **Evaluation of the toxicity of chemical compounds using digestive acini of the bivalve mollusc Pecten maximus L. maintained alive in vitro.** Aquat Toxicol 2001;53(1):1-7.
Abstract: The digestive gland of bivalve molluscs is a model of choice for experiments in ecotoxicology because of its implication in detoxification processes moreover of its classical functions in digestive phenomena. All physiological deteriorations of this organ, related or not to pollution, can lead to animal death. The recent development of a method allowing digestive acini of Pecten maximus to be maintained alive in vitro for 96 h opens up new research prospects in ecotoxicology. The action of contaminants considered to be cytotoxic or genotoxic in the literature were tested on this model. The results show the high cytotoxicity of ethylmethane sulphonate 80 and 5 mM after 2 h of contact with acini. Other compounds such as 4-nitroquinoline-N-oxide 0.1 mM, cadmium chloride 10(-5) M and atrazine 10(-4) M, which were weakly toxic after 2 h, became highly toxic after 48 h of contact. Compounds such as 4-nitroquinoline-N-oxide 1 mM, which were not cytotoxic after 2 h, proved to be the most genotoxic of all those tested. Others, such as MMS 1 mM, cadmium chloride 10(-4) and 10(-5) M, and atrazine 10(-5) M, showed an unconfirmed tendency to be genotoxic. The results obtained with this 'acinus' model seem more readily transposable to the whole organism than those obtained with 'isolated cell' models, in that acini can be considered as digestive glands 'in miniature'.

GENOTOXICITY AND MUTAGENESIS

Abe K, Matsuki N. **Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT.** Neurosci Res 2000;38(4):325-9.
Abstract: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and lactate dehydrogenase (LDH) release assay have been widely used for evaluating cell viability in culture. MTT reduction assay measures the redox activity of living cells, while LDH assay measures the activity of LDH released into the medium from dead cells. In this paper, we introduce a quick and simple method of measuring cellular MTT reduction and LDH release with the same dye, MTT. The substrate mixture for measuring LDH activity contained lactate, beta-nicotinamide adenine dinucleotide, 1-methoxyphenazine methosulfate, MTT and Triton X-100. When the medium containing LDH was mixed with the substrates, MTT was converted into MTT formazan in proportion to LDH activity. This method was successfully applied for evaluating t-butyl hydroperoxide toxicity in cultured rat cortical astrocytes and glutamate toxicity in cultured rat hippocampal neurons. Our method is economical and convenient especially for measuring cellular MTT reduction and LDH release in the same culture.

Abu-Shakra A, McQueen ET, Cunningham ML. **Rapid analysis of base-pair substitutions induced by mutagenic drugs through their oxygen radical or epoxide derivatives.** Mutat Res 2000;470(1):11-8.
Abstract: Among the drugs that induce base-pair substitution mutations in the Salmonella reversion assay are the nitric oxide (NO)-delivery drug, diethylamine NONOate (DeaNO), and the ovarian cancer chemotherapeutic drug, treosulphan (TE). The present study compared the mutation spectra generated

by DeaNO and TE in the hisG46 strains, TA1535 and TA100, the hisG428 strain, TA102, and the six Ames II 7000 series strains. Using these strains, it was feasible to conduct rapid analysis of the type and magnitude of induced mutation without resorting to DNA amplification and sequencing. A putative hydrolysis product of TE, 1,2:3,4-diepoxybutane (DEB), and hydrogen peroxide (H(2)O(2)) were included in the study to allow for further comparisons between epoxide-induced damage and that induced by the hydroxyl radical. TE (0.93 micromole/pl) induced 16. 8-fold-over-background reversion or a mutagenicity ratio (MR) of 16. 8 in TA1535. The response was weaker in TA100 (MR of 3), and negative in strain TA102. Only two Ames II strains demonstrated sensitivity to TE, and they were TA7004 (CG:AT) and TA7005 (GC:AT). Like TE, DeaNO (33 micromole/pl) was mutagenic in TA1535 (MR of 24.6), TA100 (MR of 5.3), TA7004 (MR of 13.7), and TA7005 (MR of 7.7), and non-mutagenic in TA102. These results showed a preferential sensitivity to reversion of the -CCC-target in TA100 and TA1535, and a lack of sensitivity to reversion of the -TAA-target in TA102. In addition, they elucidated the selectivity of the Ames II strains, with AT targets showing little or no sensitivity to reversion. The TE-epoxide derivative DEB was mutagenic in TA1535 and TA7004, but in contrast to TE, DEB was mutagenic in TA102. Interestingly, TA102 was reverted by DEB and H(2)O(2) but not by TE or DeaNO. This study showed that analysis of mutations is achievable using the battery of strains listed above. The fact that DNA damage can be detected by reversion at specific bases offers a tool for understanding the mechanisms through which drugs may exert their DNA and cellular damage.

Abul-Hassan K, Walmsley R, Boulton M. **Optimization of non-viral gene transfer to human primary retinal pigment epithelial cells.** Curr Eye Res 2000;20(5):361-6.
Abstract: PURPOSE: To optimise the high efficiency, non-viral transfer of DNA to retinal pigment epithelial (RPE) cells in vitro. METHODS: A mammalian expression vector (pcDNA3.1) containing a firefly luciferase (luc) cDNA was used to transfect RPE cells using different chemical methods; calcium phosphate, DEAE-dextran and, liposomes-based transfection techniques. Transfection was optimised for both dose and time of exposure. The efficiency of gene transfer and cytotoxicity was measured 48 hours post-transfection using luciferase and MTT assays, respectively. The percentage of transfected cells (using optimal conditions) was determined with a construct expressing a jellyfish green fluorescent protein (GFP) using flow cytometery. RESULTS: Calcium phosphate and DEAE-dextran techniques failed to transfect the vector and led to high cytotoxicity. Liposomes-based methods successfully transferred the vector to RPE cells, but the efficiency varied for different liposomes; Tfx-50 > Lipofectin > Lipofectamine > Cellfectin > DMRIE-C. No significant cytotoxicity was observed with any of the liposome treatments. Optimal transfection was achieved with Tfx-50 at a 3:1 ratio of DNA:liposome; between 12-15% of cells being transfected. CONCLUSIONS: Efficient and non-toxic transfer of functional genes into primary RPE cells in vitro can be successfuly achieved by liposomes-based techniques. Tfx-50 appears to be a promising non-viral vector for RPE gene transfer.

Abul-Hassan K, Walmsley R, Tombran-Tink J, Boulton M. **Regulation of tyrosinase expression and activity in cultured human retinal pigment epithelial cells.** Pigment Cell Res 2000;13(6):436-41.
Abstract: The purpose of this study was to investigate the regulation of tyrosinase gene expression and activity in cultured human retinal pigment epithelial (RPE) cells. The tyrosinase promoter (Ty.prom) region (400 bp) was PCR amplified and cloned into a modified mammalian expression vector (pcDNA3.1) upstream of a firefly luciferase (Luc) cDNA and was designated 'pcDNA3.1-Ty.prom.Luc'.

The plasmid was co-transfected into RPE cells with a second mammalian expression plasmid (pRL-TK) containing a herpes simplex virus thymidine kinase promoter region upstream of Renilla Luc in a protocol designated the 'dual luciferase assay' (DLA). After co-transfection, cells were treated with a range of potential melanogenic agents; basic fibroblast growth factor (bFGF), methyl methane sulphonate, alpha-melanocyte stimulating hormone, verapamil, phorbol myristate acetate, cholera toxin (CT), pigment epithelium derived factor (PEDF), and L-tyrosine. The expression of tyrosinase promoter and enzymatic activities were determined 48 hr post-transfection using the DLA and DOPA oxidase assays, respectively. Tyrosinase activity could not be detected in RPE cells with any of the treatments. Tyrosinase promoter activity was significantly up-regulated in RPE cells treated with bFGF, PEDF, verapamil, CT and tyrosine compared with control cells. In conclusion, the tyrosinase gene is not only expressed but can be regulated in response to different chemicals in cultured human RPE cells. However, it appears that RPE cells in culture lack a post-transcriptional and/or translational modification point(s), which are necessary for tyrosinase enzymic activity.

Acton TB, Mead J, Steiner AM, Vershon AK. **Scanning mutagenesis of Mcm1: residues required for DNA binding, DNA bending, and transcriptional activation by a MADS-box protein.** Mol Cell Biol 2000;20(1):1-11.
Abstract: MCM1 is an essential gene in the yeast Saccharomyces cerevisiae and is a member of the MADS-box family of transcriptional regulatory factors. To understand the nature of the protein-DNA interactions of this class of proteins, we have made a series of alanine substitutions in the DNA-binding domain of Mcm1 and examined the effects of these mutations in vivo and in vitro. Our results indicate which residues of Mcm1 are important for viability, transcriptional activation, and DNA binding and bending. Substitution of residues in Mcm1 which are highly conserved among the MADS-box proteins are lethal to the cell and abolish DNA binding in vitro. These positions have almost identical interactions with DNA in both the serum response factor-DNA and alpha2-Mcm1-DNA crystal structures, suggesting that these residues make up a conserved core of protein-DNA interactions responsible for docking MADS-box proteins to DNA. Substitution of residues which are not as well conserved among members of the MADS-box family play important roles in contributing to the specificity of DNA binding. These results suggest a general model of how MADS-box proteins recognize and bind DNA. We also provide evidence that the N-terminal extension of Mcm1 may have considerable conformational freedom, possibly to allow binding to different DNA sites. Finally, we have identified two mutants at positions which are critical for Mcm1-mediated DNA bending that have a slow-growth phenotype. This finding is consistent with our earlier results, indicating that DNA bending may have a role in Mcm1 function in the cell.

Adamska M, Wolff A, Kreusler M, Wittbrodt J, Braun T, Bober E. **Five Nkx5 genes show differential expression patterns in anlagen of sensory organs in medaka: insight into the evolution of the gene family.** Dev Genes Evol 2001;211(7):338-49.
Abstract: We report the identification and characterisation of five different Nkx5-related genes in medaka fish (Oryzias latipes). They constitute homologues of genes previously isolated in higher vertebrates, Nkx5--1, Nkx5--2, Hmx1/Nkx5--3 and SOHo-1, and were named accordingly: OlNkx5--1.1, OlNkx5--2, OlNkx5--3 and OlSOHo. For the Nkx5--1 gene a new, second homologue, OlNkx5--1.2, was isolated. In medaka, Nkx5 and SOHo genes are differentially expressed in three developing

sensory organs: eye, ear and lateral line and later in defined brain regions. Phylogenetic analyses of the entire Nkx5 family revealed that four paralogous Nkx5 groups, Nkx5--1, Nkx5--2, Hmx1/Nkx5--3/GH6 and SOHo, are present in vertebrates. Only some of the Nkx5 family members have been identified in singular vertebrate species so far. Here we present, for the first time, the isolation of representatives of each Nkx5 subgroup in one species, the medaka fish. Based on similarities in sequence and expression patterns, and genomic organisation we propose a model of the evolutionary history of the Nkx5 family. The model predicts that the four vertebrate Nkx5 genes arose by a tandem duplication, followed by chromosomal duplication. The two Nkx5--1 genes identified so far exclusively in medaka most probably result from an additional genome duplication in the fish lineage.

Agapakis-Causse C, Bosca F, Castell JV, Hernandez D, Marin ML, Marrot L, Miranda MA. **Tiaprofenic acid-photosensitized damage to nucleic acids: a mechanistic study using complementary in vitro approaches.** Photochem Photobiol 2000;71(5):499-505.
Abstract: In order to determine whether or not tiaprofenic acid (TPA) could cause cellular DNA damage, human fibroblasts were irradiated in the presence of the drug and subsequently examined by means of the comet assay. This led to the observation that TPA actually sensitizes cellular DNA to the subsequent irradiation. When TPA was irradiated in the presence of supercoiled plasmid DNA, it produced large amounts of single-strand breaks (SSB); this is consistent with the effects observed on cellular genomic DNA by the comet assay. More importantly, low concentrations of TPA, unable to produce direct SSB, caused photo-oxidative damage to DNA as revealed by the use of excision-repair enzymes. The fact that TPA-irradiated DNA was a substrate of formamidopyrimidine glycosylase as well as endonuclease III revealed that both purine and pyrimidine bases were oxidized. This was further supported by the TPA-photosensitized oxidation of 2'-deoxyguanosine which led to a product mixture characteristic of mixed type-I/II mechanisms. Thymidine was less reactive under similar conditions, but it also decomposed to give a typical type-I product pattern. Accordingly, the TPA triplet was quenched by the two nucleosides with clearly different rate constants (10(8) vs 10(7) M-1 s-1, respectively). As cellular RNA also contains oxidizable bases, it could be the target of similar processes, thus interfering with the biosynthesis of proteins by the cells. Extraction of total RNA from TPA-irradiated human fibroblasts, followed by gel electrophoresis and PCR analysis, confirmed this hypothesis. Finally, photosensitization experiments with Saccharomyces cerevisiae showed that, in spite of an efficient drug-yeast interaction leading to cytotoxicity, neither intergenic recombination nor gene conversion took place. Thus, while TPA-photosensitized damage to nucleic acids can result in genotoxicity, the risk of mutagenicity does not appear to be significant.

Agrawal S, Kishore MC. **MHC class I gene expression and regulation.** J Hematother Stem Cell Res 2000;9(6):795-812.
Abstract: Major histocompatibility complex (MHC) is a conglomerate of genes that play an important role in recognition of self and nonself. These genes are under tight control. In this review we have discussed the transcription processes regulating MHC gene expression. Various biological or chemical modulators can modulate MHC gene expression. The promoter region of class I genes can be activated through several pathways. Hence, these genes are not typical "domestic" genes. Extensive studies on regulation of MHC class I expression, using transfection techniques and transgenic animal models, have resulted in identification of various cis-acting sequences involved in positive and negative regulation of

class I genes. Work is in progress to identify the transacting proteins that bind to these sites and to delineate the mechanisms that regulate constitutive and inducible expression of class I genes in normal and diseased cells. It has been seen that various biological molecules (IFN, GM-CSF, IL-2) and other chemicals up-regulate the MHC expression. If the exact mechanisms are known by which the expression of class I genes is up regulated, the efforts can be made to balance the beneficial and toxic effects of biological molecules with one another, which may facilitate the use of combination of these molecules in subpharmacological doses (to eliminate toxicity) for early and better management of neoplastic diseases, as it is well-known that during malignancy MHC gene expression is down-regulated. In the future, the use of transgenic and knockout mice will be useful in acquiring a better understanding, which may further help in cancer therapy.

Albertini RJ. **HPRT mutations in humans: biomarkers for mechanistic studies.** Mutat Res 2001;489(1):1-16.
Abstract: The X-chromosomal gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT), first recognized through its human germinal mutations, quickly became a useful target for studies of somatic mutations in vitro and in vivo in humans and animals. In this role, HPRT serves as a simple reporter gene. The in vivo mutational studies have concentrated on peripheral blood lymphocytes, for obvious reasons. In vivo mutations in T cells are now used to monitor humans exposed to environmental mutagens with analyses of molecular mutational spectra serving as adjuncts for determining causation. Studies of the distributions of HPRT mutants among T cell receptor (TCR) gene-defined T cell clones in vivo have revealed an unexpected clonality, suggesting that HPRT mutations may be probes for fundamental cellular and biological processes. Use of HPRT in this way has allowed the analyses of V(D)J recombinase mediated mutations as markers of a mutational process with carcinogenic potential, the use of somatic mutations as surrogate markers for the in vivo T cell proliferation that underlies immunological processes, and the discovery and study of mutator phenotypes in non-malignant T cells. In this last application, the role of HPRT is related to its function, as well as to its utility as a reporter of mutation. Most recently, HPRT is finding use in studies of in vivo selection for in vivo mutations arising in either somatic or germinal cells.

Aldhous P. **'Saturation screen' lets zebrafish show their stripes.** Nature 2000;404(6781):910

Ameixa C, Brickell PM. **Characterization of a chicken retinoid X receptor-gamma gene promoter and identification of sequences that direct expression in retinal cells.** Biochem J 2000;347(Pt 2):485-90.
Abstract: Development of the cellular complexity of the vertebrate neural retina relies on an intricate interplay between extracellular signals and intracellular factors. In parcticular, transcription factors play a key role in determining the competence of cells to respond to extracellular signals. We have previously shown that, in the developing chick neural retina, expression of the retinoid X receptor-gamma (RXR-gamma2) nuclear receptor gene is restricted to photoreceptors. To characterize the mechanisms that regulate expression of this gene in the neural retina, we isolated a chicken RXR-gamma genomic clone containing the RXR-gamma2 promoter and mapped the transcription initiation site by means of ribonuclease protection. We analysed promoter activity by transient transfection of luciferase reporter gene constructs into cultured cells isolated from embryonic-chick neural retina or facial mesenchyme,

which does not normally express detectable RXR-gamma2 transcripts. The DNA fragment lying between nucleotides -657 and +37 with respect to the transcription initiation site had basal promoter activity in both cell types. The fragment lying between nucleotides -1198 and -991 directed 10-20-fold higher levels of luciferase activity in neural retina cells, but only basal levels in facial mesenchyme cells. This 208 bp fragment also enhanced the activity of the simian-virus-40 promoter, when placed upstream in either orientation. Electrophoretic-mobility-shift assays using this 208 bp fragment demonstrated the formation of four neural retina-specific protein-DNA complexes. These results indicate that regulation of RXR-gamma2 transcription in the developing chick neural retina involves the binding of one or more neural retina-specific protein factors to an enhancer element located approx. 1 kbp upstream of the transcription initiation site.

Amin F, Bowen ID, Szegedi Z, Mihalik R, Szende B. **Apoptotic and non-apoptotic modes of programmed cell death in MCF-7 human breast carcinoma cells.** Cell Biol Int 2000;24(4):253-60.
Abstract: Apoptosis is a specific mode of programmed cell death (PCD), recognized by characteristic morphological and molecular changes. Here we present evidence for a non-apoptotic type of PCD in human MCF-7 breast carcinoma cells. We used TNF-alpha and tyrphostin AG213 to induce apoptotic and non-apoptotic cell death respectively in vitro. Microscopic and immunohistochemical studies, together with DNA analysis and flow cytometric analysis of p53 and bcl-2 oncogene expression, revealed some novel characteristics of non-apoptotic cell death. We show here for the first time some of the biochemical features of an experimentally induced non-apoptotic PCD and emphasize the distinct biochemical events leading to apoptotic and non-apoptotic PCD.

Anderson D. **Factors that contribute to biomarker responses in humans including a study in individuals taking Vitamin C supplementation**. Mutat Res 2001;480-481:337-47.
Abstract: It is possible in many situations to identify humans exposed to potentially toxic materials in the workplace and in the environment. As in most human studies, there tends to be a high degree of interindividual variability in response to chemical insults. Some non-exposed control individuals exhibit as high a level of damage as some exposed individuals and some of these have levels of damage as low as many of the controls. Thus, it is only the mean values of the groups that can substantiate an exposure-related problem; the data on an individual basis are still of limited use. While human lymphocytes remain the most popular cell type for monitoring purposes, sperm, buccal, nasal, epithelial and placental cells are also used. However, for interpretation of responses, the issue of confounding factors must be addressed. There are endogenous confounding factors, such as age, gender, and genetic make-up and exogenous ones, including lifestyle habits (smoking, drinking, etc.) There are biomarkers of exposure, effect/response and susceptibility and the last may be influenced by the genotype and polymorphism genes existing in a population. From our own studies, confounding effects on cytogenetic damage and ras oncoproteins will be considered in relation to workers exposed to vinyl chloride and petroleum emissions and to volunteers taking Vitamin C supplementation. Smoking history, exposure and duration of employment affected the worker studies. For petroleum emissions, so did gender and season of exposure. For the non-smoking volunteer Vitamin C supplementation study, cholesterol levels, plasma Vitamin C levels, lipid peroxidation products and DNA damage in the Comet assay were also measured. Gender affected differences in Vitamin C levels, antioxidant capacity and the number of chromosome aberrations induced by bleomycin challenge in vitro. The results were the same for both high and low

cholesterol subjects. The relationship between biomarkers and the various factors which affect them is complex. Sometimes the variables are not completely independent of each other.

Ando H, Mishina M. **[Mutagenesis in zebrafish].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2829-37 [Jpn].

Angelis KJ, McGuffie M, Menke M, Schubert I. **Adaptation to alkylation damage in DNA measured by the comet assay**. Environ Mol Mutagen 2000;36(2):146-50.
Abstract: The alkylating mutagens N-methyl-N-nitrosourea (MNU) and methyl methanesulfonate (MMS) were studied for their potential to induce DNA strand breaks and abasic (AP) sites in meristematic nuclei of Vicia faba root tips by the comet assay. The alkaline unwinding/neutral electrophoresis (A/N) and alkaline unwinding/alkaline electrophoresis (A/A) protocols were used for detection of DNA damage. With the A/N comet assay, less DNA damage was seen after conditioning pretreatment with a low dose prior to a high challenging dose of alkylating mutagens as compared to application of the high dose only, whereas a nearly additive effect was seen when the A/A comet assay was used. Adaptation was even more obvious when AP sites were revealed by the AP-endonuclease activity of exonuclease III. The adaptation observed with the A/N comet assay was abolished by pretreatment with the protein synthesis inhibitor cycloheximide. These data suggest that the comet assay is able to detect on molecular level a phenomenon resembling clastogenic adaptation.

Anitha B, Chandra N, Gopinath PM, Durairaj G. **Genotoxicity evaluation of heat shock in gold fish (Carassius auratus).** Mutat Res 2000;469(1):1-8.
Abstract: Genotoxicity evaluation of heat shock was carried out in Carassius auratus. The genotoxicity end points studied were nuclear anomalies (micronucleus assay), chromosomal aberrations, DNA damage (comet assay) and cell proliferation. The heat shock temperatures used were 34 degrees C, 36 degrees C and 38 degrees C. The results demonstrated that heat shock causes the induction of micronucleus at all the three temperature studied. Heat shock also inhibited cell proliferation at 38 degrees C and caused aberrations in the metaphase chromosomes at 34 degrees C and 36 degrees C. Comet assay demonstrated single strand DNA damage at all the three temperatures. The results obtained indicate that heat shock is a genotoxicant.

Annas A, Brittebo E, Hellman B. **Evaluation of benzo(a)pyrene-induced DNA damage in human endothelial cells using alkaline single cell gel electrophoresis.** Mutat Res 2000;471(1-2):145-55.
Abstract: The alkaline version of the 'comet assay' was used to evaluate DNA damage in human umbilical vein endothelial cells (HUVEC) exposed to 0.1, 1.0, or 10 microM benzo(a)pyrene for 90min. The genotoxicity was monitored in HUVEC pretreated with the Ah-receptor agonist beta-naphthoflavone (BNF), previously shown to induce cytochrome P4501A1 (CYP1A1) activity in these cells, and in vehicle-treated HUVEC with only constitutive levels of this enzyme. Increased DNA damage was observed only in cells that had been exposed to 10 microM benzo(a)pyrene, cells exposed to BNF being subjected to the most extensive damage. The CYP1A/B-inhibitor alpha-naphthoflavone (ANF) reduced the benzo(a)pyrene-induced DNA-damage in the BNF-treated HUVEC to the same level as in the uninduced cells. The fact that benzo(a)pyrene induced DNA damage in vehicle-treated HUVEC suggests that there may be at least one alternative route of bioactivation for benzo(a)pyrene in these

cells. Consequently, judging from the present results it seems as if tobacco-related polycyclic aromatic hydrocarbons (PAHs) may disrupt the function of the endothelial lining in blood vessels with low monooxygenase activity. It is proposed that exposure to Ah receptor agonists via, for example, tobacco smoke, may enhance the DNA-damaging effects of smoke-related genotoxic PAHs in human endothelial cells. The role of PAHs in endothelial dysfunction of tobacco smokers should therefore be further studied.

Archer MC, Clarkson TW, Strain JJ. **Genetic aspects of nutrition and toxicology: report of a workshop.** J Am Coll Nutr 2001;20(2 Suppl):119-28.
Abstract: The health and resilience of humans and animals is, in large part, determined by the quality and quantity of the diet. This, in turn, may influence an individual's capability to deal with stress including toxic insult. In addition, there may be specific components of the diet that modulate the toxicity of specific toxicants whether the latter are ingested as food or absorbed via other routes. Many examples attest to the importance of interactions between dietary components and toxicants after absorption in the body. Such interactions occur at every level of biological organization from the molecular to the whole organism. Some may be synergistic, others antagonistic. Some may involve direct chemical reaction between the nutrient molecule and the toxicant, others may occur by indirect action at the cellular or organ levels. All examples point to the importance of considering diet when measuring the response to toxic agents whether in animals or humans. In order to foster interaction between the sciences of nutrition and toxicology, The Heinz Institute of Nutritional Sciences as sponsoring a series of workshops. The first of these was held in June, 1999 at the University of Ulster to address evolutionary aspects of nutrition--toxicology (for report see Eur. J. Nutr, 39, 49-52, 2000). In June, 2000, a second workshop was held at the University of Toronto to address genetic aspects, and this is a brief summary of the proceedings. We are beginning to understand the molecular basis of the regulation of gene expression by dietary factors and how genetic changes can affect response to toxicants. Recent advances in technology and a detailed understanding of disease etiology has led to the ability to study molecular determinants of disease risk. The workshop provided a forum for nutritionists, toxicologists, molecular biologists, epidemiologists and others to discuss common interests and to merge their efforts towards an integrated approach to nutrition--toxicology via genetics and genomics. The first session dealt with the mechanism by which nutrients such as fatty acids (Clarke), amino acids (Jefferson) and metal ions (Cousins) can regulate gene expression. In the second session, there were presentations on the effects of nutritional factors on genes of toxicological significance such as phase I and phase II enzymes of drug metabolism (Guengerich, Goodfellow and Grant) as well as on oxidative DNA damage and its repair (Collins, Weindruch). Session three dealt with gene-nutrient interactions in the development of chronic diseases such as diabetes (Hegele, Berdanier) and cancer (Kim, Ambrosone et al.). New developments such as DNA microarrays (McGlynn) and the use of transgenic and knockout models (Sehayek) were presented in the final session.

Armstrong MJ, Gara JP, Gealy R 3rd, Greenwood SK, Hilliard CA, Laws GM, Galloway SM. **Induction of chromosome aberrations in vitro by phenolphthalein: mechanistic studies.** Mutat Res 2000;457(1-2):15-30.
Abstract: Phenolphthalein induces tumors in rodents but because it is negative in assays for mutation in Salmonella and in mammalian cells, for DNA adducts and for DNA strand breaks, its primary

mechanism does not seem to be DNA damage. Chromosome aberration (Ab) induction by phenolphthalein in vitro is associated with marked cytotoxicity. At very high doses, phenolphthalein induces weak increases in micronuclei (MN) in mouse bone marrow; a larger response is seen with chronic treatment. All this suggests genotoxicity is a secondary effect that may not occur at lower doses. In heterozygous TSG-p53((R)) mice, phenolphthalein induces lymphomas and also MN, many with kinetochores (K), implying chromosome loss. Induction of aneuploidy would be compatible with the loss of the normal p53 gene seen in the lymphomas.Here we address some of the postulated mechanisms of genotoxicity in vitro, including metabolic activation, inhibition of thymidylate synthetase, cytotoxicity, oxidative stress, DNA damage and aneuploidy. We show clearly that phenolphthalein does not require metabolic activation by S9 to induce Abs. Inhibition of thymidylate synthetase is an unlikely mechanism, since thymidine did not prevent Ab induction by phenolphthalein. Phenolphthalein dramatically inhibited DNA synthesis, in common with many non-DNA reactive chemicals that induce Abs at cytotoxic doses. Phenolphthalein strongly enhances levels of intracellular oxygen radicals (ROS). The radical scavenger DMSO suppresses phenolphthalein-induced toxicity and Abs whereas H(2)O(2) potentiates them, suggesting a role for peroxidative activation. Phenolphthalein did not produce DNA strand breaks in rat hepatocytes or DNA adducts in Chinese hamster ovary (CHO) cells. All the evidence points to an indirect mechanism for Abs that is unlikely to operate at low doses of phenolphthalein. We also found that phenolphthalein induces mitotic abnormalities and MN with kinetochores in vitro. These are also enhanced by H(2)O(2) and suppressed by DMSO. Our findings suggest that induction of Abs in vitro is a high-dose effect in oxidatively stressed cells and may thus have a threshold. There may be more than one mechanism operating in vitro and in vivo, possibly indirect genotoxicity at high doses and also chromosome loss, both of which would likely have a threshold.

Aruna R, Jagetia GC. **Azidothymidine induces dose dependent increase in micronuclei formation in cultured HeLa cells.** Pharmazie 2001;56(6):492-500.
Abstract: Exposure of HeLa cells to azidothymidine (AZT) resulted in a concentration dependent decline in growth kinetics. 100 microM of AZT completely inhibited the cell growth. The frequency of binucleate and multinucleate cells declined with increasing concentration of AZT and the formation of multinucleate cells was completely inhibited at 20 and 30 h at higher concentrations indicating inhibition of cell division. Similarly, the clonogenicity of cells declined in a concentration dependent manner and 10 microM AZT killed 50% of the cells. Conversely, the frequency of MNBNC (micronucleated binucleate cell) increased in a concentration dependent manner and was significantly higher in the AZT treated group than the non-drug treated control group. The relationship between concentrations of AZT and micronuclei-induction was linear for all the post-treatment time periods studied. The biological response was also determined by plotting the surviving fraction of cells on the X-axis and the number of micronuclei on the Y-axis. A close and inverse correlation between the surviving fraction and micronuclei formation was observed and the data could be fitted on to a linear quadratic model.

Asch WS, Schechter N. **Plasticin, a type III neuronal intermediate filament protein, assembles as an obligate heteropolymer: implications for axonal flexibility.** J Neurochem 2000;75(4):1475-86.
Abstract: The assembly characteristics of the neuronal intermediate filament protein plasticin were studied in SW13 cells in the presence and absence of a cytoplasmic filament network. Full-length plasticin cannot polymerize into homopolymers in filament-less SW13c1.2Vim(-) cells but efficiently

coassembles with vimentin in SW13c1.1Vim(-) cells. By cotransfecting plasticin and vimentin in SW13c1.1Vim(-) cells, we show that plasticin assembly requires vimentin in noncatalytic amounts. Differing effects on assembly were seen with point mutations of plasticin monomers that were analogous to the keratin mutations that cause epidermolysis bullosa simplex (EBS). In parcticular, plasticin monomers with point mutations analogous to those in EBS do not uniformly inhibit neurofilament (NF) network formation. A point mutation in the helix termination sequence resulted in complete filament aggregation when coexpressed with vimentin but showed limited coassembly with low- and medium-molecular-weight NF proteins (NF-L and NF-M, respectively). In transfected SW13c1.1Vim(+) cells, a point mutation in the first heptad of the alpha-helical coil region formed equal amounts of filaments, aggregates, and a mixture of filaments and aggregates. Furthermore, coexpression of this point mutation with NF-L and NF-M was associated with a shift toward increased numbers of aggregates. These results suggest that there are important structural differences in assembly properties between homologous fish and mammalian intermediate filament proteins. These structural differences may contribute to the distinctive growth characteristics of the teleost visual pathway.

Ashby J, Tinwell H. **Continuing ability of the rodent bone marrow micronucleus assay to act as a predictor of the possible germ cell mutagenicity of chemicals.** Mutat Res 2001;478(1-2):211-3.

Au WW, Oberheitmann B, Heo MY, Hoffmann W, Oh HY. **Biomarker monitoring for health risk based on sensitivity to environmental mutagens.** Rev Environ Health 2001;16(1):41-64.
Abstract: Ongoing human and environmental genome programs have generated a tremendous amount of information regarding the genetic basis for human disease. The information can be used to enhance existing bioassays, as well as to develop new bioassays for improving human monitoring with the goal of disease prevention. In this review, some biomarkers that can be used for the purpose are presented, with an emphasis on using biomarkers to monitor human sensitivity to environmental mutagens. The application of biomarkers in clarifying the role of inherited and acquired susceptibility for developing environmental disease will be discussed. We emphasize the use of biomarkers that can detect mutagen sensitivity and DNA repair deficiency in the humans as an indication of susceptibility to disease. Such sensitivity can be either genetically determined or acquired from the exposure to environmental mutagens.

Ayene IS, Bernhard EJ, McKenna WG, Muschel RJ, Krisch RE, Koch CJ. **DNA as an important target in radiation-induced apoptosis of MYC and MYC plus RAS transfected rat embryo fibroblasts.** Int J Radiat Biol 2000;76(3):343-54.
Abstract: PURPOSE: This study uses a radiation chemistry approach to determine if DNA is an important target for radiation-induced apoptosis of myc (MR4) and myc plus ras (3.7) transfected rat embryo fibroblast cell lines. MATERIALS AND METHODS: The radiation protection efficiency of four thiols was compared with net molecular charge ranging from -1 to +2: mercaptopropionic acid (Z= -1), mercaptoethanol (Z=0), cysteamine (Z= +1), N(2-mercaptoethyl)-1,3-diaminopropane (Z= +2). Protection factors were determined for these thiols against radiation-induced apoptosis (Apoalert assay), mitotic cell death (clonogenic assay) and double-strand break (dsb) induction (pulse field gel electrophoresis) in MR4 and 3.7 cells. Theoretical protection factors for these thiols against dsb induction were also calculated from second-order chemical repair constants for single-strand breaks

(ssb) and the concentration of added thiols in MR4 and 3.7 cell lines. RESULTS: The charge-dependent increases observed for measured protection factors against radiation-induced apoptosis did not differ significantly between the two cell lines, nor did they differ significantly from the corresponding increases observed for radiation-induced mitotic cell killing and for induction of dsb. The calculated protection factor for dsb also showed a thiol charge-dependent increase similar to the measured protection factors for all of the other parameters studied. CONCLUSIONS: These results are consistent with the hypothesis that DNA is an important target for radiation-induced apoptosis.

Bachl J, Carlson C, Gray-Schopfer V, Dessing M, Olsson C. **Increased transcription levels induce higher mutation rates in a hypermutating cell line.** J Immunol 2001;166(8):5051-7.
Abstract: Somatic hypermutation, in addition to V(D)J recombination, is the other major mechanism that generates the vast diversity of the Ab repertoire. Point mutations are introduced in the variable region of the Ig genes at a million-fold higher rate than in the rest of the genome. We have used a green fluorescent protein (GFP)-based reversion assay to determine the role of transcription in the mutation mechanism of the hypermutating cell line 18-81. A GFP transgene containing a premature stop codon is transcribed from the inducible tet-on operon. Using the inducible promoter enables us to study the mutability of the GFP transgene at different transcription levels. By analyzing stable transfectants of a hypermutating cell line with flow cytometry, the mutation rate at the premature stop codon can be measured by the appearance of GFP-positive revertant cells. Here we show that the mutation rate of the GFP transgene correlates with its transcription level. Increased transcription levels of the GFP transgene caused an increased point mutation rate at the premature stop codon. Treating a hypermutating transfection clone with trichostatin A, a specific inhibitor of histone deacetylase, caused an additional 2-fold increase in the mutation rate. Finally, using Northern blot analysis we show that the activation-induced cytidine deaminase, an essential trans-factor for the in vivo hypermutation mechanism, is transcribed in the hypermutating cell line 18-81.

Bacova G, Hunakova LE, Chorvath M, Boljesikova E, Chorvath B, Sedlak J, Gabelova A. **Radiation-induced DNA damage and repair evaluated with 'comet assay' in human ovarian carcinoma cell lines with different radiosensitivities.** Neoplasma 2000;47(6):367-74.
Abstract: Radiation-induced DNA damage and kinetics of DNA repair was evaluated in three human ovarian carcinoma cell lines (i.e. CH-1, A-2780 and SKOV-3) with different sensitivities to ionizing radiation and radiation-induced apoptosis with the aid of single cell gel electrophoresis (SCGE, the comet assay). A good correlation was found between the initial level of DNA breaks and radiation induced apoptosis in CH-1 and SKOV-3 cell lines. While the radiation-sensitive CH-1 cell line manifested the highest level of initial DNA breakage and a significant delay in DNA break rejoining, the inverse correlation was found in the radiation-resistant cell line SKOV-3. Intermediate initial level of breaks was induced in the A-2780 cell line characterized by the intermediate sensitivity to X-ray radiation in comparison to CH-1 and SKOV-3 cells, however, the kinetics of DNA repair was comparable with radiation-resistant cell line SKOV-3. Our data suggest that the comet assay could be a promising tool for prediction of intrinsic cell radiosensitivity. This method might be considered as a supplementary technique to the more reliable but time consuming clonogenic assay.

Bacso Z, Everson RB, Eliason JF. **The DNA of annexin V-binding apoptotic cells is highly**

fragmented. Cancer Res 2000;60(16):4623-8.
Abstract: Jurkat leukemia cells induced to undergo apoptosis by treatment with an antibody against the Fas receptor have two annexin V (AV)-binding subpopulations: (a) single-positive cells that bind AV but not propidium iodide (PI); and (b) double-positive cells that bind AV and PI. The single-positive population is thought to represent an early stage of apoptosis. We have examined the relationship between AV binding and a classical characteristic of apoptosis, DNA fragmentation. Time course studies with Jurkat cells treated for 1, 2, or 4 h with anti-Fas indicated that the proportion of AV-binding cells was increased after 2 h. A significant increase in DNA fragmentation was observed only at 4 h as measured by the mean tail moment determined with the alkaline single cell gel electrophoresis (comet) assay. This correlation suggests a temporal relationship between the two parameters, but does not provide direct evidence of what happens in individual cells. We developed a method to measure fluorescent markers of cellular structure or function with a laser scanning cytometer and then perform the comet assay on the same cells. Cells in each AV-binding subpopulation were re-examined before and after electrophoresis. Most AV-/PI- cells had no DNA damage, although a few cells showed a pattern of damage characteristic for apoptosis. Double-positive cells all had damaged DNA; approximately half had the apoptotic pattern, and the rest had a pattern typical for necrosis. Nearly all of the single-positive cells had damaged DNA with the apoptotic pattern. Both AV-positive populations contained cells with little or no detectable DNA after electrophoresis, indicating that the DNA was highly fragmented. These results indicate that AV binding is an excellent marker for apoptotic cells, but that these cells already have fragmented DNA.

Baier H, Copenhagen D. **Combining physiology and genetics in the zebrafish retina.** J Physiol 2000;524 Pt 1:1
Abstract: The zebrafish has recently joined the ranks of Drosophila and C. elegans as a tractable model for genetic screens (Fishman, 1999). Zebrafish grow fast, can be kept in large numbers in a small space, and are efficiently mutagenized and screened. Genomic resources are made available at an increasing pace. These days, a mutation can be mapped and cloned in a matter of months. Because a mutant hunt is intrinsically unbiased in terms of the classes of genes that will be tagged, it holds the unique potential to discover novel genes or, in our era of genome sequencing, to identify novel functions for known genes. Zebrafish display dozens of innate behaviours in response to light, of which the optomotor and the optokinetic responses are the most widely studied (Brockerhoff et al. 1995; Easter & Nicola, 1996). Their retinae are crisply layered following the typical vertebrate pattern, and the retinal layers are tiled in an almost crystalline fashion by mosaics of different cell types. Electroretinograms are recorded routinely and therefore, not surprisingly, zebrafish are now also being used for a genetic approach to the visual system.

Barresi MJ, Stickney HL, Devoto SH. **The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity.** Development 2000;127(10):2189-99.
Abstract: Hedgehog proteins mediate many of the inductive interactions that determine cell fate during embryonic development. Hedgehog signaling has been shown to regulate slow muscle fiber type development. We report here that mutations in the zebrafish slow-muscle-omitted (smu) gene disrupt many developmental processes involving Hedgehog signaling. smu(-/-) embryos have a 99% reduction

in the number of slow muscle fibers and a complete loss of Engrailed-expressing muscle pioneers. In addition, mutant embryos have partial cyclopia, and defects in jaw cartilage, circulation and fin growth. The smu(-/-) phenotype is phenocopied by treatment of wild-type embryos with forskolin, which inhibits the response of cells to Hedgehog signaling by indirect activation of cAMP-dependent protein kinase (PKA). Overexpression of Sonic hedgehog (Shh) or dominant negative PKA (dnPKA) in wild-type embryos causes all somitic cells to develop into slow muscle fibers. Overexpression of Shh does not rescue slow muscle fiber development in smu(-/-) embryos, whereas overexpression of dnPKA does. Cell transplantation experiments confirm that smu function is required cell-autonomously within the muscle precursors: wild-type muscle cells rescue slow muscle fiber development in smu(-/-) embryos, whereas mutant muscle cells cannot develop into slow muscle fibers in wild-type embryos. Slow muscle fiber development in smu mutant embryos is also rescued by expression of rat Smoothened. Therefore, Hedgehog signaling through Slow-muscle-omitted is necessary for slow muscle fiber type development. We propose that smu encodes a vital component in the Hedgehog response pathway.

Bartell SM, Ponce RA, Takaro TK, Zerbe RO, Omenn GS, Faustman EM. **Risk estimation and value-of-information analysis for three proposed genetic screening programs for chronic beryllium disease prevention.** Risk Anal 2000;20(1):87-99.
Abstract: Genetic differences (polymorphisms) among members of a population are thought to influence susceptibility to various environmental exposures. In practice, however, this information is rarely incorporated into quantitative risk assessment and risk management. We describe an analytic framework for predicting the risk reduction and value-of-information (VOI) resulting from specific risk management applications of genetic biomarkers, and we apply the framework to the example of occupational chronic beryllium disease (CBD), an immune-mediated pulmonary granulomatous disease. One described Human Leukocyte Antigen gene variant, HLA-DP beta 1*0201, contains a substitution of glutamate for lysine at position 69 that appears to have high sensitivity (approximately 94%) but low specificity (approximately 70%) with respect to CBD among individuals occupationally exposed to respirable beryllium. The expected postintervention CBD prevalence rates for using the genetic variant (1) as a required job placement screen, (2) as a medical screen for semiannual in place of annual lymphocyte proliferation testing, or (3) as a voluntary job placement screen are 0.08%, 0.8%, and 0.6%, respectively, in a hypothetical cohort with 1% baseline CBD prevalence. VOI analysis is used to examine the reduction in total social cost, calculated as the net value of disease reduction and financial expenditures, expected for proposed CBD intervention programs based on the genetic susceptibility test. For the example cohort, the expected net VOI per beryllium worker for genetically based testing and intervention is $13,000, $1,800, and $5,100, respectively, based on a health valuation of $1.45 million per CBD case avoided. VOI results for alternative CBD evaluations are also presented. Despite large parameter uncertainty, probabilistic analysis predicts generally positive utility for each of the three evaluated programs when avoidance of a CBD case is valued at $1 million or higher. Although the utility of a proposed risk management program may be evaluated solely in terms of risk reduction and financial costs, decisions about genetic testing and program implementation must also consider serious social, legal, and ethical factors.

Bartkowiak D, Hogner S, Nothdurft W, Rottinger EM. **Cell cycle and growth response of CHO cells to X-irradiation: threshold-free repair at low doses.** Int J Radiat Oncol Biol Phys 2001;50(1):221-7.

Abstract: PURPOSE: To test the hypothesis of a threshold for induced repair of DNA damage (IR) and, secondarily, of hyperradiosensitivity (HRS) to low-dose X-irradiation. METHODS AND MATERIALS: Exponentially growing Chinese hamster ovary cells (CHO) were X-irradiated with doses from 0.2 to 8 Gy. Survival data were established by conventional colony-forming assay and flow-cytometric population counting. The early cell cycle response to radiation was studied based on DNA-profiles and bromodeoxyuridine pulse-labeling experiments. RESULTS: Colony-forming data were consistent with HRS. However, these data were of low statistic significance. Population counting provided highly reproducible survival curves that were in perfect accord with the linear-quadratic (LQ) model. The dominant cell cycle reaction was a dose-dependent delay of G2 M and late S-phase. CONCLUSION: There was no evidence for a threshold of IR and for low-dose HRS in X-irradiated CHO cells. It is suggested that DNA damage repair activity is constitutively expressed during S-phase and is additionally induced in a dose-dependent and threshold-free manner in late S-phase and G2. The resulting survival is precisely described by the LQ model.

Bartosiewicz M, Penn S, Buckpitt A. **Applications of gene arrays in environmental toxicology: fingerprints of gene regulation associated with cadmium chloride, benzo(a)pyrene, and trichloroethylene.** Environ Health Perspect 2001;109(1):71-4.
Abstract: Toxicity testing of unknown chemicals currently uses a number of short-term bioassays. These tests are costly and time consuming, require large numbers of animals, and generally focus on a single end point. The recent development of DNA arrays provides a potential mechanism for increasing the efficiency of standard toxicity testing through genome-wide assessments of gene regulation. In this study, we used DNA arrays containing 148 genes for xenobiotic metabolizing enzymes, DNA repair enzymes, heat shock proteins, cytokines, and housekeeping genes to examine gene expression patterns in the liver in response to cadmium chloride, benzo(a)pyrene (BaP), and trichloroethylene (TCE). Dose-response studies were carried out in mice for each chemical; each produced a unique pattern of gene induction. As expected, CdCl2 markedly up-regulated metallothionine I and II (5- to 10,000-fold at the highest doses) and several of the heat shock/stress response proteins and early response genes. In contrast, administration of BaP up-regulated only Cyp1a1 and Cyp1a2 genes and produced no significant increases in any of the stress response genes or any of the DNA repair genes present on the array. Likewise, TCE-induced gene induction was highly selective; only Hsp 25 and 86 and Cyp2a were up-regulated at the highest dose tested. Microarray analysis with a highly focused set of genes is capable of discriminating between different classes of toxicants and has potential for differentiating highly noxious versus more subtle toxic agents. These data suggest that use of microarrays to evaluate the potential hazards of unknown chemicals or chemical mixtures must include multiple doses and time points to provide effective assessments of potential toxicity of these substances.

Barut BA, Zon LI. **Realizing the potential of zebrafish as a model for human disease.** Physiol Genomics 2000;2(2):49-51.
Abstract: The value of the zebrafish (Danio rerio) as a model for human disease has been substantiated by a number of recently published papers. Several zebrafish mutants with "human" diseases have been found, spanning a variety of human pathologies. These successful studies utilizing the zebrafish have been made possible by the development of key reagents such as YAC, PAC, and BAC libraries, as well as radiation hybrid panels. With the further establishment of new tools and access to the newly generated

resources, the zebrafish is poised to serve as a novel model for human disease.

Basso K, Russo A. **Detection and characterization of micronuclei in a murine liver epithelial cell line, by application of the in vitro cytokinesis block MN assay and PRINS.** Mutagenesis 2000;15 (4):349-56.
Abstract: The cytokinesis block micronucleus assay was applied to murine cell line C6, derived from fetal liver, after an optimal protocol had been designed. Micronucleus frequencies were assayed after exposure to three concentrations of colcemid or diepoxybutane. Two-colour primed in situ DNA synthesis (PRINS) was applied to simultaneously label telomeric and centromeric (minor satellite DNA) sequences. Both chemicals induced a highly significant increase in MN and the effect was dose dependent. Diepoxybutane did not appear to significantly increase the frequency of centromere-positive micronuclei. Colcemid, as expected, induced high frequencies of centromere-positive micronuclei at all concentrations tested; in addition a significant increase in centromere-negative micronuclei was observed at 10(-5) M. Many centromere-positive micronuclei carried three or four telomeres, thus indicating that a duplicated (non-disjoined) chromosome with two chromatids was contained in the micronucleus. This observation leads to the conclusion that micronuclei deriving from missegregation could be due to errors occurring before the onset of anaphase. The results obtained on C6 cells are in good agreement with those obtained on other cell systems, indicating that this cell line can be considered for in vitro aneuploidy evaluation.

Bellosta P, Iwahori A, Plotnikov AN, Eliseenkova AV, Basilico C, Mohammadi M. **Identification of receptor and heparin binding sites in fibroblast growth factor 4 by structure-based mutagenesis.** Mol Cell Biol 2001;21(17):5946-57.
Abstract: Fibroblast growth factors (FGFs) comprise a large family of multifunctional, heparin-binding polypeptides that show diverse patterns of interaction with a family of receptors (FGFR1 to -4) that are subject to alternative splicing. FGFR binding specificity is an essential mechanism in the regulation of FGF signaling and is achieved through primary sequence differences among FGFs and FGFRs and through usage of two alternative exons, IIIc and IIIb, for the second half of immunoglobulin-like domain 3 (D3) in FGFRs. While FGF4 binds and activates the IIIc splice forms of FGFR1 to -3 at comparable levels, it shows little activity towards the IIIb splice forms of FGFR1 to -3 as well as towards FGFR4. To begin to explore the structural determinants for this differential affinity, we determined the crystal structure of FGF4 at a 1.8-A resolution. FGF4 adopts a beta-trefoil fold similar to other FGFs. To identify potential receptor and heparin binding sites in FGF4, a ternary FGF4-FGFR1-heparin model was constructed by superimposing the FGF4 structure onto FGF2 in the FGF2-FGFR1-heparin structure. Mutation of several key residues in FGF4, observed to interact with FGFR1 or with heparin in the model, produced ligands with reduced receptor binding and concomitant low mitogenic potential. Based on the modeling and mutational data, we propose that FGF4, like FGF2, but unlike FGF1, engages the betaC'-betaE loop in D3 and thus can differentiate between the IIIc and IIIb splice isoforms of FGFRs for binding. Moreover, we show that FGF4 needs to interact with both the 2-O- and 6-O-sulfates in heparin to exert its optimal biological activity.

Bemark M, Sale JE, Kim HJ, Berek C, Cosgrove RA, Neuberger MS. **Somatic hypermutation in the absence of DNA-dependent protein kinase catalytic subunit (DNA-PK(cs)) or recombination-**

activating gene (RAG)1 activity. J Exp Med 2000;192(10):1509-14.
Abstract: Somatic hypermutation and isotype switch recombination occur in germinal center B cells, are linked to transcription, and are similarly affected by deficiency in MutS homologue (MSH)2. Class-switch recombination is abrogated by disruption of genes encoding components of the catalytic subunit of DNA-dependent protein kinase (DNA-PK(cs))/Ku complex and likely involves nonhomologous end joining (NHEJ). That somatic hypermutation might also be associated with end joining is suggested by its association with the creation of deletions, duplications, and sites accessible to terminal transferase. However, a requirement for NHEJ in the mutation process has not been demonstrated. Here we show that somatic mutation in mice deficient in NHEJ can be tested by introduction of rearranged immunoglobulin and T cell receptor transgenes: the transgene combination not only permits reconstitution of peripheral lymphoid compartments but also allows formation of germinal centers, despite the wholly monoclonal nature of the lymphocyte antigen receptors in these animals. Using this strategy, we confirm that somatic hypermutation like class-switching can occur in the absence of recombination-activating gene (RAG)1 but show that the two processes differ in that hypermutation can proceed essentially unaffected by deficiency in DNA-PK(cs) activity.

Benitez-Bribiesca L, Sanchez P, Toledo J, Penarroja R, Flores M, Sosa J. **Differential staining of DNA strand breaks in dried comet assay slides.** J Histochem Cytochem 2001;49(7):921-2.
Abstract: The comet assay involves embedding cells in agarose on microscope slides. After lysis and electrophoresis, staining is usually performed with a fluorescent DNA-binding dye and observation is carried out on fresh wet slides through an epifluorescence microscope. We present here a simple alternative for preservation of the agarose comet slides and a fluorescent staining that allows fine differential analysis of DNA strand breaks under confocal microscopy. Lymphocytes were processed according to previous published methods. Slides were quickly dehydrated in a hot oven at 50C for 20 min. Once the agarose layer was dried and reduced to a thin film, slides were treated with RNase. Image analysis showed higher tail length, total area, and tail moment. Using confocal microscopic optical sectioning, a thickness of approximately 180 microm for wet slides and 12 microm for dehydrated gels was calculated. Acridine orange, used for DNA differential staining, allowed quantitation of metachromasia and orthochromasia with confocal scanning microscopy. Differences between alkaline and neutral comet assay with AO were clear-cut and, in principle, a metachromatic index can be calculated. (J Histochem Cytochem 49:921-922, 2001).

Bennett CM, Kanki JP, Rhodes J, Liu TX, Paw BH, Kieran MW, Langenau DM, Delahaye-Brown A, Zon LI, Fleming MD, et al. **Myelopoiesis in the zebrafish, Danio rerio.** Blood 2001;98(3):643-51.
Abstract: Genome-wide chemical mutagenesis screens in the zebrafish (Danio rerio) have led to the identification of novel genes affecting vertebrate erythropoiesis. In determining if this approach could also be used to clarify the molecular genetics of myelopoiesis, it was found that the developmental hierarchy of myeloid precursors in the zebrafish kidney is similar to that in human bone marrow. Zebrafish neutrophils resembled human neutrophils, possessing segmented nuclei and myeloperoxidase-positive cytoplasmic granules. The zebrafish homologue of the human myeloperoxidase (MPO) gene, which is specific to cells of the neutrophil lineage, was cloned and used to synthesize antisense RNA probes for in situ hybridization analyses of zebrafish embryos. Granulocytic cells expressing zebrafish mpo were first evident at 18 hours after fertilization (hpf) in the posterior intermediate cell mass (ICM)

and on the anterior yolk sac by 20 hpf. By 24 hpf, mpo-expressing cells were observed along the ICM and within the developing vascular system. Thus, the mpo gene should provide a useful molecular probe for identifying zebrafish mutants with defects in granulopoiesis. The expression of zebrafish homologues was also examined in 2 other mammalian hematopoietic genes, Pu.1, which appears to initiate a commitment step in normal mammalian myeloid development, and L-Plastin, a gene expressed by human monocytes and macrophages. The results demonstrate a high level of conservation of the spatio-temporal expression patterns of these genes between zebrafish and mammals. The morphologic and molecular genetic evidence presented here supports the zebrafish as an informative model system for the study of normal and aberrant human myelopoiesis. (Blood. 2001;98:643-651).

Berhow MA, Wagner ED, Vaughn SF, Plewa MJ. **Characterization and antimutagenic activity of soybean saponins.** Mutat Res 2000;448(1):11-22.
Abstract: An extract was prepared from a commercial soybean-processing by-product (soybean molasses) and was fractionated into purified chemical components. In previous work, this extract (phytochemical concentrate, PCC) repressed induced genomic DNA damage, whole cell clastogenicity and point mutation in cultured mammalian cells. In the current study, a chemical fraction was isolated from PCC using preparative high-performance liquid chromatography (HPLC). This fraction, PCC100, repressed 2-acetoxyacetylaminofluorene (2AAAF)-induced DNA damage in Chinese hamster ovary (CHO) cells as measured by single cell gel electrophoresis (alkaline Comet assay). Using liquid chromatography-electrospray ionization-mass spectroscopy and 1H and 13C nuclear magnetic resonance (NMR) spectroscopy, PCC100 was shown to consist of a mixture of group B soyasaponins and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) soyasaponins. These include soyasaponins I, II, III, IV, V, Be, betag, betaa, gammag and gammaa. Purified soyasapogenol B aglycone prepared from fraction PCC100 demonstrated significant antigenotoxic activity against 2AAAF. To our knowledge, these data demonstrate for the first time the antimutagenic activity of soybean saponins in mammalian cells.

Bertrand OF, Mongrain R, Thorin E, Lehnert S. **In vitro response of human and porcine vascular cells exposed to high dose-rate gamma-irradiation.** Int J Radiat Biol 2000;76(7):999-1007.
Abstract: AIM: The objective of this study was to compare the in vitro response of human and pig endothelial cells, smooth muscle cells and fibroblasts exposed to conventional high dose-rate gamma-irradiation. MATERIALS AND METHODS: Clonogenic cell survival and growth responses were obtained after irradiation of plateau-phase cells with a 60Co source at a dose-rate of 1.5 Gy/min. DNA single-strand breaks were also evaluated using an alkaline filter elution technique. RESULTS: Overall, both the pig and human cell lines showed a similar response to conventional high dose-rate irradiation. Using clonogenic assays, the human aortic smooth muscle cell line was more sensitive than the fibroblast and endothelial cell lines, whereas the pig endothelial cell line was more sensitive than smooth muscle cells and fibroblasts. Shortly after irradiation (10 days) there was a temporary growth arrest, which was similar for endothelial, smooth muscle cells and fibroblasts with doses above 6 Gy. There was also a non-linear, dose-dependent growth delay up to 4 weeks after irradiation. This effect was also consistent between the different cell lines. Using alkaline filter elution, there was no significant difference in relative elution between endothelial cells, smooth muscle cells and fibroblasts, indicating similar DNA damage among the different cell lines. CONCLUSION: The in vitro response of human

and pig endothelial cells, smooth muscle cells and fibroblasts exposed to high dose-rate irradiation appeared similar. The pig model seems well suited to evaluate the short- and long-term effects of ionizing radiation in the prevention of restenosis after vessel injury.

Bessereau JL, Wright A, Williams DC, Schuske K, Davis MW, Jorgensen EM. **Mobilization of a Drosophila transposon in the Caenorhabditis elegans germ line.** Nature 2001;413(6851):70-4.
Abstract: Transposons have been enormously useful for genetic analysis in both Drosophila and bacteria. Mutagenic insertions constitute molecular tags that are used to rapidly clone the mutated gene. Such techniques would be especially advantageous in the nematode Caenorhabditis elegans, as the entire sequence of the genome has been determined. Several different types of endogenous transposons are present in C. elegans, and these can be mobilized in mutator strains (reviewed in ref. 1). Unfortunately, use of these native transposons for regulated transposition in C. elegans is limited. First, all strains contain multiple copies of these transposons and thus new insertions do not provide unique tags. Second, mutator strains tend to activate the transposition of several classes of transposons, so that the type of transposon associated with a parcticular mutation is not known. Here we demonstrate that the Drosophila mariner element Mos1 can be mobilized in C. elegans. First, efficient mobilization of Mos1 is possible in somatic cells. Second, heritable insertions of the transposon can be generated in the germ line. Third, genes that have been mutated by insertion can be rapidly identified using inverse polymerase chain reaction. Fourth, these insertions can subsequently be remobilized to generate deletion and frameshift mutations by imperfect excision.

Bezlepkin VG, Vasil'eva GV, Lomaeva MG, Sirota NP, Gaziev AI. **[Study of genome instability using DNA fingerprinting of the offspring of male mice subjected to chronic low dose gamma irradiation].** Radiats Biol Radioecol 2000;40(5):506-12 [Rus].
Abstract: By a polymerase chain reaction with an arbitrary primer (AP-PCR), the possibility of transmission of genome instability to somatic cells of the offspring (F1 generation) from male parents of mice exposed to chronic low-level gamma-radiation was studied. Male BALB/c mice 15 days after exposure to 10-50 cGy were mated with unirradiated females. Biopsies were taken from tale tips of two month-old offspring mice and DNA was isolated. The primer in the AP-PCR was a 20-mer oligonucleotide flanking the microsatellite locus Atp1b2 on chromosome 11 of the mouse. A comparative analysis of individual fingerprints of AP-PCR products on DNA-templates from the offspring of irradiated and unirradiated male mice revealed an increased variability of microsatellite-associated sequences in the genome of the offspring of the males exposed to 25 and 50 cGy. The DNA-fingerprints of the offspring of male mice exposed to chronic irradiation with the doses 10 and 25 cGy 15 days before fertilization (at the post-meiotic stage of spermatogenesis) showed an increased frequency of "non-parent bands". The results of the study point to the possibility of transmission to the offspring somatic cells of changes increasing genome instability from male parents exposed to chronic low-level radiation prior to fertilization.

Bhaya D, Takahashi A, Shahi P, Grossman AR. **Novel motility mutants of synechocystis strain PCC 6803 generated by in vitro transposon mutagenesis.** J Bacteriol 2001;183(20):6140-3.
Abstract: We screened for transposon-generated mutants of Synechocystis sp. strain PCC 6803 that exhibited aberrant phototactic movement. Of the 300 mutants generated, about 50 have been partially

characterized; several contained transposons in genes encoding chemotaxis-related proteins, while others mapped to novel genes. These novel genes and their possible roles in motility are discussed.

Bolanos-Jimenez F, Bordais A, Behra M, Strahle U, Mornet D, Sahel J, Rendon A. **Molecular cloning and characterization of dystrophin and Dp71, two products of the Duchenne Muscular Dystrophy gene, in zebrafish.** Gene 2001;274(1-2):217-26.
Abstract: Dystrophin, the protein responsible for Duchenne Muscular Dystrophy (DMD), plays a critical role in the maintenance of the muscle membrane integrity. There are several forms of dystrophin derived from the DMD gene by alternative promoter usage. In addition to full-length dystrophin (Dp427), four shorter transcripts have been identified: Dp260, Dp140, Dp116 and Dp71. The functional role played by the different products of the DMD gene is not yet determined. To get insight into the function of dystrophin and related products, we have investigated the presence of dystrophin in zebrafish. This choice takes advantage of large-scale mutagenesis screens in zebrafish, which have led to the identification of several mutants with motility defects. The identification and characterization of the genes affected by these mutations is likely to provide relevant information for the understanding of the molecular mechanisms of muscle development and function. Two cDNA clones encoding the homologues of dystrophin and Dp71 in zebrafish were identified and characterized. Both transcripts exhibit a high degree of sequence homology with the dystrophin and Dp71 proteins described in higher vertebrates. In addition, three alternative spliced transcripts that occur at the C-terminal end of the zebrafish DMD gene have been identified. These transcripts exhibit different patterns of tissue expression. We have also determined the chromosomal localization of dystrophin on the radiation hybrid map of the zebrafish genome. Our results indicate that the dystrophin gene is localized to linkage group one. Altogether, these results give new insights on the physiological role played by dystrophin and related proteins, and provide new tools for the identification of mutated genes associated with muscle defects in zebrafish.

Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP. **Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases.** Mol Cell 2001;8(2):269-80.
Abstract: Steroid hormones have rapid nongenomic effects on cell-signaling pathways, but the receptor mechanisms responsible for this are not understood. We have identified a specific polyproline motif in the amino-terminal domain of conventional progesterone receptor (PR) that mediates direct progestin-dependent interaction of PR with SH3 domains of various cytoplasmic signaling molecules, including c-Src tyrosine kinases. Through this interaction, PR is a potent activator of Src kinases working by an SH3 domain displacement mechanism. By mutagenesis, we also show that rapid progestin-induced activation of Src and downstream MAP kinase in mammalian cells is dependent on PR-SH3 domain interaction, but not on the transcriptional activity of PR. Preliminary evidence for the biological significance of this PR signaling pathway through regulatory SH3 domains was shown with respect to an influence on progestin-induced growth arrest of breast epithelial cells and induction of Xenopus oocyte maturation.

Bowers BJ. **Applications of transgenic and knockout mice in alcohol research.** Alcohol Res Health 2000;24(3):175-84.
Abstract: Multiple genetic and environmental factors contribute to the development of alcoholism.

Researchers attempting to elucidate the roles of specific genes in alcoholism risk have benefited from advances in genetic engineering. Two important tools used by researchers include transgenic mice, in which a foreign gene is integrated into an animal's genetic material, and knockout/knock-in mice, in which targeted genes either are rendered nonfunctional or are altered. Both of these animal models are currently used in alcohol research to determine how genes may influence the development of alcoholism in humans.

Boyd M, Livingstone A, Wilson LE, Marshall EM, McCluskey AG, Mairs RJ, Wheldon TE. **Dose-response relationship for radiation-induced mutations at micro- and minisatellite loci in human somatic cells in culture.** Int J Radiat Biol 2000;76(2):169-76.
Abstract: PURPOSE: The study was designed to determine the dose-response relationship for radiation induction of mutations at mini- and microsatellite loci in human somatic cells. Mutations induced by graded doses of gamma-irradiation were quantified by screening clones derived from single irradiated cells for micro- and minisatellite alterations following irradiation with 1, 2 or 3 Gy. MATERIALS AND METHODS: After irradiation, the moderately radioresistant glioma cell line UVW was seeded at low density into Petri dishes to allow formation of discrete colonies, 100 of which were examined at each dose. All the cells within a colony were presumed to have arisen from a single irradiated cell. Radiation-induced microsatellite alterations were determined at 16 different loci, by PCR amplification and visualization on polyacrylamide gels. Minisatellite alterations were identified at four different minisatellite loci by restriction enzyme digestion and Southern blotting. RESULTS: A dose-response curve for mutation frequency was obtained by analysis of 100 clones, yielding a minisatellite mutation rate of 5.5x10(-3) mutations/locus/Gy/cell and a microsatellite mutation rate of 8.75x10(-4) mutations/ locus/ Gy/cell. At microsatellite loci, alterations were predominantly simple loss or gain of repeat units and loss of heterozygosity (LOH). The mutations in minisatellite loci resulted predominantly in LOH and variation in repeat number. The background instability at each locus was determined by analysis of non-irradiated clones. Only 2% and 1% of the micro-and minisatellite loci respectively showed altered bands. CONCLUSIONS: This is the first report of a dose-response relationship for radiation-induced micro- and minisatellite mutations in human somatic cells. Described is a sensitive method for analysis of low-dose radiation mutagenesis in somatic cells that may prove to be a useful tool for radiation protection and dosimetry.

Boyd Y, Blair HJ, Cunliffe P, Masson WK, Reed V. **A phenotype map of the mouse X chromosome: models for human X-linked disease.** Genome Res 2000;10(3):277-92.
Abstract: The identification of many of the transcribed genes in man and mouse is being achieved by large scale sequencing of expressed sequence tags (ESTs). Attention is now being turned to elucidating gene function and many laboratories are looking to the mouse as a model system for this phase of the genome project. Mouse mutants have long been used as a means of investigating gene function and disease pathogenesis, and recently, several large mutagenesis programs have been initiated to fulfill the burgeoning demand of functional genomics research. Nevertheless, there is a substantial existing mouse mutant resource that can be used immediately. This review summarizes the available information about the loci encoding X-linked phenotypic mutants and variants, including 40 classical mutants and 40 that have arisen from gene targeting.

Branza-Nichita N, Negroiu G, Petrescu AJ, Garman EF, Platt FM, Wormald MR, Dwek RA, Petrescu SM. **Mutations at critical N-glycosylation sites reduce tyrosinase activity by altering folding and quality control.** J Biol Chem 2000;275(11):8169-75.
Abstract: Tyrosinase is a copper-containing enzyme that regulates melanin biosynthesis in mammals. Mutations at a single N-glycosylation sequon of tyrosinase have been reported to be responsible for oculocutaneous albinism type IA in humans, characterized by inactive tyrosinase and the total absence of pigmentation. To probe the role that each N-glycosylation site plays in the synthesis of biologically active tyrosinase, we analyzed the calnexin mediated folding of tyrosinase N-glycosylation mutants. We have determined that four of the six potential N-glycosylation sites, including that associated with albinism, are occupied. Analysis of the folding pathway and activity of 15 tyrosinase mutants lacking one or more of the occupied N-glycosylation sites shows that glycans at any two N-glycosylation sites are sufficient to interact with calnexin and give partial activity, but a specific pair of sites (Asn(86) and Asn(371)) is required for full activity. The mutants with less than two N-glycosylation sites do not interact with calnexin and show a complete absence of enzyme activity. Copper analysis of selected mutants suggests that the observed partial activity is due to two populations with differential copper content. By correlating the degree of folding with the activity of tyrosinase, we propose a local folding mechanism for tyrosinase that can explain the mechanism of inactivation of tyrosinase N-glycosylation mutants found in certain pigmentation disorders.

Brennan RJ, Schiestl RH. **Persistent genomic instability in the yeast Saccharomyces cerevisiae induced by ionizing radiation and DNA-damaging agents.** Radiat Res 2001;155(6):768-77.
Abstract: A "hypermutable" genome is a common characteristic of cancer cells, and it may contribute to the progressive accumulation of mutations required for the development of cancer. It has been reported that mammalian cells surviving exposure to gamma radiation display several highly persistent genomic instability phenotypes which may reflect a hypermutability similar to that seen in cancer. These phenotypes include an increased mutation frequency and a decreased plating efficiency, and they continue to be observed many generations after the radiation exposure. The underlying causes of this genomic instability have not been fully determined. We show here that exposure to gamma radiation and other DNA-damaging treatments induces a similar genomic instability in the yeast Saccharomyces cerevisiae. A dose-dependent increase in intrachromosomal recombination was observed in cultures derived from cells surviving gamma irradiation as many as 50 generations after the exposure. Increased forward mutation frequencies and low colony-forming efficiencies were also observed. Persistently elevated recombination frequencies in haploid cells were dominant after these cells were mated to nonirradiated partners, and the elevated recombination phenotype was also observed after treatment with the DNA-damaging agents ultraviolet light, hydrogen peroxide, and ethyl methanesulfonate. Radiation-induced genomic instability in yeast may represent a convenient model for the hypermutability observed in cancer cells.

Broder S, Venter JC. **Sequencing the entire genomes of free-living organisms: the foundation of pharmacology in the new millennium.** Annu Rev Pharmacol Toxicol 2000;40:97-132.
Abstract: The power and effectiveness of clinical pharmacology are about to be transformed with a speed that earlier in this decade could not have been foreseen even by the most astute visionaries. In the very near future, we will have at our disposal the reference DNA sequence for the entire human genome,

estimated to contain approximately 3.5 billion bp. At the same time, the science of whole genome sequencing is fostering the computational science of bioinformatics needed to develop practical applications for pharmacology and toxicology. Indeed, it is likely that pharmacology, toxicology, bioinformatics, and genomics will merge into a new branch of medical science for studying and developing pharmaceuticals from molecule to bedside.

Brodsky MH, Sekelsky JJ, Tsang G, Hawley RS, Rubin GM. **mus304 encodes a novel DNA damage checkpoint protein required during Drosophila development.** Genes Dev 2000;14(6):666-78.
Abstract: Checkpoints block cell cycle progression in eukaryotic cells exposed to DNA damaging agents. We show that several Drosophila homologs of checkpoint genes, mei-41, grapes, and 14-3-3epsilon, regulate a DNA damage checkpoint in the developing eye. We have used this assay to show that the mutagen-sensitive gene mus304 is also required for this checkpoint. mus304 encodes a novel coiled-coil domain protein, which is targeted to the cytoplasm. Similar to mei-41, mus304 is required for chromosome break repair and for genomic stability. mus304 animals also exhibit three developmental defects, abnormal bristle morphology, decreased meiotic recombination, and arrested embryonic development. We suggest that these phenotypes reflect distinct developmental consequences of a single underlying checkpoint defect. Similar mechanisms may account for the puzzling array of symptoms observed in humans with mutations in the ATM tumor suppressor gene.

Brodt P, Fallavollita L, Khatib AM, Samani AA, Zhang D. **Cooperative regulation of the invasive and metastatic phenotypes by different domains of the type I insulin-like growth factor receptor beta subunit.** J Biol Chem 2001;276(36):33608-15.
Abstract: The receptor for the type 1 insulin-like growth factor (IGF-I) regulates multiple cellular functions impacting on the metastatic phenotype of tumor cells, including cellular proliferation, anchorage-independent growth, survival, migration, synthesis of the 72-kDa type IV collagenase and invasion. We have used site-directed mutagenesis to generate domain-specific mutants of the receptor beta subunit to analyze the role of specific tyrosines in the regulation of the invasive/metastatic phenotype. Poorly invasive M-27 carcinoma cells expressing low receptor numbers were transfected with a plasmid vector expressing IGF-I receptor cDNA in which single or multiple tyrosine codons in the kinase domain, namely Tyr-1131, Tyr-1135, and Tyr-1136 or the C-terminal tyrosines 1250 and 1251 were substituted with phenylalanine. Changes in the invasive and metastatic properties were analyzed relative to M-27 cells expressing the wild type receptor. We found that cells expressing the Y1131F,Y1135F,Y1136F or Y1135F receptor mutants lost all IGF-IR-dependent functions and their phenotypes were indistinguishable from, or suppressed relative to, the parent line. The Y1250F,Y1251F substitution abolished anchorage-independent growth, cell spreading, and the anti-apoptotic effect of IGF-I whereas all other IGF-IR-dependent phenotypes were either unperturbed (i.e. mitogenicity) or only partially reduced (migration and invasion). The results identify three types of receptor-dependent functions in this model: those dependent only on an intact kinase domain (DNA synthesis), those dependent equally on kinase domain and Tyr-1250/1251 signaling (e.g. apoptosis, soft agar cloning) and those dependent on kinase domain and enhanced through Tyr-1250/1251 signaling (migration, invasion). They suggest that signals derived from both regions of the receptor cooperate to enhance tumor metastasis.

Broomfield S, Hryciw T, Xiao W. **DNA postreplication repair and mutagenesis in Saccharomyces cerevisiae.** Mutat Res 2001;486(3):167-84.
Abstract: DNA postreplication repair (PRR) is defined as an activity to convert DNA damage-induced single-stranded gaps into large molecular weight DNA without actually removing the replication-blocking lesions. In bacteria such as Escherichia coli, this activity requires RecA and the RecA-mediated SOS response and is accomplished by recombination and mutagenic translesion DNA synthesis. Eukaryotic cells appear to share similar DNA damage tolerance pathways; however, some enzymes required for PRR in eukaryotes are rather different from those of prokaryotes. In the yeast Saccharomyces cerevisiae, PRR is centrally controlled by RAD6 and RAD18, whose products form a stable complex with single-stranded DNA-binding, ATPase and ubiquitin-conjugating activities. PRR can be further divided into translesion DNA synthesis and error-free modes, the exact molecular events of which are largely unknown. This error-free PRR is analogous to DNA damage-avoidance as defined in mammalian cells, which relies on recombination processes. Two possible mechanisms by which recombination parcticipate in PRR to resolve the stalled replication folk are discussed. Recombination and PRR are also genetically regulated by a DNA helicase and are coupled to the cell-cycle. The PRR processes appear to be highly conserved within eukaryotes, from yeast to human.

Brown CD, Barnes K, Turner AJ. **Functional significance of the isoforms of endothelin-converting enzyme-1.** J Cardiovasc Pharmacol 2000;36(5 Suppl 1):S26-7.
Abstract: The subcellular localization of endothelin-converting enzyme-1 (ECE-1) is a matter of some controversy, further complicated by the discovery of its multiple isoforms. ECE-1 is a critical enzyme in the biosynthesis of the potent vasoconstrictor peptide endothelin (ET), and, as such, represents a potential target for drug therapy in the control of disease states involving the ET system. Knowledge of the precise locations of the isoforms and their regulation would aid in the design of drugs to target specifically ECE-1. In this study, the subcellular localization and potential targeting pathways of the ECE-1 isoforms were investigated. Antipeptide antibodies were raised to the unique N-terminal sequence of ECE-1b and were then used in the investigation of its subcellular distribution. Mutagenesis of proposed targeting sequences within the cytoplasmic tails of the isoforms was carried out to determine their significance in subcellular localization.

Brown CW, Houston-Hawkins DE, Woodruff TK, Matzuk MM. **Insertion of Inhbb into the Inhba locus rescues the Inhba-null phenotype and reveals new activin functions.** Nat Genet 2000;25 (4):453-7.
Abstract: The activins (dimers of betaA or betaB subunits, encoded by the genes Inhba and Inhbb, respectively) are TGF-beta superfamily members that have roles in reproduction and development. Whereas mice homozygous for the Inhba-null allele demonstrate disruption of whisker, palate and tooth development, leading to neonatal lethality, homozygous Inhbb-null mice are viable, fertile and have eye defects. To determine if these phenotypes were due to spatiotemporal expression differences of the ligands or disruption of specific ligand-receptor interactions, we replaced the region of Inhba encoding the mature protein with Inhbb, creating the allele Inhbatm2Zuk (hereafter designated InhbaBK). Although the craniofacial phenotypes of the Inhba-null mutation were rescued by the InhbaBK allele, somatic, testicular, genital and hair growth were grossly affected and influenced by the dosage and bioactivity of the allele. Thus, functional compensation within the TGF-beta superfamily can occur if the

replacement gene is expressed appropriately. The novel phenotypes in these mice further illustrate the usefulness of insertion strategies for defining protein function.

Brown RS, Akhtar P, Akerman J, Hampel L, Kozin IS, Villerius LA, Klamer HJ. **Partition controlled delivery of hydrophobic substances in toxicity tests using poly(dimethylsiloxane) (PDMS) films.** Environ Sci Technol 2001;35(20):4097-102.
Abstract: Interpretation of toxicity test results may be hampered when doubt exists about the actual exposure concentration. Processes that are responsible for differences between the nominal and the actual concentration in aqueous test systems may include sorption, precipitation, volatilization, chemical and biological degradation, and uptake into biological or test tissue. In this study, the use of a poly (dimethylsiloxane) (PDMS) film containing the test compound is introduced as a versatile technique for partition controlled delivery of hydrophobic compounds to aqueous toxicity tests. Two methods developed produced preloaded films, having toxicant added to the PDMS prepolymer solution before film deposition and curing, and postloaded films, which are created by the addition of toxicant in a solvent to an already-polymerized PDMS film. Preloaded films were generally more easily prepared, may better accommodate larger molecules, and have a higher capacity than postloaded films. Postloaded films provided film-solution partition coefficients with higher precision and allowed for the use of films from stock and thus for a more portable technique. Chemical analysis showed that equilibrium between films and the aqueous solution was established within 7-10 min and was maintained for a suite of aromatic compounds (log Kow ranging from 2.8 to 6.1). The reliability of the film technique was demonstrated by application to the Microtox bacterial toxicity tests of solutions of polycyclic aromatic hydrocarbons (PAHs).

Bruschi GC, de Souza CC, Fagundes MR, Dani MA, Goldman MH, Morris NR, Liu L, Goldman GH. **Sensitivity to camptothecin in Aspergillus nidulans identifies a novel gene, scaA+, related to the cellular DNA damage response.** Mol Genet Genomics 2001;265(2):264-75.
Abstract: The anti-cancer drug camptothecin targets eukaryotic DNA topoisomerase I by trapping the covalent complex formed between the catalytically active enzyme and DNA. We are interested in identifying factors, other than topoisomerase I, that are involved in mediating cellular sensitivity to camptothecin. To this end, we have isolated eighteen mutants that are sensitive to camptothecin (sca) in the filamentous fungus Aspergillus nidulans and characterised one of them, sca299. The mutant sca299 is hypersensitive to camptothecin, and sensitive to several different mutagenic agents and to actinomycin D. Using temperature-sensitive mutations in genes that are known to regulate the cell cycle, we showed that the camptothecin sensitivity of the mutant sca299 is not affected by a mitotic block. The abnormal nuclear morphology observed in the sca299 mutant strain suggests that the germlings might be undergoing mitosis in the presence of unrepaired DNA damage, which would result in mitotic catastrophe. The hypersensitivity of the sca299 mutant to camptothecin does not result from elevated levels of topoisomerase I mRNA or from alterations in enzyme activity. Using DNA-mediated complementation of the sca299 mutant phenotype, the scaA+ gene was cloned. This gene encodes a 594-amino acid product; moderate structural similarity suggests that the scaA gene product may be related to the human nibrin gene which encodes a product involved in DNA double-strand break repair. Strains disrupted in the scaA gene were sensitive to the anti-topoisomerase I agent berberine, the DNA crosslinking agents mitomycin C and cis-platinum, and also to t-butyl hydroperoxide, which is an

inducer of oxidative stress.

Brylawski BP, Cohen SM, Longmire JL, Doggett NA, Cordeiro-Stone M, Kaufman DG. **Construction of a cosmid library of DNA replicated early in the S phase of normal human fibroblasts.** J Cell Biochem 2000;78(3):509-17.
Abstract: We constructed a subgenomic cosmid library of DNA replicated early in the S phase of normal human diploid fibroblasts. Cells were synchronized by release from confluence arrest and incubation in the presence of aphidicolin. Bromodeoxyuridine (BrdUrd) was added to aphidicolin-containing medium to label DNA replicated as cells entered S phase. Nuclear DNA was partially digested with Sau 3AI, and hybrid density DNA was separated in CsCl gradients. The purified early-replicating DNA was cloned into sCos1 cosmid vector. Clones were transferred individually into the wells of 96 microtiter plates (9,216 potential clones). Vigorous bacterial growth was detected in 8,742 of those wells. High-density colony hybridization filters (1, 536 clones/filter) were prepared from a set of replicas of the original plates. Bacteria remaining in the wells of replica plates were combined, mixed with freezing medium, and stored at -80 degrees C. These pooled stocks were analyzed by polymerase chain reaction to determine the presence of specific sequences in the library. Hybridization of high-density filters was used to identify the clones of interest, which were retrieved from the frozen cultures in the 96-well plates. In testing the library for the presence of 14 known early-replicating genes, we found sequences at or near 5 of them: APRT, beta-actin, beta-tubulin, c-myc, and HPRT. This library is a valuable resource for the isolation and analysis of certain DNA sequences replicated at the beginning of S phase, including potential origins of bidirectional replication.

Burczynski ME, McMillian M, Ciervo J, Li L, Parker JB, Dunn RT 2nd, Hicken S, Farr S, Johnson MD. **Toxicogenomics-based discrimination of toxic mechanism in HepG2 human hepatoma cells.** Toxicol Sci 2000;58(2):399-415.
Abstract: The rapid discovery of sequence information from the Human Genome Project has exponentially increased the amount of data that can be retrieved from biomedical experiments. Gene expression profiling, through the use of microarray technology, is rapidly contributing to an improved understanding of global, coordinated cellular events in a variety of paradigms. In the field of toxicology, the potential application of toxicogenomics to indicate the toxicity of unknown compounds has been suggested but remains largely unsubstantiated to date. A major supposition of toxicogenomics is that global changes in the expression of individual mRNAs (i.e., the transcriptional responses of cells to toxicants) will be sufficiently distinct, robust, and reproducible to allow discrimination of toxicants from different classes. Definitive demonstration is still lacking for such specific "genetic fingerprints," as opposed to nonspecific general stress responses that may be indistinguishable between compounds and therefore not suitable as probes of toxic mechanisms. The present studies demonstrate a general application of toxicogenomics that distinguishes two mechanistically unrelated classes of toxicants (cytotoxic anti-inflammatory drugs and DNA-damaging agents) based solely upon a cluster-type analysis of genes differentially induced or repressed in cultured cells during exposure to these compounds. Initial comparisons of the expression patterns for 100 toxic compounds, using all approximately 250 genes on a DNA microarray (approximately 2.5 million data points), failed to discriminate between toxicant classes. A major obstacle encountered in these studies was the lack of reproducible gene responses, presumably due to biological variability and technological limitations.

Thus multiple replicate observations for the prototypical DNA damaging agent, cisplatin, and the non-steroidal anti-inflammatory drugs (NSAIDs) diflunisal and flufenamic acid were made, and a subset of genes yielding reproducible inductions/repressions was selected for comparison. Many of the "fingerprint genes" identified in these studies were consistent with previous observations reported in the literature (e. g., the well-characterized induction by cisplatin of p53-regulated transcripts such as p21 (waf1/cip1) and PCNA [proliferating cell nuclear antigen]). These gene subsets not only discriminated among the three compounds in the learning set but also showed predictive value for the rest of the database (approximately 100 compounds of various toxic mechanisms). Further refinement of the clustering strategy, using a computer-based optimization algorithm, yielded even better results and demonstrated that genes that ultimately best discriminated between DNA damage and NSAIDs were involved in such diverse processes as DNA repair, xenobiotic metabolism, transcriptional activation, structural maintenance, cell cycle control, signal transduction, and apoptosis. The determination of genes whose responses appropriately group and dissociate anti-inflammatory versus DNA-damaging agents provides an initial paradigm upon which to build for future, higher throughput-based identification of toxic compounds using gene expression patterns alone.

Burger H, Capello A, Schenk PW, Stoter G, Brouwer J, Nooter K. **A genome-wide screening in Saccharomyces cerevisiae for genes that confer resistance to the anticancer agent cisplatin.** Biochem Biophys Res Commun 2000;269(3):767-74.
Abstract: Cisplatin is a potent DNA-damaging agent that has demonstrated anticancer activities against several tumors. However, manifestation of cellular resistance is a major obstacle in anticancer therapy that severely limits the curative potential of cisplatin. Therefore, understanding the molecular basis of cisplatin resistance could significantly improve the clinical efficacy of this anticancer agent. Here, we employed Saccharomyces cerevisiae as a model organism to study cisplatin resistance mechanisms and describe a one-step cisplatin selection to identify and characterize novel cisplatin resistance genes. Screening a multicopy yeast genomic library enabled us to isolate several yeast clones for which we could confirm that the cisplatin resistance phenotype was linked to the introduced fragment. In a first attempt, a number of open reading frames could be identified. Among these genes, PDE2 and ZDS2 were repeatedly identified as genes whose overexpression confers cellular resistance to cisplatin. PDE2, encoding cAMP-phosphodiesterase 2, is of parcticular interest because the overexpression of this yeast gene is known to induce cisplatin resistance in mammalian cells as well, providing proof of the principle of our experimental approach. In addition, the identification of PDE2 shows that our yeast screening system can directly be informative for drug resistance in mammalian cells.

Burgess S, Hopkins N. **Use of pseudotyped retroviruses in zebrafish as genetic tags.** Methods Enzymol 2000;327:145-61.

Burkey JL, Sauer JM, McQueen CA, Sipes IG. **Cytotoxicity and genotoxicity of methyleugenol and related congeners-- a mechanism of activation for methyleugenol.** Mutat Res 2000;453(1):25-33.
Abstract: Methyleugenol is a substituted alkenylbenzene found in a variety of foods, products, and essential oils. In a 2-year bioassay conducted by the National Toxicology Program, methyleugenol caused neoplastic lesions in the livers of Fischer 344 rats and B6C3F(1) mice. We were interested in the cytotoxicity and genotoxicity caused by methyleugenol and other alkenylbenzene compounds: safrole (a

known hepatocarcinogen), eugenol, and isoeugenol. The endpoints were evaluated in cultured primary hepatocytes isolated from male Fischer 344 rats and female B6C3F(1) mice. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release, while genotoxicity was determined by using the unscheduled DNA synthesis (UDS) assay. Rat and mouse hepatocytes showed similar patterns of toxicity for each chemical tested. Methyleugenol and safrole were relatively non-cytotoxic, but caused UDS at concentrations between 10 and 500 microM. In contrast, isoeugenol and eugenol produced cytotoxicity in hepatocytes with LC50s of approximately 200-300 microM, but did not cause UDS. Concurrent incubation of 2000 microM cyclohexane oxide (CHO), an epoxide hydrolase competitor, with a non-cytotoxic concentration of methyleugenol (10 microM) resulted in increased cytotoxicity but had no effect on genotoxicity. However, incubation of 15 microM pentacholorophenol, a sulfotransferase inhibitor, with 10 uM methyleugenol resulted in increased cytotoxicity but had a significant reduction of genotoxicity. These results suggest that methyleugenol is similar to safrole in its ability to cause cytotoxicity and genotoxicity in rodents. It appears that the bioactivation of methyleugenol to a DNA reactive electrophile is mediated by a sulfotransferase in rodents, but epoxide formation is not responsible for the observed genotoxicity.

Burkhart JG. **Fishing for mutations.** Nat Biotechnol 2000;18(1):21-2.

Burnouf DY, Fuchs RP. **The early detection of frameshift mutations induced by a food-borne carcinogen in rats: a new tool for molecular epidemiology.** Mutat Res 2000;462(2-3):281-91.
Abstract: The accumulation of genetic changes is considered as the main factor that determines the development of cancer. Recent progresses in genetics and molecular biology led to the discovery of many new molecular markers and to the development of techniques able to monitor these markers. As a consequence, molecular epidemiology has emerged as a powerful approach to study the ternary relationship between the environment, the behaviour and the genetic predisposition of each individual. Susceptibility to cancer is determined at different levels such as the genetic polymorphism of enzymes involved in the activation and detoxification of carcinogens, the polymorphism of genes that maintains the genome stability, like those involved in DNA repair or recombination processes, and finally the polymorphism in oncogenes or tumour suppressor genes. Consequently, the full assessment of each individual's genetic predisposition is a long and difficult task. As the accumulation of mutations in somatic cells integrates all these parameters, its measurement would facilitate the evaluation of the individual predisposition status, provided that a marker common to a large spectrum of carcinogens could be found. Our current studies on the molecular mechanisms of carcinogen-induced mutagenesis has revealed that G-rich repetitive sequences are mutational hot spots for several major classes of environmental genotoxins such as aromatic and heterocyclic amines, polycyclic hydrocarbons and oxidative agents. We thus consider the possibility that these sequences form a new class of biomarkers for carcinogen exposure. In order to validate this hypothesis, we designed a sensitive PCR-based assay able to detect specific mutations induced by a common food-borne carcinogen in the colon epithelium of rats exposed for a short period to this carcinogen. This assay is sensitive enough to allow early detection of induced mutations and therefore allows to differentiate between unexposed animal and those exposed for a period as short as 1 week.

Burz DS, Hanes SD. **Isolation of mutations that disrupt cooperative DNA binding by the**

Drosophila bicoid protein. J Mol Biol 2001;305(2):219-30.
Abstract: Cooperative DNA binding is thought to contribute to the ability of the Drosophila melanogaster protein, Bicoid, to stimulate transcription of target genes in precise sub-domains within the embryo. As a first step toward testing this idea, we devised a genetic screen to isolate mutations in Bicoid that specifically disrupt cooperative interactions, but do not disrupt DNA recognition or transcription activation. The screen was carried out in Saccharomyces cerevisiae and 12 cooperativity mutants were identified. The mutations map across most of the Bicoid protein, with some located within the DNA-binding domain (homeodomain). Four homeodomain mutants were characterized in yeast and shown to activate a single-site reporter gene to levels comparable to that of wild-type, indicating that DNA binding per se is not affected. However, these mutants failed to show cooperative coupling between high and low-affinity sites, and showed reduced activation of a reporter gene carrying a natural Drosophila enhancer. Homology modeling indicated that none of the four mutations is in residues that contact DNA. Instead, these residues are likely to interact with other DNA-bound Bicoid monomers or other parts of the Bicoid protein. In vitro, the isolated homeodomains did not show strong cooperativity defects, supporting the idea that other regions of Bicoid are also important for cooperativity. This study describes the first systematic screen to identify cooperativity mutations in a eukaryotic DNA-binding protein.

Buscemi G, Saracino F, Masnada D, Carbone ML. **The Saccharomyces cerevisiae SDA1 gene is required for actin cytoskeleton organization and cell cycle progression.** J Cell Sci 2000;113 (Pt 7):1199-211.
Abstract: The organization of the actin cytoskeleton is essential for several cellular processes. Here we report the characterization of a Saccharomyces cerevisiae novel gene, SDA1, encoding a highly conserved protein, which is essential for cell viability and is localized in the nucleus. Depletion or inactivation of Sda1 cause cell cycle arrest in G(1) by blocking both budding and DNA replication, without loss of viability. Furthermore, sda1-1 temperature-sensitive mutant cells arrest at the non-permissive temperature mostly without detectable structures of polymerized actin, although a normal actin protein level is maintained, indicating that Sda1 is required for proper organization of the actin cytoskeleton. To our knowledge, this is the first mutation shown to cause such a phenotype. Recovery of Sda1 activity restores proper assembly of actin structures, as well as budding and DNA replication. Furthermore we show that direct actin perturbation, either in sda1-1 or in cdc28-13 cells released from G (1) block, prevents recovery of budding and DNA replication. We also show that the block in G(1) caused by loss of Sda1 function is independent of Swe1. Altogether our results suggest that disruption of F-actin structure can block cell cycle progression in G(1) and that Sda1 is involved in the control of the actin cytoskeleton.

Busch E, Hohenester E, Timpl R, Paulsson M, Maurer P. **Calcium affinity, cooperativity, and domain interactions of extracellular EF-hands present in BM-40.** J Biol Chem 2000;275(33):25508-15.
Abstract: The structure and function of cytosolic Ca(2+)-binding proteins containing EF-hands are well understood. Recently, the presence of EF-hands in an extracellular protein was for the first time proven by the structure determination of the EC domain of BM-40 (SPARC (for secreted protein acidic and rich in cysteine)/osteonectin) (Hohenester, E., Maurer, P., Hohenadl, C., Timpl, R., Jansonius, J. N., and Engel, J. (1996) Nat. Struct. Biol. 3, 67-73). The structure revealed a pair of EF-hands with two bound

Ca(2+) ions. Two unusual features were noted that distinguish the extracellular EF-hands of BM-40 from their cytosolic counterparts. An insertion of one amino acid into the loop of the first EF-hand causes a variant Ca(2+) coordination, and a disulfide bond connects the helices of the second EF-hand. Here we show that the extracellular EF-hands in the BM-40 EC domain bind Ca(2+) cooperatively and with high affinity. The EC domain is thus in the Ca(2+)-saturated form in the extracellular matrix, and the EF-hands play a structural rather than a regulatory role. Deletion mutants demonstrate a strong interaction between the EC domain and the neighboring FS domain, which contributes about 10 kJ/mol to the free energy of binding and influences cooperativity. This interaction is mainly between the FS domain and the variant EF-hand 1. Certain mutations of Ca(2+)-coordinating residues changed affinity and cooperativity, but others inhibited folding and secretion of the EC domain in a mammalian cell line. This points to a function of EF-hands in extracellular proteins during biosynthesis and processing in the endoplasmic reticulum or Golgi apparatus.

Buschini A, Cassoni F, Anceschi E, Pasini L, Poli P, Rossi C. **Urban airborne parcticulate: genotoxicity evaluation of different size fractions by mutagenesis tests on microorganisms and comet assay.** Chemosphere 2001;44(8):1723-36.
Abstract: The genotoxic effects of different size fractions of airborne parcticulate (Total, PM10 and PM25), extracted with acetone or toluene, were evaluated by: the Ames plate test (TA98 and TA100 strains, w/o S9), gene conversion and reversion (w/o endogenous metabolic activation) in the Saccharomyces cerevisiae D7 strain, and the comet assay on human leukocytes. The data on human leukocytes confirm the sensitivity of the comet assay and its applicability to assess genotoxicity in environmental samples. The PM2.5 fraction of airborne parcticulate generally shows the highest concentration of DNA-damaging compounds. Genotoxic response, in all the test systems applied, is highly dependent on extraction solvent used. Acetone seems to extract compounds with more similar genotoxic responses in the three test systems used than toluene extracts. Toluene appears to extract air pollutants genotoxic on yeast and leukocytes but is mainly cytotoxic on Salmonella.

Cabrera G. **Effect of five dietary antimutagens on the genotoxicity of six mutagens in the microscreen prophage-induction assay.** Environ Mol Mutagen 2000;36(3):206-20.
Abstract: Dietary antimutagens have been studied extensively in the last two decades, using mainly bacterial and mammalian cells. These studies have shown that certain dietary antimutagens, acting individually or as mixtures, are useful in counteracting the effects of certain mutagens and/or carcinogens to which humans are commonly exposed. However, there are some inconsistencies among publications using different bioassays. The general purpose of the research presented here was to conduct a comparative study of the antigenotoxic activity of five dietary antimutagens against six mutagens, using three rather different short-term tests: the Microscreen prophage-induction assay, the Tradescantia micronucleus test, and the Salmonella/mammalian microsome test. In this study I report the results with the Microscreen prophage-induction assay. The antimutagens selected were chlorophyllin, beta-carotene, and vitamins A, C, and E. The mutagens selected were 2-aminoanthracene, benzo[a] pyrene, 2-nitrofluorene, toxaphene, dichlorvos, and nitrofen. The results show that chlorophyllin and beta-carotene inhibited the genotoxicity of all six mutagens; vitamin E inhibited all except dichlorvos; and vitamins C and A inhibited 2-aminoanthracene, benzo[a]pyrene, 2-nitrofluorene, and nitrofen.

Cain SA, Williams DM, Harris V, Monk PN. **Selection of novel ligands from a whole-molecule randomly mutated C5a library.** Protein Eng 2001;14(3):189-93.
Abstract: Novel antagonists of the proinflammatory leukocyte chemoattractant C5a have been produced from a phage display library of whole-molecule random mutants. The cDNA for the inflammatory polypeptide C5adR(74) was used as template in a PCR reaction doped with the mutagenic nucleoside triphosphates dPTP [dP: 6-(2-deoxy-beta-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazin-7-one] and 8-oxodGTP (8-oxodG: 8-oxo-2'-deoxyguanosine) to allow the introduction of mutations in a highly controlled manner throughout the cDNA. The resultant library of mutants was displayed on bacteriophage M13 using a jun/fos linker sequence. Functional polypeptides were isolated by several rounds of selection against the receptor for C5a expressed on the surface of CHO cells. From this selection procedure, a limited number of variants of C5adR(74) were obtained. When expressed as free polypeptide, the binding affinities of the selected C5adR(74) sequences were increased 5-fold relative to wild-type protein. Site-directed mutagenesis of the C-terminus of these variants resulted in the production of antagonists of C5adR(74) activity.

Call DR, Brockman FJ, Chandler DP. **Detecting and genotyping Escherichia coli O157:H7 using multiplexed PCR and nucleic acid microarrays.** Int J Food Microbiol 2001;67(1-2):71-80.
Abstract: Rapid detection and characterization of food borne pathogens such as Escherichia coli O157:H7 is crucial for epidemiological investigations and food safety surveillance. As an alternative to conventional technologies, we examined the sensitivity and specificity of nucleic acid microarrays for detecting and genotyping E. coli O157:H7. The array was composed of oligonucleotide probes (25-30 mer) complementary to four virulence loci (intimin, Shiga-like toxins I and II, and hemolysin A). Target DNA was amplified from whole cells or from purified DNA via single or multiplexed polymerase chain reaction (PCR), and PCR products were hybridized to the array without further modification or purification. The array was 32-fold more sensitive than gel electrophoresis and capable of detecting amplification products from < 1 cell equivalent of genomic DNA (1 fg). Immunomagnetic capture, PCR and a microarray were subsequently used to detect 55 CFU ml(-1) (E. coli O157:H7) from chicken rinsate without the aid of pre-enrichment. Four isolates of E. coli O157:H7 and one isolate of O91:H2, for which genotypic data were available, were unambiguously genotyped with this array. Glass-based microarrays are relatively simple to construct and provide a rapid and sensitive means to detect multiplexed PCR products; the system is amenable to automation.

Cao H, Thompson HM, Krueger EW, McNiven MA. **Disruption of Golgi structure and function in mammalian cells expressing a mutant dynamin**. J Cell Sci 2000;113 (Pt 11):1993-2002.
Abstract: The large GTPase dynamin is a mechanoenzyme that parcticipates in the scission of nascent vesicles from the plasma membrane. Recently, dynamin has been demonstrated to associate with the Golgi apparatus in mammalian cells by morphological and biochemical methods. Additional studies using a well characterized, cell-free assay have supported these findings by demonstrating a requirement for dynamin function in the formation of clathrin-coated, and non-clathrin-coated vesicles from the trans-Golgi network (TGN). In this study, we tested if dynamin parcticipates in Golgi function in living cells through the expression of a dominant negative dynamin construct (K44A). Cells co-transfected to express this mutant dynamin and a GFP-tagged Golgi resident protein (TGN38) exhibit Golgi structures that are either compacted, vesiculated, or tubulated. Electron microscopy of these mutant cells revealed

large numbers of Golgi stacks comprised of highly tubulated cisternae and an extraordinary number of coated vesicle buds. Cells expressing mutant dynamin and GFP-tagged VSVG demonstrated a marked retention (8- to 11-fold) of the nascent viral G-protein in the Golgi compared to control cells. These observations in living cells are consistent with previous morphological and in vitro studies demonstrating a role for dynamin in the formation of secretory vesicles from the TGN.

Carabeo RA, Hackstadt T. **Isolation and characterization of a mutant Chinese hamster ovary cell line that is resistant to Chlamydia trachomatis infection at a novel step in the attachment process.** Infect Immun 2001;69(9):5899-904.
Abstract: Host factors involved in Chlamydia trachomatis pathogenesis were investigated by random chemical mutagenesis of Chinese hamster ovary (CHO-K1) cells followed by selection for clones resistant to chlamydial infection. A clonal mutant cell line, D4.1-3, refractory to infection by the C. trachomatis L2 serovar was isolated. The D4.1-3 cell line appears to be lacking in a previously undescribed temperature-dependent and heparin-resistant binding step that occurs subsequent to engagement of cell surface heparan sulfate by L2 elementary bodies. This novel binding step differentiates the lymphogranuloma venereum (LGV) serovar from other serovars and may contribute the different pathologies associated with LGV and non-LGV strains.

Chaubey RC, Bhilwade HN, Rajagopalan R, Bannur SV. **Gamma ray induced DNA damage in human and mouse leucocytes measured by SCGE-Pro: a software developed for automated image analysis and data processing for Comet assay.** Mutat Res 2001;490(2):187-97.
Abstract: The studies reported in this communication had two major objectives: first to validate the in-house developed SCGE-Pro: a software developed for automated image analysis and data processing for Comet assay using human peripheral blood leucocytes exposed to radiation doses, viz. 2, 4 and 8 Gy, which are known to produce DNA/chromosome damage using alkaline Comet assay. The second objective was to investigate the effect of gamma radiation on DNA damage in mouse peripheral blood leucocytes using identical doses and experimental conditions, e.g. lyses, electrophoretic conditions and duration of electrophoresis which are known to affect tail moment (TM) and tail length (TL) of comets. Human and mouse whole blood samples were irradiated with different doses of gamma rays, e.g. 2, 4 and 8 Gy at a dose rate of 0.668Gy/min between 0 and 4 degrees C in air. After lyses, cells were electrophorased under alkaline conditions at pH 13, washed and stained with propidium iodide. Images of the cells were acquired and analyzed using in-house developed imaging software, SCGE-Pro, for Comet assay. For each comet, total fluorescence, tail fluorescence and tail length were measured. Increase in TM and TL was considered as the criteria of DNA damage. Analysis of data revealed heterogeneity in the response of leucocytes to gamma ray induced DNA damage both in human as well as in mouse. A wide variation in TM and TL was observed in control and irradiated groups of all the three donors. Data were analyzed for statistical significance using one-way ANOVA. Though a small variation in basal level of TM and TL was observed amongst human and mouse controls, the differences were not statistically significant. A dose-dependent increase in TM ($P<0.001$) and TL ($P<0.001$) was obtained at all the radiation doses (2-8 Gy) both in human and mouse leucocytes. However, there was a difference in the nature of dose response curves for human and mouse leucocytes. In human leucocytes, a linear increase in TM and TL was observed up to the highest radiation dose of 8 Gy. However, in case of mouse leucocytes, a sharp increase in TM and TL was observed only up to 4 Gy, and there after

saturation ensued. In human samples, the dose response of both TM and TL showed best fits with linear model (r(TM)=0.999 and r(TL)=0.999), where as in mouse, the best fit was obtained with Sigmoid (Boltzman) model. From the present data on leucocytes with increase in TM and TL as the criteria of DNA damage, it appears that mouse is relatively more sensitive to radiation damage than humans.

Chauvel-Lebre DJ, Auroy P, Tricot-Doleux S, Bonnaure-Mallet M. **Evaluation of the capacity of the SCGE assay to assess the genotoxicity of biomaterials.** Biomaterials 2001;22(13):1795-801.
Abstract: The comet test or SCGE assay, which is already widely used in other areas, has never been used to evaluate the mutagenic potential of medical biomaterials in the final form. The purpose of our study was thus to assess the comet test as a means of assessing the genotoxic potential of finished medical biomaterials. We used silicone elastomers with increasing concentrations of 4-nitroquinoline oxide, a genotoxic agent. Hydrogen peroxide was used as the positive control, and tissue culture polystyrene as the negative control. In our study, the comet test did not detect a significant difference in genotoxicity between the pure elastomer and the same elastomer containing 0.01 mg/ml 4-nitroquinoline oxide, but did detect a significant difference between two elastomers containing 0.01 and 0.3 mg/ml of 4-nitroquinoline oxide, respectively. Since, the surface properties of the samples were identical, only the chemical composition may have caused significant differences in mutagenicity. Whatever the cause of the genotoxicity detected by the SCGE assay, testing finished biomaterials using the comet assay makes it possible to evaluate interactions between biomaterials and living tissues that are much closer to actual application conditions.

Choi K, Meier PG. **Toxicity evaluation of metal plating wastewater employing the Microtox assay: a comparison with cladocerans and fish.** Environ Toxicol 2001;16(2):136-41.
Abstract: The relative sensitivity of the Microtox assay is closely related to the type of toxicant, and hence its utility in biomonitoring effluents is better evaluated on a case-by-case basis. The Microtox assay, employing the marine bacterium Vibrio fischeri, was evaluated for its applicability in monitoring metal plating wastewater for toxicity. The results of the Microtox assay after 5, 15, and 30 min of exposure, were compared with data obtained from conventional whole effluent toxicity testing (WET) methods that employed Daphnia magna, Ceriodaphnia dubia, and the fathead minnow (Pimephales promelas). The Microtox assay produced notably comparable EC50 values to the LC50 values of the acute fathead minnow toxicity test (< 0.5 order of difference). The Spearman's rank correlation analyses showed that the bacterial assay, regardless of exposure duration, correlated better with the acute fish than the daphnid results ($p < 0.05$). These observations were consistent to other studies conducted with inorganic contaminants. The relative sensitivity of the 30-min Microtox assay was within the range of the two frequently used acute daphnid/fish toxicity tests. In conclusion, the Microtox assay correlated well with the acute fathead minnow data and is well suited for toxicity monitoring for these types of industrial wastes.

Choucroun P, Gillet D, Dorange G, Sawicki B, Dewitte JD. **Comet assay and early apoptosis.** Mutat Res 2001;478(1-2):89-96.
Abstract: The comet assay is a single cell gel electrophoresis test currently used as a qualitative and quantitative genotoxicity test. However, some of the results from this comet assay and current knowledge on apoptosis lead us to suspect the presence of some false positive results. The aim of this

study was to ascertain if apoptotic cells can yield comet images that might distort the interpretation of the results. Using Jurkat cells, that hardly express Fas antigen, and apoptosis induction with anti-Fas antibody, it was possible to show that apoptosis can generate typical comet pictures as soon as the cells enter the apoptosis process. Therefore, comet images cannot be interpreted as a genotoxicity indicator when an apoptosis risk is present. Yopro-1 staining, that is also nearly immediate after apoptosis induction, can be used to balance comet assay results.

Claxton LD, Houk VS, Warren S. **Methods for the spiral Salmonella mutagenicity assay including specialized applications.** Mutat Res 2001;488(3):241-57.
Abstract: An automated approach to bacterial mutagenicity testing - the spiral Salmonella assay - was developed to simplify testing and to reduce the labor and materials required to generate dose-responsive mutagenicity information. This document provides the reader with an overview of the spiral assay and a discussion of its application for examining the mutagenic potential of pure compounds, complex environmental mixtures, and interactive effects. Guidelines for performing a routine spiral assay are presented, and alternative test methods intended to overcome a variety of technical difficulties (such as restricted sample availability, sample viscosity or volatility, etc.) are recommended. Methods for the computerized analysis of data and the interpretation of results are discussed.

Collins AR, Dusinska M, Horvathova E, Munro E, Savio M, Stetina R. **Inter-individual differences in repair of DNA base oxidation, measured in vitro with the comet assay.** Mutagenesis 2001;16(4):297-301.
Abstract: There is a need for a reliable, robust and sensitive assay for DNA repair, suitable for use with human lymphocyte samples in molecular epidemiological investigations. The comet assay (single cell alkaline gel electrophoresis) has been modified to measure the ability of a simple subcellular extract of lymphocytes to carry out the initial step of repair, i.e. incision, on a DNA substrate carrying specific lesions--namely, oxidized bases introduced by visible light in the presence of photosensitizer. The cell extract is free of non-specific nuclease activity, incising DNA only if the DNA has been treated with photosensitizer and light. The activity varies between individuals, but consistency is seen between samples from each individual taken on occasions several months apart. The lack of activity of extract from Ogg1(-) mouse cells (deficient in the glycosylase that excises 8-oxoguanine) in this assay confirms that the activity measured is predominantly excision repair of oxidized bases. This new DNA repair assay is simple, rapid and requires only small quantities of lymphocyte extract (obtainable from 10 ml blood).

Collins AR, Horvathova E. **Oxidative DNA damage, antioxidants and DNA repair: applications of the comet assay.** Biochem Soc Trans 2001;29(Pt 2):337-41.
Abstract: Estimates of background levels of oxidative base damage in human white blood cells vary enormously, from 300 down to 0.4 molecules of 8-oxoguanine per 10(6) guanines. An EC-funded Concerted Action, the European Standards Committee on Oxidative DNA Damage, is currently attempting to resolve the discrepancy and to agree a realistic estimate of basal endogenous oxidation. Oxidation of lymphocyte DNA is a useful marker of oxidative stress, and this can be decreased by supplementation with pure antioxidants or with foods rich in antioxidants. The steady-state level of DNA oxidation is ultimately controlled by the process of DNA repair; the extent to which this varies

between individuals has yet to be established.

De Boeck M, Touil N, De Visscher G, Vande PA, Kirsch-Volders M. **Validation and implementation of an internal standard in comet assay analysis.** Mutat Res 2000;469(2):181-97.
Abstract: The comet assay is widely used to detect DNA damage in single cells. However, only moderate attention has been paid to the experimental variability of this assay, especially during electrophoresis. To take into account this variation and to be able to compare measurements from different electrophoretic runs, as would be necessary when large numbers of samples need to be analysed, it is important to integrate an internal standard into the assay. This study presents a first step in the validation and implementation of an internal standard in the alkaline comet assay. Untreated and ethyl methanesulfonate treated cells (K562 human erythroleukemia cell line) were used as negative and positive internal standards, respectively, in each electrophoresis run. Three steps were followed: (1) assessment of the different levels of variability which may influence the damage levels of the internal standards, (2) evaluation of the variability across separate electrophoresis runs on the quantification of DNA damage in the internal standards by three experimenters involved in different studies and (3) proposal of an adequate calculation system to integrate the internal standards into test sample data. The application of the two proposed models to samples from a human biomonitoring study is presented. The model which calibrates the measurements against the negative internal standard is the most useful since this negative standard was the most stable across experiments and among the three experimenters. The percentage of DNA in the tail is the most appropriate parameter to analyse induced DNA damage, because its interelectrophoresis and interexperimenter variation is less pronounced than that of tail length.

de Souza Bueno AM, de Braganca Pereira CA, Rabello-Gay MN. **Environmental genotoxicity evaluation using cytogenetic end points in wild rodents.** Environ Health Perspect 2000;108(12):1165-9.
Abstract: We analyzed cytogenetic end points in three populations of two species of wild rodents--Akodon montensis and Oryzomys nigripes--living in an industrial, an agricultural, and a preservation area at the Itajai Valley, state of Santa Catarina, Brazil. Our purpose was to evaluate the performance of the following end points in the establishment of a genotoxic profile of each area: the polychromatic/normochromatic cell ratio; the mitotic index; the frequency of micronucleated cells both in the bone marrow and peripheral blood; and the frequency of cells with chromosome aberrations in the bone marrow. Preparations were obtained using conventional cytogenetic techniques. The results showed a) the role of the end points used as biomarkers in the early detection of genotoxic agents and in the identification of species and populations at higher risk; b) the difference in sensitivity of the species selected as bioindicators in relation to the cytogenetic end points analyzed; c) the need to use at least two sympatric species to detect the presence of genotoxins in each locality; and d) the need to use several end points when trying to establish a genotoxic profile of an area.

de Stoppelaar JM, Faessen P, Zwart E, Hozeman L, Hodemaekers H, Mohn GR, Hoebee B. **Isolation of DNA probes specific for rat chromosomal regions 19p, 19q and 4q and their application for the analysis of diethylstilbestrol-induced aneuploidy in binucleated rat fibroblasts.** Mutagenesis 2000;15(2):165-75.

Abstract: DNA probes specific for rat chromosomes 19p, 19q and 4q were isolated, characterized and used for the detection and analysis of diethylstilbestrol(DES)-induced aneuploidy. By denaturing and partially reassociating total genomic DNA a new rat repetitive DNA family was isolated, which was located on chromosome 19p21. Sequencing of a number of subclones from cos76-1 and other clones of this so-called 76-family revealed that the repeat units are interrupted with large areas of other (unique) DNA. Consequently, after fluorescence in situ hybridization (FISH) the signals in interphase nuclei are large and spread out. The other two probes, cos25 (chromosome 4q) and cos42-47 (chromosome 19q), were isolated by screening cosmid libraries with probes isolated previously in our laboratory. The repeat unit of cos25 is a 2174 bp long EcoRI unit that contains three Sau3A sites and is tandemly organized. Sequencing of subclones of cos42-47 revealed that this probe was in fact the 5S RNA gene, located on 19q12. In order to determine if these probes were suitable probes for aneuploidy detection, two series of dual colour FISH with the combinations cos25/cos76-1 (4q/19p) and cos42-47/cos76-1 (19q/19p) were carried out on slides from an in vitro micronucleus assay with DES. With all three probes used, an increase in binucleated cells with non-disjunction or chromosome loss was observed in the DES-treated cultures. Scoring of additional micronucleated cells on slides hybridized with the cos25/cos76-1 (4q/19p) probes revealed that the hybridization signal of probe cos25 (4q) was over-represented in the micronuclei of the control cultures. The simultaneous use of the 19q and 19p probes is a parcticularly valuable tool for the detection of aneuploidy, since it allows distinction between aneugenic and clastogenic events in binucleated cells. Results of this analysis showed that apart from aneuploidy, DES also induced structural chromosome aberrations, although to a lesser extent.

de Stoppelaar JM, van de Kuil T, Verharen HW, Hokse H, Opperhuizen A, Mohn GR, van Benthem J, Hoebee B. **In vivo cytokinesis blocked micronucleus assay with carbendazim in rat fibroblasts and comparison with in vitro assays.** Mutagenesis 2000;15(2):155-64.

Abstract: A successful in vivo application of the cytokinesis blocked micronucleus assay for the detection of aneuploidy induced by carbendazim (CARB) was carried out in the granuloma pouch assay. This was performed in two ways: (i) in vivo exposure of the skin fibroblasts to cytochalasin B (cytB) and CARB, by simultaneous injection of both substances into the pouch; (ii) in vivo exposure to CARB followed by in vitro culturing of the fibroblasts in the presence of cytB. Only the first assay was successful. Injection of cytB (with or without the test compound) into the pouch resulted in the induction of binucleate cells in vivo, up to a maximum of 5% at 1 mg cytB/pouch. After injection of CARB (0-50 or 0-10 mg/pouch) and cytB (1 mg) into the pouch, aneuploidy was determined in the isolated binucleate fibroblasts by fluorescence in situ hybridization with a general centromeric probe and combinations of chromosome-specific probes (19p + 19q, 4q + Yq). With all probes, the induction of chromosome loss and/or non-disjunction by CARB was very pronounced; at 10 mg CARB/pouch the total malsegregation frequency of chromosomes 4, 19 and Y was approximately 300/1000 binucleate cells. In an in vitro cytokinesis block assay with CARB (0-2.5 microg/ml) in primary skin fibroblasts the induced aneuploidy frequencies were as high as observed in the in vivo assay. The use of two probes for chromosome 19, which enabled the scoring of chromosome breaks in addition to aneuploidy, revealed no significant induction of chromosome breaks by CARB. The frequency of polyploid mononucleate and binucleate cells was decreased after CARB treatment, in both the in vivo and in vitro assays. However, in an additional in vitro assay without cytB a major induction of polyploidy from 2.5 microg/ml CARB and above was observed, showing that cytB may interfere with polyploidy induction.

den Besten PJ, Tuk CW. **Relation between responses in the neutral red retention test and the comet assay and life history parameters of Daphnia magna.** Mar Environ Res 2000;50(1-5):513-6.
Abstract: Responses of the neutral red retention (NRR) assay as test for lysosomal stability and the comet assay as test for DNA integrity were measured in the water flea, Daphnia magna, and compared with mortality and effects on population growth rate during short- or long-term exposure to seven different toxicants. The NRR test and the comet assay were performed with fresh preparations of pieces of tissue from the digestive tract or with cell preparations from whole daphnias. Five toxicants caused responses of the NRR test or the comet assay after short-term exposure at concentrations below the acute toxicity level. Preliminary results of long-term exposure experiments suggest that these biomarker responses can be related to chronic effects on survival and/or reproduction of D. magna. This type of research should provide the basis for future use of the NRR test and the comet assay as early warning biomarkers for effects of toxicants on Daphnia populations.

Diehl MS, Willaby SL, Snyder RD. **Comparison of the results of a modified miniscreen and the standard bacterial reverse mutation assays.** Environ Mol Mutagen 2000;36(1):72-7.
Abstract: The bacterial reverse mutation assay (Ames test) provides a rapid assessment of the mutagenic potential of chemicals. The assay is widely used in the pharmaceutical industry for early assessment during candidate compound selection and for regulatory drug submissions. Early in development, many candidate compounds are available in only very small quantities. The use of the standard plate incorporation bacterial reverse mutation assay for screening, using only a single petri plate per concentration, requires the use of approximately 140 mg of test compound to test up to a stock concentration of 100 mg/ml (5000 microg/plate) in five strains of bacteria. A modification of the existing Ames Miniscreen assay has been developed using six-well cell-culture dishes that requires only 21 mg of compound to test a stock concentration of up to 100 mg/ml (2000 microg/well) in three strains of bacteria. The standard plate incorporation assay and the modified Miniscreen assays conducted on proprietary compounds without and with metabolic activation have yielded a high degree of concordance in findings.

Djomo JE, Ferrier V, Bekaert C. **Amphibian micronucleus test in vivo (Jaylet test) to evaluate the genotoxicity of petrochemical waste waters.** Bull Environ Contam Toxicol 2000;65(2):168-74.

Dodd A, Curtis PM, Williams LC, Love DR. **Zebrafish: bridging the gap between development and disease.** Hum Mol Genet 2000;9(16):2443-9.
Abstract: The zebrafish has been the model of choice amongst developmental biologists for many years. This small freshwater species offers many advantages to the study of organ and tissue development that are not provided by other model systems. Against this background, modern molecular genetic approaches are being applied to expand the physical and genetic mapping of the zebrafish genome. These approaches complement the large-scale mutagenic screens that have led to the isolation of mutant phenotypes. Some of the phenotypes have been found to resemble human disease states, while mapping and sequencing data have revealed zebrafish genes with significant homology to human disease-causing genes. It is the realization that the zebrafish offers an amenable system for understanding disease, as opposed to development, that underpins this review. The adventitious identification of disease

phenotypes amongst zebrafish mutants and the important area of deliberate disease modelling using transgenesis and gene targeting should lead to a better application of the zebrafish as a vertebrate model of human diseases.

Domart-Coulon I, Auzoux-Bordenave S, Doumenc D, Khalanski M. **Cytotoxicity assessment of antibiofouling compounds and by-products in marine bivalve cell cultures.** Toxicol In Vitro 2000;14 (3):245-51.
Abstract: Short-term primary cell cultures were derived from adult marine bivalve tissues: the heart of oyster Crassostrea gigas and the gill of clam Ruditapes decussatus. These cultures were used as experimental in vitro models to assess the acute cytotoxicity of an organic molluscicide, Mexel-432, used in antibiofouling treatments in industrial cooling water systems. A microplate cell viability assay, based on the enzymatic reduction of tetrazolium dye (MTT) in living bivalve cells, was adapted to test the cytotoxicity of this compound: in both in vitro models, toxicity thresholds of Mexel-432 were compared to those determined in vivo with classic acute toxicity tests. The clam gill cell model was also used to assess the cytotoxicity of by-products of chlorination, a major strategy of biofouling control in the marine environment. The applications and limits of these new in vitro models for monitoring aquatic pollutants were discussed, in reference with the standardized Microtox test.

Dorr W, Obeyesekere MN. **A mathematical model for cell density and proliferation in squamous epithelium after single-dose irradiation.** Int J Radiat Biol 2001;77(4):497-505.
Abstract: PURPOSE: To establish a mathematical model describing changes in cell density in squamous epithelia induced by single-dose irradiation. Detailed data from previous studies in mouse tongue epithelium have been used for this study. MATERIALS AND METHODS: The major mechanisms of the epithelial regeneration response, i.e. loss of division asymmetry and accelerated proliferation of stem cells, in combination with residual, abortive proliferation of sterilized cells, have been included in a tissue compartment model. These phenomena have been incorporated via three parameters; T(delay), the duration of the cell cycle block; T(min), the minimum stem cell cycle time due to acceleration; and T (stop), the duration of abortive proliferation. The compartments introduced in the model are normal stem cells, S1; sterilized stem cells, S2; and post-mitotic, functional cells, F. The flux rats between the tissue compartments were defined by autoregulation of the stem cell population, and by overall cell numbers. The model was applied to fit experimental data on changes in oral mucosal cell density after single-dose exposure with 13 and 20 Gy. The best-fit sets of parameters were identified by L2 norm error analysis based on the total cell count. RESULTS: For 13 Gy, the best fit was achieved with T(min) = 1.0 days, T (delay) = 1.2 days and T(stop) = 7.5 days. For 20 Gy, the parameters were, T(min) =0.7 days, T(delay)= 1.0 days and T(stop) =9.5 days. In both data sets, T(min) was the most influential parameter. The resulting fluctuations in stem cell numbers were in good accordance with changes in radiation tolerance after 13 Gy. CONCLUSIONS: The model can be used to define dose-dependent parameters describing the morphological response of squamous epithelia to single-dose irradiation. Based on these parameters, post-irradiation fluctuations in radiosensitivity can be predicted. For developing more complex and reliable mathematical models, which could incorporate transit divisions or fractionated radiotherapy, further experimental data at various dose levels are required.

Ehret R, Baumann W, Brischwein M, Lehmann M, Henning T, Freund I, Drechsler S, Friedrich U,

Hubert ML, Motrescu E, et al. **Multiparametric microsensor chips for screening applications.** Fresenius J Anal Chem 2001;369(1):30-5.
Abstract: The identification of drug targets for pharmaceutical screening can be greatly accelerated by gene databases and expression studies. The identification of leading compounds from growing libraries is realized by high throughput screening platforms. Subsequently, for optimization and validation of identified leading compounds studies of their functionality have to be carried out, and just these functionality tests are a limiting factor. A rigorous preselection of identified compounds by in vitro cellular screening is necessary prior to using the drug candidates for the further time consuming and expensive stage, e.g. in animal models. Our efforts are focused to the parallel development, adaptation and integration of different microelectronic sensors into miniaturized biochips for a multiparametric, functional on-line analysis of living cells in physiologically environments. Parallel and on-line acquisition of data related to different cellular targets is required for advanced stages of drug screening and for economizing animal tests.

Erexson GL, Periago MV, Spicer CS. **Differential sensitivity of Chinese hamster V79 and Chinese hamster ovary (CHO) cells in the in vitro micronucleus screening assay.** Mutat Res 2001;495(1-2):75-80.
Abstract: Both the V79 and CHO cell lines are routinely used in the in vitro MN screening assay for the detection of possible genotoxicants. The CHO cell line is the predominant cell line currently used in the genetic toxicology testing industry. However, some laboratories routinely utilize the V79 cell line since the in vitro MN screening assay was initially developed using V79 cells. Our laboratory has historically used the CHO cell line. Therefore, our laboratory was interested in comparing the two cell lines with regard to possible similarities or differences in MN induction sensitivity after exposure to cyclophosphamide (CPA) and mitomycin C (MMC), the two standard positive control chemicals routinely used in this assay. Three exposure conditions in the presence of CPA and MMC were examined in both cell lines. Replicate cultures of CHO cells in McCoy's 5A and V79 cells in both McCoy's 5A and E-MEM were established and treated with 5 microg CPA/ml (4h exposure with S9), 0.5 microg MMC (4h exposure without S9) and 0.5 microg MMC (24h exposure without S9). A total of 400 cytochalasin B-blocked binucleated cells and 200 consecutive cells were analyzed from each culture for MN and cell cycle kinetics, respectively. Analysis of the data demonstrated that CHO cells were up to approximately five-fold more sensitive to the induction of CPA- and MMC-induced MN than V79 cells. Both cell lines exhibited similar average generation times among identical exposure groups. Therefore, the difference in MN sensitivity cannot be attributed to possible differences in cell cycle kinetics and is possibly related to inherent cellular differences in the processing of and/or repair of CPA- and MMC-induced damage by V79 and CHO cells.

Felix K, Kovalchuk AL, Park SS, Coleman AE, Ramsay ES, Qian M, Kelliher KA, Jones GM, Ried T, Bornkamm GW, et al. **Inducible mutagenesis in TEPC 2372, a mouse plasmacytoma cell line that harbors the transgenic shuttle vector lambdaLIZ.** Mutat Res 2001;473(1):121-36.
Abstract: The plasmacytoma cell line, TEPC 2372, was derived from a malignant plasma cell tumor that developed in the peritoneal cavity of a BALB/c mouse that harbored the transgenic shuttle vector for the assessment of mutagenesis in vivo, lambdaLIZ. TEPC 2372 was found to display the typical features of a BALB/c plasmacytoma. It consisted of pleomorphic plasma cells that secreted a monoclonal

immunoglobulin (IgG2b/lambda), was initially dependent on the presence of IL-6 to grow in cell culture, contained a hyperdiploid chromosome complement with a tendency to undergo tetraploidization, and harbored a constitutively active c-myc gene by virtue of a T(6;15) chromosomal translocation. TEPC 2372 was further characterized by the ability to respond to in vitro exposure with 4-NQO (4-nitroquinoline-1-oxide), an oxidative model mutagen, with a vigorous dose-dependent increase in mutagenesis that peaked at a 7.85-fold elevation of mutant rates in lambdaLIZ when compared to background mutant rates in untreated controls. Cotreatment with 4-NQO and BSO (buthionine sulfoximine), a glutathione-depleting compound that causes endogenous oxidative stress, resulted in a 9.03-fold increase in the mutant frequency in lambdaLIZ. These results demonstrated that TEPC 2372, the malignant plasma cell counterpart of the lambdaLIZ-based in vivo mutagenesis assay, may be useful as an in vitro reference point for the further elucidation of oxidative mutagenesis in lymphoid tissues.

Fenech M. **The in vitro micronucleus technique.** Mutat Res 2000;455(1-2):81-95.
Abstract: The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably. Because micronuclei can only be expressed in cells that complete nuclear division a special method was developed that identifies such cells by their binucleate appearance when blocked from performing cytokinesis by cytochalasin-B (Cyt-B), a microfilament-assembly inhibitor. The cytokinesis-block micronucleus (CBMN) assay allows better precision because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions. The method is now applied to various cell types for population monitoring of genetic damage, screening of chemicals for genotoxic potential and for specific purposes such as the prediction of the radiosensitivity of tumours and the inter-individual variation in radiosensitivity. In its current basic form the CBMN assay can provide, using simple morphological criteria, the following measures of genotoxicity and cytotoxicity: chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), cell division inhibition, necrosis and apoptosis. The cytosine-arabinoside modification of the CBMN assay allows for measurement of excision repairable lesions. The use of molecular probes enables chromosome loss to be distinguished from chromosome breakage and importantly non-disjunction in non-micronucleated binucleated cells can be efficiently measured. The in vitro CBMN technique, therefore, provides multiple and complementary measures of genotoxicity and cytotoxicity which can be achieved with relative ease within one system. The basic principles and methods (including detailed scoring criteria for all the genotoxicity and cytotoxicity end-points) of the CBMN assay are described and areas for future development identified.

Fenech M. **A mathematical model of the in vitro micronucleus assay predicts false negative results if micronuclei are not specifically scored in binucleated cells or in cells that have completed one nuclear division.** Mutagenesis 2000;15(4):329-36.
Abstract: A mathematical model is described that predicts the effect of altered nuclear/cell division kinetics and cytotoxicity on micronucleus expression in vitro when the micronucleus assay is performed without discriminating between cells that have divided once and cells that have not divided after genotoxic insult. The model is based on the probabilities of: (i) a viable cell completing nuclear division;

(ii) micronucleus expression in a cell that completes nuclear division after genotoxic insult; (iii) a cell not dividing and surviving as a mononuclear cell; (iv) a cell dying by necrosis or apoptosis. The model predicts: (i) false negative results for relatively weak chromosome damaging agents that also inhibit nuclear division, if micronuclei are scored in mononucleated cells without discriminating between divided and non-divided cells; (ii) this tendency for a false negative result when scoring micronuclei without discriminating between non-divided and once-divided mononuclear cells increases with cell lines and culture conditions that do not result in optimal rates of nuclear division (i.e. >90% of dividing cells); (iii) the absolute increment in micronucleus frequency in binucleated cells is at least 2-fold greater than that observed in mononucleated cells when nuclear division is not inhibited and this difference increases with increasing nuclear inhibition. The number of dead cells does not influence the micronucleus frequency if only viable cells are considered when determining the micronucleus frequency ratio. The results from this model suggest that the micronucleus assay when performed by scoring mononucleated cells, without restricting the score to those cells that have divided once after genotoxic insult, is prone to produce false negative results and, therefore, cannot be considered reliable or conclusive. Scoring of micronuclei in cytokinesis-blocked binucleated cells is predicted by the model to provide consistent results under all culture conditions and based on these theoretical results should be considered the preferred choice.

Fibach E. **Cell culture and animal models to screen for promising fetal hemoglobin-stimulating compounds.** Semin Hematol 2001;38(4):374-81.
Abstract: Sickle cell anemia (SCA) and the thalassemias are globally the most common class of inherited single-gene disorders. Current treatment options are limited, especially in developing countries. More practical and cheaper therapies are urgently needed. Since high fetal hemoglobin (HbF) levels ameliorate the clinical symptoms of these diseases, one current approach is to use pharmcaological agents to reactivate the gamma-globin genes and stimulate the production of HbF. Several in vitro and in vivo experimental models developed for this purpose are the subject of this review. The models include in vitro established erythroid-like cell lines and primary cultures (both in semisolid and liquid media) of erythroid progenitor cells obtained from normal donors and patients with SCA and beta-thalassemia, as well as in vivo models in genetically modified (transgenic) and unmodified animals. These experimental systems are useful for large-scale screening of compounds for HbF-stimulating potential, for determining the mechanism of action of potent compounds at the cellular and molecular levels, and for studying the pharmacology, pharmacokinetics, and toxicology of the drugs. These models are essential to find, test, and develop new drugs that will be effective and safe for clinical use in adults and children. Semin Hematol 38:374-381.

Flamand N, Meunier J, Meunier P, Agapakis-Causse C. **Mini mutagenicity test: a miniaturized version of the Ames test used in a prescreening assay for point mutagenesis assessment.** Toxicol In Vitro 2001;15(2):105-14.
Abstract: The bacterial reverse mutagenicity test on Salmonella typhimurium, known as the Ames test, is widely used by regulatory agencies, academic institutions and chemical companies to assess the mutagenic potential of raw compounds. Several attempts have been made to miniaturise the Ames test in order to fit the industrial constraint of screening more products at the low quantities available. The major limitation of these miniaturised versions of the Ames test lies in the impossibility to work with all the six

strains used in the regular Ames test, especially with those showing a low spontaneous revertant frequency. We describe here a mini version of the regulatory Ames test protocol that allows a significant reduction of the quantity of test substance needed (300 mg) but remains applicable to all Salmonella strains used in the regulatory protocol. In a preliminary study, 10 in-house chemical compounds have been evaluated in the Mini Mutagenicity Test (MMT) together with some positive control substances. A first set of historical data obtained in 1999 as well as the predictivity and the sensitivity of the MMT are presented and compared to those of the regular Ames test.

Fontana L, Lasfargues G, Ughetto S, Rogier S, Masdieu E, Lafaure M, Aublet-Cuvelier B, Catilina P. **The micronucleus assay in human exfoliated urothelial cells: application in a genotoxicity study of workers exposed to a mineral jelly containing sodium nitrite and N-phenyl-1-naphthylamine.** Mutagenesis 2001;16(5):449-52.
Abstract: Exposure to certain chemical agents in occupational settings has been identified as carcinogenic to the human bladder. Micronucleus (MN) analysis in exfoliated urothelial cells is an interesting method for biomonitoring genetic damage in human populations. However, few studies have been performed in an occupational context. The aim of this study was to examine whether the occupational use of a mineral jelly induced a genotoxic risk for workers employed at a single factory producing bearings using the MN test on exfoliated urothelial cells. The prevalence of micronucleated exfoliated urothelial cells (MNC) was determined in 35 female workers with dermal exposure to the jelly and 41 female controls. The mean percentage of MNC (expressed as percent cells with MN per 1000 cells scored) observed in the exposed worker group was 0.46 +/- 0.11% (range 0-2.8) and in the control group 0.14 +/- 0.03% (range 0-0.8). There is a significant job effect (P = 0.0018, MANCOVA) on the prevalence of MNC, whereas age and smoking habit had no significant effect (P = 0.90 and 0.91, respectively). There is no interaction between job and smoking habit (P = 0.4421). Exposure to the mineral jelly appeared to be the main factor inducing the increased prevalence of MNC. This may be due to the presence of mutagens/carcinogens in the jelly: an aromatic amine, N-phenyl-1-naphthylamine (CAS no. 90-30-2), which is carcinogenic in mice, or sodium nitrite (CAS no. 7632-00-0), which is genotoxic in human cell systems. In conclusion, these results suggest that use of the mineral jelly could present a genotoxic risk for workers. We think that the MN assay on exfoliated cells could be valuable for biological monitoring purposes in occupational contexts as a marker of significant exposure to bladder mutagenic/carcinogenic agents.

Foss EJ. **Tof1p regulates DNA damage responses during S phase in Saccharomyces cerevisiae.** Genetics 2001;157(2):567-77.
Abstract: A tof1 mutant was recovered in a screen aimed at identifying genes involved specifically in the S phase branch of the MEC1-dependent DNA damage response pathway. The screen was based on the observation that mutants missing this branch are parcticularly dependent on the cell cycle-wide branch and, therefore, on RAD9, for surviving DNA damage. tof1 and rad9 conferred synergistic sensitivity to MMS, UV, and HU, and the double mutant was incapable of slowing S phase in response to MMS, inducing RNR3 transcription in response to UV, and phosphorylating Rad53p in response to HU. TOF1's contribution to DNA damage response appeared to be restricted to S phase, since TOF1 did not contribute to UV-induced transcription during G1 or to the cdc13-1-induced block to anaphase in G2/M. I suggest a model in which Tof1p functions to link Mec1p with Rad53p.

Frenzilli G, Bosco E, Barale R. **Validation of single cell gel assay in human leukocytes with 18 reference compounds.** Mutat Res 2000;468(2):93-108.
Abstract: To validate the alkaline single cell gel (SCG) assay as a tool for the detection of DNA damage in human leukocytes, we investigated the in vitro activity of 18 chemicals. Thirteen of these chemicals (pyrene (PY), benzo(a)pyrene (BaP), cyclophosphamide (CP), 4-nitroquinoline-1-oxide (4NQO), bleomycin (BLM), methylmercury chloride (MMC), mitomycin C (MTC), hydrogen peroxide (HP), diepoxybutane (DEB), glutaraldehyde (GA), formaldehyde (FA), griseofulvin (GF), sodium azide (NA)) are genotoxic in at least one cell system, while five compounds (ascorbic acid (AA), glucose (GL), D-mannitol (MAN), O-vanillin (VAN), chlorophyllin (CHL)) are classified as non-genotoxic. In this in vitro SCG assay, PY, BaP and CP were positive with exogeneous metabolic activation (rat S9 mix) while 4NQO, BLM, MMC, MTC, hydrogen peroxide, and diepoxbutane were positive in the absence of metabolic activation. CHL and VAN were unexpectedly found to induce a dose-dependent increase in DNA migration. AA, GL, and MAN were negative in a non-toxic range of doses. GF gave equivocal results, while FA and GA increased DNA migration at low doses and decreased DNA migration at higher doses. This behaviour is consistent with the known DNA damaging and crosslinking properties of these compounds. These data support the sensitivity and specificity of this assay for identifying genotoxic agents.

Frieauff W, Hartmann A, Suter W. **Automatic analysis of slides processed in the Comet assay.** Mutagenesis 2001;16(2):133-7.
Abstract: In recent years the Comet assay (or single cell gel electrophoresis assay) has been established as a rapid and sensitive method for the detection of DNA damage. For early genotoxicity screening of new chemical entities in industrial toxicology, the Comet assay is more and more used for assessment of the DNA damaging potential of a test compound. In order to increase compound screening throughput, we have established an image analysis system for fully automated measurement of microscope slides processed in the Comet assay. For the comparative investigation various cell types, such as V79 Chinese hamster cells, mouse lymphoma cells and human leukocytes, were treated with several test compounds. Using tail moment as the quantitative parameter for comet formation, we show a very high correlation between our automatic image analysis system and a commercially available, interactive system (Comet Assay II of Perceptive Instruments). The possibility of analyzing 50 samples within 1 day and the high reproducibility of results make automated image processing a powerful tool for automatic analysis of slides processed in the Comet assay.

Fu L, Mambrini M, Perrot E, Chourrout D. **Stable and full rescue of the pigmentation in a medaka albino mutant by transfer of a 17 kb genomic clone containing the medaka tyrosinase gene.** Gene 2000;241(2):205-11.
Abstract: In the medaka Oryzias latipes, several albino strains have mutations in the tyrosinase gene that have been fully characterized at the molecular level. A genomic clone from wild-type medaka containing the 5 kb tyrosinase gene with its five exons, 10 kb of upstream sequences and 2 kb downstream sequences was introduced into fertilized eggs from a tyrosinase-negative albino strain. We show that the injection of this genomic clone predominantly conferred mosaic expression ending before the hatching stage. A minority of juveniles retained a variable number of pigmented cells, including four

individuals keeping one pigmented eye through adulthood. Two of these could be mated, and one of these transmitted the transgene resulting in complete rescue of pigmentation to 16% of its offspring. The resulting transgenic line harbors a single copy of the wild-type tyrosinase gene and all fish are wild-type with respect to pigmentation. These experiments suggest that the tyrosinase genomic clone, or a future shorter version of it, can be used in fish to routinely detect transgenic lines. The apparent faithful and systematic expression of the tyrosinase transgene is most probably due to the presence of a locus control region (LCR) in the injected clone.

Fujita K, Kamataki T. **Role of human cytochrome P450 (CYP) in the metabolic activation of N-alkylnitrosamines: application of genetically engineered Salmonella typhimurium YG7108 expressing each form of CYP together with human NADPH-cytochrome P450 reductase.** Mutat Res 2001;483(1-2):35-41.

Abstract: The role of human cytochrome P450 (CYP) in the metabolic activation of N-alkylnitrosamines was examined by Ames test using genetically engineered Salmonella typhimurium (S. typhimurium) YG7108 cells expressing each form of human CYP together with human NADPH-cytochrome P450 reductase (OR). The relationship between the structure of N-alkylnitrosamines and CYP form(s) involved in the activation was evaluated. Eleven strains of S. typhimurium YG7108 cells expressing each form of CYP (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 or CYP3A5) were employed. Eight N-alkylnitrosamines including N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosodibutylamine (NDBA), N-nitrosomethylethylamine (NMEA), N-nitrosomethylpropylamine (NMPA), N-nitrosomethylbutylamine (NMBA) and N-nitrosoethylbutylamine (NEBA) were examined. Minimal concentration (MC) value of a promutagen was defined as the concentration of a chemical giving a positive result. Mutagen-producing capacity of CYP, as indicated by induced revertants/nmol promutagen/pmol CYP, for an N-alkylnitrosamine was determined for all forms of CYP. These N-alkylnitrosamines were mainly activated by CYP2E1, CYP2A6 and CYP1A1. N-alkylnitrosamines with relatively short alkyl chains such as NDMA and NMEA were primarily activated by CYP2E1 as judged by mutagen-producing capacity. With the increase of the number of the carbon atoms of the alkyl chains, the contribution of CYP2A6 increased. CYP2A6 played major roles in the activation of NDEA, NDPA, NMPA, NMBA and NEBA. Interestingly, CYP1A1 became a molecular form of CYP playing a major role in the metabolic activation of NDBA.

Gage BM, Alroy D, Shin CY, Ponomareva ON, Dhar S, Sharma GG, Pandita TK, Thayer MJ, Turker MS. **Spontaneously immortalized cell lines obtained from adult Atm null mice retain sensitivity to ionizing radiation and exhibit a mutational pattern suggestive of oxidative stress.** Oncogene 2001;20 (32):4291-7.

Abstract: The study of Ataxia-telangiectasia (A-T) has benefited significantly from mouse models with knockout mutations for the Atm (A-T mutation) locus. While these models have proven useful for in vivo studies, cell cultures from Atm null embryos have been reported to grow poorly and then senesce. In this study, we initiated primary cultures from adult ears and kidneys of Atm homozygous mice and found that these cultures immortalized readily without loss of sensitivity to ionizing radiation and other Atm related cell cycle defects. A mutational analysis for loss of expression of an autosomal locus showed that ionizing radiation had a mutagenic effect. Interestingly, some spontaneous mutants

exhibited a mutational pattern that is characteristic of oxidative mutagenesis. This result is consistent with chronic oxidative stress in Atm null cells. In total, the results demonstrate that permanent cell lines can be established from the tissues of adult mice homozygous for Atm and that these cell lines will exhibit expected and novel consequences of this deficiency.

Gajendiran N, Tanaka K, Kamada N. **Comet assay to sense neutron 'fingerprint'.** Mutat Res 2000;452(2):179-87.
Abstract: The suitability of comet assay to identify DNA damage induced by neutrons of varying energy was tested. For this purpose, monoenergetic neutrons from Hiroshima University Radiobiological Research Accelerator (HIRRAC) were used to induce DNA damage in irradiated human peripheral blood lymphocytes. The level of damage was computed as tail moment for different doses (0.125-1 Gy) and compared with the effects resulting from irradiation with (60)Co gamma. The neutron-irradiated cells exhibited longer comet tails consisting of tiny pieces of broken DNA in contrast to the streaking tails generated by (60)Co gamma. The peak biological effectiveness occurred at 0.37 and 0.57 MeV; a further increase or decrease in neutron energy led to a reduced RBE value. The RBE values, as measured by the comet assay, were 6.3, 5.4, 4.7, 4.3, 2.6, and 1.7 for 0.37, 0.57, 0.79, 0.186, 1, and 2.3 MeV neutrons. The lower RBE value obtained by the comet assay when compared to that for other biological end points is discussed. This study reports the usefulness of the alkaline comet assay for identifying DNA damage induced by neutrons of the same radiation weighting factor. The comet assay is a potential tool for use in neutron therapy, as well as a method for the rapid screening of samples from individuals accidentally exposed to radiation.

Gaullier JM, Ronning E, Gillooly DJ, Stenmark H. **Interaction of the EEA1 FYVE finger with phosphatidylinositol 3-phosphate and early endosomes. Role of conserved residues.** J Biol Chem 2000;275(32):24595-600.
Abstract: FYVE zinc finger domains, which are conserved in multiple proteins from yeast to man, interact specifically with the membrane lipid phosphatidylinositol 3-phosphate (PtdIns(3)P). Here we have investigated the structural requirements for the interaction of the FYVE finger of the early endosome antigen EEA1 with PtdIns(3)P and early endosomes. The binding of the FYVE finger to PtdIns(3)P is Zn(2+)-dependent, and Zn(2+) could not be replaced by any other bivalent cations tested. By surface plasmon resonance, the wild-type FYVE finger was found to bind to PtdIns(3)P with an apparent K(D) of about 50 nm and a 1:1 stoichiometry. Mutagenesis of cysteines involved in Zn(2+) coordination, basic residues thought to be directly involved in ligand binding and other conserved residues, resulted in a 6- to >100-fold decreased affinity for PtdIns(3)P. A mutation in the putative PtdIns(3)P-binding pocket, R1375A, may prove parcticularly informative, because it led to a strongly decreased affinity for PtdIns(3)P without affecting the FYVE three-dimensional structure, as measured by fluorescence spectroscopy. Whereas the C terminus of EEA1 localizes to early endosomes when expressed in mammalian cells, all the FYVE mutants with reduced affinity for PtdIns(3)P were found to be largely cytosolic. Furthermore, whereas expression of the wild-type EEA1 C terminus interferes with early endosome morphology, the point mutants were without detectable effect. These results support recently proposed models for the ligand binding of the FYVE domain and indicate that PtdIns(3)P binding is crucial for the localization and function of EEA1.

Gaur M, Murphy GJ, deSauvage FJ, Leavitt AD. **Characterization of Mpl mutants using primary megakaryocyte-lineage cells from mpl(-/-) mice: a new system for Mpl structure-function studies.** Blood 2001;97(6):1653-61.
Abstract: Mpl is the thrombopoietin (TPO) receptor. The current molecular understanding of how Mpl activation stimulates proliferation of megakaryocyte-lineage cells is based largely on the engineered expression of Mpl in nonmegakaryocyte-lineage cell lines. However, the relevance of these findings to Mpl signaling in primary megakaryocyte-lineage cells remains largely unknown. Therefore, a system was developed to study Mpl function in primary mpl(-/-) megakaryocyte-lineage cells. Expressing avian retroviral receptors on the surfaces of mammalian cells overcomes their natural block to avian retroviral infection; 815 bp of human GPIIb regulatory sequence was used to generate transgenic mice with megakaryocyte-lineage expression of the subgroup A avian leukosis virus receptor, TVA. Avian retroviral infection of unfractionated bone marrow from these mice is restricted to megakaryocyte-lineage cells. The transgenic mice were crossed to an mpl(-/-) background generating GPIIb-tva+mpl (-/-) mice. By using avian retroviruses to express wild-type or mutant Mpl on the surfaces of primary megakaryocyte-lineage cells, it was demonstrated that (1) the 10 membrane-proximal, cytoplasmic amino acids of Mpl are required for TPO-induced proliferation; (2) Y582F mutation confers a proliferative advantage over wild-type Mpl and imparts a constitutive anti-apoptotic signal; (3) truncating the 50 C-terminal Mpl amino acids reduces but does not eliminate TPO-induced mitogen-activated protein kinase activation, yet it does not alter the synergistic effect of stem cell factor on TPO-induced proliferation; and (4) TPO-induced proliferation of early, primary megakaryocyte-lineage cells does not require Stat-5 phosphorylation. The system reported provides an improved approach for Mpl structure-function studies, and the method can be applied to any hematopoietic lineage.

Gluck U, Gebbers JO. **The comet assay of nasal epithelia: measurement of DNA damage for the assessment of genotoxic air pollution.** Laryngoscope 2000;110(1):123-5.
Abstract: OBJECTIVES: The alkaline single cell gel electrophoresis or "comet" assay allows measurement of DNA damage in single cells with a high degree of sensitivity, e.g., for investigations of the effect of environmental agents with DNA-damaging potential. This study aimed to adapt this test to respiratory cells of the human nasal mucosa to examine the genotoxic effect of air pollution (cigarette smoke). STUDY DESIGN: In a prospective study, nasal epithelia of 16 cigarette smokers were examined by the adapted comet assay and the results were correlated with the results of the Papanicolaou-stained nasal cytology, carried out in a blinded fashion. The control group comprised 20 non-smoking men. All subjects under investigation were healthy office workers. METHODS: Nasal epithelia were harvested from the maxilloturbinates. One part of cells was Papanicolaou stained and evaluated by cytopathologists. The comet assay was performed on the other part of the cells. The examiners were blinded to the study and control groups. RESULTS: Among cigarette smokers, a significant correlation between cytopathological cell nucleus changes (metaplasia and dysplasia) and the DNA migration (tail lengths) in the comet assay was found as a sign of DNA damage. This was not found in nonsmoking control persons. CONCLUSIONS: These results confirm the sensitivity of the comet assay and the hypothesis that cell nucleus changes in conventional nasal cytology are associated with DNA damage.

Gollapudi BB, Krishna G. **Practical aspects of mutagenicity testing strategy: an industrial**

perspective. Mutat Res 2000;455(1-2):21-8.
Abstract: Genetic toxicology studies play a central role in the development and marketing of new chemicals for pharmaceutical, agricultural, industrial, and consumer use. During the discovery phase of product development, rapid screening tests that require minimal amounts of test materials are used to assist in the design and prioritization of new molecules. At this stage, a modified Salmonella reverse mutation assay and an in vitro micronucleus test with mammalian cell culture are frequently used for screening. Regulatory genetic toxicology studies are conducted with a short list of compounds using protocols that conform to various international guidelines. A set of four assays usually constitutes the minimum test battery that satisfies global requirements. This set includes a bacterial reverse mutation assay, an in vitro cytogenetic test with mammalian cell culture, an in vitro gene mutation assay in mammalian cell cultures, and an in vivo rodent bone marrow micronucleus test. Supplementary studies are conducted in certain instances either as a follow-up to the findings from this initial testing battery and/or to satisfy a regulatory requirement. Currently available genetic toxicology assays have helped the scientific and industrial community over the past several decades in evaluating the mutagenic potential of chemical agents. The emerging field of toxicogenomics has the potential to redefine our ability to study the response of cells to genetic damage and hence our ability to study threshold phenomenon.

Gonda SR, Wu H, Pingerelli PL, Glickman BW. **Three-dimensional transgenic cell model to quantify genotoxic effects of space environment.** Adv Space Res 2001;27(2):421-30.
Abstract: In this paper we describe a three-dimensional, multicellular tissue-equivalent model, produced in NASA-designed, rotating wall bioreactors using mammalian cells engineered for genomic containment of multiple copies of defined target genes for genotoxic assessment. Rat 2 lambda fibroblasts, genetically engineered to contain high-density target genes for mutagenesis (Stratagene, Inc., Austin, TX), were cocultured with human epithelial cells on Cytodex beads in the High Aspect Ratio Bioreactor (Synthecon, Inc, Houston, TX). Multi-bead aggregates were formed by day 5 following the complete covering of the beads by fibroblasts. Cellular retraction occurred 8-14 days after coculture initiation culminating in spheroids retaining few or no beads. Analysis of the resulting tissue assemblies revealed: multicellular spheroids, fibroblasts synthesized collagen, and cell viability was retained for the 30-day test period after removal from the bioreactor. Quantification of mutation at the LacI gene in Rat 2 lambda fibroblasts in spheroids exposed to 0-2 Gy neon using the Big Blue color assay (Stratagene, Inc.), revealed a linear dose-response for mutation induction. Limited sequencing analysis of mutant clones from 0.25 or 1 Gy exposures revealed a higher frequency of deletions and multiple base sequencing changes with increasing dose. These results suggest that the three-dimensional, multicellular tissue assembly model produced in NASA bioreactors are applicable to a wide variety of studies involving the quantification and identification of genotoxicity including measurement of the inherent damage incurred in Space.

Grahame NJ. Selected lines and inbred strains. **Tools in the hunt for the genes involved in alcoholism.** Alcohol Res Health 2000;24(3):159-63.
Abstract: In their quest to elucidate the genetic influences contributing to alcoholism, researchers have long used selected lines and inbred strains of rodents. Selected lines are obtained by repeatedly mating those animals within a population that show extremely high or low values of the desired trait. Inbred strains are generated by mating male and female siblings, irrespective of any parcticular trait, over

several generations. Both of these approaches have provided researchers with extensive knowledge about the genetic and neurobiological mechanisms contributing to alcohol-related traits. However, the use of these models is associated with some limitations, mostly resulting from the inbreeding involved in generating such lines and strains. Nevertheless, these models can offer some advantages over other genetic approaches, such as the analysis of quantitative trait loci or the generation of transgenic and knockout mice.

Gutierrez JA. **Genomics: from novel genes to new therapeutics in parasitology.** Int J Parasitol 2000;30(3):247-52.
Abstract: The advent of rapid DNA sequencing technologies is generating vast quantities of raw genomic information ranging from in-depth analysis of the expressed genes to complete sequencing of genomes at an increasing rate (bioinformatics). However, it is the functional characterisation of a specific gene product that is the key limiting factor for validation as targets for high throughput assay development. The challenge is to obtain the raw genomic information from parasites of economic importance and to effectively integrate broad technologies such as gene disruption and over-expression, DNA arrays, proteomics, antisense RNAs, with bioinformatics in a timely fashion to identify relevant biological targets. Screening of validated targets in a strategy that includes large numbers of chemistries with high diversity and predictive in vitro and in vivo assays should permit the successful identification of novel chemical entities with high specificity to the target parasite. It is proposed that this rational approach will permit the identification of new antiparasitic therapies able to surpass the current toxicological, environmental, and economic challenges of the marketplace.

Hamada S, Sutou S, Morita T, Wakata A, Asanami S, Hosoya S, Ozawa S, Kondo K, Nakajima M, Shimada H, et al. **Evaluation of the rodent micronucleus assay by a 28-day treatment protocol: Summary of the 13th Collaborative Study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)-Mammalian Mutagenicity Study Group (MMS).** Environ Mol Mutagen 2001;37(2):93-110.
Abstract: To examine whether micronucleus tests can be incorporated into general toxicology assays, we performed micronucleus tests applying the treatment protocols typically used in such assays. In this 13th Collaborative Study of the CSGMT, both rats and mice were tested, although rats were used in the majority of the studies. Fifteen mutagens were tested in rats, mainly by oral (p.o.) administration. Micronucleus induction was evaluated 2, 3, and 4 days, and 1, 2, 3, and 28 days after the beginning of the treatment in the peripheral blood, and at 28 days in the bone marrow. Of the 15 chemicals that induced micronuclei in rats in short-term assays, two chemicals (1,2-dimethylhydrazine.2HCl and mitomycin C) were negative in all our experiments, possibly because of insufficient dose levels. The remaining 13 were positive within the estimated dose range of a general toxicology assay, suggesting the possibility of integrating the micronucleus assay into general toxicology assays. Three patterns were observed in micronucleus induction during the period of repeated treatment: (1) gradual increases in micronucleus frequency with sequential doses, (2) a peak at 3-5 days followed by gradual decreases in micronucleus frequency with sequential doses, and (3) a rapid increase in micronucleus frequency followed by a plateau. We evaluated factors that might have been involved in those patterns, such as the spleen function, target organ exposure, extramedullary hematopoiesis, hypothermia, and hypoxia. Another factor we considered was dosage. Because the dosages employed in a general toxicity assay are

usually lower than those used in short-term micronucleus assays, this discrepancy was considered the greatest potential problem for integrating the micronucleus assay into general toxicology assays. Our results indicate that the integration of the micronucleus assay into a 28-day toxicological assay is feasible. To serve this purpose, blood samples collected 4 days after the beginning of treatment and blood and bone marrow samples collected at autopsy should be examined. Furthermore, although it is recognized that mice may be suitable for performing independent micronucleus assays, we propose that rats can provide biologically important and relevant information regarding potential chemical mutagens that can be evaluated under conditions used in the conduct of general toxicology studies. Copyright 2001 Wiley-Liss, Inc.

Hamada S, Yamasaki KI, Nakanishi S, Omori T, Serikawa T, Hayashi M. **Evaluation of the general suitability of the rat for the micronucleus assay: the effect of cyclophosphamide in 14 strains.** Mutat Res 2001;495(1-2):127-34.
Abstract: To evaluate the general suitability of the rat for the micronucleus assay, we conducted the assay in males of 14 different strains, 13 inbred (ACI, BN, BUF, COP, DRH, F344, IS, LEW, RCS, SHR, WAG, WKYO, WTC) and 1 outbred (SD), using cyclophosphamide as the test chemical. Cyclophosphamide at 0 (vehicle), 5, 10, or 20mg/kg per day was administered orally twice, 24h apart, to five rats per dosage group. Bone marrow and peripheral blood were collected 24h after the second treatment.All 14 strains showed a positive response to cyclophosphamide, with slight differences in sensitivity. We concluded that the rat is suitable for the micronucleus assay regardless of strain.

Hamers T, van Schaardenburg, Felzel EC, Murk AJ, Koeman JH. **The application of reporter gene assays for the determination of the toxic potency of diffuse air pollution.** Sci Total Environ 2000;262 (1-2):159-74.
Abstract: Diffuse air pollution consists of a mixture of numerous compounds. It is emitted by many distributed sources and is omnipresent due to atmospheric transport. Risk assessment of the complex mixture of air pollutants on the basis of the toxicity of the individual compounds is not yet possible because the chemical identity and/or toxicity of the constituencies of a substantial fraction is unknown. In addition, no adequate procedures are available to integrate toxicity data of such complex mixtures, so that an individual risk assessment of the constituents of air pollution disregards possible combination effects. In the present study, an approach has been developed to assess the toxic potency by using in vitro bio-assay techniques. Genotoxicity was assessed in the umu-assay, a reporter gene assay using a strain of Salmonella typhimurium stably transfected with a plasmid (pSK1002) carrying the SOS-gene umuC fused to the reporter gene lacZ. Arylhydrocarbon-receptor activation was assessed in the DR-CALUX-assay, using a stably transfected H4IIE hepatoma cell line containing a plasmid for the luciferase gene under transcriptional control of dioxin-responsive elements. Samples of airborne parcticulate matter (APM) were collected with a high volume sampler next to a highway and in a natural conservation area. Both assays proved to be applicable to quantify genotoxicity and the presence of polycyclic aromatic hydrocarbons (PAHs) in small extracts from air-filter samples. Results indicate that PAHs from traffic exhausts seem to be largely responsible for an increased genotoxic activity of APM collected down-wind from the highway (western wind). APM collected at eastern wind directions seems to have a different composition of compounds, with a higher genotoxic activity that is less related to highway-emitted PAH-like compounds. At northern wind directions, APM is relatively less genotoxic

and contains less PAHs than at other wind directions. Dioxin-like compounds contribute negligibly to the Ah-receptor agonistic potency of APM. Airborne pollutants with genotoxic and/or PAH-like characteristics form an undesired mutagenic risk, which will be evaluated in further in vivo studies.

Hardouin SN, Nagy A. **Mouse models for human disease.** Clin Genet 2000;57(4):237-44.

Harries HM, Fletcher ST, Duggan CM, Baker VA. **The use of genomics technology to investigate gene expression changes in cultured human liver cells.** Toxicol In Vitro 2001;15(4-5):399-405.
Abstract: The field of genomics has great potential in toxicology; however, the technology is still in its infancy and there are many questions that need to be addressed. In this study we focus on the use of toxicogenomics for the determination of gene expression changes associated with hepatotoxicity. The human hepatoma cell line HepG2 was used to assess the toxic effects of two well-studied hepatotoxins, carbon tetrachloride (CCl(4)) and ethanol (EtOH). Replicate dishes of HepG2 cells were exposed to two concentrations of CCl(4) and EtOH - doses which caused 20% and 50% cell death (as determined by the MTT assay) were chosen [0.18% and 0.4% (v/v) CCl(4); 2.5% and 5% (v/v) EtOH] and the cells exposed for periods of 2 and 24 h. mRNA was extracted and used to probe Atlas Human Toxicology II arrays (Clontech). Preliminary data revealed that following a 2-h exposure at the low doses of both compounds, few changes in gene expression were detected. However, after 24-h exposure of the cells to the same low concentration of both compounds, multiple changes in gene expression were observed, many of which were specific to the individual hepatotoxins, presumably reflecting their different mechanisms of action. CCl(4) treatment of HepG2 cells gave rise to treatment specific up-regulation of genes involved in extracellular transport and cell signalling, whereas EtOH treatment gave rise predominantly to down-regulation of genes involved in stress response and metabolism. In addition, changes in regulation of certain genes (involved in stress response and cell cycle) were common to both treatments. Exposure of HepG2 cells to higher doses of the hepatotoxins gave rise to more changes in gene expression at lower exposure times. These results strongly suggest that different mechanisms of hepatotoxicity may be associated with specific patterns of gene expression, while some genes associated with common cellular responses may be useful as early markers of toxicity.

Hartmann A, Elhajouji A, Kiskinis E, Poetter F, Martus H, Fjallman A, Frieauff W, Suter W. **Use of the alkaline comet assay for industrial genotoxicity screening: comparative investigation with the micronucleus test.** Food Chem Toxicol 2001;39(8):843-58.
Abstract: We evaluated the suitability of the alkaline comet assay as a screening test in industrial routine testing of new chemicals. Thirty-six pharmaceutical compounds with unknown genotoxic potential were tested comparatively in the comet assay and micronucleus test (MNT) using V79 Chinese hamster cells. The comparison of results is generally based on at least two independent experiments, each with two replicate cultures at a minimum of three concentrations. We found a high degree of concordance between results of the comet assay and MNT. All compounds with negative MNT results were also negative in the comet assay. All positive compounds in the comet assay were also positive in the MNT. However, 16 of 38 positive MNT results were negative in the comet assay. Some of the contrary findings may be due to aneugenic effects, which are detected in the MNT but not in the comet assay. However, the majority of the contrary results may be a consequence of cytotoxicity, which can induce elevated micronucleus frequencies but may not lead to positive effects in the comet assay. Additional

data of 39 compounds tested in the Ames test and the comet assay were compared. Four of these compounds that were Ames positive were also positive in the comet assay. However, the comet assay also detected 16 compounds that were negative in the Ames test. We believe that the comet assay in vitro is a useful, fast screening system in mammalian cells that can be used in a test battery during drug development.

Hayashi M, MacGregor JT, Gatehouse DG, Adler ID, Blakey DH, Dertinger SD, Krishna G, Morita T, Russo A, Sutou S. **In vivo rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring.** Environ Mol Mutagen 2000;35(3):234-52.
Abstract: An expert working group on the in vivo micronucleus assay, formed as part of the International Workshop on Genotoxicity Test Procedures (IWGTP), discussed protocols for the conduct of established and proposed micronucleus assays at a meeting held March 25-26, 1999 in Washington, DC, in conjunction with the annual meeting of the Environmental Mutagen Society. The working group reached consensus on a number issues, including: (1) protocols using repeated dosing in mice and rats; (2) integration of the (rodent erythrocyte) micronucleus assay into general toxicology studies; (3) the possible omission of concurrently-treated positive control animals from the assay; (4) automation of micronucleus scoring by flow cytometry or image analysis; (5) criteria for regulatory acceptance; (6) detection of aneuploidy induction in the micronucleus assay; and (7) micronucleus assays in tissues (germ cells, other organs, neonatal tissue) other than bone marrow. This report summarizes the discussions and recommendations of this working group. In the classic rodent erythrocyte assay, treatment schedules using repeated dosing of mice or rats, and integration of assays using such schedules into short-term toxicology studies, were considered acceptable as long as certain study criteria were met. When the micronucleus assay is integrated into ongoing toxicology studies, relatively short-term repeated-dose studies should be used preferentially because there is not yet sufficient data to demonstrate that conservative dose selection in longer term studies (longer than 1 month) does not reduce the sensitivity of the assay. Additional validation data are needed to resolve this point. In studies with mice, either bone marrow or blood was considered acceptable as the tissue for assessing micronucleus induction, provided that the absence of spleen function has been verified in the animal strains used. In studies with rats, the principal endpoint should be the frequency of micronucleated immature erythrocytes in bone marrow, although scoring of peripheral blood samples gives important supplementary data about the time course of micronucleus induction. When dose concentration and stability are verified appropriately, concurrent treatment with a positive control agent is not necessary. Control of staining and scoring procedures can be obtained by including appropriate reference samples that have been obtained from a separate experiment. For studies in rats or mice, treatment/sampling regimens should include treatment at intervals of no more than 24 hr (unless the test Article has a half-life of more than 24 hr) with sampling of bone marrow or blood, respectively, within 24 or 40 hr after the last treatment. The use of a DNA specific stain is recommended for the identification of micronuclei, especially for studies in the rat. In the case of a negative assay result with a non-toxic test Article, it is desirable that systemic exposure to the test Article is demonstrated. The group concluded that successful application of automated scoring by both flow cytometry and image analysis had been achieved, and defined criteria that should be met if automated scoring is employed. It was not felt appropriate to attempt to define specific recommended protocols for automated scoring at the present time. Other

issues reviewed and discussed by the working group included micronucleus assays that have been developed in a number of tissues other than bone marrow. The group felt that these assays were useful research tools that could also be used to elucidate mechanisms in certain regulatory situations, but that these assays had not yet been standardized and validated for routine regulatory application.

He JL, Chen WL, Jin LF, Jin HY. **Comparative evaluation of the in vitro micronucleus test and the comet assay for the detection of genotoxic effects of X-ray radiation.** Mutat Res 2000;469(2):223-31.
Abstract: The genotoxic effects of X-ray radiation on human lymphocytes were measured using the single cell gel electrophoresis (SCGE) assay (comet assay) and the cytokinesis-blocked micronucleus (CBMN) test; both were carried out in vitro on isolated human lymphocytes in order to compare the relationship and sensitivity of these two detecting methods. The radiation-doses were 0.00, 0.02, 0.05, 0.10, 0.25, 0.50, 1.00 and 2.00 Gy. In the comet assay, the average comet length (38.6+/-0.8 microm) of 0.05 Gy was significantly longer than that (29.4+/-1.1 microm) of 0 Gy (P<0.01), moreover, the average comet length increased with the dose of X-ray radiation. In the CBMN, both the average micronucleus rate (MN) and micronucleated cell rate (MNC) of 0.05 Gy were 11.5+/-4.5 per thousand, which showed no difference with that (7.5+/-0.5 per thousand) of 0 Gy (P>0.05). The lowest dose, which induced significant increase of average MN and MNC, was 0.25 Gy. The average MN and MNC rates increased with radiation-dose. The results showed that there was correlation between SCGE and CBMN, and the sensitivity of SCGE was significantly higher than that of CBMN.

Helleday T, Johansson F, Jenssen D. **The DRAG test: an assay for detection of genotoxic damage.** Altern Lab Anim 2001;29(3):233-41.
Abstract: A high throughput assay (the DRAG test) is described, which could be a useful tool for the detection of repairable DNA adducts, and which is based on the inhibition of the growth of DNA repair-deficient Chinese hamster ovary (CHO) cells. The cytotoxicity of a test substance towards DNA repair-deficient CHO cell lines is compared with the corresponding cytotoxicity in the parental wild-type CHO cell line (AA8). A more pronounced toxicity toward a DNA repair-deficient cell line is interpreted as being the consequence of its inability to repair the DNA adduct induced by the compound. (+)-7beta,8alpha-Dihydroxy-9alpha,10alpha-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, camptothecin, ethyl methanesulphonate and mitomycin C were used as reference substances, and the overall results indicate that the DRAG test could be useful in the screening of compounds for the production of repairable DNA adducts. The main advantages with the DRAG test are that it provides a relevant endpoint, it is rapid, it requires small amounts of the test item, and it permits a large number of compounds to be tested.

Helma C, Uhl M. **A public domain image-analysis program for the single-cell gel-electrophoresis (comet) assay.** Mutat Res 2000;466(1):9-15.
Abstract: The single-cell gel electrophoresis (or comet) assay has gained widespread acceptance as a cheap and simple genotoxicity test, but it requires a computer-assisted image-analysis system. As commercial programs are expensive and inflexible, we decided to develop an image-analysis system based on public domain programs and make it publicly available for the scientific community. Our system is based on the scientific image-processing program NIH Image, and was written in its Pascal-like macro language. User interaction was kept as simple as possible, to enable the measurement of a large number of cells with a few keystrokes. Therefore, the time for image analysis is very low, even on

slow computers. The comet macro can be obtained from http://www.predictive-toxicology.org/comet/. NIH Image is available at http://rsb.info.nih.gov/nih-image/. Both programs are free of charge.

Henrich T, Wittbrodt J. **An in situ hybridization screen for the rapid isolation of differentially expressed genes.** Dev Genes Evol 2000;210(1):28-33.
Abstract: To rapidly isolate genes specifically expressed during medaka development we generated a cDNA library enriched for genes expressed in the head region of the developing embryo. Clones were spotted on filters automatically and preselected for abundantly expressed genes by hybridizing them with a probe derived from RNA of undifferentiated totipotent cells. Of the nonhybridizing clones 153 were chosen randomly and further analyzed by whole-mount in situ hybridization. There were 67 selected clones differentially expressed in the developing embryos, and 48 of these were expressed in the developing head. Differentially expressed genes were either of novel type or showed homology to known genes containing DNA binding motifs or to putative housekeeping genes.

Hu VW, Heikka DS, Dieffenbach PB, Ha L. **Metabolic radiolabeling: experimental tool or Trojan horse? (35)S-Methionine induces DNA fragmentation and p53-dependent ROS production.** FASEB J 2001;15(9):1562-8.
Abstract: Despite the general assumption that widely used radiolabeled metabolites such as [(35)S] methionine and (3)H-thymidine do not adversely affect or perturb cell function, we and others have shown that such low-energy beta-emitters can cause cell cycle arrest and apoptosis of proliferating cells. The goal of the present study was to elucidate the targets and mechanisms of [(35)S]methionine-induced cellular toxicity. Comet analyses (single-cell electrophoresis) demonstrated dose-dependent DNA fragmentation in rabbit smooth muscle cells within a time frame (1-4 h) well within that of most radiolabeling protocols, whereas fluorescence analyses using a peroxide/hydroperoxide-sensitive dye revealed production of reactive oxygen species (ROS). Although ROS generation was inhibitable by antioxidants, DNA fragmentation was not inhibited and was in fact observed even under hypoxic conditions, suggesting that beta-radiation-induced DNA damage can occur independently of ROS formation. Studies with p53(+/+) and p53(-/-) human colorectal carcinoma cells further demonstrated the dissociation of early DNA damage from ROS formation in that both cell types exhibited DNA fragmentation in response to radiolabeling whereas only the p53(+/+) cells exhibited significant increases in ROS formation, which occurred well after significant DNA damage was observed. These findings demonstrate that metabolically incorporated low-energy beta-emitters such as [(35)S] methionine and (3)H-thymidine can induce DNA damage, thereby initiating cellular responses leading to cell cycle arrest or apoptosis. The results of this study require a reevaluation using low-energy beta-emitters to follow not only experimental protocols in vivo processes, but also acceptable exposure levels of these genotoxic compounds in the workplace and environment.

Hyrien O, Kles V, Concordet D, Bonneu M, Laurentie M, Sanders P. **Stochastic modeling in toxicokinetics. Application to the in vivo micronucleus assay.** Math Biosci 2001;169(1):27-51.
Abstract: A stochastic model for the in vivo micronucleus assay is presented. This model describes the kinetic of the rate of micronucleated polychromatic erythrocytes induced by the administration of a mutagenic compound. For this, biological assumptions are made both on the erythropoietic system and

on the mechanisms of action of the compound. Its pharmacokinetic profile is also taken into account and it is linked to the induced toxicological effect. This model has been evaluated by analyzing the induction of micronuclei is mice bone marrow by a mutagenic compound, 6-mercaptopurine (6-mp). This analysis enabled to make interesting remarks about the induction of micronuclei by 6-mp and to put to light an unsuspected wavy kinetic by optimizing the experimental design of the in vivo micronucleus assay.

Indra AK, Li M, Brocard J, Warot X, Bornert JM, Gerard C, Messaddeq N, Chambon P, Metzger D. **Targeted somatic mutagenesis in mouse epidermis.** Horm Res 2000;54(5-6):296-300.
Abstract: Gene targeting in the mouse is a powerful tool to study mammalian gene function. The possibility to efficiently introduce somatic mutations in a given gene, at a chosen time and/or in a given cell type will further improve such studies, and will facilitate the generation of animal models for human diseases. To create targeted somatic mutations in the epidermis, we established transgenic mice expressing the bacteriophage P1 Cre recombinase or the tamoxifen-dependent Cre-ER(T2) recombinase under the control of the human keratin 14 (K14) promoter. We show that LoxP flanked (floxed) DNA segments were efficiently excised in epidermal keratinocytes of K14-Cre transgenic mice. Furthermore, Tamoxifen administration to adult K14-Cre-ER(T2) mice efficiently induced recombination in the basal keratinocytes, whereas no background recombination was detected in the absence of ligand treatment. These two transgenic lines should be very useful to analyse the functional role of a number of genes expressed in keratinocytes.

Ivancsits S, Rudiger HW. **Use of the alkaline comet assay to monitor DNA damage in technicians exposed to low-dose radiation.** J Occup Environ Med 2000;42(6):573

Jones C, Kortenkamp A. **RAPD library fingerprinting of bacterial and human DNA: applications in mutation detection.** Teratog Carcinog Mutagen 2000;20(2):49-63.
Abstract: Random amplified polymorphic DNA (RAPD) fingerprinting is a modification of the polymerase chain reaction (PCR), which utilises a single, arbitrarily-chosen primer to amplify a number of fragments from a given template DNA to generate a discrete "fingerprint" when resolved by gel electrophoresis. Alterations by as little as a single base in the primer sequence lead to marked alterations in the fingerprints generated with a given template under optimised conditions. By inference, single base alterations in the genomic template DNA may also lead to changes in the RAPD fingerprints. We have examined this potential application to detect mutations in bacteria and cultured human cells. We have utilised Escherichia coli and human lymphoblastoid cell lines exposed to UV radiation, selected for by cellular mutation assays, and compared RAPD fingerprints of mutant and non-mutant samples. Polymorphisms became evident as the presence and/or absence of DNA fragments between the two samples. A dose-dependent increase in the number of polymorphic bands was seen with UV irradiation of E. coli. To a lesser degree, polymorphisms were also evident for human lymphoblastoid DNA. The possible underlying mechanisms for these alterations in fingerprints as a result of mutation(s) in the primer binding site(s) are discussed. The ability of RAPD fingerprinting to detect a mutant in a population of non-mutants is evaluated, and whilst the lack of sensitivity inherent in the technique precludes its use as a mutation screening assay, its potential for generation of mutant and non-mutant DNA probes for other mutation detection techniques may prove to be of great merit. Teratogenesis Carcinog. Mutagen. 20:49-63, 2000.

Justice MJ. **Capitalizing on large-scale mouse mutagenesis screens.** Nat Rev Genet 2000;1(2):109-15.
Abstract: Variation is the crux of genetics. Mutagenesis screens in organisms from bacteria to fish have provided a battery of mutants that define protein functions within complex pathways. Large-scale mutation isolation has been carried out in Caenorhabditis elegans, Drosophila melanogaster and zebrafish, and has been recently reported in the mouse in two screens that have generated many new, clinically relevant mutations to reveal the power of phenotype-driven screens in a mammal.

Kalapothakis E, Jardim S, Magalhaes AC, Mendes TM, De Marco L, Afonso LC, Chavez-Olortegui C. **Screening of expression libraries using ELISA: identification of immunogenic proteins from Tityus bahiensis and Tityus serrulatus venom.** Toxicon 2001;39(5):679-85.
Abstract: The present report describes the use of ELISA with cDNA expression libraries in the identification of immunogenic proteins. The methodology described was applied using libraries constructed with mRNA isolated from Tityus serrulatus and Tityus bahiensis venom glands. In addition we describe for the first time the sequence of a neurotoxin from Tityus bahiensis venom gland named TbTx5 whose amino acid sequencing showed 93% similarity with the Tityus bahiensis TbTx IV-5 neurotoxin. The methodology described can be used for the generation of an immunogenic bank in order to contribute to genome and proteome projects.

Karlstrom A, Zhong G, Rader C, Larsen NA, Heine A, Fuller R, List B, Tanaka F, Wilson IA, Barbas CF 3rd, et al. **Using antibody catalysis to study the outcome of multiple evolutionary trials of a chemical task.** Proc Natl Acad Sci U S A 2000;97(8):3878-83.
Abstract: Catalytic aldolase antibodies generated by immunization with two different, but structurally related, beta-diketone haptens were cloned and sequenced to study similarities and differences between independently evolved catalysts. Kinetic and sequence analysis coupled with mutagenesis, structural, and modeling studies reveal that the defining event in the evolution of these catalysts was a somatic mutation that placed a lysine residue in a deep, yet otherwise unrefined, hydrophobic pocket. We suggest that covalent chemistries may be as readily selected from the immune repertoire as the traditional noncovalent interactions that have formed the basis of immunochemistry until this time. Further, we believe that these experiments recapitulate the defining events in the evolution of nature's enzymes, parcticularly as they relate to chemical mechanism, catalytic promiscuity, and gene duplication.

Kassie F, Parzefall W, Knasmuller S. **Single cell gel electrophoresis assay: a new technique for human biomonitoring studies.** Mutat Res 2000;463(1):13-31.
Abstract: Human biomonitoring using the single cell gel electrophoresis (SCGE) or comet assay is a novel approach for the assessment of genetic damage in exposed populations. This assay enables the detection of various forms of DNA damage in individual cells with ease and speed and is, therefore, well suited to the analysis of a large group in a population. Here, application of SCGE assay in the identification of dietary protective factors, in clinical studies and in monitoring the risk of DNA damage resulting from occupational, environmental or lifestyle exposures is reviewed. Also, the comparative sensitivity of SCGE assay and conventional cytogenetic tests to detect genetic damage is discussed. Finally, strengths and shortcomings of the SCGE assay are addressed.

Kawakami K. **[Insertional mutagenesis in zebrafish and a proviral insertional mutation hagoromo]**. Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2810-9 [Jpn].

Kawakami K, Amsterdam A, Shimoda N, Becker T, Mugg J, Shima A, Hopkins N. **Proviral insertions in the zebrafish hagoromo gene, encoding an F-box/WD40-repeat protein, cause stripe pattern anomalies.** Curr Biol 2000;10(8):463-6.
Abstract: The zebrafish, Danio rerio, has three types of pigment cells (melanophores, xanthophores and iridophores) and, in adult fish, these cells are organized into a stripe pattern. The mechanisms underlying formation of the stripe pattern are largely unknown. We report here the identification and characterization of a novel dominant zebrafish mutation, hagoromo (hag), which was generated by insertional mutagenesis using a pseudotyped retrovirus. The hag mutation caused disorganized stripe patterns. Two hag mutant alleles were isolated independently and proviruses were located within the fifth intron of a novel gene, which we named hag, encoding an F-box/WD40-repeat protein. The hag gene was mapped to linkage group (LG)13, close to fgf8 and pax2.1. Amino acid sequence similarity, conserved exon-intron boundaries and conserved synteny indicated that zebrafish hag is an ortholog of mouse Dactylin, the gene mutated in the Dactylaplasia (Dac) mouse [1]. The Dac mutation is dominant and causes defects in digit formation in fore- and hindlimbs. This study revealed that the hag locus is important for pattern formation in fish but is involved in distinct morphogenetic events in different vertebrates.

Kawakami K, Shima A, Kawakami N. **Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage.** Proc Natl Acad Sci U S A 2000;97(21):11403-8.
Abstract: The Tol2 element of the medaka fish Oryzias latipes belongs to the hAT family of transposons (hobo/Ac/Tam3). We report here identification of a functional transposase of Tol2 that is capable of catalyzing its transposition in the germ line of zebrafish Danio rerio. A transcript produced from Tol2 encodes a putative transposase. Zebrafish fertilized eggs were coinjected with mRNA transcribed in vitro, using cDNA of the Tol2 transcript as a template and a plasmid DNA harboring a mutant Tol2, which had a deletion in the putative transposase gene but retained necessary cis sequences. The injected fish were raised to adulthood and mated to noninjected fish, and genomic DNA of the progeny fish were analyzed by PCR and Southern hybridization. Half of F(1) fish obtained from one of eight injected fish contained the Tol2 DNA in their genomes but not the vector portion. Among these F(1) fish, Tol2 insertions at four different loci were identified, and some F(1) fish carried two or three different Tol2 insertions, indicating that the germ line of the founder fish is highly mosaic. Sequencing analyses revealed that, in all cases, Tol2 was surrounded by zebrafish genomic sequences, and an 8-bp duplication was created at the target site, indicating that Tol2 was integrated in the zebrafish genome through transposition. This study identifies an autonomous member of a DNA-based transposable element from a vertebrate genome. The Tol2 transposon system should thus be used to develop novel transgenesis and insertional mutagenesis methods in zebrafish and possibly in other fishes.

Kaya B, Yanikoglu A, Creus A, Marcos R. **Genotoxicity testing of five herbicides in the Drosophila wing spot test**. Mutat Res 2000;465(1-2):77-84.
Abstract: Four triazine herbicides: amitrole, metribuzin, prometryn and terbutryn, and the bipyridal

compound diquat dibromide have been evaluated for genotoxicity in the wing somatic mutation and recombination test of Drosophila melanogaster, following standard procedures. Third-instar larvae trans-heterozygous for the third chromosome recessive markers multiple wing hairs (mwh) and flare-3 (flr(3)) were chronically fed with different concentrations of the test compounds. Feeding ended with pupation of the surviving larvae. Genetic changes induced in somatic cells of the wing's imaginal discs lead to the formation of mutant clones on the wing blade. Point mutation, chromosome breakage and mitotic recombination produce single spots; while twin spots are produced only by mitotic recombination. Exposure to 0.5 mM and 1 mM of amitrole clearly increased the frequency of small single, large single and total spots. Terbutryn, at the concentration of 5 mM, induced a slight increase in the frequency of small single and total spots, but this result could be false positive. The other three herbicides tested did not show any genotoxic effect. When heterozygous larvae for mwh and the multiple inverted TM3 balancer chromosomes were treated, significant increases in the frequency of mutant spots were only detected for amitrole. The observed spot frequencies were lower than those found in mwh/flr(3)50%) of the total spot induction was due to mitotic recombination.

Kevekordes S, Spielberger J, Burghaus CM, Birkenkamp P, Zietz B, Paufler P, Diez M, Bolten C, Dunkelberg H. **Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line Hep-G2: results with 15 naturally occurring substances.** Anticancer Res 2001;21(1A):461-9.
Abstract: To examine the concordance of two metabolizing systems for use in genotoxocity testing with the micronucleus test, 15 naturally occurring substances (arecoline, the plant extract aristolochic acid, beta-asarone, benzyl acetate, coumarin, emodine, isatidine dihydrate, monocrotaline, psoralen, reserpine, retrorsine, safrole, sanguinarine chloride, tannin and thiourea) were tested for their genotoxicity in the cytokinesis-block micronucleus test in vitro with human lymphocytes and in the presence and the absence of an exogenous metabolizing system from rat liver S9-mix and the metabolically competent human hepatoma cell line Hep-G2. Arecoline, the plant extract aristolochic acid, psoralen and tannin caused a significant increase in the number of micronuclei in human lymphocytes in the presence and the absence of an exogenous metabolising system from rat liver S9-mix and the metabolically competent human hepatoma cell line Hep-G2. A significant increase in the number of micronuclei with beta-asarone, coumarin, monocrotaline and retrorsine could be detected in the presence of S9-mix and the cell line Hep-G2. Benzyl acetate, emodine, isatidine dihydrate, reserpine, safrole, sanguinarine chloride and thiourea did not reveal any micronucleus inducing activity in either human lymphocytes or in Hep-G2. In addition to the other Hep-G2 results in the literature, this human hepatoma cell line could have a useful potential in the in vitro micronucleus test.

Khromov-Borisov NN, Picada JN, Henriques JA. **Dose finding in the Ames Salmonella assay.** Mutat Res 2000;453(1):35-44.
Abstract: Threshold dose/concentration values, such as the lowest effective dose, minimum effective dose or the lowest effective concentration (LED, MED or LEC, respectively) are in use as an alternative to the mutagen potency measures based on the 'rate' measurements (e.g., the slope of the initial part of the dose-response curve). In this respect, several statistical procedures for the corresponding so-called 'dose finding' were proposed during the last decades. However, most of them disregard the discrete nature of responses such as the plate colony count in the Ames Salmonella assay. When the plate counts

agree with the Poisson assumption, two procedures considered here seem to be appropriate for the dose finding. One is based on the stepwise collapsing of the homogeneous control and dose counts; another consists of constructing the confidence limits for the mutation induction factor (MIF). When the dose and control counts are non-overlapping, the simple 'visual' non-parametric estimation of LED is possible. Applicability and validity of the methods is demonstrated with the two data sets on the mutagenicity of the beta-carboline alkaloid, harmine, and one of the oxidation products of apomorphine.

Kim BS, Cho M, Kim HJ. **Statistical analysis of in vivo rodent micronucleus assay.** Mutat Res 2000;469(2):233-41.
Abstract: The in vivo rodent micronucleus assay (MNC) is widely used as a cytogenetic assay to detect the clastogenic activity of a chemical in vivo. MNC is one of three tests in a battery recommended by the fourth International Conference on Harmonization (ICH4) of Genotoxicity Guidelines. As such it has been accepted by many regulatory authorities. However, the determination of a positive result in a genotoxicity test, including MNC, has been an issue of debate among toxicologists and biometricians. In this presentation we compare several statistical procedures that have been suggested for the analysis of MNC data and indicate which one is the most powerful. The standard protocol of MNC has at least three dose levels plus the control dose and uses at least four animals per group. For each animal, 2000 polychromatic erythrocytes (PCE) are counted. Two statistical procedures can be employed, either alone or jointly, for the analysis of the MNC dose-response curve. These are the Cochran-Armitage (C-A) trend test and the Dunnett type test. For performing Dunnett type tests, toxicologists often use negative historical control rate for the estimate of the concurrent negative control rate. Some toxicologists emphasize the reproducibility of assay results instead of the dose-response relationship for the important criterion [J. Ashby, H. Tinwell, Mutat. Res. 327 (1995) 49-55; for the rebuttal see M. Hayashi, T. Sofuni, Mutat. Res. 331 (1995) 173-174]. The following three procedures are currently employed in toxicology labs for the evaluation of MNC result. The assay response is deemed positive if it is detected by (i) the C-A trend test alone, (ii) both the C-A trend test and the Dunnett type test and (iii) either the C-A trend test or the Dunnett type test. Using Monte Carlo simulation, we first find for each procedure, sizes of tests which yield the experiment-wise type I error rate of 0.05 and show that the procedure (ii) is the most powerful against the alternatives of monotone increase. The procedure (ii) which originated from Hayashi's three-step procedure was coded in C and termed 'MNC'. The MNC software program is available in the public domain through the ftp.

Kim BS, Zhao B, Kim HJ, Cho M. **The statistical analysis of the in vitro chromosome aberration assay using Chinese hamster ovary cells.** Mutat Res 2000;469(2):243-52.
Abstract: The purpose of the in vitro chromosome aberration assay (ABS) is to determine whether the test compound is a clastogen, i.e. induces structural changes in chromosomes. Details of this assay can be found in Galloway et al. [S.M. Galloway, M. Aardema, M. Ishidate Jr, J.L. Ivett, D.J. Kirkland, M. Takeshi, P. Mosesso, T. Sofuni, Mutation Res. 312 (1994) 241-261]. The standard design consists of a negative control and at least three positive dose groups. At each dose, a sample, say 200, of metaphase cells is examined microscopically and cells exhibiting at least one type of chromosome aberration are identified. Using Chinese hamster ovary cells, Margolin et al. [B.H. Margolin, M.A. Resnick, J.Y. Rimpo, P. Archer, S.M. Galloway, A.D. Bloom, E. Zeiger, Environ. Mutagen. 8 (1986) 183-204] and Richardson et al. [C. Richardson, D.A. Williams, J.A. Allen, G. Amphlett, D.O. Chanter, B. Phillips,

Analysis of data from in vitro cytogenetic assays, in: D.J. Kirkland (Ed.), Statistical Evaluation of Mutagenicity Test Data, Cambridge University Press, Cambridge, 1989, pp. 141-154] demonstrated that a binomial sampling model could be used to describe the proportion of cells with chromosome aberrations.Statisticians and toxicologists have also suggested evaluation criteria for the dose response pattern of ABS. Margolin et al. [B.H. Margolin, M.A. Resnick, J.Y. Rimpo, P. Archer, S.M. Galloway, A.D. Bloom, E. Zeiger, Environ. Mutagen. 8 (1986) 183-204] suggested one use the Cochran-Armitage trend test. Sofuni et al. [T. Sofuni, A. Matsuoka, M. Sawada, M. Ishidate Jr, E. Zeiger, M.D. Shelby, Mutation Res. 241 (1990) 175-213] considered the dose response to be (strong) positive if it had two significant doses out of three dose groups and decided it was weakly positive if it had only one significant dose and there was a significant trend. The criterion of Galloway et al. for a positive response was a clear dose-related increase in cells with structural aberrations in one experiment or a reproducible single positive dose [S.M. Galloway, M. Aardema, M. Ishidate Jr, J.L. Ivett, D.J. Kirkland, M. Takeshi, P. Mosesso, T. Sofuni, Mutation Res. 312 (1994) 241-261].We formulate the above three procedures in terms of a Cochran-Armitage trend test and a Dunnett type test. We then compare the performance of these three procedures in terms of a Monte Carlo simulation study. We then develop a software program from the chosen procedure for its ease of use by statisticians and toxicologists.

Kim M, Sun ZY, Byron O, Campbell G, Wagner G, Wang J, Reinherz EL. **Molecular dissection of the CD2-CD58 counter-receptor interface identifies CD2 Tyr86 and CD58 Lys34 residues as the functional "hot spot".** J Mol Biol 2001;312(4):711-20.
Abstract: The heterophilic CD2-CD58 adhesion interface contains interdigitating residues that impart high specificity and rapid binding kinetics. To define the hot spot of this counter-receptor interaction, we characterized CD2 adhesion domain variants harboring a single mutation of the central Tyr86 or of each amino acid residue forming a salt link/hydrogen bond. Alanine mutations at D31, D32 and K34 on the C strand and K43 and R48 on the C' strand reduce affinity for CD58 by 47-127-fold as measured by isothermal titration calorimetry. The Y86A mutant reduces affinity by approximately 1000-fold, whereas Y86F is virtually without effect, underscoring the importance of the phenyl ring rather than the hydroxyl moiety. The CD2-CD58 crystal structure offers a detailed view of this key functional epitope: CD2 D31 and D32 orient the side-chain of CD58 K34 such that CD2 Y86 makes hydrophobic contact with the extended aliphatic component of CD58 K34 between CD2 Y86 and CD58 F46. The elucidation of this hot spot provides a new target for rational design of immunosuppressive compounds and suggests a general approach for other receptors.

Kim SR, Matsui K, Yamada M, Gruz P, Nohmi T. **Roles of chromosomal and episomal dinB genes encoding DNA pol IV in targeted and untargeted mutagenesis in Escherichia coli.** Mol Genet Genomics 2001;266(2):207-15.
Abstract: DNA polymerase IV (pol IV) in Escherichia coli is a member of a novel family of DNA polymerases (the DinB/UmuC/Rad30/Rev1 super-family or the DNA polymerase Y family). Although expression of the dinB gene encoding DNA pol IV is known to result in an enhancement of untargeted mutagenesis, it remains uncertain whether DNA pol IV is involved in a variety of lesion-induced mutagenesis (targeted mutagenesis), and the relationship between expression levels of dinB and the mutagenesis that DNA pol IV promotes has not been investigated thoroughly. Here, we report that DNA pol IV is involved in -1 frameshift mutagenesis induced by 4-nitroquinoline N-oxide (4-NQO) and that

the expression level of the chromosomal pol IV gene is 6-12 times higher than those for other SOS-inducible DNA polymerases in E. coli, i.e., DNA pol II (PolB) or DNA pol V (UmuDC), respectively. Interestingly, the dinB gene is present not only on the chromosome but also on the F' plasmid in the E. coli CC108 strain. In this strain, 750 molecules of DNA pol IV are expressed from the F' dinB gene in the uninduced state and 250 molecules are expressed from the chromosomal gene. These cellular expression levels strongly affect -1 frameshifts induced by 4-NQO in runs of six guanine bases: mutagenicity was highest in the strain CC108, followed by strains YG2242 (chromosome deltadinB/F' dinB+), YG2247 (chromosome dinB+/F' deltadinB) and FC1243 (chromosome deltadinB/F' deltadinB). The incidence of untargeted -1 frameshifts was reduced by two-thirds on deletion of dinB from the F' episome. The chromosomal dinB gene appeared to have little or no effect on the untargeted mutagenesis. These results suggest that DNA pol IV efficiently mediates targeted mutagenesis by 4-NQO, and that the cellular levels of expression substantially affect targeted and untargeted mutagenesis.

Kinoshita M, Tanaka M, Yamashita M. **[Development of transgenic fish strain and promoter analysis].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2954-61 [Jpn].

Kirsch-Volders M, Fenech M. **Inclusion of micronuclei in non-divided mononuclear lymphocytes and necrosis/apoptosis may provide a more comprehensive cytokinesis block micronucleus assay for biomonitoring purposes.** Mutagenesis 2001;16(1):51-8.
Abstract: Human biomonitoring of early genetic effects requires accurate, sensitive and, if possible, easy and not too time-consuming methodologies to assess mutations. One of the most promising methodologies at the present time is the cytokinesis block micronucleus (MN) assay (CBMN), which detects both chromosome breakage and chromosome loss in once-divided binucleated (BN) cells. Many studies have been published with this methodology, but before its extensive application is recommended, it is necessary to evaluate its strengths and limitations. Recently, Fenech et al. reviewed the advantages of the CBMN assay for biomonitoring purposes. However, up to now information present in mononucleated (MONO) cells has rarely been taken into account, although it might be complementary to that assessed in BN cells. Indeed, MONO cells should indicate damage which was present in vivo before the start of culture and BN cells may contain pre-existing micronuclei (MNi) plus lesions which are expressed as MNi during in vitro culture. To address this question, the objectives of this paper were as follows. (i) To situate the CBMN assay in a historical and mechanistic perspective. (ii) To consider whether impaired mitotic capacity in vitro may be responsible for false negative biomonitoring studies if MN in MONO cells are not taken into account in the CBMN test. The following factors were considered: division delay for repair and mitotic block, in vitro apoptosis and necrosis of damaged cells, mitotic slippage and correlation between MN expression in vitro versus in vivo. (iii) To analyse the factors which may cause a negative result in the CBMN assay in biomonitoring when exposure to specific genotoxins is evident. The specific effects of aneugens and of adaptive responses to chronic low level exposure were examined. (iv) To compare the sensitivity of MONO and BN cells in relation to the genotoxic mechanism. (v) To propose an adequate sampling scheme to study MN in both MONO and BN cells. It was concluded that a more comprehensive assessment of DNA damage may be achieved if the CBMN assay includes measures of: (i) MNi in MONO cells; (ii) MNi in BN cells; (iii) apoptotic cells; (iv) necrotic cells. It is probable that the 24 h post-phytohaemagglutinin time point may be the optimal time to assess the frequency of MNi in MONO cells, apoptotic cells and necrotic cells. It is also

practical to include these measures when scoring MNi in BN cells after cytokinesis block.

Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate M Jr, Lorge E, Norppa H, Surralles J, et al. **Report from the In Vitro Micronucleus Assay Working Group.** Environ Mol Mutagen 2000;35(3):167-72.
Abstract: At the Washington International Workshop on Genotoxicity Test Procedures (March 25-26, 1999), the current methodologies and data for the in vitro micronucleus test were reviewed. From this, guidelines for the conduct of specific aspects of the protocol were developed. Because there are a number of important in vitro micronucleus validation studies in progress, it was not possible to design a definitive, internationally harmonized protocol at this time. Agreement was achieved on the following topics: Cells. The choice of cells is flexible, yet the choice of cell type should be justified and take into consideration doubling time, spontaneous frequency of micronuclei, and genetic background. Slide preparation. A fixation method that preserves the cytoplasm and cytoplasmic boundaries, and minimizes clumping should be used. Use of fluorescent DNA-specific dyes is encouraged for better detection of small micronuclei. Analysis. Micronuclei should have a diameter less than one-third of the main nucleus, and should be clearly distinguishable from the main nucleus. In the cytokinesis-block method, binucleated cells selected for analysis should have two clearly distinguishable main nuclei. Cells where the main nucleus(ei) is undergoing apoptosis should not be scored for micronuclei because the assumed micronuclei may have been the result of nuclear fragmentation during the apoptotic process. Toxicity. Cytotoxicity can be measured by various methods including cell growth, cell counts, nucleation (i.e., percent binucleated), division/proliferation index, confluence. A majority of the group recommended that the highest concentration should induce at least 50% cytotoxicity (by whatever measure is selected). Cytochalasin B. There is much debate regarding the use of cytochalasin B. For human lymphocytes, the use of cytochalasin B (6 &mgr;g/ml [lymphocytes cultured from whole blood cells] and 3-6 &mgr;g/ml [isolated lymphocyte cultures]) is recommended. For cell lines, because there were no definitive data showing a clear advantage or disadvantage of the use of cytochalasin B for a variety of chemicals, the majority opinion of the group was that at this time, the use of cytochalasin B for cell lines is considered optional. Further studies (many chemicals of a variety of potencies, tested both with and without cytochalasin B) are clearly needed to resolve this issue. Number of doses. At least three concentrations should be scored for micronuclei. Treatment/harvest times. At this time, there are not enough data to define the most appropriate treatment/harvest times. Following the principles of the in vitro metaphase assay (with or without metabolic activation), it was agreed that there was a need for a short treatment followed by a recovery time in the absence of test chemical, there was a need for a long treatment (maybe with and without recovery time), and ideally, treatment should cover cells in different cell cycle stages.

Klemm M, Genschow E, Pohl I, Barrabas C, Liebsch M, Spielmann H. **Permanent embryonic germ cell lines of BALB/cJ mice - an in vitro alternative for in vivo germ cell mutagenicity tests.** Toxicol In Vitro 2001;15(4-5):447-53.
Abstract: To offer a sensitive and predictive in vitro method to assess germ cell mutagenicity, we established primordial germ (PG) cell-derived permanent female and male embryonic germ (EG) cell lines of the mouse (strain BALB/cJ). The differences in developmental sensitivity of EG cells and differentiated fibroblast cells of the mouse cell line 3T3 to genotoxicants were tested comparatively

under identical test conditions. Cytotoxicity assay was measured by the MTT test and genotoxic effects were determined by sister chromatid exchanges (SCE) rates induced by standard reference mutagens. Both methods are used to assign the chemicals to two classes of in vivo reproductive toxicity, non- and strongly genotoxic to germ cells. Applying linear discriminant analysis, a biostatistical prediction model (PM) was developed for the female cell line EG(3). This procedure identified a single variable, the Ig (SCE(200)EG(3)) as the statistically significant concentration related increase of 200% in the mean number of SCEs per metaphase spread after 3 h of exposure to be sufficient for separation into the classes: non- and strongly genotoxic to germ cells. Applying this PM to the training set of five genotoxic and three non-genotoxic test chemicals, 100% correct classifications were obtained.

Klinakis AG, Zagoraiou L, Vassilatis DK, Savakis C. **Genome-wide insertional mutagenesis in human cells by the Drosophila mobile element Minos.** EMBO Rep 2000;1(5):416-21.
Abstract: The development of efficient non-viral methodologies for genome-wide insertional mutagenesis and gene tagging in mammalian cells is highly desirable for functional genomic analysis. Here we describe transposon mediated mutagenesis (TRAMM), using naked DNA vectors based on the Drosophila hydei transposable element Minos. By simple transfections of plasmid Minos vectors in HeLa cells, we have achieved high frequency generation of cell lines, each containing one or more stable chromosomal integrations. The Minos-derived vectors insert in different locations in the mammalian genome. Genome-wide mutagenesis in HeLa cells was demonstrated by using a Minos transposon containing a lacZ-neo gene-trap fusion to generate a HeLa cell library of at least 10(5) transposon insertions in active genes. Multiple gene traps for six out of 12 active genes were detected in this library. Possible applications of Minos-based TRAMM in functional genomics are discussed.

Kocabas NA, Karahalil B, Karakaya AE, Sardas S. **Influence of GSTM1 genotype on comet assay and chromosome aberrations after induction by bleomycin in cultured human lymphocytes.** Mutat Res 2000;469(2):199-205.
Abstract: Investigators have demonstrated that the mutagen sensitivity assay, based on the quantification of bleomycin (BLM)-induced chromatid breaks in short-term cultured peripheral lymphocytes, can be a marker of cancer susceptibility. Although many factors can contribute to variability in human biomonitoring studies, genetic susceptibility (the influence of polymorphic metabolising genes on response to environmental mutagens) should be considered whenever appropriate. Glutathione-S-transferases (GSTs) encode a family of detoxifying phase II enzymes catalysing the conjugation of glutathione to electrophilic compounds. Studies on Caucasians indicate that about 45% of individuals lack the glutathione-S-transferase M1 (GSTM1, null) enzyme, and are therefore, theoretically at a higher risk to the toxic effects of chemicals. The aim of the present study was to investigate this hypothesis further by evaluating whether the GSTM1 genotype influences the backround level of DNA damage and the induction of chromosomal aberrations by BLM in peripheral-blood lymphocytes. The alkaline comet assay was used to evaluate background levels of DNA damage in unstimulated lymphocytes while standard cytogenetic techniques were used in mitogen-stimulated lymphocytes treated with BLM. Without BLM treatment, individuals with the GSTM1 null genotype had no significant difference in frequencies of damaged cells by comparison to individuals with the GSTM1 genotype. Also, no significant differences between the two groups of individuals (GSTM1 positive and GSTM1 null) were observed for BLM-induced chromosomal aberrations.

Koga A, Hori H. **Detection of de novo insertion of the medaka fish transposable element Tol2.** Genetics 2000;156(3):1243-7.
Abstract: Tol2 is a terminal-inverted-repeat transposable element of the medaka fish Oryzias latipes. It is a member of the hAT (hobo/Activator/Tam3) transposable element family that is distributed in a wide range of organisms. We here document direct evidence for de novo insertion of this element. A Tol2 clone marked with the bacterial tetracycline-resistance gene was microinjected into fertilized eggs together with a target plasmid, and the plasmid was recovered from embryos. The screening of plasmid molecules after transformation into Escherichia coli demonstrated transposition of tet into the plasmid and, by inference, precise insertion of Tol2 in medaka fish cells. De novo excision of Tol2 has previously been demonstrated. The present study provides direct evidence that the Tol2 element has the entire activity necessary for cut-and-paste transposition. Some elements of the mariner/Tc1 family, another widespread group, have already been applied to development of gene tagging systems in vertebrates. The Tol2 element of the hAT family, having different features from mariner/Tc1 family elements, also has potential as an alternative gene tagging tool in vertebrates.

Koga A, Hori H. **The Tol2 transposable element of the medaka fish: an active DNA-based element naturally occurring in a vertebrate genome.** Genes Genet Syst 2001;76(1):1-8.
Abstract: Several DNA-based transposable elements are known to be present in vertebrate genomes, but few of them have been demonstrated to be active. The Tol2 element of the medaka fish is one such element and, therefore, is potentially useful for developing a gene tagging system and other molecular biological tools applicable to vertebrates. Towards this goal, analyses of the element at the molecular, cellular and population levels are in progress. Results so far obtained are described here.

Koga A, Shimada A, Shima A, Sakaizumi M, Tachida H, Hori H. **Evidence for recent invasion of the medaka fish genome by the Tol2 transposable element.** Genetics 2000;155(1):273-81.
Abstract: Tol2 is a transposable element of the terminal-inverted-repeat class, residing in the genome of the medaka fish Oryzias latipes. The genus Oryzias contains more than 10 species for which phylogenetic relationships have previously been estimated. To infer the history of Tol2 in this genus we performed genomic Southern blots and PCR analyses of 10 of the species. It was revealed that Tol2 occurs in 2 of the 10 species (O. curvinotus and O. latipes) and that the length and the restriction map structure of Tol2 are identical in the two cases. Further, sequencing analysis revealed an extremely low level of divergence compared with that in a nuclear gene. These results suggest recent incorporation of Tol2 into one or both of the two species, implying horizontal transfer of Tol2 from one species to the other or into them both from a common source.

Kong Q, Maizels N. **Breaksite batch mapping, a rapid method for assay and identification of DNA breaksites in mammalian cells.** Nucleic Acids Res 2001;29(6):E33
Abstract: DNA breaks occur during many processes in mammalian cells, including recombination, repair, mutagenesis and apoptosis. Here we report a simple and rapid method for assaying DNA breaks and identifying DNA breaksites. Breaksites are first tagged and amplified by ligation-mediated PCR (LM-PCR), using nested PCR primers to increase the specificity and sensitivity of amplification. Breaksites are then mapped by batch sequencing LM-PCR products. This allows easy identification of

multiple breaksites per reaction without tedious fractionation of PCR products by gel electrophoresis or cloning. Breaksite batch mapping requires little starting material and can be used to identify either single- or double-strand breaks.

Koo SH, Cunningham MC, Arabshahi B, Gruss JS, Grant JH 3rd. **The transforming growth factor-beta 3 knock-out mouse: an animal model for cleft palate**. Plast Reconstr Surg 2001;108(4):938-48; discussion 949-51.
Abstract: The recent report of a transforming growth factor-beta 3 (TGF-beta 3) knock-out mouse in which 100 percent of the homozygous pups have cleft palate raised the question as to the potential usefulness of these animals as a model for cleft palate research. The specific aim in this study was to carefully document the anatomy of the cleft palate in the TGF-beta 3 knock-out mice as compared with wild type controls. Special attention was paid to the levator veli palatini muscle, the tensor veli palatini muscle, and their respective innervation.Because the TGF-beta 3 knock-out is lethal in the early perinatal period and because the heterozygotes are phenotypically normal, polymerase chain reaction was required to genotype the animals before mating. Time-mated pregnancies between proven heterozygotes were then delivered by cesarean section at gestational day 18.5 to prevent maternal cannibalism of homozygote pups. All delivered pups were killed and their tails processed by polymerase chain reaction to verify genotype. The heads were then fixed and sectioned in axial, coronal, or sagittal planes. Sections were stained with hematoxylin and eosin or processed for immunohistochemistry with nerve specific protein gene product 9.5 and calcitonin gene-related peptide antibodies. Sections were analyzed in a serial fashion. Nine wild type control animals were analyzed along with nine TGF-beta 3 knock-out homozygotes.Time matings between proven heterozygotes yielded wild type pups, heterozygote pups, and homozygote knock-out pups in the expected mendelian ratios (28 percent to 46 percent to 26 percent; n = 43). The results demonstrated 100 percent clefting in the homozygous TGF-beta 3 knock-out pups. Complete clefting of the secondary palate was seen in four of nine and incomplete clefting was seen in five of nine. The levator veli palatini and tensor veli palatini muscles were demonstrated coursing parallel to the cleft margin in all cleft mice. The orientation of these muscles differs from the normal transverse sling of the levator veli palatini muscle and the normal palatine aponeurosis of the tensor veli palatini muscle at the soft palate in control animals. Innervation of the levator veli palatini muscle by cranial nerve IX and the tensor veli palatini muscle by cranial nerve V were demonstrated in both cleft and control animals by use of immunohistochemistry with nerve-specific antibodies.Demonstration of a teratogen-free, reproducible animal model of clefting of the palate with a known, single-gene etiology is an important step in the systematic understanding of a congenital defect whose multifactorial etiology has hampered previous research efforts. This study presents a detailed anatomic description of such a model, including a description of the muscular anatomy and the innervation of the muscles of the palate. Because of early perinatal mortality, this model has limited applications for postnatal studies.

Kopjar N, Garaj-Vrhovac V. **Application of cytogenetic endpoints and Comet assay on human lymphocytes treated with vincristine in vitro.** Neoplasma 2000;47(3):162-7.
Abstract: The genotoxic potential of vincristine is assessed on human peripheral blood lymphocytes following administration of the drug at a dose 0.0875 microg/ml by use of single cell gel electrophoresis - Comet assay (SCGE), analysis of structural chromosome aberrations (CA), micronucleus assay (MN)

and sister chromatid exchange (SCE) analysis. In vitro treatment of human lymphocytes with vincristine was performed on cells in G0 phase, as well on lymphocyte cultures 24 hours after stimulation with mitogen phytohemagglutinine. For the Comet assay at 24, 48 and 72 h the treated cells were embedded in agarose on slides, lysed with alkaline lysis solution and exposed to an electric field. DNA migrated within the agarose and formed comets whose length depends on the amount of DNA damage. For the analysis of structural CA cells were grown on F-10 medium for 48 hours, and for MN and SCE analysis for 72 hours. The results on SCGE showed an increase in tail length compared to control both in cells treated in G0 and in cells treated 24 h after mitogen stimulation. The amount of DNA damage was higher in cells treated with vincristine 24 h after mitogen stimulation. Administered concentration of drug caused total inhibition of lymphocytes growth in 72-h cultures for MN and SCE analysis indicating strong microtubule distruptive effects of vincristine. Analysis of structural CA reveals chromatid breaks and acentric fragments as the main aberration types both in cells treated in G0 and in cells treated 24 h after mitogen stimulation. Number of these aberrations was higher in cells treated in G0 phase. Results obtained in this study by use of different cytogenetic endpoints confirmed that vincristine exhibits both aneugenic and clastogenic effects on human lymphocytes.

Kopjar N, Garaj-Vrhovac V. **Application of the alkaline comet assay in human biomonitoring for genotoxicity: a study on Croatian medical personnel handling antineoplastic drugs.** Mutagenesis 2001;16(1):71-8.
Abstract: The alkaline comet assay was used to evaluate the genotoxicity towards peripheral blood lymphocytes of medical personnel regularly handling various antineoplastic drugs with different safety precautions. The study population consisted of 50 exposed subjects working in the oncology, pulmonology, gynaecology and haematology units of nine Croatian hospitals and 20 unexposed control subjects. Peripheral blood lymphocytes from the subjects were embedded in agarose on a microscope slide and lysed; the DNA was unwound and subjected to electrophoresis at pH 13. Staining with a fluorescent dye was used to identify cells with DNA damage, as judged by increased migration of genetic material from the cell nucleus. DNA damage was quantified by measuring the displacement between the genetic material of the nucleus and the resulting tail using an image analysis system. Three parameters were used as indicators of DNA damage: i.e. tail length, percentage of DNA in the tail and tail moment. Statistically significant differences in all three parameters were observed between the exposed and control groups. Within the exposed group, there were marked differences between individuals in the comet tail parameters. In the majority of exposed subjects an effect on DNA damage of age or duration of occupational exposure could be excluded. In the exposed group, the highest level of DNA damage was recorded in subjects who used only latex gloves in their work with antineoplastic drugs. The observed DNA damage was lower in exposed subjects who used more than one type of protective equipment and who worked in a well-ventilated safety cabinet. No statistically significant differences were found between the mean values of comet tail parameters for smoking and non-smoking subpopulations from the exposed group. In view of the results obtained, the alkaline comet assay, as a simple, rapid and sensitive method, appears to be a promising additional test for biomonitoring purposes in human populations.

Koppen G, Verschaeve L. **The alkaline single-cell gel electrophoresis/comet assay: a way to study DNA repair in radicle cells of germinating Vicia faba.** Folia Biol (Praha) 2001;47(2):50-4.

Abstract: Dry seeds are known to accumulate DNA damage with time of storage. Repair of DNA lesions during germination of Vicia faba seeds was followed in the radicles using the alkaline single-cell gel electrophoresis/comet assay. In this assay nuclei were liberated, mixed with agarose and spread out over a microscope slide. After lysis of the nuclear membrane and unwinding of the DNA duplex, DNA was stretched during electrophoresis, giving a comet-like migration pattern. The more DNA was damaged, the higher its mobility. DNA repair took place rapidly the first hours of imbibition and more slowly until ca 33 h after onset of germination. A small amount of heavily damaged cells remained present. Labelling with BrdU provided the possibility to localize repair patches and replicated sites in the comet migration pattern. At 15 h of germination, incorporation of BrdU in radicle DNA was situated at random over the entire comet. At 33 h, DNA repair was more or less accomplished and BrdU was mainly localized in the 'heads' of most comets.

Kovalchuk I, Kovalchuk O, Hohn B. **Genome-wide variation of the somatic mutation frequency in transgenic plants.** EMBO J 2000;19(17):4431-8.
Abstract: In order to analyse the frequency of point mutations in whole plants, several constructs containing single nonsense mutations in the beta-glucuronidase (uidA) gene were used to generate transgenic Arabidopsis thaliana plants. Upon histochemical staining of transgenic plants, sectors indicative of transgene reactivation appeared. Reversion frequencies were in the range of 10(-7)-10(-8) events per base pair, exceeding the previous estimates for other eukaryotes at least 100-fold. The frequency was dependent on the position of the mutation substrate within the transgene and the position of the transgene within the Arabidopsis genome. An inverse relationship between the level of transgene transcription and mutation frequency was observed in single-copy lines. DNA-damaging factors induced the mutation frequency by a factor of up to 56 for UV-C, a factor of 3 for X-rays and a factor of 2 for methyl methanesulfonate. This novel plant mutation-monitoring system allowed us to measure the frequencies of point mutation in whole plants and may be used as an alternative or complement to study the mutagenicity of different environmental factors on the higher eukaryote's genome.

Kovalchuk O, Titov V, Hohn B, Kovalchuk I. **A sensitive transgenic plant system to detect toxic inorganic compounds in the environment.** Nat Biotechnol 2001;19(6):568-72.
Abstract: We describe a transgenic plant-based assay to study the genetic effects of heavy metals. Arabidopsis thaliana plants carrying a beta-glucuronidase (GUS) marker gene either with a point mutation or as a recombination substrate were used to analyze the frequency of somatic point mutations and homologous recombination in whole plants. Transgenic test plants sown on media contaminated by the salts of the heavy metals Cd^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, and As_2O_3 exhibited a pronounced uptake-dependent increase in the frequencies of both somatic intrachromosomal recombination and point mutation. The test was applied to monitor the genotoxicity of soils sampled in sites contaminated with several heavy metals. Our results indicate that this is a highly sensitive system for monitoring metal contamination in soils and water.

Kranendonk M, Laires A, Rueff J, Estabrook WR, Vermeulen NP. **Heterologous expression of xenobiotic mammalian-metabolizing enzymes in mutagenicity tester bacteria: an update and practical considerations.** Crit Rev Toxicol 2000;30(3):287-306.
Abstract: There is an increasing need for metabolic competent cell systems for the mechanistic studies of

biotransformation of xenobiotics in toxicology in general and in genotoxicology in parcticular. These cell systems combine the heterologous expression of a parcticular mammalian biotransformation enzyme with a specific target/ end point by which a functional analysis of the expressed gene product in the (geno)toxicity of chemicals can be performed. cDNAs of an increasing number of mammalian biotransformation enzymes is being cloned. The construction of specific expression vectors permits their heterologous expression in laboratory bacteria, such as Escherichia coli strains. This development does not only allow biochemical and enzymatic studies of (pure) enzyme preparations but also facilitates the engineering of metabolically competent mutagenicity tester bacteria, thereby providing new tools for genotoxicity testing and for studying of the roles of biotransformation in chemical carcinogenesis. In this review, we describe an update as well as an evaluation of enzymes expressed in mutagenicity tester bacteria. Four types of biotransformation enzymes are now expressed in these bacteria, namely, GSTs, CYPs, NATs, and STs. The expression of these enzymes in the tester bacteria and their subsequent application in mutagenicity assays demonstrates that heterologous expression in this type of bacteria has a number implications for the functionality of the biotransformation enzymes as well as for the functioning of the tester bacteria in mutagenicity detection. We also describe here a number of practical considerations in this regard.

Krishna G, Hayashi M. **In vivo rodent micronucleus assay: protocol, conduct and data interpretation.** Mutat Res 2000;455(1-2):155-66.
Abstract: In vivo rodent micronucleus assay has been widely used to detect genotoxicity. Evaluation of micronucleus induction is the primary in vivo test in a battery of genotoxicity tests and is recommended by the regulatory agencies around the globe to be conducted as part of product safety assessment. The assay, when performed appropriately, detects both clastogenicity and aneugenicity. Methods for performing micronucleus evaluation have evolved since its initial description in the 1970s. In recent years, the focus has been directed toward improving micronucleus detection with high efficiency by proposing data-based recommendations to the standard initial protocol design. Such improvements include, e.g., the use of appropriate harvest time(s), inclusion of one or both sexes, number of doses tested, limit dose, integrating micronucleus assessment into the routine toxicology studies, use of fluorescent staining, automation of micronucleus detection and assessment of micronuclei in multiple tissues. This protocol paper describes: the mechanism of micronucleus formation, a generalized protocol for manual detection, enumeration of micronuclei, and data interpretation in light of published information thus far, on the regulatory aspects of this assay. Certain recent protocol issues that are practical in nature are equally valid in relation to standard manual method and provide robust database, which are also included for consideration. It is expected that such improvements of the protocol will continue to drive the utility of this assay in the product safety assessment.

Krishna G, Urda G, Paulissen J. **Historical vehicle and positive control micronucleus data in mice and rats.** Mutat Res 2000;453(1):45-50.
Abstract: The rodent bone marrow micronucleus (MN) assay has been widely used as part of an in vivo genotoxicity test battery in product safety evaluation. In this assay, the historical vehicle and positive control data form an important component in the assay performance and data interpretation. Also, in light of minimizing animal use in research and still obtain required data from a study, the routine use of positive control in every MN assay has been questioned by the scientific community, especially in

laboratories which have demonstrated assay reproducibility and conduct studies under Good Laboratory Practice regulations. In this paper, mouse and rat vehicle and positive control MN data, collected manually, are described as a reference for a period of 12 years (1987-1998) in our laboratory. The vehicles generally included a variety of aqueous solutions and suspensions and cyclophosphamide dosed intraperitoneally at 20mg/kg (rats) or 40 mg/kg (mice) served as positive control, in all studies. Based on combined sex data (430 animals), for CD(1) mice, the vehicle control MN polychromatic erythrocyte (PCE) range was 0.9-3.1 with a mean of 1.75 per 1000 PCE and the positive control range (220 animals) was 8.8-42.1 with a mean of 23.1 MNPCE per 1000 PCE. Similarly, for Wistar rats, the vehicle control range (360 animals) was 1.3-5.3 with a mean of 2.6 MNPCE per 1000 PCE and the positive control range (240 animals) was 10.4-33.8 MNPCE per 1000 PCE. Vehicle control ranges reported here are comparable to the literature database and the positive control response was > or = 4-fold over vehicle control, in all studies. These data demonstrate the reproducibility of positive control response in MN assay in our laboratory and support the MN Assay Expert Panel's view that the use of positive control may not be necessary in every study.

Krul C, Luiten-Schuite A, Tenfelde A, van Ommen B, Verhagen H, Havenaar R. **Antimutagenic activity of green tea and black tea extracts studied in a dynamic in vitro gastrointestinal model.** Mutat Res 2001;474(1-2):71-85.
Abstract: An in vitro gastrointestinal model, which simulates the conditions in the human digestive tract, was used to determine potential antimutagenic activity of extracts of black tea and green tea. In this paper, results are presented on the availability for absorption of potential antimutagenic compounds present in tea and on the influence of the food matrix on this activity. Between 60 and 180min after the tea was introduced into the model, antimutagenic activity was recovered from the jejunal compartment by means of dialysis: the dialysate appeared to inhibit the mutagenicity of the food mutagen MeIQx in the direct plate assay with Salmonella typhimurium (Ames test). The maximum inhibition was measured at 2h after the start of the experiment and was comparable for black tea and green tea extract. To determine the influence of food matrices on the antimutagenic activity of tea, the model was loaded with black tea together with milk or a homogenized standard breakfast. The maximum inhibition observed with black tea was reduced by 22, 42 and 78% in the presence of whole milk, semi-skimmed milk, and skimmed milk, respectively. Whole milk and skimmed milk abolished the antimutagenic activity of green tea by more than 90%; for semi-skimmed milk the inhibition was more than 60%. When a homogenized breakfast was added into the model together with the black tea extract, the antimutagenic activity was completely eliminated. When tea and MeIQx were added together into the digestion model, MeIQx mutagenicity was efficiently inhibited, with green tea showing a slightly stronger antimutagenic activity than black tea. In this case, the addition of milk had only a small inhibiting effect on the antimutagenicity.Antioxidant capacity and the concentration of catechins were also measured in the jejunal dialysates. The reduction in antimutagenic activity corresponded with reduction in antioxidant capacity and with a decrease of concentration of three catechins, viz. catechin, epigallocatechin gallate and epigallocatechin. The in vitro gastrointestinal model appears to be a useful tool to study the antimutagenicity of food components.

Kumar A, Cheung KH, Ross-Macdonald P, Coelho PS, Miller P, Snyder M. **TRIPLES: a database of gene function in Saccharomyces cerevisiae.** Nucleic Acids Res 2000;28(1):81-4.

Abstract: Using a novel multipurpose mini-transposon, we have generated a collection of defined mutant alleles for the analysis of disruption phenotypes, protein localization, and gene expression in Saccharomyces cerevisiae. To catalog this unique data set, we have developed TRIPLES, a Web-accessible database of TRansposon-Insertion Phenotypes, Localization and Expression in Saccharomyces. Encompassing over 250 000 data points, TRIPLES provides convenient access to information from nearly 7800 transposon-mutagenized yeast strains; within TRIPLES, complete data reports of each strain may be viewed in table format, or if desired, downloaded as tab-delimited text files. Each report contains external links to corresponding entries within the Saccharomyces Genome Database and International Nucleic Acid Sequence Data Library (GenBank). Unlike other yeast databases, TRIPLES also provides on-line order forms linked to each clone report; users may immediately request any desired strain free-of-charge by submitting a completed form. In addition to presenting a wealth of information for over 2300 open reading frames, TRIPLES constitutes an important medium for the distribution of useful reagents throughout the yeast scientific community. Maintained by the Yale Genome Analysis Center, TRIPLES may be accessed at http://ygac.med.yale.edu/triples/default.htm.

Kushida H, Fujita K, Suzuki A, Yamada M, Endo T, Nohmi T, Kamataki T. **Metabolic activation of N-alkylnitrosamines in genetically engineered Salmonella typhimurium expressing CYP2E1 or CYP2A6 together with human NADPH-cytochrome P450 reductase.** Carcinogenesis 2000;21(6):1227-32.
Abstract: A Salmonella typhimurium tester strain YG7108 2E1/OR co-expressing human CYP2E1 together with human NADPH-cytochrome P450 reductase (OR) was established. The mutagen-activating capacity of human CYP2E1 for N-alkylnitrosamines was compared with that of CYP2A6 using the YG7108 2E1/OR and the YG7108 2A6/OR strains of SALMONELLA: Salmonella YG7108 2A6/OR is a derivative of YG7108 co-expressing CYP2A6 together with OR. Eight N-alkylnitrosamines, including N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosodibutylamine (NDBA), N-nitrosomethylphenylamine (NMPhA), N-nitrosopyrrolidine (NPYR), N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were examined. CYP2E1 expressed in the YG7108 2E1/OR cells showed mutagen-activating capacity, as indicated by induced revertants/min/pmol cytochrome P450, for NDMA, NDEA, NDPA, NDBA, NPYR and NNK, but not NMPhA and NNN. CYP2A6 activated NDMA, NDEA, NDPA, NDBA, NMPhA, NPYR, NNN and NNK. The ratio of the mutagen-activating capacity seen with CYP2A6 to that seen with CYP2E1 was calculated for each N-alkylnitrosamine. In the case of NDMA, NPYR and NDEA, the ratio was under 1.0, while the ratio was over 1.0 with NDPA, NDBA, NNK, NMPhA and NNN. We conclude that human CYP2E1 is mainly responsible for the metabolic activation of N-nitrosamines with a relatively short alkyl chain(s), whereas CYP2A6 was predominantly responsible for the metabolic activation of N-alkylnitrosamines possessing a relatively bulky alkyl chain(s).

Kushida H, Fujita K, Suzuki A, Yamada M, Nohmi T, Kamataki T. **Development of a Salmonella tester strain sensitive to promutagenic N-nitrosamines: expression of recombinant CYP2A6 and human NADPH-cytochrome P450 reductase in S. typhimurium YG7108.** Mutat Res 2000;471(1-2):135-43.

Abstract: We developed a new Salmonella tester strain highly sensitive to promutagenic N-nitrosamines by introducing a plasmid carrying human cytochrome P450 2A6 (CYP2A6) and NADPH-cytochrome P450 reductase (OR) cDNA into the ada- and ogt-deficient strain YG7108. The YG7108 2A6/OR cells expressed high levels of CYP2A6 (77+/-8nmol/l) and OR (470+/-20 micromol cytochrome c reduced/min/l). The expressed CYP2A6 efficiently catalyzed coumarin 7-hydroxylation. N-Nitrosodiethylamine (NDEA), N-nitrosomethylphenylamine (NMPhA), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were mutagenic in the new strain in the absence of any exogenous activation system. The concentrations of promutagen that caused a two-fold increase in revertants were 7.1, 0.14, and 1.4 microM for NDEA, NMPhA, and NNK, respectively. YG7108 2A6/OR cells showed about 10- and 100-fold higher sensitivity to NDEA and NNK, respectively, than parental YG7108 cells assayed in the presence of rat liver S9 (final concentration, 21% (v/v)). Parental YG7108 cells did not detect NMPhA mutagenicity even in the presence of rat liver S9. We believe that this is the first demonstration that CYP2A6 is responsible for the metabolic activation of NMPhA. The established tester strain may be useful to predict human activation of N-nitrosamine promutagens.

Kwak H, Lee M, Cho M. **Interrelationship of apoptosis, mutation, and cell proliferation in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced medaka carcinogenesis model.** Aquatic Toxicol 2000;50(4):317-29.

Abstract: The present study examined the interrelationship of GSH depletion, apoptosis, mutation, and cell proliferation following carcinogen exposure. Medaka (Oryzias latipes) were investigated following a 28 day, three times/week pulse exposure to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Fish (5 weeks old) were exposed to MNNG at concentrations of 0, 0.5, and 1 mg l(-1) and reared for 3, 5 and 7 more months after the last day of exposure. GSH levels were decreased in the higher concentration groups and longer-reared groups. Flow cytometric analysis revealed that fish from the groups reared 3 and 5 months showed active apoptotic changes in the dose- and time-dependent manner, but the group reared 7 months had fewer apoptotic, rather showed more necrotic and carcinogenic alterations. Mutational responses were detected by an arbitrarily primed polymerase chain reaction (AP-PCR) fingerprinting method using whole body DNA samples as templates and pBR primer. A mutational change was expressed by a loss or gain of a band. There was a time-dependent mutational change, but no distinctive concentration-dependent one. A band from normal fish sample that disappeared after treatment of MNNG was excised and sequenced. The band had an 869 base pair-long sequence, however, there was no putative protein-coding region based on an analysis by DNAsis. Spindle cell sarcomas invading muscle were detected on the whole body sections from three of ten fish examined, and immunohistochemical analysis with PCNA showed that tumor cells were actively proliferating. However, terminal deoxynucleotidyl transferase (TdT) assay showed that tumored fish still had active apoptotic cell changes in the tissues without tumor. This study shows not only the interrelationship of GSH depletion, apoptosis, mutation and cell proliferation, but also indicates that medaka is appropriate as a fish model for research on the passage of carcinogenesis.

Lamatsch DK, Steinlein C, Schmid M, Schartl M. **Noninvasive determination of genome size and ploidy level in fishes by flow cytometry: detection of triploid Poecilia formosa.** Cytometry 2000;39 (2):91-5.

Abstract: BACKGROUND: In order to understand the evolutionary significance of single triploids

among the mostly diploid Poecilia formosa we have developed a simple, noninvasive technique for DNA content and ploidy determination. METHODS: From dorsal fin clips of 14 different fish species single cell suspensions were obtained by chopping the material in 2.1% citric acid/0.5% Tween20, passing it through a 0. 6-gauge needle and incubating it for 20 min at room temperature (RT) with gentle agitation. After overnight fixation in 70% ethanol, the cells were treated with 1ml 0.5% pepsin/0.1 M HCl for 15 min at RT before adding DAPI to a final volume of 2 ml. The cells were stained for 1-3 h and then analyzed by flow cytometry. RESULTS: We obtained good measurements with CVs ranging from 1.23% to 3.36%. The poeciliid species measured contain from 1.6 to 2.0 pg/nucleus, Oryzias latipes (Medaka) exhibits a nuclear DNA content of 2.2 pg, Danio rerio (zebrafish) 4.6 pg, Tetraodon fluviatilis (freshwater fugu) 0.70 pg. All values except zebrafish are in good agreement with the literature. CONCLUSIONS: The identification of living specimens of different ploidy for breeding experiments, behavioral studies and tissue transplantations is now made possible. With slight modifications the method can be extended to a field technique, providing therefore a useful tool for a variety of researchers.

Lane TF, Lin C, Brown MA, Solomon E, Leder P. **Gene replacement with the human BRCA1 locus: tissue specific expression and rescue of embryonic lethality in mice.** Oncogene 2000;19(36):4085-90.
Abstract: We have generated transgenic mice that harbor a 140 kb genomic fragment of the human BRCA1 locus (TgN x BRCA1GEN). We find that the transgene directs appropriate expression of human BRCA1 transcripts in multiple mouse tissues, and that human BRCA1 protein is expressed and stabilized following exposure to DNA damage. Such mice are completely normal, with no overt signs of BRCA1 toxicity commonly observed when BRCA1 is expressed from heterologous promoters. Most importantly, however, the transgene rescues the otherwise lethal phenotype associated with the targeted hypomorphic allele (Brca1DeltaexllSA). Brca1-/-; TgN x BRCA1GEN bigenic animals develop normally and can be maintained as a distinct line. These results show that a 140 kb fragment of chromosome 17 contains all elements necessary for the correct expression, localization, and function of the BRCA1 protein. Further, the model provides evidence that function and regulation of the human BRCA1 gene can be studied and manipulated in a genetically tractable mammalian system. Oncogene (2000) 19, 4085 - 4090.

Lanio T, Jeltsch A, Pingoud A. **Automated purification of His6-tagged proteins allows exhaustive screening of libraries generated by random mutagenesis.** Biotechniques 2000;29(2):338-42.
Abstract: In the course of site-directed mutagenesis or directed evolution experiments, large numbers of protein variants are often generated. To characterize functional properties of individual mutant proteins in vitro, a rapid and reliable protein purification system is required. We have developed an automated method for the parallel purification of 96 different protein variants that takes about two hours. Using a 96-well format, the whole process can be performed automatically by a pipetting robot. Coupled with a suitable assay, again using a 96-well format, all variants can be functionally characterized within a few hours. The protein purification procedure described here is based on the interaction between His6-tagged proteins and Ni-NTA-coated microplates. Typical yields are 3-8 pmol purified protein/well, which is sufficient to analyze most enzymatic activities. Using this procedure, we have purified and characterized variants of the restriction endonuclease EcoRV, which were produced in an effort to enhance the selectivity of this enzyme. For this purpose, three amino acid residues were randomized in a region

known from the co-crystal structure to be located at the protein-DNA interface. From a library of about 1200 variants, predominantly single and double mutants, more than 1000 variants were purified and characterized in parallel, which corresponds to an almost complete screening of the library.

LeBlond JB, Duffy LK. **Toxicity assessment of total dissolved solids in effluent of Alaskan mines using 22-h chronic Microtox and Selenastrum capricornutum assays.** Sci Total Environ 2001;271(1-3):49-59.
Abstract: In order to overcome limitations associated with the Daphnia assay, we have explored two alternative assays, the 22-h chronic Microtox test and the 3-day S. capricornutum test, as substitutes. During this study, we compared the two assays using both a simple TDS standard solution and field water samples from two Alaskan mines. Using EC20 values, our results suggest that simple TDS standard solutions are not representative of environmental water samples of equivalent TDS concentrations. When comparing assays, our results showed that the 22-h Microtox assay was more reproducible and sensitive to effluent waters than the algal assay. Principle component analysis indicated that the 22-h Microtox test was generally more sensitive to nickel, ammonia and chloride while the S. capricornutum growth test appeared sensitive to cadmium levels.

Lehnert S. **Prediction of tumor response to therapy: molecular markers and the microenvironment.** Apoptosis and chips: an overview of the proceedings. Radiat Res 2000;154(2):121-4.

Lesser CF, Miller SI. **Expression of microbial virulence proteins in Saccharomyces cerevisiae models mammalian infection.** EMBO J 2001;20(8):1840-9.
Abstract: Bacterial virulence proteins that are translocated into eukaryotic cells were expressed in Saccharomyces cerevisiae to model human infection. The subcellular localization patterns of these proteins in yeast paralleled those previously observed during mammalian infection, including localization to the nucleus and plasma membrane. Localization of Salmonella SspA in yeast provided the first evidence that SspA interacts with actin in living cells. In many cases, expression of the bacterial virulence proteins conferred genetically exploitable growth phenotypes. In this way, Yersinia YopE toxicity was demonstrated to be linked to its Rho GTPase activating protein activity. YopE blocked polarization of the yeast cytoskeleton and cell cycle progression, while SspA altered polarity and inhibited depolymerization of the actin cytoskeleton. These activities are consistent with previously proposed or demonstrated effects on higher eukaryotes and provide new insights into the roles of these proteins in pathogenesis: SspA in directing formation of membrane ruffles and YopE in arresting cell division. Thus, study of bacterial virulence proteins in yeast is a powerful system to determine functions of these proteins, probe eukaryotic cellular processes and model mammalian infection.

Lewis PD, Harvey JS, Waters EM, Parry JM. **The mammalian gene mutation database.** Mutagenesis 2000;15(5):411-4.
Abstract: The Mammalian Gene Mutation Database (MGMD) is a comprehensive collection of published mutation data from the open literature on mammalian cell-based gene model mutation detection systems. The database currently contains approximately 30000 comprehensively described mutant spectra records and it is maintained and up- dated on a daily basis. The major objectives of the

MGMD were (i) to provide an Internet-accessible database (http://lisntweb.swan.ac.uk/cmgt/index.htm) for chemically induced and spontaneous mutation types and spectra in selected genes; (ii) to standardize the reporting of mutations within different genes where ambiguity exists in the literature; and (iii) to provide interactive and user-friendly access to the information. A multi-option search facility has been included that allows the user to search the database for parameters such as mutagen, gene or cell type of interest. The structure of the database permits easy retrieval of specific mutation data for further analysis. Thus, the MGMD should become a useful and necessary reference source and provides an analysis tool for genetic toxicologists.

Li M, Lewis B, Capuco AV, Laucirica R, Furth PA. **WAP-TAg transgenic mice and the study of dysregulated cell survival, proliferation, and mutation during breast carcinogenesis.** Oncogene 2000;19(8):1010-9.
Abstract: Understanding the process of carcinogenesis is key to developing therapies which might interrupt or reverse tumor onset and progression. Cell growth and death signals are dependent not only upon molecular mechanisms within a cell but also upon external stimuli such as hormones, cell - cell signaling, and extracellular matrix. Mouse models can be used to dissect these complex processes, to identify key signaling pathways operating at different stages of tumorigenesis, and to test the strength of specific interventions. In the WAP-TAg mouse model, carcinogenesis is initiated by expression of the Simian Virus 40 T antigen (TAg). TAg expression is triggered by hormonal stimulation, either during estrus or pregnancy. Breast adenocarcinomas (ranging from well to poorly differentiated) develop in 100% of the female mice by approximately 8 - 9 months of age. Three distinct stages of tumorigenesis are easily identified: an initial proliferation, hyperplasia, and adenocarcinoma. The mean time to first palpable tumor in mice which undergo at least one pregnancy is 6 months. The tumorigenic process is marked by a competition between proliferation and apoptosis and is characterized by cellular acquisition of genetic mutations and increased stromal fibrosis. Protein levels of cell cycle control genes cyclin D1, cdk2, and E2F-1 are increased in these adenocarcinomas. c-Fos protein levels are slightly increased in these cancers, while c-Jun levels do not change. Hormonal exposure alters progression. Estrogen plays a role during the early stages of oncogenesis although the growth of the resulting adenocarcinomas is estrogen-independent. Transient hormonal stimulation by glucocorticoids that temporarily increases the rate of cell proliferation results in tetraploidy, premature appearance of irreversible hyperplasia, and early tumor development. Tumor appearance also can be accelerated through over expression of the cell survival protein, Bcl-2. Bcl-2 over expression not only reduces apoptosis during the initial proliferative process but also decreases the total rate of cell proliferation. This block in cell proliferation is lost selectively as the cells transition to adenocarcinoma. The WAP-TAg model can be utilized to investigate how the basic processes of cell proliferation, apoptosis, DNA mutation, and DNA repair are modified by external and internal signals during mammary oncogenesis.

Lieschke GJ. **Zebrafish--an emerging genetic model for the study of cytokines and hematopoiesis in the era of functional genomics.** Int J Hematol 2001;73(1):23-31.
Abstract: Now that whole genomes are sequenced, the identification of gene function rather than gene discovery is a major challenge. Saturation mutagenesis and screening for mutant phenotypes are methods that allow sampling of the genome for lesions in genes critical for parcticular physiological processes. This approach promises to provide new insights into gene function, even for molecularly well-

characterized processes such as hematopoiesis and cytokine signaling. Animal models for such genetic approaches have traditionally included Drosophila and the mouse. Recently, the zebrafish (Danio rerio) has emerged as a flexible and informative vertebrate for genetic studies. Zebrafish hematopoiesis has a morphological and molecular complexity closer to that of mammals than does Drosophila, providing scope for recognizing mutant zebrafish phenotypes representing finely tuned lesions in these processes. Compared to mice, zebrafish represent an economical, flexible, and genetically tractable animal model for mutagenesis studies. The structure of the teleost genome creates several phylogenetic issues in assessing zebrafish and piscine orthologues and paralogues of known mammalian genes, here exemplified by a cytokine ligand (interleukin-1beta), kinase receptors (c-kit and c-fins), and a family of intracellular signaling molecules (JAK kinases). Several anemic zebrafish mutants are now genetically characterized, and others present hematopoietic phenotypes that promise novel insights into the regulation of hematopoiesis.

Lovell DP. **Dose-response and threshold-mediated mechanisms in mutagenesis: statistical models and study design.** Mutat Res 2000;464(1):87-95.
Abstract: The objective of this paper is to review the use, in mutagenesis, of various mathematical models to describe the dose-response relationship and to try to identify thresholds. It is often taken as axiomatic that genotoxic carcinogens could damage DNA at any level of exposure, leading to a mutation, and that this could ultimately result in tumour development. This has led to the assumption that for genotoxic chemicals, there is no discernible threshold. This assumption is increasingly being challenged in the case of aneugens. The distinction between 'absolute' and 'pragmatic' thresholds is made and the difficulties in determining 'absolute' thresholds using hypothesis testing approaches are described. The potential of approaches, based upon estimation rather than statistical significance for the characterization of dose-response relationships, is stressed. The achievement of a good fit of a mathematical model to experimental data is not proof that the mechanism supposedly underlying this model is operating. It has been argued, in the case of genotoxic chemicals, that any effects produced by a genotoxic chemical which augments that producing a background incidence in unexposed individuals will lead to a dose-response relationship that is non-thresholded and is linear at low doses. The assumptions underlying this presumption are explored in the context of the increasing knowledge of the mechanistic basis of mutagenicity and carcinogenicity. The possibility that exposure to low levels of genotoxic chemicals may induce and enhance defence and repair mechanisms is not easily incorporated into many of the existing mathematical models and should be an objective in the development of the next generation of biologically based dose-response (BB-DR) models. Studies aimed at detecting or characterizing non-linearities in the dose-response relationship need appropriate experimental designs with careful attention to the choice of biomarker, number and selection of dose levels, optimum allocation of experimental units and appropriate levels of replication within and repetition of experiments. The characterization of dose-response relationships with appropriate measures of uncertainty can help to identify 'pragmatic' thresholds based upon biologically relevant criteria which can help in the regulatory process.

Majumdar A, Lun K, Brand M, Drummond IA. **Zebrafish no isthmus reveals a role for pax2.1 in tubule differentiation and patterning events in the pronephric primordia.** Development 2000;127 (10):2089-98.

Abstract: Pax genes are important developmental regulators and function at multiple stages of vertebrate kidney organogenesis. In this report, we have used the zebrafish pax2.1 mutant no isthmus to investigate the role for pax2.1 in development of the pronephros. We demonstrate a requirement for pax2.1 in multiple aspects of pronephric development including tubule and duct epithelial differentiation and cloaca morphogenesis. Morphological analysis demonstrates that noi(-)larvae specifically lack pronephric tubules while glomerular cell differentiation is unaffected. In addition, pax2.1 expression in the lateral cells of the pronephric primordium is required to restrict the domains of Wilms' tumor suppressor (wt1) and vascular endothelial growth factor (VEGF) gene expression to medial podocyte progenitors. Ectopic podocyte-specific marker expression in pronephric duct cells correlates with loss of expression of the pronephric tubule and duct-specific markers mAb 3G8 and a Na(+)/K(+) ATPase (&agr;)1 subunit. The results suggest that the failure in pronephric tubule differentiation in noi arises from a patterning defect during differentiation of the pronephric primordium and that mutually inhibitory regulatory interactions play an important role in defining the boundary between glomerular and tubule progenitors in the forming nephron.

Mak HY, Parker MG. **Use of suppressor mutants to probe the function of estrogen receptor-p160 coactivator interactions.** Mol Cell Biol 2001;21(13):4379-90.
Abstract: Estrogen-dependent recruitment of coactivators by estrogen receptor alpha (ERalpha) represents a crucial step in the transcriptional activation of target genes. However, studies of the function of individual coactivators has been hindered by the presence of endogenous coactivators, many of which are potentially recruited in the presence of agonist via a common mechanism. To circumvent this problem, we have generated second-site suppressor mutations in the nuclear receptor interaction domain of p160 coactivators which rescue their binding to a transcriptionally defective ERalpha that is refractory to wild-type coactivators. Analysis of these altered-specificity receptor-coactivator combinations, in the absence of interference from endogenous coregulators, indicated that estrogen-dependent transcription from reporter genes is critically dependent on direct recruitment of a p160 coactivator in mammalian cells and that the three p160 family members serve functionally redundant roles. Furthermore, our results suggest that such a change-of-specificity mutation may act as a transposable protein-protein interaction module which provides a novel tool with which to dissect the functional roles of other nuclear receptor coregulators at the cellular level.

Malling HV, Delongchamp RR. **Direct separation of in vivo and in vitro am3 revertants in transgenic mice carrying the phiX174 am3, cs70 vector.** Environ Mol Mutagen 2001;37(4):345-55.
Abstract: Target genes in most transgenic systems have higher spontaneous mutation frequencies than do endogenous mammalian genes. Spontaneous mutations in transgenes predominantly arise from three sources: (1) mutations fixed in the animals, (2) mutations arising from replication errors caused by damage to the DNA that may have occurred in vivo or in vitro and then was fixed during amplification of the vector in vitro, and (3) mutations arising during replication of non-revertant phages in non-permissive bacteria. An assay based on single bursts was developed to directly distinguish between the in vivo and in vitro origins of revertants. The size of the aliquot is determined by mutant frequency and is adjusted so that ideally no more than 10 to 20% of the aliquots contain a bacterial cell transformed with a mutant phage. Mutations are detected as revertants of an amber mutation (am3) in phiX174 am3, cs70. The minimum burst size of non-revertant phiX am3, cs70 from splenic DNA on a permissive

bacterial strain was larger than 30 plaque-forming units (pfu). Based on this observation, a burst size of 31 plaque-forming revertants was chosen as the minimum burst size of a fixed mutation. The single burst assay was tested on DNA from spleens of animals that were treated with 150 mg/kg 1-ethyl-1 nitrosurea. Only the fraction of aliquots with single bursts of revertants (> 30) increased in the treated animals compared to the controls. In contrast, there was no difference between treated and control animals for revertant frequencies calculated for burst sizes < or =30 pfu. Among the spontaneous mutations, only 30% were caused by mutations fixed in animals (i.e. burst size >30 pfu). Total average revertant frequency measured in DNA from treated animals was less than twofold more than the average spontaneous frequency (P = 0.048). When frequencies were based on burst sizes >30, there was a 4.6-fold increase among treated animals compared with controls (P = 0.026). The single burst-assay resulted in a more sensitive test for mutagenicity because it eliminated noise from in-vitro mutations.

Malvy C, Lefrancois M, Bertrand JR, Markovits J. **Modified alkaline elution allows the measurement of intact apurinic sites in mammalian genomic DNA.** Biochimie 2000;82(8):717-21.
Abstract: The presence of apurinic/apyrimidinic (AP) sites in cell genomes is known to be toxic and mutagenic. These lesions are therefore repaired in cells by efficient enzymatic systems. However, a report (Nakamura and Swenberg, Cancer Res. 59 (1999) 2522-2526) indicates an unexpected high rate of endogenous apurinic/apyrimidinic (AP) sites in genomic DNA in mammalian tissues. The technology used does not allow the authors to distinguish between intact AP sites and 3'cleaved AP sites. The corresponding values range between 2 and 4 sites per million of nucleotides in various human and rat tissues. Using a modified alkaline elution method we show here that the stationary level of intact AP sites is about 0.16 per million of nucleotides in leukemic mouse L1210 cells.

Marangoni E, Foray N, O'Driscoll M, Douc-Rasy S, Bernier J, Bourhis J, Jeggo P. **A Ku80 fragment with dominant negative activity imparts a radiosensitive phenotype to CHO-K1 cells.** Nucleic Acids Res 2000;28(23):4778-82.
Abstract: DNA non-homologous end joining, the major mechanism for the repair of DNA double-strands breaks (DSB) in mammalian cells requires the DNA-dependent protein kinase (DNA-PK), a complex composed of a large catalytic subunit of 460 kDa (DNA-PKcs) and the heterodimer Ku70-Ku80 that binds to double-stranded DNA ends. Mutations in any of the three subunits of DNA-PK lead to extreme radiosensitivity and DSB repair deficiency. Here we show that the 283 C-terminal amino acids of Ku80 introduced into the Chinese hamster ovary cell line CHO-K1 have a dominant negative effect. Expression of Ku(449-732) in CHO cells was verified by northern blot analysis and resulted in decreased Ku-dependent DNA end-binding activity, a diminished capacity to repair DSBs as determined by pulsed field gel electrophoresis and decreased radioresistance determined by clonogenic survival. The stable modifications observed at the molecular and cellular level suggest that this fragment of Ku80 confers a dominant negative effect providing an important mechanism to sensitise radioresistant cells.

Maria DA, Ribeiro OG, Pizzocaro KF, De Franco M, Cabrera WK, Starobinas N, Gallois V, Siqueira M, Seman M, Ibanez OM. **Resistance to melanoma metastases in mice selected for high acute inflammatory response.** Carcinogenesis 2001;22(2):337-42.
Abstract: The role of innate immunity in natural resistance to tumor progression was investigated in two mouse lines, AIRmax and AIRmin, selected by bi-directional selective breeding on the basis of high or

low acute inflammatory response. Compared with AIRmin, AIRmax mice were shown to be resistant to 7,12-dimethylbenz[a]anthracene (DMBA)/12-O:-tetradecanoylphorbol-13-acetate-induced skin cancers and here we demonstrate that AIRmax are also able to restrain the development of metastases upon transfer of MHC compatible, incompatible or xenogeneic melanomas. An acute inflammatory response to melanoma cells was observed in AIRmax mice only, although both lines were found to mount similar specific immune responses to melanoma antigens. The genetically selected lines therefore represent a model system to analyze the positive correlation between multiple resistance to tumorigenesis and host inflammatory responsiveness.

Marrot L, Belaidi JP, Chaubo C, Meunier JR, Perez P, Agapakis-Causse C. **Fluoroquinolones as chemical tools to define a strategy for photogenotoxicity in vitro assessment.** Toxicol In Vitro 2001;15(2):131-42.
Abstract: Today's lifestyle is often associated with frequent exposure to sunlight, but some xenobiotics used in drugs, cosmetics or food chemicals can produce adverse biological effects when irradiated. In parcticular, they can increase the risk of photogenotoxicity already due to UV radiation itself. There is thus a need to design appropriate approaches in order to obtain relevant data at the molecular and cellular level in this field. For ethical and practical reasons, in vitro models can be very convenient at least for first evaluation tests. Here, we propose a strategy based on complementary experiments to study the photogenotoxic potential of a compound. The fluoroquinolones BAYy3118 and lomefloxacin were used as standards to demonstrate the performance of each test: photoinduced interaction with supercoiled circular DNA, photomutagenicity in the yeast Saccharomyces cerevisae, induction of DNA photodamage in cultured human skin cells as revealed by comet assay, and finally induction of specific phototoxic stress responses such as p53 activation or melanogenesis stimulation. Such a strategy should help to ensure the safety of products likely to undergo environmental sunlight exposure.

Matsuda M, Kawato N, Asakawa S, Shimizu N, Nagahama Y, Hamaguchi S, Sakaizumi M, Hori H. **Construction of a BAC library derived from the inbred Hd-rR strain of the teleost fish, Oryzias latipes.** Genes Genet Syst 2001;76(1):61-3.
Abstract: A large insert genomic bacterial artificial chromosome (BAC) library was constructed from the inbred Hd-rR strain of the medaka, Oryzias latipes. Approximately 92,000 clones were gridded on high-density replica filters. Insert analysis of randomly selected clones indicated a mean insert size of 210 kb and predicted a 24 times coverage of the medaka genome. The library was hybridized with a single locus DNA fragment, and the resulting positive clones were characterized and shown to be compatible with a 24-fold redundant library. This first large insert genomic library of the medaka should increase the speed of genomic analyses for this fish species.

McNamee JP, McLean JR, Ferrarotto CL, Bellier PV. **Comet assay: rapid processing of multiple samples.** Mutat Res 2000;466(1):63-9.
Abstract: The present study describes modifications to the basic comet protocol that increase productivity and efficiency without sacrificing assay reliability. A simple technique is described for rapidly preparing up to 96 comet assay samples simultaneously. The sample preparation technique allows thin layers of agarose-embedded cells to be prepared in multiple wells attached to a flexible film of Gelbond, which improves the ease of manipulating and processing samples. To evaluate the effect of

these modifications on assay sensitivity, dose-response curves are presented for DNA damage induced by exposure of TK6 cells to low concentrations of hydrogen peroxide (0-10 &mgr;M) and for exposure of human lymphocytes to X-irradiation (0-100 cGy). The limit of detection of DNA damage induced by hydrogen peroxide in TK6 cells was observed to be 1 uM for all parameters (tail ratio, tail moment, tail length and comet length) while the limit of detection of DNA damage in human lymphocytes was 10 cGy for tail and comet length parameters, but 50 cGy for tail ratio and tail moment parameters. These results are similar to those previously reported using the conventional alkaline comet assay. The application of SYBR Gold for detection of DNA damage was compared to that of propidium iodide. Measurements of matching samples for tail length and comet length were similar using both stains. However, comets stained with SYBR Gold persisted longer and were much brighter than those obtained with propidium iodide. SYBR Gold was found to be ideal for measuring tail length and comet length but, under present assay conditions, impractical for measuring tail ratio or tail moment due to saturation of staining in the head region of the comets.

Meetei AR, Ullas KS, Rao MR. **Identification of two novel zinc finger modules and nuclear localization signal in rat spermatidal protein TP2 by site-directed mutagenesis.** J Biol Chem 2000;275(49):38500-7.
Abstract: Spermatidal protein TP2, which appears transiently during stages 12-16 of mammalian spermiogenesis, is a DNA condensing zinc metalloprotein with a preference to GC-rich DNA. We have carried out a detailed site-directed mutagenesis analysis of rat spermatidal protein TP2 to delineate the amino acid residues involved in coordination with two atoms of zinc. Two zinc fingers modules have been identified involving 4 histidine and 4 cysteine residues, respectively. The modular structure of the two zinc fingers identified in TP2 define a new class of zinc finger proteins that do not fall into any of the known classes of zinc fingers. Transfection experiments with COS-7 cells using wild type and the two zinc finger pocket mutants have shown that TP2 preferentially localizes to nucleolus. The nuclear localization signal in TP2 was identified to be (87)GKVSKRKAV(95) present in the C-terminal third of TP2 as a part of an extended NoLS sequence.

Meintieres S, Biola A, Pallardy M, Marzin D. **Apoptosis can be a confusing factor in in vitro clastogenic assays.** Mutagenesis 2001;16(3):243-50.
Abstract: Among the tests used to determine the mutagenic potential of chemicals, the chromosomal aberrations and micronucleus assays play an important role. These tests score either chromosomal structural aberrations at metaphase or micronuclei at interphase. One of the hallmarks of apoptosis is DNA fragmentation into 50-300 kpb leading to oligonucleosomal fragmentation that can interfere with the results of clastogenic assays. In this case, apoptosis may be a confusing factor in the evaluation of the mutagenic potential of molecules and lead to false positive results. For these reasons we have developed a cell line able to demonstrate the interference of apoptosis in two mutagenicity tests: the in vitro micronucleus test and metaphase analysis in vitro. We used a murine cytotoxic T cell line, CTLL-2 Bcl2, in which a stably transfected bcl2 gene is known to protect these cells from apoptosis induced by various stimuli. A comparison between results obtained in parental CTLL-2 cells and in CTLL-2 Bcl2 cells treated with non-genotoxic apoptosis inducers, such as dexamethasone or gliotoxin, leads us to conclude that apoptosis could give false positive results due to DNA fragmentation. Moreover, with etoposide, a clastogen that also induces apoptosis, we observed that the percentages of aberrant cells and

numbers of micronuclei were significantly increased in CTLL-2 cells compared with CTLL-2 Bcl2 cells. This observation suggests that apoptosis leads to an overestimation of the genotoxic potential of chemicals. Finally, with nocodazole, an aneugen, we confirm that this model can also detect agents that have only genotoxic potential and thus allows a better estimation of the genotoxic threshold in studies with aneugens, thus avoiding overestimation of the mutagenic risk of such a compound.

Metzger D, Chambon P. **Site- and time-specific gene targeting in the mouse.** Methods 2001;24(1):71-80.
Abstract: The efficient introduction of somatic mutations in a given gene, at a given time, in a specific cell type, will facilitate studies of gene function and the generation of animal models for human diseases. We have established a conditional site-specific recombination system in mice using a new version of the Cre/lox system. The Cre recombinase has been fused to a mutated ligand binding domain of the human estrogen receptor (ER), resulting in a tamoxifen-dependent Cre recombinase, Cre-ER(T), that is activated by tamoxifen, but not by estradiol. Transgenic mice were generated expressing Cre-ER (T) under the control of a cytomegalovirus promoter. Administration of tamoxifen to these transgenic mice induced excision of a chromosomally integrated gene flanked by loxP sites in a number of tissues, whereas no excision could be detected in untreated animals. However, the efficiency of excision varied between tissues, and the highest level (approximately 40%) was obtained in the skin. To determine the efficiency of excision mediated by Cre-ER(T) in a given cell type, Cre-ER(T)-expressing mice were crossed with reporter mice in which expression of Escherichia coli beta-galactosidase can be induced through Cre-mediated recombination. The efficiency and kinetics of this recombination were analyzed at the cellular level in the epidermis of 6- to 8-week-old double transgenic mice. Site-specific excision occurred within a few days of tamoxifen treatment in essentially all epidermis cells expressing Cre-ER (T). These results indicate that cell-specific expression of Cre-ER(T) in transgenic mice can be used for efficient tamoxifen-dependent Cre-mediated recombination at loci containing loxP sites, to generate site-specific somatic mutations in a spatiotemporally controlled manner. This conditional site-specific recombination system should allow the analysis of knockout phenotypes that cannot be addressed by conventional gene targeting. Copyright 2001 Academic Press.

Mills AA, Bradley A. **From mouse to man: generating megabase chromosome rearrangements.** Trends Genet 2001;17(6):331-9.
Abstract: Experimental approaches for deciphering the function of human genes rely heavily on our ability to generate mutations in model organisms such as the mouse. However, because recessive mutations are masked by the wild-type allele in the diploid context, conventional mutagenesis and screening is often laborious and costly. Chromosome engineering combines the power of gene targeting in embryonic stem (ES) cells with Cre--loxP technology to create mice that are functionally haploid in discrete portions of the genome. Chromosome deletions, duplications and inversions can be tagged with visible markers, facilitating strain maintenance. These approaches allow for more refined mutagenesis screens that will greatly accelerate functional mouse genomics and generate mammalian models for developmental processes and cancer.

Miwa M, Hongo Y. **Application of a single-cell gel electrophoresis (comet) assay to screen the antimutagenic activity in foods.** Biosci Biotechnol Biochem 2000;64(6):1292-4.

Abstract: Three cell lines (HL60, U937 and RAW264.7) were studied for their sensitivity against mutagens by using a single-cell gel electrophoresis (comet) assay. RAW264.7, the most sensitive one, was chosen to screen the antimutagenic activity in swine and bovine offal. Aqueous extracts of the swine stomach (0.2 mg/ml) and heart (10 mg/ml) were found to have antimutagenic activity against MeIQx (+ S9mix)-treated cells.

Miyahara M, Saito A, Ito H, Toyoda M. **Capability for identification of gamma-irradiated bovine liver by new high sensitivity comet assay.** Biol Pharm Bull 2000;23(12):1399-405.
Abstract: DNA in food will sustain damage by gamma radiation. The detection capability of the high sensitivity comet assay was studied using fluorescence-microscopy. Beef liver was irradiated at a range of 1 Gy to 8 kGy. Single cells were obtained from the irradiated liver, then analyzed by agaros-gel electrophoresis. The pH of the buffer for electrophoresis was pH 13, which is generally utilized for sensitive detection of DNA damage. The pattern formed by DNA was visualized by staining with ethidium bromide. The resulting comets were evaluated with a scale we developed, and Influence Scores were calculated based on the Tice method. It is possible to detect irradiation damage to beef liver at 10 Gy. Together with Influence Score, histogram of comet type is used for detection of irradiation. We elucidated those histograms were useful for distinguishing damage caused by irradiation from that of others. DNA damage can be caused not only by irradiation, but also by the other treatments. Therefore, the respective influences of freezing, preservation, irradiating temperature, atmosphere of irradiation, cooking, and homogenizing devices were also examined. This new comet assay will be a useful method of detecting DNA damage to identify irradiated foods.

Moller P, Knudsen LE, Loft S, Wallin H. **The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors.** Cancer Epidemiol Biomarkers Prev 2000;9(10):1005-15.
Abstract: Within the last decade, the comet assay has been used with increasing popularity to investigate the level of DNA damage in terms of strand breaks and alkaline labile sites in biomonitoring studies. The assay is easily performed on WBCs and has been included in a wide range of biomonitoring studies of occupational exposures encompassing styrene, vinyl chloride, 1,3-butadiene, pesticides, hair dyes, antineoplastic agents, organic solvents, sewage and waste materials, wood dust, and ionizing radiation. Eleven of the occupational studies were positive, whereas seven were negative. Notably, the negative studies appeared to have less power than the positive studies. Also, there were poor dose-response relationships in many of the biomonitoring studies. Many factors have been reported to produce effects by the comet assay, e.g., age, air pollution exposure, diet, exercise, gender, infection, residential radon exposure, smoking, and season. Until now, the use of the comet assay has been hampered by the uncertainty of the influence of confounding factors. We argue that none of the confounding factors are unequivocally positive in the majority of the studies. We recommend that age, gender, and smoking status be used as criteria for the selection of populations and that data on exercise, diet, and recent infections be registered before blood sampling. Samples from exposed and unexposed populations should be collected at the same time to avoid seasonal variation. In general, the comet assay is considered a suitable and fast test for DNA-damaging potential in biomonitoring studies.

Mortelmans K, Zeiger E. **The Ames Salmonella/microsome mutagenicity assay.** Mutat Res 2000;455

(1-2):29-60.
Abstract: The Ames Salmonella/microsome mutagenicity assay (Salmonella test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test employs several histidine dependent Salmonella strains each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the Salmonella tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (his(+)) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner. The Ames test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs. The test is also used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides. International guidelines have been developed for use by corporations and testing laboratories to ensure uniformity of testing procedures. This review provides historical aspects of how the Ames was developed and detailed procedures for performing the test, including the design and interpretation of results.

Moshinsky DJ, Wogan GN. **UV-induced mutagenesis of human p53: analysis using a double-selection method in yeast.** Environ Mol Mutagen 2000;35(1):31-8.
Abstract: Comparison of the mutation patterns of p53 in human tumors with those of selectable genes in model systems is a powerful approach to identify potential etiological factors for specific tumor types. Recently, we validated use of a yeast assay to permit direct determination of the mutation spectrum induced in human p53 by carcinogens that would reduce uncertainties inherent in comparing spectra induced in different target genes. Here, we describe modifications in the assay designed to facilitate screening for mutants and to permit intracellular exposure of the gene instead of in vitro treatment. This was accomplished by introducing growth-based selection for transactivation-deficient p53 mutants into yeast already possessing red/white colony color selection. This improved model system was able to detect cells harboring p53 mutations among cells with wild-type p53 at a frequency of 10(-4) or less. Additionally, UV light was used to verify that the majority of mutagenized cells with the appropriate phenotype on selective medium contained mutations in p53, not elsewhere in the genome. Sequence analysis of UV-induced mutations revealed that the nature of the mutations was similar to those obtained in previous studies of this mutagen. This system will prove useful in the determination of the ability of environmental agents to mutate the human p53 gene, and thus may contribute to hazard identification.

Muller L, Sofuni T. **Appropriate levels of cytotoxicity for genotoxicity tests using mammalian cells in vitro.** Environ Mol Mutagen 2000;35(3):202-5.
Abstract: Among standard battery genotoxicity assays, the in vitro chromosome aberration test and the mouse lymphoma tk assay (MLA) yield about fourfold higher incidences of positive test results than the bacterial reverse mutation test or in vivo bone marrow tests. This is a result of experience with submissions of 335 new pharmaceuticals to the German Federal Institute for Drugs and Medical Devices. While all of the standard systems have their value in detecting relevant genotoxins, there is no supportive evidence for DNA reactivity for a considerable number of in vitro clastogens and MLA

positives. In parcticular the clastogenic response of such compounds is often associated with high cytotoxicity. This may invoke the need to change the approach to test for clastogenicity in vitro. A combination of measures such as (1) a change in the upper limits of cytotoxicity that are currently given in International Conference on Harmonisation (ICH) and Organization for Economic Co-Operation & Economic Development (OECD) guidelines, (2) the creation of a common ground of understanding for interpretation of in vitro (positive) test results, and (3) lowering the upper limits of test compound concentration irrespective of cytotoxicity may prove useful to ensure a sufficient reliability of genotoxicity testing with mammalian cells in vitro.

Nadin SB, Vargas-Roig LM, Ciocca DR. **A silver staining method for single-cell gel assay.** J Histochem Cytochem 2001;49(9):1183-6.
Abstract: The single-cell gel assay (comet assay) is a very useful microelectrophoretic technique for evaluation of DNA damage and repair in individual cells. Usually, the comets are visualized and evaluated with fluorescent DNA stains. This staining requires specific equipment (e.g., a high-quality fluorescence microscope), the slides must be analyzed immediately, and they cannot be stored for long periods of time. Here we describe, using human lymphocytes, some modifications of the silver staining for comets that significantly increase the sensitivity/reproducibility of the assay. This silver staining was compared with fluorescence staining and commercial silver stains. (J Histochem Cytochem 49:1183-1186, 2001).

Naruse K, Fukamachi S, Mitani H, Kondo M, Matsuoka T, Kondo S, Hanamura N, Morita Y, Hasegawa K, Nishigaki R, et al. **A detailed linkage map of medaka, Oryzias latipes: comparative genomics and genome evolution.** Genetics 2000;154(4):1773-84.
Abstract: We mapped 633 markers (488 AFLPs, 28 RAPDs, 34 IRSs, 75 ESTs, 4 STSs, and 4 phenotypic markers) for the Medaka Oryzias latipes, a teleost fish of the order Beloniformes. Linkage was determined using a reference typing DNA panel from 39 cell lines derived from backcross progeny. This panel provided unlimited DNA for the accumulation of mapping data. The total map length of Medaka was 1354.5 cM and 24 linkage groups were detected, corresponding to the haploid chromosome number of the organism. Thirteen to 49 markers for each linkage group were obtained. Conserved synteny between Medaka and zebrafish was observed for 2 independent linkage groups. Unlike zebrafish, however, the Medaka linkage map showed obvious restriction of recombination on the linkage group containing the male-determining region (Y) locus compared to the autosomal chromosomes.

Naruse K, Kondo S, Mitani H, Shima A, Fukamachi S, Kondo M, Mitani H, Shima A. **[Medaka linkage map--from positional cloning and comparative genomics to genome evolution].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2844-52 [Jpn].

Nasevicius A, Ekker SC. **The zebrafish as a novel system for functional genomics and therapeutic development applications.** Curr Opin Mol Ther 2001;3(3):224-8.
Abstract: Unconventional antisense technology has entered the mainstream for both therapeutic and functional genomics applications in a variety of biological settings. Further development of this approach has been hampered by the high cost and limited information obtained with standard bioassays and animal models. The embryo of the zebrafish Danio rerio offers both biologists and technologists a

new strategy that rapidly garners efficacy, toxicity and specificity data in an in vivo setting. This system has been used to optimize current antisense targeting methods, and it provides an ideal initial assay system for the development of new chemistries or other new gene targeting approaches.

Newell JG, Davies M, Bateson AN. **The use of site-directed mutagenesis, transient transfection, and radioligand binding**. A method for the characterization of receptor-ligand interactions. Mol Biotechnol 2000;14(1):25-45.
Abstract: Receptor-ligand interactions have traditionally been evaluated using a number of biochemical techniques including radioligand binding, photoaffinity labeling, crosslinking, and chemical modification. In modern biochemistry, these approaches have largely been superseded by site-directed mutagenesis in the study of protein function, owing in part to a better understanding of the chemical properties of oligonucleotides and to the ease with which mutant clones can now be generated. The Altered Sites II in vitro Mutagenesis System from the Promega Corporation employs oligonucleotides containing two mismatches to introduce specific nucleotide substitutions in the nucleic acid sequence of a target DNA. One of these mismatches will alter the primary sequence of a given protein, whereas the second will give rise to a silent restriction site that is used to screen for mutants. Transient transfection of tsA201 cells with mutant cDNA constructs using calcium phosphate as a carrier for plasmid DNA permits expression of recombinant receptors that can be characterized using radioligand binding assays. In this Article, we focus on site-directed mutagenesis, heterologous expression in eukaryotic cells, and radioligand binding as a methodology to enable the characterization of receptor-ligand interactions.

Nguyen V, Joly J, Bourrat F. **An in situ screen for genes controlling cell proliferation in the optic tectum of the medaka (Oryzias latipes).** Mech Dev 2001;107(1-2):55-67.
Abstract: The optic tectum is a dorsal, prominent and well corticalised structure of the fish brain. It grows according to a pattern exceptional in the vertebrate central nervous system, by addition of radial columns of cells at its periphery. We took advantage of this peculiar feature to readily identify genes differentially expressed in the tectal proliferative (marginal) vs. post-mitotic (central) zones. Out of 500 medaka cDNA clones screened by WMISH, more than 100 were expressed in one or the other of these zones. Unexpectedly, we also identified a small class of genes expressed between these two zones. All the characterised genes of this class encode down regulators of the cell cycle. Therefore, such a screening strategy allows in parcticular cases to raise testable hypotheses on the involvement of genes in the control of the cell cycle, in addition to characterising unknown genes with patterned expression related to cell proliferation.

Nia AB, Van Schooten FJ, Schilderman PA, De Kok TM, Haenen GR, Van Herwijnen MH, Van Agen E, Pachen D, Kleinjans JC. **A multi-biomarker approach to study the effects of smoking on oxidative DNA damage and repair and antioxidative defense mechanisms.** Carcinogenesis 2001;22 (3):395-401.
Abstract: We investigated the effects of smoking-induced oxidative stress in healthy volunteers (21 smokers versus 24 non-smokers) by quantifying various markers of oxidative DNA damage and repair, and antioxidative defense mechanisms. Lymphocytic 7-hydroxy-8-oxo-2'-deoxyguanosine (8-oxo-dG) levels measured by high performance liquid chromatography with electrochemical detection, were significantly lower in smokers as compared with non-smokers (38.6 +/- 5.2 versus 50.9 +/- 4.6/10(6) dG,

P = 0.05). The levels of oxidized pyrimidine bases in lymphocytes of smokers quantified by the endonuclease III-modified comet assay were non-significantly lower than those of non-smokers (% DNA in tail: 13 +/- 3 versus 14 +/- 2; tail length: 69 +/- 13 versus 96 +/- 10; tail moment: 6416 +/- 1220 versus 7545 +/- 1234). Urinary excretion levels of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) assessed by enzyme-linked immunosorbent assay did not differ significantly between smokers and non-smokers (197 +/- 31 versus 240 +/- 33 ng/body mass index, P = 0.3). Overall DNA repair activity expressed as unscheduled DNA synthesis in blood leukocytes, was not significantly different between smokers and non-smokers (2.9 +/- 0.3 versus 3.3 +/- 0.3, P = 0.4). Plasma antioxidative capacity measured by the Trolox equivalent antioxidant capacity assay was slightly higher in smokers as compared with non-smokers (440 +/- 16 versus 400 +/- 15 microM Trolox equivalent, P = 0.09), and it was significantly related to lymphocytic 8-oxo-dG levels (r = 0.4, P = 0.001). Genotyping of human 8-OH-dG glycosylase/apurinic lyase and glutathione S-transferase M1 showed that a polymorphism in either or both of the two genes does not affect any of the quantified biomarkers. We conclude that oxidative stress imposed by cigarette smoking has a low impact upon certain pathways involved in DNA damage and the antioxidative defense system.

Nolan PM, Peters J, Strivens M, Rogers D, Hagan J, Spurr N, Gray IC, Vizor L, Brooker D, Whitehill E, et al. **A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse.** Nat Genet 2000;25(4):440-3.
Abstract: As the human genome project approaches completion, the challenge for mammalian geneticists is to develop approaches for the systematic determination of mammalian gene function. Mouse mutagenesis will be a key element of studies of gene function. Phenotype-driven approaches using the chemical mutagen ethylnitrosourea (ENU) represent a potentially efficient route for the generation of large numbers of mutant mice that can be screened for novel phenotypes. The advantage of this approach is that, in assessing gene function, no a priori assumptions are made about the genes involved in any pathway. Phenotype-driven mutagenesis is thus an effective method for the identification of novel genes and pathways. We have undertaken a genome-wide, phenotype-driven screen for dominant mutations in the mouse. We generated and screened over 26,000 mice, and recovered some 500 new mouse mutants. Our work, along with the programme reported in the accompanying paper, has led to a substantial increase in the mouse mutant resource and represents a first step towards systematic studies of gene function in mammalian genetics.

O'Callaghan YC, Woods JA, O'Brien NM. **Limitations of the single-cell gel electrophoresis assay to monitor apoptosis in U937 and HepG2 cells exposed to 7beta-hydroxycholesterol.** Biochem Pharmacol 2001;61(10):1217-26.
Abstract: The single-cell gel electrophoresis (comet) assay is a method which allows the detection of DNA strand breaks in individual cells. It has been suggested that the single cell gel electrophoresis assay, as an index of DNA fragmentation during cell death, may be applied to monitor apoptosis. The aim of the present study was to determine if the pattern of DNA fragmentation determined by the single cell gel electrophoresis assay can be used to discriminate between the mode of cell death in two cell lines (U937, a human monocytic blood cell line and HepG2, a human hepatocarcinoma cell line) which were treated with 30 microM 7beta-hydroxycholesterol (7betaOHC) over a 48 hr period. The single cell gel electrophoresis assay was compared with more established methods for the determination of

apoptosis such as morphological examination, flow cytometry and DNA laddering. The percentage of maximally damaged nuclei as measured by the single cell gel electrophoresis assay was found to be similar at 48 hr in both U937 and HepG2 cells when treated with 7betaOHC. However, morphological examination, flow cytometry and DNA laddering techniques showed that 7betaOHC induced apoptosis in U937 cells but not in HepG2 cells. Thus, although the alkaline single cell gel electrophoresis assay detected DNA strand breaks occurring during cell death, these breaks were observed only when the process was fairly well advanced and a major part of the cells had lost membrane permeability. Therefore the present report demonstrates that the single cell gel electrophoresis assay, used in isolation, cannot accurately be used to distinguish between the mode of cell death induced by 7betaOHC in U937 cells (apoptosis), or HepG2 cells (cell lysis).

Ollmann M, Young LM, Di Como CJ, Karim F, Belvin M, Robertson S, Whittaker K, Demsky M, Fisher WW, Buchman A, et al. **Drosophila p53 is a structural and functional homolog of the tumor suppressor p53.** Cell 2000;101(1):91-101.
Abstract: The importance of p53 in carcinogenesis stems from its central role in inducing cell cycle arrest or apoptosis in response to cellular stresses. We have identified a Drosophila homolog of p53 ("Dmp53"). Like mammalian p53, Dmp53 binds specifically to human p53 binding sites, and overexpression of Dmp53 induces apoptosis. Importantly, inhibition of Dmp53 function renders cells resistant to X ray-induced apoptosis, suggesting that Dmp53 is required for the apoptotic response to DNA damage. Unlike mammalian p53, Dmp53 appears unable to induce a G1 cell cycle block when overexpressed, and inhibition of Dmp53 activity does not affect X ray-induced cell cycle arrest. These data reveal an ancestral proapoptotic function for p53 and identify Drosophila as an ideal model system for elucidating the p53 apoptotic pathway(s) induced by DNA damage.

Osswald K, Becker TW, Grimm M, Jahreis G, Pool-Zobel BL. **Inter- and intra-individual variation of faecal water - genotoxicity in human colon cells.** Mutat Res 2000;472(1-2):59-70.
Abstract: Exogenous nutritional factors modulate the faecal contents leading to an enhanced or reduced burden with toxic and cancerogenic factors. These factors are thought to contribute to colon cancer by inducing mutations or enhancing proliferation in colon cells. Faecal water more or less causes these effects in model systems and thus could be the basis for valuable biomarker approaches. Our investigations are aimed at determining geno- and cytotoxicity of faecal water in human colon cell lines in vitro. We are developing techniques for their applicability as biomarker tests during dietary intervention studies. Faecal water is isolated by centrifugation of the faeces at 25000xg and added to cultured human colon cells (HT29). Membrane damage as assessed by trypan blue exclusion is determined as a measure for cytotoxicity. Semiquantitative analysis of inducible DNA damage (breaks and alkali labile sites) are analysed with the single cell microgelelectrophoresis assay (comet-assay) and oxidised DNA bases by the additional use of repair specific enzymes. We have now determined baseline toxic activities and calculated inter- and intra-individual and -experimental coefficients of variation for faecal water from different subjects consuming similar or different diets. Most faecal water induced DNA damage and oxidised DNA bases in HT29 clone 19a cells (0.9-9.14 fold and 1.7-4.9 fold, respectively in comparison to the NaCl controls). Intra- and inter-experimental coefficients (CV) of variation, were in a similar order of magnitude and ranged from 6.9 to 31.4. In contrast both intra- and inter-individual variability were considerably higher (CV-ranges of 29.7-76.6 and 21.3-64.0,

respectively). Interestingly, these inter-individual values were not lowered when subjects consumed identical diets (CV-ranges of 28.4-126.0). However, following intervention with certain protective dietary regimens (e.g. lignan containing bread) significant reductions of faecal water-induced genotoxicity can be observed. Therefore, in spite of the expected and observed degrees of variation in this methodology, effective experimental protocols may still lead to detectable modulations of the level of toxic and genotoxic effects.

Pavanello S, Clonfero E. **Biological indicators of genotoxic risk and metabolic polymorphisms.** Mutat Res 2000;463(3):285-308.
Abstract: International scientific publications on the influence of metabolic genotypes on biological indicators of genotoxic risk in environmental or occupational exposure are reviewed. Biomarkers of exposure (substance or its metabolites in biological fluids, urinary mutagenicity, protein and DNA adducts) and of effects (chromosome aberrations (CAs), sister chromatid exchanges (SCEs), micronuclei (Mn), COMET assay, HPRT mutants) have been evaluated according to different genotypes (or phenotypes) of several activating/detoxifying metabolic activities. In less than half the studies (43 out of 95), the influence of genotype on the examined biological indicator was found, of which four report poorly reliable results (i.e., with scarce biological plausibility, because of the inconsistency of modulated effect with the type of enzymatic activity expressed). As regards urinary metabolites, the excretion of mercapturic acids (MA) is greater in subjects with high GST activity, that of 1-pyrenol and other PAH metabolites turns out to be significantly influenced by genotypes CYP1A1 or GSTM1 null, and that of exposure indicators to aromatic amines (AA) (acetylated and non-acetylated metabolites) is modulated by NAT2. In benzene exposure, preliminary results suggest an increase in urinary t, t-muconic acid (t,t-MA) in subjects with some genotypes. On urinary mutagenicity of PAH-exposed subjects, the effects of genotype GSTM1 null, alone or combined with NAT2 slow are reported. When DNA adduct levels are clearly increased in PAH-exposed group (18 out of 22), 7 out of 18 studies report the influence of GSTM1 null on this biomarker, and of the five studies which also examined genotype CYP1A1, four report the influence of genotype CYP1A1, alone or in combination with GSTM1 null. A total of 25 out of 41 publications (61%) evaluating the influence of metabolic polymorphisms on biomarkers of effect (cytogenetic markers, COMET assay, HPRT mutants) do not record any increase in the indicator due to exposure to the genotoxic agents studied, confirming the scarce sensitivity of these indicators (mainly HPRT mutants, Mn, COMET assay) for assessing environmental or occupational exposure to genotoxic substances. Concluding, in determining urinary metabolites for monitoring exposure to genotoxic substances, there is sufficient evidence that genetically-based metabolic polymorphisms must be taken into account in the future. The unfavourable association for the activating/detoxifying metabolism of PAH is also confirmed as a risk factor due to the formation of PAH-DNA adducts. The clearly protective role played by GSTT1 on DEB (and/or related compound)-induced sister chromatid exchanges (SCEs) should be noted. The modulating effects of genotypes on protein adduct levels in environmental and occupational exposure have not yet been documented, and most studies on the influence of genotype on biological indicators of early genotoxic effects report negative results.

Pavanello S, Clonfero E. **[Biomarkers of gentotoxic risk and metabolic polymorphism].** Med Lav 2000;91(5):431-69 [Ita].
Abstract: This paper reviews studies published in the international scientific literature evaluating the

influence of genetically based metabolic polymorphisms on biological indicators of genotoxic risk in environmental or occupational exposure. Exposures due to life style (i.e. diet or smoking) were not considered. Indicators are subdivided into internal dose indicators (concentration of the substance or its metabolites in biological fluids, urinary mutagenicity, adducts of hemoglobin, plasma proteins and DNA), and early biological effects (chromosome aberrations, sister chromatid exchanges, micronuclei, COMET assay, HPRT mutants). The metabolic genotypes (or phenotypes) examined by various authors are: ALDH2 (aldehyde dehydrogenase), CYP (P450 cytochrome) 1AI, CYP1A2, CYP2E1, CYP2D6, EPHX (epoxidohydrolase), NAT2 (N-acetyl transferase), NQO1 (NAD(P)H: kinone oxidoreductase), PON1 (paraoxonase), GST (glutathione S-transferase) M1, GSTT1 and GSTP1. In more than half the studies (52 out of 96), no influence of genotype was found in the biological indicator. This may be due either to the poor sensitivity of the indicator used, or to low exposure. In studies examining the effect of genotype on the indicator, the biological plausibility of the result was evaluated, i.e., whether the effect is consistent with the type of enzymatic activity expressed. Four studies reported not very reliable results and suggest either the unfavourable influence of genotype GSTM1 with high detoxifying activity, or enzymatic activity poorly involved in the metabolism of the xenobiotics in question (NAT2 in the case of PAH). As regards urinary metabolites of genotoxic agents, eight studies reported the modulating effect of genotype. The urinary excretion of mercapturic acids was greater in subjects with high GST activity. In exposure to PAH, urinary 1-pyrenol and PAH metabolites turn out to be significantly influenced by genotypes CYP1A1 or GSTM1 null; in exposure to aromatic amines, the influence of NAT2 on exposure indicators (levels of acetylated and non-acetylated metabolites) was confirmed. Exposure to benzene led to an increase in t-t-MA in some genotypes, although experimental verification is still necessary. As regards urinary mutagenicity, the effect of genotype GSTM1 null is reported, and of the same genotype combined with NAT2 slow, in non-smoking individuals subjected to high exposure to PAH and in cigarette-smoking/coke-oven workers. Lastly, the determination of urinary metabolites in monitoring exposure to genotoxic substances, provides sufficient evidence that genetically based metabolic polymorphisms must be taken into account in the future. There is still little evidence regarding the importance of genotype on the level of protein adducts in environmental and occupational exposure. A relatively large number of publications (22) dealt with DNA adduct levels in PAH exposure. In 18 studies, the biological indicator clearly increases with respect to values in control subjects. Of these studies, seven reported the influence of GSTM1 null on DNA adducts and, of the five studies which also examined genotype CYP1A1, four reported the influence on DNA adduct level of genotype CYP1A1, alone or in combination with GSTM1 null. It therefore seems as if the unfavourable association for the activating/detoxifying metabolism of PAH is a risk factor for the formation of PAH-DNA adducts. Most publications (25 out of 41; 61%) dealing with metabolic polymorphisms in effect indicators (cytogenetic markers, COMET assay, HPRT mutants) did not report any increase in the indicator due to exposure to the genotoxic agents studied. These indicators of genotoxic damage, including mainly the frequency of HPRT mutants (100%), Mn (90%) and the COMET assay (67%), are not sufficiently sensitive in revealing exposure, confirming that they are not parcticularly suitable for measuring exposure to genotoxic substances in occupational or environmental exposures. It is therefore difficult to assess the influence of metabolic genotypes by means of this type of biological indicator. The few positive results reported for SCE in occupational studies mentioned the influence of genotype ALDH2, either alone or in combination with genotype CYP2E1 in exposure to CVM, or in combination with GSTM1 null in exposure to epichlorohydrin. For CA the results showed unfavourable combinations

of genotypes CYP2E1, GSTM1 and PON1 in exposure to pesticides, and GSTM1 null in combination with NAT2 slow in exposure to urban air. All the remaining studies on the effect of genotype on biological indicators of cytogenetic damage reported negative results.

Pavlica M, Klobucar GI, Mojas N, Erben R, Papes D. **Detection of DNA damage in haemocytes of zebra mussel using comet assay.** Mutat Res 2001;490(2):209-14.
Abstract: The aim of the study was to use the comet assay on haemocytes of freshwater mussel, Dreissena polymorpha Pallas, for detection of possible DNA damage after exposure to pentachlorophenol (PCP) and to evaluate the potential application of the comet assay on mussel haemocytes for genotoxicity monitoring of freshwater environment. Zebra mussels were exposed for seven days to different concentrations (10, 80, 100, 150 microg/l) of PCP and in the river Sava downstream from Zagreb municipal wastewater outlet. Significant increase in DNA damage was observed after exposure to PCP at doses of 80 microg/l and higher and after in situ exposure in the river Sava as well. This study confirmed that the comet assay applied on zebra mussel haemocytes may be a useful tool in determining the potential genotoxicity of water pollutants.

Petersen AB, Gniadecki R, Wulf HC. **Laser scanning cytometry for comet assay analysis.** Cytometry 2000;39(1):10-5.
Abstract: BACKGROUND: The comet assay (single-cell gel electrophoresis) is a sensitive method for evaluating nuclear DNA damage. Previously used evaluation methods for the comet assay are time consuming and have an inherent risk of biased selection of comets due to manual selection and categorization of comet images. Laser scanning cytometry (LSC), the principle of which is equivalent to flow cytometry, enables quantification of fluorescence emitted from the cells on a microscope slide. In the present study, we explored whether LSC could be used to determine the degree of DNA damage demonstrated by the comet assay. METHODS: DNA damage was induced by ultraviolet A irradiation of keratinocytes and visualized by the comet assay. The evaluation included (a) LSC determination of DNA-specific fluorescence in 1,000 comet heads (undamaged DNA), (b) image acquisition of comets by rescanning of the microscope slide, and (c) digital image analysis and computation of tail moment and DNA content in the comet tails. RESULTS: Cells with damaged DNA were observed in a sub-G(1) area because the comet head loses DNA to the tail. We found a strong inverse correlation between tail moment and DNA content per nucleus. CONCLUSIONS: LSC enables an automated method for cell recognition and evaluation of the comets, thus providing quantitative information about nuclear DNA damage without subjective selection of analyzed comets.

Picer M, Kovac T, Britvic S, Picer N. **The chemical and biogenotoxic characterization of organic xenobiotics in aquatic sediment materials 1. The application and comparison of chemically non-specific and biogenotoxic methods.** Chemosphere 2001;44(8):1673-83.
Abstract: The aim of this work was to evaluate the Ames assay and mixed function oxidase (MFO)-Induct Test used in parallel with chemical group tests (ECD fingerprint and PAH estimation) for the characterization of the organic pollution of water sediment materials. Sediment materials were collected from "clean" and relatively heavily polluted locations in the Middle Adriatic Sea, and from some locations in continental Croatia polluted with wastewaters from different enterprises. Characterization of the organic extracts of the sediment materials investigated was performed chemically using UV

spectrofluorometry for the determination polyaromatic hydrocarbons (PAH) and gas chromatography for the determination of volatile EC detector sensitive materials. Genotoxic analysis of the extracts was performed using the MFO-Induct Test and mutagenicity testing using the Standard Plate Incorporation Test as described by Maron and Ames with Salmonella typhimurium TA 98. Measurement of the BaPMO enzyme activity in the livers of carp treated i.p. with total extracts of the sediment investigated confirmed that the methanol extracts generally contained more inducing matter than the petroleum ether extracts. Ames assay showed that for all the samples following the elimination of the sulfur, there was an increase in the number of revertants in comparison to the control number, which indicates that the samples contained mutagenic substances. The larger doses of extracts generally demonstrated cytotoxicity, as evidenced by a reduced number of spontaneous revertants in the SalmonellalMicrosome Test. Investigation of the correlation of the chemical parameters with the biological parameter showed that the induction of BaPMO exhibited a statistically significant correlation with the level of the ECD fingerprint of the petroleum ether sediment extract.

Piegorsch WW, Simmons SJ, Margolin BH, Zeiger E, Gidrol XM, Gee P. **Statistical modeling and analyses of a base-specific Salmonella mutagenicity assay.** Mutat Res 2000;467(1):11-9.
Abstract: Statistical features of a base-specific Salmonella mutagenicity assay are considered in detail, following up on a previous report comparing responses of base-specific Salmonella (Ames II) strains with those of traditional tester strains. In addition to using different Salmonella strains, the new procedure also differs in that it is performed as a microwell fluctuation test, as opposed to the standard plate or preincubation test. This report describes the statistical modeling of data obtained from the use of these new strains in the microwell test procedure. We emphasize how to assess any significant interactions between replicate cultures and exposure doses, and how to identify a significant increase in the mutagenic response to a series of concentrations of a test substance.

Pretsch W. **Enzyme-activity mutants in Mus musculus. I. Phenotypic description and genetic characterization of ethylnitrosourea-induced mutations.** Mamm Genome 2000;11(7):537-42.
Abstract: The specific activity of erythrocyte enzymes was measured to detect gene mutations in F(1)-offspring of male mice treatment with different doses (80, 160, or 250 mg/kg body weight) of ethylnitrosourea (ENU). Altogether 13,230 offspring were screened for 10 enzyme activities. Mutants with reduced activity as well as mutants with enhanced activity were found. Of the 36 independently observed mutations, 20 were homozygous lethal. Genetic and biochemical characterizations were routinely performed. These mutants provide insight into the mechanism of ENU mutagenesis and can serve as models for structure-function studies of the corresponding enzymes.

Reyes CM, Kollman PA. **Investigating the binding specificity of U1A-RNA by computational mutagenesis.** J Mol Biol 2000;295(1):1-6.
Abstract: The mammalian spliceosomal protein U1A binds a hairpin RNA with picomolar affinity. To examine the origin of this binding specificity, we carried out computational mutagenesis on protein and RNA residues in the U1A-RNA binding interface. Our computational mutagenesis methods calculate the relative binding affinity between mutant and wild-type as the sum of molecular mechanical energies and solvation free energies estimated with a continuum solvent model. We obtained good agreement with experimental studies and we verified mutations that abolish and improve binding. Therefore, we offer

these methods as computationally inexpensive tools for investigating and predicting the effects of site-specific mutagenesis.

Rong YS, Golic KG. **A targeted gene knockout in Drosophila.** Genetics 2001;157(3):1307-12.
Abstract: We previously described a method for targeted homologous recombination at the yellow gene of Drosophila melanogaster. Because only a single gene was targeted, further work was required to show whether the method could be extended to become generally useful for gene modification in Drosophila. We have now used this method to produce a knockout of the autosomal pugilist gene by homologous recombination between the endogenous locus and a 2.5-kb DNA fragment. This was accomplished solely by tracking the altered genetic linkage of an arbitrary marker gene as the targeting DNA moved from chromosome X or 2 to chromosome 3. The results indicate that this method of homologous recombination is likely to be generally useful for Drosophila gene targeting.

Rosenkranz HS, Cunningham AR. **The high production volume chemical challenge program: the relevance of the in vivo micronucleus assay.** Regul Toxicol Pharmacol 2000;31(2 Pt 1):182-9.
Abstract: The in vivo rodent bone marrow micronucleus assay (Mnt) has assumed a pivotal role in screening strategies for the identification of substances potentially carcinogenic to humans. The analysis of the results of the current international 5-year effort to provide toxicological data for high production volume chemicals will play a crucial role in developing future strategies for identifying health hazards. As part of that program, consideration is being given to accepting either in vitro genotoxicity data or results of the Mnt. The present analyses indicate that for hazard identification purposes that, in fact, in vitro genotoxicity test results, such as those derived from the Salmonella mutagenicity assay, may be an acceptable alternative.

Rotchell JM, Blair JB, Shim JK, Hawkins WE, Ostrander GK. **Cloning of the Retinoblastoma cDNA from the Japanese medaka (Oryzias latipes) and preliminary evidence of mutational alterations in chemically-induced retinoblastomas.** Gene 2001;263(1-2):231-7.
Abstract: We have cloned a medaka homolog of the human retinoblastoma (Rb) susceptibility gene. The medaka Rb cDNA encodes a predicted protein of 909 amino acids. DNA sequence analysis with other vertebrate Rb sequences demonstrates that the medaka Rb cDNA is highly conserved in regions of functional importance. An antibody raised against an epitope of the human pRb recognizes the protein product of the medaka Rb gene, detecting a 105 kDa protein in all tissues examined and at differential levels for the stages of embryonic development studied. The sequence reported herein, combined with the high degree of conservation observed in critical domains, has also facilitated a preliminary investigation of the molecular etiology of chemically-induced retinoblastoma. The mutational alterations characterized suggest that medaka may provide a novel model and, thus, provide additional insight into the human retinoblastoma condition.

Rotchell JM, Scoggins B, Blair JB, Ostrander GK. **Isolation and characterization of the retinoblastoma protein from fish.** Comp Biochem Physiol B Biochem Mol Biol 2001;130(3):385-91.
Abstract: The retinoblastoma (Rb) gene represents the first tumor suppressor gene characterized. The encoded protein, pRb, plays a crucial role in cell cycle control, preventing malignant cell proliferation. Recently, homologues of the Rb gene have been isolated in fish and the pocket domain, which is central

to Rb function, was conserved. In our studies, using coelocanth (Latimeria chalumnae), rainbow trout (Oncorhynchus mykiss), medaka (Oryzias latipes) and English sole (Parophrys vetulus), we have developed a simple protocol for the isolation of the Rb tumor suppressor protein and determined its' tissue and cellular localization. Fish Rb proteins display apparent molecular weights in the range of 100-110 kDa, similar to the human pRb. The protein was detected in all tissues examined, consistent with the proteins' universal role in cellular signalling. An interesting pattern of immunoreactive bands was detected in each of the cells' two main compartments, suggesting differential proteolysis. Immuno-analysis of the pRb in trout liver tumor material revealed an additional Rb reactive product that was absent in normal liver cell extracts.

Rothfuss A, Merk O, Radermacher P, Speit G. **Evaluation of mutagenic effects of hyperbaric oxygen (HBO) in vitro. II. Induction of oxidative DNA damage and mutations in the mouse lymphoma assay.** Mutat Res 2000;471(1-2):87-94.
Abstract: We recently showed that treatment of V79 cells with hyperbaric oxygen (HBO) efficiently induced DNA effects in the comet assay and chromosomal damage in the micronucleus test (MNT), but did not lead to gene mutations at the hprt locus. Using the comet assay in conjunction with bacterial formamidopyrimidine DNA glycosylase (FPG protein), we now provide indirect evidence that the same treatment leads to the induction of 8-oxoguanine, a premutagenic oxidative DNA base modification in V79 and mouse lymphoma (L5178Y) cells. We also demonstrate that HBO efficiently induces mutations in the mouse lymphoma assay (MLA). Exposure of L5178Y cells to HBO (98% O(2); 3bar) for 2h caused a clear mutagenic effect in the MLA, which was further enhanced after a 3h exposure. As this mutagenic effect was solely due to the strong increase of small colony (SC) mutants, we suggest that HBO causes mutations by induction of chromosomal alterations. Molecular characterization of induced SC mutants by loss of heterozygosity (LOH) analysis showed an extensive loss of functional tk sequences similar to the pattern found in spontaneous SC mutants. This finding confirmed that the majority of HBO-induced mutants is actually produced by a clastogenic mechanism. The induction of point mutations as a consequence of induced oxidative DNA base damage seems to be of minor importance.

Rothfuss A, Schutz P, Bochum S, Volm T, Eberhardt E, Kreienberg R, Vogel W, Speit G. **Induced micronucleus frequencies in peripheral lymphocytes as a screening test for carriers of a BRCA1 mutation in breast cancer families.** Cancer Res 2000;60(2):390-4.
Abstract: Enhanced sensitivity to the chromosome-damaging effects of ionizing radiation is a feature of many cancer-predisposing conditions. It has been suggested that women with breast cancer are deficient in the repair of radiation-induced DNA damage. We have now investigated whether mutagen sensitivity is related to mutations in the breast cancer gene BRCA1. We studied the induction and repair of DNA damage in lymphocytes of women from families with familial breast cancer and breast and ovarian cancer. The mutagens used were gamma-irradiation and hydrogen peroxide and the DNA effects were determined with the micronucleus test and the comet assay. Women with a BRCA1 mutation (n = 12) and relatives without the familial mutation (n = 10) were compared to controls (i.e., healthy women without family history of breast or ovarian cancer; n = 17). Our results indicate a close relationship between the presence of a BRCA1 mutation and sensitivity for the induction of micronuclei. Compared to a concurrent control, 10 of 11 women with a BRCA1 mutation showed elevated radiation sensitivity.

Of the 10 related women without the familial mutation, only 2 had clearly enhanced micronucleus frequencies. In addition to the sensitivity toward gamma-irradiation, hypersensitivity toward hydrogen peroxide was also observed, indicating that the mutagen sensitivity is not solely due to a defect in the repair of DNA double strand breaks. In contrast to the results with the micronucleus assay, we found no significant difference between women with and without a BRCA1 mutation with respect to the induction and repair of DNA damage in the comet assay. This finding suggests a normal rate of damage removal and points to a disturbed fidelity of DNA repair as a direct or indirect consequence of a BRCA1 mutation. Our results support the usefulness of induced micronucleus frequencies as a biomarker for cancer predisposition and suggest its application as a screening test for carriers of a BRCA1 mutation in breast cancer families.

Russo A. **In vivo cytogenetics: mammalian germ cells.** Mutat Res 2000;455(1-2):167-89.
Abstract: This chapter summarizes the most relevant methodologies available for evaluation of cytogenetic damage induced in vivo in mammalian germ cells. Protocols are provided for the following endpoints: numerical and structural chromosome aberrations in secondary oocytes or first-cleavage zygotes, reciprocal translocations in primary spermatocytes, chromosome counting in secondary spermatocytes, numerical and structural chromosome aberrations, and sister chromatid exchanges (SCE) in spermatogonia, micronuclei in early spermatids, aneuploidy in mature sperm. The significance of each methodology is discussed. The contribution of novel molecular cytogenetic approaches to the detection of chromosome damage in rodent germ cells is also considered.

Ryden E, Ekstrom C, Hellmer L, Bolcsfoldi G. **Comparison of the sensitivities of Salmonella typhimurium strains TA102 and TA2638A to 16 mutagens.** Mutagenesis 2000;15(6):495-502.
Abstract: The qualitative and quantitative sensitivity of the genetically related, histidine-auxtrophic Salmonella typhimurium strains TA102 and TA2638a to 16 compounds was examined. The compounds were mainly cross-linking and oxidising mutagens, the effects of which were known to be detected by strain TA102 preferentially or by a combination of Escherichia coli WP2 (pkM101) and uvrA/pkM101. The morphology and number of spontaneous revertants was also compared. Fourteen of the 16 compounds caused reversion in both strains. Bleomycin and streptomycin induced reversion in strain TA102 but not TA2638a. The greater sensitivity of TA102 to these compounds may be associated with the extrachromosomal location of the target genes. The overall quantitative sensitivity of the two strains was similar for the other compounds. The number of compounds that caused reversions at lower doses or produced greater proportional increases were the same in TA102 as in TA2638a. The spontaneous number of revertants, without and with metabolic activation, respectively, was 98 and 130 for TA2638a and 322 and 465 for TA102. Strain TA2638a formed larger, more uniform colonies than TA102. The present results together with those of previous studies indicate a high degree of concordance between the sensitivity of strains TA102 and TA2638 for the detection of mutagens. The uniform colony size and lower spontaneous reversion frequency seen with strain TA2638a compared with TA102 would make it more reliable and convenient for routine testing. It is concluded that strain TA2638a should be considered as an alternative to TA102 and included, as well as the two E.coli strains, in the set of bacterial strains used in the standard test battery for mutagenicity testing.

Salti GI, Das Gupta TK, Constantinou AI. **A novel use for the comet assay: detection of**

topoisomerase II inhibitors. Anticancer Res 2000;20(5A):3189-93.
Abstract: BACKGROUND: The simple and quick comet assay can quantitatively detect DNA cleavage in cells. This study aimed to determine whether the comet assay could be used to detect topoisomerase (topo) II inhibitors. MATERIALS AND METHODS: HT-29 colon cancer cells were pre-incubated with aclarubicin, a topo II antagonist, then treated with topo II poisons: etoposide (VP-16), teniposide (VM-26), 4'-(acridinylamino) methansulfon-m-anisidide (m-AMSA) and adriamycin (doxorubicin). We also tested a topo I poison (camptothecin) and a microtubule depolymerization inhibitor (taxol). RESULTS: Aclarubicin significantly reduced DNA cleavage induced by topo II poisons, but not that induced by camptothecin. In HL-60/MX2 cells (containing no topo II beta and reduced topo II alpha), DNA breakage induced by topo II poisons was lower. Also, aclarubicin antagonized topo I-mediated camptothecin-induced DNA cleavage in these resistant cells. CONCLUSIONS: The comet assay can be used to detect topo II poisons in cultured cells. Also, aclarubicin has a dual topo I and topo II antagonism, with "preferential antagonism" of topo II when topo II beta catalytic activity is normally expressed.

Sanchez P, Llorente MT, Castano A. **Flow cytometric detection of micronuclei and cell cycle alterations in fish-derived cells after exposure to three model genotoxic agents: mitomycin C, vincristine sulfate and benzo(a)pyrene.** Mutat Res 2000;465(1-2):113-22.
Abstract: The measurement of cytogenetic alterations in vitro is considered an initial step in the risk assessment procedures for genotoxic agents. The concern about genotoxic pollutants in natural fish population makes the use of fish-derived cells an useful tool for these purposes. The technological improvements in well-established cytogenetic endpoints, such as micronuclei (MN) estimations by means of flow cytometry, have been proposed in the later years using mammalian cells. In this work, we test the capability of flow cytometry to evaluate MN induction and cell cycle alterations in an established fish cell line (RTG-2) using three agent-inductor models at different concentrations and exposure periods. For mitomycin C, an inverse relationship between length of exposure period and concentrations was observed. A dose-response relationship was observed after exposing RTG-2 cells to vincristine sulfate and benzo(a)pyrene. As this study shows, RTG-2 cells respond to clastogenic and aneugenic effects of the tested chemicals through the induction of MN at similar doses to mammalian cells and without the addition of exogenous metabolic activity. The possibility to check cell cycle alterations, in the same sample, gives the opportunity to evaluate early signals of cytotoxicity. The use of flow cytometry improves the assay by means of its speed and objectivity, which makes the assay very useful for genotoxicity assessment of aquatic chemicals.

Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. **The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database.** Crit Rev Toxicol 2000;30(6):629-799.
Abstract: The comet assay is a microgel electrophoresis technique for detecting DNA damage at the level of the single cell. When this technique is applied to detect genotoxicity in experimental animals, the most important advantage is that DNA lesions can be measured in any organ, regardless of the extent of mitotic activity. The purpose of this Article is to summarize the in vivo genotoxicity in eight organs of the mouse of 208 chemicals selected from International Agency for Research on Cancer (IARC)

Groups 1, 2A, 2B, 3, and 4, and from the U.S. National Toxicology Program (NTP) Carcinogenicity Database, and to discuss the utility of the comet assay in genetic toxicology. Alkylating agents, amides, aromatic amines, azo compounds, cyclic nitro compounds, hydrazines, halides having reactive halogens, and polycyclic aromatic hydrocarbons were chemicals showing high positive effects in this assay. The responses detected reflected the ability of this assay to detect the fragmentation of DNA molecules produced by DNA single strand breaks induced chemically and those derived from alkali-labile sites developed from alkylated bases and bulky base adducts. The mouse or rat organs exhibiting increased levels of DNA damage were not necessarily the target organs for carcinogenicity. It was rare, in contrast, for the target organs not to show DNA damage. Therefore, organ-specific genotoxicity was necessary but not sufficient for the prediction of organ-specific carcinogenicity. It would be expected that DNA crosslinkers would be difficult to detect by this assay, because of the resulting inhibition of DNA unwinding. The proportion of 10 DNA crosslinkers that was positive, however, was high in the gastrointestinal mucosa, stomach, and colon, but less than 50% in the liver and lung. It was interesting that the genotoxicity of DNA crosslinkers could be detected in the gastrointestinal organs even though the agents were administered intraperitoneally. Chemical carcinogens can be classified as genotoxic (Ames test-positive) and putative nongenotoxic (Ames test-negative) carcinogens. The Ames test is generally used as a first screening method to assess chemical genotoxicity and has provided extensive information on DNA reactivity. Out of 208 chemicals studied, 117 are Ames test-positive rodent carcinogens, 43 are Ames test-negative rodent carcinogens, and 30 are rodent noncarcinogens (which include both Ames test-positive and negative noncarcinogens). High positive response ratio (110/117) for rodent genotoxic carcinogens and a high negative response ratio (6/30) for rodent noncarcinogens were shown in the comet assay. For Ames test-negative rodent carcinogens, less than 50% were positive in the comet assay, suggesting that the assay, which detects DNA lesions, is not suitable for identifying nongenotoxic carcinogens. In the safety evaluation of chemicals, it is important to demonstrate that Ames test-positive agents are not genotoxic in vivo. This assay had a high positive response ratio for rodent genotoxic carcinogens and a high negative response ratio for rodent genotoxic noncarcinogens, suggesting that the comet assay can be used to evaluate the in vivo genotoxicity of in vitro genotoxic chemicals. For chemicals whose in vivo genotoxicity has been tested in multiple organs by the comet assay, published data are summarized with unpublished data and compared with relevant genotoxicity and carcinogenicity data. Because it is clear that no single test is capable of detecting all relevant genotoxic agents, the usual approach should be to carry out a battery of in vitro and in vivo tests for genotoxicity. The conventional micronucleus test in the hematopoietic system is a simple method to assess in vivo clastogenicity of chemicals. Its performance is related to whether a chemical reaches the hematopoietic system. Among 208 chemicals studied (including 165 rodent carcinogens), 54 rodents carcinogens do not induce micronuclei in mouse hematopoietic system despite the positive finding with one or two in vitro tests. Forty-nine of 54 rodent carcinogens that do not induce micronuclei were positive in the comet assay, suggesting that the comet assay can be used as a further in vivo test apart from the cytogenetic assays in hematopoietic cells. In this review, we provide one recommendation for the in vivo comet assay protocol based on our own data.

Sastre MP, Vernet M, Steinert S. **Single-cell gel/comet assay applied to the analysis of UV radiation-induced DNA damage in Rhodomonas sp. (Cryptophyta).** Photochem Photobiol 2001;74(1):55-60.
Abstract: The single-cell gel/comet assay is an electrophoretic technique used to detect single-strand

breaks in DNA. Damage is assessed examining individual cells under an epifluorescent microscope. UV-induced DNA damage consists mostly of the formation of pyrimidine dimers; therefore, most of the damage cannot be detected using a standard comet assay. The enzyme T4 endonuclease V breaks DNA strands at sites of pyrimidine dimers. The main objective of this work is to evaluate the comet assay to detect UV-induced damage in DNA after an initial treatment of cells with T4 endonuclease V. This work was conducted on Rhodomonas sp. (Cryptophyta), a marine unicellular flagellate. Cells of Rhodomonas sp. were exposed to 12 h visible + ultraviolet-A + ultraviolet-B (VIS + UVA + UVB) and VIS (control), with and without T4 endonuclease V. Cells exposed to VIS + UVA + UVB showed approximately 200% more damage than control if these were treated with T4 endonuclease V. Rhodomonas sp. were exposed to 3, 6, 9 and 12 h of VIS, VIS + UVA and VIS + UVA + UVB. Damage induced by VIS + UVA + UVB as detected by the comet assay increased along with exposure time. However, damage caused by VIS and VIS + UVA remained relatively constant at all times. Results of this study indicate that the comet assay is more sensitive to UV radiation damage when used in conjunction with T4 endonuclease V. This modification of the comet assay can be used as an alternative technique to detect DNA damage in single cells caused by UV radiation.

Sauer G, Weber KJ, Peschke P, Eble MJ. **Measurement of hypoxia using the comet assay correlates with preirradiation microelectrode pO2 histography in R3327-AT rodent tumors.** Radiat Res 2000;154(4):439-46.

Abstract: Polarographic determination of tumor oxygenation by Eppendorf histography is currently under investigation as a possible predictor of radiotherapy outcome. Alternatively, the alkaline comet assay has been proposed as a radiobiological approach for the detection of hypoxia in clinical tumor samples. Direct comparisons of these methods are scarce. One earlier study with different murine tumors could not establish a correlation, whereas a weak correlation was reported for a variety of human tumors. Considering the different end points and spatial resolution of the two methods, a direct comparison for a single tumor entity appeared desirable. Anaplastic R3327-AT Dunning prostate tumors were grown on Copenhagen rats to volumes of 1-6 cm(3). Eppendorf histography (100-200 readings in 5 parallel tracks) for 8 different tumors revealed various degrees of oxygenation, with median pO(2) values ranging from 1.1 to 23 mmHg. Within 5 min after an acute exposure to 8 Gy (60)Co gamma rays, tumors were excised from killed animals and rapidly cooled to limit repair, and a single cell suspension was prepared for use with the comet assay. The resulting comet moment distributions did not exhibit two subpopulations (one hypoxic and the other aerobic), and a hypoxic fraction could not be calculated. Instead, the average comet moment distribution was taken as a parameter of overall strand break induction. Corresponding experiments with tumor cells grown in vitro allowed us to derive the relationship between the oxygen enhancement ratio (OER) for the average comet moment and oxygen partial pressure (Howard-Flanders and Alper formula). The validity of this relationship was inferred for cells exposed in situ, and the convolution of a pO(2) distribution with the formula of Howard-Flanders and Alper yielded an array of expected OER values for each tumor. The average expected OER correlated well with the average comet moment ($r = 0.89$, $P < 0.01$), and the in situ comet moment distributions could be predicted from the Eppendorf data when 50% repair was taken into account, assuming a 5-min damage half-life. The findings confirm the potential of interstitial polarography to reflect radiobiologically relevant intracellular oxygenation, but also underscore the confounding influence of differences in repair that may occur when cells are prepared from irradiated tissues for use

with the comet assay.

Scacheri PC, Crabtree JS, Novotny EA, Garrett-Beal L, Chen A, Edgemon KA, Marx SJ, Spiegel AM, Chandrasekharappa SC, Collins FS. **Bidirectional transcriptional activity of PGK-neomycin and unexpected embryonic lethality in heterozygote chimeric knockout mice.** Genesis 2001;30(4):259-63.
Abstract: Summary: In an effort to create a conventional knockout mouse model for multiple endocrine neoplasia type 1 (MEN1), we targeted disruption of the mouse Men1 gene through homologous recombination in ES cells. Men1 exons 2-4 were replaced by a PGK-neomycin cassette inserted in the opposite direction of Men1 transcription (Men1(MSK/+)). Unexpectedly, the Men1 conventional knockout was lethal in heterozygous, chimeric animals. Analysis of embryos revealed late gestational lethality with some embryos showing omphalocele. This was a very surprising phenotype, given that humans and mice that are heterozygotes for loss of function mutations in MEN1 are phenotypically normal except for a risk of endocrine tumors. Northern analysis of Men1(MSK/+) embryonic stem cell RNA revealed the presence of an abundant, novel transcript of 2.1 kb, in addition to the expected wild-type transcripts of 2.7 kb and 3.1 kb. RT-PCR analysis identified this aberrant transcript as arising from the antisense strand of the PGK promoter. We hypothesize that this transcript is producing either a toxic effect at the RNA level, or a dominant negative effect through the production of an amino-terminal truncated protein product. This example serves as a cautionary reminder that mouse knockouts using PGK-neo may sometimes display phenotypes that reflect more than just the loss of function of the targeted gene. genesis 30:259-263, 2001. Published 2001 Wiley-Liss, Inc.

Scheffler IE, Yadava N. **Molecular genetics of the mammalian NADH-ubiquinone oxidoreductase.** J Bioenerg Biomembr 2001;33(3):243-50.
Abstract: A serendipitous observation led to the first characterization of a respiration-deficient Chinese hamster mutant cell line. It has guided the design of an enrichment scheme for the isolation of additional mutant cell lines. Several complementation groups were identified with mutations affecting complex I. The X-linked NDUFA1 gene encoding the MWFE protein represents one group. Several mutant alleles isolated independently are described that yield very low activities and demonstrate that the MWFE protein is essential for activity. A phylogenetic sequence analysis of this highly conserved protein has directed attention to species-specific differences that make the primate MWFE protein inactive in hamster cells. Based on such comparisons, mutant alleles made by site-directed mutagenesis were expressed in a null mutant and reduced complex I activities were observed, with the mutant protein assembled into the complex. These and other mutants promise to be valuable for structure-function analyses, especially in conjunction with a high-resolution structure to be expected in the future. The possibility for transgenic and knock-in mice as models for mitochondrial diseases is being explored.

Schindewolf C, Lobenwein K, Trinczek K, Gomolka M, Soewarto D, Fella C, Pargent W, Singh N, Jung T, Hrabe de Angelis M. **Comet assay as a tool to screen for mouse models with inherited radiation sensitivity.** Mamm Genome 2000;11(7):552-4.
Abstract: Recent in vivo and in vitro data of patients analyzed for genetic susceptibility to radiation during cancer therapy have shown structural changes in the chromosomes to be prevalent both in the patients being treated and in their immediate family members. As structural changes in chromosomes

frequently lead to activation of proto-oncogenes and elimination of tumor-suppressor genes, they represent important mechanisms for the initiation of DNA repair processes and tumorigenesis. With the exception of rare genetic syndromes such as AT (Ataxia telangiectasia) or NBS (Nijmegen Breakage Syndrome), the background for the inheritance of genetic susceptibility to radiation is unknown. Recently, a large-scale genetic screen of mouse mutants has been established within the German Human Genome Project (Hrabe de Angelis and Balling 1998). The goal of this ENU (ENU: ethylnitrosourea) mutagenesis screen is the generation of mutant mice that will serve as animal models for human diseases and genetic susceptibility. In order to fully utilize the potential of a genetic screen of this magnitude, in which exploration for genes responsible for genomic instability and radiation sensitivity is to occur, it is necessary to establish a simple assay system that is amenable to automation. Hence, we are using the single-cell gel electrophoresis (comet assay) to detect mouse mutants that display a genetic susceptibility to ionizing radiation. We have established the analysis parameters in the comet assay which are currently used to detect radiation-sensitive mouse mutants and to control the variance within the mouse population in the ENU screen. The assay can be used to isolate genes that are responsible for DNA repair and radiation sensitivity in mouse and human.

Schwartz JL, Jordan R, Sun J, Ma H, Hsieb AW. **Dose-dependent changes in the spectrum of mutations induced by ionizing radiation**. Radiat Res 2000;153(3):312-7.
Abstract: We examined the influence of dose on the spectrum of mutations induced at the hypoxanthine guanine phosphoribosyltransferase (Hprt) locus in Chinese hamster ovary (CHO) cells. Independent CHO-K1 cell mutants at the Hprt locus were isolated from cells exposed to 0, 0.5, 1.5, 3.0 and 6.0 Gy (137)Cs gamma rays, and the genetic changes responsible for the mutations were determined by multiplex polymerase chain reaction (PCR)-based exon deletion analysis. We observed dose-dependent changes in mutation spectra. At low doses, the principal radiation-induced mutations were point mutations. With increasing dose, multibase deletion mutations became the predominant mutation type such that by 6.0 Gy, there were almost three times more deletion mutations than point mutations. The dose response for induction of point mutations was linear while that for multibase deletions fit a linear-quadratic response. There was a biphasic distribution of deletion sizes, and different dose responses for small compared to large deletions. The frequency of large (>36 kb) total gene deletions increased exponentially, implying that they develop from the interaction between two independent events. In contrast, the dose response for deletion mutations of less than 10 kb was nearly linear, suggesting that these types of mutations develop mostly from single events and not the interactions between two independently produced lesions. The observation of dose-dependent changes in radiation-induced mutation spectra suggests that the types of alterations and therefore the risks from low-dose radiation exposure cannot be easily extrapolated from high-dose effects.

Scoglio ME, Di Pietro A, Anzalone C, Calimeri S, Lo Giudice D, Trimarchi GR. **Direct viable count as test for toxicity assessment: the effects of four metals on a Salmonella enteritidis strain.** Ann Ig 2000;12(6):457-68.
Abstract: The toxicity of synthetic sewage containing increasing concentrations of arsenic (.125, .25, .5, 1.0 mg L-1), cadmium (.02, .05, .1, .2 mg L-1), lead (.2, .5, 1.0, 2.0 mg L-1) and nickel (.5, 1.0, 2.0, 4.0 mg L-1) has been investigated by determining the total direct count (TDC) and the direct viable count (DVC) of Salmonella enteritidis by means of an immunofluorescence technique (IFA). This has been

done in order to evaluate the possibility of using the IFA technique to estimate the toxicity of complex effluents. Arsenic, cadmium and nickel produced a concentration-dependent reduction in the number of viable bacterial cells. This was more clear when the viable bacterial cells were considered than when only the culturable part was used. Lead did not show a concentration-dependent and reproducible effect. At the highest concentrations allowed by the Italian wastewater regulations, lead, cadmium, arsenic and nickel reduced the viable/total bacterial cells ratio to 74.5%, 68.5%, 28.4% and 6.9%, respectively. The toxic effects of the metals were also tested using the standard Microtox assay.

Sekihashi K, Sasaki T, Yamamoto A, Kawamura K, Ikka T, Tsuda S, Sasaki YF. **A comparison of intraperitoneal and oral gavage administration in comet assay in mouse eight organs.** Mutat Res 2001;493(1-2):39-54.
Abstract: One of the important advantages of the comet assay is its ability to detect genotoxicity in many different organs. Since the exposure route of the test compounds is likely to influence the genotoxicity detected in a given organ, it is an important factor to consider when conducting the assay. In this study, we compared the effects of numerous model compounds on eight organs when administered to mice by intraperitoneal (i.p.) injection and oral (p.o.) gavage.Groups of four mice were treated once i.p. or p.o. at the identical proportion of LD50 for each route, and the stomach, colon, liver, kidney, bladder, lung, brain, and bone marrow were sampled 3, 8, and 24h after treatment. For 19 of the 20 tested mutagens with various modes of action, genotoxicity in some organs varied with treatment route; only the genotoxicity of methyl methane sulfonate was not affected. Treatment route, however, did not produce a qualitative difference in the genotoxicity of promutagens at the sites of conversion to ultimate mutagens, with aromatic hydrocarbons as the exception. When chemicals with positive responses in at least one organ were considered to be comet assay-positive, the administration route made no difference. Since azo reduction is mediated by azo reductase synthesized in the gastrointestinal wall and by gut microflora and i.p.-administered azo dyes bypass their activation site (colon), the administration route is expected to make a difference in their in vivo genotoxicity. Direct-acting mutagens are expected to affect the mucosa of the gastrointestinal tract when given p.o. For those mutagens, however, the administration route did not make a qualitative difference in gastrointestinal tract genotoxicity. Moreover, although the gastrointestinal mucosa is the first site to be exposed to p.o. administered agents, the peak times in the stomach tended to be the same as in most other organs. Based on those results, we concluded that the genotoxicity at high exposures was due to a systemic effect, and that both routes are acceptable for the comet assay when the liver and gastrointestinal organs are sampled, so long as appropriate dose levels for systemic exposure are selected for each route.

Selvakumaran M, Bao R, Crijns AP, Connolly DC, Weinstein JK, Hamilton TC. **Ovarian epithelial cell lineage-specific gene expression using the promoter of a retrovirus-like element.** Cancer Res 2001;61(4):1291-5.
Abstract: We have isolated 462 bp of sequence termed ovarian-specific promoter 1 (OSP-1) that is part of a retrovirus-like element specifically expressed in the rat ovary. We have evaluated the ability of OSP-1 to activate gene expression in normal and neoplastic cell lines derived from the ovaries of rats and women. We have found that there was marked specificity in the ability of OSP-1 to drive reporter gene expression in an ovarian epithelial cell lineage manner. The expression of herpes simplex virus thymidine kinase (HSV-TK) under OSP-1 control was sufficiently ovarian cancer cell line specific to

render ganciclovir approximately 50-fold more toxic in the A2780 human ovarian cancer cell line compared with clones of the HCT-116 and HT-29 colon cancer cell lines. Furthermore, ganciclovir had marked antitumor efficacy in vivo in severe combined immunodeficient mice bearing A2780OSP-1-HSV-TK as a s.c. xenograft. We suggest that these data support the use of OSP-1 as a tool to provide specificity to the gene therapy of ovarian cancer and to drive ovarian-specific oncogene expression for the creation of transgenic mouse models of ovarian cancer.

Seong S, Park TH. **Swimming characteristics of magnetic bacterium, Magnetospirillum sp. AMB-1, and implications as toxicity measurement.** Biotechnol Bioeng 2001;76(1):11-6.
Abstract: To develop a novel toxicity measurement system using the persistent swimming property of magnetic bacteria along an externally applied magnetic field, certain characteristics of Magnetospirillum sp. AMB-1 cells were examined, including their growth pattern, motility, magnetosensitivity, swimming speed, and cell length distribution. In addition, the effect of toxic compounds on the swimming speed was assessed relative to application as a toxicity sensor. With an inoculum of 1.0 x 10(8) cells/mL, the cells reached the stationary phase with a concentration of about 5 x 10(8) cells/mL after 20 h, under both aerobic and anaerobic conditions. The distribution of the cell length did not vary significantly during the growth period, and both aerobically and anaerobically growing cells showed a similar cell length distribution. Although the cells showed similar growth patterns under both conditions, the anaerobically grown cells exhibited higher motility and magnetosensitivity. Actively growing cells under anaerobic conditions had an average swimming speed of 49 microm/s with a standard deviation of 20 microm/s. When the anaerobically growing cells were exposed to various concentrations of toxic compounds, such as 1-propanol and acetone, the swimming speed decreased with an increased concentration of the toxic compound. Accordingly, the relationship between swimming speed and toxicity can be used as an effective quantitative toxicity measurement; furthermore, the relative sensitivity of the proposed system was comparable to Microtox, which is commercially available. Copyright 2001 John Wiley & Sons, Inc.

Shen MM, Schier AF. **The EGF-CFC gene family in vertebrate development.** Trends Genet 2000;16 (7):303-9.
Abstract: EGF-CFC genes encode extracellular proteins that play key roles in intercellular signaling pathways during vertebrate embryogenesis. Mutations in zebrafish and mouse EGF-CFC genes lead to defects in germ-layer formation, anterior-posterior axis orientation and left-right axis specification. In addition, members of the EGF-CFC family have been implicated in carcinogenesis. Although formerly regarded as signaling molecules that are distant relatives of epidermal growth factor (EGF), recent findings indicate that EGF-CFC proteins act as essential cofactors for Nodal, a member of the transforming growth factor beta (TGF-beta) family. Here, we review molecular genetic evidence from mouse and zebrafish on biological and biochemical roles of the EGF-CFC family, and discuss differing models for EGF-CFC protein function.

Shettlemore MG, Bundy KJ. **Examination of in vivo influences on bioluminescent microbial assessment of corrosion product toxicity.** Biomaterials 2001;22(16):2215-28.
Abstract: The composition of ionically dissolved and precipitated corrosion products from both free corrosion of ASTM F75 Co-Cr-Mo and galvanostatic polarization of Co-Cr-Mo and F138 316L stainless steel was determined using differential pulse polarography and inductively coupled plasma atomic

emission spectroscopy. A bacterial bioluminescence assay, Microtox, was used to assess the toxicity of the solid and dissolved corrosion products produced by galvanostatic polarization and the individual ions within them. The role of in vivo salinity, temperature, and protein content as modulators of corrosion product toxicity assessment was investigated empirically and mechanistically. Co-Cr-Mo products were found to be more toxic than those of 316L, and the most toxic ions were Cr6+, Ni2+, and Co2+. Ringer's solution potentiated the toxicity of the more toxic metal ions and reduced the toxicity of the less toxic ions. Using theoretical analysis in conjunction with experimental measurements, the ions in both alloys were found to interact in an antagonistic fashion. The presence of albumin was found to decrease metal toxicity, presumably by chelation.

Slebos RJ, Taylor JA. **A novel host cell reactivation assay to assess homologous recombination capacity in human cancer cell lines.** Biochem Biophys Res Commun 2001;281(1):212-9.
Abstract: Repair of DNA double-strand breaks (DSB) is essential for cell viability and genome stability. Homologous recombination repair plays an important role in DSB repair and impairment of this repair mechanism may lead to loss of genomic integrity, which is one of the hallmarks of cancer. Recent research has shown that the tumor suppressor genes p53 and BRCA1 and -2 are involved in the proper control of homologous recombination, suggesting a role of this type of repair in human cancer. We developed a novel assay based on recombination between two Green Fluorescent Protein (GFP) sequences in transiently transfected plasmid DNA. The plasmid construct contains an intact, emission-shifted, "blue" variant of GFP (BFP), with a 300 nucleotide stretch of homology to a nonfunctional copy of GFP. In the absence of homologous recombination only BFP is present, but homologous recombination can create a functional GFP. The homologous regions in the plasmid were constructed in both the direct and the inverted orientation of transcription to detect possible differences in the recombination mechanisms involved. A panel of human tumor cell lines was chosen on the basis of genetic background and chromosome integrity and tested for homologous recombination using this assay. The panel included cell lines with varying levels of karyotypic abnormalities, isogenic cell lines with normal and mutant p53, isogenic cell lines with or without DNA mismatch repair, BRCA1 and -2 mutant cell lines, and the lymphoma cell line DT40. With this assay, the observed differences between cell lines with the lowest and highest levels of recombination were about 100-fold. Increased levels of recombination were associated with mutant p53, whereas a low level of recombination was found in the BRCA1 mutant cell line. In the cell line HT1080TG, a mutagenized derivative of HT1080 with two mutant alleles of p53, high levels of recombination were found with the direct orientation but not with the inverted orientation plasmid. No difference in recombination was detected between two isogenic cell lines that only differed in DNA mismatch repair capability. We conclude that this assay can detect differences in homologous recombination capacity in cultured cell lines and that these differences follow the patterns that would be expected from the different genotypes of these cell lines. Future application in normal cells may be useful to identify genetic determinants controlling genomic integrity or to detect differences in DNA repair capacity in individuals.

Smilenov LB, Brenner DJ, Hall EJ. **Modest increased sensitivity to radiation oncogenesis in ATM heterozygous versus wild-type mammalian cells.** Cancer Res 2001;61(15):5710-3.
Abstract: Subpopulations that are genetically predisposed to radiation-induced cancer could have significant public health consequences. Individuals homozygous for null mutations at the ataxia

telangiectasia gene are indeed highly radiosensitive, but their numbers are very small. Ataxia Telangiectasia heterozygotes (1-2% of the population) have been associated with somewhat increased radiosensitivity for some end points, but none directly related to carcinogenesis. Here, intralitter comparisons between wild-type mouse embryo fibroblasts and mouse embryo fibroblasts carrying ataxia telangiectasia mutated (ATM) null mutation indicate that the heterozygous cells are more sensitive to radiation oncogenesis than their normal, litter-matched, counterparts. From these data we suggest that Ataxia Telangiectasia heterozygotes could indeed represent a societally-significant radiosensitive human subpopulation.

Smolewski P, Bedner E, Gorczyca W, Darzynkiewicz Z. **"Liquidless" cell staining by dye diffusion from gels and analysis by laser scanning cytometry: potential application at microgravity conditions in space.** Cytometry 2001;44(4):355-60.
Abstract: BACKGROUND: Conventional staining of cells or tissue sections on microscope slides involves immersing the slides into solutions of dyes then rinsing to remove the unbound dye. There are instances, however, when use of stain solutions is undesirable-e.g., at microgravity conditions in space, where the possibility of accidental spill (many dyes are known carcinogens) introduces health hazard. Likewise, transporting bulk of liquid stains and rinses may be burdensome in certain situations such as field expeditions or combat. METHODS: The "liquidless" staining procedure is proposed in which the dyes are contained in thin strips of hydrated polyacrylamide or gelatin gels that have been presoaked in the stain solutions. Fluorochromes that have affinity to DNA (propidium iodide, PI; 4,6-diamidino-2-phenylindole, DAPI, Hoechst 33342) or to protein (sulforhodamine 101) were used to saturate the gels. The gel strips were placed over the prefixed cells or tissue sections deposited on microscope slides and relatively low (20 g/cm2) pressure was applied to ensure the contact. The cells were also stained by using commercially available mounting media into which DAPI or PI were admixed. Intensity of fluorescence of the PI stained cells was measured by laser scanning cytometry (LSC). RESULTS: Satisfactory cell and tissue staining, with minimal background, was achieved after 10-20 min contact between the cells and gels. Optimal concentrations of the dyes in the solutions used to presoak the gels was found to be 2-4-fold higher than the concentrations used routinely in cytometry. The measurements of intensity of cellular fluorescence by LSC revealed that the staining of DNA was stoichiometric as reflected by the characteristic cellular DNA content frequency histograms with distinct G1, S, and G2/M cell populations and 2:1 ratio of G2/M to G1 peak fluorescence. Individual gels can be saturated with more than a single dye-e.g., to obtain differential DNA and protein staining. Cell staining with DAPI or PI in the gelatin-based mounting media led to high fluorescence background while staining with DAPI in "aqueous" medium was satisfactory. CONCLUSIONS: Relatively fast staining of cells or tissue sections on microscope slides can be achieved by nonconvective dye diffusion using hydrated gels permeated with the dyes, applied to cells at low pressure. The quality of the staining provided by this methodology is comparable to conventional cell staining in dye solutions.

Snyder RD. **Use of catalytic topoisomerase II inhibitors to probe mechanisms of chemical-induced clastogenicity in Chinese hamster V79 cells.** Environ Mol Mutagen 2000;35(1):13-21.
Abstract: Determination of the clastogenic potential of new chemical entities, parcticularly pharmaceuticals, is an important part of the overall safety assessment of such drugs. It is appreciated that clastogenicity can arise from perturbation of many different cellular processes distinct from direct DNA/

drug interactions. One such alternative clastogenic process is inhibition of DNA topoisomerase II, during which process the topoisomerase/DNA/drug ternary complex forms stable DNA double-strand breaks (cleavable complex), which become templates for recombinational, mutagenic, and chromosomal fragmentation events. Without extensive experimentation, it is generally not possible to distinguish clastogenicity arising from direct drug/DNA interaction from that arising from inhibition of topoisomerase II. In the present investigation, we demonstrate that specific catalytic inhibitors of DNA topoisomerase II reduce the clastogenicity of topoisomerase poisons but not that arising via non-topoisomerase-dependent mechanisms. In parcticular, it is shown that catalytic topoisomerase II inhibitors such as chloroquine, sodium azide, and A-74932, as well as certain intercalating agents such as 9-aminoacridine and ethidium bromide, strongly antagonize the formation of micronuclei induced by the DNA gyrase inhibitor clinafloxacin and the antitumor topoisomerase II poison etoposide. These catalytic inhibitors are also shown to antagonize the clastogenicity of experimental compounds and novel pharmaceuticals presumed to be DNA intercalating agents by virtue of their response in a cell-based bleomycin amplification assay. We extend our previous hypothesis, suggesting that the clastogenicity of some nonstructurally alerting drugs may be due to an as yet unappreciated propensity for DNA intercalation. It is further proposed that intercalation-dependent inhibition of DNA topoisomerase II may be responsible for this clastogenicity and that this may be detected in intact mammalian cells with the use of catalytic topoisomerase inhibitors.

Song HL, Jenkins GJ, Ashby J, Tinwell H, Parry JM. **The application of the restriction site mutation assay to compare 1-ethyl-1-nitrosourea-induced mutations between the endogenous p53 gene and the transgenic LacZ gene in MutaMouse testes.** Mutagenesis 2001;16(1):59-64.
Abstract: Transgenic mouse modelling has provided a new approach to study the various steps involved in spontaneous and induced mutagenesis in rodent somatic and germline tissues in vivo. However, the important question arises as to whether mutations occur at the same rate in transgenes as in endogenous genes. Here, the restriction site mutation (RSM) assay was used to study mutations induced in the endogenous p53 gene and LacZ transgene of MutaMouse testes treated with 1-ethyl-1-nitrosourea (ENU). The aim of these experiments was to compare mutation susceptibility between the endogenous p53 gene and the integrated LacZ gene in the transgenic mouse. ENU-treated and control testes were analysed 102 days after treatment; a total of 297 RSM analyses were performed on ENU-treated and untreated testis DNA. Ten mutational events were detected in the p53 gene (exon 5 and intron 8), two of which occurred in untreated animals and probably represent spontaneous events. Only a single mutation was detected in the LacZ gene of an ENU-treated animal by the RSM assay. Thus the RSM assay can readily detect ENU-induced mutations in the p53 gene, but not in the LacZ transgene. Comparison of the LacZ RSM mutation data with results from a previous study of identically dosed MutaMice in the transgenic selection assay [Ashby, J., Gorelick,N.J. and Shelby,M.D. (1997) Mutat. Res., 388, 111-122] showed that LacZ mutations were far more readily recovered with the MutaMouse transgenic selection assay than by RSM analysis. The reason for the relative inability of the RSM assay to detect LacZ mutations may be the smaller target size of the RSM analysis compared with the transgenic selection assay (16 bases compared with 3000 bases). Taking into account the different target sizes by calculating the mutation frequency per base allowed the RSM data regarding p53 and LacZ to be compared with previously published data from transgenic selection assays. These studies demonstrated that the p53 mutations were present at mutation frequencies (per base) 5- to 70-fold higher than the LacZ gene

mutations. In addition, the LacZ mutation frequency per base found in the RSM was an order of magnitude higher than that found in the transgenic selection assay. The transgenic selection assay is more sensitive per locus (due to the larger target of the LacZ gene), as evidenced by ability to detect ENU-induced testes mutations readily.

Sotomayor RE, Sega GA. **Unscheduled DNA synthesis assay in mammalian spermatogenic cells: an update.** Environ Mol Mutagen 2000;36(4):255-65.
Abstract: The unscheduled DNA synthesis (UDS) assay measures DNA repair in response to DNA damage. To date, 59 chemicals plus UV and X rays have been tested for UDS in spermatogenic cells of humans, rabbits, rats, and mice. In vivo, in vitro, and combined in vivo/in vitro procedures have been used. UDS has been shown to occur in spermatogonia, meiotic spermatocytes, and early spermatid stages. Fifty-nine percent of the agents tested gave a positive UDS response in one or more germ-cell stages. Results show 95% concordance (positive or negative) between different mammalian species. Some well-known genotoxic chemicals, for example, aflatoxin B(1) (AFB(1)), benzo[a]pyrene (B[a]P), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), did not induce significant levels of UDS. Possible explanations are discussed. Results from the UDS assay were compared with those from the mouse specific-locus mutation (SLM) test to determine correlations between the two assays. Only two chemicals, ethyl- and methyl-nitrosourea (ENU and MNU), have been tested for UDS and SLM induction in spermatogonial stages. Results show full concordance between the two assays. In postspermatogonial stages, 25 chemicals and X rays have been tested for UDS and SLM induction. Seventy-seven percent of these agents showed similar results (positive or negative) in these germ-cell stages. Although the UDS assay cannot replace the SLM test, the strong correlations between the two assays suggest the usefulness of the UDS assay as a predictor of germ-cell mutations in mammalian systems.

Sram RJ, Binkova B. **Molecular epidemiology studies on occupational and environmental exposure to mutagens and carcinogens, 1997-1999.** Environ Health Perspect 2000;108 Suppl 1:57-70.
Abstract: Molecular epidemiology is a new and evolving area of research, combining laboratory measurement of internal dose, biologically effective dose, biologic effects, and influence of individual susceptibility with epidemiologic methodologies. Biomarkers evaluated were selected according to basic scheme: biomarkers of exposure--metabolites in urine, DNA adducts, protein adducts, and Comet assay parameters; biomarkers of effect--chromosomal aberrations, sister chromatid exchanges, micronuclei, mutations in the hypoxanthine-guanine phosphoribosyltransferase gene, and the activation of oncogenes coding for p53 or p21 proteins as measured on protein levels; biomarkers of susceptibility--genetic polymorphisms of genes CYP1A1, GSTM1, GSTT1, NAT2. DNA adducts measured by 32P-postlabeling are the biomarker of choice for the evaluation of exposure to polycyclic aromatic hydrocarbons. Protein adducts are useful as a biomarker for exposure to tobacco smoke (4-aminobiphenyl) or to smaller molecules such as acrylonitrile or 1,3-butadiene. Of the biomarkers of effect, the most common are cytogenetic end points. Epidemiologic studies support the use of chromosomal breakage as a relevant biomarker of cancer risk. The use of the Comet assay and methods analyzing oxidative DNA damage needs reliable validation for human biomonitoring. Until now there have not been sufficient data to interpret the relationship between genotypes, biomarkers of exposure, and biomarkers of effect for assessing the risk of human exposure to mutagens and carcinogens.

Styles JA, Clark H, Festing MF, Rew DA. **Automation of mouse micronucleus genotoxicity assay by laser scanning cytometry.** Cytometry 2001;44(2):153-5.
Abstract: BACKGROUND: The evaluation of the safety of drugs and other chemicals is an important aspect of toxicology work. The mouse micronucleus assay is a standard in vivo genotoxicity assay. Chromosomal damage is an indicator of genotoxicity, which manifests in the formation of micronuclei in polychromatic erythrocytes from bone marrow and in peripheral blood erythrocytes. The assay is laborious to perform by manual counting. The laser scanning cytometer allows automated and rapid quantitation of cellular and subcellular fluorescence in monodisperse cell samples on a microscope slide. The object of this study was to evaluate the application of this new technology in the mouse micronucleus genotoxicity assay. Materials and Methods One hundred forty-four mice of various strains were dosed with combinations of carcinogens and antioxidants. Duplicate blood films were prepared 3 days later. One set of slides was stained with acridine orange, and the proportion of micronucleated erythrocytes was counted in 5,000 cells per slide. The duplicates were stained with propidium iodide (40 microg/ml). Five thousand cells per sample were examined using a laser scanning cytometer. The proportion of micronucleated erythrocytes was measured. RESULTS: A coefficient of correlation of 0.96 was found between the data from the two assays. The automation of the assay on the LSC produced a considerable time saving and efficiency gain. CONCLUSIONS: We conclude that with further development, laser scanning cytometry is likely to become the preferred modality for the performance of standard genotoxicity assays.

Takahashi M, Keicho K, Takahashi H, Ogawa H, Schultz RM, Okano A. **Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay.** Theriogenology 2000;54(1):137-45.
Abstract: The correlation of oxidative stress on development and DNA damage in bovine embryos was investigated by the comet assay (single-cell microgel electrophoresis), an effective technique for detecting single-strand DNA breakage. After in vitro maturation and fertilization, one-cell stage embryos without cumulus cells were cultured for 8 days in SOF medium containing amino acids plus 5% FCS under low (5%) and atmospheric (20%) oxygen concentration. After 8 days of culture, the extent of blastocyst formation was significantly decreased (P<0.001) when embryos were cultured under 20% oxygen concentration (5.8 +/- 2.4%) when compared to embryos cultured under 5% oxygen concentration (35.1 +/- 6.7%). At the day 3 of development, DNA damage of individual embryos cultured under 5% or 20% oxygen concentration was measured by the comet assay, which entails microgel electrophoresis that can readily detect damaged DNA. After measuring the DNA damage in individual embryos by the comet assay, the length (149.9 +/- 15.3 microm) of the migrating DNA fragment that is indicative of damaged DNA was significantly increased (P<0.001) in the embryos cultured under 20% oxygen concentration when compared to embryos cultured in 5% oxygen concentration (42.3 +/- 7 microm). The length of damaged DNA in more than 50% of embryos was less than 50 microm. when embryos were cultured under 5% oxygen concentration. In contrast, the distribution of damaged DNA shifted to the more damaged extent when embryos were cultured under 20% oxygen concentration. These results demonstrate that the retardation in bovine embryo development than in likely due oxidative stress as a consequence of the higher atmospheric oxygen concentration is positively correlated with an increase in the extent of DNA damage. Moreover, these

results demonstrate that the comet assay is a useful method to evaluate embryo culture conditions.

Talbot WS, Hopkins N. **Zebrafish mutations and functional analysis of the vertebrate genome.** Genes Dev 2000;14(7):755-62.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. **Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing.** Environ Mol Mutagen 2000;35(3):206-21.
Abstract: Atthe International Workshop on Genotoxicity Test Procedures (IWGTP) held in Washington, DC, March 25-26, 1999, an expert panel met to develop guidelines for the use of the single-cell gel (SCG)/Comet assay in genetic toxicology. The expert panel reached a consensus that the optimal version of the Comet assay for identifying agents with genotoxic activity was the alkaline (pH > 13) version of the assay developed by Singh et al. [1988]. The pH > 13 version is capable of detecting DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites. Relative to other genotoxicity tests, the advantages of the SCG assay include its demonstrated sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, its flexibility, its low costs, its ease of application, and the short time needed to complete a study. The expert panel decided that no single version of the alkaline (pH > 13) Comet assay was clearly superior. However, critical technical steps within the assay were discussed and guidelines developed for preparing slides with agarose gels, lysing cells to liberate DNA, exposing the liberated DNA to alkali to produce single-stranded DNA and to express ALS as SSB, electrophoresing the DNA using pH > 13 alkaline conditions, alkali neutralization, DNA staining, comet visualization, and data collection. Based on the current state of knowledge, the expert panel developed guidelines for conducting in vitro or in vivo Comet assays. The goal of the expert panel was to identify minimal standards for obtaining reproducible and reliable Comet data deemed suitable for regulatory submission. The expert panel used the current Organization for Economic Co-operation and Development (OECD) guidelines for in vitro and in vivo genetic toxicological studies as guides during the development of the corresponding in vitro and in vivo SCG assay guidelines. Guideline topics considered included initial considerations, principles of the test method, description of the test method, procedure, results, data analysis and reporting. Special consideration was given by the expert panel to the potential adverse effect of DNA degradation associated with cytotoxicity on the interpretation of Comet assay results. The expert panel also discussed related SCG methodologies that might be useful in the interpretation of positive Comet data. The related methodologies discussed included: (1) the use of different pH conditions during electrophoreses to discriminate between DNA strand breaks and ALS; (2) the use of repair enzymes or antibodies to detect specific classes of DNA damage; (3) the use of a neutral diffusion assay to identify apoptotic/necrotic cells; and (4) the use of the acellular SCG assay to evaluate the ability of a test substance to interact directly with DNA. The alkaline (pH > 13) Comet assay guidelines developed by the expert panel represent a work in progress. Additional information is needed before the assay can be critically evaluated for its utility in genetic toxicology. The information needed includes comprehensive data on the different sources of variability (e.g., cell to cell, gel to gel, run to run, culture to culture, animal to animal, experiment to experiment) intrinsic to the alkaline (pH > 3) SCG assay, the generation of a large database based on in vitro and in vivo testing using these guidelines, and the results of appropriately designed multilaboratory international validation studies.

Tinwell H, Brinkworth MH, Ashby J. **Further evidence for the rodent bone marrow micronucleus assay acting as a sensitive predictor of the possible germ cell mutagenicity of chemicals.** Mutat Res 2001;473(2):259-61.

Torous DK, Dertinger SD, Hall NE, Tometsko CR. **Enumeration of micronucleated reticulocytes in rat peripheral blood: a flow cytometric study.** Mutat Res 2000;465(1-2):91-9.
Abstract: Micronuclei (MN) are routinely enumerated in mouse peripheral blood to index genotoxicity. Recent data from the Collaborative Study Group for the Micronucleus Test (CSGMT) [CSGMT (The Collaborative Study Group for the Micronucleus Test), Evaluation of the rat micronucleus test with bone marrow and peripheral blood: summary of the 9th collaborative study by CSGMT/JEMS MMS, Environ. Mol. Mutagen. 32 (1998) 84-100] suggest that rat peripheral blood may also be appropriate for the enumeration of MN, if scoring is limited to the youngest fraction of reticulocytes. The experiments described herein were designed to test whether modifications to a flow cytometric scoring procedure for measuring micronucleated reticulocytes (MN-RET) in mouse peripheral blood could be extended to accurately enumerate MN in rat peripheral blood. Rats were treated with saline or one of three genotoxic agents (6-mercaptopurine, ethyl methanesulfonate or propane sultone) in an acute dosing protocol. Peripheral blood samples were subsequently collected for both microscopic and flow cytometric analysis. Micronucleus frequencies were scored in the youngest fraction of reticulocytes: scoring by microscopy was restricted to the types I and II reticulocytes based on RNA content utilizing acridine orange supravital staining; flow cytometric measurements were restricted to the youngest fraction of reticulocytes based on transferrin receptor (CD71) staining. A statistically significant dose-related increase in the incidence of MN was observed, irrespective of scoring method. A higher level of statistical discrimination between control and genotoxin-treated groups was observed for the flow cytometric data and can most likely be explained by the increased number of cells scored (10x more than microscopy) and the lower scoring variability. Together, these data suggest that (i) rat peripheral blood represents an appropriate compartment for evaluating genotoxin-induced MN when the analysis is restricted to young reticulocytes, and (ii) the measurement of MN in rat peripheral blood reticulocytes benefits from the high throughput methodology of flow cytometry.

Torres de Lemos C, Erdtmann B. **Cytogenetic evaluation of aquatic genotoxicity in human cultured lymphocytes.** Mutat Res 2000;467(1):1-9.
Abstract: This paper presents the results of a cytogenetic study to evaluate the quality of Cai river water at the area under the influence of the Petrochemical Complex of the State of Rio Grande do Sul. Cytokinesis-block micronucleus assay (CBMN) was performed on cultured human lymphocytes exposed to stream water samples. The Cai River is an important tributary to the Guaiba basin that includes Porto Alegre, the capital of Rio Grande do Sul. The Cai river water is the source of drinking water after conventional treatment, and is also used for irrigation and primary contact recreation according to Federal regulations. Water samples were collected in the Cai River and some tributaries at four sites for 20 months with a bimonthly frequency, in a total of 11 samplings, from November 1993 to July 1995. It was possible to detect the presence of substances with clastogenic and/or aneugenic potential in vitro human lymphocytes at the different sites analyzed. As to the four sites studied, site B presents nearly half of the total positive results (44%), followed by sites A (28%), D (17%) and C

(11%). The CBMN assay in human lymphocytes was a sensitive cytogenetic approach for aquatic environmental studies, and should be better exploited to monitor industrial areas.

Tsuda S, Matsusaka N, Madarame H, Miyamae Y, Ishida K, Satoh M, Sekihashi K, Sasaki YF. **The alkaline single cell electrophoresis assay with eight mouse organs: results with 22 mono-functional alkylating agents (including 9 dialkyl N-nitrosoamines) and 10 DNA crosslinkers.** Mutat Res 2000;467(1):83-98.
Abstract: The genotoxicity of 22 mono-functional alkylating agents (including 9 dialkyl N-nitrosoamines) and 10 DNA crosslinkers selected from IARC (International Agency for Research on Cancer) groups 1, 2A, and 2B was evaluated in eight mouse organs with the alkaline single cell gel electrophoresis (SCGE) (comet) assay. Groups of four mice were treated once intraperitoneally at the dose at which micronucleus tests had been conducted, and the stomach, colon, liver, kidney, bladder, lung, brain, and bone marrow were sampled 3, 8, and/or 24 h later. All chemicals were positive in the SCGE assay in at least one organ. Of the 22 mono-functional alkylating agents, over 50% were positive in all organs except the brain and bone marrow. The two subsets of mono-functional alkylating agents differed in their bone marrow genotoxicity: only 1 of the 9 dialkyl N-nitrosoamines was positive in bone marrow as opposed to 8 of the 13 other alkylating agents, reflecting the fact that dialkyl N-nitrosoamines are poor micronucleus inducers in hematopoietic cells. The two groups of mono-functional alkylating agents also differ in hepatic carcinogenicity in spite of the fact that they are similar in hepatic genotoxicity. While dialkyl N-nitrosoamines produce tumors primarily in mouse liver, only one (styrene-7,8-oxide) out of 10 of the other type of mono-functional alkylating agents is a mouse hepatic carcinogen. Taking into consideration our previous results showing high concordance between hepatic genotoxicity and carcinogenicity for aromatic amines and azo compounds, a possible explanation for the discrepancy might be that chemicals that require metabolic activation show high concordance between genotoxicity and carcinogenicity in the liver. A high percent of the 10 DNA crosslinkers were positive in the SCGE assay in the gastrointestinal mucosa, but less than 50% were positive in the liver and lung. In this study, we allowed 10 min alkali-unwinding to obtain low and stable control values. Considering that DNA crosslinking lesions can be detected as lowering of not only positive but also negative control values, low control values by short alkali-treatment might make it difficult to detect DNA crosslinking lesions. In conclusion, although both mono-functional alkylating agents and DNA crosslinkers are genotoxic in mouse multiple organs, the genotoxicity of DNA crosslinkers can be detected in the gastrointestinal organs even though they were given intraperitoneally followed by the short alkali-treatment.

Tsuda S, Matsusaka N, Madarame H, Ueno S, Susa N, Ishida K, Kawamura N, Sekihashi K, Sasaki YF. **The comet assay in eight mouse organs: results with 24 azo compounds.** Mutat Res 2000;465(1-2):11-26.
Abstract: The genotoxicity of 24 azo compounds selected from IARC (International Agency for Research on Cancer) groups 2A, 2B, and 3 were determined by the comet (alkaline single cell gel electrophoresis, SCG) assay in eight mouse organs. We treated groups of four mice once orally at the maximum tolerated dose (MTD) and sampled stomach, colon, liver, kidney, bladder, lung, brain, and bone marrow 3, 8, and 24 h after treatment. For the 17 azo compounds, the assay was positive in at least one organ; (1) 14 and 12 azo compounds induced DNA damage in the colon and liver, respectively, (2)

the genotoxic effect of most of them was greatest in the colon, and (3) there were high positive responses in the gastrointestinal organs, but those organs are not targets for carcinogenesis. One possible explanation for this discrepancy is that the assay detects DNA damage induced shortly after administration of a relatively high dose, while carcinogenicity is detected after long treatment with relatively low doses. The metabolic enzymes may become saturated following high doses and the rates and pathways of metabolic activation and detoxification may differ following high single doses vs. low long-term doses. Furthermore, considering that spontaneous colon tumors are very rare in rats and mice, the ability to detect tumorigenic effects in the colon of those animals might be lower than the ability to detect genotoxic events in the comet assay. The in vivo comet assay, which has advantage of reflecting test chemical absorption, distribution, and excretion as well as metabolism, should be effective for estimating the risk posed by azo dyes to humans in spite of the difference in dosage regimen.

Turner SD, Wijnhoven SW, Tinwell H, Lashford LS, Rafferty JA, Ashby J, Vrieling H, Fairbairn LJ. **Assays to predict the genotoxicity of the chromosomal mutagen etoposide -- focussing on the best assay.** Mutat Res 2001;493(1-2):139-47.
Abstract: The topoisomerase II inhibitor etoposide is used routinely to treat a variety of cancers in patients of all ages. As a result of its extensive use in the clinic and its association with secondary malignancies it has become a compound of great interest with regard to its genotoxic activity in vivo. This paper describes a series of assays that were employed to determine the in vivo genotoxicity of etoposide in a murine model system. The alkaline comet assay detected DNA damage in the bone marrow mononuclear compartment over the dose range of 10--100mg/kg and was associated with a large and dose dependent rise in the proportion of cells with severely damaged DNA. In contrast, the bone marrow micronucleus assay was found to be sensitive to genotoxic damage between the doses of 0.1--1mg/kg without any corresponding increases in cytotoxicity. An increase in the mutant frequency was undetectable at the Hprt locus at administered doses of 1 and 10mg/kg of etoposide, however, an increase in the mutant frequency was seen at the Aprt locus at these doses. We conclude that the BMMN assay is a good short-term predictor of the clastogenicity of etoposide at doses that do not result in cytotoxic activity, giving an indication of potential mutagenic effects. Moreover, the detection of mutants at the Aprt locus gives an indication of the potential of etoposide to cause chromosomal mutations that may lead to secondary malignancy.

Uhl M, Helma C, Knasmuller S. **Evaluation of the single cell gel electrophoresis assay with human hepatoma (Hep G2) cells.** Mutat Res 2000;468(2):213-25.
Abstract: Human Hep G2 cells have retained the activities of phase I and phase II enzymes which are involved in the metabolism of environmental genotoxins. The present study describes the results of single cell gel electrophoresis (SCGE) assays with a panel of different model compounds with these cells. With genotoxic carcinogens such as aflatoxin B(1) (AFB(1)), benzo(a)pyrene (B(a)P), nitrosodimethylamine (NDMA) and cyclophosphamide (CP), statistically significant dose dependent induction of DNA migration was measured. With the two heterocyclic amines, 2-amino-3-methyl-3H-imidazo[4, 5-f]quinoline (IQ) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), and also with rodent carcinogens such as safrole, hexamethylphosphoramide (HMPA) and the pyrrolizidine alkaloid isatidine, which give negative results in other in vitro genotoxicity tests, positive results were obtained in Hep G2/SCGE assays. Nitrosomethylurea (NMU) was the only directly acting compound

tested in the study and was by far (ca. 10(3)-fold) more active than the corresponding nitrosamine. The exposure concentrations required to cause significant effects varied over a broad range. The most pronounced effect was seen with AFB(1) (0.008 microM) followed by HMPA (15 microM), B(a)P (25 microM), NMU (100 microM), isatidin (500 microM), CP (900 microM), IQ (1200 microM), safrol (4000 microM), and NDMA (90x10(3) microM). Numbers in parenthesis give the lowest concentrations, which caused a significant increase of DNA migration. With two compounds, namely, the non-carcinogen pyrene and the synthetic hormone tamoxifen (TF), negative results were obtained under all test conditions. These findings are in agreement with the results of recent investigations which indicated that human hepatocytes are unable to convert TF to DNA reactive metabolites, whereas it is activated by rat liver cells and causes DNA adducts in these cells. Comparisons of the present results with data from earlier experiments indicate that the Hep G2/SCGE assay enables the detection of genotoxins including compounds which give misleading results in other in vitro genotoxicity tests and appears to be an alternative to tests with primary liver cells from laboratory animals.

Umegaki K, Fenech M. **Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils.** Mutagenesis 2000;15(3):261-9.
Abstract: We have developed a method that can detect the DNA-damaging and cytotoxic effects of physiological levels of reactive oxygen species (ROS) and activated human neutrophils. This was achieved using WIL2-NS cells, a human B lymphoblastoid cell line, as target cells and the cytokinesis-block micronucleus (CBMN) assay. With this method, we observed a 4- and a 30-fold increase in the frequency of micronucleated binucleated cells (MNed BNC) when cells were exposed to 10 and 30 microM hydrogen peroxide, for 1 h, respectively. A dose-dependent increase in the frequency of MNed BNC was also detected when cells were exposed to hypoxanthine (HX)/xanthine oxidase (XO), a superoxide generating system: a 50-fold increase in the frequency of MNed BNC was observed at the highest XO dose (12.5 mU/ml). In this CBMN assay, nucleoplasmic bridges (NPB) in BNC and necrotic cells were also readily detected, especially at the higher exposure doses of hydrogen peroxide or HX/XO. When WIL2-NS cells were exposed to neutrophils stimulated with phorbol 12-myristate acetate (PMA) for 1 h, the frequencies of MNed BNC in WIL2-NS cells increased in a dose-dependent manner (30-fold increase at 100 nM PMA) and with an increasing neutrophil:WIL2-NS co-culture ratio. The frequencies of MNed BNC were closely related to the production of ROS, especially hydrogen peroxide, by the neutrophils. Differentiated HL60 cells (DMSO-treated HL60) also produced ROS in response to PMA. In this case, we used a 'Transwell' system to expose WIL2-NS cells to DMSO-treated HL60 cells, because direct contact with DMSO-treated HL60 cells impaired cell division in WIL2-NS target cells. Exposure to PMA-stimulated DMSO-treated HL60 cells resulted in a PMA dose-dependent increase in the frequency of MNed BNC in WIL2-NS cells. MNed BNC frequencies were positively correlated with NPB ($r = 0.61$-0.93) and necrosis ($r = 0.55$-0.86) and negatively correlated with nuclear division index ($r = -0.72$ to -0.91) in all of the above experiments. These results suggest that the CBMN assay using WIL2-NS cells is a sensitive assay system to examine ROS-induced chromosomal damage and necrosis by activated human neutrophils.

van Duijn-Goedhart A, Zdzienicka MZ, Sankaranarayanan K, van Buul PP. **Differential responses of Chinese hamster mutagen sensitive cell lines to low and high concentrations of calicheamicin and**

neocarzinostatin. Mutat Res 2000;471(1-2):95-105.
Abstract: To shed light on the mechanism underlying the cellular response to the radiomimetic agents calicheamicin Y(1)(1) (CAL) and neocarzinostatin (NCS), several hamster cell mutants defective in different DNA repair pathways were used. Two X-ray sensitive Chinese hamster V79 mutant cell lines, XR-V9B and V-E5 were studied for their response to the induction of cell killing, micronuclei, and G2-chromosomal aberrations relative to that of parental wild-type cells. In addition, effects of CAL and NCS on bleomycin sensitive BL-V40 cells and on UV sensitive V-H1 cells were analyzed.In general, the radiosensitive cell lines showed the highest sensitivities to CAL and NCS, but also the other mutants demonstrated differences in their responses compared to wild-type cells. With respect to cell killing, expressed as D(10)-value, enhanced sensitivities of mutants with factors up to 4.4 were recorded. For the induction of micronuclei (MN) and chromosomal aberrations (CA) all cell lines, including the parental cells, show a steep increase in the frequencies at the lowest tested doses and a leveling off at higher concentrations. Probably toxic effects at the higher exposure levels are responsible for these biphasic dose effect curves. Enhanced sensitivities of the various mutants were primarily observed at the higher exposure levels. With respect to the induction of MN increased sensitivities up to a factor of 18.1 were observed for the radiosensitive mutants, whereas for CA the mutant cell lines showed a variation from resistance (0.3) of VH-1 cells up to a 3.8-fold higher sensitivity to the radiomimetic agents. However, at the lowest tested concentrations for both MN and CA, the differences between the sensitive mutants and wild-type clearly diminished, suggesting the existence of residual and/or alternative DNA repair pathways in these mutants.

van Gestel CA, van der Waarde JJ, Derksen JG, van der Hoek EE, Veul MF, Bouwens S, Rusch B, Kronenburg R, Stokman GN. **The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils.** Environ Toxicol Chem 2001;20(7):1438-49.
Abstract: To compare the effectiveness of acute and chronic bioassays for the ecological risk assessment of polluted soils, soil samples from a site with an historical mineral oil contamination (< 50-3,300 mg oil/kg dry soil) at the Petroleum Harbour in Amsterdam, The Netherlands, were screened for ecological effects using acute and chronic bioassays. A two-step 0.001 M Ca(NO3)2 extraction at a final solution-to-soil ratio of 1:1 was used to prepare extracts for the acute bioassays. Acute bioassays (< or = 5 d) applied to the 0.001 M Ca(NO3)2 extracts from the polluted and reference soils included growth tests with bacteria (Bacillus sp.), algae (Raphidocelis subcapitata), and plants (Lactuca sativa), immobility tests with nematodes (Plectus acuminatus), springtails (Folsomia candida), and cladocerans (Daphnia magna), and the Microtox test (Vibrio fischeri). Chronic bioassays (four weeks) performed on the same soil samples included tests with L. sativa, F. candida, and earthworms (Eisenia fetida) and the bait-lamina test (substrate consumption). The acute bioassays on Microtox showed a response that corresponded with the level of oil pollution. All other acute bioassays did not show such a consistent response, probably because pollutant levels were too low to cause acute effects. All chronic bioassays showed sublethal responses according to the contaminant levels (oil and in some soils also metals). This shows that chronic bioassays on soil samples are more sensitive in assessing the toxicity of mineral oil contamination in soil than acute bioassays on soil extracts. A pilot scale bioremediation study on soils taken from the two most polluted sites and a control site showed a rapid decline of oil concentrations to reach a stable level within eight weeks. Acute bioassays applied to the soils, using Microtox, algae, and D. magna, and chronic bioassays, using plants, Collembola, earthworms, and bait-lamina consumption,

in all cases showed a rapid reduction of toxicity, which could be attributed to the degradation of light oil fractions.

van Steeg H, Mullenders LH, Vijg J. **Mutagenesis and carcinogenesis in nucleotide excision repair-deficient XPA knock out mice.** Mutat Res 2000;450(1-2):167-80.
Abstract: Mice with a defect in the xeroderma pigmentosum group A (XPA) gene have a complete deficiency in nucleotide excision repair (NER). As such, these mice mimic the human XP phenotype in that they have a >1000-fold higher risk of developing UV-induced skin cancer. Besides being UV-sensitive, XPA(-/-) mice also develop internal tumors when they are exposed to chemical carcinogens. To investigate the effect of a total NER deficiency on the induction of gene mutations and tumor development, we crossed XPA(-/-) mice with transgenic lacZ/pUR288 mutation-indicator mice. The mice were treated with various agents and chemicals like UV-B, benzo[a]pyrene and 2-aceto-amino-fluorene. Gene mutation induction in several tumor target- and non-target tissues was determined in both the bacterial lacZ reporter gene and in the endogenous Hprt gene. Furthermore, alterations in the p53- and ras genes were determined in UV-induced skin tumors of XPA(-/-) mice. In this work, we review these results and discuss the applicability and reliability of enhanced gene mutant frequencies as early indicators of tumorigenesis.

Vanhauwaert A, Vanparys P, Kirsch-Volders M. **The in vivo gut micronucleus test detects clastogens and aneugens given by gavage.** Mutagenesis 2001;16(1):39-50.
Abstract: A general testing battery for pharmaceuticals includes a bacterial gene mutation assay, an in vitro chromosomal aberration or a gene mutation test on mammalian cells and an in vivo test for chromosome/genome mutations. The aim of this study was to determine whether the in vivo mouse gut micronucleus assay could be a more sensitive method to detect direct clastogens and/or aneugens given orally by gavage than the in vivo bone marrow micronucleus assay (which can also detect indirect genotoxins). Two laboratories collaborated in this project, one analysing bone marrow cells and the other analysing gut cells from the same animals. The reference substances tested in this study were colchicine (COL), carbendazim (CAR), tubulazole (TUB) and griseofulvin (GRI), all known aneugens, and 1,2-dimethylhydrazine (DMH), a colon carcinogen with clastogenic activity. For all substances tested, the in vivo gut micronucleus test was as sensitive as or more sensitive than the in vivo bone marrow micronucleus assay: COL and TUB induced micronuclei in both gut and bone marrow cells; DMH, CAR and GRI induced micronuclei only in gut cells. The results show that the micronucleus test on gut cells is able to detect clastogens and aneugens given orally by gavage, some of which were not detected by the bone marrow micronucleus test.

Vargas VM, Migliavacca SB, de Melo AC, Horn RC, Guidobono RR, de Sa Ferreira IC, Pestana MH. **Genotoxicity assessment in aquatic environments under the influence of heavy metals and organic contaminants.** Mutat Res 2001;490(2):141-58.
Abstract: The genotoxicity of river water and sediment including interstitial water was evaluated by microscreen phage-induction and Salmonella/microsome assays. Different processes used to fractionate the sediment sample were compared using solvents with different polarities. The results obtained for mutagenic activity using the Salmonella/microsome test were negative in the water and interstitial water samples analysed using the direct concentration method. The responses in the microscreen phage-

induction assay showed the presence of genotoxic or indicative genotoxic activity for at least one water sample of each site analysed using the same concentration method. Similar results were obtained for interstitial water samples, i.e. absence of mutagenic activity in the Salmonella/microsome test and presence of genotoxic activity in the microscreen phage-induction assay. Metal contamination, as evidenced by the concentrations in stream sediments, may also help explain some of these genotoxic results. Stream sediment organic extracts showed frameshift mutagenic activity in the ether extract detected by Salmonella/microsome assay. The concentrates evaluated by microscreen phage-induction assay identified the action of organic compounds in the non-polar, medium polar and polar fractions. Thus, the microscreen phage-induction assay has proven to be a more appropriate methodology than the Salmonella/microsome test to analyse multiple pollutants in this ecosystem where both organic compounds and heavy metals are present.

Villani P, Altavista PL, Castaldi L, Leter G, Cordelli E. **Analysis of DNA oxidative damage related to cell proliferation.** Mutat Res 2000;464(2):229-37.
Abstract: In vivo and in vitro cell populations exhibit a different sensitivity and a heterogeneous response to many genotoxic agents. Several studies have been carried out to evaluate the possibility that the different sensitivity of the cells is related to their proliferative status. In this study, the sensitivity of proliferating (P) and quiescent (Q) C3H10T1/2 cells to oxidative damage and their repair capability has been investigated by single cell gel electrophoresis (SCGE) and micronucleus test. Furthermore the possibility to simultaneously detect DNA damage and cell cycle position has been evaluated. Our results showed a dose-related increase of DNA damage in exponential and plateau phase cells treated with hydrogen peroxide (doses ranging between 2.5 and 100 microM). DNA damage was almost completely repaired within 2 h after treatment in both culture conditions. The percentage of cells in the various phases of the cell cycle has been determined by comet assay and by flow cytometry, and a good agreement between the results of the two techniques was found. Untreated exponentially growing cells in G1 phase showed a lower tail moment than S and G2/M cells. The same cell cycle dependence was evidenced in cells treated with low doses of H(2)O(2), while, at the higher doses, all cells showed a similar level of damage. These results confirm the sensitivity of the Comet Assay in assessing DNA damage, and support its usefulness in evaluating cell cycle-related differential sensitivity to genotoxic agents.

Visvardis E, Haveles KS, Pataryas TA, Margaritis LH, Sophianopoulou V, Sideris EG. **Diversity of peripheral blood mononuclear cells as revealed by a novel multiple microgel "comet assay".** Environ Mol Mutagen 2000;36(1):32-9.
Abstract: Multiple microgel comet assay (MMCA) is a metho-dological adaptation of the single-cell gel electrophoresis assay in which we have introduced the use of standard agarose plug molds in an attempt to improve and expand the applications of the assay. We focused on the study of the heterogeneity of peripheral blood mononuclear cells (PBMC) at the level of the basal single-strand breakage and the DNA damage induction caused by ionizing radiation. Differences among subpopulations were also investigated at the level of chromatin organization and methylation after NotI digestion of microgel-embedded cells. In parallel experiments, the NotI-digested nucleoids were also analyzed with the use of pulsed-field gel electrophoresis (PFGE) and the DNA migration patterns were compared with the corresponding patterns from the MMCA. Significant heterogeneity in the distribution of the oxidative

DNA damage, as well as intracellular variations in the NotI digestion patterns were observed in the cell population of PBMC. The combined use of both the comet assay and PFGE provides a useful model for analysis of variation in DNA damage in individual cells as well as information on size of DNA fragments.

Voigt CA, Kauffman S, Wang ZG. **Rational evolutionary design: the theory of in vitro protein evolution**. Adv Protein Chem 2000;55:79-160.
Abstract: Directed evolution uses a combination of powerful search techniques to generate proteins with improved properties. Part of the success is due to the stochastic element of random mutagenesis; improvements can be made without a detailed description of the complex interactions that constitute function or stability. However, optimization is not a conglomeration of random processes. Rather, it requires both knowledge of the system that is being optimized and a logical series of techniques that best explores the pathways of evolution (Eigen et al., 1988). The weighing of parameters associated with mutation, recombination, and screening to achieve the maximum fitness improvement is the beginning of rational evolutionary design. The optimal mutation rate is strongly influenced by the finite number of mutants that can be screened. A smooth fitness landscape implies that many mutations can be accumulated without disrupting the fitness. This has the effect of lowering the required library size to sample a higher mutation rate. As the sequence ascends the fitness landscape, the optimal mutation rate decreases as the probability of discovering improved mutations also decreases. Highly coupled regions require that many mutations be simultaneously made to generate a positive mutant. Therefore, positive mutations are discovered at uncoupled positions as the fitness of the parent increases. The benefit of recombination is twofold: it combines good mutations and searches more sequence space in a meaningful way. Recombination is most beneficial when the number of mutants that can be screened is limited and the landscape is of an intermediate ruggedness. The structure of schema in proteins leads to the conclusion that many cut points are required. The number of parents and their sequence identity are determined by the balance between exploration and exploitation. Many disparate parents can explore more space, but at the risk of losing information. The required screening effort is related to the number of uphill paths, which decreases more rapidly for rugged landscapes. Noise in the fitness measurements causes a dramatic increase in the required mutant library size, thus implying a smaller optimal mutation rate. Because of strict limitations on the number of mutants that can be screened, there is motivation to optimize the content of the mutant library. By restricting mutations to regions of the gene that are expected to show improvement, a greater return can be made with the same number of mutants. Initial studies with subtilisin E have shown that structurally tolerant positions tend to be where positive activity mutants are made during directed evolution. Mutant fitness information is produced by the screening step that has the potential to provide insight into the structure of the fitness landscape, thus aiding the setting of experimental parameters. By analyzing the mutant fitness distribution and targeting specific regions of the sequence, in vitro evolution can be accelerated. However, when expediting the search, there is a trade-off between rapid improvement and the quality of the long-term solution. The benefit of neutrality has yet to be captured with in vitro protein evolution. Neutral theory predicts the punctuated emergence of novel structure and function, however, with current methods, the required time scale is not feasible. Utilizing neutral evolution to accelerate the discovery of new functional and structural solutions requires a theory that predicts the behavior of mutational pathways between networks. Because the transition from neutral to adaptive evolution requires a multi-mutational switch, increasing the mutation

rate decreases the time required for a punctuated change to occur. By limiting the search to the less coupled region of the sequence (smooth portion of the fitness landscape), the required larger mutation rate can be tolerated. Advances in directed evolution will be achieved when the driving forces behind such proce.

Vooijs M, Jonkers J, Berns A. **A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent.** EMBO Rep 2001;2(4):292-7.
Abstract: Conditional gene inactivation using the Cre/loxP system is widely used, but the difficulty in properly regulating Cre expression remains one of the bottlenecks. One approach to regulate Cre activity utilizes a mutant estrogen hormone-binding domain (ERT) to keep Cre inactive unless the non-steroidal estrogen analog 4-hydroxytamoxifen (OHT) is present. Here we describe a mouse strain expressing Cre-ERT from the ubiquitously expressed ROSA26 (R26) locus. We demonstrate efficient temporal and spatial regulation of Cre recombination in vivo and in primary cells derived from these mice. We show the existence of marked differences in recombination frequencies between different substrates within the same cell. This has important consequences when concurrent switching of multiple alleles within the same cell is needed, and highlights one of the difficulties that may be encountered when using reporter mice as indicator strains.

Ward TH, Marples B. **Technical report: SYBR Green I and the improved sensitivity of the single-cell electrophoresis assay**. Int J Radiat Biol 2000;76(1):61-5.
Abstract: The single-cell electrophoresis (comet) assay is an established method for measuring radiation-induced strand breaks in DNA. The extent of damage is quantified in individual cells by assessing the intensity and distribution of a fluorescent DNA-binding dye using image analysis software. Using the Kinetic Imaging Komet3 system, it is demonstrated that the fluorochrome SYBR Green I improves the resolution and sensitivity of the comet assay, parcticularly for measuring radiation-induced double strand breaks.

Waring JF, Ciurlionis R, Jolly RA, Heindel M, Ulrich RG. **Microarray analysis of hepatotoxins in vitro reveals a correlation between gene expression profiles and mechanisms of toxicity.** Toxicol Lett 2001;120(1-3):359-68.
Abstract: A rate-limiting step that occurs in the drug discovery process is toxicological evaluation of new compounds. New techniques that use small amounts of the experimental compound and provide a high degree of predictivity would greatly improve this process. The field of microarray technology, which allows one to monitor thousands of gene expression changes simultaneously, is rapidly advancing and is already being applied to numerous areas in toxicology. However, it remains to be determined if compounds with similar toxic mechanisms produce similar changes in transcriptional expression. In addition, it must be determined if gene expression changes caused by an agent in vitro would reflect those produced in vivo. In order to address these questions, we treated rat hepatocytes with 15 known hepatoxins (carbon tetrachloride, allyl alcohol, aroclor 1254, methotrexate, diquat, carbamazepine, methapyrilene, arsenic, diethylnitrosamine, monocrotaline, dimethyl-formamide, amiodarone, indomethacin, etoposide, and 3-methylcholanthrene) and used microarray technology to characterize the compounds based on gene expression changes. Our results showed that gene expressional profiles for compounds with similar toxic mechanisms indeed formed clusters, suggesting a similar effect on

transcription. There was not complete identity, however, indicating that each compound produced a unique signature. These results show that large-scale analysis of gene expression using microarray technology has promise as a diagnostic tool for toxicology.

Winn RN, Norris MB, Brayer KJ, Torres C, Muller SL. **Detection of mutations in transgenic fish carrying a bacteriophage lambda cII transgene target.** Proc Natl Acad Sci U S A 2000;97(23):12655-60.
Abstract: To address the dual needs for improved methods to assess potential health risks associated with chemical exposure in aquatic environments and for new models for in vivo mutagenesis studies, we developed transgenic fish that carry multiple copies of a bacteriophage lambda vector that harbors the cII gene as a mutational target. We adapted a forward mutation assay, originally developed for lambda transgenic rodents, to recover cII mutants efficiently from fish genomic DNA by lambda in vitro packaging. After infecting and plating phage on a hfl- bacterial host, cII mutants were detected under selective conditions. We demonstrated that many fundamental features of mutation analyses based on lambda transgenic rodents are shared by transgenic fish. Spontaneous mutant frequencies, ranging from 4.3 x 10(-5) in liver, 2.9 x 10(-5) in whole fish, to 1.8 x 10(-5) in testes, were comparable to ranges in lambda transgenic rodents. Treatment with ethylnitrosourea resulted in concentration-dependent, tissue-specific, and time-dependent mutation inductions consistent with known mechanisms of action. Frequencies of mutants in liver increased insignificantly 5 days after ethylnitrosourea exposure, but increased 3.5-, 5.7- and 6. 7-fold above background at 15, 20, and 30 days, respectively. Mutants were induced 5-fold in testes at 5 days, attaining a peak 10-fold induction 15 days after treatment. Spontaneous and induced mutational spectra in the fish were also consistent with those of lambda transgenic rodent models. Our results demonstrate the feasibility of in vivo mutation analyses using transgenic fish and illustrate the potential value of fish as important comparative animal models.

Witt KL, Knapton A, Wehr CM, Hook GJ, Mirsalis J, Shelby MD, MacGregor JT. **Micronucleated erythrocyte frequency in peripheral blood of B6C3F(1) mice from short-term, prechronic, and chronic studies of the NTP carcinogenesis bioassay program.** Environ Mol Mutagen 2000;36(3):163-94.
Abstract: The mouse peripheral blood micronucleus (MN) test was performed on samples collected from 20 short-term, 67 subchronic, and 5 chronic toxicity and carcinogenicity studies conducted by the National Toxicology Program (NTP). Data are presented for studies not previously published. Aspects of protocol that distinguish this test from conventional short-term bone marrow MN tests are duration of exposure, and absence of repeat tests and concurrent positive controls. Furthermore, in contrast to short-term bone marrow MN tests where scoring is limited to polychromatic erythrocytes (PCE), longer term studies using peripheral blood may evaluate MN in both, or either, the normochromatic (NCE) or PCE populations. The incidence of MN-PCE provides an index of damage induced within 72 hr of sampling, whereas the incidence of MN in the NCE population at steady state provides an index of average damage during the 30-day period preceding sampling. The mouse peripheral blood MN test has been proposed as a useful adjunct to rodent toxicity tests and has been effectively incorporated as a routine part of overall toxicity testing by the NTP. Data derived from peripheral blood MN analyses of dosed animals provide a useful indication of the in vivo potential for induced genetic damage and supply an important piece of evidence to be considered in the overall assessment of toxicity and health risk of a

parcticular chemical. Although results indicate that the test has low sensitivity for prediction of carcinogenicity, a convincingly positive result in this assay appears to be highly predictive of rodent carcinogenicity.

Witt KL, Zeiger E, Tice RR, van Birgelen AP. **The genetic toxicity of 3,3',4,4'-tetrachloroazobenzene and 3,3',4, 4'-tetrachloroazoxybenzene: discordance between acute mouse bone marrow and subchronic mouse peripheral blood micronucleus test results.** Mutat Res 2000;472(1-2):147-54.
Abstract: 3,3',4,4'-Tetrachloroazobenzene (TCAB) and 3,3',4, 4'-tetrachloroazoxybenzene (TCAOB) are dioxin-like chemicals that were investigated for toxicity in 13-week gavage studies in male and female B6C3F(1) mice and F344N rats by the National Toxicology Program. As part of the comprehensive toxicological investigation of these chemicals, peripheral blood smears from mice treated 5 days per week for 13 weeks with 0.1-30mg/kg/day TCAB or TCAOB were analyzed for the frequency of micronucleated (MN) normochromatic erythrocytes (NCE). Both chemicals produced significant increases in MN-NCE in male and female mice. In contrast to these positive results in subchronic exposure studies, no significant increases were seen in acute bone marrow MN tests in male mice administered three daily injections of 50-200mg/kg/day TCAB and TCAOB. The results with TCAB and TCAOB suggest that the routine integration of MN tests with subchronic toxicity studies may allow detection of mutagenic activity for some chemicals that fail to elicit responses in short-term, high dose tests. In addition, the integration of mutagenicity tests into general toxicity tests reduces the use of laboratory animals and the cost of the testing.

Wodnicka M, Guarino RD, Hemperly JJ, Timmins MR, Stitt D, Pitner JB. **Novel fluorescent technology platform for high throughput cytotoxicity and proliferation assays.** J Biomol Screen 2000;5(3):141-52.
Abstract: We have developed a novel fluorescent Oxygen BioSensor technology platform adaptable to many applications in the area of drug discovery and development, particularly cell-based assays. This biosensor technology requires no additional reagents or incubations, and affords continuous real-time readout of dissolved oxygen concentrations. Since the level of oxygen dissolved in an assay's medium correlates to the number and viability of the cells in the medium, this technology is ideally suited for monitoring cell viability, proliferation, or death. The technology is particularly well suited to investigating cells' kinetic responses to proliferative or toxic stimuli, such as drugs. When incorporated into a 96- or 384-well microplate format, it is compatible with standard laboratory automation systems. Here we present data illustrating the application of the Oxygen BioSensor technology for rapid, homogeneous detection and evaluation of metabolic activity of a variety of eukaryotic and prokaryotic cells, including mammalian cells, insect cells, yeast, and bacteria. In the absence of toxic substances, we find a good correlation between cell number and signal over a wide range of cell concentrations and growth times. To evaluate the usefulness of the Oxygen BioSensor for cytotoxicity assays, we have performed a series of experiments using a range of toxic agents and cell types, including both bacteria and mammalian cell lines. In a side-by-side comparison to standard MTT assays using HL60 cells, comparable IC(50) values were found with the Oxygen BioSensor for five different toxins or drugs. This assay method does not have the need for additional reagents, handling steps, or incubation periods required by the MTT assays.

Wright WD, Lagroye I, Zhang P, Malyapa RS, Roti JL. **Cytometric methods to analyze ionizing-radiation effects.** Methods Cell Biol 2001;64:251-68.
Abstract: Four cytometric assays for the assessment of radiation-induced DNA damage in individual cells are presented. Two of these, the alkaline and neutral comet assays, are useful for the detection of DNA damage due to very low radiation doses and promise to be useful for the quantitation of genomic damage after clinically or environmentally relevant exposures. The other two, the halo and halo-comet assays, reveal aspects of chromatin structure in the presence of DNA damage that reflect differences in intrinsic cellular radiosensitivity. Further development of these assays used alone, or in combination, should eventually lead to the definition of readily measurable cytometric parameters that will be useful as predictive markers for cellular responses to DNA damaging agents.

Wu RW, Panteleakos FN, Kadkhodayan S, Bolton-Grob R, McManus ME, Felton JS. **Genetically modified Chinese hamster ovary cells for investigating sulfotransferase-mediated cytotoxicity and mutation by 2-amino-1-methyl-6- phenylimidazo[4,5-b]pyridine.** Environ Mol Mutagen 2000;35 (1):57-65.
Abstract: To test the hypothesis that the sulfotransferase gene plays a role in the phase II bioactivation of PhIP, a heterocyclic amine found in cooked meats, we transfected the UV5P3 cell line with cDNA plasmids of human aryl sulfotransferases (HAST1 and HAST3). UV5P3 is a nucleotide excision repair-deficient and P4501A2-expressing CHO cell line that we have previously developed. Functionally transformed clones were identified by the differential cytotoxicity (DC) assay that used PhIP as the cytotoxic agent. Two clones designated 5P3H1 and 5P3H3, expressing HAST1 and HAST3, respectively, were chosen for further characterization. Correct fragment sizes of the sulfotransferase cDNAs were identified in both cell lines by polymerase chain reaction. Immunoblot analysis confirmed the expression of the sulfotransferase proteins. The addition of the sulfotransferase inhibitor DCNP decreased the cytotoxic effects of PhIP in a dose-dependent manner. The increase in cell growth was 6. 5-fold for 5P3H1 and 2.4-fold for 5P3H3, relative to values obtained without DCNP. Based on D(50) values, the dose that reduced the survival to 50% relative to untreated controls, the cytotoxic effect of PhIP was increased threefold for 5P3H1 and 1.87-fold for 5P3H3 cell lines over the parental UV5P3 line. There was also a small increase in the mutation response at the aprt locus. These newly established 5P3H1 and 5P3H3 sulfotransferase-expressing cells provide valuable mechanistic information of the bioactivation of PhIP and related compounds. Environ. Mol. Mutagen. 35:57-65, 2000. Published 2000 Wiley-Liss, Inc.

Xu D, Shen H, Wang J. **[Detection of DNA strand breakage in human spermatozoa by use of single-cell gel electropheresis].** Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2000;17(4):281-4 [Chi].
Abstract: OBJECTIVE: To establish the single-cell gel electropherosis (SCGE) protocol for detection of DNA strand breakage in human spermatozoa. METHODS: The slides on which sperm cells and agarose were layered were immersed in a cold lysing solution of pH10 to lyse the sperm cells. Sperm nuclei were then pretreated with 10 mmol/L of DTT for 1 h, 10 microg/ml of RNase A for 4 h and 200 microg/ml of proteinase K for 15 h. Lastly, electrophoresis was performed in electrophoresis running buffer (pH10) at 12V (0.46V/cm) and 100 mA for 1 h. Sperm nuclei were stained with 15 microg/ml of EtBr for 5 min. The percentage of comet cells was counted. The in vitro hydrogen peroxide-induced DNA damage in human spermatozoa was measured with SCGE established by this laboratory. RESULTS:

The comet sperm cells in human spermatozoa ranged from 2% to 38%. There was a significant variance on the percentage of comet cells between different subjects. Hydrogen peroxide increased the percentage of comet sperm in a dose- and time-dependent manner. CONCLUSION: SCGE may be used to detect DNA strand breakage in human spermatozoa. Hydrogen peroxide-induced DNA damage in human sperm cells was detected successfully using SCGE protocol established by this laboratory.

Yamamura K. **[Receptor and disease: genetic perspective]**. Rinsho Byori 2000;48(5):416-22 [Jpn].
Abstract: Cells in higher animals communicate with each other using hundreds of kinds signaling molecules. Most of these signaling molecules are secreted from the signaling cell by exocytosis or diffusion. Regardless of the nature of the signal, the target cell responds by a specific protein called a receptor. It specifically binds the signaling molecule and then initiates a response in the target cell. Many genes for receptor molecules have been identified and mutations in many receptors are shown to be related to human diseases. In general, both genetic and environmental factors are involved in determining phenotypes including disease susceptibility. As the relative significance of genetic and environmental factors varies from disease to disease, we need animal models for human diseases to analyze these factors. It is now possible to manipulate mouse embryos to produce transgenic and knockout mice. These mice are quite useful for analyses of receptor functions and pathologic processes of disease development. As we only have about 6,000 mutant mice, we still need to produce at lease 94,000 other kinds of mice. To achieve this goal, we have to carry out random mutagenesis, instead of homologous recombination in ES cells.

Yen GC, Chen HY, Peng HH. **Evaluation of the cytotoxicity, mutagenicity and antimutagenicity of emerging edible plants.** Food Chem Toxicol 2001;39(11):1045-53.
Abstract: This study evaluates the toxic, mutagenic and antimutagenic effects of emerging edible plants that are consumed as new leafy vegetables in Taiwan. Among eight plant extracts, only the extracts of Sol (Solanum nigrum L.) showed cytotoxicity to Salmonella typhimurium TA100 in the absence of S9 mix. The toxicity of extracts from different parts of the Sol plant, such as leaf and stem, immature fruit and mature fruit, towards S. typhimurium TA100 and human lymphocytes was also assayed. The immature fruit extracts of Sol exhibited strong cytotoxicity with dose dependence and induced significant DNA damage in human lymphocytes based on the comet assay. However, no mutagenicity was found in eight plant extracts to TA98 or TA100 either with or without the S9 mixture. Sol and Sec [Sechium edule (Jacq.) Swartz] extracts showed the strongest inhibitory effect towards the mutagenicity of 2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ) in S. typhimurium TA98 and TA100; the ID(50) was less then 1 mg/plate. Cra [Crassocephalum creidioides (Benth.) S. Moore] extracts also expressed moderate antimutagenic activities towards IQ and benzo[a]pyrene (B[a]P) either in TA98 or in TA100; the ID(50) was 1.63-2.41 mg/plate. The extracts from Bas (Basella alba L.), Bou (Boussingaultia gracilis Miers var. pseudobaselloides Bailey), Cen (Centella asiatica L. Urban), Cor (Corchorus olitorius L.) and Por (Portulaca oleracea L.) showed weak to moderate inhibition of mutagenicity of IQ. However, the potential antimutagenicity of these plant extracts towards B[a]P was weaker than that towards IQ. For a direct mutagen, 4-nitroquinoline-N-oxide (NQNO), only the Sol extracts showed strong inhibitory effects in the TA100 system. The antimutagenic activity of water extracts of Sec was partly reduced by heating at 100 degrees C for 20 min. The heat-stable antimutagens in Sec extracts could be produced in the plant extract preparation process. Fractions with molecular weights above 30,000 showed the

strongest antimutagenicity and peroxidase activity in all the fractions of the Sec extracts.

Yesilada E. **Genotoxicity testing of some metals in the Drosophila wing somatic mutation and recombination test.** Bull Environ Contam Toxicol 2001;66(4):464-9.

Yi BA, Lin YF, Jan YN, Jan LY. **Yeast screen for constitutively active mutant G protein-activated potassium channels.** Neuron 2001;29(3):657-67.
Abstract: GIRK2 is a major contributor to G protein-activated inward rectifier potassium channels in the mammalian brain. How GIRK channels open upon contact with Gbetagamma remains unknown. Using a yeast genetic screen to select constitutively active mutants from a randomly mutagenized GIRK2 library, we identified five gating mutations at four residues in the transmembrane domain. Further mutagenesis indicates that GIRK channel opening involves a rotation of the transmembrane segments, bringing one of these residues (V188) to a pore-lining position in the open conformation. Combined with double-mutant studies, these findings suggest that GIRK channels gate by moving from the open conformation inferred from our yeast study of Kir2.1 to a closed conformation perhaps resembling the known KcsA structure.

Yoder JA, Litman GW. **The zebrafish fth1, slc3a2, men1, pc, fgf3 and cycd1 genes define two regions of conserved synteny between linkage group 7 and human chromosome 11q13.** Gene 2000;261(2):235-42.
Abstract: In addition to being an excellent model system for studying vertebrate development, the zebrafish has become a great tool for gene discovery by mutational analysis. The recent availability of the zebrafish EST database and radiation hybrid mapping panels has dramatically expanded the framework for genomic research in this species. Developing comparative maps of the zebrafish and human genomes is of particular importance for zebrafish mutagenesis studies in which human orthologs are sought for zebrafish genes. However, only partial cDNA sequences are determined routinely for mapped ESTs, leaving the identity of the EST in question. It previously had been reported that zebrafish linkage group 7 shares conserved synteny with human chromosome 11q13. In an effort to further define this relationship, five full-length zebrafish cDNAs, fth1, slc3a2, prkri, cd81, and pc, as well as one putative human gene, DBX were identified and their map positions ascertained. These six genes, along with men1, fgf3 and cycd1 define two regions of conserved synteny between linkage group 7 and 11q13.

Yoshikawa T, Nakanishi F, Ogura Y, Oi D, Omasa T, Katakura Y, Kishimoto M, Suga KI. **Flow cytometry: an improved method for the selection of highly productive gene-amplified CHO cells using flow cytometry.** Biotechnol Bioeng 2001;74(5):435-42.
Abstract: In previous work, we clarified the relationship between the productivity and stability of gene-amplified cells and the location of the amplified gene. The location of the amplified gene enabled us to classify resistant cells into two types. One type of resistant cell group, in which the amplified genes were observed near the telomeric region, was named the "telomere type." The other type of cell group, in which the amplified genes were observed in other chromosomal regions, was named the "other type." The phenotypes of these two types of cells are very different. In this experiment, using a fluorescein isothiocyanate-labeled methotrexate (F-MTX) reagent with flow cytometry, we were easily able to distinguish between highly productive cells and the other types of cells. The level of fluorescence

differed according to the difference in resistance to MTX. Based on this new finding, highly productive gene-amplified cells could be isolated from heterogeneous gene-amplified cell pools more easily than by the method of limiting-dilution assay. The limiting-dilution method requires several months to obtain highly productive gene-amplified cells, while our flow-cytometry-based method of selection requires only a few weeks.

Zhang H, Buchholz TA, Hancock D, Spitz MR, Wu X. **Gamma-radiation-induced single cell DNA damage as a measure of susceptibility to lung cancer: a preliminary report.** Int J Oncol 2000;17 (2):399-404.
Abstract: The comet assay is a sensitive and rapid method for detecting DNA single-strand and double-strand breaks and the individual cell's DNA repair profile. This pilot study was designed to determine whether the comet assay could measure inherited susceptibility to lung cancer. We applied the comet assay in the alkaline condition to test the DNA damage in gamma-irradiated and untreated cultured peripheral blood lymphocytes of 31 cases with previously untreated lung cancer and 39 controls. For each culture, 200 consecutive cells were examined and the number of cells with DNA uncoiling under electrophoresis (Ñcomet cells>) was recorded. The comet tail length (the radius of the nucleus plus the length of the migrated DNA) at 400-fold magnification was measured for the first 50 identified comet cells. The mean number of induced comet cells was significantly higher in cases (96.0+/-45.7) than matched controls (68.9+/-35.8) (P<0.05). However, no significant difference was observed in induced comet tail length between cases and controls. When we categorized the number of comet cells by the 75th percentile value in the controls, a higher number of comet cells was associated with significantly increased risk for lung cancer [odds ratio = 4.8 (confidence intervals of 1.5, 15.2)] after adjustment for age, sex, ethnicity and smoking status. The number of gamma-irradiation-induced comet cells (r=0.499, P<0.05) and comet tail length (r=0.520, P<0.05) correlated with the results on a previously reported lung cancer susceptibility marker, bleomycin sensitivity. Also, the number of gamma-irradiation-induced comet cells correlated with the results of the benzo[alpha]pyrene diol epoxide mutagen sensitivity assay, which quantifies induced chromatid breaks (r=0.275, P<0.05). The comet assay might be a simple and inexpensive tool for detecting genetic susceptibility to lung cancer.

Zhao Z, Cao Y, Li M, Meng A. **Double-stranded RNA injection produces nonspecific defects in zebrafish.** Dev Biol 2001;229(1):215-23.
Abstract: We have investigated the ability of dsRNA to inhibit gene functions in zebrafish using sequences targeted to the maternal gene pouII-1, the transgene GFP, and an intron of the zebrafish gene terra. We found that embryos injected with all of these dsRNAs at approximately 7.5 pg/embryo or higher had general growth arrest during gastrulation and displayed various nonspecific defects at 24 h postfertilization, although embryonic development was unaffected before the midblastula stage. Reducing dsRNA concentration could alleviate the global defects. Injection of GFP dsRNA (7.5-30 pg/embryo) did not inhibit GFP expression in transgenic fish, although abnormal embryos were induced. Co-injection of GFP mRNA with either GFP or non-GFP dsRNA caused reduction of GFP expression. Whole-mount in situ hybridization clearly showed that embryos injected with dsRNA degraded co-injected and endogenous mRNA without sequence specificity, indicating that dsRNA has a nonspecific effect at the posttranscriptional level. It appears that RNAi is not a viable technique for studying gene function in zebrafish embryos.

Zheng B, Sage M, Sheppeard EA, Jurecic V, Bradley A. **Engineering mouse chromosomes with Cre-loxP: range, efficiency, and somatic applications.** Mol Cell Biol 2000;20(2):648-55.
Abstract: Chromosomal rearrangements are important resources for genetic studies. Recently, a Cre-loxP-based method to introduce defined chromosomal rearrangements (deletions, duplications, and inversions) into the mouse genome (chromosome engineering) has been established. To explore the limits of this technology systematically, we have evaluated this strategy on mouse chromosome 11. Although the efficiency of Cre-loxP-mediated recombination decreases with increasing genetic distance when the two endpoints are on the same chromosome, the efficiency is not limiting even when the genetic distance is maximized. Rearrangements encompassing up to three quarters of chromosome 11 have been constructed in mouse embryonic stem (ES) cells. While larger deletions may lead to ES cell lethality, smaller deletions can be produced very efficiently both in ES cells and in vivo in a tissue- or cell-type-specific manner. We conclude that any chromosomal rearrangement can be made in ES cells with the Cre-loxP strategy provided that it does not affect cell viability. In vivo chromosome engineering can be potentially used to achieve somatic losses of heterozygosity in creating mouse models of human cancers.

Zhong Y, Feng SL, Luo Y, Zhang GD, Kong ZM. **Evaluating the genotoxicity of surface water of Yangzhong City using the Vicia faba micronucleus test and the comet assay.** Bull Environ Contam Toxicol 2001;67(2):217-24.

Zuo J, Niu QW, Moller SG, Chua NH. **Chemical-regulated, site-specific DNA excision in transgenic plants.** Nat Biotechnol 2001;19(2):157-61.
Abstract: We have developed a chemical-inducible, site-specific DNA excision system in transgenic Arabidopsis plants mediated by the Cre/loxP DNA recombination system. Expression of the Cre recombinase was tightly controlled by an estrogen receptor-based fusion transactivator XVE. Upon induction by beta-estradiol, sequences encoding the selectable marker, Cre, and XVE sandwiched by two loxP sites were excised from the Arabidopsis genome, leading to activation of the downstream GFP (green fluorescent protein) reporter gene. Genetic and molecular analyses indicated that the system is tightly controlled, showing high-efficiency inducible DNA excision in all 19 transgenic events tested with either single or multiple T-DNA insertions. The system provides a highly reliable method to generate marker-free transgenic plants after transformation through either organogenesis or somatic embryogenesis.

Kuroda N, Naruse K, Shima A, Nonaka M, Sasaki M. **Molecular cloning and linkage analysis of complement C3 and C4 genes of the Japanese medaka fish.** Immunogenetics 2000;51(2):117-28.
Abstract: The thioester-containing complement components, C3 and C4, are believed to have arisen by gene duplication from a common ancestor, and the mammalian C4 gene resides in the vicinity of the C2 and B genes within the major histocompatibility complex (MHC) class III region. To analyze the evolution of both the complement system and the MHC, we determined the complete primary structures of two C3 genes, termed Orla C3-1 and Orla C3-2, and one C4 gene, termed Orla C4, of a teleost, Japanese medaka fish (Oryzias latipes), by analyzing cDNA clones isolated from a liver library constructed using the inbred AA2 strain. The deduced basic structures of Orla C3-1, C3-2, and C4, such

as the subunit chain structure, the thioester site, and the proteolytic activation site, are similar to their mammalian counterparts. However, the catalytic His residue which greatly increases the rate of thioester reaction, is replaced by Ala in Orla C3-2, implying functional differentiation between two C3 molecules. Mapping analysis revealed a close linkage between the C3-1 and C3-2 genes, indicating that they arose by a local duplication rather than by a genome-wide tetraploidization. The C4 gene belongs to a different linkage group, and no linkage was observed among the C3, C4, Bf/C2, MHC class I, and MHC class II loci. These results suggest that the MHC class III complement region was established in the tetrapod lineage, or lost in the teleost lineage.

Afanassiev V, Sefton M, Anantachaiyong T, Barker G, Walmsley R, Wolfl S. **Application of yeast cells transformed with GFP expression constructs containing the RAD54 or RNR2 promoter as a test for the genotoxic potential of chemical substances.** Mutat Res 2000;464(2):297-308.
Abstract: Yeast strains transformed with high copy number plasmids carrying the gene encoding a green fluorescent protein optimised for yeast (yEGFP3) under the control of the RAD54 or RNR2 promoter were used to investigate the activity of potentially DNA-damaging substances. The assays were performed on 96-well microtitre plates in the presence of different concentrations of the test substances. The synthesis of GFP protein was measured through the fluorescence signal and cell growth was monitored by absorption. Here, we demonstrate that this system can be used as a biosensor to assess the genotoxic potential of drugs and other chemical substances. The use of microtitre plates will enable full automation of the system and allows the inclusion of internal reference standards in each assay.

Akkineni LK, Zeisig M, Baranczewski P, Ekstrom LG, Moller L. **Formation of DNA adducts from oil-derived products analyzed by 32P-HPLC.** Arch Toxicol 2001;74(11):720-31.
Abstract: The aim of this study was to investigate the genotoxic potential of DNA adducts and to compare DNA adduct levels and patterns in petroleum vacuum distillates, coal tar distillate, bitumen fume condensates, and related substances that have a wide range of boiling temperatures. An in vitro assay was used for DNA adduct analysis with human and rat S-9 liver extract metabolic activation followed by 32P-postlabeling and 32P-high-performance liquid chromatography (32p-HPLC). For petroleum distillates originating from one crude oil there was a correlation between in vitro DNA adduct formation and mutagenic index, which showed an increase with a distillation temperature of 250 degrees C and a peak around a distillation point of approximately 400 degrees C. At higher temperatures, the genotoxicity (DNA adducts and mutagenicity) rapidly declined to very low levels. Different petroleum products showed a more than 100-fold range in DNA adduct formation, with severely hydrotreated base oil and bitumen fume condensates being lowest. Coal tar distillates showed ten times higher levels of DNA adduct formation than the most potent petroleum distillate. A clustered DNA adduct pattern was seen over a wide distillation range after metabolic activation with liver extracts of rat or human origin. These clusters were eluted in a region where alkylated aromatic hydrocarbons could be expected. The DNA adduct patterns were similar for base oil and bitumen fume condensates, whereas coal tar distillates had a wider retention time range of the DNA adducts formed. Reference substances were tested in the same in vitro assay. Two- and three-ringed nonalkylated aromatics were rather low in genotoxicity, but some of the three- to four-ringed alkylated aromatics were very potent inducers of DNA adducts. Compounds with an amino functional group showed a 270-fold higher level of DNA adduct formation than the same structures with a nitro functional group. The most potent DNA adduct

inducers of the 16 substances tested were, in increasing order, 9,10-dimethylanthracene, 7,12-dimethylbenz[a]anthracene and 9-vinylanthracene. Metabolic activation with human and rat liver extracts gave rise to the same DNA adduct clusters. When bioactivation with material from different human individuals was used, there was a significant correlation between the CYP 1A1 activity and the capacity to form DNA adducts. This pattern was also confirmed using the CYP 1A1 inhibitor ellipticine. The 32P-HPLC method was shown to be sensitive and reproducible, and it had the capacity to separate DNA adduct-forming substances when applied to a great variety of petroleum products.

Api AM, Gudi R. **An in vivo mouse micronucleus assay on musk ketone.** Mutat Res 2000;464(2):263-7.
Abstract: Musk ketone (3,5-dinitro-2,6-dimethyl-4-tert-butyl-acetophenone) was evaluated in an in vivo mouse micronucleus assay. Male and female mice were dosed with 250, 500 or 1000 mg musk ketone/kg body weight by a single intraperitoneal injection in corn oil. Results of the assay showed that under the conditions of this test evaluated at 24, 48 and 72 h after dosing, musk ketone did not induce a significant increase in micronucleated polychromatic erythrocytes in either male or female mice at any dose or any time period. Musk ketone was considered to be negative in the mouse in vivo micronucleus test as well as in a battery of previously published in vitro genotoxicity tests. Based on the total weight of evidence available, it was concluded that musk ketone does not have significant potential to act as a genotoxic carcinogen.

Aryal P, Terashita T, Guengerich FP, Shimada T, Oda Y. **Use of genetically engineered Salmonella typhimurium OY1002/1A2 strain coexpressing human cytochrome P450 1A2 and NADPH-cytochrome P450 reductase and bacterial O-acetyltransferase in SOS/umu assay.** Environ Mol Mutagen 2000;36(2):121-6.
Abstract: The major pathway of bioactivation of procarcinogenic heterocyclic aromatic amines (HCAs) is cytochrome P450 1A2 (CYP1A2)-catalyzed N-hydroxylation and subsequent esterification by O-acetyltransferase (O-AT). We have previously reported that an umu tester strain, Salmonella typhimurium OY1001/1A2, endogenously coexpressing human CYP1A2 and NADPH-P450 reductase (reductase), is able to detect the genotoxicity of some aromatic amines [Aryal et al., 1999, Mutat Res 442:113-120]. To further enhance the sensitivity of the strain toward HCAs, we developed S. typhimurium OY1002/1A2 by introducing pCW"/1A2:hNPR (a bicistronic construct coexpressing human P450 1A2 and the reductase) and pOA102 (constructed by subcloning the Salmonella O-AT gene in the pOA101-expressing umuC"lacZ gene) in S. typhimurium TA1535. In addition, as an O-AT-deficient strain, we developed the OY1003/1A2 strain by introducing pCW"/1A2:hNPR and pOA101 into O-AT-deficient S. typhimurium TA1535/1,8-DNP. Strains OY1001/1A2, OY1002/1A2, and OY1003/1A2 expressed, respectively, about 150, 120, and 140 nmol CYP1A2/l culture (in whole cells), and respective cytosolic preparations acetylated 15, 125, and > or = 0 nmol isoniazid/min/mg protein as the O-AT activities of cytosolic preparations, respectively. We compared the induction of umuC gene expression as a measure of genotoxicity and observed that the OY1002/1A2 strain was more sensitive than OY1001/1A2 strain toward the genotoxicity of 2-amino-1,4-dimethylimidazo[4,5-f]quinol ine (MeIQ), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ),2-amino-3, 8-dimethylimidazo[4,5-f] quinoxaline (MeIQx),2-aminoanthracene, 2-amino-6-methyldipyrido[1,2-a::3,2'-d]i midazole,3-amino-1, 4-dimethyl-5H-pyrido[4,3-b]indole, and 3-amino-1-methyl-5H-pyrido[4, 3-a]indole. However, the

genotoxicity of MeIQ, IQ, and MeIQx was not detected with the OY1003/1A2 strain. These results indicate that the newly developed strain OY1002/1A2 can be employed in detecting potential genotoxic aromatic amines requiring bioactivation by CYP1A2 and O-acetyltransferase.

Barbin A. **Etheno-adduct-forming chemicals: from mutagenicity testing to tumor mutation spectra.** Mutat Res 2000;462(2-3):55-69.
Abstract: During the past 25 years, ethenobases have emerged as a new class of DNA lesions with promutagenic potential. Ethenobases were first investigated as DNA reaction products of vinyl chloride, an occupational carcinogen causing angiosarcoma of the liver (ASL). They were subsequently shown to be formed by several carcinogenic agents, including urethane (ethyl carbamate), and more recently, to occur in various tissues of unexposed humans and rodents. The endogenous source of ethenobases in DNA is thought to be a lipid peroxidation (LPO) product. Initial studies on metabolic activation, mutagenicity and carcinogenicity moved to the analyses of the formation of ethenobases in vivo and to the determination of their promutagenic properties. Quantification of etheno adducts in vivo became possible with the development of ultrasensitive techniques of analysis. To study the miscoding properties of ethenobases, the initial assays on the fidelity of replication or of transcription were replaced by site-directed mutagenesis assays in vivo. Ethenobases generate mainly base pair substitution mutations. With the advent of new techniques of molecular biology, mutations were investigated in the ras and p53 genes of tumors induced by vinyl chloride and urethane. In liver tumors induced by vinyl chloride, specific mutational patterns were found in the Ki-ras gene in human ASL, in the Ha-ras gene in hepatocellular carcinoma (HCC) in rats, and in the p53 gene in human and rat ASL. In tumors induced by urethane in mice, codon 61 of the Ha-ras gene (liver, skin) and of the Ki-ras gene (lung) seems to be a characteristic target. These tumor mutation spectra are compatible with the promutagenic properties of etheno adducts and with their formation in target tissues, suggesting that ethenobases can be initiating lesions in carcinogenesis. Another recent focus has been given to the repair of etheno adducts, and DNA glycosylases able to excise these adducts in vitro have been identified. The last two decades have brought ethenobases to light as potentially important DNA lesions in carcinogenesis. More research is needed to better understand the environmental and genetic factors that affect the formation and persistence of ethenobases in vivo.

Bernardini S, Hirvonen A, Jarventaus H, Norppa H. **Trans-stilbene oxide-induced sister chromatid exchange in cultured human lymphocytes: influence of GSTM1 and GSTT1 genotypes.** Mutagenesis 2001;16(3):277-81.
Abstract: About 50% and 15% of Caucasians lack the glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) genes and the corresponding enzyme activity, respectively. Both of these polymorphisms have been shown to affect the genotoxicity of some epoxides in cultured human lymphocytes. Especially GSTT1 appears to be important in whole-blood cultures, probably because GSTT1 activity is high in erythrocytes. The in vitro genotoxicity of trans-stilbene oxide (TSO), a model substrate for GSTM1, has been shown to depend on individual GSTM1 activity. The potential role of GSTM1 genotype, and the possible interference of GSTT1 genotype, has not previously been examined in this context. We have studied TSO-induced sister chromatid exchanges (SCEs) in 72 h whole-blood lymphocyte cultures from 24 healthy human donors, representing different combinations of GSTM1 and GSTT1 positive and null genotypes. TSO clearly increased SCEs in cultures of all donors. The mean number of SCEs per cell

induced by 75 and 150 microM TSO was, respectively, 1.5- and 1.3-times higher in cultures of GSTM1 null than GSTM1 positive donors. In another experiment, GSTM1 null individuals showed, in comparison with GSTM1 positive subjects, a 1.8-fold SCE induction by 50 microM TSO. GSTT1 genotype did not have an unequivocal effect. Our findings suggest that the lack of the GSTM1 gene, resulting in reduced detoxification capacity, increases individual sensitivity to the genotoxic effects of TSO.

Burchiel SW, Knall CM, Davis JW 2nd, Paules RS, Boggs SE, Afshari CA. **Analysis of genetic and epigenetic mechanisms of toxicity: potential roles of toxicogenomics and proteomics in toxicology.** Toxicol Sci 2001;59(2):193-5.
Abstract: The article highlighted in this issue is "An Aryl Hydrocarbon Receptor Independent Mechanism of JP-8 Jet Fuel Immunotoxicity in Ah-Responsive and Ah-Nonresponsive Mice" by Andrew C. Dudley, Margie M. Peden-Adams, Jackie EuDaly, Richard S. Pollenz, and Deborah E. Keil (pp. 251-259).

Cain SA, Ratcliffe CF, Williams DM, Harris V, Monk PN. **Analysis of receptor/ligand interactions using whole-molecule randomly-mutated ligand libraries.** J Immunol Methods 2000;245(1-2):139-45.
Abstract: We report a novel method for the analysis of protein ligands using a whole molecule mutagenesis/phage display system. The cDNA for the inflammatory polypeptide C5a was used as template in a PCR reaction doped with mutagenic nucleoside triphosphates (dP and 8-oxo-2'deoxyguanosine (8-oxodG)) to allow introduction of mutations in a highly controlled manner throughout the cDNA. The resultant library of mutants was displayed on the surface of phage and functional polypeptides were selected by several rounds of selection against the cells bearing the receptor for C5a. Following selection only a limited number of residues in C5a were found to be mutated, suggesting that mutations in key residues involved in the maintenance of structure and in receptor binding had been eliminated. The selected C5a sequences had a higher affinity for receptor than wild type phage-C5a conjugates. As this method for analysing the functional characteristics of proteins does not rely on knowledge a priori of structure, it may be useful for affinity maturation or analysis in a wide range of protein ligand/receptor systems.

Cipak L, Miadokova E, Dingova H, Kogan G, Novotny L, Rauko P. **Comparative DNA protectivity and antimutagenicity studies using DNA-topology and Ames assays.** Toxicol In Vitro 2001;15 (6):677-81.
Abstract: Two experimental techniques, the DNA-topology assay and the Ames assay, were proved to be suitable for monitoring compounds with a genotoxic potential and/or with an antimutagenic effect. Both procedures were used in assaying the acid-mine water (AMW) containing toxic metals and sulfoethyl chitin-glucan (SE-Ch-G), a derivative of chitin-glucan, in which bioprotective activities were detected earlier. It was shown that after toxic metal concentrations were decreased due to AMW dilution to the limits that correspond with those set by the Slovak Technical Norm (STN) for drinking water, AMW was not genotoxic in the Ames assay. As it is possible to detect any single-strand DNA (ssDNA) break in the DNA-topology assay, the SE-Ch-G protective effect against the ssDNA breaks induced by $Fe(2+)$ in the DNA-topology assay was recorded. SE-Ch-G exhibited the antimutagenic potential after its application simultaneously with diagnostic mutagens in the Ames assay. These results demonstrate

the complementarity of both experimental systems.

Clements J. **The mouse lymphoma assay.** Mutat Res 2000;455(1-2):97-110.
Abstract: In this paper, the current status of the protocol for the Mouse Lymphoma Assay is discussed. A brief history describes the events leading to current protocol recommendations. Areas for further development such as cytotoxicity, 24-h treatments, acceptability criteria and statistical analysis are also considered. Recent guidelines are reviewed, and consensus issues from the Mouse Lymphoma workgroup assembled as part of the International Workshop on Genotoxicity Test Procedures (IWGTP) are included. There are two versions of the assay - soft agar and microwell - and both will be discussed. For assay procedures, the emphasis will be on a typical microwell protocol but an attempt will be made to highlight protocol variations between laboratories and between the microwell and agar versions of the assay.

Crebelli R. **Threshold-mediated mechanisms in mutagenesis: implications in the classification and regulation of chemical mutagens.** Mutat Res 2000;464(1):129-35.
Abstract: Chemical mutagens are currently regulated and labelled on the basis of their hazardous properties defined in hazard classification schemes. The strength and type of experimental evidence is used as the only criterion for classification in categories which express different levels of concern for the possibility of adverse effects - notably transmissible genetic alterations - in humans. Differently from the classification of carcinogens, no consideration is given to potency, nor to the mechanism of action. The rationale of such hazard based classification is that the hazardous property of a chemical is an intrinsic feature, which is expressed independently of dosing. Changing of dose level results in a mere change in the probability to observe an adverse effect, but not in its potential occurrence. The lack of theoretical threshold underlying this approach can be envisaged, in principle, for stochastic processes such as DNA damage, which can be triggered by single molecular interactions. On the other hand, indirect mechanisms of genotoxicity, involving multiple interactions with non-DNA targets, are expected to show a threshold. At variance to DNA reactive agents, chemicals acting with threshold-mediated mechanism do change also qualitatively their toxic properties depending on the dose level. Possible problems arising in the application of hazard based schemes for the evaluation of chemicals with threshold-mediated mechanism of action are discussed, using the spindle poisons benzimidazole fungicides as an example.

Davidov Y, Rozen R, Smulski DR, Van Dyk TK, Vollmer AC, Elsemore DA, LaRossa RA, Belkin S. **Improved bacterial SOS promoter∷lux fusions for genotoxicity detection.** Mutat Res 2000;466(1):97-107.
Abstract: Escherichia coli strains containing plasmid-borne fusions of Vibrio fischeri lux to the recA promoter-operator region were previously shown to be potentially useful for detecting genotoxicants. In an attempt to improve past performance, the present study examines several modifications and variations of this design, singly or in various combinations: (1) modifying the host cell's toxicant efflux capacity via a tolC mutation; (2) incorporating the lux fusion onto the bacterial chromosome, rather then on a plasmid; (3) changing the reporter element to a different lux system (Photorhabdus luminescens), with a broader temperature range; (4) using Salmonella typhimurium instead of an E. coli host. A broad spectrum of responses to pure chemicals as well as to industrial wastewater samples was observed.

Generally, fastest responses were exhibited by Sal94, a S. typhimurium strain harboring a plasmid-borne fusion of V. fischeri lux to the E. coli recA promoter. Highest sensitivity, however, was demonstrated by DPD3063, an E. coli strain in which the same fusion was integrated into the bacterial chromosome, and by DPD2797, a plasmid-bearing tolC mutant. Overall, the two latter strains appeared to perform better and seemed preferable over the others. The sensor strains retained their sensitivity following a 2-month incubation after alginate-embedding, but at the cost of a significantly delayed response.

George SE, Huggins-Clark G, Brooks LR. **Use of a Salmonella microsuspension bioassay to detect the mutagenicity of munitions compounds at low concentrations.** Mutat Res 2001;490(1):45-56.
Abstract: Past production and handling of munitions has resulted in soil contamination at various military facilities. Depending on the concentrations present, these soils pose both a reactivity and toxicity hazard and the potential for groundwater contamination. Many munitions-related chemicals have been examined for mutagenicity in the Ames test, but because the metabolites may be present in low environmental concentrations, a more sensitive method is needed to elucidate the associated mutagenicity. RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), TNT (2,4,6-trinitrotoluene), tetryl (N-methyl-N-2,4,6-tetranitroaniline), TNB (1,3,5-trinitrobenzene) and metabolites were examined for mutagenicity in a microsuspension modification of the Salmonella histidine reversion assay with and without metabolic activation. TNB and tetryl were positive in TA98 (32.5, 5.2revertants/nmole) and TA100 (7.4, 9.5revertants/nmole) without metabolic activation and were more potent than TNT (TA98, 0.3revertants/nmole; TA100, 2.4revertants/nmole). With the exception of the tetranitroazoxytoluene derivatives, TNT metabolites were less mutagenic than TNT. RDX and two metabolites were negative in both strains, however, hexahydro-1,3,5-trinitroso-1,3,5-triazine was positive in TA100 with and without S9. Microsuspension bioassay results tend to correlate well with published Ames test data, however, there are discrepancies among the published data sets and the microsuspension assay results.

Gocke E, Muller L, Guzzie PJ, Brendler-Schwaab S, Bulera S, Chignell CF, Henderson LM, Jacobs A, Murli H, Snyder RD, et al. **Considerations on photochemical genotoxicity: report of the International Workshop on Genotoxicity Test Procedures Working Group.** Environ Mol Mutagen 2000;35(3):173-84.
Abstract: Recent toxicological observations have caused concern regarding the need to test, for example, pharmaceuticals and cosmetic products for photochemical genotoxicity. The objective of this report is to give assistance on how to adapt existing test methods to investigate the potential of light-absorbing compounds to induce genotoxic effects on photoactivation. In general, the Organization for Economic Co-Operation & Economic Development (OECD) draft guideline on in vitro phototoxicity testing served as a basis for consideration. Concomitant exposure of the cells to the test compound and solar simulated light was considered appropriate as the initial, basic test condition. Optimization of the exposure scheme, e.g., a change of the irradiation spectrum, might be indicated depending on the initial test results. Selection of test compound concentrations should be based on results obtained with the dark version of the respective test system but might have to be modified if phototoxic effects are observed. Selection of the irradiation dose has to be performed individually for each test system based on dose-effect studies. The irradiation should induce per se a small, reproducible toxic or genotoxic effect. The report includes a specification of necessary controls, discusses factors that might have an impact on the irradiation characteristics, and gives a rationale for the omission of an external metabolic activation

system. It also addresses the question that physicochemical and pharmacokinetic properties might trigger the need to test a chemical for photochemical genotoxicity. Relevant experimental observations are presented to back up the recommendations. The working group did not reach a consensus as to whether a single, adequately perfomed in vitro test for clastogenicity would be sufficient to exclude a photogenotoxic liability or whether a test battery including a gene mutation assay would be needed for product safety testing regarding photochemical genotoxicity.

Jenkins GJ, Burlinson B, Parry JM. **The polymerase inhibition assay: A methodology for the identification of DNA-damaging agents.** Mol Carcinog 2000;27(4):289-97.
Abstract: We report here the development of the polymerase inhibition assay (PI assay), a methodology capable of simultaneously identifying multiple DNA-damaging agents. The PI assay was developed in order to fulfil a requirement for the screening of new pharmaceuticals for potential DNA-damaging effects. The assay has the potential to screen hundreds of new compounds per week because of the microtiter plate format employed. We review previous descriptions of the phenomenon and provide researchers with the necessary methodology to obtain optimum polymerase inhibition effects. The assay is based on the inhibition of DNA polymerases (including those used in the polymerase chain reaction (PCR)) encountering damaged DNA bases. Hence, DNA-damaging agents can be identified by a corresponding reduction in PCR amplification after exposure. We demonstrate the detection of polymerase inhibition induced by a range of model genotoxic agents (N-methyl-N-nitrosourea, N-ethyl-N-nitrosourea, and ultraviolet (UV) C radiation), illustrating the successful application of the methodology. In addition, the PI assay is shown to be capable of detecting DNA damaging agents of biological relevance, i.e., known human carcinogens. These were N-OH-PhIP (from cooked meat) and UV-B (from sunlight). In addition to its employment in the detection of putative DNA damaging agents, the PI assay may also be applied as a research tool in carcinogenicity studies.

Kappers WA, van Och FM, de Groene EM, Horbach GJ. **Comparison of three different in vitro mutation assays used for the investigation of cytochrome P450-mediated mutagenicity of nitro-polycyclic aromatic hydrocarbons.** Mutat Res 2000;466(2):143-59.
Abstract: Three different in vitro mutation assays were used to investigate the involvement of cytochrome P450 enzymes in the activation of the nitro-polycyclic aromatic hydrocarbons (nitroPAHs) 1-nitropyrene and 2-nitrofluorene and their reduced metabolites amino-polycyclic aromatic hydrocarbons (aminoPAHs) 1-aminopyrene and 2-aminofluorene. Mutagenicity was investigated at the HPRT locus in Chinese hamster V79 cells with (V79-NH) or without (V79-MZ) endogenous acetyltransferase activity, stably expressing human cytochrome P450 cDNAs; in NIH/3T3 control or stably expressing human CYP1A2 cells, in combination with a shuttle vector containing a reporter gene; and in Salmonella typhimurium TA98, by inhibition of cytochrome P450 enzymes in rat liver S9 mix. Both the HPRT assay and the Ames test did not show any involvement of CYP3A in the activation of 1-nitropyrene to a mutagenic metabolite. In addition, a clear involvement of CYP1A2 in the activation of the nitroPAH 1-nitropyrene was demonstrated in both mutation assays using eukaryotic cells. However, no activation of 1-nitropyrene was seen in the eukaryotic cell lines when expressing only CYP1A2 (V79-MZ1A2) or acetyltransferase (V79-NH, 3T3-LNCX). The reduced metabolite of 1-nitropyrene, 1-aminopyrene, was also shown to be activated to a mutagenic metabolite by CYP1A2, using 3T3-1A2 cells in combination with a shuttle vector, and the Amestest in combination with the specific CYP1A2

inhibitor furafylline. No clear involvement of cytochrome P450 could be demonstrated for activation of 2-nitrofluorene to a mutagenic metabolite, whereas a role for CYP1A2 in the bioactivation of 2-aminofluorene is suggested.In the present study, we have demonstrated the complementary value of the three in vitro mutation assays in the examination of promutagen activation pathways.

Kind JA, Winn RN, Boerrigter ME, Jagoe CH, Glenn TC, Dallas CE. **Investigation of the radioadaptive response in brain and liver of pUR288 lacZ transgenic mice.** J Toxicol Environ Health A 2001;63(3):207-20.
Abstract: The radioadaptive response, where a small priming dose of ionizing radiation can lessen the effects of subsequent exposure to a higher radiation challenge dose, was investigated in brain and liver within transgenic mice. Although it is well characterized in models in vitro, current radioadaptive response research has focused on particular cell types (i.e., lymphocytes) and does not provide comparative data for responses of multiple tissues within an organism. Transgenic animals are useful for such comparisons, because the transgene is integrated into all cells in the body. The pUR288 lacZ plasmid-based transgenic mouse model utilizes a plasmid vector allowing highly efficient recovery of mutational targets, including large size-change mutations that result from radiation exposure. Female C57BI/6 pUR288 lacZ mice were exposed to priming doses of 0.075- to 0.375-Gy x-rays over a 3-d period. After 3 wk, they received an acute challenge dose of 2.5-Gy x-rays. Spontaneous mutant frequencies in lacZ were significantly higher in liver than in brain (6.62 x 10(-5) vs. 3.51 x 10(-5)). In the absence of a priming dose, the 2.5-Gy challenge doubled the mutant frequency of both liver and brain (13.38 x 10(-5), and 7.63 x 10(-5) respectively). Priming doses of 0.15, 0.225, and 0.375 Gy significantly reduced (by 40%) the mutagenic effects of the 2.5-Gy challenge in brain. Restriction enzyme analysis of rescued mutant plasmids revealed a decrease in large size-change mutations at the three priming doses in brain. This study demonstrates the utility of this model for the investigation of radiological processes of large size-change mutations, as well as showing a radioadaptive response in brain, but not liver, of mice in vivo.

Kirkland DJ, Muller L. **Interpretation of the biological relevance of genotoxicity test results: the importance of thresholds.** Mutat Res 2000;464(1):137-47.
Abstract: Despite recent improvements in genotoxicity protocols, we have observed an increase in the occurrence of positive results, particularly in chromosomal aberration tests in vitro, yet very few of these are accompanied by positive responses in vivo. Thus, the positive results may not be biologically relevant either for rodents or humans in vivo, but how should we determine "biological relevance"? Chemicals that produce thresholded dose-responses may well not pose a genotoxic risk at low (relevant to human) exposures, but thresholds should not just be "seen"; there must be an explanation and understanding of the underlying mechanism. In addition to extremes of pH, ionic strength and osmolality, as have been identified previously, such mechanisms include indirect genotoxicity resulting from interaction with non-DNA targets, chemicals/metabolites which are inherently genotoxic but which, at low concentrations, are effectively conjugated and unable to form adducts, and production of specific metabolites under in vitro conditions that are not formed in rodents or humans in vivo. If such thresholded mechanisms can be identified at exposures which are well in excess of expected human exposure, then there may be a strong argument that the positive results are not biologically relevant.

Kovalchuk I, Kovalchuk O, Hohn B. **Biomonitoring the genotoxicity of environmental factors with transgenic plants.** Trends Plant Sci 2001;6(7):306-10.
Abstract: All organisms must react to constantly changing surroundings. Environmental factors are thus powerful forces continuously shaping the genomes of all species. Induced genetic changes can be followed using a biomonitor - a living organism that reacts to a given compound in the environment. A vital but challenging task is identifying organisms with which to study the influence of changing environmental conditions. Plants are especially valuable biomonitors. Here, we describe the use of transgenic plant systems to evaluate the genotoxicity of chemical and radiological compounds. We evaluate the potential of further transgene-based systems for studying somatic and germ-line mutations.

Kraemer SM, Kronenberg A, Ueno A, Waldren CA. **Measuring the spectrum of mutation induced by nitrogen ions and protons in the human-hamster hybrid cell line A(L)C.** Radiat Res 2000;153 (6):743-51.
Abstract: Astronauts can be exposed to charged particles, including protons, alpha particles and heavier ions, during space flights. Therefore, studying the biological effectiveness of these sparsely and densely ionizing radiations is important to understanding the potential health effects for astronauts. We evaluated the mutagenic effectiveness of sparsely ionizing 55 MeV protons and densely ionizing 32 MeV/nucleon nitrogen ions using cells of two human-hamster cell lines, A(L) and A(L)C. We have previously characterized a spectrum of mutations, including megabase deletions, in human chromosome 11, the sole human chromosome in the human-hamster hybrid cell lines A(L)C and A(L). CD59(-) mutants have lost expression of a human cell surface antigen encoded by the CD59 gene located at 11p13. Deletion of genes located on the tip of the short arm of 11 (11p15.5) is lethal to the A(L) hybrid, so that CD59 mutants that lose the entire chromosome 11 die and escape detection. In contrast, deletion of the 11p15.5 region is not lethal in the hybrid A(L)C, allowing for the detection of chromosome loss or other chromosomal mutations involving 11p15.5. The 55 MeV protons and 32 MeV/nucleon nitrogen ions were each about 10 times more mutagenic per unit dose at the CD59 locus in A(L)C cells than in A(L) cells. In the case of nitrogen ions, the mutations observed in A(L)C cells were predominantly due to chromosome loss events or 11p deletions, often containing a breakpoint in the pericentromeric region. The increase in the CD59(-) mutant fraction for A(L)C cells exposed to protons was associated with either translocation of portions of 11q onto a hamster chromosome, or discontinuous or "skipping" mutations. We demonstrate here that A(L)C cells are a powerful tool that will aid in the understanding of the mutagenic effects of different types of ionizing radiation.

Krul C, Luiten-Schuite A, Baandagger R, Verhagen H, Mohn G, Feron V, Havenaar R. **Application of a dynamic in vitro gastrointestinal tract model to study the availability of food mutagens, using heterocyclic aromatic amines as model compounds.** Food Chem Toxicol 2000;38(9):783-92.
Abstract: The TNO gastro-Intestinal tract Model (TIM) is a dynamic computer-controlled in vitro system that mimics the human physiological conditions in the stomach and small intestine. In the current TIM physiological parameters such as pH, temperature, peristaltic movements, secretion of digestion enzymes, bile and pancreatic juices, and absorption of digested products-by removal through dialysis-was simulated. Heterocyclic aromatic amines (HAA; viz. IQ, MeIQ, MeIQx and PhIP) were used as model compounds for food mutagens, and the passage through TIM was investigated for each of these compounds separately. Subsequently, the influence of a matrix and different rates of passage on the

availability for absorption and distribution were studied in experiments with prepared meat, supplemented with MeIQx. Samples taken at various time points from the jejunal and ileal dialysates and from the lumen at the end of the small intestine (ileal delivery) were tested for the presence of mutagenic activity in the Ames test with Salmonella typhimurium strain TA98 as indicator, in the presence of mammalian metabolic activation (rat S9 mix). The results show that, comparable with the human in vivo situation, all four HAA are quickly removed (approx. 50% in 2 hr; approx. 95% in 6 hr) and mainly recovered from the lumen into the jejunal and ileal dialysates (94% of recovery). Only 5+/-1.5% is recovered in the chyme at the end of the small intestine. When MeIQx was added to meat, its availability for absorption was slower, although the influence of the gastrointestinal passage time on the availability of MeIQx was more pronounced than this matrix effect. More MeIQx was found in the jejunal dialysate (23%; $P<0.01$) and less in the ileal delivery (8%; $P<0.01$) when simulating the gastrointestinal passage of solid meals was compared to simulating that of liquid meals. The present experiments demonstrate that TIM can be applied to study in vitro the availability of heterocyclic aromatic amines in the gastrointestinal tract. More generally, these studies indicate that TIM shows promise as a useful tool for various research purposes dealing with the availability for absorption of mutagenic as well as antimutagenic components in food.

Lauerman JF. **Arrays cast toxicology in a new light.** Environ Health Perspect 2001;109(1):A20-1.

Liu SX, Athar M, Lippai I, Waldren C, Hei TK. **Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity.** Proc Natl Acad Sci U S A 2001;98(4):1643-8.
Abstract: Although arsenic is a well-established human carcinogen, the mechanisms by which it induces cancer remain poorly understood. We previously showed arsenite to be a potent mutagen in human-hamster hybrid (A(L)) cells, and that it induces predominantly multilocus deletions. We show here by confocal scanning microscopy with the fluorescent probe 5',6'-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate that arsenite induces, within 5 min after treatment, a dose-dependent increase of up to 3-fold in intracellular oxyradical production. Concurrent treatment of cells with arsenite and the radical scavenger DMSO reduced the fluorescent intensity to control levels. ESR spectroscopy with 4-hydroxy-2,2,6,6-tetramethyl-1-hydroxypiperidine (TEMPOL-H) as a probe in conjunction with superoxide dismutase and catalase to quench superoxide anions and hydrogen peroxide, respectively, indicates that arsenite increases the levels of superoxide-driven hydroxyl radicals in these cells. Furthermore, reducing the intracellular levels of nonprotein sulfhydryls (mainly glutathione) in A(L) cells with buthionine S-R-sulfoximine increases the mutagenic potential of arsenite by more than 5-fold. The data are consistent with our previous results with the radical scavenger DMSO, which reduced the mutagenicity of arsenic in these cells, and provide convincing evidence that reactive oxygen species, particularly hydroxyl radicals, play an important causal role in the genotoxicity of arsenical compounds in mammalian cells.

Lovell DP, Yoshimura I, Hothorn LA, Margolin BH, Soper K. **Report and summary of the major conclusions from statistics in genotoxicity testing working group from the International Workshop on Genotoxicity Test Procedures (IWGTP), March 1999.** Environ Mol Mutagen 2000;35(3):260-3.
Abstract: A working group of five statisticians experienced in the use of statistical methods in mutagenicity reviewed aspects of the statistical analysis of genotoxicity test procedures. Issues discussed

included methods for integrating biological importance and statistical significance, the relationship of the experimental unit to the experimental design, and the impact of new developments in statistics and computing. Three major recommendations were made relating to the need for: (1) the effective use of statistical advice in designing interlaboratory and intralaboratory investigations; (2) the development of appropriate experimental designs for new assays; and (3) education and training in the use of statistical methodology in mutagenicity testing. Environ. Mol. Mutagen. 35:260-263, 2000 Published 2000 Wiley-Liss, Inc.

Martinez A, Urios A, Blanco M. **Mutagenicity of 80 chemicals in Escherichia coli tester strains IC203, deficient in OxyR, and its oxyR(+) parent WP2 uvrA/pKM101: detection of 31 oxidative mutagens.** Mutat Res 2000;467(1):41-53.
Abstract: Strain IC203, deficient in OxyR, and its oxyR(+) parent WP2 uvrA/pKM101 (denoted IC188) are the basis of a new bacterial reversion assay, the WP2 Mutoxitest, which has been used in the evaluation of 80 chemicals for oxidative mutagenicity. The following 31 oxidative mutagens were recognized by their greater mutagenic response in IC203 than in IC188: (1) peroxides: hydrogen peroxide (HP), t-butyl hydroperoxide (BOOH) and cumene hydroperoxide (COOH); (2) benzoquinones (BQ): 2-methyl-1,4-BQ, 2,6-dimethyl-1,4-BQ and 2,3, 5,6-tetramethyl-1,4-BQ; (3) naphthoquinones (NQ): 1,4-NQ, 2-methyl-1, 4-NQ and 2-hydroxy-1,4-NQ; (4) phenol derivatives: catechol, hydroquinone, pyrogallol, 1,2,4-benzenetriol, t-butylhydroquinone, gallic acid and 4-aminophenol; (5) catecholamines: DL- and L-dopa, DL- and L-epinephrine, dopamine and L-norepinephrine; (6) thiols: L-cysteine methyl ester, L-cysteine ethyl ester, L-penicillamine and dithiothreitol; (7) diverse: 3,4-dihydroxyphenylacetic acid, hypoxanthine and xanthine, both in the presence of xanthine oxidase, L-ascorbic acid plus copper (II) and phenazine methosulfate. Among these oxidative mutagens, 25 were found to be uniquely positive in IC203. With the exception of BOOH and COOH, mutagenesis by all oxidative mutagens was inhibited by catalase present in rat liver S9, indicating that it is mediated by HP generation, probably in autoxidation reactions. These catalase-sensitive oxidative mutagens were poor inducers of mutations derived from 8-oxoguanine lesions, whereas such mutations were efficiently induced by organic hydroperoxides. The results support the usefulness of incorporating IC203 in the bacterial battery for testing of chemicals. The well-characterized oxidative mutagens available with the use of the WP2 Mutoxitest may serve as a reference in studies on the genotoxicity of oxidative stress.

Martinez A, Urios A, Felipo V, Blanco M. **Mutagenicity of nitric oxide-releasing compounds in Escherichia coli: effect of superoxide generation and evidence for two mutagenic mechanisms.** Mutat Res 2001;497(1-2):159-67.
Abstract: The mutagenicity of three nitric oxide (NO) donors, 3-morpholinosydnonimine (SIN-1), a compound generating the precursors of peroxynitrite NO and superoxide, diethylamine/NO (DEA/NO) and spermine/NO (SPER/NO), both releasing authentic NO was analyzed using Escherichia coli tester strains IC203, carrying a deletion of the oxyR gene, and its oxyR(+) parent IC188 (the alternative name of WP2 uvrA/pKM101). The OxyR protein is a redox-sensitive transcriptional activator of genes encoding antioxidant enzymes. Strains IC203 and IC188 contain error-prone DNA polymerases polV, encoded by the chromosomal umuDC genes, and polRI, encoded by mucAB genes carried by pKM101. SIN-1 was determined to be an oxidative mutagen giving a positive response only in IC203, whereas DEA/NO and SPER/NO induced similar positive responses in IC203 and IC188 and were considered as

non-oxidative mutagens. The spectrum of ochre suppressors in Trp(+) revertants induced by SIN-1 in IC203 was characterized by a higher number of TA-->AT transversions and GC-->AT transitions, and a lower number of GC-->TA transversions, with respect to the untreated control. The mutagenicity of SIN-1 in IC203, probably induced by peroxynitrite through reactive derivatives, was enhanced in the presence of plumbagin (PLB), a superoxide generator. Superoxide generation by PLB, as well as formation of peroxynitrite in cells treated with SIN-1, evaluated by monitoring the oxidation, respectively, of dihydroethidium and dihydrorhodamine 123, were greater in IC203 than in IC188. Formation of peroxynitrite in IC203 treated with SIN-1 was stimulated by PLB. After treatment with DEA/NO and SPER/NO the number of revertants scored in IC188 was higher than in strains IC187, containing only polV, and IC204, deficient in both polV and polRI. For these compounds, induced suppressor revertants in IC187 and IC204 were almost exclusively GC-->AT transitions, whereas in IC188 significant levels of GC-->TA and TA-->AT transversions were also induced. Mutagenesis by both DEA/NO and SPER/NO was partially inhibited in the presence of PLB. The results show the usefulness of the new tester strain IC203 to differentiate NO-promoted mutagenic mechanisms that involve or do not involve oxygen radicals.

Monteiro C, Marcelino LA, Conde AR, Saraiva C, Giphart-Gassler M, De Nooij-van Dalen AG, Van Buuren-van Seggelen V, Van der Keur M, May CA, Cole J, et al. **Molecular methods for the detection of mutations.** Teratog Carcinog Mutagen 2000;20(6):357-86.
Abstract: We report the results of a collaborative study aimed at developing reliable, direct assays for mutation in human cells. The project used common lymphoblastoid cell lines, both with and without mutagen treatment, as a shared resource to validate the development of new molecular methods for the detection of low-level mutations in the presence of a large excess of normal alleles. As the "gold standard, " hprt mutation frequencies were also measured on the same samples. The methods under development included i) the restriction site mutation (RSM) assay, in which mutations lead to the destruction of a restriction site; ii) minisatellite length-change mutation, in which mutations lead to alleles containing new numbers of tandem repeat units; iii) loss of heterozygosity for HLA epitopes, in which antibodies can be used to direct selection for mutant cells; iv) multiple fluorescence-based long linker arm nucleotides assay (mf-LLA) technology, for the detection of substitutional mutations; v) detection of alterations in the TP53 locus using a (CA) array as the target for the screening; and vi) PCR analysis of lymphocytes for the presence of the BCL2 t(14:18) translocation. The relative merits of these molecular methods are discussed, and a comparison made with more "traditional" methods.

Nohmi T. **[Development of novel genotoxicity assays by genetic engineering methods].** Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku 2000;(118):1-20 [Jpn].
Abstract: Novel genotoxicity assays have been developed to efficiently detect the genotoxicity of various environmental chemicals, and to evaluate the potential hazards to man. Salmonella typhimurium strains harboring plasmids carrying the genes encoding drug metabolizing enzymes, such as acetyl Co-A O-acetyltransferase, or the strains lacking DNA repair enzymes, such as O6-methylguanine DNA methyltransferase, are highly sensitive to the mutagenicity of particular classes of mutagens. Thus, they are widely used for the efficient detection of genotoxicity of complex mixtures. Transgenic mice, named gpt delta, have been established for the detection and molecular analysis of mutations in various organs of rodents induced by chemicals. Future perspective of the genotoxicity assays using genetically

engineered organisms is discussed.

Nohmi T, Suzuki T, Masumura K. **Recent advances in the protocols of transgenic mouse mutation assays**. Mutat Res 2000;455(1-2):191-215.
Abstract: Transgenic mutation assays were developed to detect gene mutations in multiple organs of mice or rats. The assays permit (1) quantitative measurements of mutation frequencies in all tissues/organs including germ cells and (2) molecular analysis of induced and spontaneous mutations by DNA sequencing analysis. The protocols of recently developed selections in the lambda phage-based transgenic mutation assays, i.e. cII, Spi(-) and 6-thioguanine selections, are described, and a data set of transgenic mutation assays, including those using Big Blue and Muta Mouse, is presented.

Oh YB, Lee JS, Park EH. **Fish cell line as an in vitro test system for analyzing chromosome aberrations.** Bull Environ Contam Toxicol 2001;67(1):6-11.

Phillips DH, Farmer PB, Beland FA, Nath RG, Poirier MC, Reddy MV, Turteltaub KW. **Methods of DNA adduct determination and their application to testing compounds for genotoxicity.** Environ Mol Mutagen 2000;35(3):222-33.
Abstract: At the International Workshop on Genotoxicity Test Procedures (IWGTP) held in Washington, DC (March 25-26, 1999), a working group considered the uses of DNA adduct determination methods for testing compounds for genotoxicity. When a drug or chemical displays an unusual or inconsistent combination of positive and negative results in in vitro and in vivo genotoxicity assays and/or in carcinogenicity experiments, investigations into whether or not DNA adducts are formed may be helpful in assessing whether or not the test compound is a genotoxin. DNA adduct determinations can be carried out using radiolabeled compounds and measuring radioactive decay (scintillation counting) or isotope ratios (accelerator mass spectrometry) in the isolated DNA. With unlabeled compounds adducts may be measured by (32)P-postlabeling analysis of the DNA, or by physicochemical methods including mass spectrometry, fluorescence spectroscopy, or electrochemical detection, or by immunochemical methods. Each of these approaches has different strengths and limitations, influenced by sensitivity, cost, time, and interpretation of results. The design of DNA binding studies needs to be on a case-by-case basis, depending on the compound's profile of activity. DNA purity becomes increasingly important the more sensitive, and less chemically specific, the assay. While there may be adduct levels at which there is no observable biological effect, there are at present insufficient data on which to set a threshold level for biological significance.

Quinones A, Rainov NG. **Identification of genotoxic stress in human cells by fluorescent monitoring of p53 expression.** Mutat Res 2001;494(1-2):73-85.
Abstract: The tumor suppressor protein p53 is induced upon DNA damage essentially by post-translational regulatory mechanisms, which lead to a substantial increase of p53 levels. To exploit this essential property of p53, we developed a novel reporter system for monitoring accumulation and subcellular translocation of p53 protein, which is able to function as a simple test for detecting mutagenic and genotoxic stress in human cells. For this purpose, we constructed a plasmid with a specific translational TP53::EGFP gene fusion and selected stable transfected clones in the human cell line HEK293, in which p53 is functionally stabilized due to the expression of the transgenic adenoviral

E1A oncoproteins. HEK293-TP53::EGFP clones may be used as a living cell system for monitoring not only of the induction of p53 protein in the cell, but also of its subcellular localization. Using this human reporter cell system, we examined levels of p53 by fluorescence microscopy and by FACS analysis following treatment with several classes of genotoxic and carcinogenic compounds. All tested DNA damaging agents caused a significant increase of intracellular p53-EGFP levels in a concentration-dependent manner. On the other hand, non-genotoxic carcinogens and stress conditions that cannot damage DNA were not able to induce p53-EGFP accumulation. The induction effect caused by genotoxic stress was found to be dependent on the endogenous p53 status, because it was not observed in p53-deficient cell lines. This corroborates the notion that p53 may be used as an universal sensor for genotoxic stress and demonstrates the usefulness of HEK293-p53-EGFP cells as a reporter system for identification of mutagens and genotoxic carcinogens in human cells by means of visualizing and monitoring intracellular p53 levels and localization.

Rosenkranz HS, Cunningham AR. **A new approach to evaluate mechanistic relationships among genotoxic phenomena: validation.** Mutagenesis 2000;15(4):325-8.
Abstract: In order to determine its applicability for the study of genotoxicity, a recently developed method to probe for possible mechanistic relationships among toxicological phenomena was applied to the induction of mutations in Salmonella typhimurium. Since the basis of this phenomenon is understood, this would provide a test of the applicability of the new method to DNA-based mechanisms. The results presented indicate that significant relationships are indeed found among phenomena involving damage to or modification of DNA but not between them and non-genotoxic phenomena. The present results suggest that the newly developed approach could be applied to test mechanistic hypotheses involving genotoxic phenomena.

Sawano A, Miyawaki A. **Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis.** Nucleic Acids Res 2000;28(16):E78
Abstract: To develop a simple, speedy, economical and widely applicable method for multiple-site mutagenesis, we have substantially modified the Quik-Change Site-Directed Mutagenesis Kit protocol (Stratagene, La Jolla, CA). Our new protocol consists of (i) a PCR reaction using an in vitro technique, LDA (ligation-during-amplification), (ii) a DPN:I treatment to digest parental DNA and to make megaprimers and (iii) a synthesis of double-stranded plasmid DNA for bacterial transformation. While the Quik Change Kit protocol introduces mutations at a single site, requiring two complementary mutagenic oligonucleotides, our new protocol requires only one mutagenic oligonucleotide for a mutation site, and can introduce mutations in a plasmid at multiple sites simultaneously. A targeting efficiency >70% was consistently achieved for multiple-site mutagenesis. Furthermore, the new protocol allows random mutagenesis with degenerative primers, because it does not use two complementary primers. Our mutagenesis strategy was successfully used to alter the fluorescence properties of green fluorescent protein (GFP), creating a new-color GFP mutant, cyan-green fluorescent protein (CGFP). An eminent feature of CGFP is its remarkable stability in a wide pH range (pH 4-12). The use of CGFP would allow us to monitor protein localization quantitatively in acidic organelles in secretory pathways.

Smolewski P, Ruan Q, Vellon L, Darzynkiewicz Z. **Micronuclei assay by laser scanning cytometry.** Cytometry 2001;45(1):19-26.

Abstract: BACKGROUND: The micronuclei (MN) assay is used to assess the chromosomal/mitotic spindle damage induced by ionizing radiation or mutagenic agents in vivo or in vitro. Because visual scoring of MN is cumbersome semi-automatic procedures that relay either on flow cytometry or image analysis were developed: both offer some advantages but also have shortcomings. METHODS: In the present study laser scanning cytometer (LSC), the instrument that combines analytical capabilities of flow and image cytometry, has been adapted for quantitative analysis of MN. The micronucleation of human breast carcinoma MCF-7 and leukemic HL-60 and U-937 cells was induced by in vitro treatment with mitomycin C. Cellular DNA was stained with propidium iodide (PI), protein was counterstained with fluorescein isothiocyanate (FITC). Two approaches were used to detect MN: (a) the threshold contour was set based on the data from the photosensor measuring red fluorescence of PI and MN were identified on the bivariate PI versus PI/FITC fluorescence distributions by their characteristic position; (b) the threshold contour was set on the data from the sensor measuring FITC fluorescence which made it possible, using the LSC software dedicated for FISH analysis, to assay both the frequency and DNA content of individual MN within each measured cell. RESULTS: The capability of LSC to relocate MN for visual examination was useful to confirm their identification. Visual identification of MN combined with their multiparameter characterization that took into an account their DNA content and protein/DNA ratio made it possible establish the gating parameters that excluded objects that were not MN; 93.3+/-3.3 events within the selected gate were MN. It was also possible to successfully apply FISH software to characterize individual cells with respect to quantity of MN residing in them. The percentage of MN assayed by LSC correlated well with that estimated visually by microscopy, both for MCF-7 (r = 0.93) and HL-60 cells (r = 0.87). CONCLUSIONS: LSC can be used to obtain unbiased estimate of MN frequencies. Unlike flow cytometry, it also allows one to characterize individual cells with respect to frequency and DNA content of MN residing in these cells. These analytical capabilities of LSC may be helpful not only to score MN but also to study mechanisms by which clastogenic agents induce MN.

Snyder RD, Green JW. **A review of the genotoxicity of marketed pharmaceuticals.** Mutat Res 2001;488(2):151-69.

Abstract: Information in the 1999 Physician's Desk Reference as well as from the peer-reviewed published literature was used to evaluate the genotoxicity of marketed pharmaceuticals. This survey is a compendium of genotoxicity information and a means to gain perspective on the inherent genotoxicity of structurally diverse pharmaceuticals. Data from 467 marketed drugs were collected. Excluded from analysis were anti-cancer drugs and nucleosides, which are expected to be genotoxic, steroids, biologicals and peptide-based drugs. Of the 467 drugs, 115 had no published gene-tox data. This group was comprised largely of acutely administered drugs such as antibiotics, antifungals, antihistamines decongestants and anesthetics. The remaining 352 had at least one standard gene-tox assay result. Of these, 101 compounds (28.7%) had at least one positive assay result in the pre-ICH/OECD standard four-test battery (bacterial mutagenesis, in vitro cytogenetics, mouse lymphoma assay (MLA), in vivo cytogenetics). Per assay type, the percentage of positive compounds was: bacterial mutagenesis test, 27/323 (8.3%); in vitro cytogenetics 55/222 (24.8%); MLA 24/96 (25%); in vivo cytogenetics 29/252 (11.5%). Of the supplemental genetic toxicology test findings reported, the sister chromatid exchange (SCE) assay had the largest percentage of positives 17/39 (43.5%) and mammalian mutagenesis assays (excluding MLA) had the lowest percentage of positives 2/91 (2.2%). The predictive value of genetic toxicology findings for 2-year bioassay outcomes is difficult to assess since carcinogenicity can occur

via non-genotoxic mechanisms. Nevertheless, the following survey findings were made: 201 drugs had both gene-tox data and rodent carcinogenicity data. Of these, 124 were negative and 77 were equivocal or positive for carcinogenicity in at least 1 gender/1 species. Of the 124 non-carcinogens, 100 had no positive gene-tox findings. Of the remaining 24, 19 were positive in in vitro cytogenetics assays. Among the 77 compounds that exhibited equivocal or positive effects in carcinogenesis studies, 26 were positive in gene-tox assays and 51 were negative. Of the 51 negatives, 47 had multiple negative gene-tox assay results suggesting that these are probably non-genotoxic carcinogens. Statistical analyses suggested that no combination of gene-tox assays provided a higher predictivity of rodent carcinogenesis than the bacterial mutagenicity test itself.

Sofuni T, Hayashi M, Nohmi T, Matsuoka A, Yamada M, Kamata E. **Semi-quantitative evaluation of genotoxic activity of chemical substances and evidence for a biological threshold of genotoxic activity.** Mutat Res 2000;464(1):97-104.
Abstract: In Japan, the Chemical Substances Control Law requires evaluation of the genotoxic potential of chemical substances semi-quantitatively by application of a ranking system. During the past 10 years under the law, 1049 new chemical substances were evaluated by a reverse mutation assay in bacteria (RMA) and a chromosome aberration test in cultured mammalian cells (CAT). Of them, 130 (12.4%) were positive in the RMA and 402 (38.3%) were positive in the CAT. Eighty (7.6%) were positive in both tests. Fifty (4.8%) were positive only in the RMA, 322 (30.7%) were positive only in the CAT, and 452 (43.1%) were positive in either the RMA or the CAT. Thus, the tests complement each other in detecting genotoxic substances in vitro. To explore the "threshold" concept, we compared the genotoxic responses of Salmonella typhimurium tester strains with and without DNA repair capacity. Recently constructed strains of TA1535 lacking O(6)-methylguanine DNA methyltransferase genes (ogt(ST) or ada(ST) and ogt(ST)) showed dose-related increases in the number of revertants induced by N-ethyl-N'-nitro-N-nitrosoguanidine, methyl methanesulfonate, dimethylnitrosamine, and ethylnitrosourea, while in the same dose ranges the parental strain TA1535 did not. This finding suggests that there is a threshold at which all DNA damage induced by low dose levels of genotoxic chemicals are repaired. That biological threshold seems to exist for both DNA and non-DNA targeting chemicals.

Tohe A, Oguchi T. **An improved integration replacement/disruption method for mutagenesis of yeast essential genes.** Genes Genet Syst 2000;75(1):33-9.
Abstract: We improved the integration replacement/disruption method (Shortle, D., Novic, P., and Botstein, D. Proc. Natl. Acad. Sci. USA 81: 4889-4893, 1984) for isolating mutants in any of essential genes of the yeast Saccharomyces cerevisiae by integrating mutagenized DNA into the wild type gene of interest. We adopted this method to isolate temperature-sensitive mutants of the MPC1 gene encoding the YLL031C ORF. To facilitate integration of the mutagenic plasmid at a site near the 5' end of the ORF, a BamHI site was created at 300 bp downstream of the 5' end of the truncated ORF to be mutagenized. The MPC1 gene was disrupted in the wild type haploid strain by integrating a 5'-truncated derivative of the gene with mutations induced by in vitro mutagenesis. Transformants thus obtained were subjected for diagnosis of conditional lethality by replica-plating onto an appropriate selection medium to detect mutants. A primary mutant isolated by this method reverted in a high frequency due to a tandem repeat created by mutagenic integration. We deviced a method to obtain a stable temperature-sensitive strain by disrupting the tandem duplication. Two stable temperature-sensitive mutants thus

obtained were found to be remedial either with 1 M sorbitol or with 0.1 M Mg2+ and to be sensitive to local anestheticum, tetracaine, at 25 degrees C.

van Brabant AJ, Stan R, Ellis NA. **DNA helicases, genomic instability, and human genetic disease.** Annu Rev Genomics Hum Genet 2000;1:409-59.
Abstract: DNA helicases are a highly conserved group of enzymes that unwind DNA. They function in all processes in which access to single-stranded DNA is required, including DNA replication, DNA repair and recombination, and transcription of RNA. Defects in helicases functioning in one or more of these processes can result in characteristic human genetic disorders in which genomic instability and predisposition to cancer are common features. So far, different helicase genes have been found mutated in six such disorders. Mutations in XPB and XPD can result in xeroderma pigmentosum, Cockayne syndrome, or trichothiodystrophy. Mutations in the RecQ-like genes BLM, WRN, and RECQL4 can result in Bloom syndrome, Werner syndrome, and Rothmund-Thomson syndrome, respectively. Because XPB and XPD function in both nucleotide excision repair and transcription initiation, the cellular phenotypes associated with a deficiency of each one of them include failure to repair mutagenic DNA lesions and defects in the recovery of RNA transcription after UV irradiation. The functions of the RecQ-like genes are unknown; however, a growing body of evidence points to a function in restarting DNA replication after the replication fork has become stalled. The genomic instability associated with mutations in the RecQ-like genes includes spontaneous chromosome instability and elevated mutation rates. Mouse models for nearly all of these entities have been developed, and these should help explain the widely different clinical features that are associated with helicase mutations.

von der Hude W, Kalweit S, Engelhardt G, McKiernan S, Kasper P, Slacik-Erben R, Miltenburger HG, Honarvar N, Fahrig R, Gorlitz B, et al. **In vitro micronucleus assay with Chinese hamster V79 cells - results of a collaborative study with in situ exposure to 26 chemical substances.** Mutat Res 2000;468(2):137-63.
Abstract: A collaborative study with 10 participating laboratories was conducted to evaluate a test protocol for the performance of the in vitro micronucleus (MN) test using the V79 cell line with one treatment and one sampling time only. A total of 26 coded substances were tested in this study for MN-inducing properties. Three substances were tested by all 10 laboratories and 23 substances were tested by three or four laboratories in parallel. Six aneugenic, 7 clastogenic and 6 non-genotoxic chemicals were uniformly recognised as such by all laboratories. Three chemicals were tested uniformly negative by three laboratories although also clastogenic properties have been reported for these substances. Another set of three clastogenic substances showed inconsistent results and one non-clastogenic substance was found to be positive by one out of three laboratories. Within the study, the applicability of the determination of a proliferation index (PI) as an internal cytotoxicity parameter in comparison with the determination of the mitotic index (MI) was also evaluated. Both parameters were found to be useful for the interpretation of the MN test result with regard to the control of cell cycle kinetics and the mode of action for MN induction. The MN test in vitro was found to be easy to perform and its results were mainly in accordance with results from chromosomal aberration tests in vitro.

Winn RN. **Trangenic fish as models in environmental toxicology.** ILAR J 2001;42(4):322-9.
Abstract: Historically, fish have played significant roles in assessing potential risks associated with

exposure to chemical contamination in aquatic environments. Considering the contributions of transgenic rodent models to biomedicine, it is reasoned that the development of transgenic fish could enhance the role of fish in environmental toxicology. Application of transgenic fish in environmental studies remains at an early stage, but recent introduction of new models and methods demonstrates progress. Rapid advances are most evident in the area of in vivo mutagenesis using fish carrying transgenes that serve as recoverable mutational targets. These models highlight many advantages afforded by fish as models and illustrate important issues that apply broadly to transgenic fish in environmental toxicology. Development of fish models carrying identical transgenes to those found in rodents is beneficial and has revealed that numerous aspects of in vivo mutagenesis are similar between the two classes of vertebrates. Researchers have revealed that fish exhibit frequencies of spontaneous mutations similar to rodents and respond to mutagen exposure consistent with known mutagenic mechanisms. Results have demonstrated the feasibility of in vivo mutation analyses using transgenic fish and have illustrated their potential value as a comparative animal model. Challenges to development and application of transgenic fish relate to the needs for improved efficiencies in transgenic technology and in aspects of fish husbandry and use. By taking advantage of the valuable and unique attributes of fish as test organisms, it is anticipated that transgenic fish will make significant contributions to studies of environmentally induced diseases.

Zavolan M, Kepler TB. **Statistical inference of sequence-dependent mutation rates.** Curr Opin Genet Dev 2001;11(6):612-5.
Abstract: Several lines of research are now converging towards an integrated understanding of mutational mechanisms and their evolutionary implications. Experimentally, crystal structures reveal the effect of sequence context on polymerase fidelity; large-scale sequencing projects generate vast amounts of sequence polymorphism data; and locus-specific databases are being constructed. Computationally, software and analytical tools have been developed to analyze mutational data, to identify mutational hot spots, and to compare the signatures of mutagenic agents.

HEPATIC AND RENAL TOXICITY

Alvarez-Barrientos A, O'Connor JE, Nieto Castillo R, Moreno Moreno AB, Prieto P. **Use of flow cytometry and confocal microscopy techniques to investigate early CdCl(2)-induced nephrotoxicity in vitro**. Toxicol In Vitro 2001;15(4-5):407-12.
Abstract: CdCl(2) is a well-known toxic compound for the kidney in vivo and in vitro. We report here part of the results of an ECVAM (European Centre for the Validation of Alternative Methods) contract study, aimed at establishing and assessing several flow cytometric and confocal microscopic endpoints for use in an in vitro nephrotoxicity model. Three renal tubule cell lines, OK (opossum, proximal tubule origin), LLC-PK1 (pig, proximal tubule origin) and MDCK (dog, distal tubule origin) were exposed for 1, 5 and 24 h to 25 &mgr;M and 100 &mgr;M CdCl(2). The results obtained for mitochondrial membrane potential showed a decrease in all the cell lines after 5 h of treatment with both CdCl(2) concentrations. In some cases, this decrease was detected by flow cytometry after a 1-h exposure. On the contrary, intracellular Ca(2+) increased in a time-dependent and concentration-dependent fashion. This increase was especially high in the MDCK cell line after a 24-h exposure to 100 &mgr;M CdCl(2). However, cell viability was not affected by 25 &mgr;M CdCl(2). Our results demonstrate early changes

in mitochondrial membrane potential and cytoplasmic Ca(2+) levels in renal tubular epithelial cell lines treated with CdCl(2).

Ayala-Fierro F, Carter DE. **LLC-PK1 cells as a model for renal toxicity caused by arsine exposure.** J Toxicol Environ Health A 2000;60(1):67-79.
Abstract: The mechanisms of arsine (AsH3) toxicity are not completely understood. Studies were undertaken to determine AsH3 and arsenite [As(III)] toxicity in a renal tubular epithelial cell line to model kidney dysfunction caused by AsH3 exposure. The hypothesis was that As(III) is the toxic metabolite responsible for the renal toxicity of AsH3. There was a concentration- and time-dependent toxic response after As(III) incubation. As(III) produced significant LDH leakage as early as 1 h and intracellular potassium loss at 5 h. AsH3 produced no changes in these parameters. AsH3 affected neither potassium nor LDH levels over 24 h and up to 1 mM AsH3 concentration. In this system, As(III) induced LDH leakage before K+ loss. Oxidative stress-like toxicity effects were also studied by determining levels of glutathione (GSH), glutathione disulfide (GSSG), and heat-shock protein 32 (Hsp32) levels. GSH levels were not markedly affected by any arsenical over a 6-h period or up to 100 microM concentration of the arsenical. However, 100 microM AsH3 significantly increased GSSG levels as early as 30 min and reached a maximum at 2.5 h. Incubation with 10 microM AsH3 was sufficient to significantly increase GSSG levels. As(III) had no marked effect on GSSG. Both arsenicals (50 microM) produced a slight increase (about threefold) in Hsp32 levels after 4-h incubation. These results showed that unchanged AsH3 produced oxidative stress-like toxic effects without producing cell death. However, similar As(III) concentrations induced the stress response and were toxic to the cells. These data indicated that AsH3 is not directly toxic to LLC-PK1 cells.

Behrens A, Schirmer K, Bols NC, Segner H. **Polycyclic aromatic hydrocarbons as inducers of cytochrome P4501A enzyme activity in the rainbow trout liver cell line, RTL-W1, and in primary cultures of rainbow trout hepatocytes.** Environ Toxicol Chem 2001;20(3):632-43.
Abstract: In order to investigate cell-specific differences in the response of in vitro models to environmental toxicants, we compared the capacity of nine polycyclic aromatic hydrocarbons (PAHs) to induce cytochrome P4501A (CYPIA) in primary rainbow trout (Oncorhynchus mykiss) hepatocytes and a rainbow trout liver cell line, RTL-W1. Induction of CYPIA was estimated from the catalytic activity of 7-ethoxyresorufin-O-deethylase (EROD) and compared by median effective concentration (EC50) values, induction spans, and benzo[a]pyrene induction equivalency factors for inducing PAHs. The influence of culture conditions was investigated with respect to the presence or absence of serum and varying exposure times. Both in vitro systems lead to an identical classification of the PAHs in noninducing (anthracene, fluoranthene, phenanthrene, and pyrene) and inducing compounds with a similar ranking of inducing PAHs. Mean EC50 values in RTL-W1 cells were, respectively, 343 and 266 nM for benzo[a]anthracene, 57 and 92 nM for BaP, 134 and 283 nM for benzo[b]fluoranthene, 455 and 270 nM for chrysene, and 98 and 116 nM for 3-methylcholanthrene. Compared to primary hepatocytes, the RTL-W1 cell line was more sensitive in its EROD response to the presence or absence of serum and to the increase in exposure time, which led to higher EC50 values.

Cihlar T, Ho ES. **Fluorescence-based assay for the interaction of small molecules with the human renal organic anion transporter 1.** Anal Biochem 2000;283(1):49-55.

Abstract: Secretion of small molecules from the systemic blood circulation into urine is one of the physiologically essential functions of the kidney. The human organic anion transporter (hOAT1) is a key component in the renal tubular secretion of negatively charged molecules including a variety of important therapeutics. In some cases, compounds interacting with hOAT1 may induce pharmacokinetic drug-drug interactions or cause nephrotoxicity. We developed a fluorescence-based, 96-well format assay using CHO cells stably expressing hOAT1, which allows for the evaluation of interactions between small molecules and hOAT1. The assay is based on the inhibition of the transport of 6-carboxyfluorescein, a high-affinity hOAT1 substrate (Km = 3.9 microM), which was identified as one of several fluorescent organic anions. The relative inhibition potency of various known hOAT1 substrates determined using the 6-carboxyfluorescein-based inhibition assay correlated well with their Km values, indicating that the fluorescent assay exhibits a proper specificity. This in vitro assay can be employed to evaluate the mechanism of renal clearance of organic anions, to assess potential drug-drug interactions and/or nephrotoxic effects of various therapeutics, and to screen for novel hOAT1 inhibitors that could serve as efficient nephroprotectants.

Coleman R, Roma MG. **Hepatocyte couplets.** Biochem Soc Trans 2000;28(2):136-40.
Abstract: Hepatocyte couplets, by retaining functional and morphological polarity similar to that of hepatocytes in situ, are a valuable in vitro model to study mechanisms of bile secretion, cholestasis and hepatocellular injury. They have been useful in studies of the hormonal control of bile formation and are suitable for morphological studies. The availability of periportal- and perivenous-enriched couplet populations now allows a zonal perspective. Their contribution to our understanding of regulatory aspects of hepatobiliary dysfunction due to toxicological or cholestatic insult, as well as its reversion by using hepatoprotective agents, is still at an early stage. The next few years should see further exiting contributions to our understanding of hepatobiliary function and dysfunction.

Dilworth C, Hamilton GA, George E, Timbrell JA. **The use of liver spheroids as an in vitro model for studying induction of the stress response as a marker of chemical toxicity.** Toxicol In Vitro 2000;14 (2):169-76.
Abstract: Stress protein induction has been advocated as a sensitive indicator of compound-induced toxicity. In monolayer cultures of primary hepatocytes, however, the two stress proteins, Hsp25 and Hsp72/3 are up-regulated, probably due to the effect of the isolation procedure and adaptation of the cells to the culture conditions. The aim of the current studies was to determine whether liver spheroids would provide an improved experimental model for the study of heat shock protein induction in vitro. Primary rat hepatocytes were cultured as liver spheroids and the expression of Hsp25 and Hsp72/3 measured along with the levels of ATP, GSH and albumin secretion. Hsp72/3 was initially increased in spheroid culture but returned to in vivo levels after 3 days of culture. Hsp25 was maintained at in vivo levels until day 6 of culture, after which levels increased slightly. The effects of the two hepatotoxins, hydrazine and cadmium chloride (CdCl(2)), were therefore measured on day 6 of spheroid culture. CdCl (2) had no effect on Hsp25 but increased Hsp72/3 at concentrations that affected other biochemical parameters. Hydrazine caused a rapid reduction in ATP levels and albumin secretion, but did not affect Hsp72/3. Hsp25 was slightly induced by hydrazine at later sampling times at concentrations, however, that affected other biochemical parameters. It can be concluded that liver spheroids provide a model for studying stress protein expression. However, the increase in stress proteins appears to be a relatively

insensitive parameter compared to other more conventionally used toxicity endpoints and the response appears to vary with individual toxins under study.

Follmann W, Guhe C, Weber S, Birkner S, Mahler S. **Cultured porcine urinary bladder epithelial cells as a screening model for genotoxic effects of aromatic amines: characterisation and application of the cell culture model.** Altern Lab Anim 2000;28(6):833-54.
Abstract: Isolated epithelial cells from porcine urinary bladders were maintained in dividing long-term monolayer cultures, and were used as a model system for the urinary bladder in toxicological studies in vitro. To examine the state of differentiation during the culture period, the culture system was characterised morphologically by light and transmission electron microscopy and by immune fluorescence labelling with antibodies against cytokeratins 7,13 and pan. The cultured cells were identified as urothelial epithelium by their polarised structure, and by their expression of several uroepithelial specific morphological features, such as fusiform vesicles, tight junctions and an asymmetric apical cell membrane. Additionally, the cells were labelled with anti-cytokeratin 7,13 and pan antibodies, and negatively with anti-vimentin antibodies. The maintenance of suitable culture conditions was shown by the stable enzyme activities of (gamma-glutamyltranspeptidase, alkaline phosphatase and acid phosphatase over a culture period of 4 weeks. A good viability of the cultured cells under the chosen culture conditions was shown by the presence of low amounts of lactate dehydrogenase (< of = 5%) in the culture medium. The activities of the chosen marker enzymes for cell differentiation (gamma-glutamyltranspeptidase), lysosomes (acid phosphatase) and luminal membranes (alkaline phosphatase) were relatively stable over the observed culture period. Enzyme activities involved in metabolism of xenobiotics were determined, to define the ability for metabolism in cultured cells compared with bladder tissue in situ. Several constitutive phase I and II enzyme activities were found to be stable during the culture period, indicating that the cultured cells should be able to metabolise xenobiotics in a comparable manner to the urothelium in vivo. The cytotoxic effects of xenobiotics were investigated and IC50 values were determined by means of lactate dehydrogenase leakage and inhibition of neutral red uptake. The induction of sister chromatid exchanges was used as a parameter for the genotoxic effects of several xenobiotics. This cell culture system was found to be a very good screening system for the testing of substances that affect the bladder, especially aromatic amines.

Fujiyama C, Jones A, Fuggle S, Bicknell R, Cranston D, Harris AL. **Human bladder cancer invasion model using rat bladder in vitro and its use to test mechanisms and therapeutic inhibitors of invasion.** Br J Cancer 2001;84(4):558-64.
Abstract: As well as being a passive support, the extracellular matrix also regulates key biological processes such as invasion, differentiation and angiogenesis. We have therefore developed an in vitro model of bladder cancer invasion using de-epithelialized rat bladder to allow for tumour cell-extracellular matrix interactions. Onto this we have seeded a panel of human bladder cancer cell lines (RT4, RT112, 253J and EJ28 (T24)) representing progression from well to poorly differentiated phenotypes and used as models of superficial to invasive bladder cancer. The better differentiated cell lines RT4 and RT112 reproducibly grew as stratified epithelium, whereas poorly differentiated EJ28 cells invaded across a broad front. Invasion was not simply related to proliferation rate, measured either as doubling time on plastic (non-invasive 253J and invasive EJ28 having the same doubling time) or by Ki-67 proliferation index within the model. We used the model to test the ability of 4 compounds that

interfere with tumour cell-extracellular matrix interactions (suramin, N-acetylcysteine and the urokinase plasminogen activator pathway antagonists A5 compound and monoclonal antibody Mab 3936) to inhibit invasion. At non-toxic concentrations, all significantly inhibited invasion ($P< 0.05$), although to varying degrees, suramin and A5 almost completely and N-acetylcysteine the least. In conclusion, this model shows the urokinase system is important for bladder invasion and can be used to investigate other mechanisms of bladder cancer invasion and also for the testing of intravesical drugs. Copyright 2001 Cancer Research Campaign.

Hartung T. **Summary: EU standards measurements and testing project PL95-3407. Development of a standardised in vitro methodology for hepatic and renal toxicity testing.** Altern Lab Anim 2001;29 (4):493-5.

Hoebe KH, Monshouwer M, Witkamp RF, Fink-Gremmels J, van Miert AS. **Cocultures of porcine hepatocytes and Kupffer cells as an improved in vitro model for the study of hepatotoxic compounds**. Vet Q 2000;22(1):21-5.
Abstract: In this study primary hepatocyte cultures (HC cultures) and cocultures comprised of hepatocytes and Kupffer cells (HC/KC cocultures) were compared to investigate the inflammatory response induced by lipopolysaccharide (LPS). In addition both culture types were compared to study the hepatotoxic effects of two frequently used drugs: tiamulin and chlorpromazine. The inflammatory response in both culture types was determined by measurement of tumour necrosis factor-alpha (TNF-alpha), interleukin 6 (IL-6) and nitric oxide (NO). The drug-induced hepatotoxic effects were determined by measuring production of intracellular reactive oxygen species (ROS) and cytotoxicity. Exposure of both cultures to LPS resulted in a significantly increased production of TNF-alpha, IL-6 and NO. However, the production of TNF-alpha, IL-6 and NO was substantially increased in culture supernatant of cocultures, compared to single HC-cultures. Both tiamulin and chlorpromazine were potent inducers of intracellular ROS production at concentrations > or = 50 microM. High ROS production was paralleled by increased cytotoxicity as observed in both culture types. Incubation of cocultures with chlorpromazine resulted in a significant increased ROS production as compared to HC cultures. In contrast, no significant differences between HC-cultures and HC/KC cocultures were observed for tiamulin induced ROS production or cytotoxicity. The present study demonstrates that cocultures between Kupffer cells and hepatocytes provide an excellent model for the study of hepatotoxic compounds which exert (part) of their toxic effects via the activation of Kupffer cells. Furthermore they offer a valuable tool to study increased susceptibility to intoxication from xenobiotic agents in case of a concurrent or pre-existing inflammation.

Hogemann D, Baumann A, Rocker D, Bader A, Galanski M. **In vitro model of the human liver parenchyma to study hepatotoxic side effects by Dy-EOB-DTPA.** Invest Radiol 2000;35(6):373-9.
Abstract: RATIONALE AND OBJECTIVES: In vivo studies have shown species-specific toxicity after application of the liver-specific contrast agent Dy-ethoxybenzyl (EOB)-DTPA. To predict species differences in the laboratory, an in vitro model of the liver was used to examine the divergent results. METHODS: Rat, canine, porcine, and human hepatocytes were isolated and embedded between layers of collagen. During and after 48 hours of incubation with different concentrations of Dy-EOB-DTPA (maximum concentration 50 mmol/L), morphological changes and enzyme leakage were determined.

RESULTS: The response to the contrast agent varied for hepatocytes from different species. For canine cells, morphological changes and cell death were evident with as little as 5 mmol/L Dy-EOB-DTPA. Rat hepatocytes tolerated up to 50 mmol/L Dy-EOB-DTPA, and enzyme leakage was transient. Only after incubation with 50 mmol/L Dy-EOB-DTPA was the formation of intracellular vacuoles evident. In contrast, even the highest concentration of Dy-EOB-DTPA did not cause an enzyme leakage of porcine or human hepatocytes, although similar vacuoles were seen. CONCLUSIONS: These data demonstrate a species-dependent toxicity for Dy-EOB-DTPA in vitro, with similar responses in porcine and human hepatocytes.

Koebe HG, Deglmann CJ, Metzger R, Hoerrlein S, Schildberg FW. **In vitro toxicology in hepatocyte bioreactors-extracellular acidification rate (EAR) in a target cell line indicates hepato-activated transformation of substrates.** Toxicology 2000;154(1-3):31-44.
Abstract: In this article we introduce an in vitro model for hepato-mediated toxicity testing consisting of a Hepatocyte-Bioreactor connected to a microphysiometer system for monitoring of the extracellular acidification rate (EAR) of cells. The EAR in this system represented the metabolic activity of a tested cell line under the influence of bioreactor supernatant. Cyclophosphamide (CYCL), a well-known hepato-activated cytostatic drug was used as a model substrate because of its widespread clinical use. The predrug CYCL needed CYP 450 dependent activation to its active cytotoxic metabolite 4-OH cyclophosphamide. Primary pig hepatocytes from slaughterhouse organs were cultured in a collagen sandwich configuration in specially designed flasks and after 3 days introduced into a 50 ml recirculating perfusion system including 30 microg/ml CYCL. In a parallel open circuit, this bioreactor was connected to three perfusion chambers of a microphysiometer system housing 1.5 x 10(5) ZR 751 cells (breast tumor cell line). Bioreactor supernatant including CYCL was pumped at 150 microl/min into the microphysiometer. The recorded EARs under CYCL influence were correlated to controls, which were set to be 100%. After 1 and 7 h of bioreactor supernatant perfusion, including activated CYCL, the ZR 751 cell line showed an EAR of 98.99%+/-3.15 (mean+/-SD) and 81. 32%+/-10.18 ($P<0.05$), respectively, as compared to controls (bioreactor supernatant from the identical set-up without CYCL). The inactivated predrug CYCL showed no effect on the EAR: Perfusion of medium with 30 microg/ml CYCL alone, excluding the bioreactor activation, resulted in an EAR of 100. 11%+/-4.74 (mean+/-SD) after 7 h. Thus the presented model of hepato-activated toxicity showed an EAR decrease in the ZR 751 cell line that reflected the toxic activation of the predrug by the bioreactor.

Nebert DW, Dalton TP, Stuart GW, Carvan MJ 3rd. **"Gene-swap knock-in" cassette in mice to study allelic differences in human genes.** Ann N Y Acad Sci 2000;919:148-70.
Abstract: Genetic differences in environmental toxicity and cancer susceptibility among individuals in a human population often reflect polymorphisms in the genes encoding drug-metabolizing enzymes (DMEs), drug transporters, and receptors that control DME levels. This field of study is called "ecogenetics", and a subset of this field--concerning genetic variability in response to drugs--is termed "pharmacogenetics". Although human-mouse differences might be 3- to perhaps 10-fold, human interindividual differences can be as great as 20-fold or more than 40-fold. It would be helpful, therefore, to study toxicokinetics/pharmacokinetics of particular environmental agents and drugs in mice containing these "high-" and "low-extreme" human alleles. We hope to use transgenic "knock-in" technology in order to insert human alleles in place of the orthologous mouse gene. However, the knock-

in of each gene has normally been a separate event requiring the following: (a) construction of the targeting vector, (b) transfection into embryonic stem (ES) cells, (c) generation of a targeted mouse having germline transmission of the construct, and (d) backcross breeding of the knock-in mouse (at least 6-8 times) to produce a suitable genetically homogeneous background (i.e., to decrease "experimental noise"). These experiments require 1 1/2 to 2 years to complete, making this very powerful technology inefficient for routine applications. If, on the other hand, the initial knock-in targeting vector might include sequences that would allow the knocked-in gene to be exchanged (quickly and repeatedly) for one new allele after another, then testing distinctly different human polymorphic alleles in transgenic mice could be accomplished in a few months instead of several years. This "gene-swapping" technique will soon be done by zygotic injection of a "human allele cassette" into the sperm or fertilized ovum of the parental knock-in mouse inbred strain or by the cloning of whole mice from cumulus ovaricus cells or tail-snip fibroblasts containing the nucleus wherein each new human allele has already been "swapped." In mouse cells in culture using heterotypic lox sites, we and others have already succeeded in gene swapping, by exchanging one gene, including its regulatory regions, with a second gene (including its regulatory regions). It is anticipated that mouse lines carrying numerous human alleles will become commonplace early in the next millennium.

Rodriguez H, Bustos-Obregon E. **An in vitro model to evaluate the effect of an organophosphoric agropesticide on cell proliferation in mouse seminiferous tubules.** Andrologia 2000;32(1):1-5.
Abstract: Recently, there has been public concern about the toxic effects of organophosphoric pesticides (OP) upon human and animal populations. Since the seminiferous epithelium is an actively proliferating tissue, it was of interest to study germ cell proliferation in the isolated seminiferous tubules of mice that were cultured in the presence of Parathion or paraoxon, its metabolite. Eighteen 3-month-old CF-1 male mice were used. Paraoxon (PO) and Parathion (PT) were added to cultures of seminiferous tubules in the following groups: (1) Ham F-10 medium pH 7.4 (M) (control); groups 2 to 5, M + decreasing doses of either PO or PT (0.8; 0.4; 0.04; 0.004; 0.0004 mM). Each group consisted of six mice. Incubation of the tubules was carried out for 5 h at 35 degrees C, 5% CO2, 95% air. One hour before the end of incubation 5 microCi of 3H-thymidine was added to the cultures. DNA uptake was measured by scintillation counting. PO and PT at concentrations of 0.8 mM elicited a sharp decrease in testicular DNA synthesis. Recuperation at concentrations under 0.4 mM was different. With PO it was rapid, possibly due to the high detoxificative ability of the testis, which contains high quantities of the enzyme 'type A esterases' that hydrolyses PO. At concentrations lower than PO, PT has an inhibitory effect upon germ cell proliferation, which deserves further research.

Tian W, Zhang Z, Cohen DM. **MAPK signaling and the kidney.** Am J Physiol Renal Physiol 2000;279(4):F593-604.
Abstract: Following an overview of the biochemistry of mitogen-activated protein kinase (MAPK) pathways, the relevance of these signaling events to specific models of renal cell function and pathophysiology, both in vitro and in vivo, will be emphasized. In in vitro model systems, events activating the principal MAPK families [extracellular signal-regulated and c-Jun NH(2)-terminal kinase and p38] have been best characterized in mesangial and tubular epithelial cell culture systems and include peptide mitogens, cytokines, lipid mediators, and physical stressors. Several in vivo models of proliferative or toxic renal injury are also associated with aberrant MAPK regulation. It is anticipated

that elucidation of downstream effector signaling mechanisms and a clearer understanding of the immediate and remote upstream activating pathways, when applied to these highly clinically relevant model systems, will ultimately provide much greater insight into the basis for specificity now seemingly absent from these signaling events.

Yang JW, Shin JS, Lee JJ, Chang HI, Kim CW. **In vitro model using mouse hepatocytes for study of alcohol stress**. Biosci Biotechnol Biochem 2001;65(7):1528-33.
Abstract: In this study, the effects of ethanol and allyl alcohol on primary mouse hepatocytes were investigated. No cytotoxicity was observed by ethanol treatments, but more toxicity to cells was found in the response to allyl alcohol treatment. The expression of cytochrome P450 2E1 (CYP2E1), phase I enzyme was examined in response to ethanol and allyl alcohol. Both xenobiotics induced CYP2E1 up to 1.5 to approximately 5 fold at the protein level. The effects of insulin on CYP2E1 expression were also measured. Insulin, which has been regarded as an essential hormone for primary hepatocytes, was shown to decrease the level of CYP2E1 protein, and did not affect cell viability. These results on CYP2E1 induction demonstrate that primary mouse hepatocytes, when using ethanol and allyl alcohol as substrates and in insulin-free medium, provide a suitable system for the studies of the role of CYP2E1 in xenobiotic metabolism and toxicity.

Binda D, Nicod L, Viollon-Abadie C, Rodriguez S, Berthelot A, Coassolo P, Richert L. **Strain difference (WKY, SPRD) in the hepatic antioxidant status in rat and effect of hypertension (SHR, DOCA). Ex vivo and in vitro data.** Mol Cell Biochem 2001;218(1-2):139-46.
Abstract: We assessed the hepatic antioxidant status of spontaneously (SHR) and desoxicorticosterone acetate (DOCA)-induced hypertensive rats and that of respective normotensive Wistar Kyoto (WKY) and Sprague-Dawley (SPRD) rats. For this we evaluated, ex vivo in liver cytosols, reduced glutathione (GSH) content, glutathione-related enzyme (peroxidase, reductase and transferase) activities as well as the rate of lipid peroxidation in 9-11 week-old rats. The antioxidant status and the cytotoxicity of acetaminophen, a radical- and hydrogen peroxide-mediated hepatotoxic compound, were also assessed in vitro in cultured hepatocytes isolated from hypertensive (SHR, DOCA) and normotensive control (WKY, SPRD) rats. Our results suggest that a difference exists in the hepatic antioxidant status between rat strains, with GSH levels being lower (-15%) and lipid peroxidation rate higher (+30%) in WKY compared to SPRD rats. In hepatocyte cultures from WKY rats, both GSH content and catalase activity were lower (-30 and -70% respectively) compared to hepatocyte cultures from SPRD rats. This was associated with a 35% higher cytotoxicity of acetaminophen in cultured hepatocytes from WKY rats compared to that in hepatocytes from SPRD rats. Hypertension in DOCA rats (mmHg: 221+/-9 vs. 138 +/-5 in control SPRD rats) was associated with decreases (about 30%) in both glutathione peroxidase (GSH-Px) and catalase activities, ex vivo in livers and in vitro in hepatocyte cultures. Hypertension in SHR (mmHg: 189+/-7 vs. 130+/-5 in control WKY rats) was also associated with decreases (about 50%) in GSH-Px activity, ex vivo in livers and in vitro in hepatocyte cultures but catalase activity was not modified. The IC50 of acetaminophen was also lower in hepatocytes from hypertensive rats compared to respective controls, which could be related to the weakened antioxidant status in hepatocytes from hypertensive rats. Our data thus suggest that hepatocyte cultures are appropriated tools in which to assess hepatotoxicity and hepatoprotection in hypertension.

Ganey PE, Roth RA. **Concurrent inflammation as a determinant of susceptibility to toxicity from xenobiotic agents.** Toxicology 2001;169(3):195-208.
Abstract: Sensitivity to the toxic effects of xenobiotic agents is influenced by a number of factors. Recent evidence derived from studies using experimental animals suggests that inflammation is one of these factors. For example, induction of inflammation by coexposure to bacterial endotoxin, vitamin A or Corynebacterium parvum increases injury in response to a number of xenobiotic agents that target liver. These agents are diverse in chemical nature and in mechanism of hepatotoxic action. Factors critical to the augmentation of liver injury by inflammation include Kupffer cells, neutrophils, cytokines such as tumor necrosis factor-alpha (TNF-alpha) and lipid mediators such as prostaglandins, but these may vary depending on the xenobiotic agent and the mechanisms by which it alters hepatocellular homeostasis. In addition, the timing of inflammagen exposure can qualitatively alter the toxic response to chemicals. Inflammation-induced increases in susceptibility to toxicity are not limited to liver. Concurrent inflammation also sensitizes animals to the toxic effects of agents that damage the respiratory tract, kidney and lymphoid tissue. It is concluded that inflammation should be considered as a determinant of susceptibility to intoxication by xenobiotic exposure.

Gomez-Lechon MJ, Ponsoda X, Bort R, Castell JV. **The use of cultured hepatocytes to investigate the metabolism of drugs and mechanisms of drug hepatotoxicity.** Altern Lab Anim 2001;29(3):225-31.
Abstract: Hepatotoxins can be classified as intrinsic when they exert their effects on all individuals in a dose-dependent manner, and as idiosyncratic when their effects are the consequence of an abnormal metabolism of the drug by susceptible individuals (metabolic idiosyncrasy) or of an immune-mediated injury to hepatocytes (allergic hepatitis). Some xenobiotics are electrophilic, and others are biotransformed by the liver into highly reactive metabolites that are usually more toxic than the parent compound. This activation process is the key to many hepatotoxic phenomena. Mitochondria are a frequent target of hepatotoxic drugs, and the alteration of their function has immediate effects on the energy balance of cells (depletion of ATP). Lipid peroxidation, oxidative stress, alteration of Ca^{2+} homeostasis, and covalent binding to cell macromolecules are the molecular mechanisms that are frequently involved in the toxicity of xenobiotics. Against these potential hazards, cells have their own defence mechanisms (for example, glutathione, DNA repair, suicide inactivation). Ultimately, toxicity is the balance between bioactivation and detoxification, which determines whether a reactive metabolite elicits a toxic effect. The ultimate goal of in vitro experiments is to generate the type of scientific information needed to identify compounds that are potentially toxic to man. For this purpose, both the design of the experiments and the interpretation of the results are critical.].

Rodriguez-Barbero A, L'Azou B, Cambar J, Lopez-Novoa JM. **Potential use of isolated glomeruli and cultured mesangial cells as in vitro models to assess nephrotoxicity.** Cell Biol Toxicol 2000;16 (3):145-53.
Abstract: The purpose of this short review is to present the potential of using isolated glomeruli and cultured mesangial cells as two different in vitro models to assess the glomerular effect of molecules with nephrotoxic properties. The advantage of using isolated renal glomeruli is that they conserve the architecture of this anatomical region of the kidney; moreover, they are free of any vascular, nervous or humoral influences derived from other regions of the kidney. Mesangial cells are perivascular pericytes located within the central portion of the glomerular tuft between capillary loops. Mesangial cells have a

variety of functions including synthesis and assembly of the mesangial matrix, endocytosis and processing of plasma macromolecules, and control of glomerular hemodynamics, mainly the ultrafiltration coefficient Kf, via mesangial cell contraction or release of vasoactive hormones. Most authors agree that mesangial cells play a major role in glomerular contraction, filtration surface area, and Kf regulation. One of the major effects of toxicants on glomerular structures is contraction. We can assess quantitatively the degree of toxicant-induced mesangial cell contraction or glomerular contraction by measuring the changes in planar cell surface area or apparent glomerular cross-sectional area after exposition to the toxicant. These in vitro models can also reveal glomerular effects of xenobiotics that are difficult or impossible to observe in vivo. In addition, these studies permit a fundamental examination of the mechanism of action of xenobiotics on glomerular cells, including the possibility that at least a part of their effects are mediated by local mediators released by glomerular cells. We review the effects and the mechanisms of action of several toxicants such as gentamicin, cyclosporin, cisplatin, and cadmium on isolated glomeruli or cultured mesangial cells. As such in vitro results confirm in vivo renal hemodynamic changes caused by toxicants, we conclude that these models are fruitful tools for the study of renal toxicity. These in vitro systems might also serve as a predictive tool in the evaluation of drugs inducing changes in glomerular filtration rate and as a way to propose protective agents against these dramatic hemodynamic effects.

IMMUNOTOXICITY

Akira S, Hoshino K, Kaisho T. **The role of Toll-like receptors and MyD88 in innate immune responses.** J Endotoxin Res 2000;6(5):383-7.
Abstract: Toll-like receptors (TLRs) are phylogenetically conserved receptors that recognize pathogen associated molecular patterns (PAMPS). We previously generated mice lacking TLR2 and TLR4 and showed the differential role of TLR2 and TLR4 in microbial recognition. TLR4 functions as the transmembrane component of the lipopolysaccharide (LPS) receptor, while TLR2 recognizes peptidoglycan from Gram-positive bacteria and lipoprotein. We also generated mice lacking MyD88, an adaptor involved in IL-1R/TLR signalings. The responses to a variety of bacterial components were completely abrogated in MyD88-deficient cells. However, unlike the signaling mediated by other bacterial components such as lipoprotein and bacterial DNA, activation of NF-kappaB and MAP kinases was induced in response to LPS even in the absence of MyD88, which indicates the existence of a MyD88-independent pathway. We have recently found that the MyD88-independent pathway is involved in LPS-induced maturation of dendritic cells (DCs).

Ban M, Hettich D. **Relationship between IgE positive cell numbers and serum total IgE levels in mice treated with trimellitic anhydride and dinitrochlorobenzene**. Toxicol Lett 2001;118(3):129-37.
Abstract: Although increased total serum IgE levels have been suggested as being predictive parameters of airway hypersensitivity caused by low molecular weight chemicals, it is not yet clear what level of serum total IgE in chemically-treated animals would translate to potential risk of inducing an immediate type hypersensitivity in human beings. Quantitative determination of IgE-bearing positive cells induced by chemicals in the tissue, particularly in respiratory airway, could help to resolve this problem. In BALB/c mice, serum total IgE concentrations and tissue IgE-bearing positive cell numbers were investigated following topical exposure to the chemicals, trimellitic anhydride (TMA) and

dinitrochlorobenzene (DNCB), known in human as a respiratory and dermal sensitizer, respectively. In groups of mice 8 and 10 weeks of age, there were less individual variations in 25% TMA-induced serum total IgE increase than in other groups of mice 6, 12 and 16 weeks of age. When chemical concentrations of 1, 2 and 3% DNCB and of 6.25, 12.5 and 25% TMA were studied, we observed that the increase in IgE was dose-dependent for both chemicals, r=0.96; P=0.03 for DNCB and r=0.99; P=0.002 for TMA. However, the increase in serum total IgE induced by TMA was at least twice that induced by DNCB and was associated with the numbers of IgE bearing cells in the tracheal lamina propria (r=0.67, P=0.0003). A respiratory hypersensitivity caused by TMA, but not by DNCB, may be partly due to the presence of immuno effector cells bearing IgE at their surface in airway microenvironments, and their presence might be related to the higher level of serum total IgE. The IgE bearing positive cells could, therefore, help to identify chemicals which have the potential risk of inducing an immediate type hypersensitivity in humans.

Birner P, Ritzi M, Musahl C, Knippers R, Gerdes J, Voigtlander T, Budka H, Hainfellner JA. **Immunohistochemical detection of cell growth fraction in formalin-fixed and paraffin-embedded murine tissue.** Am J Pathol 2001;158(6):1991-6.
Abstract: Monoclonal antibody MIB-1 is a reliable tool for determining proliferating cells in human tissues, but does not react with the homologous mouse antigen and is therefore useless in experimental pathology using mice as model systems. Standard method for assessment of cellular proliferation in formalin-fixed, paraffin-embedded murine tissues is immunohistochemical detection of DNA synthesis using antibodies against exogenously injected 5-bromodeoxyuridine (BrdU), which is a tedious procedure and not useful for routine investigations. We tested monoclonal antibody MIB-5 and monoclonal and polyclonal anti-MCM3 antibodies as immunohistochemical proliferation markers for paraffin-embedded nonneoplastic and neoplastic tissues of wild-type and transgenic mice, compared to anti-BrdU immunostaining. Percentage of proliferating cells was determined with continuously decreasing antibody dilutions. Percentages of MIB-5 and anti-BrdU immunostained cells correlated strongly, as well as percentage of MIB-5-decorated cells and frequency of mitotic figures. Anti-MCM3 antibodies labeled significantly higher percentages of cells than anti-BrdU or MIB-5, and showed a linear decrease with increasing antibody dilutions. We conclude that MIB-5 detects reliably the cell growth fraction in formalin fixed, paraffin-embedded murine tissues, bypassing methodological drawbacks of BrdU. Anti-MCM3 antibodies are less useful for determination of proliferating cells although they might detect the fraction of cells remaining competent for proliferation.

Bishop GR, Jaso-Friedmann L, Evans DL. **Activation-induced programmed cell death of nonspecific cytotoxic cells and inhibition by apoptosis regulatory factors.** Cell Immunol 2000;199(2):126-37.
Abstract: Nonspecific cytotoxic cells (NCC) are the teleost equivalent of mammalian lymphokine-activated natural killer cells. The cytotoxic activities of NCC are enhanced by stress-activated serum factors (SASF) present in tilapia acute-phase serum. In the present study purified NCC and xenogeneic target HL-60 tumor cells and nuclei were distinguishable in mixtures determined by flow cytometry. NCC activated by target HL-60 cells undergo activation-induced programmed cell death (AIPCD) during 12- to 16-h killing assays as shown by Annexin-V binding and nuclear DNA fragmentation results. Annexin-V binding studies also demonstrated that NCC kill HL-60 cells by an apoptotic mechanism. NCC are protected from AIPCD by 4-h preincubation in 50% SASF. Pretreatment also

produced more than a fourfold increase in NCC cytotoxicity (effector/target (E:T) ratio = 100:1). In the absence of SASF preincubation, the percentage of apoptotic NCC increased from 8 to 91% at E:T ratios of 1:0 and 1:1, respectively. Kinetic studies (E:T = 10:1) demonstrated that the percentage of NCC exhibiting HL-60-dependent AIPCD increased between 0.1 and 12 h and then decreased inversely with total cell necrosis over the next 60 h. Preincubation of NCC with SASF protected NCC from AIPCD for over 72 h. Crosslinkage of the NCCRP-1 receptor with monoclonal antibody (mab) 5C6 produced AIPCD between 1 and 100 microg/mL mab concentrations. Preincubation with SASF completely protected NCC from mab 5C6-dependent AIPCD. SASF-mediated protection of NCC from AIPCD was dependent upon divalent cations, as demonstrated by increases in DNA hypoploidy of 38, 67, and 88% following preincubation in the presence of 10, 100, and 1000 &mgr;M EDTA, respectively. SASF also protected NCC from glucocorticoid- (i. e., dexamethasone) induced apoptosis. Combined, these results demonstrated that NCC activity is down-regulated by AIPCD. Release of SASF into the peripheral circulation may prevent negative regulation of NCC by AIPCD by increasing recycling capacity. Results are discussed in the context of the effects of acute stressors on innate immunity.

Boder ET, Midelfort KS, Wittrup KD. **Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity.** Proc Natl Acad Sci U S A 2000;97(20):10701-5.
Abstract: Single-chain antibody mutants have been evolved in vitro with antigen-binding equilibrium dissociation constant K(d) = 48 fM and slower dissociation kinetics (half-time > 5 days) than those for the streptavidin-biotin complex. These mutants possess the highest monovalent ligand-binding affinity yet reported for an engineered protein by over two orders of magnitude. Optimal kinetic screening of randomly mutagenized libraries of 10(5)-10(7) yeast surface-displayed antibodies enabled a >1,000-fold decrease in the rate of dissociation after four cycles of affinity mutagenesis and screening. The consensus mutations are generally nonconservative by comparison with naturally occurring mouse Fv sequences and with residues that do not contact the fluorescein antigen in the wild-type complex. The existence of these mutants demonstrates that the antibody Fv architecture is not intrinsically responsible for an antigen-binding affinity ceiling during in vivo affinity maturation.

Borenstein SH, Graham J, Zhang XL, Chamberlain JW. **CD8+ T cells are necessary for recognition of allelic, but not locus-mismatched or xeno-, HLA class I transplantation antigens.** J Immunol 2000;165(5):2341-53.
Abstract: Although HLA transgenic mice (HLA TgM) could provide a powerful approach to investigate human MHC-specific T cell responsiveness, the extent to which these molecules are recognized by the mouse immune system remains unclear. We established TgM expressing HLA class I alleles A2, B7, or B27 in their fully native form (HLAnat) or as hybrid molecules (HLAhyb) of the HLA alpha1/alpha2 domains linked to the H-2Kb alpha3, transmembrane, and cytoplasmic domains (i.e., to maintain possible species-specific interactions). Comparison of each as xeno- (i.e., by non-TgM) vs allo- (i.e., by TgM carrying an alternate HLA allele) transplantation Ags revealed the following: 1) Although HLAhyb molecules induced stronger xeno-CD8+ T cell responses in vitro, additional effector mechanisms must be active in vivo because HLAnat skin grafts were rejected faster by non-TgM; 2) gene knockout recipients showed that xenorejection of HLAnat and, unexpectedly, HLAhyb grafts doesn't depend on CD8+ or CD4+ T cells or B cells; 3) each HLAhyb strain developed tolerance to "self" but rejected allele- (-B27 vs -B7) and locus- (-B vs -A) mismatched grafts, the former requiring CD8+ T cells, the

latter by CD8+ T cell-independent mechanisms. The finding that recognition of xeno-HLAhyb does not require CD8+ T cells while recognition of the identical molecule in a strictly allo context does, demonstrates an alpha1/alpha2 domain-dependent difference in effector mechanism(s). Furthermore, the CD8+ T cell-independence of locus-mismatched rejection suggests the degree of similarity between self and non-self alpha1/alpha2 determines the effector mechanism(s) activated. The HLA Tg model provides a unique approach to characterize these mechanisms and develop tolerance protocols in the context of human transplantation Ags.

Costagliola S, Many MC, Denef JF, Pohlenz J, Refetoff S, Vassart G. **Genetic immunization of outbred mice with thyrotropin receptor cDNA provides a model of Graves' disease.** J Clin Invest 2000;105(6):803-11.
Abstract: We performed genetic immunization of outbred NMRI mice, using a cDNA encoding the human thyrotropin receptor (TSHr). All mice produced antibodies capable of recognizing the recombinant receptor expressed at the surface of stably transfected Chinese hamster ovary (CHO) cells, and sera from most of the immunized mice blocked TSH-dependent stimulation of cAMP accumulation in cells expressing the TSHr. Five out of 29 female mice showed sign of hyperthyroidism including elevated total T4 and suppressed TSH levels. The serum of these mice contained thyroid-stimulating activity, as measured in a classic assay using CHO cells expressing recombinant TSHr. In contrast, only 1 male out of 30 had moderately elevated serum total T4 with undetectable TSH values. The hyperthyroid animals had goiters with extensive lymphocytic infiltration, characteristic of a Th2 immune response. In addition, these animals displayed ocular signs reminiscent of Graves' ophthalmopathy, including edema, deposit of amorphous material, and cellular infiltration of their extraocular muscles. Our results demonstrate that genetic immunization of outbred NMRI mice with the human TSHr provides the most convincing murine model of Graves' disease available to date.

Hammond-McKibben D, Lake P, Zhang J, Tart-Risher N, Hugo R, Weetall M. **A high-capacity quantitative mouse model of drug-mediated immunosuppression based on rejection of an allogeneic subcutaneous tumor.** J Pharmacol Exp Ther 2001;297(3):1144-51.
Abstract: We describe a high-capacity in vivo assay to measure drug-mediated transplant immunosuppression using a mouse model of Sa1 tumor rejection. Sa1 grew poorly and was rejected by 14 days in immunocompetent allogeneic recipient mice. In nude (nu/nu) mice, Sa1 grew more rapidly and was not rejected, confirming the T cell dependence of this response. In immunocompetent animals, administration of immunosuppressive agents resulted in increased tumor growth relative to vehicle-treated animals. Treatment with immunosuppressive drugs such as cyclosporin A (CsA), 40-O-(2-hydroxyethyl)-rapamycin (SDZ RAD), or 2-amino-2-[2-(4-octylphenyl)ethyl]-1,2-propanediol hydrochloride (FTY720) produced dose-dependent inhibition of tumor rejection. By contrast, the drugs did not affect Sa1 tumor growth in nu/nu mice, which is consistent with their predicted indirect effect on tumor size by suppressing immunity, rather than by directly stimulating Sa1 growth. Drug potency, which is usually not described for immunosuppressive agents, was calculated from the linear relationship between drug dose and tumor volume. The potency of CsA was inversely related to the stringency of the histocompatibility barrier. Another advantage of this assay is that the endpoint is an objective size measurement over a short time period, compared with transplant models where the endpoint may not be reached for many weeks and may be more subjective. In addition, this model can

measure the potency of combination drug treatments and compare new immunosuppressive drug regimens. For example, the administration of SDZ RAD or FTY720 with CsA resulted in a more than additive increase in potency, compared with the sum of the drugs as single agents.

Herouet C, Cottin M, LeClaire J, Enk A, Rousset F. **Contact sensitizers specifically increase MHC class II expression on murine immature dendritic cells**. In Vitr Mol Toxicol 2000;13(2):113-23.
Abstract: Contact sensitivity is a T-cell-mediated immune disease that can occur when low-molecular-weight chemicals penetrate the skin. In vivo topical application of chemical sensitizers results in morphological modification of Langerhans cells (LC). Moreover, within 18 h, LC increase their major histocompatibility complex (MHC) class II antigens expression and migrate to lymph nodes where they present the sensitizer to T lymphocytes. We wanted to determine if such an effect could also be observed in vitro. However, because of the high genetic diversity encountered in humans, assays were performed with dendritic cells (DC) obtained from a Balb/c mouse strain. The capacity of a strong sensitizer, DNBS (2,4-dinitrobenzene sulfonic acid), to modulate the phenotype of bone marrow-derived DC in vitro, was investigated. A specific and marked increase of MHC class II molecules expression was observed within 18 h. To eliminate the use of animals in sensitization studies, the XS52 DC line was tested at an immature stage. A 30-min contact with the strong sensitizers DNBS and oxazolone, or the moderate mercaptobenzothiazole, resulted in upregulation of MHC class II molecules expression, analyzed after 18-h incubation. This effect was not observed with irritants (dimethyl sulfoxide and sodium lauryl sulfate) nor with a neutral molecule (sodium chloride). These data suggested the possibility of developing an in vitro model for the identification of the sensitizing potential of chemicals, using a constant and non animal-consuming material.

Langezaal I, Coecke S, Hartung T. **Whole blood cytokine response as a measure of immunotoxicity.** Toxicol In Vitro 2001;15(4-5):313-8.
Abstract: Immunotoxicity, although increasingly recognized as a potential hazard, still lacks standardized in vivo and in vitro models. The considerable species differences and species-specific effects in immune responses prompt the development of human in vitro test systems. Immunotoxic reactions comprise activation (inflammatory processes, autoaggressive processes, pyrogenicity), sensitization (priming, idiosyncratic reactions) and impairment of immune responses (anergy, immunosuppression). We have previously studied a human whole blood system which allows the study of the release of inflammatory cytokines in response to a variety of stimuli. This model allows the assessment of this basic immune mechanism without preparation artefacts and relatively small interindividual variances. We have used this model previously to assess pyrogens, namely type (1) immunotoxic reactions. The model also proved to be suitable for immunopharmacological studies in vitro as well as ex vivo. Here, we studied the suitability of the test system to study type (3) immunotoxic effects. In order to also allow ex vivo studies, we have transferred the system to murine blood. This report summarizes our own use of this model with special emphasis on immunotoxicological studies. Our own listed bibliography gives access to the variety of applications of the human whole blood model since its introduction in 1982.

Marano F. **[Physiopathology of particle attack: animal and in vitro models]**. Allerg Immunol (Paris) 2001;33(2);64-5 [Fre]. Moser R, Quesniaux V, Ryffel B. **Use of transgenic animals to investigate**

drug hypersensitivity. Toxicology 2001;158(1-2):75-83.
Abstract: Hypersensitivity reactions to drugs and environmental agents are often due to exaggerated humoral (Th(2)) or cell mediated (Th(1)) immune responses with typical cytokine profiles. Overexpression of Th(2) cytokines, such as IL-4, IL-5 or IL-13 in mice, enhances an IgE antibody mediated response, while deletion of these cytokines attenuates and/or prevents allergic responses. Conversely, modulation of Th(1) cytokine gene expression may affect cell-mediated immune responses. Therefore, cytokine transgenic mice are used as investigative tools to study potential chemicals and/or drug allergies. In addition to cytokines and chemokines, other factors are important for the development of allergic responses, such as IgE, Fc receptors, vasopressin and several other factors, which can be tested in transgenic mice.

Pichler WJ. **Predictive drug allergy testing: an alternative viewpoint.** Toxicology 2001;158(1-2):31-41.
Abstract: T- and B-cells recognise drugs when bound as haptens to carrier molecules. Recent studies suggest that drugs might also bind in a non-covalent form to MHC-peptide complexes and T cell receptors, and are thereby able to stimulate T cells. This has, however, only been shown for drug-specific T cell clones. Functional analysis revealed that drug-reactive T cells secrete high amounts of IL-5 and are cytotoxic. Cytotoxicity is mediated by drug-specific CD4(+) and CD8(+) cells and, as revealed by the immunohistochemical analysis of drug-induced exanthems, might be involved in the killing of keratinocytes thus explaining the drug-induced exanthem. Further work is needed to clarify the type and exact location of the rather labile drug binding to MHC and T cell receptors, and to evaluate what drug allergies might be caused by such an unusual presentation and immune stimulation. This new model as well as findings from the analysis of clinical drug allergies may have major implications on how to test and predict the allergenic potential of drugs. A change and expansion of currently performed test procedures is required to predict the allergenic potential of drugs.

Rininsland FH, Helms T, Asaad RJ, Boehm BO, Tary-Lehmann M. **Granzyme B ELISPOT assay for ex vivo measurements of T cell immunity.** J Immunol Methods 2000;240(1-2):143-55.
Abstract: A major goal in immunodiagnostics has been the development of assay systems that can measure CD8(+) T cell immunity in humans, directly ex vivo, at high resolution, and with high throughput. We established granzyme B (grB) enzyme-linked immunospot assay (ELISPOT) in conjunction with image analysis to this end. Using grB transfected and untransfected Chinese hamster ovary (CHO) cells and T cell lines, we show that the antibody pair utilized was grB-specific and that only activated T cells secrete grB. GrB release began within 4 h after antigen stimulation and stopped within 40 h. Side-by-side comparison showed grB ELISPOT assays to have a higher resolution than classic chromium-release assays in terms of signal-to-noise ratio. The linearity of the relation of the number of CD8(+) effector T cells plated to grB spots detected suggests that grB ELISPOT assays measure the frequencies of grB-secreting cells directly. Reactivity to HIV peptides was seen in grB ELISPOT assays of freshly isolated PBMC from HIV patients, consistent with the detection of peptide-specific memory cells. The higher resolution and lower labor and material investment should make grB ELISPOT assays an attractive alternative to chromium-release assays in monitoring the clonal sizes of specific CD8 memory cells in vivo.

Rodriguez-Burford C, Barnes MN, Berry W, Partridge EE, Grizzle WE. **Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention.** Gynecol Oncol 2001;81(3):373-9.
Abstract: OBJECTIVE: A significant obstacle confronting the evaluation of potential chemopreventive compounds in ovarian carcinoma is the absence of an animal model of spontaneous ovarian carcinogenesis. A potential model of adenocarcinoma has been described in the laying hen (Gallus domesticus). The purpose of this study was to evaluate the immunohistochemical expression of available antibodies that have been utilized in chemoprevention studies in this potential model of epithelial carcinoma. METHODS: Two hundred 2-year-old hens were sacrificed at Auburn University in accordance with IUACUC guidelines. Of these hens, 8 animals were thought grossly to have ovarian carcinoma and ascites. The tumors from these 8 hens were fixed in neutral-buffered formalin and processed to paraffin blocks. Hematoxylin and eosin stains were used to document the histologic presence of adenocarcinoma. Immunohistochemical evaluation for expression of antigen was performed using the following antibodies: CA125, CEA, cytokeratin, EGFR, erbB-2, Ki-67, Lewis Y, p27, PCNA, Tag 72, TGF-alpha, Muc 1, and Muc 2. RESULTS: Upon microscopic examination by a pathologist eight specimens were documented as adenocarcinomas. Several antibodies to antigens that are frequently expressed in human ovarian cancer were cross-reactive in the laying hen. Of these, cytokeratin AE1/AE3, pan cytokeratin, EGFR, Lewis Y, CEA, Tag 72, and erbB-2 stained the chicken carcinomas. EGFR and p185erbB-2 stained diffusely, and cytokeratin AE1/AE3, pan cytokeratin, Lewis Y, CEA, and Tag 72 were focally positive in the tumor. The aforementioned antibodies which have been useful as surrogate endpoints in chemoprevention trials and which also stained the chicken carcinomas included PCNA, p27, and TGF-alpha Antibodies that were not cross-reactive include CA 125, Ki-67, Muc 1, and Muc 2. CONCLUSION: The data presented in this pilot study support the potential utility of an avian model of spontaneously arising adenocarcinoma in which to study chemopreventive agents. More importantly, the influence of chemoprevention protocols on the expression of relevant antigens can be determined using available antibodies that are cross-reactive in this model. Thus, changes in the phenotypic expression of surrogate endpoint biomarkers as identified by cross-reactive antibodies can aid in the development of chemoprevention trials for human ovarian cancer. Copyright 2001 Academic Press.

Steiling W, Basketter D, Berthold K, Butler M, Garrigue JL, Kimber I, Lea L, Newsome C, Roggeband R, Stropp G, et al. **Skin sensitisation testing--new perspectives and recommendations.** Food Chem Toxicol 2001;39(4):293-301.
Abstract: Various methodological aspects of skin sensitisation testing have been explored, particularly in the context of animal welfare considerations and reliability and sensitivity of test methods. Recommendations are made for the conduct of current and proposed OECD skin sensitisation tests with respect to appropriate test configurations for the purposes of hazard identification and labelling, and the requirement for positive controls. Specifically, the following aspects of guinea pig sensitisation test methods have been addressed: (1) the number of test and control animals required; (2) the option of using joint positive controls between independent laboratories; (3) the choice of positive control chemicals; (4) the optimal conduct and interpretation of rechallenge; and (5) the requirement for pretreatment with sodium lauryl sulfate. In addition, the use of the murine local lymph node assay (LLNA) has been considered. A number of conclusions have been drawn and recommendations made as

follows: In many instances, particularly with the conduct of the guinea pig maximisation test, it is acceptable to halve the number of test and control animals used. An optional scheme for the conduct of joint positive control studies within a co-ordinated group of laboratories is appropriate. Only one positive control chemical (alpha-hexyl cinnamic aldehyde) is necessary for the routine assessment of assay sensitivity. The proper conduct and interpretation of rechallenge can provide valuable information and confirmation of results in guinea pig sensitisation tests. Sodium lauryl sulfate should no longer be used as a pretreatment in the guinea pig maximisation test. The LLNA is a viable and complete alternative to traditional guinea pig test methods for the purposes of skin sensitisation hazard identification. These recommendations provide the opportunity for both animal welfare benefits and improved hazard identification.

Ulrich P, Streich J, Suter W. **Intralaboratory validation of alternative endpoints in the murine local lymph node assay for the identification of contact allergic potential: primary ear skin irritation and ear-draining lymph node hyperplasia induced by topical chemicals.** Arch Toxicol 2001;74 (12):733-44.

Abstract: We validated a two-tiered murine local lymph node assay (LLNA) with a panel of standard contact (photo)allergens and (photo)irritants with the aim of improving the discrimination between contact (photo) allergenic potential and true skin (photo)irritation potential. We determined ear weights to correlate chemical-induced skin irritation with the ear-draining lymph node (LN) activation potential. During tier I LLNAs, a wide range of concentrations were applied on three consecutive days to the dorsum of both ears. Mice were exposed to UVA light immediately after topical application to determine the photoreactive potential of some test chemicals. Mice were killed 24 h after the last application to determine ear and LN weights and LN cell counts. It was possible to classify the tested chemicals into three groups according to their threshold concentrations for LN activation and skin irritation: (1) chemicals with a low LN activation potential and no or very low skin irritation potential; (2) chemicals with a marked LN activation potential higher than a distinct skin irritation potential; and (3) chemicals with LN activation potential equal to or lower than their skin irritation potential. Group 1 consisted only of contact allergens, indicating that LN activation in the absence of skin irritation points to a contact allergenic activity. Since groups 2 and 3 comprised irritants and contact allergens, a tier II LLNA protocol was used to finally differentiate between true irritants and contact allergens. Briefly, mice were pretreated with mildly to moderately irritating concentrations of the chemical to the shaved back and after 12 days were challenged on the ears as described above in order to elicit a contact allergenic response in the ear skin and the ear-draining LN. With this approach, tier II LLNAs have to be conducted only in cases for which skin irritation potential is in the range of LN activation potential and no structure-activity relationship data indicating a contact allergenic hazard are available.

Zelikoff JT, Raymond A, Carlson E, Li Y, Beaman JR, Anderson M. **Biomarkers of immunotoxicity in fish: from the lab to the ocean.** Toxicol Lett 2000;112-113:325-31.

Abstract: Historically, host immunocompetence has been monitored using a battery of immune parameters. Recently, many of these same assays have been employed as biomarkers for predicting chemical-induced immunotoxicity in wildlife species. In this laboratory, assays measuring immunopathology, immune cell function, and host resistance against bacteria have been used successfully to assess immunotoxicity in laboratory-reared Japanese medaka (Oryzias latipes) and in

feral fish populations. As an example of the latter, smallmouth bass collected from a PCB-contaminated site demonstrated significantly reduced phagocyte function and antioxidant activity compared to reference site fish. Taken together, these studies along with those from other investigators demonstrate the usefulness of immune assays as indicators to predict the toxicological risk associated with 'real-world' polluted aquatic environments.

Garthwaite I, Ross KM, Miles CO, Briggs LR, Towers NR, Borrell T, Busby P. **Integrated enzyme-linked immunosorbent assay screening system for amnesic, neurotoxic, diarrhetic, and paralytic shellfish poisoning toxins found in New Zealand.** J AOAC Int 2001;84(5):1643-8.
Abstract: Enzyme-linked immunosorbent assays (ELISAs) were developed for amnesic, neurotoxic, and diarrhetic shellfish poisoning (ASP, NSP, and DSP) toxins and for yessotoxin. These assays, along with a commercially available paralytic shellfish poisoning (PSP) ELISA, were used to test the feasibility of an ELISA-based screening system. It was concluded that such a system to identify suspect shellfish samples, for subsequent analysis by methods approved by international regulatory authorities, is feasible. The assays had sufficient sensitivity and can be used on simple shellfish extracts. Alcohol extraction gave good recovery of all toxin groups. The ease of ELISAs permits the ready expansion of the system to screen for other toxins, as new ELISAs become available.

Hinton DM. **US FDA "Redbook II" immunotoxicity testing guidelines and research in immunotoxicity evaluations of food chemicals and new food proteins.** Toxicol Pathol 2000;28(3):467-78.
Abstract: The rapid advances in the field of immunology and an understanding of the potential adverse effects of xenobiotics on the immune system have resulted in the development of a discipline in toxicology now referred to as immunotoxicology. This discipline has evolved steadily over the last 2 decades as a result of research in the national and international communities. Various US, European, and Japanese regulatory agencies have recognized a need to promulgate testing guidelines for immunotoxicity in support of the approval process involving toxicological testing. The US Food and Drug Administration "Redbook II" guidelines and some of the research conducted in support of the concepts and testing strategies are presented here. Concerns raised with regard to these guidelines are included, as are on-going initiatives in development of experimental approaches for assessing allergic potential and/or hypersensitivity responses to new foods and food constituents.

Johnson CW, Williams WC, Copeland CB, DeVito MJ, Smialowicz RJ. **Sensitivity of the SRBC PFC assay versus ELISA for detection of immunosuppression by TCDD and TCDD-like congeners.** Toxicology 2000;156(1):1-11.
Abstract: The splenic antibody plaque forming cell (PFC) assay is a widely used assay in immunotoxicity testing. A recent revision of the Federal Insecticide, Fungicide and Rodenticide (FIFRA) Immunotoxicity test guidelines by the EPA recommended that either the PFC assay or the sheep red blood cell (SRBC) specific serum IgM enzyme-linked immunosorbent assay (ELISA) be used to assess the primary humoral response to SRBCs. The PFC assay quantifies the number of plasma cells in the spleen producing SRBC-specific antibody, while the ELISA measures SRBC-specific IgM antibody in the serum. Because these two assays measure different endpoints, there is a need for comparison of their sensitivity and reliability. The purpose of this project was to determine if these two

assays are equally sensitive to suppression of the SRBC response in B6C3F1 female mice. Female B6C3F1 mice were given a single oral exposure to different doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or four TCDD-like congeners. One week later, two sets of mice were immunized with SRBC. The first set was evaluated for the PFC response and the second for the ELISA response, on day 4 or 5 post-immunization, respectively. The four TCDD-like congeners tested were: 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD), 1,2,3,4,7-pentachlorodibenzofuran (4PeCDF), 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3',4,4',5-pentachlrorbiphenyl (PCB118). The results were used to generate dose-response curves for the determination of the ED(50) for TCDD and each TCDD-like congener. For all chemicals tested, measuring the level of SRBC-specific IgM antibody by ELISA was more sensitive than the PFC assay to detect immunosuppression, as indicated by lower ED(50) values. These results indicate that the SRBC-specific IgM ELISA is a more sensitive assay for detecting the T-cell mediated immunotoxicity of dioxin-like chemicals in this rodent model.

Koller LD. **A perspective on the progression of immunotoxicology.** Toxicology 2001;160(1-3):105-10.
Abstract: Immunotoxicology originated in the early 1970s, when scientists began investigating chemicals. During the 1970s and early 1980s, a few investigators determined that chemicals were immunotoxic, developed and/or refined immunoassays, and began to characterize immunotoxic responses. In the 1980s, many new investigators entered the field, graduates were being trained as immunotoxicologists, the immune system was identified as a primary target organ, mechanisms of action studies proliferated, a comprehensive immunotoxicological panel was validated, the discipline gained universal credibility, and human studies emerged. The 1990s were ushered in with the concept of biological markers in immunotoxicology, a better understanding of "immune function", inclusion of immunotoxicology in risk assessment analysis, and a focus on molecular immunology. Future investigations will continue to improve and expand this foundation, pursue the relationship of immunotoxic chemicals and adverse health effects in humans, utilize genetically altered rodent models, and use gene expression technology to better understand the pathogenesis of immunotoxicological processes. Immunotoxicology has not only matured since its inception nearly 30 yr ago, but has become a prominent and respected discipline with global recognition; one that has made significant contributions to the advancement of the biomedical sciences.

Pieters R. **The popliteal lymph node assay: a tool for predicting drug allergies.** Toxicology 2001;158 (1-2):65-9.
Abstract: A considerable number of drugs is able to induce systemic hypersensitivity in man. Systemic hypersensitivity can be drug- or autoantigen-specific, but in either case a complex of immunological processes and predisposing factors are involved and it is rarely if ever noticed in standard toxicity testing. The popliteal lymph node assay (PLNA) is regarded a suitable test to screen for the immunostimulating ability of a chemical, which may indicate its immunosensitizing potential. The most simple, primary PLNA measures popliteal lymph node hyperplasia after subcutaneous injection of a chemical into the footpad of the hindpaw of a mouse or rat. In order to assess the involvement of T cells, and hence immunosensitizating potential of a chemical, anamnestic immune reactions to a chemical or its metabolite can be measured in previously exposed (and sensitized) animals or in naive animals that received an adoptive transfer of syngeneic T cells from previously exposed animals. In the recently

introduced modified PLNA, defined reporter antigens TNP-OVA (T cell-dependent antigen) and TNP-Ficoll (T cell-independent antigen) are used to distinguish between sensitizing and non-sensitizing (IgG1-response or not to TNP-Ficoll, respectively) and between mere inflammatory and complete innocent (no IgG1-response to TNP-Ficoll and an IgG1-response or not to TNP-OVA, respectively) drugs. Results with about 130 compounds (drugs and environmental pollutants) with the various types of the PLNA show a good correlation with documented immunostimulating (both autoimmunogenic and allergic) potential and no false negative chemicals were detected if metabolism was considered. The PLNA awaits further validation before this test can be recommended as a tool for prediction of drug allergy.

Pieters R. **The popliteal lymph node assay in predictive testing for autoimmunity**. Toxicol Lett 2000;112-113:453-9.
Abstract: A large number of chemicals or metabolites thereof is known to induce or exacerbate autoimmune disease (AID) in man. Due to the complex immunological processes involved, chemical-induced autoimmunity is hardly if ever detected in standard toxicity testing and generally applicable animal models that detect a chemical's potential to induce AID do not exist. The popliteal lymph node assay (PLNA) focusses on the chemical's ability to initiate an immune response rather than on inducing or exacerbating autoimmune reactions, and is regarded a suitable test for pre-screening of immunostimulating and -sensitizing potential. The most simple primary PLNA measures enlargement of the popliteal lymph node (PLN) 6-8 days after subcutaneous injection of a chemical into the footpad distinguishes between immunostimulating and innocent chemicals. The primary PLNA is however unable to assess the involvement of T cells and thus the immunosensitizing potential of a chemical. For this, the secondary and/or modified PLNA is appropriate. The secondary PLNA detects challenge reactions in the PLN to non-sensitizing doses of a chemical in pre-sensitized animals or in unsensitized animals that received an adoptive transfer of pre-sensitized syngeneic T cells. The modified PLNA uses the defined reporter antigens TNP-OVA (T cell-dependent antigen) and TNP-Ficoll (T cell-independent antigen) to distinguish sensitizing from non-sensitizing (IgG1-response or not to TNP-Ficoll) and mere inflammatory from complete innocent (IgG1-response or not to TNP-OVA) chemicals. To date, about 130 compounds (drugs and environmental pollutants) have been tested in either one or more of these PLNAs. Results show a good correlation with documented immunostimulating (both autoimmunogenic and allergic) potential and no false negative chemicals were detected if metabolism was considered. In particular the modified PLNA awaits validation before it can be recommended as a standard test for autoimmunogenic potential.

Vohr HW, Ruhl-Fehlert C. **Industry experience in the identification of the immunotoxic potential of agrochemicals.** Sci Total Environ 2001;270(1-3):123-33.
Abstract: During recent years immunotoxicity has been increasingly recognized as an important endpoint in rodent short-time studies. This has been documented by FDA, OECD, and just recently in a new EPA guideline. This guideline is confined to the immunosuppressive effects of chemicals. Various parameters to detect immunotoxic effects exist, including cell counts, cell subpopulation analysis, functional tests, and/or advanced pathology. Their validity in detecting immunotoxic effects has been demonstrated to different degrees. Our experience with some of these parameters is reported here. Due to the recommendation of the guideline, it is necessary to differentiate from the context of the study data

between primary and secondary immunotoxicity, the latter being an unspecific sequel of toxicity to other organs. In our studies, we found examples for both mechanisms. For primary immunotoxic substances, immunosuppression is markedly more frequent than immunostimulation, although primary effects, on the whole, occur relatively seldom during toxicological screening. In both cases, we found a good correlation between cell analysis and functional parameters on one hand and pathology on the other, thus warranting that overt immunotoxicity would not remain undetected in routine studies with high dose levels. However, the higher predictivity of functional parameters and the analysis of special subpopulations is necessary for the determination of the no-effect level and for fine differentiation during the screening of comparable immunotoxic compounds. Cyclosporin A is an example for the former, and the screening of different agrochemicals is an example for the latter aspect. As verified by the collaboration studies, an advanced histopathology of lymphoid organs, combined with flow cytometry of immune competent cells and a functional assay, is able to discriminate between primary and secondary effects as well as immunosuppression and immunostimulation, and thus to identify an immunotoxic hazard.

NEUROTOXICITY

Adamchik Y, Frantseva MV, Weisspapir M, Carlen PL, Perez Velazquez JL. **Methods to induce primary and secondary traumatic damage in organotypic hippocampal slice cultures.** Brain Res Brain Res Protoc 2000;5(2):153-8.
Abstract: Organotypic brain slice cultures have been used in a variety of studies on neurodegenerative processes [K.M. Abdel-Hamid, M. Tymianski, Mechanisms and effects of intracellular calcium buffering on neuronal survival in organotypic hippocampal cultures exposed to anoxia/aglycemia or to excitotoxins, J. Neurosci. 17, 1997, pp. 3538-3553; D.W. Newell, A. Barth, V. Papermaster, A.T. Malouf, Glutamate and non-glutamate receptor mediated toxicity caused by oxygen and glucose deprivation in organotypic hippocampal cultures, J. Neurosci. 15, 1995, pp. 7702-7711; J.L. Perez Velazquez, M.V. Frantseva, P.L. Carlen, In vitro ischemia promotes glutamate mediated free radical generation and intracellular calcium accumulation in pyramidal neurons of cultured hippocampal slices, J. Neurosci. 23, 1997, pp. 9085-9094; L. Stoppini, L.A. Buchs, D. Muller, A simple method for organotypic cultures of nervous tissue, J. Neurosci. Methods 37, 1991, pp. 173-182; R.C. Tasker, J.T. Coyle, J.J. Vornov, The regional vulnerability to hypoglycemia induced neurotoxicity in organotypic hippocampal culture: protection by early tetrodotoxin or delayed MK 801, J. Neurosci. 12, 1992, pp. 4298-4308.]. We describe two methods to induce traumatic cell damage in hippocampal organotypic cultures. Primary trauma injury was achieved by rolling a stainless steel cylinder (0.9 g) on the organotypic slices. Secondary injury was followed after dropping a weight (0.137 g) on a localised area of the organotypic slice, from a height of 2 mm. The time course and extent of cell death were determined by measuring the fluorescence of the viability indicator propidium iodide (PI) at several time points after the injury. The initial localised impact damage spread 24 and 67 h after injury, cell death being 25% and 54%, respectively, when slices were kept at 37 degrees C. To validate these methods as models to assess neuroprotective strategies, similar insults were applied to slices at relatively low temperatures (30 degrees C), which is known to be neuroprotective [F.C. Barone, G.Z. Feuerstein, R.F. White, Brain cooling during transient focal ischaemia provides complete neuroprotection, Neurosci. Biobehav. Rev. 1, 1997, pp. 31-44; V.M. Bruno, M.P. Goldberg, L.L. Dugan, R.G. Giffard, D.W. Choi,

Neuroprotective effect of hypothermia in cortical cultures exposed to oxygen glucose deprivation or excitatory aminoacids, J. Neurochem. 4, 1994, pp. 387-392; G.C. Newman, H. Qi, F.E. Hospod, K. Grundhmann, Preservation of hippocampal brain slices with in vivo or in vitro hypothermia, Brain Res. 1, 1992, pp. 159-163; J.Y. Yager, J. Asseline, Effect of mild hypothermia on cerebral energy metabolism during the evolution of hypoxic ischaemic brain damage in the immature rat, Stroke, 5, 1996, pp. 919-925.]. Low temperature incubation significantly reduced cell death, now being 9% at 24 h and 14% at 67 h. Our results show that these models of moderate mechanical trauma using organotypic slice cultures can be used to study neurodegeneration and neuroprotective strategies.

Andreassen OA, Ferrante RJ, Dedeoglu A, Albers DW, Klivenyi P, Carlson EJ, Epstein CJ, Beal MF. **Mice with a partial deficiency of manganese superoxide dismutase show increased vulnerability to the mitochondrial toxins malonate, 3-nitropropionic acid, and MPTP.** Exp Neurol 2001;167(1):189-95.
Abstract: There is substantial evidence implicating mitochondrial dysfunction and free radical generation as major mechanisms of neuronal death in neurodegenerative diseases. The major free radical scavenging enzyme in mitochondria is manganese superoxide dismutase (SOD2). In the present study we investigated the susceptibility of mice with a partial deficiency of SOD2 to the neurotoxins 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), 3-nitropropionic acid (3-NP), and malonate, which are commonly used animal models of Parkinson's and Huntington's disease. Heterozygous SOD2 knockout (SOD2(+/-)) mice showed no evidence of neuropathological or behavioral abnormalities at 2-4 months of age. Compared to littermate wild-type mice, mice with partial SOD2 deficiency showed increased vulnerability to dopamine depletion after systemic MPTP treatment and significantly larger striatal lesions produced by both 3-NP and malonate. SOD2(+/-) mice also showed an increased production of "hydroxyl" radicals after malonate injection measured with the salicylate hydroxyl radical trapping method. These results provide further evidence that reactive oxygen species play an important role in the neurotoxicity of MPTP, malonate, and 3-NP. These findings show that a subclinical deficiency in a free radical scavenging enzyme may act in concert with environmental toxins to produce selective neurodegeneration.

Ault DT, Werling LL. **SH-SY5Y cells as a model for sigma receptor regulation of potassium-stimulated dopamine release.** Brain Res 2000;877(2):354-60.
Abstract: Previous studies in our laboratory using rat brain tissue have shown that neuropeptide Y (NPY) can enhance NMDA- and potassium-stimulated dopamine release from various brain regions and that this enhancement is reversed by sigma (sigma) receptor antagonists. In the current study, we sought to determine whether SH-SY5Y cells are suitable for investigating sigma receptor effects and whether any sigma receptors present are of the subtype responsive to NPY. We compare mechanisms by which the prototypical sigma receptor agonist (+)-pentazocine, and the proposed endogenous sigma receptor ligand NPY regulate potassium-stimulated [(3)H]dopamine release from SH-SY5Y cells. Both (+)-pentazocine and NPY inhibit potassium-stimulated [(3)H]dopamine release. Unlike our studies in rat brain tissue, the effect of NPY on [(3)H]dopamine release is not reversed by sigma receptor antagonists. SH-SY5Y cells appear to be an appropriate model to study the regulation of dopamine release by sigma receptors or by NPY receptors, but this population is not identical to that population identified in brain slices.

Backstrom E, Chambers BJ, Kristensson K, Ljunggren HG. **Direct NK cell-mediated lysis of syngenic dorsal root ganglia neurons in vitro**. J Immunol 2000;165(9):4895-900.
Abstract: In contrast to extensive studies on the role of T and B lymphocytes in the pathogenesis of autoimmune diseases of the nervous system, little is known about NK cells and their potential role in the destruction of neural tissue. NK cells have been implicated in the selective death of sympathetic neurons resident in the superior cervical ganglia of rats after exposure to the drug guanethidine. This observation suggests that NK cells may function as principle effectors in immunological diseases of the nervous system. However, the direct mechanism of action of NK cells in this model is not known. In particular, it is not known whether NK cells can kill autologous neurons directly. The aim of the present study was to examine whether NK cells can kill directly dorsal root ganglia neurons cultured in vitro. We demonstrate that C57BL/6 (B6)-derived dorsal root ganglia neurons can be killed directly by syngenic IL-2-activated NK cells, and that this nerve cell lysis is dependent on the expression of perforin in the NK cells. NK cells were less effective in destroying neurons grown in the presence of glial cells. These observations indicate a potential role for NK cells in nerve cell degeneration in inflammatory diseases of the nervous system.

Baker KA, Hong M, Sadi D, Mendez I. **Intrastriatal and intranigral grafting of hNT neurons in the 6-OHDA rat model of Parkinson's disease**. Exp Neurol 2000;162(2):350-60.
Abstract: The clinical findings on neural transplantation for Parkinson's disease (PD) reported thus far are promising but many issues must be addressed before neural transplantation can be considered a routine therapeutic option for PD. The future of neural transplantation for the treatment of neurological disorders may rest in the discovery of a suitable alternative cell type for fetal tissue. One such alternative may be neurons derived from a human teratocarcinoma (hNT). hNT neurons have been shown to survive and integrate within the host brain following transplantation and provide functional recovery in animal models of stroke and Huntington's disease. In this study, we describe the transplantation of hNT neurons in the substantia nigra (SN) and striatum of the rat model for PD. Twenty-seven rats were grafted with one of three hNT neuronal products; hNT neurons, hNT-DA neurons, or lithium chloride (LiCl) pretreated hNT-DA neurons. Robust hNT grafts could be seen with anti-neural cell adhesion molecule and anti-neuron-specific enolase immunostaining. Immunostaining for tyrosine hydroxylase (TH) expression revealed no TH-immunoreactive (THir) neurons in any animals with hNT neuronal grafts. THir cells were observed in 43% of animals with hNT-DA neuronal grafts and all animals with LiCl pretreated hNT-DA neuronal grafts (100%). The number of THir neurons in these animals was low and not sufficient to produce significant functional recovery. In summary, this study has demonstrated that hNT neurons survive transplantation and express TH in the striatum and SN. Although hNT neurons are promising as an alternative to fetal tissue and may have potential clinical applications in the future, further improvements in enhancing TH expression are needed.

Barc S, Page G, Fauconneau B, Barrier L, Huguet F. **A new in vitro approach for investigating the MPTP effect on DA uptake.** Neurochem Int 2001;38(3):243-8.
Abstract: Previous studies have shown that dopamine (DA) uptake was decreased after preincubation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 1-methyl-4-phenylpyridinium (MPP(+)) in in vitro slice and synaptosome models. The present study, conducted with and without preincubation,

attempted to determine whether inhibition results from a direct effect of neurotoxins on neuronal DA transporter or from an alteration of the transporter secondary to other toxic events. DA uptake was inhibited about 50% in the presence of MPTP+O(2) or MPP(+) (0.1, 1 and 5 mM) in rat striatal slices and synaptosomes. Such inhibition was obtained in synaptosomes preincubated for 150 min with MPP (+) and then washed. Inhibition of DA uptake was lower in slices preincubated with MPTP (5 mM)+O (2) and then washed (30%). Experiments in synaptosomes prepared from slices preincubated with MPTP or MPP(+) showed greater inhibition of DA uptake with MPTP. The results suggest that the inhibition of DA uptake in vitro by MPTP or MPP(+) results initially from a direct effect on the transporter during its penetration in nerve endings and subsequently from a transporter alteration related to toxic events. Thus, the preincubation of striatal slices followed by DA uptake measurement in synaptosomes would appear to be a good in vitro model for studying the dopaminergic toxicity of MPTP.

Beattie CE, Melancon E, Eisen JS. **Mutations in the stumpy gene reveal intermediate targets for zebrafish motor axons.** Development 2000;127(12):2653-62.
Abstract: Primary motoneurons, the earliest developing spinal motoneurons in zebrafish, have highly stereotyped axon projections. Although much is known about the development of these neurons, the molecular cues guiding their axons have not been identified. In a screen designed to reveal mutations affecting motor axons, we isolated two mutations in the stumpy gene that dramatically affect pathfinding by the primary motoneuron, CaP. In stumpy mutants, CaP axons extend along the common pathway, a region shared by other primary motor axons, but stall at an intermediate target, the horizontal myoseptum, and fail to extend along their axon-specific pathway during the first day of development. Later, most CaP axons progress a short distance beyond the horizontal myoseptum, but tend to stall at another intermediate target. Mosaic analysis revealed that stumpy function is needed both autonomously in CaP and non-autonomously in other cells. stumpy function is also required for axons of other primary and secondary motoneurons to progress properly past intermediate targets and to branch. These results reveal a series of intermediate targets involved in motor axon guidance and suggest that stumpy function is required for motor axons to progress from proximally located intermediate targets to distally located ones.

Blader P, Strahle U. **Zebrafish developmental genetics and central nervous system development.** Hum Mol Genet 2000;9(6):945-51.
Abstract: The central nervous system (CNS) is the most complex tissue of vertebrates. Recently, the zebrafish has emerged as a powerful genetic system for studying early development, and large-scale mutagenic screens for embryonic patterning defects have been accomplished. Mutants isolated in these screens are proving helpful in unravelling the molecular hierarchies involved in the development of the CNS. We review here recent studies in zebrafish which shed light on the genetic pathways involved in induction and regionalization of the CNS.

Bogler O, Furnari FB, Kindler-Roehrborn A, Sykes VW, Yung R, Huang HJ, Cavenee WK. **SETA: a novel SH3 domain-containing adapter molecule associated with malignancy in astrocytes.** Neuro-oncol 2000;2(1):6-15.
Abstract: Differential display polymerase chain reaction analysis was used to compare five differentiation states of the O-2A progenitor-like cell line CG4: progenitor cells and cells at 12 h or 4

days after the induction of differentiation into oligodendrocytes or astrocytes. This led to the identification of 52 sequence tags that were expressed differentially with cellular phenotype. One sequence was upregulated during differentiation of CG4 cells and represented a novel gene that we named SETA (SH3 domain-containing gene expressed in tumorigenic astrocytes). This gene encodes an SH3 domain-containing adapter protein with sequence similarity to the CD2AP (CD2 adapter protein) and CMS (Cas ligand with multiple Src homology) genes. SETA mRNA was expressed at high levels in the developing rat brain but was barely detectable in the normal adult rat or human brain. However, SETA mRNA was found in approximately one half of the human gliomas tested, including astrocytomas grades II, III, and IV, as well as oligodendrogliomas, mixed oligoastrocytomas, and human glioma-derived cell lines. A rat glioma generated by treatment with the alkylating carcinogen ethylnitrosourea on postnatal day 1 and a derived cell line also expressed SETA mRNA. Furthermore, in an in vitro model of astrocytoma progression based on p53-/- astrocytes, expression of SETA was restricted to cells that are tumorigenic.

Bohrmann B, Adrian M, Dubochet J, Kuner P, Muller F, Huber W, Nordstedt C, Dobeli H. **Self-assembly of beta-amyloid 42 is retarded by small molecular ligands at the stage of structural intermediates.** J Struct Biol 2000;130(2-3):232-46.
Abstract: Assemblyof the amyloid-beta peptide (Abeta) into fibrils and its deposition in distinct brain areas is considered responsible for the pathogenesis of Alzheimer's disease (AD). Thus, inhibition of fibril assembly is a potential strategy for therapeutic intervention. Electron cryomicroscopy was used to monitor the initial, native assembly structure of Abeta42. In addition to the known fibrillar intermediates, a nonfibrillar, polymeric sheet-like structure was identified. A temporary sequence of supramolecular structures was revealed with (i) polymeric Abeta42 sheets during the onset of assembly, inversely related to the appearance of (ii) fibril intermediates, which again are time-dependently replaced by (iii) mature fibrils. A cell-based primary screening assay was used to identify compounds that decrease Abeta42-induced toxicity. Hit compounds were further assayed for binding to Abeta42, radical scavenger activity, and their influence on the assembly structure of Abeta42. One compound, Ro 90-7501, was found to efficiently retard mature fibril formation, while extended polymeric Abeta42 sheets and fibrillar intermediates are accumulated. Ro 90-7501 may serve as a prototypic inhibitor for Abeta42 fibril formation and as a tool for studying the molecular mechanism of fibril assembly.

Bolaris S, Bozas E, Benekou A, Philippidis H, Stylianopoulou F. **In utero radiation-induced apoptosis and p53 gene expression in the developing rat brain.** Int J Radiat Biol 2001;77(1):71-81.
Abstract: PURPOSE: This study addressed the question of the role of the p53 gene in prenatal low-dose radiation-induced apoptosis in the neuroepithelium, in an effort to elucidate molecular mechanisms involved in the extreme radiosensitivity of the developing brain. MATERIALS AND METHODS: Pregnant Wistar rats were exposed to a single dose of 10, 20 or 40 cGy of X-rays on day 15 or 17 of gestation. Animals were sacrificed 4 or 24h after exposure. Apoptosis was studied by gel electrophoresis of isolated DNA and in situ by the TUNEL reaction. Expression of the p53 gene was studied by immunocytochemistry and Western analysis, as well as Northern analysis, for the detection of the protein and mRNA respectively. RESULTS: In utero low-dose irradiation led to apoptosis and an increase of p53 gene expression in the developing rat brain. Apoptotic as well as p53 immunopositive cells were detected among proliferating, migratory and post-mitotic neurones in the developing

neuroepithelium following prenatal irradiation, even after only l0 cGy. In addition to the p53 protein, p53 mRNA brain levels were also increased following prenatal irradiation. CONCLUSIONS: Low-dose prenatal irradiation of the developing brain led to p53 induction and cell death by apoptosis.

Calingasan NY, Huang PL, Chun HS, Fabian A, Gibson GE. **Vascular factors are critical in selective neuronal loss in an animal model of impaired oxidative metabolism.** J Neuropathol Exp Neurol 2000;59(3):207-17.
Abstract: Thiamine deficiency (TD) models the cellular and molecular mechanisms by which chronic oxidative deficits lead to death of select neurons in brain. Region- and cell-specific oxidative stress and vascular changes accompany the TD-induced neurodegeneration. The current studies analyzed the role of oxidative stress in initiating these events by testing the role of intercellular adhesion molecule-1 (ICAM-1) and endothelial nitric oxide synthase (eNOS) in the selective neuronal loss that begins in the submedial thalamic nucleus of mice. Oxidative stress to microvessels is known to induce eNOS and ICAM-1. TD increased ICAM-1 immunoreactivity in microvessels within the submedial nucleus and adjacent regions 1 day prior to the onset of neuronal loss. On subsequent days, the pattern of ICAM-1 induction overlapped that of neuronal loss, and of induction of the oxidative stress marker heme oxygenase-1 (HO-1). The intensity and extent of ICAM-1 and HO-1 induction progressively spread in parallel with the neuronal death in the thalamus. Targeted disruption of ICAM-1 or eNOS gene, but not the neuronal NOS gene, attenuated the TD-induced neurodegeneration and HO-1 induction. TD induced ICAM-1 in eNOS knockout mice, but did not induce eNOS in mice lacking ICAM-1. These results demonstrate that in TD, an ICAM-1-dependent pathway of eNOS induction leads to oxidative stress-mediated death of metabolically compromised neurons. Thus, TD provides a useful model to help elucidate the role of ICAM-1 and eNOS in the selective neuronal death in diseases in which oxidative stress is implicated.

Chan PH. **Reactive oxygen radicals in signaling and damage in the ischemic brain.** J Cereb Blood Flow Metab 2001;21(1):2-14.
Abstract: Reactive oxygen species have been implicated in brain injury after ischemic stroke. These oxidants can react and damage the cellular macromolecules by virtue of the reactivity that leads to cell injury and necrosis. Oxidants are also mediators in signaling involving mitochondria, DNA repair enzymes, and transcription factors that may lead to apoptosis after cerebral ischemia. Transgenic or knockout mice with cell- or site-specific prooxidant and antioxidant enzymes provide useful tools in dissecting the events involving oxidative stress in signaling and damage in ischemic brain injury.

Chang PY, Kanazawa N, Lutze-Mann L, Winegar RA. **p53 deficiency alters the yield and spectrum of radiation-induced lacZ mutants in the brain of transgenic mice.** Mutagenesis 2001;16(1):7-15.
Abstract: Exposure to heavy particle radiation in the galacto-cosmic environment poses a significant risk in space exploration and the evaluation of radiation-induced genetic damage in tissues, especially in the central nervous system, is an important consideration in long-term manned space missions. We used a plasmid-based transgenic mouse model system, with the pUR288 lacZ transgene integrated in the genome of every cell of C57Bl/6(lacZ) mice, to evaluate the genetic damage induced by iron particle radiation. In order to examine the importance of genetic background on the radiation sensitivity of individuals, we cross-bred p53 wild-type lacZ transgenic mice with p53 nullizygous mice, producing

lacZ transgenic mice that were either hemizygous or nullizygous for the p53 tumor suppressor gene. Animals were exposed to an acute dose of 1 Gy of iron particles and the lacZ mutation frequency (MF) in the brain was measured at time intervals from 1 to 16 weeks post-irradiation. Our results suggest that iron particles induced an increase in lacZ MF (2.4-fold increase in p53+/+ mice, 1.3-fold increase in p53 +/- mice and 2.1-fold increase in p53-/- mice) and that this induction is both temporally regulated and p53 genotype dependent. Characterization of mutants based on their restriction patterns showed that the majority of the mutants arising spontaneously are derived from point mutations or small deletions in all three genotypes. Radiation induced alterations in the spectrum of deletion mutants and reorganization of the genome, as evidenced by the selection of mutants containing mouse genomic DNA. These observations are unique in that mutations in brain tissue after particle radiation exposure have never before been reported owing to technical limitations in most other mutation assays.

Figlewicz DA, Dong L, Mlodzienski M, Turcotte JC. **Culture models of neurodegenerative disease.** Ann N Y Acad Sci 2000;919:106-18.
Abstract: In order to investigate how mutant SOD1 protein or environmental exogenous stressors lead to the death of motor neurons, we have established several in vitro model systems. We describe some features of the various models in order to demonstrate the advantages and shortcomings of each system.

Fletcher L, Mcfarlane E, Fletcher K, Grant PA, Campbell IC. **A mathematical model for assessing changes in neurofilament protein levels in neurites and cell bodies of differentiating neuroblastoma cells.** J Theor Biol 2000;205(2):241-52.
Abstract: A mathematical model which allows the calculation of the level of neurofilament protein in the cell body (x) and in the neurites (y) of differentiating SK-N-SH cells is presented. The model considers the changes in cell number (proliferating cells) and the number of cells with neurites (differentiating cells). It takes into account the fact that (i) when cells are cultured in differentiating conditions, an increase in cell number is initially observed and (ii) in a non-synchronized population of differentiating cells, the length of neurite extended by individual cells varies within the population. Total neurofilament protein levels in a population of cells were measured by enzyme-linked immunoabsorbant assay and application of the model to the data allowed values for x and y to be calculated. The validity of the model is supported by the fact that the predicted total neurofilament protein levels are highly correlated with the experimentally derived neurofilament protein levels. The model should be of use in temporal studies of cytoskeletal proteins involved in neuronal growth/differentiation and also in studies in which the system is a target of toxic insult.

Frantseva MV, Velazquez JL, Hwang PA, Carlen PL. **Free radical production correlates with cell death in an in vitro model of epilepsy.** Eur J Neurosci 2000;12(4):1431-9.
Abstract: Free radical (FR) production, a major step in calcium-dependent neurodegeneration, has been linked to the generation of epileptiform activity and seizure-induced cell death. However, direct evidence of FR production in neurons during seizures has never been presented. Using hippocampal cultured slices we demonstrate that FRs are produced in CA3 but not CA1 pyramidal neurons during the rhythmic synchronous activity induced by the GABAA receptor antagonist bicuculline. The production of FRs (measured as changes in the fluorescence emission of dihydrorhodamine 123) was correlated with an increase in the baseline levels of intracellular calcium ([Ca2+]i) estimated by fluo-3 injected into

individual neurons via a patch pipette. [Ca2+]i increased during spike bursting and returned to baseline levels after the burst termination in CA1, but not in CA3, pyramidal neurons where 'interburst' calcium concentrations progressively increased. Measurement of cell death, performed with propidium iodide 48 h after a 30-min exposure to bicuculline, revealed most prominent degeneration of pyramidal neurons in the CA3 pyramidal layer. The FR scavengers vitamin E and glutathione significantly reduced the seizure-induced neurodegeneration without supressing spontaneous epileptiform activity. These observations indicate that FR overproduction is related to seizure-induced neuronal death.

Friedman SD, Dager SR, Richards TL, Petropoulos H, Posse S. **Modeling brain compartmental lactate response to metabolic challenge: a feasibility study.** Psychiatry Res 2000;98(1):55-66.
Abstract: Magnetic resonance spectroscopy has been used to characterize abnormal brain lactate response in panic disorder (PD) subjects following lactate infusion. The present study integrated water quantification and tissue segmentation to evaluate compartmental lactate response within brain and cerebrospinal fluid (CSF). As there is evidence of brain parenchymal pH changes during lactate infusion, water scans were collected at baseline and post-infusion to address brain water stability. Water levels remained essentially stable across the protocol suggesting internal water provides an improved reference signal for measuring dynamic changes in response to metabolic challenge paradigms such as lactate infusion. To model brain lactate changes by compartments, we took the null hypothesis that lactate rises occur only in tissue. The approach referenced lactate amplitude (potentially from both compartments) to 'voxel' water (water scan corrected for differential T(2) between CSF brain at long-echo times - synonymous to a short-echo water scan). If the magnitude of lactate rise in CSF was equal to or greater than brain, voxels with substantial CSF fractions should demonstrate an equivalent or elevated response to voxels comprised only of tissue. The magnitude of lactate increases paralleled voxel tissue fraction suggesting the abnormal lactate rise observed in PD is tissue-based. The feasibility of lactate quantification and compartmental modeling are discussed.

Gainetdinov RR, Mohn AR, Caron MG. **Genetic animal models: focus on schizophrenia.** Trends Neurosci 2001;24(9):527-33.
Abstract: The neurobiology of schizophrenia remains poorly understood. Symptoms of schizophrenia are classically thought to be associated with an imbalance of the dopaminergic system. However, the contribution of other neurotransmitters, in particular glutamate, has been increasingly appreciated. The role of individual components of neurotransmitter systems in aberrant behaviors can be experimentally tested in transgenic animals. Dopamine transporter knockout mice display persistently elevated dopaminergic tone and therefore might be appropriate substrates to evaluate the dopamine hypothesis. Similarly, NMDA receptor-deficient mice can be used to evaluate the glutamate hypothesis of schizophrenia. In this review we discuss how such animal models might be relevant for understanding the neurochemical underpinnings of certain manifestations of schizophrenia.

Gerlai R, Lahav M, Guo S, Rosenthal A. **Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects.** Pharmacol Biochem Behav 2000;67(4):773-82.
Abstract: Zebra fish may be an ideal vertebrate model system for numerous human diseases with which the genetics and biological mechanisms of the disease may be studied. Zebra fish has been successfully used in developmental genetics, and recently, neurobiologists have also started to study this species. A

potentially interesting target disease amenable for analysis with zebra fish is drug addiction, e.g. alcoholism. Although genetic tools to manipulate the genome of zebra fish are available, appropriate phenotypical testing methods are often lacking. In this paper, we describe basic behavioral tests to investigate the acute effects of alcohol on zebra fish. These behavioral paradigms will be useful for the genetic and biological analysis of acute and chronic drug effects as well as addiction. In addition to presenting findings for the acute effects of alcohol, we briefly describe our strategy for generating and screening mutants. We hope that our pilot work will facilitate the future development of behavioral tests and the use of zebra fish in the genetic analysis of the biological effects of drugs of abuse.

Goldman D, Hankin M, Li Z, Dai X, Ding J. **Transgenic zebrafish for studying nervous system development and regeneration**. Transgenic Res 2001;10(1):21-33.
Abstract: Alpha1 tubulin gene expression is induced in the developing and regenerating CNS of vertebrates. Therefore, alpha1 tubulin gene expression may serve as a good probe for mechanisms underlying CNS development and regeneration. One approach to identify these mechanisms is to work backwards from the genome. This requires identification of alpha1 tubulin DNA sequences that mediate its developmental and regeneration-dependent expression pattern. Therefore, we generated transgenic zebrafish harboring a fragment of the alpha1 tubulin gene driving green fluorescent protein expression (GFP). In these fish, and similar to the endogenous gene, transgene expression was dramatically induced in the developing and regenerating nervous system. Although transgene expression generally declined during maturation of the nervous system, robust GFP expression was maintained in progenitor cells in the retinal periphery, lining brain ventricles and surrounding the central canal of the spinal cord. When these cells were cultured in vitro they divided and gave rise to new neurons. We also show that optic nerve crush in adult fish re-induced transgene expression in retinal ganglion cells. These studies identified a relatively small region of the alpha1 tubulin promoter that mediates its regulated expression pattern in developing and adult fish. This promoter will be extremely useful to investigators interested in targeting gene expression to the developing or regenerating nervous system. As adult transgenic fish maintain transgene expression in neural progenitors, these fish also provide a valuable resource of labeled adult neural progenitor cells that can be studied in vivo or in vitro. Finally, these fish should provide a unique in vivo system for investigating mechanisms mediating CNS development and regeneration.

Gray M, Moens CB, Amacher SL, Eisen JS, Beattie CE. **Zebrafish deadly seven functions in neurogenesis.** Dev Biol 2001;237(2):306-23.
Abstract: In a genetic screen, we isolated a mutation that perturbed motor axon outgrowth, neurogenesis, and somitogenesis. Complementation tests revealed that this mutation is an allele of deadly seven (des). By creating genetic mosaics, we demonstrate that the motor axon defect is non-cell autonomous. In addition, we show that the pattern of migration for some neural crest cell populations is aberrant and crest-derived dorsal root ganglion neurons are misplaced. Furthermore, our analysis reveals that des mutant embryos exhibit a neurogenic phenotype. We find an increase in the number of primary motoneurons and in the number of three hindbrain reticulospinal neurons: Mauthner cells, RoL2 cells, and MiD3cm cells. We also find that the number of Rohon-Beard sensory neurons is decreased whereas neural crest-derived dorsal root ganglion neurons are increased in number supporting a previous hypothesis that Rohon-Beard neurons and neural crest form an equivalence group during development.

Mutations in genes involved in Notch-Delta signaling result in defects in somitogenesis and neurogenesis. We found that overexpressing an activated form of Notch decreased the number of Mauthner cells in des mutants indicating that des functions via the Notch-Delta signaling pathway to control the production of specific cell types within the central and peripheral nervous systems.

Griffiths R, Grieve A, Scollon J, Scott M, Williams C, Meredith C. **Preliminary evaluation of an in vitro test for assessment of excitotoxicity by measurement of early gene (c-fos mRNA) levels.** Toxicol In Vitro 2000;14(5):447-58.
Abstract: Using primary cultures of mouse cerebellar granule cells as an in vitro model system, it has been demonstrated that different profiles of temporal expression of the c-fos proto-oncogene are observed under non-excitotoxic and excitotoxic conditions. A ratio has been derived previously for the steady-state level of c-fos mRNA after 30 min and 240 min which suggests that a 240 min/30 min ratio of greater than 1 correlates with excitotoxicity, whereas a ratio of less than 1 correlates with a non-excitotoxic outcome. Moreover, a positive correlation is seen with abrogation of excitotoxicity in response to selective excitatory amino acid receptor antagonists. This test, proposed as a specific biomarker for excitotoxicity is undergoing prevalidation. Excitotoxicity is defined as neuronal cell death mediated by hyperactivation of glutamate receptor subtypes and therefore might be expected to be prevented by selective glutamate receptor antagonists. In preliminary evaluation studies, we have conducted work under the direction of the European Center for Validation of Alternate Methods (ECVAM) using compounds specified by ECVAM that have been subdivided into four groups based on known or presumed actions. These groups comprise: Group 1-endogenous/synthetic excitotoxins; Group 2-environmental, putative excitotoxins; Group 3-neurotoxic but non-excitotoxic compounds, and Group 4-non-toxic compounds. The results of this study support the proposal that the c-fos mRNA time-ratio test is a specific biomarker of excitotoxicity. Just as importantly, this test has the potential for application in screening newly-designed EAA receptor antagonists in the search for clinically relevant drugs to treat a variety of neuropathologies.

Hoffman PL, Yagi T, Tabakoff B, Phillips TJ, Kono H, Messing RO, Choi DS. **Transgenic and gene "knockout" models in alcohol research.** Alcohol Clin Exp Res 2001;25(5 Suppl ISBRA):60S-6S.
Abstract: This article represents the proceedings of a symposium at the 2000 ISBRA Meeting in Yokohama, Japan. The chairs were Paula L. Hoffman and Takeshi Yagi. The presentations were (1) cAMP signaling in ethanol sensitivity and tolerance, by Boris Tabakoff; (2) Synaptic signaling pathways of Fyn-tyrosine kinase, by Takeshi Yagi; (3) Ethanol drinking and sensitization in dopaminergic and serotonergic receptor knockouts, by Tamara J. Phillips; (4) ICAM-1 is involved in early alcohol-induced liver injury in the mouse given enteral alcohol, by Hiroshi Kono; and (5) Strategies for targeted and regulated knockouts, by Robert O. Messing and Doo-Sup Choi.

Honore P, Rogers SD, Schwei MJ, Salak-Johnson JL, Luger NM, Sabino MC, Clohisy DR, Mantyh PW. **Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons.** Neuroscience 2000;98(3):585-98.
Abstract: The aim of this investigation was to determine whether murine models of inflammatory, neuropathic and cancer pain are each characterized by a unique set of neurochemical changes in the spinal cord and sensory neurons. All models were generated in C3H/HeJ mice and hyperalgesia and

allodynia behaviorally characterized. A variety of neurochemical markers that have been implicated in the generation and maintenance of chronic pain were then examined in spinal cord and primary afferent neurons.Three days after injection of complete Freund's adjuvant into the hindpaw (a model of persistent inflammatory pain) increases in substance P, calcitonin gene-related peptide, protein kinase C gamma, and substance P receptor were observed in the spinal cord. Following sciatic nerve transection or L5 spinal nerve ligation (a model of persistent neuropathic pain) significant decreases in substance P and calcitonin gene-related peptide and increases in galanin and neuropeptide Y were observed in both primary afferent neurons and the spinal cord. In contrast, in a model of cancer pain induced by injection of osteolytic sarcoma cells into the femur, there were no detectable changes in any of these markers in either primary afferent neurons or the spinal cord. However, in this cancer-pain model, changes including massive astrocyte hypertrophy without neuronal loss, increase in the neuronal expression of c-Fos, and increase in the number of dynorphin-immunoreactive neurons were observed in the spinal cord, ipsilateral to the limb with cancer.These results indicate that a unique set of neurochemical changes occur with inflammatory, neuropathic and cancer pain in C3H/HeJ mice and further suggest that cancer induces a unique persistent pain state. Determining whether these neurochemical changes are involved in the generation and maintenance of each type of persistent pain may provide insight into the mechanisms that underlie each of these pain states.

Hosohata Y, Varga EV, Stropova D, Li X, Knapp RJ, Hruby VJ, Rice KC, Nagase H, Roeske WR, Yamamura HI. **Mutation W284L of the human delta opioid receptor reveals agonist specific receptor conformations for G protein activation.** Life Sci 2001;68(19-20):2233-42.
Abstract: Intrinsic activities of different delta opioid agonists were determined in a [35S]GTPgammaS binding assay using cell membranes from Chinese hamster ovary (CHO) cells stably expressing the wild type (hDOR/CHO) or W284L mutant human delta opioid receptor (W284L/CHO). Agonist binding affinities were regulated more robustly by sodium and guanine nucleotide in W284L/CHO than in hDOR/ CHO cell membranes. The W284L mutation selectively reduced the affinity of SNC 80 while having moderate effect ((-) TAN 67) or no effect (DPDPE) on the affinities of other delta selective agonists. The mutation had opposite effects on the intrinsic activities of agonists belonging to different chemical classes. The effects of the mutation on agonist affinities and potencies were independent from its effects on the intrinsic activities of the agonists. Maximal stimulation of [35S]GTPgammaS binding by SNC 80 was 2-fold higher in W284L mutant cell membranes than in wild type hDOR/CHO cell membranes, despite lower receptor expression levels in the W284L/CHO cells. The binding affinity of SNC 80 however, was significantly reduced (15-fold and 30-fold in the absence or presence of sodium +GDP respectively) in W284L/CHO cell membranes relative to wild type hDOR/CHO membranes. Conversely, the Emax of (-)TAN 67 in the [35S]GTPgammaS binding assay was markedly reduced (0.6-fold of that of the wild type) with only a slight (6-fold) reduction in its binding affinity. The affinity and intrinsic activity of DPDPE on the other hand remained unchanged at the W284L mutant hDOR. The mutation had similar effects on the affinities potencies and intrinsic activities of (-)TAN 67 and SB 219825. The results indicate that delta opioid agonists of different chemical classes use specific conformations for G protein activation.

Hudson NJ, Evans AT, Yeung CK, Hewitt PJ. **Effect of process parameters upon the dopamine and lipid peroxidation activity of selected MIG welding fumes as a marker of potential neurotoxicity.**

Ann Occup Hyg 2001;45(3):187-92.
Abstract: There is growing concern over the neurotoxic effects of chronic occupational exposure to metal fume produced by welding. Elevated iron and manganese levels in the brain have been linked to an increase in lipid peroxidation, dopamine depletion and predisposition to the development of a Parkinson's type condition in advanced cases. Chemical and toxicological analysis of selected welding fumes, generated by model processes, were used in order to evaluate their potential to release solutes that promote oxidation of dopamine and peroxidation of brain lipids in cell free assays. This study compared the effect of shield gas, electrode type and voltage/currect upon the dopamine and brain lipid peroxidation potential of selected welding fume, obtained from metal inert gas (MIG) welding systems. Overall, fume extracts were found to enhance dopamine oxidation and inhibit lipid peroxidation. Significant differences were also found in the oxidising potential of fume generated under differing process conditions; it may therefore be possible to determine the potential neurotoxicity of fumes using this system.

Kellner T, Sanborn J, Wilson B. **In vitro and in vivo assessment of the effect of impurities and chirality on methamidophos-induced neuropathy target esterase aging.** Toxicol Sci 2000;54(2):408-15.
Abstract: In vitro and in vivo studies evaluated neuropathy target esterase (NTE) inhibition and aging (i. e., loss of reactivation potential) by analytical and technical grade racemic and resolved L-(-) and D-(+) isomers of methamidophos (O,S-dimethyl phosphoramidothioate). For studies in vitro, microsomal protein from phenobarbital-induced livers was isolated from chick embryos and NTE inhibition assays were performed using chick embryo brain homogenate treated with 1 or 5 mM methamidophos (with and without metabolic enzymes); for studies in vivo, hens received 30 to 35 mg/kg methamidophos injected into the pectoral muscle. NTE aging in hens was assessed 24 h later or after 30 min to 1 h incubation in vitro using solutions of potassium fluoride (KF) reactivator. Technical methamidophos produced significantly higher levels of aged-inhibited NTE than analytical methamidophos or isolated optical isomers. In vivo, technical methamidophos produced 61% total NTE inhibition with 18% aged and 43% unaged NTE; hens receiving analytical grade averaged 6% aged, 52% unaged, and 58% total NTE inhibition. Results for 1 mM analytical methamidophos in vitro were 5% aged, 54% unaged, and 59% total inhibition; for 1 mM technical methamidophos, values averaged 11% aged, 50% unaged, and 60% total NTE inhibition. The degree of NTE aging obtained both in vivo and in vitro for the isolated D-(+) and L-(-) isomers never exceeded that obtained using analytical grade. These data indicate that impurities in methamidophos could contribute to OPIDN potential. The in vitro methodology described could be applied to first tier screening for detection of NTE inhibition and aging, thus reducing the need for whole-animal testing for OPIDN.

Kim S, Westphalen R, Callahan B, Hatzidimitriou G, Yuan J, Ricaurte GA. **Toward development of an in vitro model of methamphetamine-induced dopamine nerve terminal toxicity.** J Pharmacol Exp Ther 2000;293(2):625-33.
Abstract: To develop an in vitro model of methamphetamine (METH)-induced dopamine (DA) neurotoxicity, striatal synaptosomes were incubated at 37 degrees C with METH for different periods of time (10-80 min), washed once, then tested for DA transporter function at 37 degrees C. METH produced time- and dose-dependent reductions in the V(max) of DA uptake, without producing any

change in K(m). Incubation of synaptosomes with the DA neurotoxins 1-methyl-4-phenyl-pyridinium ion, 6-hydroxydopamine, and amphetamine under similar conditions produced comparable effects. In contrast, incubation with fenfluramine, a serotonin neurotoxin, did not. METH-induced decreases in DA uptake were selective, insofar as striatal glutamate uptake was unaffected. Various DA transporter blockers (cocaine, methylphenidate, and bupropion) afforded complete protection against METH-induced decreases in DA uptake, without producing any effect themselves. METH's effects were also temperature dependent, with greater decreases in DA uptake occurring at higher temperatures. Tests for residual drug revealed small amounts (0.1-0.2 microM) of remaining METH, but kinetic studies indicated that decreases in DA uptake were not likely to be due to METH acting as a competitive inhibitor of DA uptake. Decreases in the V(max) of DA uptake were not accompanied by decreases in B (max) of [(3)H]WIN 35,428 binding, possibly because there is no mechanism for removing damaged DA nerve endings from the in vitro preparation Collectively, these results give good support to the development of a valid in vitro model that may prove helpful for elucidating the mechanisms underlying METH-induced DA neurotoxicity.

Maharajan P, Maharajan V, Ravagnan G, Paino G. **The weaver mutant mouse: a model to study the ontogeny of dopamine transmission systems and their role in drug addiction.** Prog Neurobiol 2001;64(3):269-76.
Abstract: Dopaminergic neurons and their projection-systems are important in some fundamental human activities like locomotion, feeding and sex, essential for survival and procreation, and are relevant to pathologies like Parkinson's disease and drug abuse. Three main dopaminergic projection-systems, namely the nigrostriatal, mesocortical and mesolimbic pathways are the major targets of the neuropharmacological actions of psychomotor stimulants such as cocaine and amphetamine. Studies on knockout mice for dopamine or its receptors provide substantial information but fail to reveal the role of individual dopaminergic projection-systems. Mutant animals with defects specific to one or more projection-systems might be useful for studying the role of individual dopaminergic projection-systems. We propose the weaver mutant mouse, with a defective nigrostriatal dopaminergic projection-system and dopamine depletion in the dorsal striatum but with intact mesocorticolimbic projection-systems, as a suitable model to study the role of individual dopaminergic systems in diverse biological processes including Parkinson's disease and drug abuse.

Marubio LM, Changeux J. **Nicotinic acetylcholine receptor knockout mice as animal models for studying receptor function.** Eur J Pharmacol 2000;393(1-3):113-21.
Abstract: Nicotinic acetylcholine receptors are pentameric ligand-gated ion channels, which are involved in a wide range of neuronal functions. During the past decade, a large number of nicotinic acetylcholine receptor subunits have been cloned and showed a discreet yet overlapping distribution pattern. Recently, several groups have produced mutant mice lacking specific nicotinic acetylcholine receptor subunits. In this review, we focus on how the study of these knockout mouse models has advanced our understanding of the role individual nicotinic acetylcholine receptor subunits play in the function and composition of endogenous receptors and the diverse pharmacological actions of nicotine in the mammalian nervous system.

Parsa AT, Chakrabarti I, Hurley PT, Chi JH, Hall JS, Kaiser MG, Bruce JN. **Limitations of the C6/**

Wistar rat intracerebral glioma model: implications for evaluating immunotherapy. Neurosurgery 2000;47(4):993-9; discussion 999-1000.
Abstract: OBJECTIVE: Intracranial rat glioma models are a useful method for evaluating the efficacy and toxicity of novel therapies for malignant glioma. The C6/Wistar model has been used extensively as a reproducible in vivo model for studying primary brain tumors including anti-glioma immune responses. The objective of the present study is to provide in vivo evidence that the C6 rat glioma model is allogeneic within Wistar rats and is therefore inappropriate for evaluating immune responses. METHODS: Growth patterns and immune responses of C6 cells implanted into the brain and flank of Wistar rats were analyzed and compared to an immunogenic syngeneic model (9L/Fischer). RESULTS: Wistar rats with C6 tumors developed a potent humoral and cellular immune response to the tumor. Wistar rats given simultaneous flank and intracerebral tumors had a survival rate of 100% compared to an 11% survival rate in control animals receiving only intracranial C6 cells. CONCLUSION: The C6 rat glioma induces a vigorous immune reaction that may mimic a specific anti-tumor response in Wistar rats. Efficacy of immunotherapy within this model must be cautiously interpreted.

Qiu Z, Naten DL, Liston JC, Yess J, Rebeck GW. **A novel approach for studying endogenous abeta processing using cultured primary neurons isolated from APP transgenic mice**. Exp Neurol 2001;170(1):186-94.
Abstract: The central component of senile amyloid plaques in Alzheimer's disease (AD) is the beta-amyloid peptide (Abeta), derived from proteolytic processing of the amyloid precursor protein (APP). In this study, we developed an in vitro model to measure and identify soluble Abeta from primary cortical neurons. Neurons were isolated from mice transgenic for human APP695 containing the K670N, M671L double mutation. We characterized soluble Abeta using Western blot and ELISA assays. We found that the Abeta levels in conditioned media from these neurons were readily detectable and almost five times higher than in CSF. The majority of Abeta in the media was Abeta1-40; however, Abeta1-42 was also detectable. When the neurons were exposed to Phorbol 12-myristate 13-acetate (PMA), alpha1-antichymotrypsin, or alpha1-antitrypsin, the alterations of soluble Abeta levels were consistent with other models reported. Most importantly, the soluble Abeta in our model was remarkably stable, and aliquots were unchanged after prolonged incubations or repeated freeze/thaw cycles. The Abeta appeared to be monomeric by Western blot analysis. Soluble Abeta coimmunoprecipitated with endogenous mouse apolipoprotein E from the primary cultures. Taken together, our data demonstrated that using a Western blot assay to detect soluble Abeta from transgenic mouse overexpressing APP695 is sensitive, specific, and reliable and provides an accessible model for examining the neuronal metabolism of APP and Abeta. Copyright 2001 Academic Press.

Sakolsky DJ, Ashby B. **Determination of D1 and D2 dopamine receptor expression by Ntera-2 cells.** Brain Res Mol Brain Res 2000;84(1-2):158-61.
Abstract: There is evidence that D1 and D2 dopamine receptors are co-expressed on some neurons. As a potential model of co-expression we examined Ntera-2 cells using RT-PCR, and showed that they express D2 but not D1 receptors. D2 dopamine receptor expression was confirmed by quinpirole inhibition of forskolin-stimulated cAMP formation. Absence of D1 dopamine receptors was confirmed by the inability of dopamine or SKF 81297 to increase cAMP.

Schmuck G, Ahr HJ, Schluter G. **Rat cortical neuron cultures: an in vitro model for differentiating mechanisms of chemically induced neurotoxicity.** In Vitr Mol Toxicol 2000;13(1):37-50.
Abstract: Various structurally unrelated chemicals [2,5 hexandione, acrylamide, organophosphates like mipafox, beta,beta iminodipropionnitrile (IDPN), 3-nitropropionic acid (3-NP), potassium cyanide (KCN), paraquat, and NMDA (N-methyl-D-apartic acid)] are known to cause degenerative damage of the peripheral or central nervous system. Differentiated neuronal cell cultures obtained from fetal rats have been used to differentiate the mechanisms underlying this type of neurotoxicity. Cytotoxicity as measured by a viability assay was not sensitive enough and had to be supplemented by further endpoints covering effects on cytoskeleton and on the energy state of the cells [glucose consumption, mitochondrial membrane potential and adenosine 5'-triphosphate (ATP) concentration]. Compounds like the delayed neurotoxic organophosphates, exert a selective direct effect on cytoskeleton elements in this model at concentrations distinctly below cytotoxic concentrations. Other compounds, like KCN, paraquat, and 3-NP selectively disrupt the balance between energy supply and demand of the neurons either by interacting with mitochondrial respiration or glycolysis. For these compounds cytoskeletal damage seemed to be secondary to the energy depletion. For NMDA, 2,5 hexandione and acrylamide, both mechanisms may contribute to the neuronal damage. In conclusion, primary cortical neuronal cultures of the rat are well suited to detect a neurotoxic potential and to differentiate its underlying mechanisms. Damage of the cytoskeleton may be considered as an endpoint mechanistically related to degenerative neuropathic effects.

Schoniger S, Wehming S, Gonzalez C, Schobitz K, Rodriguez E, Oksche A, Yulis CR, Nurnberger F. **The dispersed cell culture as model for functional studies of the subcommissural organ: preparation and characterization of the culture system.** J Neurosci Methods 2001;107(1-2):47-61.
Abstract: The subcommissural organ (SCO) is an enigmatic secretory gland of the brain, which is believed to be derived from ependymal (glial) precursor cells. We here developed a dispersed cell culture system of the bovine SCO as an approach to functional analyses of this brain gland. Tissue of the bovine SCO obtained from the slaughterhouse was papain dissociated either directly after dissection or after preparation of SCO explants. The latter had been maintained for 4-6 weeks in organ culture. The dispersed cells were cultured for up to 14 days and continuously tested for their secretory state by immunostaining of their secretory product. With respect to the morphology of the SCO cells (shape, processes, nucleus), no difference was found between the culture of freshly dissociated SCOs and that of dissociated SCO explants. In all cases, the dissociation caused a dedifferentiation; typical elongated cells were formed increasingly after 1 day of culture. Thereafter, only the cellular size increased, whereas the shape and the viability of the cells remained unchanged. Proliferating SCO cells were never observed. The culture obtained from fresh SCO tissue contained more glia cells and fibrocytes than the culture prepared from SCO explants. The proliferation of glia cells and fibrocytes was suppressed by blocking the mitotic activity with cytosine-beta-D-arabino furanoside (CAF). The cytophysiological features of the cultured dispersed cells of both origins did not differ as demonstrated by classical histology, by immunocytochemistry for the secretory products of the SCO, by the characteristics of calcium influx into the cytoplasm ([Ca2+]i) and cyclic adenosine monophosphate (cAMP) after stimulation with adenosine-5-triphosphate, substance P or serotonin, and by the activation of the transcription factor cAMP-responsive element-binding protein. Because of the maintenance of their viability, their capacity to release the secretory product into the culture medium, their receptive capacity, and their signal

transduction pathways, we conclude that the dispersed cell culture system, especially that obtained from SCO explants, represents an appropriate and useful model for functional studies of the mammalian SCO.

Shibata N. **Transgenic mouse model for familial amyotrophic lateral sclerosis with superoxide dismutase-1 mutation.** Neuropathology 2001;21(1):82-92.
Abstract: Familial amyotrophic lateral sclerosis (ALS) with mutations in the gene for superoxide dismutase-1 (SOD1) is clinicopathologically reproduced by transgenic mice expressing mutant forms of SOD1 detectable in familial ALS patients. Motor neuron degeneration associated with SOD1 mutation has been thought to result from a novel neurotoxicity of mutant SOD1, but not from a reduction in activity of this enzyme, based on autosomal dominant transmission of SOD1 mutant familial ALS and its transgenic mouse model, clinical severity of the ALS patients independent to enzyme activity, no ALS-like disease in SOD1 knockout or wild-type SOD1-overexpressing mice, and clinicopathological severity of mutant SOD1 transgenic mice dependent on transgene copy numbers. Proposed mechanisms of motor neuron degeneration such as oxidative injury, peroxynitrite toxicity, cytoskeletal disorganization, glutamate excitotoxicity, disrupted calcium homeostasis, SOD1 aggregation, carbonyl stress and apoptosis have been discussed. Intracytoplasmic vacuoles, indicative of increased oxidative damage to the mitochondria and endoplasmic reticulum, in the neuropil and motor neurons appear in high expressors of mutant SOD1 transgenic mice but not in low expressors of the mice or familial ALS patients, suggesting that overexpression of mutant SOD1 in mice may enhance oxidative stress generation from this enzyme. Thus, transgenic mice carrying small transgene copy numbers of mutant SOD1 would provide a beneficial animal model for SOD1 mutant familial ALS. Such a model would contribute to elucidating the pathomechanism of this disease and establishing new therapeutic agents.

Stariha RL, Kim SU. **Mitogen-activated protein kinase signalling in oligodendrocytes: a comparison of primary cultures and CG-4**. Int J Dev Neurosci 2001;19(4):427-37.
Abstract: Oligodendrocytes play a significant role in the central nervous system, as these cells are responsible for myelinating axons and allowing for the efficient conduction of nerve impulses. Therefore, any understanding we can gain about the functional biology of oligodendrocytes will give us important insights into demyelinating diseases such as multiple sclerosis, where oligodendrocytes and myelin are damaged or destroyed. Currently, much attention has focussed on the role of a family of mitogen-activated protein kinases in OL. This kinase family includes the extracellular signal-regulated protein kinases (ERKs), the stress-activated c-Jun N-terminal kinase (JNK), and the 38 kDa high osmolarity glycerol response kinase (p38). The actions of mitogen-activated protein kinases in oligodendrocytes appear to range from proliferation and cell survival to differentiation and cell death. In the past, studies on oligodendrocytes have been hampered by the difficulties inherent in producing large enough quantities of these cells for experimentation. This problem arises in large part due to the post-mitotic nature of mature oligodendrocytes. Over the years, a cell line known as Central Glia-4 (CG-4) has become a popular oligodendrocyte model due to its potentially unlimited capacity for self-renewal. In this review, we will look at the suitability of the Central Glia-4 cell line as an oligodendrocyte model, specifically in respect to studies on mitogen-activated protein kinase signalling in oligodendrocytes.

Stoop R, Pralong E. **Functional connections and epileptic spread between hippocampus, entorhinal cortex and amygdala in a modified horizontal slice preparation of the rat brain.** Eur J Neurosci

2000;12(10):3651-63.
Abstract: The hippocampus, the entorhinal cortex and the amygdala are interconnected structures of the limbic system that are implicated in memory and emotional behaviour. They demonstrate synaptic plasticity and are susceptible to development of temporal lobe epilepsy, which may lead to emotional and psychological disturbances. Their relative anatomical disposition has limited the study of neurotransmission and epileptic spread between these three regions in previous in vitro preparations. Here we describe a novel, modified-horizontal slice preparation that includes in the same plane the hippocampus, entorhinal cortex and amygdala. We found that, following application of bicuculline, each region in our preparation could generate spontaneous bursts that resembled epileptic interictal spikes. This spontaneous activity initiated in the hippocampal CA3/2 region, from where it propagated and controlled the activity in the entorhinal cortex and the amygdala. We found that this spontaneous bursting activity could spread via two different pathways. The first pathway comprises the well-known subiculum-entorhinal cortex-perirhinal cortex-amygdala route. The second pathway consists of a direct connection between the CA1 region and perirhinal cortex, through which the hippocampal bursting activity can spread to the amygdala while bypassing the entorhinal cortex. Thus, our experiments provide a new in vitro model of initiation and spread of epileptic-like activity in the ventral part of the limbic system, which includes a novel, fast and functional connection between the CA1 region and perirhinal cortex.

Thielemans L, Depoortere I, Van Assche G, Bender E, Peeters TL. **Demonstration of a functional motilin receptor in TE671 cells from human cerebellum.** Brain Res 2001;895(1-2):119-28.
Abstract: BACKGROUND: Our laboratory has described the presence of motilin receptors in the rabbit cerebellum. We discovered its presence in the human TE671 cell line, which is of cerebellar origin. METHODS: Cytosolic Ca(2+) fluxes were monitored on a confocal microscope in cells loaded with Indo-1 and stimulated with motilin under various conditions. Binding studies were performed with 125I-[Nle(13)]porcine motilin. Using primers, PCR for the motilin receptor was performed. RESULTS: Cells responded to motilin after 45+/-20 s. At different concentrations of motilin (10(-8), 10(-7), 10(-6.5), 10(-6) and 10(-5) M) the percentage of responding cells was 0+/-0, 0.6+/-1.5, 4.9+/-4.7, 21.7+/-15 and 35.7 +/-12, respectively. The response was blocked by the motilin antagonists [Phe(3), Nle(13)]po-motilin (0.8+/-1.8%) and GM-109 (0.0+/-0.0%) and mimicked by the agonist ABT-229 (23.6+/-15%). After stimulation with motilin, ABT-229 or [Phe(3),Leu(13)]po-motilin, but not with the antagonist GM-109, cells were desensitized. The response to motilin persisted in Ca(2+)-free solution (22.8+/-14.7%), was not affected by nifedipine (44+/-11%) but was abolished by incubation with thapsigargin (0+/-0%). Neither ryanodine, nor a previous stimulation with caffeine (0+/-0%) in Ca(2+)-free Krebs, nor both could block the response to motilin (28, 32.0+/-5.7, 41.3+/-6.1%, respectively). Binding studies revealed two binding sites for motilin, with a pK(d) of 8.9+/-0.05 and 6.11+/-0.61 (n=4). There were 100 times more low than high affinity receptors per cell. The presence of receptor mRNA was confirmed by PCR. CONCLUSION: Functional motilin receptors are present in TE671 cells. The response requires intracellular IP(3)-sensitive Ca(2+) stores. These cells may serve as a model of the central motilin receptor.

Tjalkens RB, Ewing MM, Philbert MA. **Differential cellular regulation of the mitochondrial permeability transition in an in vitro model of 1,3-dinitrobenzene-induced encephalopathy.** Brain

Res 2000;874(2):165-77.
Abstract: Exposure to 1,3-dinitrobenzene (DNB) is associated with neuropathologic changes in specific brainstem nuclei, mediated by oxidative stress and mitochondrial dysfunction. The expression of Bcl-2-family proteins as a function of sensitivity to 1, 3-dinitrobenzene (DNB)-induced mitochondrial permeability transition (MPT) was examined in C6 glioma and SY5Y neuroblastoma cells. Neuroblastoma cells were 10-fold more sensitive than glioma cells to DNB-induced decreases in mitochondrial reducing potential, measured by reduction of the tetrazolium compound, 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The IC(50) values for DNB-related inhibition of MTT reduction were 107+/-25 microM in SY5Y cells and 1047+/-101 microM in C6 cells. Levels of reactive oxygen species (ROS) were increased in both SY5Y and C6 cells following DNB exposure by 4.6- and 6.0-fold above control, respectively. DNB caused abrupt depolarization of mitochondria in both neuroblastoma and glioma cells that was inhibited by trifluoperazine. The first order rate constants for mitochondrial depolarization were: C6, k=0.31+/-0.02 min(-1); SY5Y, k=0.14+/-0.01 min(-1). Onset of MPT occurred at 10-fold lower concentration of DNB in SY5Y cells than in C6 cells. The antioxidants, deferoxamine and alpha-tocopherol, effectively prevented DNB-induced MPT in C6 and SY5Y cells, suggesting involvement of ROS in the initiation of MPT. Exposure to DNB resulted in decreased cellular ATP content in SY5Y cells and efflux of mitochondrial calcium in both SY5Y and C6 cells, concurrent with onset of MPT. The expression of Bcl-2, Bcl-X(L), and Bax was evaluated in both cell types by Western blot analysis. C6 glioma cells strongly expressed Bcl-X(L) and only weakly expressed Bcl-2 and Bax, whereas SY5Y neuroblastoma cells expressed lower levels of Bcl-X(L) and higher levels of both Bcl-2 and Bax. Collectively, these results suggest that higher constitutive expression of Bcl-X(L), rather than Bcl-2, correlates with resistance to DNB-induced MPT in SY5Y and C6 cells and that differential regulation of the permeability transition pore may underlie the cell-specific neurotoxicity of DNB.

Aschner M. **Interactions between pesticides and glia: an unexplored experimental field.** Neurotoxicology 2000;21(1-2):175-80.
Abstract: It is now well established that the role of astrocytes extends well beyond passive cytoskeletal structural support to neurons. In fact, astrocytes and neurons establish a highly dynamic reciprocal relationship that influences the growth, morphology, behavior, and central nervous system (CNS) repair. It is also well established that acute exposure to the organophosphorous insecticides leads to inhibition of neuronal acetylcholinesterase activity, an enzyme responsible for the inactivation of the neurotransmitter acetylcholine. Although astrocytes are known to express transport systems for choline, as well as acetylcholinesterase activity, little information is available on the potential interactions between the anticholinesterase class organophosphorous insecticides and these cells. This review will focus on astrocytic cholinergic receptors, choline uptake and metabolism, and address the potential importance of astrocytes in organophosphorous insecticide mediated neurotoxicity.

Bolton SJ, Jones DN, Darker JG, Eggleston DS, Hunter AJ, Walsh FS. **Cellular uptake and spread of the cell-permeable peptide penetratin in adult rat brain.** Eur J Neurosci 2000;12(8):2847-55.
Abstract: Investigation of normal and pathological diseases of the central nervous system (CNS) has been hampered by the inability to effectively manipulate protein function in vivo. In order to address this important topic, we have evaluated the ability of penetratin, a novel cell-permeable peptide consisting of

a 16-amino acid sequence derived from a Drosophila homeodomain protein, to act as a carrier system to introduce a cargo into brain cells. Fluorescently tagged penetratin was injected directly into rat brain, either into the striatum or the lateral ventricles, and rats were perfusion-fixed 24 h later in order to assess the brain response to the peptide. Immunohistochemistry following intrastriatal injection showed that injection of 10 microg penetratin caused neurotoxic cell death and triggered recruitment of inflammatory cells in a dose-dependent fashion. Doses of 1 microg or less resulted in reduced toxicity and recruitment of inflammatory cells, but interestingly, there was some spread of the penetratin. Injections of an inactive peptide sequence, derived from the same homeodomain, caused little toxicity but could still, however, trigger an inflammatory response. Intraventricular injections showed extensive inflammatory cell recruitment but minimal spread of either peptide. These results suggest that a dose of 1 microg of penetratin peptide is suitable for directing agents to small, discrete areas of the brain and as such is an interesting new system for analysing CNS function.

Cappelletti G, Pedrotti B, Maggioni MG, Maci R. **Microtubule assembly is directly affected by mpp (+)in vitro.** Cell Biol Int 2001;25(10):981-4.
Abstract: The microtubular system is emerging as a cell target in neurodegeneration evoked by the Parkinsonism-inducing neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its toxic metabolite N-methyl-4-phenylpyridinium (MPP(+)). Looking for a direct effect of the neurotoxin on microtubules, we have undertaken an in vitro study by using microtubule protein purified from bovine brain. We show that MPP(+), but not MPTP, modifies the initial rate and the critical concentration of assembly without affecting microtubule ultrastructure. These findings strengthen the hypothesis for the role of microtubules in the transduction of MPP(+)neurotoxic effect and, probably, in neuronal cell death.

Cory-Slechta DA, Crofton KM, Foran JA, Ross JF, Sheets LP, Weiss B, Mileson B. **Methods to identify and characterize developmental neurotoxicity for human health risk assessment. I: behavioral effects.** Environ Health Perspect 2001;109 Suppl 1:79-91.
Abstract: Alterations in nervous system function after exposure to a developmental neurotoxicant may be identified and characterized using neurobehavioral methods. A number of methods can evaluate alterations in sensory, motor, and cognitive functions in laboratory animals exposed to toxicants during nervous system development. Fundamental issues underlying proper use and interpretation of these methods include a) consideration of the scientific goal in experimental design, b) selection of an appropriate animal model, c) expertise of the investigator, d) adequate statistical analysis, and e) proper data interpretation. Strengths and weaknesses of the assessment methods include sensitivity, selectivity, practicality, and variability. Research could improve current behavioral methods by providing a better understanding of the relationship between alterations in motor function and changes in the underlying structure of these systems. Research is also needed to develop simple and sensitive assays for use in screening assessments of sensory and cognitive function. Assessment methods are being developed to examine other nervous system functions, including social behavior, autonomic processes, and biologic rhythms. Social behaviors are modified by many classes of developmental neurotoxicants and hormonally active compounds that may act either through neuroendocrine mechanisms or by directly influencing brain morphology or neurochemistry. Autonomic and thermoregulatory functions have been the province of physiologists and neurobiologists rather than toxicologists, but this may change as

developmental neurotoxicology progresses and toxicologists apply techniques developed by other disciplines to examine changes in function after toxicant exposure.

John HA, Laffling AJ, Marrs J, Baird A, Jat PS, Holdstock NB, Rossdale PD. **Establishment and characterization of equine autonomic ganglion cell lines to enable direct testing of candidate toxins involved in equine dysautonomia (grass sickness).** Cell Biol Toxicol 2000;16(1):63-74.
Abstract: To enable direct testing of a range of potential toxins or pathogens that might be involved in grass sickness, equine thoracic sympathetic chain ganglion cell lines were established from primary cell cultures by retroviral-mediated transduction of the temperature-sensitive mutant of the establishment oncogene encoding SV40 large T antigen. Morphological and behavioral features, temperature dependence, and immunocytochemical characteristics of the cell lines were investigated. The majority of cells were noradrenergic neurons in which dopamine-beta-hydroxylase, the enzyme that catalyzes norepinephrine synthesis, and neuropeptide Y coexisted. Cells treated with plasma from grass sickness cases that had previously been shown to induce autonomic nervous system damage when injected into normal horses showed significantly decreased mitochondrial function after 1 day. After 3 days exposure most cells showed severe degeneration in contrast to those treated with normal plasma. Liver and lung cell lines were also susceptible to plasma, suggesting that the toxin is not specifically neurotoxic.

Keefer EW, Norton SJ, Boyle NA, Talesa V, Gross GW. **Acute toxicity screening of novel AChE inhibitors using neuronal networks on microelectrode arrays.** Neurotoxicology 2001;22(1):3-12.
Abstract: Spontaneously active neuronal networks grown from embryonic murine frontal cortex on substrate integrated electrode arrays with 64 recording sites were used to assess acute neurobiological and toxic effects of a series of seven symmetrical, bifunctional alkylene-linked bis-thiocarbonate compounds designed to possess anticholinesterase activity. Acute functional neurotoxicity in the absence of cytotoxicity was defined as total collapse of spontaneous activity. All of the compounds were characterized as mixed inhibitors of AChE, with K(i)'s in the 10(-7)-10(-6) M range. The neuronal network assays revealed high repeatability for each compound, but surprisingly diverse effects among these closely related compounds. Six of the seven compounds produced changes in network activity at concentrations of 10-350 microM. Three of the compounds were excitatory, two were biphasic (excitatory at lower concentrations, inhibitory at higher), and one was solely inhibitory. Two of the inhibitory compounds produced irreversible inhibition of activity. Responses of cortical cultures to eserine were compared to the effects produced by the test compounds, with only one of seven providing a close match to the eserine profile. Matching of response patterns allows the classification of new drugs according to their response similarity to well-characterized agents. Spontaneously active neuronal networks reflect the interactions of multiple neurotransmitter and receptor systems, and can reveal unexpected side effects due to secondary binding. Utilizing such networks holds the promise of greater research efficiency through a more rapid recognition of physiological tissue responses.

Manzo L, Castoldi AF, Coccini T, Prockop LD. **Assessing effects of neurotoxic pollutants by biochemical markers.** Environ Res 2001;85(1):31-6.
Abstract: Neurotoxins cause biochemical and molecular events which indicate early stage effects in exposed persons well before or well below the induction of overt disease. Monitoring these early events may represent a valid approach to developing markers of neurotoxicity in individuals exposed to

environmental chemicals. In neurotoxicology, the use of biochemical markers is more problematic compared to other fields due to the complexity of central nervous system function, the multistage nature of neurotoxic events, and the inaccessibility of target tissue. Nevertheless, new biochemical assays have been developed in recent years to assess exposure, subclinical effects, and susceptibility to neurotoxic disorders. This paper reviews novel biomarkers of neurotoxicity and discusses perspectives and limitations of their use in occupational and environmental medicine. Copyright 2000 Academic Press.

Mooslehner KA, Chan PM, Xu W, Liu L, Smadja C, Humby T, Allen ND, Wilkinson LS, Emson PC. **Mice with very low expression of the vesicular monoamine transporter 2 gene survive into adulthood: potential mouse model for parkinsonism.** Mol Cell Biol 2001;21(16):5321-31.
Abstract: We have created a transgenic mouse with a hypomorphic allele of the vesicular monoamine transporter 2 (Vmat2) gene by gene targeting. These mice (KA1) have profound changes in monoamine metabolism and function and survive into adulthood. Specifically, these animals express very low levels of VMAT2, an endogenous protein which sequesters monoamines intracellularly into vesicles, a process that, in addition to being important in normal transmission, may also act to keep intracellular levels of the monoamine neurotransmitters below potentially toxic thresholds. Homozygous mice show large reductions in brain tissue monoamines, motor impairments, enhanced sensitivity to dopamine agonism, and changes in the chemical neuroanatomy of the striatum that are consistent with alterations in the balance of the striatonigral (direct) and striatopallidal (indirect) pathways. The VMAT2-deficient KA1 mice are also more vulnerable to the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in terms of nigral dopamine cell death. We suggest that the mice may be of value in examining, long term, the insidious damaging consequences of abnormal intracellular handling of monoamines. On the basis of our current findings, the mice are likely to prove of immediate interest to aspects of the symptomatology of parkinsonism. They may also, however, be of use in probing other aspects of monoaminergic function and dysfunction in the brain, the latter making important contributions to the pathogenesis of schizophrenia and addiction.

nells JT, Henry MM, Lewandowski MF, Seme MT, Murray TG. **Development and characterization of a rodent model of methanol-induced retinal and optic nerve toxicity.** Neurotoxicology 2000;21 (3):321-30.
Abstract: Methanol is an important public health and environmental concern because of the selective actions of its neurotoxic metabolite, formic acid, on the retina, optic nerve and central nervous system. Humans and non-human primates are uniquely sensitive to methanol-induced neurotoxicity as a consequence of the limited capacity of primate species to oxidize and thus detoxify formic acid. The toxic syndrome in primates is characterized by formic acidemia, metabolic acidosis and blindness or serious visual impairment. Nonprimate species are normally resistant to the accumulation of formate and associated metabolic and visual toxicity. We have characterized retinal and optic nerve toxicity in a nonprimate model of methanol toxicity using rats in which folate-dependent formate oxidation has been selectively inhibited, allowing formate to accumulate to toxic concentrations following methanol administration. Methanol-intoxicated rats developed formic acidemia, metabolic acidosis and visual toxicity analogous to the human methanol poisoning syndrome. Visual dysfunction was manifested as reductions in the electroretinogram and the flash-evoked cortical potential which occurred coincident with blood formate accumulation. Histological studies revealed mitochondrial disruption and

vacuolation in the retinal pigment epithelium, photoreceptor inner segments and optic nerve. The temporal relationship between methanol administration and the onset and development of ocular toxicity, as well as, the degree of metabolic acidosis and extent of formic acidemia in this rodent model are remarkably similar to that documented in human methanol intoxication. Moreover, the functional and morphologic findings in methanol-intoxicated rats are consistent with the hypothesis that formate acts as a mitochondrial toxin in the retina and optic nerve. The establishment and characterization of this nonprimate animal model of methanol intoxication will facilitate research into the mechanistic aspects of methanol toxicity and the development and testing of treatments for human methanol poisoning.

Ouary S, Bizat N, Altairac S, Menetrat H, Mittoux V, Conde F, Hantraye P, Brouillet E. **Major strain differences in response to chronic systemic administration of the mitochondrial toxin 3-nitropropionic acid in rats: implications for neuroprotection studies.** Neuroscience 2000;97(3):521-30.
Abstract: Chronic systemic treatment with 3-nitropropionic acid in rats produces persistent dystonia and bradykinesia, and striatal lesions reminiscent of Huntington's disease. However, the interpretation of results obtained with this model are complicated by a heterogeneous distribution of the response to a given toxic dose of 3-nitropropionic acid: approximately half of the animals develop selective striatal lesions, which in certain cases are associated with extrastriatal lesions, and the other half are apparently spared. Thus, the chronic 3-nitropropionic acid lesion model can be difficult for neuroprotection studies in which a consistent response to neurotoxic treatment is prerequisite. We hypothesized that some of the variability in the model was related to the use of Sprague-Dawley rats, since inter-individual variability in response to various stressful conditions has been described previously in this rat strain. We therefore compared 3-nitropropionic acid toxicity in rat strains known to be highly (Fisher 344) or poorly (Lewis) responsive to stress and compared the distribution of responses to that of Sprague-Dawley rats. In a protocol of intraperitoneal injection, toxicity of 3-nitropropionic acid was highest in Fisher rats, intermediate in Sprague-Dawley rats and lowest in Lewis rats. In addition, survival curves showed a more heterogeneous response to 3-nitropropionic acid toxicity in Sprague-Dawley rats than that observed in Lewis and Fisher rats. These differences between Sprague-Dawley and Lewis rats were confirmed in a protocol of subcutaneous 3-nitropropionic acid intoxication using osmotic minipumps, where doses up to 36-45mg/kg per day for five days were necessary to induce striatal lesions in Lewis rats as compared to 12-14mg/kg per day for five days in Sprague-Dawley rats. The selectivity of the striatum to lesions, and homogeneous progression of symptoms and neurodegeneration, were more consistently observed in Lewis as compared to Sprague-Dawley rats. These results suggest that vulnerability to 3-nitropropionic acid may depend on genetic factors, which could also influence the physiological response to stress. The present findings also establish an improved model of progressive striatal degeneration in the rat adapted for the testing of new neuroprotective strategies.

Schmued LC, Hopkins KJ. **Fluoro-Jade: novel fluorochromes for detecting toxicant-induced neuronal degeneration**. Toxicol Pathol 2000;28(1):91-9.
Abstract: Two anionic fluorescein derivatives can be used for the simple and definitive localization of neuronal degeneration in brain tissue sections. Initial work on the first generation fluorochrome, Fluoro-Jade, demonstrated the utility of this compound for the detection of neuronal degeneration induced by a variety of well-characterized neurotoxicants, including kainic acid, 3-nitropropionic acid, isoniazid,

ibogaine, domoic acid, and dizocilpine maleate (MK-801). After validation, the tracer was used to reveal previously unreported sites of neuronal degeneration associated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), methamphetamine, and d-fenfluramine. Preliminary findings with a second generation fluorescein derivative, Fluoro-Jade B, suggest that this tracer results in staining of optimal contrast and resolution in animals dosed with kainic acid. These 2 tracers can be combined with other histologic methods, including immunofluoresence and fluorescent Nissl stains. Recent preliminary findings on a number of specialized applications of Fluoro-Jade include the detection of apoptosis, amyloid plaques, astrocytes, and dead cells in tissue culture.

Zurich MG, Honegger P, Schilter B, Costa LG, Monnet-Tschudi F. **Use of aggregating brain cell cultures to study developmental effects of organophosphorus insecticides.** Neurotoxicology 2000;21 (4):599-605.
Abstract: Aggregating brain cell cultures of fetal rat telencephalon can be grown in a chemically defined medium for extended periods of time. After a phase of intense mitotic activity, these three-dimensional cell cultures undergo extensive morphological differentiation, including synaptogenesis and myelination. To study the developmental toxicity of organophosphorus compounds (OP), aggregating brain cell cultures were treated with parathion. Protein content and cell type-specific enzyme activities were not affected up to a concentration of 10(5) M. Gliosis, characterized by an increased staining for glial fibrillary acidic protein (GFAP), was observed in immature and in differentiated cells. In contrast, uridine incorporation and myelin basic protein (MBP) immunoreactivity revealed strong differences in sensitivity between these two developmental stages. These results are in agreement with the view that in vivo the development-dependent toxicity is not only due to changes in hepatic detoxification, but also to age-related modifications in the susceptibility of the different populations of brain cells. Furthermore, they underline the usefulness of histotypic culture systems with a high developmental potential, such as aggregating brain cell cultures, and stress the importance of applying a large range of criteria for testing the developmental toxicity of potential neurotoxicants.

OCULAR TOXICITY

Adriaens E, Dierckens K, Bauters TG, Nelis HJ, van Goethem F, Vanparys P, Remon JP. **The mucosal toxicity of different benzalkonium chloride analogues evaluated with an alternative test using slugs.** Pharm Res 2001;18(7):937-42.
Abstract: PURPOSE: The objective of this study was to evaluate the mucosal toxicity of different benzalkonium chloride (BAC) analogues using slugs as the alternative test organism. METHODS: The effect of different BAC analogues on the mucosal tissue of slugs was determined from the protein, lactate dehydrogenase, and alkaline phosphatase released from the foot mucosa after treatment. Additionally, mucus production and reduction in body weight of the slugs were measured. The eye irritation potency of the molecules was evaluated with the Bovine Corneal Opacity and Permeability (BCOP) assay. The antimicrobial activity of the different BAC analogues was also assessed. RESULTS: All BAC analogues induced severe damage to the mucosal epithelium of the slugs, and the irritation increased with decreasing alkyl chain length: BAC-C16 < BAC-C14 < BAC-C12 approximately BAC-mix. A similar ranking was obtained with the BCOP assay for eye irritation. The relative order of activities among the three BAC analogues was the same, i.e., BAC-C14 > or = BAC-C16 > BAC-C12.

The BAC-C14 exhibited higher activity than the BAC-mix. CONCLUSIONS: The toxicity and activity of BAC analogues depend on the alkyl chain length. The use of BAC-C14 as a conservative agent in pharmaceutical preparations instead of the BAC-mix should be considered.

Aleo MD, Avery MJ, Beierschmitt WP, Drupa CA, Fortner JH, Kaplan AH, Navetta KA, Shepard RM, Walsh CM. **The use of explant lens culture to assess cataractogenic potential.** Ann N Y Acad Sci 2000;919:171-87.
Abstract: Explanted cultures of crystalline lenses have been used to investigate mechanisms of xenobiotic-induced cataract formation. However, very few studies have utilized mechanistic information to predict the cataractogenic potential of structurally diverse xenobiotics. The present investigation outlines how visual assessment of lens clarity, biochemical endpoints of toxicity, and mechanisms of lenticular opacity formation can be used to select compounds with a lower probability of causing cataract formation in vivo. The rat lens explant culture system has been used to screen thiazolidinediones against ciglitazone for their direct cataractogenic potential in vitro. The two compounds that were selected as development candidates (englitazone and darglitazone) did not produce cataracts in rats exposed daily for 3 months. The culture system has also been used to illustrate that the lens is capable of metabolizing compounds to reactive intermediates. In this example, the toxicity of S-(1,2-dichlorovinyl)-L-cysteine (DCVC), a model cataractogen, was attenuated by inhibiting lenticular cysteine conjugate beta-lyase metabolism using aminooxyacetic acid. Finally, this model was used retrospectively to investigate the cataractogenic potential of CJ-12,918 and CJ-13,454 in rats. These compounds showed differences in the incidence of cataract formation in vivo based on differences in hepatic metabolism and penetration of parent drug and metabolites into the lens. The rank order of cataractogenic potential in vitro correlated better with in vivo results when an induced S9 microsomal fraction was added to the culture media. However, the model did not correctly predict the cataractogenic potential of ZD2138, a structurally similar compound. These studies illustrate the use of explant culture to assess mechanisms of cataract formation and outline its use and limitations for predicting cataractogenic potential in vivo.

Ando A, Ueda M, Uyama M, Masu Y, Okumura T, Ito S. **Heterogeneity in ornithine cytotoxicity of bovine retinal pigment epithelial cells in primary culture**. Exp Eye Res 2000;70(1):89-96.
Abstract: Gyrate atrophy of the choroid and retina is a chorioretinal degeneration caused by hyperornithinemia and a deficiency of ornithine-delta-aminotransferase (OAT). We recently showed that ornithine exhibits cytotoxicity to human retinal pigment epithelial (RPE) cell lines treated with the OAT inhibitor, 5-fluoromethylornithine (5-FMOrn), and suggested that this system may be an in vitro model of gyrate atrophy. In the present study, in order to apply this system to primary cultured RPE cells, we freshly prepared RPE cells from bovine eyes and studied the effect of ornithine on cell damage. Two phenotypes, epithelioid and fusiform, which coexisted in the primary culture and epithelioid phenotype cells, but not fusiform ones, were severely damaged and partially detached from the substrate by 10 m m ornithine and 0.5 m m 5-FMOrn. Neither ornithine nor 5-FMOrn alone exhibited such cytotoxicity to both phenotypes of RPE cells. Proline significantly prevented the ornithine-induced cytotoxicity. Epithelioid and fusiform phenotypes isolated from the primary culture showed different distribution of actin filaments. A combination of ornithine and 5-FMOrn time-dependently inhibited [(3)H]thymidine incorporation in the epithelioid, but not fusiform, cells. Proline prevented the inhibition of [(3)H]

thymidine incorporation by ornithine in 5-FMOrn-treated epithelioid cells. Furthermore, l -azetidine-2-carboxylic acid, a collagen synthesis inhibitor, reduced [(3)H]thymidine incorporation in epithelioid, but not fusiform, cells, which was reversed by proline. These results demonstrate that the epithelioid phenotype of bovine RPE cells becomes susceptible to ornithine following inactivation of OAT. The phenotypic cells and its prevention by proline may provide insight into biochemical triggers that induce gyrate atrophy.

Blakely EA, Bjornstad KA, Chang PY, McNamara MP, Chang E, Aragon G, Lin SP, Lui G, Polansky JR. **Growth and differentiation of human lens epithelial cells in vitro on matrix.** Invest Ophthalmol Vis Sci 2000;41(12):3898-907.
Abstract: PURPOSE: To characterize the growth and maturation of nonimmortalized human lens epithelial (HLE) cells grown in vitro. METHODS: HLE cells, established from 18-week prenatal lenses, were maintained on bovine corneal endothelial (BCE) extracellular matrix (ECM) in medium supplemented with basic fibroblast growth factor (FGF-2). The identity, growth, and differentiation of the cultures were characterized by karyotyping, cell morphology, and growth kinetics studies, reverse transcription-polymerase chain reaction (RT-PCR), immunofluorescence, and Western blot analysis. RESULTS: HLE cells had a male, human diploid (2N = 46) karyotype. The population-doubling time of exponentially growing cells was 24 hours. After 15 days in culture, cell morphology changed, and lentoid formation was evident. Reverse transcription-polymerase chain reaction (RT-PCR) indicated expression of alphaA- and betaB2-crystallin, fibroblast growth factor receptor 1 (FGFR1), and major intrinsic protein (MIP26) in exponential growth. Western analyses of protein extracts show positive expression of three immunologically distinct classes of crystallin proteins (alphaA-, alphaB-, and betaB2-crystallin) with time in culture. By Western blot analysis, expression of p57(KIP2), a known marker of terminally differentiated fiber cells, was detectable in exponential cultures, and levels increased after confluence. MIP26 and gamma-crystallin protein expression was detected in confluent cultures, by using immunofluorescence, but not in exponentially growing cells. CONCLUSIONS: HLE cells can be maintained for up to 4 months on ECM derived from BCE cells in medium containing FGF-2. With time in culture, the cells demonstrate morphologic characteristics of, and express protein markers for, lens fiber cell differentiation. This in vitro model will be useful for investigations of radiation-induced cataractogenesis and other studies of lens toxicity.

Budai P, Varnagy L. **In vitro ocular irritation toxicity study of some pesticides.** Acta Vet Hung 2000;48(2):221-8.
Abstract: The use of animals in toxicological screening is a controversial issue. The Draize eye irritation test receives particular criticism because of the injuries inflicted on the test animals. In recent years various in vitro methods have been developed to replace the heavily criticised Draize rabbit eye test for irritation testing. One of the best-studied alternative methods is the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM). In the present studies comparative screening was performed with a set of pesticides to establish parallel data on in vitro (HET-CAM) and in vivo (Draize) results. The tested pesticides included Arelon 500 FW (isoproturon), Banvel 480 (dicamba), Dikamin D (2.4 D), Karathane LC (dinocap), Ronstar (oxadiazon) and Modown 4 F (bifenox). In most cases a good correlation was found between the HET-CAM assessment and results of the Draize rabbit eye test. Although the current form of the HET-CAM test is a valuable prescreen method for predicting the ocular irritation potential

of chemicals, and can be used for reducing the number of experimental animals, a number of technical problems must still be addressed before these systems can replace whole animal tests. The HET-CAM test can be a useful component of a battery of tests needed for replacing the Draize rabbit eye test.

Burgos JS, Barat A, Ramirez G. **Ca2+-dependent kainate excitotoxicity in the chick embryonic neural retina ex vivo.** Neuroreport 2000;11(17):3855-8.
Abstract: The chick embryonic neural retina ex vivo has been singled out as a unique example of Cl(-)-dependent/Ca2+-independent excitotoxicity. However, after continuous incubation with 100 microM kainate, we have demonstrated the susceptibility of the chick retina to Ca2+-mediated damage, which becomes apparent after 12 h of exposure to the agonist in the absence of Cl-. Of the 20.8% lactate dehydrogenase released after 24 h incubation with kainate, some 11% is Cl(-)-dependent and the rest (9.8%) is presumably Ca2+-dependent. Upon omission of both Cl- and Ca2+, a 5% residual toxicity can still be detected after 24 h. This can be overcome by inclusion of EGTA in the incubation medium to neutralize Ca2+ released during incubation. A Ca2+-dependent toxicity mechanism is then operative in the embryonic chick retina ex vivo.

Burgos JS, Barat A, Ramirez G. **Cl(-)-dependent excitotoxicity is associated with 3H2O influx in chick embryonic retina.** Neuroreport 2000;11(17):3779-82.
Abstract: The aim of this study was to show that Cl(-)-dependent excitotoxicity, with its characteristic cell swelling, involves actual water influx into the intracellular compartment. Taking advantage of the Ca2+ omission paradigm of Cl(-)-dependent excitotoxicity, in the chick embryonic neural retina ex vivo, which is associated with toxicity levels (lactate dehydrogenase (LDH) release) considerably higher than those seen after simple exposure of the retinas to glutamate agonists, we have demonstrated that an intracellular water intake of 4.2 microl into retinal cells is associated with 13.3% total retinal LDH release. The fact that mannitol blocks both water inflow and LDH release appears to link both events from a pathogenic point of view.

Cooper KJ, Earl LK, Harbell J, Raabe H. **Prediction of ocular irritancy of prototype shampoo formulations by the isolated rabbit eye (IRE) test and bovine corneal opacity and permeability (BCOP) assay.** Toxicol In Vitro 2001;15(2):95-103.
Abstract: The isolated rabbit eye (IRE) test and bovine corneal opacity and permeability (BCOP) assay were evaluated for their ability to predict the eye irritation potential of a range of hair shampoo formulations, some containing a novel non-surfactant ingredient known to be an ocular irritant. The additional endpoints of corneal swelling and histological examination were incorporated into the standard BCOP protocol. Historic Draize data were available for several of the formulations and served as a reference. The standard BCOP assay (without histology) failed to distinguish between shampoos of low and high irritant potential, when exposure times of 10 and 60 min were employed (for undiluted and 10% dilution of the shampoos, respectively) and the in vitro score classified the majority of formulations as mild. The incorporation of the histological endpoint to the BCOP protocol allowed discrimination between formulations of differing irritancy, and should be included to augment the standard BCOP protocol. Corneal swelling values did not, however, correlate with the irritant potential of the shampoos tested. The IRE which includes the endpoints of corneal swelling and histopathological scoring produced classifications of irritancy that were fairly consistent with in vivo data and distinguished

between the high and low irritant potential shampoos.

Geerling G, Daniels JT, Dart JK, Cree IA, Khaw PT. **Toxicity of natural tear substitutes in a fully defined culture model of human corneal epithelial cells.** Invest Ophthalmol Vis Sci 2001;42(5):948-56.
Abstract: PURPOSE: Serum and saliva have recently been advocated as natural tear substitutes for intractable aqueous-deficient dry eyes, but the effects of these fluids on corneal epithelium have not been well characterized. A laboratory study was performed in a defined test model to compare the toxicity of natural and pharmaceutical tear substitutes and to identify potentially toxic factors in natural tear substitutes, such as amylase, hypotonicity, and variations in preparation. METHODS: Primary human corneal epithelial cells were cultured with defined keratinocyte serum-free medium. The cells were incubated with hypromellose (hydroxypropylmethylcellulose 0.3%) with and without benzalkonium chloride 0.01%, saliva with differing osmolalities, 100% serum, and 50% serum (1:1 vol/vol with chloramphenicol 0.5%) for varying times and concentrations. Toxicity was examined in four ways. Microvillous density was assessed with scanning electron microscopy. Cell membrane permeability and intracellular esterase activity were analyzed after staining with fluorescent calcein-AM/ethidium homodimer and cellular adenosine triphosphate (ATP) was quantified using a luciferin-luciferase-based assay. RESULTS: The toxicity ranking of the tear substitutes correlated in all assays. The ATP assay was the most sensitive, followed by ethidium cell permeability, and finally the esterase activity. Preserved hypromellose was more toxic than the unpreserved preparation. Among natural tear substitutes, natural saliva was most toxic. Isotonic saliva and 50% serum were of similar toxicity, and 100% serum was least toxic. Natural tear substitutes were-except for natural saliva-less toxic than unpreserved hypromellose. Hypotonicity, but not amylase, was the major toxic effect associated with saliva. The dilution of serum with chloramphenicol induced toxicity. CONCLUSIONS: This is the first toxicity study using human primary corneal epithelial cells cultured under fully defined conditions as an in vitro model. Cellular ATP is a sensitive parameter for quantifying toxicity. Isotonic saliva and serum offer greater therapeutic potential for severely aqueous-deficient dry eyes than do pharmaceutical tear substitutes.

Iwig M, Glasser D, Struck HG. [Human cultured lens cells. II. **Characterization of established lens cell lines and test of their suitability for cytotoxicity tests].** Klin Monatsbl Augenheilkd 2001;218 (4):251-61 [Ger].
Abstract: BACKGROUND: Human lens cells in culture may be valuable tools to discover cataractogenic risk factors. Here we report on the characterization of established human lens cells and their use in cytotoxicity tests. MATERIAL AND METHODS: Adhesion dependence was tested by an autoradiographic method. Soft agar test was performed to secure anchorage-independent growth. Laser scan microscopy was used for measuring the nucleus-cytoplasmic relationship. The cytoskeleton was visualized by fluorescence microscopy. Investigations on cytotoxicity were done by neutral red cytotoxicity assay and 3H-thymidine incorporation. The labeling index was determined by the BrdU-method. RESULTS: Two lens cell lines were transformed into fast growing cells. They are characterized by a partial loss of adhesion dependence, cell growth in soft agar, high cloning efficiency and reduced serum requirement. However, the nucleus-cytoplasmic relationship did not change very much in comparison to non-transformed lens cells. The organization of the cytoskeleton was cell shape

dependent. The intermediate filaments were from the vimentin type. The established cell lines proved to be highly sensitive to ethanol and mitomycin C. CONCLUSIONS: The established human lens cell lines are well suited to screen for cytotoxic substances in vitro which might be cataractogenic risk factors in vivo.

Jester JV, Li L, Molai A, Maurer JK. **Extent of initial corneal injury as a basis for alternative eye irritation tests**. Toxicol In Vitro 2001;15(2):115-30.
Abstract: Based on studies that have characterized the extent of injury occurring with irritants of differing type and severity, we have proposed that extent of initial injury is the principal mechanism underlying ocular irritation. We report here our efforts to apply this hypothesis, as a mechanistic basis, to the development of an alternative eye irritation assay using an ex vivo rabbit corneal model. Rabbit eyes were obtained immediately after sacrifice or from an abattoir and 8.5-mm diameter corneal buttons were removed and cultured overnight at an air-liquid interface under serum-free conditions. Buttons were exposed to materials of differing type (surfactant, acid, base, alcohol and aldehyde) and irritancy (slight to severe) that had been previously characterized microscopically in the rabbit low-volume eye test. Exposure was accomplished by applying 1.5 microl of an irritant to a sterile, 3 mm diameter, filter paper disk and then placing the disk on the center of the corneal button for 10 s. After removal of the disk, buttons were washed and cultured for 3, 24 or 48 h. Buttons were then evaluated for extent of injury using a Live/Dead staining kit and fluorescent microscopy to measure cell size of live surface epithelial cells, area of epithelial denudation and depth of stromal injury. Ex vivo exposure to slight irritants generally reduced surface epithelial cell size (i.e. erosion) while exposure to mild irritants produced epithelial denudation with variable injury to the corneal stroma. Severe irritants generally produced extensive epithelial denudation and damaged the corneal stroma and endothelium. Overall, ex vivo extent of injury significantly correlated with in vivo extent of injury as measured in previous animal tests ($r=0.81$, $P<0.001$). These findings indicate that extent of corneal injury, as shown to be associated with ocular irritation occurring in vivo, can be applied to the development of a mechanistically-based alternative eye irritation model. We believe that this approach may ultimately lead to an alternative assay to replace the use of animals in ocular irritation testing.

Kulkarni A, Hopfinger AJ, Osborne R, Bruner LH, Thompson ED. **Prediction of eye irritation from organic chemicals using membrane-interaction QSAR analysis.** Toxicol Sci 2001;59(2):335-45.
Abstract: Eye irritation potency of a compound or mixture has traditionally been evaluated using the Draize rabbit-eye test (Draize et al., 1944). In order to aid predictions of eye irritation and to explore possible corresponding mechanisms of eye irritation, a methodology termed "membrane-interaction QSAR analysis" (MI-QSAR) has been developed (Kulkarni and Hopfinger 1999). A set of Draize eye-irritation data established by the European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) (Bagley et al., 1992) was used as a structurally diverse training set in an MI-QSAR analysis. Significant QSAR models were constructed based primarily upon aqueous solvation-free energy of the solute and the strength of solute binding to a model phospholipid (DMPC) monolayer. The results demonstrate that inclusion of parameters to model membrane interactions of potentially irritating chemicals provides significantly better predictions of eye irritation for structurally diverse compounds than does modeling based solely on physiochemical properties of chemicals. The specific MI-QSAR models reported here are, in fact, close to the upper limit in both significance and robustness that can be

expected for the variability inherent to the eye-irritation scores of the ECETOC training set. The MI-QSAR models can be used with high reliability to classify compounds of low- and high-predicted eye irritation scores. Thus, the models offer the opportunity to reduce animal testing for compounds predicted to fall into these two extreme eye-irritation score sets. The MI-QSAR paradigm may also be applicable to other toxicological endpoints, such as skin irritation, where interactions with cellular membranes are likely.

Link BA, Fadool JM, Malicki J, Dowling JE. **The zebrafish young mutation acts non-cell-autonomously to uncouple differentiation from specification for all retinal cells.** Development 2000;127(10):2177-88.
Abstract: Embryos from mutagenized zebrafish were screened for disruptions in retinal lamination to identify factors involved in vertebrate retinal cell specification and differentiation. Two alleles of a recessive mutation, young, were isolated in which final differentiation and normal lamination of retinal cells were blocked. Early aspects of retinogenesis including the specification of cells along the inner optic cup as retinal tissue, polarity of the retinal neuroepithelium, and confinement of cell divisions to the apical pigmented epithelial boarder were normal in young mutants. BrdU incorporation experiments showed that the initiation and pattern of cell cycle withdrawal across the retina was comparable to wild-type siblings; however, this process took longer in the mutant. Analysis of early markers for cell type differentiation revealed that each of the major classes of retinal neurons, as well as non-neural Muller glial cells, are specified in young embryos. However, the retinal cells fail to elaborate morphological specializations, and analysis of late cell-type-specific markers suggests that the retinal cells were inhibited from fully differentiating. Other regions of the nervous system showed no obvious defects in young mutants. Mosaic analysis demonstrated that the young mutation acts non-cell-autonomously within the retina, as final morphological and molecular differentiation was rescued when genetically mutant cells were transplanted into wild-type hosts. Conversely, differentiation was prevented in wild-type cells when placed in young mutant retinas. Mosaic experiments also suggest that young functions at or near the cell surface and is not freely diffusible. We conclude that the young mutation disrupts the post-specification development of all retinal neurons and glia cells.

Liu PT. **Extremely small sample size in some toxicity studies: an example from the rabbit eye irritation test**. Regul Toxicol Pharmacol 2001;33(2):187-91.
Abstract: The conventional sample-size equations based on either the precision of estimation or the power of testing a hypothesis may not be appropriate to determine sample size for a "diagnostic" testing problem, such as the eye irritant Draize test. When the animals' responses to chemical compounds are relatively uniform and extreme and the objective is to classify a compound as either irritant or nonirritant, the test using just two or three animals may be adequate.

Loosli F, Winkler S, Burgtorf C, Wurmbach E, Ansorge W, Henrich T, Grabher C, Arendt D, Carl M, Krone A, et al. **Medaka eyeless is the key factor linking retinal determination and eye growth.** Development 2001;128(20):4035-44.
Abstract: The complete absence of eyes in the medaka fish mutation eyeless is the result of defective optic vesicle evagination. We show that the eyeless mutation is caused by an intronic insertion in the Rx3 homeobox gene resulting in a transcriptional repression of the locus that is rescued by injection of

plasmid DNA containing the wild-type locus. Functional analysis reveals that Six3- and Pax6-dependent retina determination does not require Rx3. However, gain- and loss-of-function phenotypes show that Rx3 is indispensable to initiate optic vesicle evagination and to control vesicle proliferation, by that regulating organ size. Thus, Rx3 acts at a key position coupling the determination with subsequent morphogenesis and differentiation of the developing eye.

Patlewicz GY, Rodford RA, Ellis G, Barratt MD. **A QSAR model for the eye irritation of cationic surfactants.** Toxicol In Vitro 2000;14(1):79-84.
Abstract: A QSAR model for the eye irritation of cationic surfactants has been constructed using a dataset consisting of the maximum average scores (MAS-accordance to Draize) for 29 in vivo rabbit eye irritation tests on 19 different cationic surfactants. The parameters used were logP (log [octanol/water partition coefficient]) and molecular volume (to model the partition of the surfactants into the membranes of the eye), logCMC (log critical micelle concentration-a measure of the reactivity of the surfactants with the eye) together with surfactant concentration. The model was constructed using neural network analysis. MAS showed strongly positive, non-linear correlations with surfactant concentration and logCMC and a strongly negative, non-linear correlation with logP. The Pearson correlation between the actual and predicted values of MAS was 0.838 showing that around 70% (r(2)=0.702) of the variance in the dataset is explained by the model. This value is consistent with levels of biological variability reported historically for the Draize rabbit eye test. The relationship provides a potentially useful prediction model for the eye irritation potential of new or untested cationic surfactants with physicochemical properties lying within the parameter space of the model.

Pinto TJ, de Azevedo JC, Cruz AS. **Comparative study of epithelial and fibroblastic cell lines as an alternative cytotoxicity test to the Draize method**. J AOAC Int 2000;83(3):665-8.
Abstract: Several methods are being used with considerable advantage as alternatives to the Draize test, although some technical difficulties still persist. This work compared the sensitivity of HeLa and NCTC L 929 cells to evaluate the cytotoxicity of shampoos used by adults and children (undiluted and diluted to 25, 5, 1, and 0.1%), and eye drops and their containers and surfactants (diluted to 30, 10, 1, and 0.1%). Nondiluted adult shampoos and their 25 and 5% dilutions were cytotoxic for both cell lines. When diluted to 1%, only one of the shampoos was noncytotoxic, whereas among those diluted to 0.1%, only one was cytotoxic. Children's shampoos were cytotoxic when not diluted or diluted to 25%. From those diluted to 5%, only one was noncytotoxic for both cell lines. The cytotoxic tests showed that the eye drops and their containers were noncytotoxic. Surfactants were cytotoxic when diluted to 30 and 10% and noncytotoxic when diluted to 1 and 0.1%. An excellent correlation (r = 0.95) was demonstrated between the sensitivity of the HeLa and NCTC L929 cells in the evaluation of cytotoxicity reactions.

Pospisil H, Holzhutter HG. **A compartment model to calculate time-dependent concentration profiles of topically applied chemical compounds in the anterior compartments of the rabbit eye.** Altern Lab Anim 2001;29(3):347-65.
Abstract: Hitherto, none of the existing in vitro methods has been convincingly demonstrated to be suitable as a replacement for the Draize rabbit eye irritation test. We examine the hypothesis that one reason for this is that insufficient consideration has been given to the differences in the effective concentrations at which chemicals operate in vitro and in vivo. When a chemical is applied topically to

the eye, the strength of the observed irritation that it elicits depends both on its toxic potential toward cells or tissues, and its effective concentration in the tissues of the eye. Most of the existing in vitro methods are based on isolated cells or tissues, and thus may be useful in assessing the cytotoxic potentials of chemicals. However, a reliable approach to assessing the effective concentrations of chemicals within the various tissues of the eye is lacking. A simplified compartment model is presented for calculating the time-dependent, intra-ocular concentration profiles of topically applied chemicals. The model encompasses the outer surface of the eye, three distinct segments of the cornea (subdivided into the epithelium, stroma and endothelium) and the conjunctiva. Transport through the membranes of these compartments is described as passive diffusion. For the transport coefficients, rate equations are established that contain, as free parameters, the molecular size and the partition coefficient of the chemical, as well as some intrinsic membrane parameters, such as thickness, viscosity and pore density. Numerical values for the unknown membrane parameters were estimated by fitting the theoretical rate equations to measured permeability coefficients. The compartment model was applied to an independent set of 52 test chemicals compiled from the European Commission/UK Home Office validation study. The calculated passage times (required to let 95% of the chemical reach the posterior eye tissues) varied between 0.33 minutes and 50.6 minutes, and are generally much shorter than the typical duration of observed impairments in the cornea or conjunctiva. This finding suggests that short-term contacts of the eye tissues with a chemical are sufficient to elicit long-term eye irritation. An example is given, showing how the conventional approach of using in vitro endpoints as predictors of eye irritation can be improved significantly by incorporating into the prediction the calculated intra-ocular concentration of a chemical.

Ubels JL, Pruis RM, Sybesma JT, Casterton PL. **Corneal opacity, hydration and endothelial morphology in the bovine cornea opacity and permeability assay using reduced treatment times.** Toxicol In Vitro 2000;14(4):379-86.
Abstract: The purpose of this study was to determine whether the standard bovine cornea opacity and permeability (BCOP) assay exposure time of 10 minutes overestimates the ocular irritancy of chemical substances. Corneas were subjected to BCOP protocol following 30-second and 1-minute exposures to irritants. Corneal opacity and hydration (mg H(2)O/mg cornea) were then measured and compared to data obtained after 10 minute irritant treatments. For most test substances corneal opacity and hydration were lower following reduced exposure times. It is suggested that using shorter exposure times in BCOP protocol may be more predictive of human response to ocular irritants, since irritants are usually in brief contact with the ocular surface during accidental exposure. A second purpose of this study was to examine effects of irritants on the corneal endothelium. Corneas were treated according to BCOP protocol following exposure to irritants for 1 or 10 minutes. The endothelium was stained with Alizarin Red and trypan blue, and examined using light microscopy. Severe irritants, such as NaOH and trichloroacetic acid, cause endothelial cell death. It was also determined that simply mounting the cornea in the BCOP assay holders caused damage to 20% of the endothelial cells. Because the endothelium is essential for normal corneal transparency and hydration, it is suggested that examination of the endothelium be added to the BCOP assay and that optimization of the assay will require modification of the cornea holders.

Vinardell MP, Garcia L. **The quantitive chlorioallantoic membrane test using trypan blue stain to**

predict the eye irritancy of liquid scintillation cocktails. Toxicol In Vitro 2000;14(6):551-5.
Abstract: The chorioallantoic membrane-trypan blue staining assay (CAM-TBS) is used to evaluate the potential ocular irritation caused by liquid scintillation cocktails constituted by complex mixtures, including surfactants and other potential irritants. The harmful effect of these substances is determined by the amount of trypan blue adsorbed by the CAM. In the membrane previously treated with the scintillation liquids this amount was concentration dependent only in certain cases, irrespective of the water solubility of the mixtures. In general, it showed a high correlation (r=0.986) with the scores obtained in the Draize eye irritation test. In the present study, only two cocktails presented values of trypan blue adsorption higher than those recorded for their eye irritation in vivo, irrespective of the solvent nature. Unlike the classical HET-CAM procedure, this assay is objective and allows the evaluation of opaque and coloured substances without interfering in the determination of irritancy. Despite these advantages, the method is not suitable for complex mixtures of products that induce ocular irritation in small quantities.

Wilhelmus KR. **The Draize eye test.** Surv Ophthalmol 2001;45(6):493-515.
Abstract: Hundreds of substances are used daily that can damage eyesight. People's eyes are open to accidental or intentional exposure during the production, transportation, use, and disposal of chemical preparations. Ensuring the safety of consumer products was born during the mid twentieth century in the aftermath of chemical warfare research, and was motivated by the hazards of unsafe cosmetics. Justified by an exigency for public protection, the Draize eye test became a governmentally endorsed method to evaluate the safety of materials meant for use in or around the eyes. The test involves a standardized protocol for instilling agents onto the cornea and conjunctiva of laboratory animals. A sum of ordinal-scale items of the outer eye gives an index of ocular morbidity. Advances in ocular toxicology are challenging the validity, precision, relevance, and need of the Draize eye test. Preclinical product-safety tests with rabbits and other mammals also raise ethical concerns of animal wellbeing. Some use the Draize test as a rallying point for how animals are treated in science and industry. A battery of cellular systems and computer models aim to reduce and ultimately to replace whole-animal testing. Molecular measures of ocular toxicity may eventually allow comprehensive screening in humans. The Draize eye test was created and refined for humanitarian reasons and has assuredly prevented harm. Its destiny is to be progressively supplanted as in vitro and clinical alternatives emerge for assessing irritancy of the ocular surface.

Zhao J, Wollmer P. **Air pollutants and tear film stability--a method for experimental evaluation**. Clin Physiol 2001;21(3):282-6.
Abstract: Instability of the pre-ocular tear film causes symptoms of dry eyes. Tear film instability may be caused by exposure to air pollutants, but identification of causative agents is often difficult. We describe an in vitro technique for assessment of putative agents on the surface activity of Meibomian lipids. The lipids were obtained from bovine eyelids and dissolved in chloroform. Surface activity of the solution was assessed on a Wilhelmy balance. After addition of benzalkonium chloride, minimum surface tension of the lipids increased and maximum surface tension fell. After addition of quartz particles, both maximum and minimum surface tension increased. The method can be used to screen substances suspected to cause dry eyes, providing a basis for selection of substances for in vivo studies.

Bee WH. **Standardized electroretinography in primates: a non-invasive preclinical tool for predicting ocular side effects in humans.** Curr Opin Drug Discov Devel 2001;4(1):81-91.
Abstract: This paper reviews the use of a standard protocol for electroretinography in drug safety evaluation studies with non-human primates. The architecture and physiology of the eye of humans and higher primates are very similar, thus making the non-human primate a preferred animal model for evaluating potential ocular side effects of drugs in humans. Using the technical and procedural features of the human protocol, i.e., the standard for clinical electroretinography (SCE), electroretinography can be routinely performed from early- to late-phases of drug development as a toxicodynamic endpoint for retinal toxicity and potential neurotoxicity in non-human primates. Hence, scientists can collect clinically relevant data using a standardized testing procedure found in the SCE. This is important in drug safety evaluations since the SCE is an established protocol that lends itself easily for incorporation into studies requiring adherence to international Good Laboratory Practice regulations. The technique is non-invasive, painless, and confers no adverse effects or damage to the eye.

PHARMACOKINETIC AND MECHANISTIC STUDIES

Andersen M, Sarangapani R, Gentry R, Clewell H, Covington T, Frederick CB. **Application of a hybrid CFD-PBPK nasal dosimetry model in an inhalation risk assessment: an example with acrylic acid.** Toxicol Sci 2000;57(2):312-25.
Abstract: The available inhalation toxicity information for acrylic acid (AA) suggests that lesions to the nasal cavity, specifically olfactory degeneration, are the most sensitive end point for developing a reference concentration (RfC). Advances in physiologically based pharmacokinetic (PBPK) modeling, specifically the incorporation of computational fluid dynamic (CFD) models, now make it possible to estimate the flux of inhaled chemicals within the nasal cavity of experimental species, specifically rats. The focus of this investigation was to apply an existing CFD-PBPK hybrid model in the estimation of an RfC to determine the impact of incorporation of this new modeling technique into the risk assessment process. Information provided in the literature on the toxicity and mode of action for AA was used to determine the risk assessment approach. A comparison of the approach used for the current U.S. Environmental Protection Agency (U.S. EPA) RfC with the approach using the CFD-PBPK hybrid model was also conducted. The application of the CFD-PBPK hybrid model in a risk assessment for AA resulted in an RfC of 79 ppb, assuming a minute ventilation of 13.8 l/min (20 m(3)/day) in humans. This value differs substantially from the RfC of 0.37 ppb estimated for AA by the U.S. EPA before the PBPK modeling advances became available. The difference in these two RfCs arises from many factors, with the main difference being the species selected (mouse vs. rat). The choice to conduct the evaluation using the rat was based on the availability of dosimetry data in this species. Once these data are available in the mouse, an assessment should be conducted using this information. Additional differences included the methods used for estimating the target tissue concentration, the uncertainty factors (UFs) applied, and the application of duration and uncertainty adjustments to the internal target tissue dose rather than the external exposure concentration.

Andersen ME, Dennison JE. **Mode of action and tissue dosimetry in current and future risk assessments.** Sci Total Environ 2001;274(1-3):3-14.
Abstract: Two fundamental concepts have emerged to organize contemporary approaches to chemical

risk assessment - mode of action and tissue dosimetry. Mode of action specifies the nature of the interactions between the chemical and the body that lead to toxic responses and should, under optimal circumstances, also specify the form of the tissue dose that leads to these effects. This paper highlights recent development of biologically based dose response (BBDR) models for specific toxic endpoints that use knowledge on mode of action to specify measures of dose. These dose measures then are used to support low dose and interspecies extrapolations. We first focus on a series of dose response models developed for several compounds that produce nasal toxicity. These examples demonstrate a range of model structures from simple dosimetry models (methylmethacrylate) to linkage of dosimetry with specific biological processes involved in carcinogenesis (formaldehyde). Two BBDR models with dioxin illustrate the organization of biological and dosimetry information into specific testable hypotheses that could distinguish these different models and lead to a more uniform approach to risk assessment for this compound. A final section discusses the impact of molecular biology and the genomic revolution in relation to development of BBDR models for specific toxic endpoints.

Andersen ME, Jarabek AM. **Nasal tissue dosimetry-issues and approaches for "Category 1" gases: a report on a meeting held in Research Triangle Park, NC, February 11-12, 1998.** Inhal Toxicol 2001;13(5):415-35.

Abstract: Three organizations, the Basic Acrylic Monomer Manufacturers (BAMM), Methacrylate Producers Association (MPA), and Vinyl Acetate Toxicology Group (VATG), have sponsored development of physiologically based pharmacokinetic (PBPK) models for nasal tissue dosimetry with, respectively, acrylic acid (AA), methyl methacrylate (MMA), and vinyl acetate (VA). These compounds cause lesions in nasal epithelial tissues and are classified as "Category 1" gases within the U.S. EPA (1994) classification scheme. The National Center for Environmental Assessment in the U.S. EPA Office of Research and Development also has continuing interests in refining its methods for dosimetry adjustments when data on mode of action are available for Category 1 gases. A round-table discussion was held in Research Triangle Park, NC, on 11-12 February 1998, to develop a broader appreciation of the key processes and parameters required in developing nasal tissue dosimetry models. The discussions at the round table drew on these three case studies and several background presentations to assess the manner in which chemical-specific and mode-of-action data can be incorporated into nasal dosimetry models. The round table had representation from the U.S. EPA, academia, and industry. This article outlines the presentations and topical areas discussed at the round table and notes recommendations made by participants to extend models for nasal dosimetry and to develop improved data for modeling. The contributions of several disciplines-toxicology, engineering, and physiologically based pharmacokinetic (PBPK) modeling-were evident in the discussions. The integration of these disciplines in creating opportunities for dosimetry model applications in risk assessments has several advantages in the breadth of skills upon which to draw in model development. A disadvantage is in the need to provide venues and develop cross-discipline dialogue necessary to ensure the understanding of cultural attitudes, terminology, and methods. The round-table discussions were fruitful in achieving such enhanced understanding and communication. Subsequent elaboration of these models will benefit from the interactions of these groups at the round table. The round-table discussions have already led to model improvements-as noted in several recently published articles. Participants emphasized several generic data needs in relation to nasal vapor uptake studies in human subjects, to broader discussion of tissue diffusion models, and to extensions to other classes of gases. The round-table articles that are published

separately in this issue and the discussions, captured in this overview, provide a glimpse of the state of the science in nasal dosimetry modeling and a clear indication of the growth of and continuing opportunities in this important research area.

Andersen ME, Krewski D. **Biologically based risk assessment model for cyclophosphamide hematotoxicity in animal species: concluding comments.** J Toxicol Environ Health A 2000;61(5-6):543-52.

Andersen ME, Sarangapani R, Reitz RH, Gallavan RH, Dobrev ID, Plotzke KP. **Physiological modeling reveals novel pharmacokinetic behavior for inhaled octamethylcyclotetrasiloxane in rats.** Toxicol Sci 2001;60(2):214-31.
Abstract: Octamethylcyclotetrasiloxane (D4) is an ingredient in selected consumer and precision cleaning products. Workplace inhalation exposures may occur in some D4 production operations. In this study, we analyzed tissue, plasma, and excreta time-course data following D4 inhalation in Fischer 344 rats (K. Plotzke et al., 2000, Drug Metab. Dispos. 28, 192-204) to assess the degree to which the disposition of D4 is similar to or different from that of volatile hydrocarbons that lack silicone substitution. We first applied a basic physiologically based pharmacokinetic (PBPK) model (J. C. Ramsey and M. E. Andersen, 1984, Toxicol. Appl. Pharmacol. 73, 159-175) to characterize the biological determinants of D4 kinetics. Parameter estimation techniques indicated an unusual set of characteristics, i.e., a low blood:air (P(b:a) congruent with 0.9) and a high fat:blood partition coefficient (P(f:b) congruent with 550). These parameters were then determined experimentally by equilibrating tissue or liquid samples with saturated atmospheres of D4. Consistent with the estimates from the time-course data, blood:air partition coefficients were small, ranging from 1.9 to 6.9 in six samples. Perirenal fat:air partition coefficients were large, from 1400 to 2500. The average P(f:b) was determined to be 485. This combination of partitioning characteristics leads to rapid exhalation of free D4 at the cessation of the inhalation exposure followed by a much slower redistribution of D4 from fat and tissue storage compartments. The basic PK model failed to describe D4 tissue kinetics in the postexposure period and had to be expanded by adding deep-tissue compartments in liver and lung, a mobile chylomicron-like lipid transport pool in blood, and a second fat compartment. Model parameters for the refined model were optimized using single-exposure data in male and female rats exposed at three concentrations: 7, 70, and 700 ppm. With inclusion of induction of D4 metabolism at 700 ppm (3-fold in males, 1-fold in females), the parameter set from the single exposures successfully predicted PK results from 14-day multiple exposures at 7 and 700 ppm. A common parameter set worked for both genders. Despite its very high lipophilicity, D4 does not show prolonged retention because of high hepatic and exhalation clearance. The high lipid solubility, low blood:air partition coefficient, and plasma lipid storage with D4 led to novel distributional characteristics not previously noted for inhaled organic hydrocarbons. These novel characteristics were only made apparent by analysis of the time-course data with PBPK modeling techniques.

Andreeva AV, Zheng H, Saint-Jore CM, Kutuzov MA, Evans DE, Hawes CR. **Organization of transport from endoplasmic reticulum to Golgi in higher plants.** Biochem Soc Trans 2000;28(4):505-12.
Abstract: In plant cells, the organization of the Golgi apparatus and its interrelationships with the

endoplasmic reticulum differ from those in mammalian and yeast cells. Endoplasmic reticulum and Golgi apparatus can now be visualized in plant cells in vivo with green fluorescent protein (GFP) specifically directed to these compartments. This makes it possible to study the dynamics of the membrane transport between these two organelles in the living cells. The GFP approach, in conjunction with a considerable volume of data about proteins participating in the transport between endoplasmic reticulum and Golgi in yeast and mammalian cells and the identification of their putative plant homologues, should allow the establishment of an experimental model in which to test the involvement of the candidate proteins in plants. As a first step towards the development of such a system, we are using Sar1, a small G-protein necessary for vesicle budding from the endoplasmic reticulum. This work has demonstrated that the introduction of Sar1 mutants blocks the transport from endoplasmic reticulum to Golgi in vivo in tobacco leaf epidermal cells and has therefore confirmed the feasibility of this approach to test the function of other proteins that are presumably involved in this step of endomembrane trafficking in plant cells.

Andrews NC. **Iron homeostasis: insights from genetics and animal models.** Nat Rev Genet 2000;1 (3):208-17.
Abstract: Disorders that perturb iron balance are among the most prevalent human diseases, but until recently iron transport remained poorly understood. Over the past five years, genetic studies of patients with inherited iron homeostasis disorders and the analysis of mutant mice, rats and zebrafish have helped to identify several important iron-transport proteins. With information being mined from the genomes of four species, the study of iron metabolism has benefited enormously from positional-cloning efforts. Complementing the genomic strategy, targeted mutagenesis in mice has produced new models of human iron diseases. The animal models described in this review offer valuable tools for investigating iron homeostasis in vivo.

Annas A, Brunstrom B, Brittebo EB. **CYP1A-dependent activation of xenobiotics in endothelial linings of the chorioallantoic membrane (CAM) in birds.** Arch Toxicol 2000;74(6):335-42.
Abstract: Metabolic activation of the heterocyclic amine 3-amino -1,4-dimethyl-5 H-pyrido[4,3-b]indole (Trp-P-1) and 7-ethoxyresorufin O-deethylase (EROD) activity were examined in the chorioallantoic membrane (CAM) of 15-day-old chicken and 18-day-old eider duck embryos. The embryos were pretreated with an Ah receptor agonist, i.e. beta-naphthoflavone (BNF) or 3,3',4,4',5-pentachlorobiphenyl (PCB 126), or vehicle in ovo. BNF and PCB 126 induced EROD activity and covalent binding of [3H]Trp-P-1 seven- to tenfold in the CAM of chicken embryos. In the CAM of eider duck embryos, which are known to be nonresponsive to coplanar PCBs, PCB 126 treatment had no effect on EROD activity or covalent binding of [3H]Trp-P-1 whereas BNF treatment increased these activities five- and threefold, respectively. Light microscopic autoradiography was used to identify the cellular localization of covalent binding of [3H]Trp-P-1 in the CAM. Preferential binding was observed in endothelial cells in intraepithelial capillaries in the chorionic epithelium and in blood vessels in the mesenchymal layer. The addition of the CYP1A inhibitor ellipticine abolished the covalent binding of [3H]Trp-P-1 in the CAM of BNF- and PCB 126-treated chicken and eider duck embryos. The results suggest that CYP1A-dependent metabolic activity can be induced in blood vessel endothelia in the CAM of bird embryos following exposure to Ah receptor agonists and that the CAM may be a target tissue for CYP1A-activated environmental pollutants. Furthermore, the highly vascularized CAM could be used as

a model for studies of Ah receptor-mediated alterations in the vasculature.

Arazi T, Kaplan B, Sunkar R, Fromm H. **Cyclic-nucleotide- and Ca2+/calmodulin-regulated channels in plants: targets for manipulating heavy-metal tolerance, and possible physiological roles.** Biochem Soc Trans 2000;28(4):471-5.
Abstract: Recently we discovered a tobacco protein (designated NtCBP4) that modulates heavy-metal tolerance in transgenic plants. Structurally, NtCBP4 is similar to mammalian cyclic-nucleotide-gated non-selective cation channels containing six putative transmembrane domains, a predicted pore region, a conserved cyclic-nucleotide-binding domain, and a high-affinity calmodulin-binding site that coincides with its cyclic-nucleotide-binding domain. Transgenic tobacco expressing the plasma-membrane-localized NtCBP4 exhibit improved tolerance to Ni(2+) and hypersensitivity to Pb(2+), which are associated with a decreased accumulation of Ni(2+) and an enhanced accumulation of Pb(2+) respectively. Transgenic plants expressing a truncated version of NtCBP4, from which regulatory domains had been removed, have a different phenotype. Here we describe our approach to studying the involvement of NtCBP4 in heavy-metal tolerance and to elucidate its physiological role.

arrington JS, Shader RI, von Moltke LL, Greenblatt DJ. **In vitro biotransformation of sildenafil (Viagra): identification of human cytochromes and potential drug interactions.** Drug Metab Dispos 2000;28(4):392-7.
Abstract: The in vitro biotransformation of sildenafil to its major circulating metabolite, UK-103,320, was studied in human liver microsomes and in microsomes containing heterologously expressed human cytochromes. In human liver microsomes, the mean K(m) (+/-S.E.) was 14.4 +/- 2.0 microM. A screen of the chemical inhibitors omeprazole (10 microM), quinidine (10 microM), sulfaphenazole (10 microM), and ketoconazole (2.5 microM) only revealed detectable inhibition with ketoconazole. Sildenafil biotransformation (36 microM) was inhibited by increasing concentrations of ketoconazole and ritonavir (IC(50) values less than 0.02 microM), which are established cytochrome P450 (CYP) 3A4 inhibitors. Using microsomes containing cDNA-expressed cytochromes, UK-103,320 formation was found to be mediated by four cytochromes: CYP3A4, -2C9, -2C19, and -2D6. Estimated relative contributions to net intrinsic clearance were 79% for CYP3A4 and 20% for CYP2C9; for CYP2C19 and -2D6, estimated contributions were less than 2%. These results demonstrate that CYP3A4 is the primary cytochrome mediating UK-103,320 formation and that drugs that inhibit CYP3A4 are likely to impair sildenafil biotransformation.

Bae DS, Gennings C, Carter WH Jr, Yang RS, Campain JA. **Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes.** Toxicol Sci 2001;63(1):132-42.
Abstract: To evaluate health effects of chemical mixtures, such as multiple heavy metals in drinking water, we have been developing efficient and accurate hazard identification strategies. Thus, in this study, we determine the cytotoxicity of arsenic, cadmium, chromium, and lead, and characterize interactions among these metals in human epidermal keratinocytes. Three immortal keratinocyte cell lines (RHEK-1, HaCaT, and NM1) and primary keratinocytes (NHEK) were used. A statistical approach applying an additivity response surface methodology was used to test the validity of the additivity concept for a 4-metal mixture. Responses of the 4 keratinocyte strains to the metal mixture were highly dose-dependent. A growth stimulatory effect (hormesis) was observed in RHEK-1, NM1, and NHEK

cells with the metal mixture at low concentrations (low ppb range). This hormesis effect was not significant in HaCaT. As the mixture concentration increased, a trend of additivity changed to synergistic cytotoxicity in all 4 cell strains. However, in NHEK, RHEK-1, and HaCaT, at the highest mixture concentrations tested, the responses to the metal mixtures were antagonistic. In NM1, no significant antagonistic interaction among the metals was observed. To explore a mechanistic basis for these differential sensitivities, levels of glutathione and metallothioneins I and II were determined in the keratinocyte cell strains. Initial data are consistent with the suggestion that synergistic cytotoxicity turned to antagonistic effects because at highest mixture exposure concentrations cellular defense mechanisms were enhanced.

Bakowski D, Glitsch MD, Parekh AB. **An examination of the secretion-like coupling model for the activation of the Ca2+ release-activated Ca2+ current I(CRAC) in RBL-1 cells.** J Physiol 2001;532 (Pt 1):55-71.

Abstract: One popular model for the activation of store-operated Ca2+ influx is the secretion-like coupling mechanism, in which peripheral endoplasmic reticulum moves to the plasma membrane upon store depletion thereby enabling inositol 1,4,5-trisphosphate (InsP3) receptors on the stores to bind to, and thus activate, store-operated Ca2+ channels. This movement is regulated by the underlying cytoskeleton. We have examined the validity of this mechanism for the activation of I(CRAC), the most widely distributed and best characterised store-operated Ca2+ current, in a model system, the RBL-1 rat basophilic cell line. Stabilisation of the peripheral cytoskeleton, disassembly of actin microfilaments and disaggregation of microtubules all consistently failed to alter the rate or extent of activation of I(CRAC). Rhodamine-phalloidin labelling was used wherever possible, and revealed that the cytoskeleton had been significantly modified by drug treatment. Interference with the cytoskeleton also failed to affect the intracellular calcium signal that occurred when external calcium was re-admitted to cells in which the calcium stores had been previously depleted by exposure to thapsigargin/ionomycin in calcium-free external solution. Application of positive pressure through the patch pipette separated the plasma membrane from underlying structures (cell ballooning). However, I(CRAC) was unaffected irrespective of whether cell ballooning occurred before or after depletion of stores. Pre-treatment with the membrane-permeable InsP3 receptor antagonist 2-APB blocked the activation of I(CRAC). However, intracellular dialysis with 2-APB failed to prevent I(CRAC) from activating, even at higher concentrations than those used extracellularly to achieve full block. Local application of 2-APB, once I(CRAC) had been activated, resulted in a rapid loss of the current at a rate similar to that seen with the rapid channel blocker La3+. Studies with the more conventional InsP3 receptor antagonist heparin revealed that occupation of the intracellular InsP3-sensitive receptors was not necessary for the activation or maintenance of I(CRAC). Similarly, the InsP3 receptor inhibitor caffeine failed to alter the rate or extent of activation of I(CRAC). Exposure to Li+, which reduces InsP3 levels by interfering with inositol monophosphatase, also failed to alter I(CRAC). Caffeine and Li+ did not affect the size of the intracellular Ca2+ signal that arose when external Ca2+ was re-admitted to cells which had been pre-exposed to thapsigargin/ionomycin in Ca2+-free external solution. Our findings demonstrate that the cytoskeleton does not seem to regulate calcium influx and that functional InsP3 receptors are not required for activation of I(CRAC). If the secretion-like coupling model indeed accounts for the activation of I(CRAC) in RBL-1 cells, then it needs to be revised significantly. Possible modifications to the model are discussed.

Banerjee BD, Seth V, Ahmed RS. **Pesticide-induced oxidative stress: perspectives and trends.** Rev Environ Health 2001;16(1):1-40.
Abstract: Pesticide-induced oxidative stress as a possible mechanism of toxicity has been a focus of toxicological research for the last decade. Yet for certain pesticides, mechanisms leading to oxidative stress are only partly understood. Pesticide-induced oxidative stress is the final manifestation of a multi-step pathway, resulting in an imbalance between pro-oxidant and antioxidant defense mechanisms. Concomitantly, pesticide intoxication induces a derangement of certain antioxidant mechanisms in different tissues, including alterations in antioxidant enzymes and the glutathione redox system. In this article, we discuss the impact of certain factors that are important in the potentiation of pesticide-induced oxidative stress, immunotoxicity, and apoptosis. Understanding risk factors largely depends upon the cellular and molecular events underlying pesticide-induced stress in experimental animals. These factors must be considered in the safety/toxicity evaluation of any pesticide. The identification and characterization of plant products/drugs might be helpful for understanding the mechanisms of compensation and repair that are due to oxidative stress-induced injury. This paper reviews the nature of such damage, the cellular conditions in which it occurs, and oxidative-stress data that may be applied to the development of risk-assessment methods and models that are designed to reduce some of these uncertainties.

Barber DS, Ehrich M. **Esterase inhibition in SH-SY5Y human neuroblastoma cells following exposure to organophosphorus compounds for 28 days.** In Vitr Mol Toxicol 2001;14(2):129-35.
Abstract: Esterase inhibition was determined in SH-SY5Y human neuroblastoma cells grown in serum-free media and exposed to 10(-11) to 10(-7) M concentrations of organophosphorus (OP) compounds for 28 days. To examine metabolic activation in these exposures, pairs of pro- and active toxicants were studied, including chlorpyrifos and its oxon, parathion and paraoxon, and tri-ortho-tolyl phosphate and phenyl saligenin phospahte. Inhibition of acetylcholinesterase was greater in cells treated for 28 days with all active organophosphorus compounds than it was in cells treated only once with the same concentration of a given OP compound. The protoxicants chlorpyrifos and parathion produced acetylcholinesterase inhibition after multiple exposures although no inhibition was seen following a single exposure to these agents. Exacerbation of neurotoxic esterase inhibition by multiple exposures to the test compounds was not as pronounced as that of acetylcholinesterase. Exposure to the test compounds for 28 days did not significantly enhance esterase inhibition produced by a subsequent exposure to 10(-9) M chlorpyrifos-oxon. The results indicate that in vitro methods can be used to study the effect of multiple OP exposures on esterase activity.

Barton HA, Clewell HJ 3rd. **Evaluating noncancer effects of trichloroethylene: dosimetry, mode of action, and risk assessment.** Environ Health Perspect 2000;108 Suppl 2:323-34.
Abstract: Alternatives for developing chronic exposure limits for noncancer effects of trichloroethylene (TCE) were evaluated. These alternatives were organized within a framework for dose-response assessment--exposure:dosimetry (pharmacokinetics):mode of action (pharmacodynamics): response. This framework provides a consistent structure within which to make scientific judgments about available information, its interpretation, and use. These judgments occur in the selection of critical studies, internal dose metrics, pharmacokinetic models, approaches for interspecies extrapolation of

pharmacodynamics, and uncertainty factors. Potentially limiting end points included developmental eye malformations, liver effects, immunotoxicity, and kidney toxicity from oral exposure and neurological, liver, and kidney effects by inhalation. Each end point was evaluated quantitatively using several methods. Default analyses used the traditional no-observed adverse effect level divided by uncertainty factors and the benchmark dose divided by uncertainty factors methods. Subsequently, mode-of-action and pharmacokinetic information were incorporated. Internal dose metrics were estimated using a physiologically based pharmacokinetic (PBPK) model for TCE and its major metabolites. This approach was notably useful with neurological and kidney toxicities. The human PBPK model provided estimates of human exposure doses for the internal dose metrics. Pharmacodynamic data or default assumptions were used for interspecies extrapolation. For liver and neurological effects, humans appear no more sensitive than rodents when internal dose metrics were considered. Therefore, the interspecies uncertainty factor was reduced, illustrating that uncertainty factors are a semiquantitative approach fitting into the organizational framework. Incorporation of pharmacokinetics and pharmacodynamics can result in values that differ significantly from those obtained with the default methods.

Barton HA, Deisinger PJ, English JC, Gearhart JN, Faber WD, Tyler TR, Banton MI, Teeguarden J, Andersen ME. **Family approach for estimating reference concentrations/doses for series of related organic chemicals.** Toxicol Sci 2000;54(1):251-61.
Abstract: The family approach for related compounds can be used to evaluate hazard and estimate reference concentrations/doses using internal dose metrics for a group (family) of metabolically related compounds. This approach is based upon a simple four-step framework for organizing and evaluating toxicity data: 1) exposure, 2) tissue dosimetry, 3) mode of action, and 4) response. Expansion of the traditional exposure-response analysis has been increasingly incorporated into regulatory guidance for chemical risk assessment. The family approach represents an advancement in the planning and use of toxicity testing that is intended to facilitate the maximal use of toxicity data. The result is a methodology that makes toxicity testing and the development of acceptable exposure limits as efficient and effective as possible. An example is provided using butyl acetate and its metabolites (butanol, butyraldehyde, and butyrate), widely used chemicals produced synthetically by the industrial oxo process. A template pharmacokinetic model has been developed that comprises submodels for each compound linked in series. This preliminary model is being used to coordinately plan toxicity studies, pharmacokinetic studies, and analyses to obtain reference concentrations/doses. Implementation of the family approach using pharmacokinetic modeling to obtain tissue dose metrics is described and its applications are evaluated.

Beamish HJ, Jessberger R, Riballo E, Priestley A, Blunt T, Kysela B, Jeggo PA. **The C-terminal conserved domain of DNA-PKcs, missing in the SCID mouse, is required for kinase activity.** Nucleic Acids Res 2000;28(7):1506-13.
Abstract: DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase (DNA-PK), has a phosphoinositol 3-kinase (PI 3-K) domain close to its C-terminus. Cell lines derived from the SCID mouse have been utilised as a model DNA-PKcs-defective system. The SCID mutation results in truncation of DNA-Pkcs at the extreme C-terminus leaving the PI 3-K domain intact. The mutated protein is expressed at low levels in most SCID cell lines, leaving open the question of whether the mutation abolishes kinase activity. Here, we show that a SCID cell line that expresses the mutant protein

normally has dramatically impaired kinase activity. We estimate that the residual kinase activity typically present in SCID fibroblast cell lines is at least two orders of magnitude less than that found in control cells. Our results substantiate evidence that DNA-PKcs kinase activity is required for DSB rejoining and V(D)J recombination and show that the extreme C-terminal region of DNA-PKcs, present in PI 3-K-related protein kinases but absent in bona fide PI 3 lipid kinases, is required for DNA-PKcs to function as a protein kinase. We also show that expression of mutant DNA-PKcs protein confers a growth disadvantage, providing an explanation for the lack of DNA-PKcs expression in most SCID cell lines.

Beck BD, Mattuck RL, Bowers TS, Cohen JT, O'Flaherty E. **The development of a stochastic physiologically-based pharmacokinetic model for lead.** Sci Total Environ 2001;274(1-3):15-9.
Abstract: This presentation describes the development of a prototype Monte Carlo module for the physiologically-based pharmacokinetic (PBPK) model for lead, created by Dr Ellen O'Flaherty. The module uses distributions for the following: exposure parameters (soil and dust concentrations, daily soil and ingestion rate, water lead concentration, water ingestion rate, air lead concentration, inhalation rate and dietary lead intake); absoption parameters; and key pharmacokinetic parameters (red blood binding capacity and half saturation concentration). Distributions can be specified as time-invariant or can change with age. Monte Carlo model predicted blood levels were calibrated to empirically measured blood lead levels for children living in Midvale, Utah (a milling/smelting community). The calibrated model was then evaluated using blood lead data from Palmerton, Pennsylvania (a town with a former smelter) and Sandy, Utah, (a town with a former smelter and slag piles). Our initial evaluation using distributions for exposure parameters showed that the model accurately predicted geometric (GM) blood lead levels of Palmerton and Sandy and slightly over predicted the GSD. Consideration of uncertainty in red blood cell parameters substantially inflated the GM. Future model development needs to address the correlation among parameters and the use of parameters for long-term exposure derived from short-term studies.

Berglund MM, Lundell I, Eriksson H, Soll R, Beck-Sickinger AG, Larhammar D. **Studies of the human, rat, and guinea pig Y4 receptors using neuropeptide Y analogues and two distinct radioligands.** Peptides 2001;22(3):351-6.
Abstract: The neuropeptide Y-family receptor Y4 differs extensively between human and rat in sequence, receptor binding, and anatomical distribution. We have investigated the differences in binding profile between the cloned human, rat, and guinea pig Y4 receptors using NPY analogues with single amino acid replacements or deletion of the central portion. The most striking result was the increase in affinity for the rat receptor, but not for human or guinea pig, when amino acid 34 was replaced with proline; [Ahx(8-20),Pro(34)]NPY bound to the rat Y4 receptor with 20-fold higher affinity than [Ahx(8-20)]NPY. Also, the rat Y4 tolerates alanine in position 34 since p[Ala(34)]NPY bound with similar affinity as pNPY while the affinity for hY4 and gpY4 decreased about 50-fold. Alanine substitutions in position 33, 35, and 36 as well as the large loop-deletion, [Ahx(5-24)]NPY, reduced the binding affinity to all three receptors more than 100-fold. NPY and PYY competed with (125)I-hPP at Y4 receptors expressed in CHO cells according to a two-site model. This was investigated for gpY4 by saturation with either radiolabeled hPP or pPYY. The number of high-affinity binding-sites for (125)I-pPYY was about 60% of the receptors recognized by (125)I-hPP. Porcine [Ala(34)]NPY and [Ahx(8-20)]NPY

bound to rY4 (but not to hY4 or gpY4) according to a two-site model. These results suggest that different full agonists can distinguish between different active conformations of the gpY4 receptor and that Y4 may display functional differences in vivo between human, guinea pig, and rat.

Bernillon P, Bois FY. **Statistical issues in toxicokinetic modeling: a bayesian perspective.** Environ Health Perspect 2000;108 Suppl 5:883-93.
Abstract: Determining the relationship between an exposure and the resulting target tissue dose is a critical issue encountered in quantitative risk assessment (QRA). Classical or physiologically based toxicokinetic (PBTK) models can be useful in performing that task. Interest in using these models to improve extrapolations between species, routes, and exposure levels in QRA has therefore grown considerably in recent years. In parallel, PBTK models have become increasingly sophisticated. However, development of a strong statistical foundation to support PBTK model calibration and use has received little attention. There is a critical need for methods that address the uncertainties inherent in toxicokinetic data and the variability in the human populations for which risk predictions are made and to take advantage of a priori information on parameters during the calibration process. Natural solutions to these problems can be found in a Bayesian statistical framework with the help of computational techniques such as Markov chain Monte Carlo methods. Within such a framework, we have developed an approach to toxicokinetic modeling that can be applied to heterogeneous human or animal populations. This approach also expands the possibilities for uncertainty analysis. We present a review of these efforts and other developments in these areas. Appropriate statistical treatment of uncertainty and variability within the modeling process will increase confidence in model results and ultimately contribute to an improved scientific basis for the estimation of occupational and environmental health risks.

Besset V, Scott RP, Ibanez CF. **Signaling complexes and protein-protein interactions involved in the activation of the Ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase.** J Biol Chem 2000;275(50):39159-66.
Abstract: Proximal signaling events and protein-protein interactions initiated after activation of the c-Ret receptor tyrosine kinase by its ligand, glial cell line-derived neurotrophic factor (GDNF), were investigated in cells carrying native and mutated forms of this receptor. Mutation of Tyr-1062 (Y1062F) in the cytoplasmic tail of c-Ret abolished receptor binding and phosphorylation of the adaptor Shc and eliminated activation of Ras by GDNF. Phosphorylation of Erk kinases was also greatly attenuated but not eliminated by this mutation. This residual wave of Erk phosphorylation was independent of the kinase activity of c-Ret. Mutation of Tyr-1096 (Y1096F), a binding site for the adaptor Grb2, had no effect on Erk activation by GDNF. Activation of phosphatidylinositol-3 kinase (PI3K) and its downstream effector Akt was also reduced in the Y1062F mutant but not completely abolished unless Tyr-1096 was also mutated. Ligand stimulation of neuronal cells induced the assembly of a large protein complex containing c-Ret, Grb2, and tyrosine-phosphorylated forms of Shc, p85(PI3K), the adaptor Gab2, and the protein-tyrosine phosphatase SHP-2. In agreement with Ras-independent activation of PI3K by GDNF in neuronal cells, survival of sympathetic neurons induced by GDNF was dependent on PI3K but was not affected by microinjection of blocking anti-Ras antibodies, which did compromise neuronal survival by nerve growth factor, suggesting that Ras is not required for GDNF-induced survival of sympathetic neurons. These results indicate that upon ligand stimulation, at least two distinct protein

complexes assemble on phosphorylated Tyr-1062 of c-Ret via Shc, one leading to activation of the Ras/Erk pathway through recruitment of Grb2/Sos and another to the PI3K/Akt pathway through recruitment of Grb2/Gab2 followed by p85(PI3K) and SHP-2. This latter complex can also assemble directly onto phosphorylated Tyr-1096, offering an alternative route to PI3K activation by GDNF.

Biaglow JE, Donahue J, Tuttle S, Held K, Chrestensen C, Mieyal J. **A method for measuring disulfide reduction by cultured mammalian cells: relative contributions of glutathione-dependent and glutathione-independent mechanisms.** Anal Biochem 2000;281(1):77-86.
Abstract: A method is described for measuring bioreduction of hydroxyethyl disulfide (HEDS) or alpha-lipoate by human A549 lung, MCF7 mammary, and DU145 prostate carcinomas as well as rodent tumor cells in vitro. Reduction of HEDS or alpha-lipoate was measured by removing aliquots of the glucose-containing media and measuring the reduced thiol with DTNB (Ellman's reagent). Addition of DTNB to cells followed by disulfide addition directly measures the formation of newly reduced thiol. A549 cells exhibit the highest capacity to reduce alpha-lipoate, while Q7 rat hepatoma cells show the highest rate of HEDS reduction. Millimolar quantities of reduced thiol are produced for both substrates. Oxidized dithiothreitol and cystamine were reduced to a lesser degree. DTNB, glutathione disulfide, and cystine were only marginally reduced by the cell cultures. Glucose-6-phosphate deficient CHO cells (E89) do not reduce alpha-lipoate and reduce HEDS at a much slower rate compared to wild-type CHO-K1 cells. Depletion of glutathione prevents the reduction of HEDS. The depletion of glutathione inhibited reduction of alpha-lipoate by 25% and HEDS by 50% in A549 cells, while GSH depletion did not inhibit alpha-lipoate reduction in Q7 cells but completely blocked HEDS reduction. These data suggest that the relative participation of the thioltransferase (glutaredoxin) and thioredoxin systems in overall cellular disulfide reduction is cell line specific. The effects of various inhibitors of the thiol-disulfide oxidoreductase enzymes (1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), arsenite, and phenylarsine oxide) support this conclusion.

Bird MG, Lewis DF, Whitman FT, Lewis RJ, Przygoda RT, Witz G. **Application of process chemistry and SAR modelling to the evaluation of health findings of lower olefins.** Chem Biol Interact 2001;135-136:571-84.
Abstract: Epidemiology studies show increased leukemia mortality among styrene butadiene rubber (SBR) workers but not among butadiene monomer production employees. A detailed review of the SBR manufacturing process indicates that sodium dimethyldithiocarbamate (DMDTC) introduced into the SBR manufacturing process for a period in the 1950s coincides with increased leukemia mortality. Using the Computer-Optimized Molecular Parametric Analysis of Chemical Toxicity (COMPACT), we assessed the enzyme (cytochrome P450) substrate specificity of an olefin series including 1,3-butadiene (BD) and also modeled its interaction with DMDTC. These analyses showed correlation of a structural/electronic parameter - the COMPACT radius - with the presence or absence of cytogenetic activity and also found that DMDTC would inhibit the oxidative metabolism of BD at least at high concentrations. Both DMDTC and its diethyl analog (DEDTC) bind with CYP 2E1 and CYP 2A6. Both of these isoforms are important in the initial oxidative metabolism of butadiene and other olefins. In co-exposure studies in mice of DMDTC with BD or with epoxybutene (EB), we found that there was a reduced increase in genotoxic activity based on micronuclei induction compared with BD or EB exposure alone. Treatment with DMDTC significantly increased the protein carbonyl contents of hepatic microsomes

compared with that of controls, a finding that may be related to DMDTC's activity as a prooxidant. Co-exposure with DMDTC and EB increased hepatic microsomal carbonyls to levels significantly greater than those of DMDTC-treated mice, while EB administration in the absence of DMDTC did not change protein carbonyls relative to those of controls. The increase in hepatic microsomal protein carbonyls suggests that DMDTC may modulate EB metabolism towards the formation of reactive intermediates that react with proteins. The present molecular modeling and mechanistic studies suggest that co-exposure of BD and DMDTC is a plausible biological hypothesis regarding increased leukemia risk among SBR workers.

Blaauboer BJ. **Toxicodynamic modelling and the interpretation of in vitro toxicity data.** Toxicol Lett 2001;120(1-3):111-23.
Abstract: The results of in vitro toxicity experiments are not easily extrapolated to 'toxicological risk' for an intact organism. One of the most obvious differences between the situation in vitro and in vivo is the absence of the processes of absorption, distribution, metabolism and excretion that govern the exposure of the target tissues of the organism in vivo. The development of biokinetic models is aimed at estimating the relevant target tissue concentration of a compound. In our study, biokinetic models were constructed, where possible, solely on the basis of in vitro derived parameters for biotransformation as well as on partition coefficients determined or calculated from physicochemical structures. Another requirement is the existence of appropriate in vitro biological systems for the measurement of relevant effects. This requires a thorough knowledge of the possible mechanisms of toxic action, and of the physiology of the target organs. When these prerequisites are met (i.e. when the appropriate parameters can be quantified in a non-animal system), then an estimate of the dynamics in vitro can be made (e.g. as a critical active concentration). This will then result in a model describing a compound's dynamics. Eventually, the result of biokinetic and toxicodynamic models will need to be integrated in a compound's hazard and/or risk evaluation. A study carried out in the ECITTS programme showed promising results for the estimation of the acute and chronic systemic toxicity of a number of neurotoxic compounds.

Blasina MF, Faria AC, Gardino PF, Hokoc JN, Almeida OM, de Mello FG, Arruti C, Dajas F. **Evidence for a noncholinergic function of acetylcholinesterase during development of chicken retina as shown by fasciculin.** Cell Tissue Res 2000;299(2):173-84.
Abstract: Fasciculin 2 (FAS), an acetylcholinesterase (AChE) peripheral site ligand that inhibits mammalian AChE in the picomolar range and chicken AChE only at micromolar concentrations, was used in chick retinal cell cultures to evaluate the influence of AChE on neuronal development. The effects of other AChE inhibitors that bind the active and/or the peripheral site of the enzyme [paraoxon, eserine, or 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51)] were also studied. Morphological changes of cultured neurons were observed with the drugs used and in the different cell culture systems studied. Cell aggregates size decreased by more than 35% in diameter after 9 days of FAS treatment, mainly due to reduction in the presumptive plexiform area of the aggregates. Eserine showed no effect on the morphology of the aggregates, although it fully inhibited the activity of AChE. In dense stationary cell culture, cluster formation increased after 3 days and 6 days of FAS treatment. However, FAS, at concentrations in which changes of morphological parameters were observed, did not inhibit the AChE activity as measured histochemically. In contrast, paraoxon treatment

produced a slight morphological alteration of the cultures, while a strong inhibition of enzyme activity caused by this agent was observed. BW284c51 showed a harmful, probably toxic effect, also causing a slight AChE inhibition. It is suggested that the effect of an anticholinesterase agent on the morphological modifications of cultured neurons is not necessarily associated with the intensity of the AChE inhibition, especially in the case of FAS. Moreover, most of the effects of AChE on culture morphology appear to be independent of the cholinolytic activity of the enzyme. The results obtained demonstrate that FAS is not toxic for the cells and suggest that regions of the AChE molecule related to the enzyme peripheral site are likely to be involved with the nonclassical role of AChE.

Bloch KO, Zemel R, Bloch OV, Grief H, Vardi P. **Streptozotocin and alloxan-based selection improves toxin resistance of insulin-producing RINm cells.** Int J Exp Diabetes Res 2000;1(3):211-9.
Abstract: The aim of our study was to develop a method for selection of subpopulations of insulin producing RINm cells with higher resistance to beta cell toxins. Cells, resistant to streptozotocin (RINmS) and alloxan (RINmA), were obtained by repeated exposure of parental RINm cells to these two toxins, while the defense capacity was estimated by the MTT colorimetric method, and [3H]-thymidine incorporation assay. We found that RINmS and RINmA displayed higher resistance to both streptozotocin (STZ) and alloxan (AL) when compared to the parental RINm cells. In contrast, no differences in sensitivity to hydrogen peroxide were found between toxin selected and parental cells. Partial protection from the toxic effect of STZ and AL was obtained only in the parental RINm cells after preincubation of cells with the unmetabolizable 3-O-methyl-glucose. The possibility that GLUT-2 is involved in cell sensitivity to toxins was confirmed by Western blot analysis, which showed higher expression of GLUT-2 in parental RINm compared to RINmS and RINmA cells. In addition to the higher cell defense property evidenced in the selected cells, we also found higher insulin content and insulin secretion in both RINmS and RINmA cells when compared to the parental RINm cells. In conclusion, STZ and AL treatment can be used for selection of cell sub-populations with higher cell defense properties and hormone production. The different GLUT-2 expression in parental and resistant cells suggest involvement of GLUT-2 in mechanisms of cell response to different toxins.

Blondelle SE, Lohner K. **Combinatorial libraries: a tool to design antimicrobial and antifungal peptide analogues having lytic specificities for structure-activity relationship studies.** Biopolymers 2000;55(1):74-87.
Abstract: In the race for supremacy, microbes are sprinting ahead. This warning by the World Health Organization clearly demonstrates that the spread of antibiotic-resistant bacteria leads to a global health problem and that antibiotics never seen before by bacteria are urgently needed. Antimicrobial peptides represent such a source for novel antibiotics due to their rapid lytic activity (within minutes) through disruption of cell membranes. However, due to the similarities between bacterial, fungal, and mammalian plasma cell membranes, a large number of antimicrobial peptides have low lytic specificities and exhibit a broad activity spectrum and/or significant toxic effect toward mammalian cells. Mutation strategies have allowed the development of analogues of existing antimicrobial peptides having greater lytic specificities, although such methods are lengthy and would be more efficient if the molecular mechanisms of action of antimicrobial peptides were clearly elucidated. Synthetic combinatorial library approaches have brought a new dimension to the design of novel biologically active compounds. Thus, a set of peptide analogues were generated based on the screening of a library

built around an existing lytic peptide, and on a deconvolution strategy directed toward activity specificity. These peptide analogues also served as model systems to further study the effect of biomembrane mimetic systems on the peptides structural behavior relevant to their biological activities.

Boatright JH, Nickerson JM, Borst DE. **Site-specific DNA hypomethylation permits expression of the IRBP gene**. Brain Res 2000;887(1):211-21.
Abstract: Interphotoreceptor retinoid binding protein (IRBP), a putative component of the visual cycle, is expressed selectively in the retina and pineal gland. This study examined whether site-specific DNA hypomethylation plays a role in this expression regulation. Southern blotting of HpaII and MspI digests of DNA from various bovine and murine tissues (whole brain, retina, pineal gland, superior colliculus, cortex, thymus, habenular nucleus, cornea, liver, tail, and kidney) revealed that specific CpG dinucleotides in the IRBP gene promoter are hypomethylated in DNA from retinal photoreceptor cells and pineal gland compared to DNA from other tissues. These sites are methylated in DNA from non-photoreceptor retinal cells. Exogenous methylation of these sites diminished DNA:protein binding in electrophoretic mobility shift assays. HpaII methylation of chloramphenicol acetyltransferase reporter constructs suppressed IRBP but not SV40 promoter activity in transiently transfected primary cultures of embryonic chick retinal cells. These data indicate that specific cytosines in the bovine and murine IRBP promoters are unmethylated in photoreceptive cells but methylated in other tissues. This differential DNA methylation may modulate IRBP gene expression since exogenous methylation of the murine sites suppresses reporter gene transcription, apparently by inhibiting DNA:protein binding events.

Boettiger D, Huber F, Lynch L, Blystone S. **Activation of alpha(v)beta3-vitronectin binding is a multistage process in which increases in bond strength are dependent on Y747 and Y759 in the cytoplasmic domain of beta3 integrin.** Mol Biol Cell 2001;12(5):1227-37.
Abstract: Integrin receptors serve as mechanical links between the cell and its structural environment. Using alpha(v)beta3 integrin expressed in K562 cells as a model system, the process by which the mechanical connection between alpha(v)beta3 and vitronectin develops was analyzed by measuring the resistance of these bonds to mechanical separation. Three distinct stages of activation, as defined by increases in the alpha(v)beta3-vitronectin binding strength, were defined by mutational, biochemical, and biomechanical analyses. Activation to the low binding strength stage 1 occurs through interaction with the vitronectin ligand and leads to the phosphorylation of Y747 in the beta3 subunit. Stage 2 is characterized by a 4-fold increase in binding strength and is dependent on stage1 and the phosphorylation of Y747. Stage 3 is characterized by a further 2.5-fold increase in binding strength and is dependent on stage 2 events and the availability of Y759 for interaction with cellular proteins. The Y747F mutant blocked the transition from stage 1 to stage 2, and the Y759F blocked the transition from stage 2 to stage 3. The data suggest a model for tension-induced activation of alpha(v)beta3 integrin.

Bogaards JJ, Freidig AP, van Bladeren PJ. **Prediction of isoprene diepoxide levels in vivo in mouse, rat and man using enzyme kinetic data in vitro and physiologically-based pharmacokinetic modelling.** Chem Biol Interact 2001;138(3):247-65.
Abstract: The present study was designed to explain the differences in isoprene toxicity between mouse and rat based on the liver concentrations of the assumed toxic metabolite isoprene diepoxide. In addition, extrapolation to the human situation was attempted. For this purpose, enzyme kinetic

parameters K(m) and V(max) were determined in vitro in mouse, rat and human liver microsomes/cytosol for the cytochrome P450-mediated formation of isoprene mono- and diepoxides, epoxide hydrolase mediated hydrolysis of isoprene mono- and diepoxides, and the glutathione S-transferases mediated conjugation of isoprene monoepoxides. Subsequently, the kinetic parameters were incorporated into a physiologically-based pharmacokinetic model, and species differences regarding isoprene diepoxide levels were forecasted. Almost similar isoprene diepoxide liver and lung concentrations were predicted in mouse and rat, while predicted levels in humans were about 20-fold lower. However, when interindividual variation in enzyme activity was introduced in the human model, the levels of isoprene diepoxide changed considerably. It was forecasted that in individuals having both an extensive oxidation by cytochrome P450 and a low detoxification by epoxide hydrolase, isoprene diepoxide concentrations in the liver increased to similar concentrations as predicted for the mouse. However, the interpretation of the latter finding for human risk assessment is ambiguous since species differences between mouse and rat regarding isoprene toxicity could not be explained by the predicted isoprene diepoxide concentrations. We assume that other metabolites than isoprene diepoxide or different carcinogenic response might play a key role in determining the extent of isoprene toxicity. In order to confirm this, in vivo experiments are required in which isoprene epoxide concentrations will be established in rats and mice.

Bogdanffy MS, Plowchalk DR, Sarangapani R, Starr TB, Andersen ME. **Mode-of-action-based dosimeters for interspecies extrapolation of vinyl acetate inhalation risk**. Inhal Toxicol 2001;13 (5):377-96.
Abstract: Vinyl acetate is used in the manufacture of many polymers. The Clean Air Act Amendments of 1990 require that an inhalation risk assessment be conducted to assess risks to human health from ambient exposures. Vinyl acetate is a nasal carcinogen in rats and induces olfactory degeneration in rats and mice. Because of the many unique aspects of the rodent nasal cavity compared to that of humans, conventional means for extrapolating dosimetry between species are not appropriate. Physiologically based pharmacokinetic (PBPK) and pharmacodynamic (PD) modeling can address many of these unique aspects. A PBPK/PD model has been developed for vinyl acetate, but the choice of appropriate dosimeter (s) to use for interspecies extrapolation depends on a hypothesis regarding mode of action. This article summarizes the key studies that formulate a mode of action hypothesis for vinyl acetate. Dose-response relationships for vinyl acetate-induced nonneoplastic and neoplastic responses are highly nonlinear, suggesting complex kinetic processes. Carboxylesterase-dependent metabolism of vinyl acetate forms acetic acid, a potent cytotoxicant, and acetaldehyde, a weak clastogen. Cell death, proposed to be the result of intracellular acidification, results in restorative cell proliferation. In conjunction with sufficient genetic damage, induced by spontaneous mutation and acetaldehyde-induced DNA-protein cross-links (DPX), olfactory degeneration progresses to a state of elevated proliferation and eventually, at high vinyl acetate concentrations, to neoplastic transformation. Thus, reduction in intracellular pH (pHi) is proposed as the dosimeter most closely linked to the earliest stages of vinyl acetate toxicity. Consequently, risk assessments that are based on protection of nasal epithelium from intracellular acidification will be protective of all subsequent pathological responses related to vinyl acetate exposure. Proposing a reasonable mode of action is an important step in any risk assessment and is critical to the choice of dosimeter(s) to be used for interspecies dosimetry extrapolation.

Bogman K, Peyer AK, Torok M, Kusters E, Drewe J. **HMG-CoA reductase inhibitors and P-glycoprotein modulation.** Br J Pharmacol 2001;132(6):1183-92.
Abstract: 1. Five 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), (e. g. atorvastatin, fluvastatin, lovastatin, pravastatin and simvastatin), were investigated for their ability to reverse P-glycoprotein (P-gp) mediated rhodamine 123 (R123) transport in a murine monocytic leukaemia cell line that over-expresses the multi-drug resistance protein 1a/b (mdr1a/1b). 2. P-gp modulation was studied by a fluorimetric assay and confocal microscopy by means of R123 efflux and uptake experiments, respectively. 3. Atorvastatin acid, methyl ester and lactone, lovastatin lactone and simvastatin lactone inhibited R123 transport in a concentration-dependent manner. Lovastatin acid, simvastatin acid, fluvastatin and pravastatin did not show a significant inhibition of the R123 transport in our cell system. Atorvastatin methyl ester and lactone showed the highest affinities for P-gp and results were comparable for both methods. 4. In conclusion, monitoring of R123 transport in living cells by confocal microscopy in addition to fluorimetric assay is a sensitive tool to study P-gp affinity in drug screening that is especially useful for early phases of drug development.

Bois FY. **Statistical analysis of Clewell et al. PBPK model of trichloroethylene kinetics.** Environ Health Perspect 2000;108 Suppl 2:307-16.
Abstract: A physiologically based pharmacokinetic model for trichloroethylene (TCE) in rodents and humans was calibrated with published toxicokinetic data sets. A Bayesian statistical framework was used to combine previous information about the model parameters with the data likelihood, to yield posterior parameter distributions. The use of the hierarchical statistical model yielded estimates of both variability between experimental groups and uncertainty in TCE toxicokinetics. After adjustment of the model by Markov chain Monte Carlo sampling, estimates of variability for the animal or human metabolic parameters ranged from a factor of 1.5-2 (geometric standard deviation [GSD]). Uncertainty was of the same order as variability for animals and higher than variability for humans. The model was used to make posterior predictions for several measures of cancer risk. These predictions were affected by both uncertainties and variability and exhibited GSDs ranging from 2 to 6 in mice and rats and from 2 to 10 for humans.

Bois FY. **Statistical analysis of Fisher et al. PBPK model of trichloroethylene kinetics.** Environ Health Perspect 2000;108 Suppl 2:275-82.
Abstract: Two physiologically based pharmacokinetic models for trichloroethylene (TCE) in mice and humans were calibrated with new toxicokinetic data sets. Calibration is an important step in model development, essential to a legitimate use of models for research or regulatory purposes. A Bayesian statistical framework was used to combine prior information about the model parameters with the data likelihood to yield posterior parameter distributions. For mice, these distributions represent uncertainty. For humans, the use of a population statistical model yielded estimates of both variability and uncertainty in human toxicokinetics of TCE. After adjustment of the models by Markov chain Monte Carlo sampling, the mouse model agreed with a large part of the data. Yet, some data on secondary metabolites were not fit well. The posterior parameter distributions obtained for mice were quite narrow (coefficient of variation [CV] of about 10 or 20%), but these CVs might be underestimated because of the incomplete fit of the model. The data fit, for humans, was better than for mice. Yet, some improvement of the model is needed to correctly describe trichloroethanol concentrations over long time

periods. Posterior uncertainties about the population means corresponded to 10-20% CV. In terms of human population variability, volumes and flows varied across subject by approximately 20% CV. The variability was somewhat higher for partition coefficients (between 30 and 40%) and much higher for the metabolic parameters (standard deviations representing about a factor of 2). Finally, the analysis points to differences between human males and females in the toxicokinetics of TCE. The significance of these differences in terms of risk remains to be investigated.

Boll M, Weber LW, Becker E, Stampfl A. **Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites.** Z Naturforsch [C] 2001;56(7-8):649-59.
Abstract: CCl4-induced liver damage was modeled in monolayer cultures of rat primary hepatocytes with a focus on involvement of covalent binding of CCl4 metabolites to cell components and/or peroxidative damage as the cause of injury. (1) Covalent binding of 14C-labeled metabolites was detected in hepatocytes immediately after exposure to CCl4. (2) Low oxygen partial pressure increased the reductive metabolism of CCl4 and thus covalent binding. (3) [14C]-CCl4 was bound to lipids and to proteins throughout subcellular fractions. Binding occurred preferentially to triacylglycerols and phospholipids, with phosphatidylcholine containing the highest amount of label. (4) The lipid peroxidation potency of CCl4 revealed subtle differences compared to other peroxidative substances, viz., ADP-Fe3+ and cumol hydroperoxide, respectively. (5) CCl4, but not the other peroxidative substances, decreased the rate of triacylglycerol secretion as very low density lipoproteins. (6) The anti-oxidant vitamin E (alpha-tocopherol) blocked lipid peroxidation, but not covalent binding, and secretion of lipoproteins remained inhibited. (7) The radical scavenger piperonyl butoxide prevented CCl4-induced lipid peroxidation as well as covalent binding of CCl4 metabolites to cell components, and also restored lipoprotein metabolism. The results confirm that covalent binding of the CCl3* radical to cell components initiates the inhibition of lipoprotein secretion and thus steatosis, whereas reaction with oxygen, to form CCl3-OO*, initiates lipid peroxidation. The two processes are independent of each other, and the extent to which either process occurs depends on partial oxygen pressure. The former process may result in adduct formation and, ultimately, cancer initiation, whereas the latter results in loss of calcium homeostasis and, ultimately, apoptosis and cell death.

Borde-Chiche P, Diederich M, Morceau F, Wellman M, Dicato M. **Phorbol ester responsiveness of the glutathione S-transferase P1 gene promoter involves an inducible c-jun binding in human K562 leukemia cells.** Leuk Res 2001;25(3):241-7.
Abstract: Overexpression of the glutathione S-transferase P1 (GSTP1) gene is related to drug resistance in human cancer cells. However, the mechanisms of the transcriptional activation of this gene remain unclear. In this study, we examined the molecular mechanisms underlying phorbol ester mediated gene regulation using human K562 leukemia cells as a model. Promoter deletion analyses revealed that the activator protein-1 (AP-1) transcription factor site was crucial for 12-O-tetradecanoyl phorbol 13-acetate (TPA)-mediated GSTP1 gene transcription. Electrophoretic mobility shift assays and transient transfection analysis demonstrated that both DNA binding and transactivation activities of AP-1 were induced by TPA. By supershift analysis, we identified transcription factors c-jun and fra-1 as well as NF-E2p45 as components of the induced binding complex. These results show for the first time that the phorbol ester TPA is involved in the molecular mechanism(s) mediating the activation of the GSTP1

promoter in a human leukemia model.

Bouilleret V, Schwaller B, Schurmans S, Celio MR, Fritschy JM. **Neurodegenerative and morphogenic changes in a mouse model of temporal lobe epilepsy do not depend on the expression of the calcium-binding proteins parvalbumin, calbindin, or calretinin.** Neuroscience 2000;97(1):47-58.
Abstract: The functional role of the calcium-binding proteins parvalbumin, calretinin, and calbindin D-28k for epileptogenesis and long-term seizure-related alterations of the hippocampal formation was assessed in single- and double-knockout mice, using a kainate model of mesial temporal lobe epilepsy. The effects of a unilateral intrahippocampal injection of kainic acid were assessed at one day, 30 days, and four months post-injection, using various markers of GABAergic interneurons (GABA-transporter type 1, GABA(A)-receptor alpha1 subunit, calretinin, calbindin D-28k, somatostatin, and neuropeptide Y). Parvalbumin-deficient, parvalbumin/calbindin-deficient, and parvalbumin/calretinin-deficient mice exhibited no difference in cytoarchitecture of the hippocampal formation and in the number, distribution, or morphology of interneurons compared to wild-type mice. Likewise, mutant mice were not more vulnerable to acute kainate-induced excitotoxicity or to long-term effects of recurrent focal seizures, and exhibited the same pattern of neurochemical alterations (e.g., bilateral induction of neuropeptide Y in granule cells) and morphogenic changes (enlargement and dispersion of dentate gyrus granule cells) as wild-type animals. Quantification of interneurons revealed no significant difference in neuronal vulnerability among the genotypes.These results indicate that the calcium-binding proteins investigated here are not essential for determining the neurochemical phenotype of interneurons. Furthermore, they are not protective against kainate-induced excitotoxicity in this model, and do not appear to modulate the overall level of excitability of the hippocampus. Finally, seizure-induced changes in gene expression in granule cells, which normally express high levels of calcium-binding proteins, apparently were not affected by the gene deletions analysed.

Bouzom F, Laveille C, Merdjan H, Jochemsen R. **Use of nonlinear mixed effect modeling for the meta-analysis of preclinical pharmacokinetic data: application to S 20342 in the rat.** J Pharm Sci 2000;89(5):603-13.
Abstract: The standard two-stage analysis of separate preclinical pharmacokinetic (PK) and toxicokinetic (TK) studies may lead to good information on the bioavailability in the rat at a low (pharmacologic) dose but only an idea on the dose/exposure relationship, on gender, and on time effect. In view of these drawbacks, we decided therefore to explore the usefulness of the implementation of a meta-analysis in preclinical studies in a given species (the rat in this case) taking as an example S 20342, an investigational new drug with potential antipsychotic properties. A nonlinear mixed-effect PK model was built from all intravenous (IV) and oral (PO) data collected until the completion of the 4-week toxicity study. The database included data from 201 Wistar rats (161 males and 40 females). Forty animals received the drug IV and 161 PO. The treatment duration ranged from 1 day to 4 weeks. IV doses were 3, 5, and 20 mg/kg, and 11 different oral doses were tested in the range of 5 to 200 mg/kg. Three different salts were administered PO: hydrochloride, sulfate, and mesylate. The modeling was performed with NONMEM IV. The best pharmacokinetic model was a two-compartment model with simultaneous first-order and zero-order absorption. The combination of these two input functions allowed the model to fit the peak plasma concentrations observed in the first hour (first order), especially

after oral administration of low doses, and to take into account the prolonged absorption phase when the dose increased (zero order). A significant gender effect was found on CL. In addition, significant positive correlations were found between weight and CL, weight and Vc, and dose and the dose fraction after a zero-order absorption. No covariate significantly influenced the other parameters. In conclusion, the meta-analysis of preclinical data allowed for an objective assessment of statistically significant effects throughout the model-building process, leading to a better knowledge (and thus a better understanding) of preclinical PK in the rat. Moreover, the model obtained could be used to interpret further preclinical specific studies involving a sparse sampling design (e.g., further TK studies and PK/PD studies). Although this meta-analysis is more complicated than the noncompartmental approach and requires a case-by-case effort, it could be very useful to integrate this approach in the preclinical development process.

Boyes WK, Bushnell PJ, Crofton KM, Evans M, Simmons JE. **Neurotoxic and pharmacokinetic responses to trichloroethylene as a function of exposure scenario.** Environ Health Perspect 2000;108 Suppl 2:317-22.
Abstract: Strategies are needed for assessing the risks of exposures to airborne toxicants that vary over concentrations and durations. The goal of this project was to describe the relationship between the concentration and duration of exposure to inhaled trichloroethylene (TCE), a representative volatile organic chemical, tissue dose as predicted by a physiologically based pharmacokinetic model, and neurotoxicity. Three measures of neurotoxicity were studied: hearing loss, signal detection behavior, and visual function. The null hypothesis was that exposure scenarios having an equivalent product of concentration and duration would produce equal toxic effects, according to the classic linear form of Haber's Rule ((italic)C(/italic) times t = k), where C represents the concentration, t, the time (duration) of exposure, and k, a constant toxic effect. All experiments used adult male, Long-Evans rats. Acute and repeated exposure to TCE increased hearing thresholds, and acute exposure to TCE impaired signal detection behavior and visual function. Examination of all three measures of neurotoxicity showed that if Haber's Rule were used to predict outcomes across exposure durations, the risk would be overestimated when extrapolating from shorter to longer duration exposures, and underestimated when extrapolating from longer to shorter duration exposures. For the acute effects of TCE on behavior and visual function, the estimated concentration of TCE in blood at the time of testing correlated well with outcomes, whereas cumulative exposure, measured as the area under the blood TCE concentration curve, did not. We conclude that models incorporating dosimetry can account for differing exposure scenarios and will therefore improve risk assessments over models considering only parameters of external exposure.

Boyle WA 3rd, Parvathaneni LS, Bourlier V, Sauter C, Laubach VE, Cobb JP. **iNOS gene expression modulates microvascular responsiveness in endotoxin-challenged mice.** Circ Res 2000;87(7):E18-24.
Abstract: Septic shock is characterized by vasodilation and decreased responsiveness to vasoconstrictors. Recent studies suggest this results from nitric oxide (NO) overproduction after expression of the calcium-independent isoform of NO synthase (iNOS) in smooth muscle cells. However, direct evidence linking iNOS (NOS2) expression and decreased microvascular responsiveness after septic stimuli is lacking. In the present study, we determined the effect of bacterial lipopolysaccharide (LPS, 20 mg/kg, IP) on smooth muscle contraction and endothelial relaxation in

mesenteric resistance arteries from wild-type and iNOS knockout mice. Four hours after challenge with LPS or saline in vivo, concentration-dependent responses to norepinephrine (NE) and acetylcholine (NE +ACh) were measured in cannulated, pressurized vessels ex vivo. In vessels from wild-type mice, NE-induced contraction was markedly impaired after LPS, and pretreatment with the iNOS inhibitor aminoguanidine (AG) partly restored the NE contraction. In contrast, NE contraction in microvessels from iNOS knockout mice was unaffected by LPS. ACh-induced relaxation was unaffected by LPS in vessels from either genotype. These data provide direct evidence that iNOS gene expression mediates the LPS-induced decrease in microvascular responsiveness to vasoconstrictors. Moreover, the observation that AG did not fully restore NE contraction after LPS, whereas iNOS gene deficiency did, suggests that iNOS expression plays a central role in the development of the NO-independent effect of LPS on microvascular responsiveness. Finally, our data indicate that LPS or iNOS expression has little effect on endothelium-dependent relaxation, and eNOS activity does not appear to play a role in the decreased smooth muscle responsiveness after LPS in this model. The full text of this article is available at http://www.circresaha.org.

Bravo-Zehnder M, Orio P, Norambuena A, Wallner M, Meera P, Toro L, Latorre R, Gonzalez A. **Apical sorting of a voltage- and Ca2+-activated K+ channel alpha -subunit in Madin-Darby canine kidney cells is independent of N-glycosylation.** Proc Natl Acad Sci U S A 2000;97(24):13114-9. Abstract: The voltage- and Ca(2+)-activated K(+) (K(V,Ca)) channel is expressed in a variety of polarized epithelial cells seemingly displaying a tissue-dependent apical-to-basolateral regionalization, as revealed by electrophysiology. Using domain-specific biotinylation and immunofluorescence we show that the human channel K(V,Ca) alpha-subunit (human Slowpoke channel, hSlo) is predominantly found in the apical plasma membrane domain of permanently transfected Madin-Darby canine kidney cells. Both the wild-type and a mutant hSlo protein lacking its only potential N-glycosylation site were efficiently transported to the cell surface and concentrated in the apical domain even when they were overexpressed to levels 200- to 300-fold higher than the density of intrinsic Slo channels. Furthermore, tunicamycin treatment did not prevent apical segregation of hSlo, indicating that endogenous glycosylated proteins (e.g., K(V,Ca) beta-subunits) were not required. hSlo seems to display properties for lipid-raft targeting, as judged by its buoyant distribution in sucrose gradients after extraction with either detergent or sodium carbonate. The evidence indicates that the hSlo protein possesses intrinsic information for transport to the apical cell surface through a mechanism that may involve association with lipid rafts and that is independent of glycosylation of the channel itself or an associated protein. Thus, this particular polytopic model protein shows that glycosylation-independent apical pathways exist for endogenous membrane proteins in Madin-Darby canine kidney cells.

Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B, Attramadal H. **Regulation and intracellular trafficking pathways of the endothelin receptors.** J Biol Chem 2000;275(23):17596-604. Abstract: The effects of endothelin (ET) are mediated via the G protein-coupled receptors ET(A) and ET (B). However, the mechanisms of ET receptor desensitization, internalization, and intracellular trafficking are poorly understood. The aim of the present study was to investigate the molecular mechanisms of ET receptor regulation and to characterize the intracellular pathways of ET-stimulated ET (A) and ET(B) receptors. By analysis of ET(A) and ET(B) receptor internalization in transfected Chinese hamster ovary cells in the presence of overexpressed betaARK, beta-arrestin-1, beta-arrestin-2,

or dynamin as well as dominant negative mutants of these regulators, we have demonstrated that both ET receptor subtypes follow an arrestin- and dynamin/clathrin-dependent mechanism of internalization. Fluorescence microscopy of Chinese hamster ovary and COS cells expressing green fluorescent protein (GFP)-tagged ET receptors revealed that the ET(A) and ET(B) subtypes were targeted to different intracellular routes after ET stimulation. While ET(A)-GFP followed a recycling pathway and colocalized with transferrin in the pericentriolar recycling compartment, ET(B)-GFP was targeted to lysosomes after ET-induced internalization. Both receptor subtypes colocalized with Rab5 in classical early endosomes, indicating that this compartment is a common early intermediate for the two ET receptors during intracellular transport. The distinct intracellular routes of ET-stimulated ET(A) and ET (B) receptors may explain the persistent signal response through the ET(A) receptor and the transient response through the ET(B) receptor. Furthermore, lysosomal targeting of the ET(B) receptor could serve as a biochemical mechanism for clearance of plasma endothelin via this subtype.

Brubacher JL, Bols NC. **Chemically de-acetylated 2',7'-dichlorodihydrofluorescein diacetate as a probe of respiratory burst activity in mononuclear phagocytes.** J Immunol Methods 2001;251(1-2):81-91.
Abstract: 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) is a fluorogenic probe commonly used to detect cellular production of reactive oxygen species (ROS), for example in the respiratory burst of granulocytes and mononuclear phagocytes. This method depends on the de-acetylation of H2DCFDA by cellular esterases, to form the oxidant-sensitive compound, 2',7'-dichlorodihydrofluorescein (H2DCF). Importantly, however, not all cells possess sufficient esterase activity to produce the H2DCF needed for accurate measurement of ROS. In this study, we used chemically de-acetylated probe (H2DCF) to assess the phorbol-ester-triggered respiratory burst of rainbow trout macrophages, which, like some mammalian mononuclear phagocytes, appear to have low probe-esterase activity. We compared this approach to the use of intact H2DCFDA and the cytochrome c reduction assay. The H2DCF and cytochrome c reduction assays gave similar portrayals of the kinetics of the macrophage respiratory burst, while H2DCFDA did not. We therefore recommend the use of H2DCF over H2DCFDA for quantification of the production of reactive oxygen species. Additionally, we stress the need to test reaction buffers or culture media used with H2DCF(DA) for their ability to oxidize the probe directly or indirectly. As an example, we have observed that tyrosine combined with ubiquitous metal contaminants of physiological buffers can result in high levels of oxidation, which may be incorrectly interpreted as cellular activity.

Bruckner JV. **Differences in sensitivity of children and adults to chemical toxicity: the NAS panel report.** Regul Toxicol Pharmacol 2000;31(3):280-5.
Abstract: The National Academy of Sciences (NAS) Committee on Pesticides in the Diets of Infants and Children worked for some 4 years to evaluate the extent and the health-related consequences of exposure of infants and children to pesticides. The focus of this paper is on deliberations and recommendations of the committee relevant to protection of infants and children from toxic effects of pesticides. The most comprehensive data available for contrasting the toxicity of chemicals in the young and adults were compilations of rodent mortality studies. Age-dependent differences in chemical lethality were less than 1 order of magnitude and usually varied no more than 2- to 3-fold. Findings in studies of pesticides and other chemicals revealed that toxicity was age- and compound-dependent. The younger and more

immature the subject, the more different its response from that of an adult. Substantial anatomical, biochemical, and physiological changes occur during infancy, childhood, and adolescence. These maturational changes can substantially affect the absorption, distribution, metabolism, and elimination of chemicals. The net effect of immaturity on pharmacokinetics and pharmacodynamics is difficult to predict. Measurements of physiological functions in different age groups can be made and input into physiologically based pharmacokinetic (PBPK) models. The committee felt that PBPK models could be effectively utilized for different exposure scenarios, to predict the time course of potentially toxic chemicals and metabolites in different organs of children. The committee recognized that maturing organ systems of infants and children may be susceptible to injury by chemicals. There may be developmental periods (i.e., windows of vulnerability) when the endocrine, reproductive, immune, visual, or nervous systems are particularly sensitive to certain chemicals. The committee recommended early assessments using sensitive indices of injury to these organ systems of test animals. Only limited information was available on the therapeutic efficacy and toxicity of drugs in pediatric populations. The most definitive data were maximally tolerated doses (MTDs) of chemotherapeutic agents. MTDs were frequently higher for children than adults, though the differences between age groups were usually < or =2. It was concluded by the NAS committee that immaturity does not necessarily entail greater sensitivity to chemical toxicity; age-dependent toxicity is chemical-dependent; and the existing 10-fold interspecies uncertainty factor provides adequate protection of infants and children, based on current knowledge.

Bu HZ, Magis L, Knuth K, Teitelbaum P. **High-throughput cytochrome P450 (CYP) inhibition screening via a cassette probe-dosing strategy. VI. Simultaneous evaluation of inhibition potential of drugs on human hepatic isozymes CYP2A6, 3A4, 2C9, 2D6 and 2E1.** Rapid Commun Mass Spectrom 2001;15(10):741-8.
Abstract: The inhibition potential of drugs towards five major human hepatic cytochrome P450 (CYP) isozymes (CYP2A6, 3A4, 2C9, 2D6, and 2E1) was investigated via cassette dosing of the five probe substrates (coumarin, midazolam, tolbutamide, dextromethorphan, and chlorzoxazone) in human liver microsomes using a 96-well plate format. After microsomal incubations had been terminated with formic acid, the five marker metabolites (7-hydroxycoumarin, 1'-hydroxymidazolam, 4-hydroxytolbutamide, dextrorphan, and 6-hydroxychlorzoxazone) were simultaneously quantified using direct injection/online guard cartridge extraction/tandem mass spectrometry (DI-GCE/MS/MS). Several advantages resulted from the use of a short C(18) guard cartridge (4 mm in length) for DI-GCE/MS/MS, including minimal sample preparation, fast online extraction, short analysis time (2.5 min), and minimal source contamination. In addition, this method demonstrated an inter-day accuracy range from -8.7 - 7.4% with a precision less than 8.3% for the quantification of all the marker metabolites. The inhibition assay for the five CYP isozymes was evaluated using their known selective inhibitors via individual and cassette dosing of the probe substrates. The IC(50) values measured via cassette dosing were consistent with those observed via individual dosing, which were all in agreement with the reported values. In addition, the validated assay was used to evaluate the inhibitory potential of 23 generic drugs (randomly selected) towards the five CYP isozymes. The results suggest the integration of the cassette dosing strategy and the DI-GCE/MS/MS method can provide a reliable in vitro approach to screening the inhibitory potential of new chemical entities, with maximal throughput and cost-effectiveness, in support of drug discovery and development. Copyright 2001 John Wiley & Sons, Ltd.

Bu HZ, Magis L, Knuth K, Teitelbaum P. **High-throughput cytochrome P450 (CYP) inhibition screening via cassette probe-dosing strategy. I. Development of direct injection/on-line guard cartridge extraction/tandem mass spectrometry for the simultaneous detection of CYP probe substrates and their metabolites.** Rapid Commun Mass Spectrom 2000;14(17):1619-24.
Abstract: A highly efficient direct injection/on-line guard cartridge extraction/tandem mass spectrometry (DI-GCE/MS/MS) method utilizing electrospray polarity switching was developed for the simultaneous detection of probe substrates and marker metabolites of seven human hepatic cytochrome P450 (CYP) isozymes: CYP1A2, 2A6, 3A4, 2C9, 2C19, 2D6 and 2E1. Microsomal incubations were terminated with formic acid, centrifuged, and the resulting supernatants were injected for analysis by DI-GCE/MS/MS. This method employed an extremely short C(18) cartridge (4 mm in length) which allowed rapid cleanup of sample matrices while retaining the analytes an appropriate time (2. 0-2.2 min). From 1.5 to 2.7 min the effluent was directed to the mass spectrometer for detection otherwise diverted to waste. As a result of the efficient on-line extraction, matrix (e.g., salts and proteins) suppression was minimized. In addition, no visible source contamination was observed and system performance (chromatographic and mass spectrometric) did not significantly deteriorate after 500 consecutive injections. Electrospray polarity switching was strategically executed on a Micromass Quattro II mass spectrometer by establishing dummy ion transitions to protect the analytes from the interference of the overwhelming noise which was unavoidable for the first transition scanned following each polarity switch. This unique strategy led to the simultaneous detection of seven CYP probe substrates and seven corresponding marker metabolites (12 by positive mode and 2 by negative mode).

Budzinski JW, Foster BC, Vandenhoek S, Arnason JT. **An in vitro evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures.** Phytomedicine 2000;7 (4):273-82.
Abstract: Serial dilutions of 21 commercial ethanolic herbal extracts and tinctures, and 13 related pure plant compounds have been analyzed for their in vitro cytochrome P450 3A4 (CYP3A4) inhibitory capability via a fluorometric microtitre plate assay. Roughly 75% of the commercial products and 50% of the pure compounds showed significant inhibition of CYP3A4 metabolite formation. For each herbal product and pure compound exhibiting dose-dependency, the inhibition values were used to generate median inhibitory concentration (IC50) curves using linear regression. Among the commercial extracts, Hydrastis canadensis (goldenseal), Hypericum perforatum (St. John's wort), and Uncaria tomentosa (cat's claw) had the lowest IC50 values at < 1% full strength, followed by Echinacea angustifolia roots, Trifolium pratense (wild cherry), Matricaria chamomilla (chamomile), and Glycyrrhiza glabra (licorice), which had IC50 values ranging from 1%-2% of full strength. Dillapiol, hypericin, and naringenin had the lowest IC50 values among the pure plant compounds at < 0.5 mM; dillapiol was the most potent inhibitor at 23.3 times the concentration of the positive CYP3A4 inhibitor ketoconazole. Utilizing high-throughput screening methodologies for assessing CYP3A4 inhibition by natural products has important implications for predicting the likelihood of potential herbal-drug interactions, as well as determining candidates for further in-depth analyses.

Bull S, Langezaal I, Clothier R, Coecke S. **A genetically engineered cell-based system for detecting metabolism-mediated toxicity.** Altern Lab Anim 2001;29(6):703-16.

Abstract: Xenobiotics undergong bioactivation by CYP450 enzymes form reactive metabolites that may exert direct metabolism-mediated toxicity. An in vitro model was developed to study the direct toxic effects that follow the metabolic activation of chemicals. The model uses monolayer cultures of genetically engineered NIH-3T3 or V79 cells that express individual human or rat CYP450 isoforms, respectively. Following exposure to 1,3-dichloropropanol or cyclophosphamide, basal cytotoxicity endpoints, including neutral red uptake and Alamar Blue(reduction were used to assess changes in cell number and functional viability resulting from the formation of metabolites. Cell lines that express cytochrome P450 enzymes metabolised the test compounds, leading to increased toxicity compared with that observed in the control cell line. The use of specific inhibitors confirmed that the formation of reactive metabolites was CYP450-isoform dependent. These results indicate that a panel of genetically engineered cell lines expressing various cytochrome P450 enzyme isoforms can be used to reveal measurable metabolising capabilities, and could become a useful tool for the detection and possible determination of CYP450 isoforms in human liver metabolism-mediated toxicity.

Burgard AP, Maranas CD. **Probing the performance limits of the Escherichia coli metabolic network subject to gene additions or deletions.** Biotechnol Bioeng 2001;74(5):364-75.
Abstract: An optimization-based procedure for studying the response of metabolic networks after gene knockouts or additions is introduced and applied to a linear flux balance analysis (FBA) Escherichia coli model. Both the gene addition problem of optimally selecting which foreign genes to recombine into E. coli, as well as the gene deletion problem of removing a given number of existing ones, are formulated as mixed-integer optimization problems using binary 0-1 variables. The developed modeling and optimization framework is tested by investigating the effect of gene deletions on biomass production and addressing the maximum theoretical production of the 20 amino acids for aerobic growth on glucose and acetate substrates. In the gene deletion study, the smallest gene set necessary to achieve maximum biomass production in E. coli is determined for aerobic growth on glucose. The subsequent gene knockout analysis indicates that biomass production decreases monotonically, rendering the metabolic network incapable of growth after only 18 gene deletions. In the gene addition study, the E. coli flux balance model is augmented with 3,400 non-E. coli reactions from the KEGG database to form a multispecies model. This model is referred to as the Universal model. This study reveals that the maximum theoretical production of six amino acids could be improved by the addition of only one or two genes to the native amino acid production pathway of E. coli, even though the model could choose from 3,400 foreign reaction candidates. Specifically, manipulation of the arginine production pathway showed the most promise with 8.75% and 9.05% predicted increases with the addition of genes for growth on glucose and acetate, respectively. The mechanism of all suggested enhancements is either by: 1) improving the energy efficiency and/or 2) increasing the carbon conversion efficiency of the production route.

Buschini A, Anceschi E, Carlo-Stella C, Regazzi E, Rizzoli V, Poli P, Rossi C. **Amifostine (WR-2721) selective protection against melphalan genotoxicity.** Leukemia 2000;14(9):1642-51.
Abstract: Amifostine (WR-2721) is an aminothiol compound dephosphorylated at the tissue site by alkaline phosphatase to the active metabolite, which is able to inactivate electrophilic substances and scavenge free radicals. Amifostine effects against melphalan-induced DNA strand breaks were studied in normal human white blood cells (WBC) and K562 leukemic cells using the single cell gel

electrophoresis (SCGE) or Comet assay, a reported method for measuring DNA damage in individual cells. Prior to treatment (1 h, 37 degrees C) with increasing doses of melphalan, with or without S9, the cells were treated (15 min, 37 degrees C) with a control medium or amifostine (3 mg/ml). Treatment of normal and leukemic cells with melphalan induced a dose-dependent 'comet formation'. Melphalan-induced DNA damage follows a normal distribution in WBC. On the other hand, in K562, a significant proportion of undamaged cells remains even with doses at which mean DNA damage is serious. Pretreatment with WR-2721 protects WBC, but not K562, against the genotoxic effect of melphalan. Amifostine might even strengthen the action of the antiblastic drug against K562 cells. S9 addition appears to enhance melphalan effectiveness. SCGE appears as a suitable primary screening method for in vitro and in vivo studies on drug-DNA interactions and their modulations by endogenous/exogenous factors.

Carrier G, Bouchard M, Brunet RC, Caza M. **A toxicokinetic model for predicting the tissue distribution and elimination of organic and inorganic mercury following exposure to methyl mercury in animals and humans. II. Application and validation of the model in humans.** Toxicol Appl Pharmacol 2001;171(1):50-60.
Abstract: The objective of this study was to develop a biologically based dynamical model describing the disposition kinetics of methyl mercury and its inorganic mercury metabolites in humans following different methyl mercury exposure scenarios. The model conceptual and functional representation was similar to that used for rats but relevant data on humans served to determine the critical parameters of the kinetic behavior. It was found that the metabolic rate of methyl mercury was on average 3 to 3.5 times slower in humans than in rats. Also, excretion rates of organic mercury from the whole body into feces and hair were 100 and 40 times smaller in humans, respectively, and urinary excretion of organic mercury in humans was found to be negligible. The human transfer rate of inorganic mercury from blood to hair was found to be 5 times lower than that of rats. On the other hand, retention of inorganic mercury in the kidney appeared more important in humans than in rats: the transfer rate of inorganic mercury from blood to kidney was 19 times higher than in rats and that from kidney to blood 19 times smaller. The excretion rate of inorganic mercury from the kidney to urine in humans was found to be twice that of rats. With these model parameters, simulations accurately predicted human kinetic data available in the published literature for different exposure scenarios. The model relates quantitatively mercury species in biological matrices (blood, hair, and urine) to the absorbed dose and tissue burden at any point in time. Thus, accessible measurements on these matrices allow inferences of past, present, and future burdens. This could prove to be a useful tool in assessing the health risks associated with various circumstances of methyl mercury exposure.

Carrier G, Brunet RC, Caza M, Bouchard M. **A toxicokinetic model for predicting the tissue distribution and elimination of organic and inorganic mercury following exposure to methyl mercury in animals and humans. I. Development and validation of the model using experimental data in rats.** Toxicol Appl Pharmacol 2001;171(1):38-49.
Abstract: The objective of this study was to develop a biologically based dynamic model for predicting the distribution and elimination of methyl mercury and its metabolite, inorganic mercury, under a variety of exposure scenarios in rats. A model is proposed based on a multicompartment approach; each compartment represents an organ or a group of organs or an excreta. The model translates into a set of

coupled differential equations taking into account interorgan rates of exchanges and excretion together with the biotransformation process. The free parameters of the model are determined from statistical fits to the experimental data of the Farris et al. (Toxicol. Appl. Pharmacol. 119, 74-90, 1993) study on the time profiles of blood and tissue concentrations and cumulative excretions. The vast range of time scales that govern tissue absorption, distribution, biotransformation, and excretion served to solve the model step by step. This interplay of time scales in the rates explains the buildups and slow attrition of inorganic mercury in certain key organs such as the brain and the kidney, which are also the sites of the more important toxic effects. The model was validated on additional experimental data provided by Norseth and Clarkson (Arch. Environ. Health 21, 717-727, 1970) and Thomas et al. (Environ. Res. 41, 219-234, 1986; Environ. Res. 43, 203-216, 1987). This approach, when adapted to humans, allows the reconstruction of the time course of blood and tissue concentrations, starting from easily accessible data on hair, urine, and feces.

Carvan MJ 3rd, Sonntag DM, Cmar CB, Cook RS, Curran MA, Miller GL. **Oxidative stress in zebrafish cells: potential utility of transgenic zebrafish as a deployable sentinel for site hazard ranking.** Sci Total Environ 2001;274(1-3):183-96.
Abstract: In order to quickly assess potential environmental hazards of forwardly deployed military bases, we have focussed our efforts on biochemical and molecular changes in vertebrate cells following exposure to aqueous soil extracts. To this end, we are designing a series of deployable transgenic fish. Fish exhibit many of the same general defenses against toxic chemicals as do mammals, including enzyme induction, and the generation of oxidative stress. In response to many foreign compounds that generate oxidative stress, the transcription of certain protective genes is induced via specific DNA motifs called electrophile response elements (EPREs). We have made a plasmid construct containing a single murine EPRE fused to a minimal promoter and the cDNA encoding firefly luciferase (EPRE-LUC). In this paper, we have shown that the treatment of zebrafish cell line ZEM2S with a variety of chemicals known to induce EPRE-dependent transcription in cultured mammalian cells, results in dose-dependent induction of the transiently-transfected EPRE-LUC reporter construct. Compounds tested include aromatic hydrocarbons, heavy metals, and organophosphates. We observed similar dose-dependent responses when we treated ZEM2S and human cells in vitro with identical aqueous extracts of soil from hazardous waste sites. This suggests that the mechanism by which these compounds activate transcription is well conserved between mammals and zebrafish, and that transgenic zebrafish lines containing EPRE-driven reporter constructs might be useful as sentinels for the early detection of oxidative stress-inducing chemicals.

Chabot S, Charlet D, Wilson TL, Yong VW. **Cytokine production consequent to T cell--microglia interaction: the PMA/IFN gamma-treated U937 cells display similarities to human microglia.** J Neurosci Methods 2001;105(2):111-20.
Abstract: Cognate interactions between human adult microglia and activated T lymphocytes induce the production of inflammatory cytokines. Since this interaction can occur in a non-antigen-dependent manner, it is relevant to a variety of CNS diseases where activated T cells, regardless of specificities, come into contact with microglia; these disorders include multiple sclerosis, trauma, stroke and Alzheimer's disease. A model cell line would facilitate studies of the engagement between T cells and human adult microglia, since the latter are difficult to obtain in substantial quantity or frequency. This

study shows that the PMA/IFN gamma-treated U937 cell line shows similarities to microglia in its interaction with activated T lymphocytes, in that the production of tumor necrosis factor (TNF)-alpha, interleukin (IL)-4, IL-10 and IL-12 is induced. Morphological features and mechanisms of cytokine production resemble those observed in microglia--T cell co-cultures since CTLA-4 and CD40--CD40L blockades reduce TNF-alpha and IL-10 levels, while anti-CD23 inhibits IL-10 only in U937--T cell interactions. We propose that PMA/IFN gamma-treated U937 cells can serve as a model of human adult microglia to study cytokine generation in response to interactions with activated T cells.

Chen S, Gong J, Liu F, Mohammed U. **Naturally occurring polyphenolic antioxidants modulate IgE-mediated mast cell activation.** Immunology 2000;100(4):471-80.
Abstract: Reactive oxygen species (ROS) are known to modulate activities of a host of kinases, phosphatases and transcription factors. Rutin and chlorogenic acid (CGA) are the major polyphenolic antioxidants present in the small molecular fraction of smokeless tobacco leaf extracts, as ascertained by reverse-phase high-pressure liquid chromatography (HPLC) and mass spectrometry. Levels of intracellular ROS in resting versus antigen-immunoglobulin E (IgE)-challenged murine mast cells were measured at 510 nm by fluorescence-activated cell sorting (FACS) using carboxy-dichlorofluorescein (DCFH-DA). Enhanced ROS production was observed in IgE-sensitized mast cells following antigenic challenge. Rutin and CGA reduced ROS levels in antigen-IgE-activated mast cells. Concomitantly, they also profoundly inhibited histamine release by these activated mast cells. In contrast, rutin and CGA augmented the inducible cytokine messages, i.e. interleukin (IL)-10, IL-13, interferon-gamma (IFN-gamma), IL-6 and tumour necrosis factor-alpha (TNF-alpha) in IgE-sensitized mast cells following antigen challenge. This study indicates that tobacco polyphenolic antioxidants that quench intracellular ROS, differentially affect two effector functions of antigen-IgE-activated mast cells. This model system may be employed to determine the molecular target of polyphenols. The potential role of these polyphenolic antioxidants on IgE-mediated allergy in vivo depends on a balance of their differential effects on mast cell activation.

Cheng L, Wang LE, Spitz MR, Wei Q. **Cryopreserving whole blood for functional assays using viable lymphocytes in molecular epidemiology studies.** Cancer Lett 2001;166(2):155-63.
Abstract: There is an increasing need for viable lymphocytes in performing phenotypic assays for biomarker studies. Both fresh and cryopreserved lymphocytes have been used for cell culture-based functional assays. However, fresh lymphocytes do not allow assays to be done in batches and cryopreservation of isolated lymphocytes results in a considerable loss of viable cells. To investigate the feasibility of using cryopreserved whole blood as a source of viable lymphocytes in molecular epidemiology studies, two well-established biomarkers, the host-cell reactivation (HCR) and mutagen sensitivity assays, were used to compare the method of cryopreserving whole blood with the traditional methods. In 25 paired blood samples assayed for DNA repair capacity (DRC) by the HCR assay, the DRC values of frozen whole blood (mean +/- SD, 11.59 +/- 3.07) were similar to those of frozen isolated lymphocytes (11.08 +/- 3.50). The correlation between the paired DRC values was 0.77 ($P < 0.001$). In 31 paired blood samples assayed for the gamma-radiation-induced chromatid breaks by the mutagen sensitivity assay, there was no significant difference between the baseline level of chromatid breaks in lymphocytes from frozen blood (0.05 +/- 0.03) and fresh blood (0.06 +/- 0.03). The blastogenic rate and mitotic index of the cells used for the two assays were compared between the

different processing methods. The lymphocytes from frozen whole blood were more sensitive to gamma-radiation, with a higher mean level of chromatid breaks (0.68 +/- 0.21) than that in fresh blood (0.42 +/- 0.12, $P < 0.01$), and the correlation between the numbers of chromatid breaks in the paired samples was statistically significant ($r = 0.61$, $P < 0.001$). These data suggest that within the limits of the parameters investigated here, cryopreserved whole blood is a good source of viable lymphocytes for biomarker assays in molecular epidemiological studies.

Cheng Q, Thomas SM, Kostichka K, Valentine JR, Nagarajan V. **Genetic analysis of a gene cluster for cyclohexanol oxidation in Acinetobacter sp. Strain SE19 by in vitro transposition.** J Bacteriol 2000;182(17):4744-51.
Abstract: Biological oxidation of cyclic alcohols normally results in formation of the corresponding dicarboxylic acids, which are further metabolized and enter the central carbon metabolism in the cell. We isolated an Acinetobacter sp. from an industrial wastewater bioreactor that utilized cyclohexanol as a sole carbon source. A cosmid library was constructed from Acinetobacter sp. strain SE19, and oxidation of cyclohexanol to adipic acid was demonstrated in recombinant Escherichia coli carrying a SE19 DNA segment. A region that was essential for cyclohexanol oxidation was localized to a 14-kb fragment on the cosmid DNA. Several putative open reading frames (ORFs) that were expected to encode enzymes catalyzing the conversion of cyclohexanol to adipic acid were identified. Whereas one ORF showed high homology to cyclohexanone monooxygenase from Acinetobacter sp. strain NCIB 9871, most of the ORFs showed only moderate homology to proteins in GenBank. In order to assign functions of the various ORFs, in vitro transposon mutagenesis was performed using the cosmid DNA as a target. A set of transposon mutants with a single insertion in each of the ORFs was screened for cyclohexanol oxidation in E. coli. Several of the transposon mutants accumulated a variety of cyclohexanol oxidation intermediates. The in vitro transposon mutagenesis technique was shown to be a powerful tool for rapidly assigning gene functions to all ORFs in the pathway.

Cheung JO, Hillarby MC, Ayad S, Hoyland JA, Jones CJ, Denton J, Thomas JT, Wallis GA, Grant ME. **A novel cell culture model of chondrocyte differentiation during mammalian endochondral ossification.** J Bone Miner Res 2001;16(2):309-18.
Abstract: Endochondral ossification (EO) occurs in the growth plate where chondrocytes pass through discrete stages of proliferation, maturation, hypertrophy, and calcification. We have developed and characterized a novel bovine cell culture model of EO that mirrors these events and will facilitate in vitro studies on factors controlling chondrocyte differentiation. Chondrocytes derived from the epiphyses of long bones of fetal calves were treated with 5-azacytidine (aza-C) for 48 h. Cultures were maintained subsequently without aza-C and harvested at selected time points for analyses of growth and differentiation status. A chondrocytic phenotype associated with an extensive extracellular matrix rich in proteoglycans and collagen types II and VI was observed in aza-C-treated and -untreated cultures. aza-C-treated cultures were characterized by studying the expression of several markers of chondrocyte differentiation. Parathyroid hormone-related protein (PTHrP) and its receptor, both markers of maturation, were expressed at days 5-9. Type X collagen, which is restricted to the stage of hypertrophy, was expressed from day 11 onward. Hypertrophy was confirmed by a 14-fold increase in cell size by day 15 and an increased synthesis of alkaline phosphatase during the hypertrophic period (days 14-28). The addition of PTHrP to aza-C-treated cultures at day 14 led to the down-regulation of type X collagen

by 6-fold, showing type X collagen expression is under the control of PTHrP as in vivo. These findings show that aza-C can induce fetal bovine epiphyseal chondrocytes to differentiate in culture in a manner consistent with that which occurs during the EO process in vivo.

Choudhury H, Harvey T, Thayer WC, Lockwood TF, Stiteler WM, Goodrum PE, Hassett JM, Diamond GL. **Urinary cadmium elimination as a biomarker of exposure for evaluating a cadmium dietary exposure--biokinetics model.** J Toxicol Environ Health A 2001;63(5):321-50.
Abstract: The Cadmium Dietary Exposure Model (CDEM) utilizes national survey data on food cadmium concentrations and food consumption patterns to estimate dietary intakes in the U.S. population. The CDEM has been linked to a modification of the cadmium biokinetic model of Kjellstrom and Nordlberg (KNM) to derive predictions of kidney and urinary cadmium that reflect U.S. dietary cadmium intake and related variability. Variability in dietary cadmium intake was propagated through the KNM using a Monte Carlo approach. The model predicts a mean peak kidney cadmium burden of approximately 3.5 mg and a 5th-95th percentile range of 2.2-5.1 mg in males. The corresponding peak renal cortex cadmium concentration in males is 15 microg/g wet cortex (10-22, 5th-95th percentile). Predicted kidney cadmium levels in females were higher than males: 5.1 (3.3-7.6) mg total kidney, 29 (19-43) microg/g wet cortex. Predicted urinary cadmium in males and females agreed with empirical estimates based on the NHANES III, with females predicted and observed to excrete approximately twice the amount of cadmium in urine than males. An explanation for the higher urinary cadmium excretion in females is proposed that is consistent with the NHANES III data as well as experimental studies in humans and animals. Females may absorb a larger fraction of ingested dietary cadmium than males, and this difference may be the result of lower iron body stores in females compared to males. This would suggest that females may be at greater risk of developing cadmium toxicity than males. The predicted 5th-95th percentile values for peak kidney cadmium burden are approximately 60% of the peak kidney burden (8-11 mg) predicted for a chronic intake at the U.S. Environmental Protection Agency (EPA) chronic reference dose of 1 microg/kg-d.

Christopoulos A, Grant MK, El-Fakahany EE. **Transducer abstraction: a novel approach to the detection of partial agonist efficacy in radioligand binding studies.** J Pharmacol Toxicol Methods 2000;43(1):55-67.
Abstract: The properties of the ternary complex model (TCM) of drug action at G protein-coupled receptors (GPCRs) were examined, using theoretical computer simulations, with regard to the predicted effects of the presence of a fixed concentration of one agonist on the competition binding profile of another. Subsequently, the binding properties of the full muscarinic acetylcholine receptor (mAChR) agonists acetylcholine (ACh) and carbachol (CCh), and the partial agonists pilocarpine and McN-A-343, were investigated in competition experiments against [(3)H]N-methylscopolamine using homogenate preparations from Chinese hamster ovary cells, stably expressing the human M(1) or M(2) mAChR. At the M(2) mAChR, all agonists displayed biphasic binding curves and were readily modulated by the non-hydrolyzable GTP analogue, Gpp(NH)p, in accordance with previously established experimental observations. In contrast, agonist binding at the M(1) mAChR showed no significant change in the presence of Gpp(NH)p, even in the case of a full agonist. This phenomenon precludes using the "GTP-shift" to assess agonist efficacy at the M(1) mAChR. When the ACh competition curves were reconstructed in the presence of graded concentrations of either a full or a partial agonist, a significant

redistribution of the fraction of the high-affinity state recognized by ACh was observed. However, when the procedure was repeated using the antagonist, atropine, no significant effect on the fraction of either the high or low affinity ACh binding components at the mAChR was observed. Taken together, these results indicate that changes in the profile of full agonist binding isotherms, when constructed in the presence of a partial agonist, may be more sensitive indicators of partial agonist efficacy than regular assays that directly measure partial agonist binding.

Clewell HJ, Gentry PR, Gearhart JM, Allen BC, Andersen ME. **Comparison of cancer risk estimates for vinyl chloride using animal and human data with a PBPK model.** Sci Total Environ 2001;274(1-3):37-66.

Abstract: Vinyl chloride (VC) is a trans-species carcinogen, producing tumors in a variety of tissues, from both inhalation and oral exposures, across a number of species. In particular, exposure to VC has been associated with a rare tumor, liver angiosarcoma, in a large number of studies in mice, rats, and humans. The mode of action for the carcinogenicity of VC appears to be a relatively straightforward example of DNA adduct formation by a reactive metabolite, leading to mutation, mistranscription, and neoplasia. The objective of the present analysis was to investigate the comparative potency of a classic genotoxic carcinogen across species, by performing a quantitative comparison of the carcinogenic potency of VC using data from inhalation and oral rodent bioassays as well as from human epidemiological studies. A physiologically-based pharmacokinetic (PBPK) model for VC was developed to support the target tissue dosimetry for the cancer risk assessment. Unlike previous models, the initial metabolism of VC was described as occurring via two saturable pathways, one representing low capacity-high affinity oxidation by CYP2E1 and the other (in the rodent) representing higher capacity-lower affinity oxidation by other isozymes of P450, producing in both cases chloroethylene oxide (CEO) and chloroacetaldehyde (CAA) as intermediate reactive products. Depletion of glutathione by reaction with CEO and CAA was also described. Animal-based risk estimates for human inhalation exposure to VC using total metabolism estimates from the PBPK model were consistent with risk estimates based on human epidemiological data, and were lower than those currently used in environmental decision-making by a factor of 80.

Clewell HJ 3rd, Gentry PR, Covington TR, Gearhart JM. **Development of a physiologically based pharmacokinetic model of trichloroethylene and its metabolites for use in risk assessment.** Environ Health Perspect 2000;108 Suppl 2:283-305.

Abstract: A physiologically based pharmacokinetic (PBPK) model was developed that provides a comprehensive description of the kinetics of trichloroethylene (TCE) and its metabolites, trichloroethanol (TCOH), trichloroacetic acid (TCA), and dichloroacetic acid (DCA), in the mouse, rat, and human for both oral and inhalation exposure. The model includes descriptions of the three principal target tissues for cancer identified in animal bioassays: liver, lung, and kidney. Cancer dose metrics provided in the model include the area under the concentration curve (AUC) for TCA and DCA in the plasma, the peak concentration and AUC for chloral in the tracheobronchial region of the lung, and the production of a thioacetylating intermediate from dichlorovinylcysteine in the kidney. Additional dose metrics provided for noncancer risk assessment include the peak concentrations and AUCs for TCE and TCOH in the blood, as well as the total metabolism of TCE divided by the body weight. Sensitivity and uncertainty analyses were performed on the model to evaluate its suitability for use in a pharmacokinetic

risk assessment for TCE. Model predictions of TCE, TCA, DCA, and TCOH concentrations in rodents and humans are in good agreement with a variety of experimental data, suggesting that the model should provide a useful basis for evaluating cross-species differences in pharmacokinetics for these chemicals. In the case of the lung and kidney target tissues, however, only limited data are available for establishing cross-species pharmacokinetics. As a result, PBPK model calculations of target tissue dose for lung and kidney should be used with caution.

Cole CE, Tran HT, Schlosser PM. **Physiologically based pharmacokinetic modeling of benzene metabolism in mice through extrapolation from in vitro to in vivo**. J Toxicol Environ Health A 2001;62(6):439-65.
Abstract: Benzene (C6H6) is a highly flammable, colorless liquid. Ubiquitous exposures result from its presence in gasoline vapors, cigarette smoke, and industrial processes. Benzene increases the incidence of leukemia in humans when they are exposed to high doses for extended periods; however, leukemia risks in humans at low exposures are uncertain. The exposure-dose-response relationship of benzene in humans is expected to be nonlinear because benzene undergoes a series of metabolic transformations, detoxifying and activating, in the liver, resulting in multiple metabolites that exert toxic effects on the bone marrow. We developed a physiologically based pharmacokinetic model for the uptake and elimination of benzene in mice to relate the concentration of inhaled and orally administered benzene to the tissue doses of benzene and its key metabolites, benzene oxide, phe nol, and hydroquinone. As many parameter values as possible were taken from the literature; in particular, metabolic parameters obtained from in vitro studies with mouse liver were used since comparable parameters are also available for humans. Parameters estimated by fitting the model to published data were first-order rate constants for pathways lacking in vitro data and the concentrations of microsomal and cytosolic protein, which effectively alter overall enzyme activity. The model was constrained by using the in vitro metabolic parameters (maximum velocities, first-order rate constants, and saturation parameters), and data from multiple laboratories and experiments were used. Despite these constraints and sources of variability, the model simulations matched the data reasonably well in most cases, showing that in vitro metabolic constants can be successfully extrapolated to predict in vivo data for benzene metabolism and dosimetry. Therefore in vitro metabolic constants for humans can subsequently be extrapolated to predict the dosimetry of benzene and its metabolites in humans. This will allow us to better estimate the risks of adverse effects from low-level benzene exposures.

Conolly RB. **Biologically motivated quantitative models and the mixture toxicity problem.** Toxicol Sci 2001;63(1):1-2.
Abstract: The article highlighted in this issue is "A PBPK Modeling-Based Approach to Account for Interactions in the Health Risk Assessment of Chemical Mixtures" by Sami Haddad, Martin Beliveau, Robert Tardif, and Kannan Krishnan (pp. 125-131).

Corley RA, English JC, Hill TS, Fiorica LA, Morgott DA. **Development of a physiologically based pharmacokinetic model for hydroquinone.** Toxicol Appl Pharmacol 2000;165(2):163-74.
Abstract: Hydroquinone (HQ) produces nephrotoxicity and renal tubular adenomas in male F344 rats following 2 years of oral dosing. Female F344 and SD rats are comparatively resistant to these effects. Nephrotoxicity and tumorigenicity have been associated with a minor glutathione conjugation pathway

following the oxidation of HQ to benzoquinone (BQ). The majority of administered doses (90-99%) consists of glucuronide and sulfate conjugates of HQ. An initial physiologically based pharmacokinetic model was developed to characterize the role of kinetics in the strain differences observed in HQ-induced renal toxicity and tumorigenicity. Partition coefficients, protein-binding, and metabolic rate constants were determined directly or estimated from a series of in vivo and in vitro studies. Metabolism was confined to the liver and GI tract. The total flux through the glutathione pathway represented the "internal dose" of HQ for nephrotoxicity. Simulations were compared to a variety of data from male and female F344 rats, male SD rats, and a single male human volunteer. Simulations of intraperitoneal administration resulted in higher amounts of glutathione conjugates than comparable oral doses. This was consistent with protein-binding and toxicity studies and emphasized the importance of first-pass GI tract metabolism. In addition, male F344 rats were predicted to form more total glutathione conjugates than SD rats at equivalent dose levels, which was also consistent with the observed strain differences in renal toxicity. This model represents the first stage in the development of a biologically based dose-response model for improving the scientific basis for human health risk assessments of HQ.

Crespi CL, Stresser DM. **Fluorometric screening for metabolism-based drug--drug interactions.** J Pharmacol Toxicol Methods 2000;44(1):325-31.
Abstract: Inhibition of cytochromes P-450 (CYP) is a principal mechanism for metabolism-based drug interactions. In vitro methods for quantitatively measuring the extent of CYP inhibition are well-established. Classical methods use drug molecules as substrates and HPLC-based analysis. However, methodologies, which do not require HPLC separations for data acquisition generally offer higher throughputs and lower costs. Multiwell plate-based, direct, fluorometric assays for the activities of the five principal drug-metabolizing enzymes are available and parameters for the use of these substrates to measure CYP inhibition have been established. This methodology is quantitative, rapid, reproducible, and compatible with common high throughput screening instrumentation. This article describes approaches to establishing this methodology in a drug-discovery support program.

Crump KS. **Resolved: biologically based models are useful for analyzing radiation epidemiological data.** Con. Radiat Res 2000;154(6):717

Darvas F, Dorman G, Papp A. **Diversity measures for enhancing ADME admissibility of combinatorial libraries.** J Chem Inf Comput Sci 2000;40(2):314-22.
Abstract: For general screening libraries, structural diversity descriptors and drug-likeness indicators still do not guarantee the in vivo bioavailability for the candidates, which is considered a major bottleneck in drug development. Early prediction of pharmacokinetics (log P, log D), metabolism, and toxicity makes it possible to deal with ADME (adsorption, distribution, metabolism, excretion) related diversity as an extension to the classical diversity concepts. It opens several new possibilities for optimization of a discovery library before doing any experimental screening. This new diversity concept is demonstrated on a subset of MeDiverse, which is a diverse collection of pharmacologically relevant compounds selected from our in-house library. From consideration of the ADME interface in living systems, virtual secondary libraries of metabolites and retrometabolites (prodrugs) can be generated. These additional libraries readily enhance both the structural and ADME related diversity. This new opportunity in library design can substantially improve the success rate for in vivo lead generation from

in vitro hits.

Delic JI, Lilly PD, MacDonald AJ, Loizou GD. **The utility of PBPK in the safety assessment of chloroform and carbon tetrachloride.** Regul Toxicol Pharmacol 2000;32(2):144-55.
Abstract: Occupational exposure limits (OELs) for individual substances are established on the basis of the available toxicological information at the time of their promulgation, expert interpretation of these data in light of industrial use, and the framework in which they sit. In the United Kingdom, the establishment of specific OELs includes the application of uncertainty factors to a defined starting point, usually the NOAEL from a suitable animal study. The magnitude of the uncertainty factors is generally determined through expert judgment including a knowledge of workplace conditions and management of exposure. PBPK modeling may help in this process by informing on issues relating to extrapolation between and within species. This study was therefore designed to consider how PBPK modeling could contribute to the establishment of OELs. PBPK models were developed for chloroform (mouse and human) and carbon tetrachloride (rat and human). These substances were chosen for examination because of the extent of their toxicological databases and availability of existing PBPK models. The models were exercised to predict the rate (chloroform) or extent (carbon tetrachloride) of metabolism of these substances, in both rodents and humans. Monte Carlo analysis was used to investigate the influence of variability within the human and animal model populations. The ratio of the rates/extent of metabolism predicted for humans compared to animals was compared to the uncertainty factors involved in setting the OES. Predictions obtained from the PBPK models indicated that average rat and mouse metabolism of carbon tetrachloride and chloroform, respectively, are much greater than that of the average human. Application of Monte Carlo analysis indicated that even those people who have the fastest rates or most extensive amounts of metabolism in the population are unlikely to generate the levels of metabolite of these substances necessary to produce overt toxicity in rodents. This study highlights the value that the use of PBPK modeling may add to help inform and improve toxicological aspects of a regulatory process.

Dourson ML, Andersen ME, Erdreich LS, MacGregor JA. **Using human data to protect the public's health.** Regul Toxicol Pharmacol 2001;33(2):234-56.
Abstract: The value of using human data in the assessment and management of risk is evaluated. Although the use of such data has a long and successful history with environmental contaminants and the development of drugs and commercial chemicals, recent deliberations within the Environmental Protection Agency (EPA) have questioned this practice in part. Specifically, we evaluate the degree to which reference doses (RfDs) and reference concentrations (RfCs) derived from human data on EPA's Integrated Risk Information System (IRIS) differ with RfDs and RfCs that we estimate from experimental animal data. We also use several minimal risk levels of the Agency for Toxic Substances and Disease Registry (ATSDR) and tolerable intakes of Health Canada in this comparison. Human-based RfDs are more than threefold lower than the corresponding animal-based RfDs for 23% of the comparisons. Human- based RfDs or RfCs are lower than corresponding animal-based RfDs or RfCs for 36% of the comparisons. Furthermore, for 10 of 43 possible comparisons, insufficient experimental animal data are readily available or data are inappropriate to estimate either RfDs or RfCs. We also discuss human pharmacokinetic data from volunteer studies and mechanistic studies with human tissues in vitro and demonstrate through a series of case discussions that utilization of such data is important

when making decisions to protect exposed individuals. Moreover, physiologically based pharmacokinetic (PBPK) modeling evaluates critical information in assessing interindividual variability and identifying at-risk populations. Within the limits of our analysis, we conclude that the direct use and interpretation of human data, in conjunction with data gathered from experimental animals, are public health protective policies that should be encouraged. Copyright 2001 Academic Press.

Douziech M, Coin F, Chipoulet JM, Arai Y, Ohkuma Y, Egly JM, Coulombe B. **Mechanism of promoter melting by the xeroderma pigmentosum complementation group B helicase of transcription factor IIH revealed by protein-DNA photo-cross-linking.** Mol Cell Biol 2000;20 (21):8168-77.
Abstract: The p89/xeroderma pigmentosum complementation group B (XPB) ATPase-helicase of transcription factor IIH (TFIIH) is essential for promoter melting prior to transcription initiation by RNA polymerase II (RNAPII). By studying the topological organization of the initiation complex using site-specific protein-DNA photo-cross-linking, we have shown that p89/XPB makes promoter contacts both upstream and downstream of the initiation site. The upstream contact, which is in the region where promoter melting occurs (positions -9 to +2), requires tight DNA wrapping around RNAPII. The addition of hydrolyzable ATP tethers the template strand at positions -5 and +1 to RNAPII subunits. A mutation in p89/XPB found in a xeroderma pigmentosum patient impairs the ability of TFIIH to associate correctly with the complex and thereby melt promoter DNA. A model for open complex formation is proposed.

Ehmer PB, Jose J, Hartmann RW. **Development of a simple and rapid assay for the evaluation of inhibitors of human 17alpha-hydroxylase-C(17,20)-lyase (P450cl7) by coexpression of P450cl7 with NADPH-cytochrome-P450-reductase in Escherichia coli.** J Steroid Biochem Mol Biol 2000;75 (1):57-63.
Abstract: P450c17 is a microsomal enzyme catalyzing the last step in androgen biosynthesis. As inhibitors of P450c17 are promising drug candidates for the treatment of prostate cancer, it was our goal to develop a new cellular assay for the in vitro evaluation of potential inhibitors. Human P450c17 was expressed in E. coli and hydroxylase activity was determined using 1,2[3H]-progesterone. As the activity was low (1.7 pmol/min/mg protein), due to a lack of the requisite electron transfer partner NADPH-cytochrome-P450-reductase (NADPH-P450-reductase), coexpression of both the enzymes had to be performed. For that purpose, a plasmid was constructed which encoded human P450c17 and rat NADPH-P450-reductase in a transcriptional unit. This strategy led to a 100-fold increase in P450cl7 activity (175 pmol/min/mg protein). Time, pH and temperature dependence of progesterone conversion of this new monooxygenase system was determined. The K(M) of progesterone was 2.75 microM. An assay procedure for the evaluation of inhibitors was established and modified for high throughput screening using 96-well plates. Selected compounds were tested for their inhibitory activity using this whole cell assay. The data was compared to the results obtained in microsomal testicular preparations.

Ekins S, Ring BJ, Grace J, McRobie-Belle DJ, Wrighton SA. **Present and future in vitro approaches for drug metabolism.** J Pharmacol Toxicol Methods 2000;44(1):313-24.
Abstract: The 1980s through 1990s witnessed the widespread incorporation of in vitro absorption, distribution, metabolism, and excretion (ADME) approaches into drug development by drug companies.

This has been exemplified by the integration of the basic science of cytochrome P450s (CYPs) into most drug metabolism departments so that information on the metabolic pathways of drugs and drug-drug interactions (DDIs) is no longer an academic exercise, but essential for regulatory submission. This has come about due to the application of a variety of new technologies and in vitro models. For example, subcellular fractions have been widely used in metabolism studies since the 1960s. The last two decades has seen the increased use of hepatocytes as the reproducibility of cell isolations improved. The 1990s saw the rejuvenation of liver slices (as new slicers were developed) and the utilization of cDNA expressed enzymes as these technologies matured. In addition, there has been considerable interest in extrapolating in vitro data to in vivo for parameters such as absorption, clearance and DDIs. The current philosophy of drug development is moving to a 'fail early--fail cheaply' paradigm. Therefore, in vitro ADME approaches are being applied to drug candidates earlier in development since they are essential for identifying compounds likely to present ADME challenges in the latter stages of drug development. These in vitro tools are also being used earlier in lead optimization biology, in parallel with approaches for optimizing target structure activity relationships, as well as identification of DDI and the involvement of metabolic pathways that demonstrate genetic polymorphisms. This would suggest that the line between discovery and development drug metabolism has blurred. In vitro approaches to ADME are increasingly being linked with high-throughput automation and analysis. Further, if we think of perhaps the fastest available way to screen for successful drugs with optimal ADME characteristics, then we arrive at predictive computational algorithms, which are only now being generated and validated in parallel with in vitro and in vivo methods. In addition, as we increase the number of ADME parameters determined early, the overall amount of data generated for both discovery and development will increase. This will present challenges for the efficient and fast interpretation of such data, as well as incorporation and communication to chemistry, biology, and clinical colleagues. This review will focus on and assess the nature of present in vitro metabolism approaches and indicate how they are likely to develop in the future.

Ekins S, Waller CL, Swaan PW, Cruciani G, Wrighton SA, Wikel JH. **Progress in predicting human ADME parameters in silico.** J Pharmacol Toxicol Methods 2000;44(1):251-72.
Abstract: Understanding the development of a scientific approach is a valuable exercise in gauging the potential directions the process could take in the future. The relatively short history of applying computational methods to absorption, distribution, metabolism and excretion (ADME) can be split into defined periods. The first began in the 1960s and continued through the 1970s with the work of Corwin Hansch et al. Their models utilized small sets of in vivo ADME data. The second era from the 1980s through 1990s witnessed the widespread incorporation of in vitro approaches as surrogates of in vivo ADME studies. These approaches fostered the initiation and increase in interpretable computational ADME models available in the literature. The third era is the present were there are many literature data sets derived from in vitro data for absorption, drug-drug interactions (DDI), drug transporters and efflux pumps [P-glycoprotein (P-gp), MRP], intrinsic clearance and brain penetration, which can theoretically be used to predict the situation in vivo in humans. Combinatorial synthesis, high throughput screening and computational approaches have emerged as a result of continual pressure on pharmaceutical companies to accelerate drug discovery while decreasing drug development costs. The goal has become to reduce the drop-out rate of drug candidates in the latter, most expensive stages of drug development. This is accomplished by increasing the failure rate of candidate compounds in the preclinical stages and

increasing the speed of nomination of likely clinical candidates. The industry now understands the reasons for clinical failure other than efficacy are mainly related to pharmacokinetics and toxicity. The late 1990s saw significant company investment in ADME and drug safety departments to assess properties such as metabolic stability, cytochrome P-450 inhibition, absorption and genotoxicity earlier in the drug discovery paradigm. The next logical step in this process is the evaluation of higher throughput data to determine if computational (in silico) models can be constructed and validated from it. Such models would allow an exponential increase in the number of compounds screened virtually for ADME parameters. A number of researchers have started to utilize in silico, in vitro and in vivo approaches in parallel to address intestinal permeability and cytochrome P-450-mediated DDI. This review will assess how computational approaches for ADME parameters have evolved and how they are likely to progress.

Elms J, Allan LJ, Pengelly I, Fishwick D, Beckett PN, Curran AD. **Colophony: an in vitro model for the induction of sensitization.** Clin Exp Allergy 2000;30(2):209-13.
Abstract: BACKGROUND: The potential of colophony fumes from soldering flux to induce asthma has been known since the 1970s, however, no direct in vitro or in vivo evidence has been reported. The present study investigated the potential of colophony to stimulate human phagocytic cells to produce reactive oxygen species. METHODS: The human cell line HL-60 was differentiated to produce cells with a monocyte-like and a neutrophil-like phenotypes. A number of procedures were used to confirm the phenotype of these differentiated cells including morphology, esterase activity, flow cytometry and phagocytosis. The potential of colophony to stimulate human phagocytic cells to produce reactive oxygen species was monitored using flow cytoenzymology. RESULTS: We were able to show that intracellular peroxide levels were increased in both monocyte-like and neutrophil-like cells, but not in undifferentiated HL-60 cells following the addition of colophony. CONCLUSIONS: The resin acid epoxides and hydroperoxides which have been suggested to be sensitizers in contact allergy, are degraded during the soldering process. However, conditions for the oxidation of colophony may occur in vivo as a result of the colophony-induced oxidative burst from neutrophils and monocytes. These oxidation products may then interact with body proteins to further initiate immune responses. Therefore for the preparation of low molecular weight chemical (LMWC)-protein conjugates, consideration must be taken to determine whether the LMWC is undergoing a reaction in vivo before it is interacting with body proteins.

Eu JP, Liu L, Zeng M, Stamler JS. **An apoptotic model for nitrosative stress.** Biochemistry 2000;39(5):1040-7.
Abstract: Nitric oxide overproduction has been implicated in the pathogenesis of many disorders, including artherosclerosis, neurodegenerative diseases, inflammatory and autoimmune diseases, and cancer. The common view holds that nitric oxide-induced cellular injury is caused by oxidative stress. This theory predicts that interactions between reactive nitrogen species and reactive oxygen species produce powerful oxidants that initiate cell death programs. Cytokine-treated murine macrophages are the prototype of this form of cellular injury. Here we report that generation of reactive nitrogen species upon lipopolysacharide/interferon-gamma stimulation of RAW 264.7 cells is largely divorced from production of reactive oxygen species, and that oxidative stress is not principally responsible for cell death (in this model). Rather, the death program is induced mainly by a nitrosative challenge,

characterized by the accrual of nitrosylated proteins without a major alteration in cellular redox state. Moreover, interactions between reactive oxygen and nitrogen species may alter the balance between pathways that yield nitrite and nitrate, without impacting the level of S-nitrosylation or extent of cell death. Our results thus (1) provide new insights into NO-related metabolic pathways, (2) demonstrate that apoptotic injury can be caused by nitrosative mechanisms, and (3) establish a model for nitrosative stress in mammalian cells.

Fannon M, Forsten KE, Nugent MA. **Potentiation and inhibition of bFGF binding by heparin: a model for regulation of cellular response.** Biochemistry 2000;39(6):1434-45.
Abstract: Basic fibroblast growth factor (bFGF) binds to cell surface tyrosine kinase receptor proteins and to heparan sulfate proteoglycans. The interaction of bFGF with heparan sulfate on the cell surface has been demonstrated to impact receptor binding and biological activity. bFGF receptor binding affinity is reduced on cells that do not express heparan sulfate. The addition of soluble heparin or heparan sulfate has been demonstrated to rescue the bFGF receptor binding affinity on heparan sulfate deficient cells yet has also been shown to inhibit binding under some conditions. While the chemical requirements of the heparin-bFGF-receptor interactions have been studied in detail, the possibility that heparin enhances bFGF binding in part by physically associating with the cell surface has not been fully evaluated. In the study presented here, we have investigated the possibility that heparin binding to the cell surface might play a role in modulating bFGF receptor binding and activity. Balb/c3T3 cells were treated with various concentrations of sodium chlorate, so as to express a range of endogenous heparan sulfate sites, and [(125)I]bFGF binding was assessed in the presence of a range of heparin concentrations. Low concentrations of heparin (0.1-30 nM) enhanced bFGF receptor binding to an extent that was inversely proportional to the amount of endogenous heparan sulfate sites present. At high concentrations (10 microM), heparin inhibited bFGF receptor binding in cells under all conditions. The ability of heparin to stimulate and inhibit bFGF-receptor binding correlated with altered bFGF-stimulated tyrosine kinase activity and cell proliferation. Under control and chlorate-treated conditions, [(125) I]heparin was observed to bind with a high affinity to a large number of binding sites on the cells (K(d) = 57 and 50 nM with 3.5 x 10(6) and 3.6 x 10(6) sites/cell for control and chlorate-treated cells, respectively). A mathematical model of this process revealed that the dual functions of heparin in bFGF binding were accurately represented by heparin cell binding-mediated stimulation and soluble heparin-mediated inhibition of bFGF receptor binding.

Fennell TR, Brown CD. **A physiologically based pharmacokinetic model for ethylene oxide in mouse, rat, and human.** Toxicol Appl Pharmacol 2001;173(3):161-75.
Abstract: Ethylene oxide (EO) is widely used as a gaseous sterilant and industrial intermediate and is a direct-acting mutagen and carcinogen. The objective of these studies was to develop physiologically based pharmacokinetic (PB-PK) models for EO to describe the exposure-tissue dose relationship in rodents and humans. We previously reported results describing in vitro and in vivo kinetics of EO metabolism in male and female F344 rats and B6C3F1 mice. These studies were extended by determining the kinetics of EO metabolism in human liver cytosol and microsomes. The results indicate enzymatically catalyzed GSH conjugation via cytosolic glutathione S-transferase (cGST) and hydrolysis via microsomal epoxide hydrolase (mEH) occur in both rodents and humans. The in vitro kinetic constants were scaled to account for cytosolic (cGST) and microsomal (mEH) protein content and

incorporated into PB-PK descriptions for mouse, rat, and human. Flow-limited models adequately predicted blood and tissue EO levels, disposition, and elimination kinetics determined experimentally in rats and mice, with the exception of testis concentrations, which were overestimated. Incorporation of a diffusion-limited description for testis improved the ability of the model to describe testis concentrations. The model accounted for nonlinear increases in blood and tissue concentrations that occur in mice on exposure to EO concentrations greater than 200 ppm. Species differences are predicted in the metabolism and exposure-dose relationship, with a nonlinear relationship observed in the mouse as a result of GSH depletion. These models represent an essential step in developing a mechanistically based EO exposure-dose-response description for estimating human risk from exposure to EO. Copyright 2001 Academic Press.

Filippi MD, Porteu F, Le Pesteur F, Rameau P, Nogueira MM, Debili N, Vainchenker W, de Sauvage FJ, Kupperschmitt AD, Sainteny F. **Embryonic stem cell differentiation to hematopoietic cells: A model to study the function of various regions of the intracytoplasmic domain of cytokine receptors in vitro.** Exp Hematol 2000;28(12):1363-72.
Abstract: To examine whether the in vitro model of embryonic stem (ES) cell hematopoietic differentiation is suitable to study the function of intracytoplasmic regions of cytokine receptors, we used the thrombopoietin receptor Mpl as a typical cytokine receptor.ES cells deficient in c-mpl (mpl (-/)-) were transfected with genes encoding the full-length or two mutated forms of the intracytoplasmic domain of Mpl using the pEF-BOS expression vector. The mutated forms lack box1 or box2.pEF-BOS was able to maintain protein production during ES cell differentiation. Reintroduction of full-length-c-mpl into mpl(-/)- ES cells restored the response of megakaryocyte progenitors to a truncated form of human Mpl-ligand conjugated to polyethylene glycol (PEG-rhuMGDF) and the formation of platelets, for which mpl(-/)- ES cells are defective. In addition, enforced expression of Mpl resulted in the development of all myeloid progenitors and mature cells in the presence of PEG-rhuMGDF. Blast colony-forming cells, the in vitro equivalent of the hemangioblast, also generated blast cell colonies with a hematopoietic potential equivalent to that of the wild type in the presence of PEG-rhuMGDF, although its growth is normally dependent on vascular endothelial cell growth factor (VEGF). Thus, Mpl acts as a substitute for other cytokine receptors and for a tyrosine kinase receptor, Flk-1, indicating that Mpl has no instructive role in hematopoietic cell commitment and differentiation. The Mpl mutant forms lacking box1 or box2 prevented response of ES cell-derived blast colony-forming cells or progenitors to PEG-rhuMGDF. Therefore, these two regions, essential for signaling by cytokine receptors, are required for the responses of ES cell-derived hematopoietic cells to PEG-rhuMGDF.These results show that the in vitro hematopoietic differentiation of ES cells is suitable for studying the role of various intracytoplasmic regions of cytokine receptors.

Fisher JW. **Physiologically based pharmacokinetic models for trichloroethylene and its oxidative metabolites.** Environ Health Perspect 2000;108 Suppl 2:265-73.
Abstract: Trichloroethylene (TCE) pharmacokinetics have been studied in experimental animals and humans for over 30 years. Compartmental and physiologically based pharmacokinetic (PBPK) models have been developed for the uptake, distribution, and metabolism of TCE and the production, distribution, metabolism, and elimination of P450-mediated metabolites of TCE. TCE is readily taken up into systemic circulation by oral and inhalation routes of exposure and is rapidly metabolized by the

hepatic P450 system and to a much lesser degree, by direct conjugation with glutathione. Recent PBPK models for TCE and its metabolites have focused on the major metabolic pathway for metabolism of TCE (P450-mediated metabolic pathway). This article briefly reviews selected published compartmental and PBPK models for TCE. Trichloroacetic acid (TCA) is considered a principle metabolite responsible for TCE-induced liver cancer in mice. Liver cancer in mice was considered a critical effect by the U.S. Environmental Protection Agency for deriving the current maximum contaminant level for TCE in water. In the literature both whole blood and plasma measurements of TCA are reported in mice and humans. To reduce confusion about disparately measured and model-predicted levels of TCA in plasma and whole blood, model-predicted outcomes are compared for first-generation (plasma) and second-generation (whole blood) PBPK models published by Fisher and colleagues. Qualitatively, animals and humans metabolize TCE in a similar fashion, producing the same metabolites. Quantitatively, PBPK models for TCE and its metabolites are important tools for providing dosimetry comparisons between experimental animals and humans. TCE PBPK models can be used today to aid in crafting scientifically sound public health decisions for TCE.

Fisher JW, Dorman DC, Medinsky MA, Welsch F, Conolly RB. **Analysis of respiratory exchange of methanol in the lung of the monkey using a physiological model.** Toxicol Sci 2000;53(2):185-93.
Abstract: A physiologically based pharmacokinetic (PBPK) model was developed for the monkey, to account for fractional systemic uptake of inhaled methanol vapors in the lung. Fractional uptake of inhaled [14C]-methanol was estimated using unreported exhaled breath time course measurements of [14C]-methanol from the D.C. Dorman et al. (1994, Toxicol Appl Pharmacol. 128, 229-238) lung-only exposure study. The cumulative amount of [14C]-methanol exhaled was linear with respect to exposure duration (0.5 to 2 h) and concentration (10 to 900 ppm). The model estimated that forty to eighty-one percent of the of inhaled [14C]-methanol delivered to the lung was taken into systemic circulation in female Cynomolgus monkeys exposed for two h to 10-900 ppm of [14C]-methanol. There was no apparent trend between the percent of inhaled [14C]-methanol absorbed systemically and the [14C]-methanol exposure concentration. Model simulations were conducted using a single saturable Michaelis-Menten equation with Vmaxc, the metabolic capacity set to 15.54 mg/kg/h and Km, the affinity constant, to 0.66 mg/l. The [14C]-methanol blood concentrations were variable across [14C]-methanol exposure groups and the PBPK model tended to over-predict systemic clearance of [14C]-methanol. Accounting for fractional uptake of inhaled polar solvents is an important consideration for risk assessment of inhaled polar solvents.

Fouchecourt MO, Beliveau M, Krishnan K. **Quantitative structure-pharmacokinetic relationship modelling.** Sci Total Environ 2001;274(1-3):125-35.
Abstract: This article presents the current methods in quantitative structure-pharmacokinetic relationship (QSPkR) modelling along with examples using chemicals of toxicological significance. The common method involves: (i) collecting pharmacokinetic data or determining pharmacokinetic parameters (e.g. elimination half-life, volume of distribution) by fitting to experimental data; and (ii) associating them with the structural features of chemicals using a Free-Wilson model. Such QSPkRs have been developed for a few series of chemicals but their usefulness is limited to the exposure scenario and conditions under which the experimental data were originally collected. The alternative approach involves the development of quantitative structure-property relationship (QSPR) models for parameters, blood:air

partition coefficient, tissue:blood partition coefficient, maximal velocity for metabolism and Michaelis affinity constant, of physiologically-based pharmacokinetic (PBPK) models which are useful for conducting species, route, dose and scenario extrapolations of the tissue dose of chemicals. Mechanistic QSPRs are available for predicting tissue:blood and blood:air partition coefficients from molecular structure information of chemicals, whereas such approaches are not currently available for hepatic metabolism parameters. However, at the present time, the pharmacokinetics of inhaled volatile organic chemicals can be simulated adequately by considering the physiological limits of the hepatic extraction ratio (0-1) and molecular structure-based estimates of partition coefficients in the PBPK model. This current state-of-the-art of structure-based modelling of pharmacokinetics will advance with the development of QSPRs for other chemical-specific parameters of PBPK models. Integrated QSPR-PBPK modelling should facilitate the identification of chemicals of a family that possess desired properties of bioaccumulation and blood concentration profile in both test animals and humans.

Frederick CB, Gentry PR, Bush ML, Lomax LG, Black KA, Finch L, Kimbell JS, Morgan KT, Subramaniam RP, Morris JB, et al. **A hybrid computational fluid dynamics and physiologically based pharmacokinetic model for comparison of predicted tissue concentrations of acrylic acid and other vapors in the rat and human nasal cavities following inhalation exposure.** Inhal Toxicol 2001;13(5):359-76.
Abstract: To assist in interspecies dosimetry comparisons for risk assessment of the nasal effects of organic acids, a hybrid computational fluid dynamics (CFD) and physiologically based pharmacokinetic (PBPK) dosimetry model was constructed to estimate the regional tissue dose of inhaled vapors in the rat and human nasal cavity. Application to a specific vapor would involve the incorporation of the chemical-specific reactivity, metabolism, partition coefficients, and diffusivity (in both air and tissue phases) of the vapor. This report describes the structure of the CFD-PBPK model and its application to a representative acidic vapor, acrylic acid, for interspecies tissue concentration comparisons to assist in risk assessment. By using the results from a series of short-term in vivo studies combined with computer modeling, regional nasal tissue dose estimates were developed and comparisons of tissue doses between species were conducted. To make these comparisons, the assumption was made that the susceptibilities of human and rat olfactory epithelium to the cytotoxic effects of organic acids were similar, based on similar histological structure and common mode of action considerations. Interspecies differences in response were therefore assumed to be driven primarily by differences in nasal tissue concentrations that result from regional differences in nasal air flow patterns relative to the species-specific distribution of olfactory epithelium in the nasal cavity. The results of simulations with the seven-compartment CFD-PBPK model suggested that the olfactory epithelium of the human nasal cavity would be exposed to tissue concentrations of acrylic acid similar to that of the rat nasal cavity when the exposure conditions are the same. Similar analysis of CFD data and CFD-PBPK model simulations with a simpler one-compartment model of the whole nasal cavities of rats and humans provides comparable results to averaging over the compartments of the seven-compartment model. These results indicate that the general structure of the hybrid CFD-PBPK model applied in this assessment would be useful for target tissue dosimetry and interspecies dose comparisons for a wide variety of vapors. Because of its flexibility, this CFD-PBPK model is envisioned to be a platform for the construction of case-specific inhalation dosimetry models to simulate in vivo exposures that do not involve significant histopathological damage to the nasal cavity.

Friedberg I I. **Recombinant in vitro tools to predict drug metabolism and safety.** 2000;3(3):99-105.
Abstract: Drug metabolism determines several pharmacological and toxicological properties of pharmaceuticals and is catalysed by drug metabolizing enzymes. Prediction of drug metabolism in humans based on animal experiments is complicated by species differences in the catalytic properties of these enzymes. This review describes and evaluates the use of recombinant models that contain human drug metabolizing enzymes to facilitate the prediction of pharmacokinetic properties of candidate drugs in humans.

Gastel JA. **Early indicators of response in biologically based risk assessment for nongenotoxic carcinogens.** Regul Toxicol Pharmacol 2001;33(3):393-8.
Abstract: The proposed existence of dose-response thresholds for nongenotoxic carcinogens has led to a major controversy in the risk extrapolation process. To resolve this debate, there has been a significant investment in mechanism-based risk assessment research. The ability to utilize this mechanistic research for risk assessment procedures is still limited and may not warrant the expense. Alternatively, an approach can be used to identify dose-response thresholds through the utilization of sensitive indicators of biological response. This approach does not rely upon a mechanistic framework for the development of pathology, is solely dependent on already existing technology, and takes into account the possibility of background levels of pathway activation. For this approach, sensitive biochemical responses need to be identified and linked to the introduction of the toxicant through dose response, by time of response, and, when possible, through a proposed biochemical mechanism. The weakness of this approach is that more sensitive unidentified responses may exist requiring that a safety factor of 10 be used to define a NOEL. For dioxin-like compounds, using a surrogate marker of response CYP1A1 induction, this approach yields an estimate of the acceptable daily intake of 5-50 fg/kg/day. This limit is remarkably similar to the results of the original EPA linear extrapolation (6 fg/kg/day). A similar approach can be used for other nongenotoxic carcinogens and the analysis can be completed within 1 year.

George S, Riley C, McEvoy J, Wright J. **Development of a fish in vitro cell culture model to investigate oxidative stress and its modulation by dietary vitamin E.** Mar Environ Res 2000;50(1-5):541-4.
Abstract: When cultured in Dulbecco's minimal essential medium the established epithelioma papulosum cyprini cell line from carp was found to be vitamin E-deficient due to the very low level of vitamin E in the medium and the foetal calf serum used as supplement. The toxicity of oxidative stressors to this cell line was evaluated by means of the neutral red cytotoxicity assay and it was found that an organic hydroperoxide, t-butylhydroperoxide was extremely cytotoxic and that the redox-cycling agents diquat and menadione were less toxic. When grown under vitamin E supplementation (25 microM), the toxicity of these chemicals was reduced by at least an order of magnitude in concentration demonstrating the protective effect of vitamin E. These data show the importance of vitamin E status for interpretation of in vitro and in vivo data and that this in vitro system is useful for mechanistic studies.

Ghanem A, Shuler ML. **Characterization of a perfusion reactor utilizing mammalian cells on microcarrier beads.** Biotechnol Prog 2000;16(3):471-9.
Abstract: Our overall objective is to develop a cell culture analogue bioreactor (CCA) that can be used

together with a corresponding physiologically based pharmacokinetic model (PBPK) to evaluate molecular mechanisms of toxicity. The PBPK is a mathematical model that divides the body into compartments representing organs, integrating the kinetic, thermodynamic, and anatomical parameters of the animal. The CCA bioreactor is a physical replica of the PBPK; where the PBPK specifies organs, the CCA bioreactor contains compartments with a corresponding cell type that mimics some of the characteristic metabolism of that organ. The device is a continuous, dynamic system composed of multiple cell types that interact through a common circulating cell culture medium. The CCA bioreactor and the model can be coupled to evaluate the plausibility of the molecular mechanism that is input into the model. This paper focuses on the design, development, and characterization of a CCA bioreactor to be used in naphthalene dose response studies. A CCA bioreactor prototype developed previously is improved upon by culturing the cells on microcarrier beads. Microcarrier beads with cells attached can form packed beds with cell culture medium perfusing the beds. In this study, two packed beds of cells, one with L2 cells (rat lung) and one with H4IIE cells (rat hepatoma), are linked in a physiologically relevant arrangement by a common recirculating cell culture medium. Studies of this CCA bioreactor presented here include mixing profiles, effect of reactor environment on cell viability and intracellular glutathione, naphthalene distribution profile, and initial naphthalene dosing studies. Unlike the prototype system there is no detectable response to naphthalene addition; in a companion paper we show that this discrepancy can be explained by differences in liquid residence times in the organ compartments. The perfusion reactor design is shown to have significant operating improvements over prototype designs.

Ghanem A, Shuler ML. **Combining cell culture analogue reactor designs and PBPK models to probe mechanisms of naphthalene toxicity.** Biotechnol Prog 2000;16(3):334-45.
Abstract: An alternative method of evaluating the toxicology of a chemical is to use cultured mammalian cells in a novel cell culture analogue reactor (CCA) together with a corresponding physiologically based pharmacokinetic model (PBPK). The PBPK is a mathematical model that divides the body into compartments representing organs, integrating the kinetic, thermodynamic, and anatomical parameters of the animal. The bioreactor is a physical replica of the PBPK; where the PBPK specifies an organ or tissue compartment, the bioreactor contains compartments with a corresponding cell type. The device is a continuous, dynamic system composed of multiple cell types that interact through a common circulating cell culture medium. The bioreactor and the model are coupled to evaluate the plausibility of the molecular mechanism that is input into the model. This concept is tested with naphthalene as a model of PAH (polycyclic aromatic hydrocarbons) toxicants. Two physically different CCA reactors were tested with naphthalene, and different results were observed. In the prototype system using cells attached to glass dilution bottles, naphthalene dosing resulted in generation of a circulating metabolite from the "liver" compartment (based on H4IIE cells from a rat hepatoma) that caused cell death in the "lung" compartment (L2 cells from a rat lung), as well as depletion of glutathione in the L2 cells. An improved CCA using packed bed reactors of microcarrier cultured cells did not show differences between naphthalene-dosed and nondosed controls. To explain the different responses of the two CCA designs, PBPKs of the two reactors were tested with variations in physical and kinetic parameters, and toxic mechanism. When the toxic metabolite of naphthalene was naphthoquinone rather than naphthalene epoxide as initially assumed, the PBPK results were consistent with the results of the two CCA designs. This result indicates that the mechanism of naphthalene toxicity in the CCAs may be mediated through naphthoquinone formation. The CCA-PBPK concept is demonstrated to be applicable

to the study of toxic mechanisms. In particular, use of this approach suggests that in vitro naphthalene toxicity is mediated through the naphthoquinone metabolite.

Giannakakou P, Gussio R, Nogales E, Downing KH, Zaharevitz D, Bollbuck B, Poy G, Sackett D, Nicolaou KC, Fojo T. **A common pharmacophore for epothilone and taxanes: molecular basis for drug resistance conferred by tubulin mutations in human cancer cells.** Proc Natl Acad Sci U S A 2000;97(6):2904-9.
Abstract: The epothilones are naturally occurring antimitotic drugs that share with the taxanes a similar mechanism of action without apparent structural similarity. Although photoaffinity labeling and electron crystallographic studies have identified the taxane-binding site on beta-tubulin, similar data are not available for epothilones. To identify tubulin residues important for epothilone binding, we have isolated two epothilone-resistant human ovarian carcinoma sublines derived in a single-step selection with epothilone A or B. These epothilone-resistant sublines exhibit impaired epothilone- and taxane-driven tubulin polymerization caused by acquired beta-tubulin mutations (beta274(Thr-->Ile) and beta282(Arg-->Gln)) located in the atomic model of alphabeta-tubulin near the taxane-binding site. Using molecular modeling, we investigated the conformational behavior of epothilone, which led to the identification of a common pharmacophore shared by taxanes and epothilones. Although two binding modes for the epothilones were predicted, one mode was identified as the preferred epothilone conformation as indicated by the activity of a potent pyridine-epothilone analogue. In addition, the structure-activity relationships of multiple taxanes and epothilones in the tubulin mutant cells can be fully explained by the model presented here, verifying its predictive value. Finally, these pharmacophore and activity data from mutant cells were used to model the tubulin binding of sarcodictyins, a distinct class of microtubule stabilizers, which in contrast to taxanes and the epothilones interact preferentially with the mutant tubulins. The unification of taxane, epothilone, and sarcodictyin chemistries in a single pharmacophore provides a framework to study drug-tubulin interactions that should assist in the rational design of agents targeting tubulin.

Goodwin TJ, Coate-Li L, Linnehan RM, Hammond TG. **Selected contribution: a three-dimensional model for assessment of in vitro toxicity in balaena mysticetus renal tissue**. J Appl Physiol 2000;89 (6):2508-17.
Abstract: This study established two- and three-dimensional renal proximal tubular cell cultures of the endangered species bowhead whale (Balaena mysticetus), developed SV40-transfected cultures, and cloned the 61-amino acid open reading frame for the metallothionein protein, the primary binding site for heavy metal contamination in mammals. Microgravity research, modulations in mechanical culture conditions (modeled microgravity), and shear stress have spawned innovative approaches to understanding the dynamics of cellular interactions, gene expression, and differentiation in several cellular systems. These investigations have led to the creation of ex vivo tissue models capable of serving as physiological research analogs for three-dimensional cellular interactions. These models are enabling studies in immune function, tissue modeling for basic research, and neoplasia. Three-dimensional cellular models emulate aspects of in vivo cellular architecture and physiology and may facilitate environmental toxicological studies aimed at elucidating biological functions and responses at the cellular level. Marine mammals occupy a significant ecological niche (72% of the Earth's surface is water) in terms of the potential for information on bioaccumulation and transport of terrestrial and

marine environmental toxins in high-order vertebrates. Few ex vivo models of marine mammal physiology exist in vitro to accomplish the aforementioned studies. Techniques developed in this investigation, based on previous tissue modeling successes, may serve to facilitate similar research in other marine mammals.

Haber LT, Maier A, Zhao Q, Dollarhide JS, Savage RE, Dourson ML. **Applications of mechanistic data in risk assessment: the past, present, and future.** Toxicol Sci 2001;61(1):32-9.
Abstract: Mechanistic data, when available, have long been considered in risk assessment, such as in the development of the nitrate RfD based on effects in a sensitive group (infants). Recent advances in biology and risk assessment methods have led to a tremendous increase in the use of mechanistic data in risk assessment. Toxicokinetic data can improve extrapolation from animals to humans and characterization of human variability. This is done by the development of improved tissue dosimetry, by the use of uncertainty factors based on chemical-specific data, and in the development of physiologically based pharmacokinetic (PBPK) models. The development of the boron RfD illustrates the use of chemical-specific data in the improved choice of uncertainty factors. The draft cancer guidelines of the U.S. Environmental Protection Agency emphasize the use of mode of action data. The first choice under the guidelines is to use a chemical-specific, biologically based dose-response (BBDR) model. In the absence of a BBDR model, mode of action data are used to determine whether low-dose extrapolation is done using a linear or nonlinear (margin of exposure) approach. Considerations involved in evaluating a hypothesized mode of action are illustrated using 1,3-dichloropropene, and use of a BBDR model is illustrated using formaldehyde. Recent developments in molecular biology, including transgenic animals, microarrays, and the characterization of genetic polymorphisms, have significant potential for improving risk assessments, although further methods development is needed. Overall, use of mechanistic data has significant potential for reducing the uncertainty in assessments, while at the same time highlighting the areas of uncertainty.

Haddad S, Beliveau M, Tardif R, Krishnan K. **A PBPK modeling-based approach to account for interactions in the health risk assessment of chemical mixtures.** Toxicol Sci 2001;63(1):125-31.
Abstract: The objectives of the present study were: (1) to develop a risk assessment methodology for chemical mixtures that accounts for pharmacokinetic interactions among components, and (2) to apply this methodology to assess the health risk associated with occupational inhalation exposure to airborne mixtures of dichloromethane, benzene, toluene, ethylbenzene, and m-xylene. The basis of the proposed risk assessment methodology relates to the characterization of the change in tissue dose metrics (e.g., area under the concentration-time curve for parent chemical in tissues [AUCtissue], maximal concentration of parent chemical or metabolite [Cmax], quantity metabolized over a period of time) in humans, during mixed exposures using PBPK models. For systemic toxicants, an interaction-based hazard index was calculated using data on tissue dose of mixture constituents. Initially, the AUCtarget tissue (AUCtt) corresponding to guideline values (e.g., threshold limit value [TLV]) of individual chemicals were obtained. Then, the AUCtt for each chemical during mixed exposure was obtained using a mixture PBPK model that accounted for the binary and higher order interactions occurring within the mixture. An interaction-based hazard index was then calculated for each toxic effect by summing the ratio of AUCtt obtained during mixed exposure (predefined mixture) and single exposure (TLV). For the carcinogenic constituents of the mixture, an interaction-based response additivity approach was applied.

This method consisted of adding the cancer risk for each constituent, calculated as the product of q*tissue dose and AUCtt. The AUCtt during mixture exposures was obtained using an interaction-based PBPK model. The approaches developed in the present study permit, for the first time, the consideration of the impact of multichemical pharmacokinetic interactions at a quantitative level in mixture risk assessments.

Haddad S, Charest-Tardif G, Krishnan K. **Physiologically based modeling of the maximal effect of metabolic interactions on the kinetics of components of complex chemical mixtures.** J Toxicol Environ Health A 2000;61(3):209-23.
Abstract: The objective of this study was to predict and validate the theoretically possible, maximal impact of metabolic interactions on the blood concentration profile of each component in mixtures of volatile organic chemicals (VOCs) [dichloromethane (DCM), benzene (BEN), trichloroethylene (TCE), toluene (TOL), tetrachloroethylene (PER), ethylbenzene (EBZ), styrene (STY), as well as para, ortho-, and meta-xylene (p-XYL, o-XYL, m-XYL)] in the rat. The methodology consisted of: (1) obtaining the validated, physiologically based toxicokinetic (PBTK) model for each of the mixture components from the literature, (2) substituting the Michaelis-Menten description of metabolism with an equation based on the hepatic extraction ratio (E) for simulating the maximal impact of metabolic interactions (i.e., by setting E to 0 or 1 for simulating maximal inhibition or induction, respectively), and (3) validating the PBTK model simulations by comparing the predicted boundaries of venous blood concentrations with the experimental data obtained following exposure to various mixtures of VOCs. All experimental venous blood concentration data for 9 of the 10 chemicals investigated in the present study (PER excepted) fell within the boundaries of the maximal impact of metabolic inhibition and induction predicted by the PBTK model. The modeling approach validated in this study represents a potentially useful tool for screening/identifying the chemicals for which metabolic interactions are likely to be important in the context of mixed exposures and mixture risk assessment.

Haddad S, Charest-Tardif G, Tardif R, Krishnan K. **Validation of a physiological modeling framework for simulating the toxicokinetics of chemicals in mixtures.** Toxicol Appl Pharmacol 2000;167(3):199-209.
Abstract: The objective of this study was to investigate the usefulness of a physiologically based toxicokinetic (PBTK) modeling framework for simulating the kinetics of chemicals in mixtures of varying complexities and composition. The approach involved the simulation of the kinetics of components in two situations: (i) when one of the mixture components was substituted with another (i.e., benzene in the benzene (B)-toluene (T)-ethyl benzene (E)-m-xylene (X) mixture was substituted with dichloromethane (D)), and (ii) when another chemical was added to the existing four-chemical mixture model (i.e., when D was added to the existing BTEX mixture model). In both cases, differing compositions of mixtures were used to obtain simulations and to generate experimental data on kinetics for validation purposes. Since the quantitative and qualitative mechanisms of interaction among B, T, E, and X have already been established, the mechanisms of binary interactions between D and the BTEX components (e.g., competitive, noncompetitive, or uncompetitive metabolic inhibition) were investigated in the present study. The analysis of rat blood kinetic data (4-h inhalation exposures, 50-200 ppm each) to all binary combinations (D-B, D-T, D-E, and D-X) investigated in the present study was suggestive of competitive metabolic inhibition as the plausible interaction mechanism. By incorporating

the newly estimated values of metabolic inhibition constant (K(i)) for each of these binary combinations within the five-chemical PBTK model (i.e., for the DBTEX mixture), the model adequately predicted the venous blood kinetics of chemicals in rats following a 4-h inhalation exposure to various mixtures (mixture 1:100 ppm of D and 50 ppm each of T, E, and X; mixture 2: 100 ppm each of D, T, E, and X; mixture 3: 100 ppm of D and 50 ppm each of B, T, E, and X; mixture 4: 100 ppm each of D, B, T, E, and X). The results of the present study suggest that the PBTK model framework is useful for conducting extrapolations of the kinetics of chemicals from one mixture to another differing in complexity and composition, based on mechanistic considerations of interactions elucidated at the binary level.

Hamza-Chaffai A, Amiard JC, Pellerin J, Joux L, Berthet B. **The potential use of metallothionein in the clam Ruditapes decussatus as a biomarker of in situ metal exposure.** Comp Biochem Physiol C Toxicol Pharmacol 2000;127(2):185-97.
Abstract: This work aimed to validate the relationship between metallothioneins (MTs) and metals (Cd, Cu and Zn) in field conditions. Specimens of the marine bivalve Ruditapes decussatus (Linne, 1758) from Gargour were transferred in two sites: Gargour and Sidi Mansour, both situated along the south-eastern coast of Tunisia. The bivalves were removed from pairs of cages at day 0 (date of transplantation), day 62 and day 132. Metals (Cd, Cu and Zn) and MTs were determined in the subcellular fractions of the digestive gland. In Gargour, metal and MT levels increased significantly after 62 days of transplantation. However, they showed modest and non-significant variations in Sidi Mansour. Zn was mainly associated with the insoluble fraction, whereas Cd and Cu percentages in the soluble and the insoluble fractions were equivalent. Simple correlation analysis showed a positive and significant relationship between MTs and each metal. If all metals were taken together, multiple correlations showed that MTs were significantly correlated with Cd and Zn, with an important coefficient for Cd, but no significant relationship was observed for Cu. Gel filtration chromatography showed that in the heat stable fraction, the only cytosolic SH rich compounds have an apparent low molecular mass (about 15 kDa), which could correspond to metallothioneins. In the digestive gland of R. decussatus MTs responded to moderate increases of metal contamination, without interference with other factors, and could be a promising biochemical indicator of metal exposure.

Hashimoto H, Shintani N, Nishino A, Okabe M, Ikawa M, Matsuyama S, Itoh K, Yamamoto K, Tomimoto S, Fujita T, et al. **Mice with markedly reduced PACAP (PAC(1)) receptor expression by targeted deletion of the signal peptide.** J Neurochem 2000;75(5):1810-7.
Abstract: In an attempt to study the pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 (PAC(1)) receptor (PAC(1)R) function in vivo and to produce a mouse model with altered expression of PAC(1)R, we have used gene targeting in embryonic stem cells to disrupt exon 2 of the PAC(1)R gene, which contains the ATG translation start site and the signal peptide. Un-expectedly, active transcription of PAC(1)R mRNA was detected in the mutant mice; however, exon 1 was spliced to exon 3 (skipping exon 2), and (125)I-PACAP27 binding in brain was greatly reduced. PAC(1)R exon 2(-/-) mice were viable, fertile, and morphologically and histologically indistinguishable from their wild-type counterparts. We next examined the ligand binding and cell surface expression of the mutant receptor lacking the signal peptide in transfected COS-7 cells. (125)I-PACAP27 binding of the mutant receptor was approximately one-tenth of that in the wild-type receptor. Although the wild-type receptor was

expressed abundantly in both the plasma membrane and the cytoplasm around the nucleus, the mutant receptor was expressed in the plasma membrane with a markedly reduced level. Digestion of the membranes with endoglycosidase F greatly reduced the size of the wild-type receptor but only slightly reduced that of the mutant receptor. These results demonstrate that the signal peptide is required for efficient cell surface expression and N-linked glycosylation of the PAC(1)R. However, the mutant receptors still functionally coupled to adenylate cyclase in COS-7 cells, suggesting the presence of sufficient spare receptors such that the mutant receptors are capable of activating the second messenger system. We suggest that the mutant mice with markedly reduced PAC(1)R expression can serve as a useful animal model or cell culture system for further studies in PAC(1)R function.

Hathway DE. **Toxic action/toxicity.** Biol Rev Camb Philos Soc 2000;75(1):95-127.
Abstract: Some six or so physiological systems, essential to normal mammalian life, are involved in poisoning; an intoxication that causes severe injury to any one of them could be life threatening. Reversible chemical reactions showing Scatchard-type binding are exemplified by CO, CN- and cyclodiene neurotoxin insecticide intoxications, and by antigen-antibody complex formation. Haemoglobin (Hb) molecular biology accounts for the allosteric co-operativity and other characteristics of CO poisoning, CN- acts as a powerful cytochrome oxidase inhibitor, and antigen binding in a deep antibody cleft between two domains equipped with epitopes for antigen-binding groups explains hapten-specific immune reactions. Covalent chemical reactions with second-order (SN2) kinetics characterize Hg and Cd poisonings, the reactions of organophosphates and phosphonates with acetylcholinesterase and neurotoxic esterase and the reaction sequence whereby Paraquat accepts electrons and generates superoxide under aerobic conditions. Indirect carcinogens require cytochrome P450 activation to form DNA adducts in target-organ DNA and cause cancer, but a battery of detoxifying enzymes clustered with the P450 system must be overcome. Thus, S-metabolism competes ineffectively with target DNA for reactive vinyl chloride (VC) metabolites, epoxide hydrolase is important to the metabolism and carcinogenicity of alfatoxins and polycyclic aromatic hydrocarbons (benzo[a]pyrene, etc.), and the non-toxic 2-naphthylhydroxylamine N-glucuronide acts as a transport form in 2-naphthylamine bladder cancer. VC liver-cancer pathogenesis is explicable in terms of the presence of the glutathione S-transferase detoxifying system in hepatocytes and its absence from the fibroblastic elements, and of the VC concentrations reaching the liver by different administrative routes. In VC carcinogenicity, chemical reactions give imidazo-cyclization products with nucleoside residues of target DNA, and in benzene leukaemia, Z,Z-muconaldehyde forms cyclic products containing a pyrrole residue linked to purine. Increased HbCO concentrations reduce the O2-carrying capacity of the blood, and the changed shape of the O2-Hb dissociation curve parallels disturbance in O2 unloading. CN- acts on electron transport and paralyses respiration. In telodrin poisoning, preconvulsive glutamine formation abstracts tricarboxylic acid intermediates incommensurately with normal cerebral respiration. Antigen-antibody complexing depletes the antibody titre, available against infection. At high doses of Cd, Cd-thionein filtered through the kidneys is reabsorbed and tubular lesions produced. Some organophosphate insecticides promote irreversible acetylcholinesterase phosphorylation and blockade nerve function, and others react with neurotoxic esterase to cause delayed neuropathy. The evidence for Paraquat pulmonary poisoning suggests a radical mechanism involving three interrelated cyclic reaction stages. The action of N- and O8 (O substituent in 6-position of the purine) demethylases explains deletion mechanisms for DNA-alkyl adducts. DNA-directed synthesis in the presence of ultimate carcinogens provides for an

estimation of misincorporations, which implicate the same transversions as those found by direct mutagenicity testing. Chemical carcinogens recognize tissue-sensitive cells and modify their heritable genetic complement. Oncoproteins encoded by activated oncogenes signal the transformation of normal cells into cancer cells. The importance of the H-ras oncogene and p53 tumour-suppressor gene is stressed. Antidotal action is analysed; for example, parenteral glutamine administration to telodrin-intoxicated rats restores the depleted cerebral glutamate level and prevents seizures. Glutamate acts as anticonvulsant in petit mal epilepsy. In general, therefore, the reaction of the toxicant-related substance with the relevant target-tissue macromolecule accounts for the biochemical/biological events at a cellular level a.

Headlam HA, Mortimer A, Easton CJ, Davies MJ. **beta-Scission of C-3 (beta-carbon) alkoxyl radicals on peptides and proteins: a novel pathway which results in the formation of alpha-carbon radicals and the loss of amino acid side chains.** Chem Res Toxicol 2000;13(11):1087-95.
Abstract: Exposure of proteins to radicals in the presence of O(2) brings about multiple changes in the target molecules. These alterations include oxidation of side chains, fragmentation, cross-linking, changes in hydrophobicity and conformation, altered susceptibility to proteolytic enzymes, and formation of new reactive groups, including hydroperoxides. These processes can result in the loss of structural or enzymatic activity. Backbone fragmentation is known to occur via a number of mechanisms, most of which involve hydrogen abstraction from the alpha-carbon site on the backbone. In this study, we demonstrate that initial attack at a side chain site, the beta-position (C-3), can give rise to formation of alpha-carbon radicals, and hence backbone cleavage, via the formation and subsequent beta-scission of C-3 alkoxyl radicals. This beta-scission reaction is rapid (k estimated to be >10(7) s(-)(1)) even with primary alkoxyl radicals derived from Ala residues, and occurs when the alkoxyl radicals are generated from a variety of precursors, including hydroperoxides and nitrate esters. These reactions release the former side chain as a reactive aldehyde or ketone; thus, Ala peptides release high yields of methanal (formaldehyde). This product has been quantified with a number of oxidized peptides and proteins, and can account for up to 64% of the initial attacking radicals with some Ala peptides. When quantified together with the hydroperoxide precursors, these species account for up to 80% of the initial radicals, confirming that this is a major process. Methanal causes cell toxicity and DNA damage and is an animal carcinogen and a genotoxic agent in human cells. Thus, the formation and subsequent reaction of alkoxyl radicals formed at the C-3 position on aliphatic amino acid side chains on peptides and proteins can give rise to both backbone fragmentation and the release of further reactive species which can cause cell toxicity and mutagenicity.

Heinrich-Hirsch B, Madle S, Oberemm A, Gundert-Remy U. **The use of toxicodynamics in risk assessment.** Toxicol Lett 2001;120(1-3):131-41.
Abstract: Risk assessment of xenobiotics is a qualitative and quantitative assessment of toxic properties conventionally based on data resulting from tests in animals exposed to the substance. The assessment of dose-effect relationship includes evaluation of exposure at the site of action. More recently, emphasis is put on understanding the relationship between exposure at the site of action and the resulting effect, i.e. toxicodynamic. In this respect, results from genotoxicity studies may be a measure for exposure and at the same time of an effect. Results of toxicodynamic endpoints such as binding to receptors or release of hormones have been used when replacing default values for interspecies extrapolation. It may also be

envisaged to use toxicodynamic endpoints in order to get an estimate of intraspecies variability. It was demonstrated that this approach may be helpful only if the relationship between the toxicodynamic endpoint and the definite endpoint is known by using the example of bisphenol A. Whereas there are clear effects of bisphenol A in in vitro and ex vivo studies, the classical two generation study has not been able to detect an effect on reproduction and/or fertility. Looking in the future development of toxicodynamic endpoints, gene profiling and the analysis of proteins ('proteomics') may be helpful tools employed in screening and being related to the mode of action are explored for their suitability in terms of toxicodynamic endpoints.

Hengstler JG, Utesch D, Steinberg P, Platt KL, Diener B, Ringel M, Swales N, Fischer T, Biefang K, Gerl M, et al. **Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction.** Drug Metab Rev 2000;32 (1):81-118.
Abstract: The use of primary hepatocytes is now well established for both studies of drug metabolism and enzyme induction. Cryopreservation of primary hepatocytes decreases the need for fresh liver tissue. This is especially important for research with human hepatocytes because availability of human liver tissue is limited. In this review, we summarize our research on optimization and validation of cryopreservation techniques. The critical elements for successful cryopreservation of hepatocytes are (1) the freezing protocol, (2) the concentration of the cryoprotectant [10% dimethyl-sulfoxide (DMSO)], (3) slow addition and removal of DMSO, (4) carbogen equilibration during isolation of hepatocytes and before cryopreservation, and (5) removal of unvital hepatocytes by Percoll centrifugation after thawing. Hepatocytes of human, monkey, dog, rat, and mouse isolated and cryopreserved by our standard procedure have a viability > or = 80%. Metabolic capacity of cryopreserved hepatocytes determined by testosterone hydroxylation, 7-ethoxyresorufin-O-de-ethylase (EROD), 7-ethoxycoumarin-O-deethylase (ECOD), glutathione S-transferase, UDP-glucuronosyl transferase, sulfotransferase, and epoxide hydrolase activities is > or = 60% of freshly isolated cells. Cryopreserved hepatocytes in suspension were successfully applied in short-term metabolism studies and as a metabolizing system in mutagenicity investigations. For instance, the complex pattern of benzo[a]pyrene metabolites including phase II metabolites formed by freshly isolated and cryopreserved hepatocytes was almost identical. For the study of enzyme induction, a longer time period and therefore cryopreserved hepatocyte cultures are required. We present a technique with cryopreserved hepatocytes that allows the induction of testosterone metabolism with similar induction factors as for fresh cultures. However, enzyme activities of induced hepatocytes and solvent controls were smaller in the cryopreserved cells. In conclusion, cryopreserved hepatocytes held in suspension can be recommended for short-term metabolism or toxicity studies. Systems with cryopreserved hepatocyte cultures that could be applied for studies of enzyme induction are already in a state allowing practical application, but may be further optimized.

Hildenbrand S, Gfrorer W, Schmahl FW, Dartsch PC. **New methods for determination of 2-butoxyethanol, butoxyacetaldehyde and butoxyacetic acid in aqueous systems, with special reference to cell culture conditions.** Arch Toxicol 2000;74(2):72-8.
Abstract: Ethylene glycol ethers, especially 2-ethoxyethanol and 2-butoxyethanol (BE) are frequently used in industry and household as solvents and detergents because of their excellent hydrophilic and lipophilic properties. BE and its oxidation products, butoxyacetaldehyde (BAL) and butoxyacetic acid

(BAA), are mainly associated with haemolytic toxicity. No method to determine BAL in aqueous systems (e.g. urine or blood) has been published up to now. BAL was synthesized by dehydration of BE and identified by gas chromatography-mass spectrometry. For determination of BAL and BE with head space-capillary gas chromatography, water and HCl or sodium dihydrogen phosphate were added to the sample. No further extraction or derivatization were necessary. For BAA determination after adding HCl and sodium dihydrogen phosphate the samples were extracted with ethyl acetate and derivatized with 2,2,2-trichloroethanol/HCl. The analytical methods presented here are reliable, sensitive and rapid. The new methods were developed for mammalian cell culture systems, because such in vitro systems are especially useful for metabolic studies and have the advantage of choosing species and organ specificity. In the cell culture experiments presented here it was demonstrated that Opossum kidney cells are able to metabolize BAL to BAA within 24 h. After this interval, in the cells neither BAL nor BAA were accumulated, whereas BAA was found in the cell culture media.

Holmes E, Nicholls AW, Lindon JC, Connor SC, Connelly JC, Haselden JN, Damment SJ, Spraul M, Neidig P, Nicholson JK. **Chemometric models for toxicity classification based on NMR spectra of biofluids**. Chem Res Toxicol 2000;13(6):471-8.
Abstract: 1H NMR spectroscopic and pattern recognition (PR)-based methods were used to investigate the biochemical variability in urine obtained from control rats and from rats treated with a hydrazine (a model hepatotoxin) or HgCl(2) (a model renal cortical toxin). The 600 MHz (1)H NMR spectra of urine samples obtained from vehicle- or toxin-treated Han-Wistar (HW) and Sprague-Dawley (SD) rats were acquired, and principal components analysis (PCA) and soft independent modeling of class analogy (SIMCA) analysis were used to investigate the (1)H NMR spectral data. Variation and strain differences in the biochemical composition of control urine samples were assessed. Control urine (1)H NMR spectra obtained from the two rat strains appeared visually similar. However, chemometric analysis of the control urine spectra indicated that HW rat urine contained relatively higher concentrations of lactate, acetate, and taurine and lower concentrations of hippurate than SD rat urine. Having established the extent of biochemical variation in the two populations of control rats, PCA was used to evaluate the metabolic effects of hydrazine and HgCl(2) toxicity. Urinary biomarkers of each class of toxicity were elucidated from the PC loadings and included organic acids, amino acids, and sugars in the case of mercury, while levels of taurine, beta-alanine, creatine, and 2-aminoadipate were elevated after hydrazine treatment. SIMCA analysis of the data was used to build predictive models (from a training set of 416 samples) for the classification of toxicity type and strain of rat, and the models were tested using an independent set of urine samples (n = 124). Using models constructed from the first three PCs, 98% of the test samples were correctly classified as originating from control, hydrazine-treated, or HgCl (2)-treated rats. Furthermore, this method was sensitive enough to predict the correct strain of the control samples for 79% of the data, based upon the class of best fit. Incorporation of these chemometric methods into automated NMR-based metabonomics analysis will enable on-line toxicological assessment of biofluids and will provide a tool for probing the mechanistic basis of organ toxicity.

Holmes E, Nicholson JK, Tranter G. **Metabonomic characterization of genetic variations in toxicological and metabolic responses using probabilistic neural networks.** Chem Res Toxicol 2001;14(2):182-91.
Abstract: Current emphasis on efficient screening of novel therapeutic agents in toxicological studies has

resulted in the evaluation of novel analytical technologies, including genomic (transcriptomic) and proteomic approaches. We have shown that high-resolution 1H NMR spectroscopy of biofluids and tissues coupled with appropriate chemometric analysis can also provide complementary data for use in in vivo toxicological screening of drugs. Metabonomics concerns the quantitative analysis of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification [Nicholson, J. K., Lindon, J. C., and Holmes, E. (1999) Xenobiotica 11, 1181-1189]. In this study, we have used 1H NMR spectroscopy to characterize the time-related changes in the urinary metabolite profiles of laboratory rats treated with 13 model toxins and drugs which predominantly target liver or kidney. These 1H NMR spectra were data-reduced and subsequently analyzed using a probabilistic neural network (PNN) approach. The methods encompassed a database of 1310 samples, of which 583 comprised a training set for the neural network, with the remaining 727 (independent cases) employed as a test set for validation. Using these techniques, the 13 classes of toxicity, together with the variations associated with strain, were distinguishable to >90%. Analysis of the 1H NMR spectral data by multilayer perceptron networks and principal components analysis gave a similar but less accurate classification than PNN analysis. This study has highlighted the value of probabilistic neural networks in developing accurate NMR-based metabonomic models for the prediction of xenobiotic-induced toxicity in experimental animals and indicates possible future uses in accelerated drug discovery programs. Furthermore, the sensitivity of this tool to strain differences may prove to be useful in investigating the genetic variation of metabolic responses and for assessing the validity of specific animal models.

Horowitz D, King AG. **Colorimetric determination of inhibition of hematopoietic progenitor cells in soft agar.** J Immunol Methods 2000;244(1-2):49-58.
Abstract: In vitro colony forming unit (CFU) assays have been used to measure the effects of compounds that regulate the growth of hematopoietic progenitor cells. These assays are time consuming and subjective and are therefore not amenable to high throughput of large numbers of compounds. Here we have shown that the traditional murine bone marrow CFU assay can be modified into a robust non-subjective colorimetric assay format. 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was added after colony formation in an agar based 96-well plate culture system. Optical density correlated with increasing cell input concentrations in the presence of growth factor. The linearity of this response was equivalent to the standard CFU assay. Several hematopoietic inhibitors were tested in both assays. Effects on colony number and size were compared to optical density. Compounds that reduced colony numbers with little effect on colony size had identical IC(50) values in both the colorimetric assay and CFU assay. The IC(50) values of compounds that also decreased colony size did not correlate in the two assays. These results demonstrate the utility of the colorimetric assay to rapidly screen for compounds that specifically inhibit hematopoietic progenitor cell colony formation in vitro.

Hosaka N, Ichikawa Y, Ishikawa T, Nagashima Y, Kunisaki C, Takahashi M, Moriwaki Y, Akiyama H, Yamaguchi S, Ota M, et al. **Correlation of immunohistochemical p53 labeling index with inhibition rate in chemosensitivity test in gastric and colon cancer.** Anticancer Res 2001;21(1A):229-35.
Abstract: To determine whether the expression of p53, p21, bcl-2 or Ki-67 in cancer cells is predictive of chemosensitivity, immunohistochemical examination of these factors and chemosensitivity assays were performed on colon and gastric cancer specimens. Chemosensitivity tests were performed using CDDP, 5-FU, MMC, or ADR and inhibition rate (IR) was calculated by MTT assay. Before exposure to

anticancer drugs, the samples were investigated immunohistochemically for expression of the above factors and after anticancer drug exposure by TUNNEL staining, for the presence of apoptotic cells. With 5-FU and MMC, the apoptotic index was well correlated with IR, so their effects were related to apoptosis. Moreover, with these two agents, the p53 labeling index (LI) was inversely correlated with IR and p21-LI showed a good correlation with IR. We therefore concluded that immunohistochemical studies for p53 and p21 were useful for predicting the chemosensitivities of colon and gastric cancer to MMC and 5-FU.

Hou DX, Kunitake T, Kusuda J, Fujii M. **Primary culture of chicken hepatocytes as an in vitro model for determining the influence of dioxin.** Biosci Biotechnol Biochem 2001;65(1):218-21.
Abstract: An easy method for primary culture of chicken hepatocytes was developed to study the influence of dioxin on birds. Chicken hepatocytes could maintain gene expression and protein secretion of albumin for a long period in serum-free medium with free atmosphere exchange at 37 degrees C. Moreover, the cells showed a sensitive response to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) by monitoring the expression of P450 1A, theta GST (theta-GST) and albumin genes.

Hu HL, Forsey RJ, Blades TJ, Barratt ME, Parmar P, Powell JR. **Antioxidants may contribute in the fight against ageing: an in vitro model**. Mech Ageing Dev 2000;121(1-3):217-30.
Abstract: Elderly humans have altered cellular redox levels and dysregulated immune responses, both of which are key events underlying the progression of chronic degenerative diseases of ageing, such as atherosclerosis and Alzeimer's disease. Poorly maintained cellular redox levels lead to elevated activation of nuclear transcription factors such as NFkB and AP-1. These factors are co-ordinately responsible for a huge range of extracellular signalling molecules responsible for inflammation, tissue remodelling, oncogenesis and apoptosis, progessess that orchestrate many of the degenerative processess associated with ageing. It is now clear that levels of endogenous anti-oxidants such as GSH decrease with age. This study aimed to investigate the potential of exogenous anti-oxidants to influence inflammatory responses and the ageing process itself. We investigated the potential of the dietary antioxidant, quercetin, to reverse the age related influences of GSH depletion and oxidative stress using in vitro human umbilical vein endothelial cells (HUVEC) and human skin fibroblast (HSF) cell models. Oxidative stress-induced inflammatory responses were investigated in a GSH depletion and a Phorbol 12-myristate 13-acetate (PMA)-induced stress model. As measured with a sensitive HPLC fluorescence method, GSH in HUVEC was depleted by the addition of L-buthionine-[S,R]-sulfoxiniine (BSO), a gamma-glutamylcysteine synthetase inhibitor, to the culture medium at a concentration of 0.25 mM. Time course studies revealed that the GSH half-life was 4.6 h in HUVEC. GSH depletion by BSO for 24 h led to a slight increase in intracellular adhesion molecule - 1 (ICAM1) expression and prostaglandin E2 (PGE2) secretion in both types of cells. However, GSH depletion markedly enhanced PMA-induced ICAM and PGE2 production in HUVEC. Responses were progressively elevated following prolonged BSO treatment. Inhibition studies showed that 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H7), a protein kinase C (PKC) inhibitor, not only abolished most of PMA-induced ICAM-1 expression and PGE2, production, but also eliminated GSH depletion-enhanced PMA stimulation. This enhancement was also inhibited by supplementation with quercetin. The results clearly demonstrate that GSH depletion increased the susceptibility of vascular endothelial cells and fibroblasts to oxidative stress associated inflammatory stimuli. This increased in vitro susceptibility may be extrapolated to the in vivo

situation of ageing, providing a useful model to study the influence of micronutrients on the ageing process. In conclusion, these data suggest that dietary antioxidants could play a significant role in the reduction of inflammatory responses.

Hu TL. **Kinetics of azoreductase and assessment of toxicity of metabolic products from azo dyes by Pseudomonas luteola.** Water Sci Technol 2001;43(2):261-9.
Abstract: This is a continuous study on a decolorization strain, Pseudomonas luteola, which involves treating seven azo dyes with different structures. This study focuses mainly on determining both the mechanism of decolorization by P. luteola and the activity of azoreductase from P. luteola as well as identifying and assessing the toxicity of metabolic products of azo dyes. The growth of P. luteola reached the stationary phase after shaking incubation for 24 hours. Then, while being kept static, the color of seven tested azo dyes (100 mg/l) could be removed. The proportion of color removal was between 59-99%, which figure is related to the structure of the dye. Monoazo dyes (RP2B, V2RP and Red 22) showed the fastest rate of decolorization, i.e. from 0.23-0.44 mg dye-mg cell-1 hr-1. P. luteola could remove the color of V2RP and a leather dye at a concentration of 200 mg/l, and as to the rest of the azo dyes, it could remove at a concentration of up to 100 mg/l. Decolorization of RP2B and Red 22 required activation energy of 7.00 J/mol and 6.63 J/mole, respectively, indicating that it was easier for azoreductase to decolorize structurally simple dyes. The kinetics of azoreductase towards seven azo dyes suggested a competitive inhibition model be applied. Microtox was used to analyze the toxicity of the metabolic products of azo dyes. EC50 showed differences in toxicity before and after the azo dyes had been metabolized. Analysis revealed significant differences between the results obtained by EC50 with Blue 15 and those obtained with the leather dye, indicating that the toxicities of the metabolic products were increased. The differences obtained by EC50 with Red 22, RP2P and V2RP were small, and Black 22 showed no such difference. Sulfanic acid and orthanilic acid may be the intermediate products of Violet 9 and RP2B, respectively. However, according to FT-IR analysis, aromatic amines were present in the metabolic product.

Hu Y, Moraes CT, Savaraj N, Priebe W, Lampidis TJ. **Rho(0) tumor cells: a model for studying whether mitochondria are targets for rhodamine 123, doxorubicin, and other drugs.** Biochem Pharmacol 2000;60(12):1897-905.
Abstract: A human osteosarcoma cell line devoid of mitochondrial DNA (rho(0)) and its wild-type parental cell counterpart (wt) are presented as a model to investigate drug targeting. By virtue of the absence of mitochondrial DNA, rho(0) cells cannot perform electron transport or oxidative phosphorylation. Since most of the drugs studied are transported by the efflux pumping systems controlled by the MDR1 and MRP1 genes, both cell lines were examined for the expression of these genes, and it was found that no MDR1 and only low amounts of MRP1 were expressed. Growth inhibition experiments indicated that doxorubicin (Dox), vinblastine, and paclitaxel were equitoxic in these cell lines. On the other hand, the IC(50) for rhodamine 123 (Rho 123) in rho(0) cells was 50 times higher than in wt cells. This result correlates with a lower accumulation of Rho 123 in rho(0) cells as measured by fluorescence microscopy and flow cytometry (3 times less than in wt cells). In contrast, when stained with Dox, both cell types accumulated similar amounts. Surprisingly, in these non-P-glycoprotein expressing cells, verapamil increased both Dox and Rho 123 retention. Overall, these data suggest that: (i) functional mitochondria do not appear to be targets for the growth inhibitory activities

of Dox, paclitaxel, or vinblastine; (ii) for lipophilic cations like Rho 123, however, normal functioning mitochondria and maintenance of a normal mitochondrial membrane potential (Deltapsi(mt)) appear to play a critical role in the intracellular accumulation and subsequent cytotoxicities of these compounds; and (iii) verapamil increases drug accumulation in non-P-glycoprotein expressing cell lines, most likely by direct action on Deltapsi(mt) for Rho 123 and safranin O, and on heretofore unidentified plasma membrane transporters, as well as via interaction with low levels of MRP1, for Dox. These results should be considered when Rho 123 and verapamil are used to detect P-glycoprotein.

Isukapalli SS, Roy A, Georgopoulos PG. **Efficient sensitivity/uncertainty analysis using the combined stochastic response surface method and automated differentiation: application to environmental and biological systems.** Risk Anal 2000;20(5):591-602.
Abstract: Estimation of uncertainties associated with model predictions is an important component of the application of environmental and biological models. "Traditional" methods for propagating uncertainty, such as standard Monte Carlo and Latin Hypercube Sampling, however, often require performing a prohibitive number of model simulations, especially for complex, computationally intensive models. Here, a computationally efficient method for uncertainty propagation, the Stochastic Response Surface Method (SRSM) is coupled with another method, the Automatic Differentiation of FORTRAN (ADIFOR). The SRSM is based on series expansions of model inputs and outputs in terms of a set of "well-behaved" standard random variables. The ADIFOR method is used to transform the model code into one that calculates the derivatives of the model outputs with respect to inputs or transformed inputs. The calculated model outputs and the derivatives at a set of sample points are used to approximate the unknown coefficients in the series expansions of outputs. A framework for the coupling of the SRSM and ADIFOR is developed and presented here. Two case studies are presented, involving (1) a physiologically based pharmacokinetic model for perchloroethylene for humans, and (2) an atmospheric photochemical model, the Reactive Plume Model. The results obtained agree closely with those of traditional Monte Carlo and Latin hypercube sampling methods, while reducing the required number of model simulations by about two orders of magnitude.

Jagadeeswaran P, Gregory M, Johnson S, Thankavel B. **Haemostatic screening and identification of zebrafish mutants with coagulation pathway defects: an approach to identifying novel haemostatic genes in man.** Br J Haematol 2000;110(4):946-56.
Abstract: Zebrafish were used as a model to study haemostasis, a vertebrate function of paramount importance. A limitation of the zebrafish model is the difficulty in assaying small amounts of blood to detect coagulation mutants. We report the use of a rapid total coagulation activity (TCA) assay to screen for coagulation defects in individual adult zebrafish. We screened the TCA in 1000 gynogenetic half-tetrad diploids derived from 86 clutches. Each clutch was from a single F1 female offspring of males mutagenized with ethylnitrosourea (ENU). We found 30-50% defective zebrafish among six clutches, consistent with a heritable defect. The assay developed here provided a rapid screen to detect overall coagulation defects. However, because of the limited amounts of plasma, we could not detect defects in specific pathways. Therefore, a novel, ultra-sensitive kinetic method was developed to identify specific pathway defects. To test whether the kinetic assay could be used as a screening tool, 1500 Florida wild-type zebrafish pairs were analysed for naturally occurring coagulation defects. We detected 30 fish with extrinsic pathway defects, but with intact common and intrinsic pathways. We conclude that it is now

possible to identify specific coagulation pathway defects in zebrafish.

Janik M, Kleinhans FW, Hagedorn M. **Overcoming a permeability barrier by microinjecting cryoprotectants into zebrafish embryos (Brachydanio rerio).** Cryobiology 2000;41(1):25-34.
Abstract: The goal of this research was to examine the developmental effects on zebrafish embryos (Brachydanio rerio) when cryoprotectants were directly microinjected into the yolk. Our objectives were to: (i) determine the final concentration of propylene glycol (PG) and dimethyl sulfoxide (Me(2)SO) that the embryos could tolerate without causing teratogenic effects; (ii) determine if the toxicity of Me(2)SO could be reduced by the simultaneous presence of various proportions of amides; and (iii) examine whether this intracellular cryoprotectant incorporation could reduce the cryodamage to the yolk syncytial layer (YSL) after vitrification trials. The rationale for conducting these microinjection experiments was to overcome the permeability barrier of the YSL. Intracellular PG produced better survival than Me(2)SO ($P < 0.05$). Embryos tolerated both 10- and 30-nl microinjections of PG, yielding final concentrations of 2.3 and 5.0 M within the yolk, resulting in 70 +/- 3 and 35 +/- 4% survival at day 5, respectively. In similar experiments with Me(2)SO, survival was lower than PG at 60 +/- 4 and 14 +/- 4% at 2.4 and 5.2 M. Unlike other cellular systems, the presence of amides, specifically acetamide or formamide, did not reduce the toxicity of Me(2)SO in zebrafish embryos ($P > 0.05$). During vitrification trials, we estimated a 25% dehydration of the yolk, yielding an effective PG concentration of 5.9 M. However, the incorporation of this vitrifiable concentration of PG was not sufficient to improve the postthaw morphology of the YSL ($P > 0.05$). Clearly, other factors need to be examined in establishing a successful vitrification protocol for zebrafish embryos.

Jones LC, Hoban PW. **Treatment plan comparison using equivalent uniform biologically effective dose (EUBED).** Phys Med Biol 2000;45(1):159-70.
Abstract: With the continuing improvement in computer speed, dose distributions can be calculated quickly with confidence. However, the resulting biological effect is known with much less certainty, despite its critical importance when assessing treatment plans. To assess plans accurately, biologically based methods of ranking plans are necessary. Many authors have suggested the use of dose volume histograms with reduction schemes and Niemierko has recently introduced another method based on the cell kill occurring in the tumour. This study presents an investigation into this value and suggests a use in prescribing dose. Equivalent uniform dose (EUD) can obviously be used for assessing treatment plans, although in its current form it is not adequate for assessing normal tissues; however, it can also be used to adjust the prescription dose ensuring all plans deliver the same EUD to the tumour. Once this is performed, plans can more easily be assessed on the effects to the normal tissues. In calculating the EUD another concept is introduced--the equivalent uniform biologically effective dose (EUBED). This value considers the distribution of dose and dose per fraction when comparing plans. Reduced dose per fraction at the edge of the target volume will exacerbate the effect of reduced dose on cell kill. Two methods are suggested for calculating the necessary prescription dose: one using an iterative method and one using the gradient of the EUBED function. A comparison was made for a series of stereotactic cases using different collimator sizes. Interestingly, using this method, although the maximum doses were different, the dose volume histograms (DVHs) for the brainstem were similar in all cases.

Jonsson F, Bois F, Johanson G. **Physiologically based pharmacokinetic modeling of inhalation**

exposure of humans to dichloromethane during moderate to heavy exercise. Toxicol Sci 2001;59 (2):209-18.
Abstract: Dichloromethane (methylene chloride, DCM) is metabolized via two pathways in humans: mixed-function oxidases (MFO) and glutathione-S:-transferase (GST). Most likely, the carcinogenicity for DCM is related to metabolic activation of DCM via the GST pathway. However, as the two pathways are competing, the metabolic capacity for the MFO pathway in vivo is also of interest in risk assessment for DCM. Past estimates of MFO metabolism are based on the in vitro activity of tissue samples. The aim of the present study was to develop a population model for DCM in order to gain more knowledge on the variability of DCM inhalation toxicokinetics in humans, with main emphasis on the MFO metabolic pathway. This was done by merging published in vitro data on DCM metabolism and partitioning with inhalation toxicokinetic data (Astrand et al., 1975, Scand. J. Work.Environ. Health 1, 78-94) from five human volunteers, using the MCMC technique within a population PBPK model. Our results indicate that the metabolic capacity for the MFO pathway in humans is slightly larger than previously estimated from four human liver samples. Furthermore, the interindividual variability of the MFO pathway in vivo is smaller among our five subjects than indicated by the in vitro samples. We also derive a Bayesian estimate of the population distribution of the MFO metabolism (median maximum metabolic rate 28, 95% confidence interval 12-66 micromol/min) that is a compromise between the information from the in vitro data and the toxicokinetic information present in the experimental data.

Jonsson F, Bois FY, Johanson G. **Assessing the reliability of PBPK models using data from methyl chloride-exposed, non-conjugating human subjects.** Arch Toxicol 2001;75(4):189-99.
Abstract: Physiologically based pharmacokinetic (PBPK) models are often optimized by adjusting metabolic parameters so as to fit experimental toxicokinetic data. The estimates of the metabolic parameters are then conditional on the assumed values for all other parameters. Meanwhile, the reliability of other parameters, or the structural model, is usually not questioned. Inhalation exposures with human volunteers in our laboratory show that non-conjugators lack metabolic capacity for methyl chloride entirely, and that elimination in these subjects takes place via exhalation only. Therefore, data from these methyl chloride exposures provide an excellent opportunity to assess the general reliability of standard inhalation PBPK models for humans. A hierarchical population PBPK model for methyl chloride was developed. The model was fit to the experimental data in a Bayesian framework using Markov chain Monte Carlo (MCMC) simulation. In a Bayesian analysis, it is possible to merge a priori knowledge of the physiological, anatomical and physicochemical parameters with the information embedded in the experimental toxicokinetic data obtained in vivo. The resulting estimates are both statistically and physiologically plausible. Model deviations suggest that a pulmonary sub-compartment may be needed in order to describe the inhalation and exhalation of volatile adequately. The results also indicate that there may be significant intra-individual variability in the model parameters. To our knowledge, this is the first time that the toxicokinetics of a non-metabolized chemical is used to assess population PBPK parameters. This approach holds promise for more elaborate experiments in order to assess the reliability of PBPK models in general.

Jonsson F, Johanson G. A **Bayesian analysis of the influence of GSTT1 polymorphism on the cancer risk estimate for dichloromethane.** Toxicol Appl Pharmacol 2001;174(2):99-112.
Abstract: The carcinogenicity of dichloromethane (DCM) is related to metabolic activation mediated by

glutathione transferase theta 1 (GSTT1), whereas oxidation serves as a detoxification pathway. The aim of this study was to calculate the excess cancer risk from DCM, using Bayesian statistics. In a first step, a previously developed population physiologically based pharmacokinetic (PBPK) model for DCM was simultaneously fitted to extensive human toxicokinetic data from 27 male volunteers exposed to 250-1000 ppm DCM (Astrand et al. Scand. J. Work Environ. Health 1, 78-94, 1975; Engstrom and Bjurstrom, Scand. J. Work Environ. Health 7, 215-224, 1977) using Markov chain Monte Carlo simulation. Improved population estimates were obtained for the PBPK model parameters. In a second step, excess cancer risk was calculated for lifelong exposure to 1-1000 ppm DCM by Monte Carlo simulation. Data on GSTT1 gene frequencies in the Swedish population were used, including all three genotypes. Estimated mean and median excess risks were in general agreement with those previously derived (El-Masri et al. Toxicol. Appl. Pharmacol. 158, 221-230, 1999). However, we estimate higher excess risks at the upper confidence limits. Furthermore, our simulations suggest that 1% of the Swedish population is not covered by a factor 4.2-7.1 away from the mean target dose. The majority of the fraction of the population not covered was classified as GSTT1 homozygote. This indicates that a higher uncertainty factor than the commonly used 3.16 should be considered in noncancer risk assessment for substances with polymorphic bioactivation. Copyright 2001 Academic Press.

Kaal EC, Vlug AS, Versleijen MW, Kuilman M, Joosten EA, Bar PR. **Chronic mitochondrial inhibition induces selective motoneuron death in vitro: a new model for amyotrophic lateral sclerosis.** J Neurochem 2000;74(3):1158-65.
Abstract: Evidence is increasing that mitochondrial dysfunction is involved in amyotrophic lateral sclerosis, a neurodegenerative disease characterized by selective motoneuron death. To study the role of mitochondrial dysfunction in the pathways leading to motoneuron death, we developed an in vitro model of chronic motoneuron toxicity, based on malonate-induced inhibition of complex II in the mitochondrial electron transport chain. Treatment with malonate resulted in a dose-dependent decrease in cellular ATP levels. We observed that motoneurons were significantly more vulnerable to mitochondrial inhibition than control neurons in the dorsal horn. We could reproduce this dose-dependent phenomenon with the complex IV inhibitor sodium azide. The free radical scavenger alpha-phenyl-N-tert-butylnitrone, the AMPA/kainate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione, and riluzole, a drug that is currently used for the treatment of amyotrophic lateral sclerosis, were protective against malonate-induced motoneuron death. Furthermore, the caspase inhibitors N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone and z-Asp-Glu-Val-Asp-fluoromethyl ketone were both protective against malonate toxicity. Our model shows that chronic mitochondrial inhibition leads to selective motoneuron death, which is most likely apoptotic.

Kanamitsu S, Ito K, Green CE, Tyson CA, Shimada N, Sugiyama Y. **Prediction of in vivo interaction between triazolam and erythromycin based on in vitro studies using human liver microsomes and recombinant human CYP3A4.** Pharm Res 2000;17(4):419-26.
Abstract: PURPOSE: To quantitatively predict the in vivo interaction between triazolam and erythromycin, which involves mechanism-based inhibition of CYP3A4, from in vitro studies using human liver microsomes (HLM) and recombinant human CYP3A4 (REC). METHODS: HLM or REC was preincubated with erythromycin in the presence of NADPH and then triazolam was added. alpha- and 4-hydroxy (OH) triazolam were quantified after a 3 min incubation and the kinetic parameters for

enzyme inactivation (k(inact) and K('app)) were obtained. Drug-drug interaction in vivo was predicted based on a physiologically-based pharmacokinetic (PBPK) model, using triazolam and erythromycin pharmacokinetic parameters obtained from the literature and kinetic parameters for the enzyme inactivation obtained in the in vitro studies. RESULTS: Whichever enzyme was used, triazolam metabolism was not inhibited without preincubation, even if the erythromycin concentration was increased. The degree of inhibition depended on preincubation time and erythromycin concentration. The values obtained for k(inact) and K('app) were 0.062 min(-1) and 15.9 microM (alpha-OH, HLM), 0.055 min(-1) and 17.4 microM (4-OH, HLM), 0.173 min(-1) and 19.1 microM (alpha-OH, REC), and 0.097 min(-1) and 18.9 microM (4-OH, REC). Based on the kinetic parameters obtained using HLM and REC, the AUCpo of triazolam was predicted to increase 2.0- and 2.6-fold, respectively, following oral administration of erythromycin (333 mg t.i.d. for 3 days), which agreed well with the reported data. CONCLUSIONS: In vivo interaction between triazolam and erythromycin was successfully predicted from in vitro data based on a PBPK model involving a mechanism-based inhibition of CYP3A4.

Kanamitsu SI, Ito K, Okuda H, Ogura K, Watabe T, Muro K, Sugiyama Y. **Prediction of in vivo drug-drug interactions based on mechanism-based inhibition from in vitro data: inhibition of 5-fluorouracil metabolism by (E)-5-(2-Bromovinyl)uracil.** Drug Metab Dispos 2000;28(4):467-74.
Abstract: The fatal drug-drug interaction between sorivudine, an antiviral drug, and 5-fluorouracil (5-FU) has been shown to be caused by a mechanism-based inhibition. In this interaction, sorivudine is converted by gut flora to (E)-5-(2-bromovinyl)uracil (BVU), which is metabolically activated by dihydropyrimidine dehydrogenase (DPD), and the activated BVU irreversibly binds to DPD itself, thereby inactivating it. In an attempt to predict this interaction in vivo from in vitro data, inhibition of 5-FU metabolism by BVU was investigated by using rat and human hepatic cytosol and human recombinant DPD. Whichever enzyme was used, increased inhibition was observed that depended on the preincubation time of BVU and enzyme in the presence of NADPH and BVU concentration. The kinetic parameters obtained for inactivation represented by k(inact) and K'(app) were 2.05 +/- 1.52 min(-1), 69.2 +/- 60.8 microM (rat hepatic cytosol), 2.39 +/- 0.13 min(-1), 48.6 +/- 11.8 microM (human hepatic cytosol), and 0.574 +/- 0.121 min(-1), 2.20 +/- 0.57 microM (human recombinant DPD). The drug-drug interaction in vivo was predicted quantitatively based on a physiologically based pharmacokinetic model, using pharmacokinetic parameters obtained from the literature and kinetic parameters for the enzyme inactivation obtained in the in vitro studies. In rats, DPD was predicted to be completely inactivated by administration of BVU and the area under the curve of 5-FU was predicted to increase 11-fold, which agreed well with the reported data. In humans, a 5-fold increase in the area under the curve of 5-FU was predicted after administration of sorivudine, 150 mg/day for 5 days. Mechanism-based inhibition of drug metabolism is supposed to be very dangerous. We propose that such in vitro studies should be carried out during the drug-developing phase so that in vivo drug-drug interactions can be predicted.

Katsuyama R, Morioka A, Oka S, Kawasaki T. **Expression of macrophage asialoglycoprotein-binding protein is induced through MAPK classical pathway**. Biochem Biophys Res Commun 2001;280(5):1269-73.
Abstract: Macrophage asialoglycoprotein-binding protein (M-ASGP-BP) is a Gal/GalNAc-specific lectin, which functions as an endocytosis receptor. We found that the expression of M-ASGP-BP mRNA

in bone marrow cells was induced during the differentiation into macrophages. To investigate the mechanism by which M-ASGP-BP mRNA expression is induced, we used U937 cells as a model. Treatment of U937 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) resulted in M-ASGP-BP mRNA expression within 6 h. This induction was completely inhibited by PKC inhibitors, calphostin C, and staurosporine. Furthermore, MAP kinase inhibitors PD98059, but not SB202190, blocked M-ASGP-BP mRNA expression. These data indicate that M-ASGP-BP mRNA expression occurs through the activation of PKC and the MAPK classical pathway in the course of cell differentiation into macrophages.

Kehrer JP. **The Haber-Weiss reaction and mechanisms of toxicity**. Toxicology 2000;149(1):43-50.
Abstract: The concept that the highly reactive hydroxyl radical (HO) could be generated from an interaction between superoxide (O(2)(-)) and hydrogen peroxide (H(2)O(2)) was proposed (with Joseph Weiss) in Professor Haber's final paper published in 1934. Until it was recognized that free radicals are produced in biological systems, this finding seemed to have no relevance to biology. However, following the discovery that O(2)(-) was a normal cellular metabolite, it was quickly recognized that the Haber-Weiss reaction (O(2)(-)+H(2)O(2) -->HO+O(2)+HO(-)) might provide a means to generate more toxic radicals. Although the basic reaction has a second order rate constant of zero in aqueous solution and thus cannot occur in biological systems, the ability of iron salts to serve as catalysts was discussed by these authors. Because transition metal ions, particularly iron, are present at low levels in biological systems, this pathway (commonly referred to as the iron-catalyzed Haber-Weiss reaction) has been widely postulated to account for the in vivo generation of the highly reactive HO. Recent data documenting the importance of redox regulation of various cellular signaling pathways makes it clear that free radicals are essential for normal cellular function. However, this also makes it obvious that disruptions of free radical production or defenses at many different levels can lead to adverse effects on cells. While the generation of HO, which is by far the most reactive oxygen species, is generally indicative of an overtly toxic event, it is through studies at this level that we have reached a better understanding of free radicals as both signaling molecules and toxic species.

Kelly SP, Fletcher M, Part P, Wood CM. **Procedures for the preparation and culture of 'reconstructed' rainbow trout branchial epithelia.** Methods Cell Sci 2000;22(2-3):153-63.
Abstract: Techniques for the in vitro 'reconstruction' of freshwater rainbow trout branchial epithelia using the primary culture of gill cells on permeable polyethylene terephthalate cell culture filter supports are described. Representing models of the freshwater fish gill, epithelia grown by two separate techniques are composed of branchial pavement cells with or without the inclusion of mitochondria-rich (MR) cells. The generation of epithelia consisting of pavement cells only (via a method called single seeded inserts = SSI) involves an initial period of flask culture during which time MR cells, that appear unable to attach to the culture flask base, are excluded from the general cell populace. Alternately, the generation of a heterogeneous epithelia consisting of both pavement cells and MR cells (via a method called double seeded inserts = DSI) is facilitated by the direct seeding of cells into cell culture filter inserts. Critical to this second procedure is the repeat seeding of filter inserts over a two day period. Repeat seeding appears to allow MR cells to nest amongst the attached cell layer generated by the first day's seeding. The use of cell culture filter supports allows free access to both the apical and basolateral compartment of the epithelium and is ideal for experimental manipulation. Cells are grown under

symmetrical conditions (apical media/basolateral media) and epithelium growth is measured as a function of transepithelial resistance (TER). When the epithelia exhibit a plateau in growth they can be subjected to asymmetrical conditions (freshwater apical/media basolateral) in order to assess gill cell function as in vivo.

Keys DA, Wallace DG, Kepler TB, Conolly RB. **Quantitative evaluation of alternative mechanisms of blood disposition of di(n-butyl) phthalate and mono(n-butyl) phthalate in rats.** Toxicol Sci 2000;53(2):173-84.
Abstract: Phthalate esters are ubiquitous, low-level environmental contaminants that induce testicular toxicity in laboratory animals. The diester is rapidly metabolized in the gut to the monoester, which causes the testicular toxicity. Several physiologically based pharmacokinetic (PBPK) model structures have been evaluated for di(2-ethylhexyl) phthalate (DEHP) and mono(2-ethylhexyl) phthalate (MEHP). The objective of this study was to test these PBPK models for a less lipophilic phthalate diester, di(n-butyl) phthalate (DBP), and monoester, mono(n-butyl) phthalate (MBP). Alternate models describing enterohepatic circulation, diffusion-limitation, tissue pH gradients (pH trapping), and a simpler, flow-limited model were evaluated. A combined diffusion-limited and pH trapping model was also tested. MBP tissue:blood partition coefficients were similar when determined either experimentally by a nonvolatile, vial equilibration technique or algorithmically. All other parameters were obtained from the literature or estimated from MBP blood concentrations following intravenous or oral exposure to DBP or MBP. A flow-limited model was unable to predict MBP blood levels, whereas each alternative model had statistically better predictions. The combined diffusion-limited and pH trapping model was the best overall, having the highest log-likelihood function value. This result is consistent with a previous finding that the pH trapping model was the best model for describing DEHP and MEHP blood dosimetry, though it was necessary to extend the model to include diffusion-limitation. The application of the pH trapping model is a step toward developing a generic model structure for all phthalate esters, though more work is required before a generic structure can be identified with confidence. Development of a PBPK model structure applicable to all phthalate esters would support more realistic assessments of risk to human health from exposure to one or more members of this class of compounds.

Kim CS, Sandberg JA, Slikker W, Binienda Z, Schlosser PM, Patterson TA. **Quantitative exposure assessment: application of physiologically-based pharmacokinetic (PBPK) modeling of low-dose, long-term exposures of organic acid toxicant in the brain.** 2001;9(4):153-60.
Abstract: Our objective was to construct a physiologically-based pharmacokinetic (PBPK) model describing the kinetic behavior of 2,4-dichlorophenoxyacetic acid (2,4-D) on rats after long-term exposures to low doses. Our study demonstrated the model's ability to simulate uptake of 2,4-D in discrete areas of the rat brain. The model was derived from the generic PBPK model that was first developed for high-dose, single exposures of 2,4-D to rats or rabbits (Kim, C.S., Gargas, M.L., Andersen, M.E., 1994. Pharmacokinetic modeling of 2,4-dichlorophenoxyacetic acid (2,4-D) in rats and rabbits brain following single dose administration. Toxicol. Lett. 74, 189-201; Kim, C.S., Slikker, W., Jr., Binienda, Z., Gargas, M.L., Andersen, M.E., 1995. Development of a physiologically based pharmacokinetic (PBPK) model for 2,4-dichlorophenoxyacetic acid (2,4-D) dosimetry in discrete areas of the brain following a single intraperitoneal or intravenous dose. Neurotox. Teratol. 17, 111-120.), to which a subcutaneous (hypodermal) compartment was incorporated for low-dose, long-term infusion. It

consisted of two body compartments, along with compartments for venous and arterial blood, cerebrospinal fluid, brain plasma and six brain regions. Uptake of the toxin was membrane-limited by the blood-brain barrier with clearance from the brain provided by cerebrospinal fluid 'sink' mechanisms. This model predicted profiles of 2,4-D levels in brain and blood over a 28-day period that compared well with concentrations measured in vivo with rats that had been given 2,4-D (1 or 10 mg/kg per day) with [14C]-2,4-D subcutaneously (s.c.) for 7, 14, or 28 days, respectively. This PBPK model should be an effective tool for evaluating the target tissue doses that may produce the neurotoxicity of organic acid toxicants after low-dose, long-term exposures to contaminated foods or the environment.

Kimbell JS, Subramaniam RP. **Use of computational fluid dynamics models for dosimetry of inhaled gases in the nasal passages.** Inhal Toxicol 2001;13(5):325-34.
Abstract: Computational fluid dynamics (CFD) models of the nasal passages of a rat, monkey, and human are being used (1) to determine important factors affecting nasal uptake, (2) to make interspecies dosimetric comparisons, (3) to provide detailed anatomical information for the rat, monkey, and human nasal passages, and (4) to provide estimates of regional air-phase mass transport coefficients (a measure of the resistance to gas transport from inhaled air to airway walls) in the nasal passages of all three species. For many inhaled materials, lesion location in the nose follows patterns that are both site and species specific. For reactive, water-soluble (Category 1) gases, regional uptake can be a major factor in determining lesion location. Since direct measurement of airflow and uptake is experimentally difficult, CFD models are used here to predict uptake patterns quantitatively in three-dimensional reconstructions of the F344 rat, rhesus monkey, and human nasal passages. In formaldehyde uptake simulations, absorption processes were assumed to be as rapid as possible, and regional flux (transport rate) of inhaled formaldehyde to airway walls was calculated for rats, primates, and humans. For uptake of gases like vinyl acetate and acrylic acid vapors, physiologically based pharmacokinetic uptake models incorporating anatomical and physical information from the CFD models were developed to estimate nasal tissue dose in animals and humans. The use of biologically based models in risk assessment makes sources of uncertainty explicit and, in doing so, allows quantification of uncertainty through sensitivity analyses. Limited resources can then be focused on reduction of important sources of uncertainty to make risk estimates more accurate.

Kimura S, Gonzalez FJ. **Applications of genetically manipulated mice in pharmacogenetics and pharmacogenomics.** Pharmacology 2000;61(3):147-53.
Abstract: Gene knockout mice have proven to be very useful in understanding the role of xenobiotic-metabolizing enzymes in chemical toxicity and carcinogenesis. The combination of gene knockout technology with transgenic mouse technology should provide more versatile and suitable animal models to study the risks of chemical exposures in humans in terms of toxicity and carcinogenesis, as well as development and design of new therapeutic drugs. Recent studies using genetically manipulated mice are summarized.

Kirman CR, Hays SM, Kedderis GL, Gargas ML, Strother DE. **Improving cancer dose-response characterization by using physiologically based pharmacokinetic modeling: an analysis of pooled data for acrylonitrile-induced brain tumors to assess cancer potency in the rat.** Risk Anal 2000;20 (1):135-51.

Abstract: Historically, U.S. regulators have derived cancer slope factors by using applied dose and tumor response data from a single key bioassay or by averaging the cancer slope factors of several key bioassays. Recent changes in U.S. Environmental Protection Agency (EPA) guidelines for cancer risk assessment have acknowledged the value of better use of mechanistic data and better dose-response characterization. However, agency guidelines may benefit from additional considerations presented in this paper. An exploratory study was conducted by using rat brain tumor data for acrylonitrile (AN) to investigate the use of physiologically based pharmacokinetic (PBPK) modeling along with pooling of dose-response data across routes of exposure as a means for improving carcinogen risk assessment methods. In this study, two contrasting assessments were conducted for AN-induced brain tumors in the rat on the basis of (1) the EPA's approach, the dose-response relationship was characterized by using administered dose/concentration for each of the key studies assessed individually; and (2) an analysis of the pooled data, the dose-response relationship was characterized by using PBPK-derived internal dose measures for a combined database of ten bioassays. The cancer potencies predicted for AN by the contrasting assessments are remarkably different (i.e., risk-specific doses differ by as much as two to four orders of magnitude), with the pooled data assessments yielding lower values. This result suggests that current carcinogen risk assessment practices overestimate AN cancer potency. This methodology should be equally applicable to other data-rich chemicals in identifying (1) a useful dose measure, (2) an appropriate dose-response model, (3) an acceptable point of departure, and (4) an appropriate method of extrapolation from the range of observation to the range of prediction when a chemical's mode of action remains uncertain.

Kobayashi T, Zinchuk VS, Okada T, Wakiguchi H, Kurashige T, Takatsuji H, Seguchi H. **A simple approach for the analysis of intracellular movement of oxidant-producing intracellular compartments in living human neutrophils**. Histochem Cell Biol 2000;113(4):251-7.
Abstract: In human neutrophils, superoxide is generated primarily within specialized oxidant-producing intracellular compartments. The present study employs a simple methodological approach to evaluate the intracellular movement of these structures in living human neutrophils. Using a CCD camera system, we monitored fluorescence in cells loaded with the succinimidyl ester of dichlorodihydrofluorescein diacetate, which is nonfluorescent until oxidized by reactive oxygen species. Fluorescence-positive intracellular compartments became detectable after neutrophils were stimulated with phorbol myristate acetate for 1 min. Further stimulation increased the intracellular compartments in both number and size in a time-dependent manner. Upon stimulation with phorbol myristate acetate, no fluorescence was seen in intracellular compartments of neutrophils isolated from patients with X-linked chronic granulomatous disease lacking gp91-phox, a membrane component of NADPH oxidase. The method enables tracking of the movement of a single oxidant-producing intracellular compartment following cell stimulation and visualization of the intracellular structures formed by fusion of oxidant-producing intracellular compartments with endocytotic vesicles and phagosomes. Therefore, it is considered to be an informative tool for evaluation of the intracellular dynamics of oxidant-producing intracellular compartments in living human neutrophils and may have a diagnostic value.

Kohn MC. **Current directions in physiological modeling for environmental health sciences: an overview.** Environ Health Perspect 2000;108 Suppl 5:857-9.

Kohn MC, Walker NJ, Kim AH, Portier CJ. **Physiological modeling of a proposed mechanism of enzyme induction by TCDD.** Toxicology 2001;162(3):193-208.
Abstract: A physiological model was previously constructed to facilitate extrapolation of surrogates for the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rat liver to doses comparable to human environmental exposures. The model included induction of P450 isozymes and suggested the presence of multiple binding sites with different affinities for the TCDD-liganded Ah receptor at CYP1A1 dioxin responsive elements. The model also indicated that protein synthesis on the mRNA template exhibited saturation kinetics with respect to message levels. In the present work the earlier model was revised to include the increased proteolysis of the Ah receptor on binding TCDD, more realistic representations of gene transcription and mRNA translation, and different stability for each mRNA. The revised model includes multiple TCDD-liganded Ah receptor binding sites for CYP1A1 and CYP1B1 genes, a lag of 0.2 day for production of mRNA and induced proteins, and stabilization of mRNA by a poly(A) tail. The model reproduced the transient depletion of the Ah receptor subsequent to binding ligand and the dose-response of the receptor in rats treated with biweekly oral doses of TCDD in corn oil. The model reproduced tissue TCDD concentrations observed for several dosing scenarios. Such robustness indicates the utility of the model in estimating internal dose. The model also reproduced the observed dose-response patterns for mRNA and protein for CYP1A1, CYP1A2, and CYP1B1 after repeated dosing. Neither of the two dissociation constants for the Ah receptor bound to the CYP1B1 gene is negligible, supporting the assumption of multiple response elements for this gene. The poorer induction of CYP1B1 was predicted to be due to lower affinity of the dioxin responsive elements for binding the liganded Ah receptor, suggesting the involvement of other regulatory factors, and a shorter poly(A) tail on CYP1B1 mRNA, leading to a shorter lifetime. Saturation in the kinetics of protein synthesis was linked to the limited number of ribosomes that could bind to each message molecule, resulting in fewer ribosomes bound per message at higher doses. Predicted induction at low doses was found to vary widely with the assumptions used in the construction of a model. More detailed descriptions of biological processes might provide more reliable predictions of enzyme induction.

Kono H, Bradford BU, Rusyn I, Fujii H, Matsumoto Y, Yin M, Thurman RG. **Development of an intragastric enteral model in the mouse: studies of alcohol-induced liver disease using knockout technology.** J Hepatobiliary Pancreat Surg 2000;7(4):395-400.
Abstract: The establishment of a continuous intragastric enteral feeding protocol in the rat by Tsukamoto and French was a major development in research of alcohol-induced liver disease. Unlike other models which only produce fat, with this model, inflammation, necrosis, and fibrosis can now be studied. However, much of what has been learned to date involves inhibitors or nutritional manipulation which may not be specific. Knockout technology could avoid these potential problems. Therefore, we have adapted a rat long-term intragastric protocol to the mouse so that the knockout technology can be used to study the mechanism of alcohol-induced liver injury. Reactive free radicals are involved in the mechanisms of early alcohol-induced liver injury; however, the key source of these species remains unclear. Cytochrome P450 (CYP) 2E1 is induced predominantly in hepatocytes by ethanol and could be one source of reactive oxygen species leading to liver injury. On the other hand, NADPH oxidase or xanthine oxidase is also a potent source of free radicals. In studies using CYP2E1 and p47phox (NADPH oxidase-deficient) knockout mice with this enteral model, it was reported that oxidants from CYP2E1 play only a small role in the mechanisms of early alcohol-induced liver injury in the mouse.

Further, free radicals from NADPH oxidase in Kupffer cells play an important role in early alcohol-induced liver injury. Thus, this new enteral mouse model using knockout technology will provide a powerful tool in alcohol research.

Kostrzewski P, Jalowiecki P. **[Use of the ACSL simulation language for physiologic toxicokinetic models].** Med Pr 2000;51(5):447-56 [Pol].
Abstract: For the description of the processes of absorption, excretion or elimination of chemicals, the open one- or two-compartment models have been used thus far. The latter consist mainly of the fast (central) and slow (peripheral) compartments. The toxicological studies were based on an assumption that the organic processes develop according to is the first order kinetic reaction. However, the absorption, elimination or excretion of toxic chemicals are in fact much more complicated processes that should be explained using, e.g. the physiologically-based toxicokinetic (PBTK) models, covering physiological, biochemical and metabolic parameters, as well as the allometric calibration of selected parameters for interspecies extrapolations, and in vitro/in vivo extrapolations of metabolic parameters. Simulation languages, e.g. ACSL (Advanced Continuous Simulation Language) are indispensable application tools to be operated with PBTK models. They have been developed for modelling systems described by time-dependent non-linear differential equations and/or transfer functions. ACSL with its interfaces (ACSL Builder, ACSL Graphic Modeller, ACSL Math) ensures data input and communication inside the model by the control, transfer and computed parameters. The physiologically-based toxicokinetic models employ a large number of different parameters, which enables, e.g. forecasting the dose/effect or dose/response relationship absorption rate, metabolic pathways, excretion or elimination according to the absorbed dose of xenobiotic; evaluation of risk assessment; extrapolation from high to low doses characteristic of environmental exposure or setting biological exposure limits.

Kramer HJ, Drenth H, vandenBerg M, Seinen W, DeJongh J. **Physiologically based pharmacokinetic model for tetrachlorobenzyltoluenes in rat: comparison of in vitro and in vivo metabolic rates.** Toxicol Sci 2001;63(1):22-8.
Abstract: Ugilec 141 is a technical mixture of tetrachlorobenzyltoluenes (TCBTs). It was introduced in the early 1980s as a replacement for polychlorinated biphenyls (PCBs). Based on physicochemical properties and accumulation in the environment, the use of this mixture was prohibited. To gain more insight in the toxicokinetics of these compounds in mammals, rats were exposed to a single iv bolus injection of a mixture of 3 TCBTs. At different time points after dosing, the tissue and blood concentrations of the TCBTs were determined. The adipose tissue is the main storage compartment, followed by skin and muscle. The TCBTs were rapidly eliminated from the liver and the blood, with half lives ranging from 65 to 72 h. Additionally, the tissue concentration data for all 3 TCBTs were analyzed using a physiologically based pharmacokinetic (PB-PK) model. Sensitivity analysis illustrated that the elimination of the TCBTs was not influenced by metabolism only, but also by the blood flow through the liver. Furthermore, the metabolic rates derived from the model were compared to previously reported in vitro metabolic rates. The in vitro values for the TCBTs were only a factor 2 to 3 smaller than the in vivo metabolic rates, indicating the value of in vitro techniques for a priori parameterization of PB-PK models.

Kunz-Schughart LA, Doetsch J, Mueller-Klieser W, Groebe K. **Proliferative activity and tumorigenic**

conversion: impact on cellular metabolism in 3-D culture. Am J Physiol Cell Physiol 2000;278(4): C765-80.
Abstract: Oxygen consumption, glucose, lactate, and ATP concentrations, as well as glucose and lactate turnover rates, have been studied in a three-dimensional carcinogenesis model of differently transformed rat embryo fibroblasts (spontaneously immortalized Rat1 and myc-transfected M1, and the ras-transfected, tumorigenic descendants Rat1-T1 and MR1) to determine metabolic alterations that accompany tumorigenic conversion. Various bioluminescence techniques, thymidine labeling, measurement of PO(2) distributions with microelectrodes, and determination of cellular oxygen uptake rates (Qc(O(2))) have been applied. In the ras-transfected, tumorigenic spheroid types, the size dependencies of some of the measured parameters exhibited sharp breaks at diameters of approximately 830 microm for Rat1-T1 and approximately 970 microm for MR1 spheroids, respectively, suggesting that some fundamental change in cell metabolism occurred at these characteristic diameters (denoted as "metabolic switch"). Qc(O(2)) decreased and lactate concentration increased as functions of size below the characteristic diameters. Concomitantly, glucose and lactate turnover rates decreased in MR1 spheroids and increased in Rat1-T1. Spheroids larger than the characteristic diameters (exhibiting cell quiescence and lactate accumulation) showed an enhancement of Qc(O(2)) with size. Systematic variations in the ATP and glucose levels in the viable cell rim were observed for Rat1-T1 spheroids only. Proliferative activity, Qc(O(2)), and ATP levels in small, nontumorigenic Rat1 and M1 aggregates did not differ systematically from those recorded in the largest spheroids of the corresponding ras transfectants. Unexpectedly, respiratory activity was present not only in viable but also in the morphologically disintegrated core regions of M1 aggregates. Our data suggest that myc but not ras transfection exerts major impacts on cell metabolism. Moreover, some kind of switch has been detected that triggers profound readjustment of tumor cell metabolism when proliferative activity begins to stagnate, and that is likely to initiate some other, yet unidentified energy-consuming process.

Larisch-Bloch S, Danielpour D, Roche NS, Lotan R, Hsing AY, Kerner H, Hajouj T, Lechleider RJ, Roberts AB. **Selective loss of the transforming growth factor-beta apoptotic signaling pathway in mutant NRP-154 rat prostatic epithelial cells.** Cell Growth Differ 2000;11(1):1-10.
Abstract: Retroviral insertional mutagenesis was used to select mutant NRP-154 rat prostate carcinoma cells resistant to transforming growth factor (TGF)-beta-induced cell death. Similar to the parental cells, a mutant clone, M-NRP1, expressed TGF-beta receptors and was still responsive to induction both of direct target genes by TGF-beta and of apoptosis by staurosporine or okadaic acid. In contrast, indicators of cell growth, strongly suppressed by TGF-beta in the parental cells, were unaffected in M-NRP1 cells. M-NRP1 cells overexpress the antiapoptotic protein, Bcl-xL, and show dysregulated expression and localization of a protein related to a novel human septin, ARTS (designation of apoptotic response to TGF-beta signals), cloned by homology to an exonic sequence flanked by the viral long terminal repeats in M-NRP1 cells and shown to make cells competent to undergo apoptosis in response to TGF-beta. We propose that ARTS might operate within the same apoptotic pathway as Bcl-xL and that M-NRP1 cells could serve as a useful model for characterization of this pathway.

Lau C, Andersen ME, Crawford-Brown DJ, Kavlock RJ, Kimmel CA, Knudsen TB, Muneoka K, Rogers JM, Setzer RW, Smith G, et al. **Evaluation of biologically based dose-response modeling for developmental toxicity: a workshop report.** Regul Toxicol Pharmacol 2000;31(2 Pt 1):190-9.

Abstract: Biologically based dose-response (BBDR) modeling represents a novel approach for quantitative assessment of health risk by incorporating pharmacokinetic and pharmacodynamic characteristics of a chemical and by relating the immediate cellular responses to a cascade of aberrant biological actions that leads to detectable adverse outcomes. The quantitative relationship of each of the intervening events can be described in mathematical forms that are amenable for adjustment and extrapolation over a range of doses and across species. A team of investigators at the Reproductive Toxicology Division of the U.S. Environmental Protection Agency has explored the feasibility of BBDR modeling by examining the developmental toxicity of a known teratogen, 5-fluorouracil. A panel of researchers from academic and industrial laboratories, biomathematical modelers, and risk assessment scientists was convened in a workshop to evaluate the approaches undertaken by the EPA team and to discuss the future prospects of BBDR modeling. This report summarizes the lessons learned from one approach to BBDR modeling and comments from the panelists: while it is possible to incorporate mechanistic information into quantitative dose-response models for the assessment of health risks, the process is enormously data-intensive and costly; in addition, the confidence of the model is directly proportional to our current understanding of basic biology and can be enhanced only through the ongoing novel discoveries. More importantly, the extent of "uncertainty" (inherent with the default assumptions associated with the NOAEL or benchmark approach) reducible by BBDR modeling requires further scrutiny and comparison.

Lau C, Mole ML, Copeland MF, Rogers JM, Kavlock RJ, Shuey DL, Cameron AM, Ellis DH, Logsdon TR, Merriman J, et al. **Toward a biologically based dose-response model for developmental toxicity of 5-fluorouracil in the rat: acquisition of experimental data.** Toxicol Sci 2001;59(1):37-48.
Abstract: Biologically based dose-response (BBDR) models represent an emerging approach to improving the current practice of human health-risk assessment. The concept of BBDR modeling is to incorporate mechanistic information about a chemical that is relevant to the expression of its toxicity into descriptive mathematical terms, thereby providing a quantitative model that will enhance the ability for low-dose and cross-species extrapolation. Construction of a BBDR model for developmental toxicity is particularly complicated by the multitude of possible mechanisms. Thus, a few model assumptions were made. The current study illustrates the processes involved in selecting the relevant information for BBDR modeling, using an established developmental toxicant, 5-fluorouracil (5-FU), as a prototypic example. The primary BBDR model for 5-FU is based on inhibition of thymidylate synthetase (TS) and resultant changes in nucleotide pools, DNA synthesis, cell-cycle progression, and somatic growth. A single subcutaneous injection of 5-FU at doses ranging from 1 to 40 mg/kg was given to pregnant Sprague-Dawley rats at gestational day 14; controls received saline. 5-FU was absorbed rapidly into the maternal circulation, and AUC estimates were linear with administered doses. We found metabolites of 5-FU directly incorporated into embryonic nucleic acids, although the levels of incorporation were low and lacked correlation with administered doses. On the other hand, 5-FU produced dose-dependent inhibition of thymidylate synthetase in the whole embryo, and recovery from enzyme inhibition was also related to the administered dose. As a consequence of TS inhibition, embryonic dTTP and dGTP were markedly reduced, while dCTP was profoundly elevated, perhaps due to feedback regulation of intracellular nucleotide pools. The total contents of embryonic macromolecules (DNA and protein) were also reduced, most notably at the high doses. Correspondingly, dose-related reductions of fetal weight were seen as early as GD 15, and these deficits persisted for the remainder of gestation. These detailed

dose-response parameters involved in the expression of 5-FU developmental toxicity were incorporated into mathematical terms for BBDR modeling. Such quantitative models should be instrumental to the improvement of high-to-low dose and cross-species extrapolation in health-risk assessment.

Lau C, Setzer RW. **Biologically based risk assessment models for developmental toxicity.** Methods Mol Biol 2000;136:271-81.
Abstract: It is obvious that the task of incorporating mechanistic information into dose-response assessment for developmental toxicity is, by and large, still at its conceptual stage. Our immature understanding of embryogenesis and teratogenesis forces us to make simplifying biological assumptions that may turn out to be erroneous; therefore, these mechanistically based models should be constructed so as to be easily modified as new information becomes available. The data-intensive (and costly) nature of these modeling efforts may also limit their practice to extraordinary situations where, for instance, large segments of the human population are exposed to low levels of a compound and the determination of a safe level of exposure is of utmost importance, or where compounds are of such immense economic or therapeutic value that their use would warrant a concerted effort to minimize the uncertainties inherent in the current methods of extrapolation. Nevertheless, this chapter has presented several attempts to translate the concept of BBDR into practice. When these applications become successful, these models should provide risk assessors with more reliable response indicators at low doses (where human exposures are realistic) and more accurate cross-strain/cross-species comparisons as well as extrapolations across exposure conditions. Although the BBDR models do not necessarily redefine the current practice of risk assessment using either the NOAEL or the benchmark approach, information derived from these efforts should provide a credible scientific basis for the estimates of RfD.

Li X, Angst MS, Clark JD. **A murine model of opioid-induced hyperalgesia.** Brain Res Mol Brain Res 2001;86(1-2):56-62.
Abstract: Controversies surround the possible long-term physiological and psychological consequences of opioid use. Analgesic tolerance and addiction are commonly at the center of these controversies, but other concerns exist as well. A growing body of evidence suggests that hyperalgesia caused by the chronic administration of opioids can occur in laboratory animals and in humans. In these studies we describe a murine model of opioid-induced hyperalgesia (OIH). After the treatment of mice for 6 days with implanted morphine pellets followed by their removal, both thermal hyperalgesia and mechanical allodynia were documented. Additional experiments demonstrated that prior morphine treatment also increased formalin-induced licking behavior. These effects were intensified by intermittent abstinence accomplished through administration of naloxone during morphine treatment. Experiments designed to determine if the mu-opioid receptor mediated OLH in our model revealed that the relatively-selective mu-opioid receptor agonist fentanyl induced the thermal hyperalgesia and mechanical allodynia characteristic of OIH when administered in intermittent boluses over 6 days. In complimentary experiments we found that CXBK mice which have reduced mu-opioid receptor binding displayed no significant OIH after morphine treatment. Finally, we explored the pharmacological sensitivities of OIH. We found that the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801, the nitric oxide synthase (NOS) inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) and the heme oxygenase (HO) inhibitor tin protoporphyrin (Sn-P) dose-dependently reduced OIH in this model while the NSAID indomethacin had no effect. Thus we have characterized a murine model of OIH which will be useful in the pursuit of

the molecular mechanisms underlying this phenomenon.

Lien GJ, McKim JM, Hoffman AD, Jenson CT. **A physiologically based toxicokinetic model for lake trout (Salvelinus namaycush).** Aquat Toxicol 2001;51(3):335-50.
Abstract: A physiologically based toxicokinetic (PB-TK) model for fish, incorporating chemical exchange at the gill and accumulation in five tissue compartments, was parameterized and evaluated for lake trout (Salvelinus namaycush). Individual-based model parameterization was used to examine the effect of natural variability in physiological, morphological, and physico-chemical parameters on model predictions. The PB-TK model was used to predict uptake of organic chemicals across the gill and accumulation in blood and tissues in lake trout. To evaluate the accuracy of the model, a total of 13 adult lake trout were exposed to waterborne 1,1,2,2-tetrachloroethane (TCE), pentachloroethane (PCE), and hexachloroethane (HCE), concurrently, for periods of 6, 12, 24 or 48 h. The measured and predicted concentrations of TCE, PCE and HCE in expired water, dorsal aortic blood and tissues were generally within a factor of two, and in most instances much closer. Variability noted in model predictions, based on the individual-based model parameterization used in this study, reproduced variability observed in measured concentrations. The inference is made that parameters influencing variability in measured blood and tissue concentrations of xenobiotics are included and accurately represented in the model. This model contributes to a better understanding of the fundamental processes that regulate the uptake and disposition of xenobiotic chemicals in the lake trout. This information is crucial to developing a better understanding of the dynamic relationships between contaminant exposure and hazard to the lake trout.

Lilling G, Hacohen H, Nordenberg J, Livnat T, Rotter V, Sidi Y. **Differential sensitivity of MCF-7 and LCC2 cells, to multiple growth inhibitory agents: possible relation to high bcl-2/bax ratio?** Cancer Lett 2000;161(1):27-34.
Abstract: Comparison of LCC2, the E(2)-independent, tamoxifen-resistant subline of the MCF-7 human breast cancer cell line with its parent line, disclosed that it is more resistant to growth inhibition and apoptosis induction by a variety of agents acting by diverse mechanisms. Thus, LCC2 cells can serve as a useful in-vitro model for the study of the molecular mechanisms of this resistance. It was found that bcl-2 protein and mRNA were elevated and that bax protein and mRNA were reduced in LCC2 compared with MCF-7 cells. Incubation of both lines in the presence of bcl-2 antisense caused growth inhibition and reduced bcl-2 protein levels only in MCF-7 cells, suggesting the involvement of bcl-2 in the regulation of normal growth of breast cancer cells. Increased bcl-2 expression in breast cancer cells may correlate with their resistance to growth inhibitory agents. Bcl-2 is a useful target for enhancing the effects of growth inhibitory agents.

Lin K, Ricciardi RP. **A rapid plate assay for the screening of inhibitors against herpesvirus DNA polymerases and processivity factors.** J Virol Methods 2000;88(2):219-25.
Abstract: Kaposi's sarcoma-associated herpesvirus (KSHV) is a newly identified human pathogen with tumorigenic potential. The DNA polymerase (Pol-8) and processivity factor (PF-8) of KSHV were cloned recently. It was shown that PF-8 forms specifically a complex with Pol-8 in vitro and allows it to synthesize fully-extended DNA. Since both Pol-8 and PF-8 are apparently essential for viral DNA replication and since they cannot be substituted by any other cellular or viral proteins, they are

potentially excellent antiviral targets. The development of a mechanistic plate assay is now described, which is suitable for rapid high-throughput screening of antiviral agents against Pol-8 and PF-8. The assay allows the measurement of not only total DNA synthesis activity (i.e. nucleotide incorporation) but also processivity (i.e. fully-extended DNA product). In this plate assay, any of the screen-compounds with an inhibitory effect against the total DNA synthesis activity and/or the processivity could be potential antiviral agents that target Pol-8 and/or PF-8. Particularly, since PF-8 is highly specific for Pol-8, the discovery of inhibitory agents against PF-8 may lead to specific antiviral therapies with minimal toxicity to host cells. This assay should be suitable for screening for inhibitory compounds against polymerases and processivity factors of other herpesviruses as well.

Lindsten K, Uhlikova T, Konvalinka J, Masucci MG, Dantuma NP. **Cell-based fluorescence assay for human immunodeficiency virus type 1 protease activity.** Antimicrob Agents Chemother 2001;45 (9):2616-22.
Abstract: The human immunodeficiency virus type 1 (HIV-1) protease is essential for production of infectious virus and is therefore a major target for the development of drugs against AIDS. Cellular proteins are also cleaved by the protease, which explains its cytotoxic activity and the consequent failure to establish convenient cell-based protease assays. We have exploited this toxicity to develop a new protease assay that relies on transient expression of an artificial protease precursor harboring the green fluorescent protein (GFP-PR). The precursor is activated in vivo by autocatalytic cleavage, resulting in rapid elimination of protease-expressing cells. Treatment with therapeutic doses of HIV-1 protease inhibitors results in a dose-dependent accumulation of the fluorescent precursor that can be easily detected and quantified by flow cytometric and fluorimetric assays. The precursor provides a convenient and noninfectious model for high-throughput screenings of substances that can interfere with the activity of the protease in living cells.

Ma Q. Induction of CYP1A1. **The AhR/DRE paradigm: transcription, receptor regulation, and expanding biological roles.** Curr Drug Metab 2001;2(2):149-64.
Abstract: The CYP1A1 gene encodes microsomal cytochrome P4501A1 that catalyzes the metabolism of many xenobiotics, including the oxygenation of polycyclic aromatic hydrocarbons (PAH). Induction of CYP1A1 enhances the metabolism of PAHs, and therefore, represents an adaptive response to chemical exposure in mammalian cells. Mechanistic studies reveal an AhR/DRE paradigm for the induction, which involves activation of the aryl hydrocarbon receptor (AhR) by an agonist, dimerization of AhR with the Ah recceptor nuclear translocator (Arnt), followed by binding of the AhR/Arnt heterodimer to the dioxin-responsive enhancer (DRE) and transcription of the gene. The AhR mediated transcription is tightly regulated through, at least, two mechanisms: (a) the cytoplasmic AhR interacts with hsp90 and an immunophilin chaperone AIP for proper folding and receptivity, and (b) the agonist-activated, nuclear AhR is degraded through the ubiquitin-26S proteasome mediated protein turnover, such that the transcription by AhR is controlled at a physiologically adequate level. In addition to CYP1A1 induction, AhR mediates a broad range of biological responses to CYP1A1 inducers, typified by the environmental contaminant dioxin, via modulating gene expression. Thus, mechanistic studies of CYP1A1 induction have provided insights into P450 induction, PAH carcinogenesis, dioxin action, AhR function, and receptor-mediated mammalian gene expression.

MacGregor JT, Collins JM, Sugiyama Y, Tyson CA, Dean J, Smith L, Andersen M, Curren RD, Houston JB, Kadlubar FF, et al. **In vitro human tissue models in risk assessment: report of a consensus-building workshop.** Toxicol Sci 2001;59(1):17-36.
Abstract: Advances in the technology of human cell and tissue culture and the increasing availability of human tissue for laboratory studies have led to the increased use of in vitro human tissue models in toxicology and pharmacodynamics studies and in quantitative modeling of metabolism, pharmacokinetic behavior, and transport. In recognition of the potential importance of such models in toxicological risk assessment, the Society of Toxicology sponsored a workshop to evaluate the current status of human cell and tissue models and to develop consensus recommendations on the use of such models to improve the scientific basis of risk assessment. This report summarizes the evaluation by invited experts and workshop attendees of the current status of such models for prediction of human metabolism and identification of drug-drug interactions, prediction of human toxicities, and quantitative modeling of pharmacokinetic and pharmaco-toxicodynamic behavior. Consensus recommendations for the application and improvement of current models are presented.

Marique T, Werenne J. **Control of 92 kDa collagenase secretion in mammalian cells by modulation of AP-1 activity: an experimentally based theoretical study.** J Theor Biol 2001;209(1):3-8.
Abstract: Collagenolytic enzymes control cell migration through connective tissues. They appear to be of crucial importance for angiogenesis, tumor metastasis or wound repair. A well-documented stimulation pathway of collagenase secretion, either by natural (cytokines) or synthetic (phorbol esters) molecules, acts through activation of the proto-oncogene activating protein 1 (AP-1). Interestingly, this nuclear factor enhances its own synthesis. It also modulates the activity of different genes, including the one coding for 92 kDa gelatinase. We developed a mathematical model to describe this pathway. It led us to conjecture the existence of an hysteresis cycle for PMA-stimulated collagenase secretion, which was experimentally demonstrated later in MDBK cells in culture. We also modified our model to simulate the behavior of tumoral cells expressing AP-1. In this case, the system becomes highly unstable and, once stimulated, cannot be brought back to rest. This approach paved the way for the understanding and the control of mammalian cell processes, connective tissue maintenance or metastasis dissemination.
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Martin C, Berridge G, Higgins CF, Mistry P, Charlton P, Callaghan R. **Communication between multiple drug binding sites on P-glycoprotein**. Mol Pharmacol 2000;58(3):624-32.
Abstract: P-glycoprotein, a member of the ATP-binding cassette transporter family, is able to confer resistance on tumors against a large number of functionally and chemically distinct cytotoxic compounds. Several recent investigations suggest that P-glycoprotein contains multiple drug binding sites rather than a single site of broad substrate specificity. In the present study, radioligand-binding techniques were used to directly characterize drug interaction sites on P-glycoprotein and how these multiple sites interact. The drugs used were classified as either 1) substrates, which are known to be transported by P-glycoprotein (e.g., vinblastine) or 2) modulators, which alter P-glycoprotein function but are not themselves transported by the protein (e.g., XR9576). Drug interactions with P-glycoprotein were either competitive, at a common site, or noncompetitive, and therefore at distinct sites. Based on these data, we can assign a minimum of four drug binding sites on P-glycoprotein. These sites fall into two categories: transport, at which translocation of drug across the membrane can occur, and regulatory

sites, which modify P-glycoprotein function. Intriguingly, however, some modulators interact with P-glycoprotein at a transport site rather than a regulatory site. The pharmacological data also demonstrate that both transport and regulatory sites are able to switch between high- and low-affinity conformations. The multiple sites on P-glycoprotein display complex allosteric interactions through which interaction of drug at one site switches other sites between high- or low-affinity conformations. The data are discussed in terms of a model for the mechanism of transport by P-glycoprotein.

Masimirembwa CM, Thompson R, Andersson TB. **In vitro high throughput screening of compounds for favorable metabolic properties in drug discovery.** Comb Chem High Throughput Screen 2001;4 (3):245-63.
Abstract: Drug metabolism can have profound effects on the pharmacological and toxicological profile of therapeutic agents. In the pharmaceutical industry, many in vitro techniques are in place or under development to screen and optimize compounds for favorable metabolic properties in the drug discovery phase. These in vitro technologies are meant to address important issues such as: (1) is the compound a potent inhibitor of drug metabolising enzymes (DMEs)? (2) does the compound induce the expression of DMEs? (3) how labile is the compound to metabolic degradation? (4) which specific enzyme(s) is responsible for the compound's biotransformation? and (5) to which metabolites is the compound metabolized? Answers to these questions provide a basis for judging whether a compound is likely to have acceptable pharmacokinetic properties in vivo. To address these issues on the increasing number of compounds inundating the drug discovery programs, high throughput assays are essential. A combination of biochemical advances in the understanding of the function and regulation of DMEs (in particular, cytochromes P450, CYPs) and automated analytical technologies are revolutionizing drug metabolism research. Automated LC-MS based metabolic stability, fluorescence, radiometric and LC-MS based CYP inhibition assays are now in routine use. Automatible models for studying CYP induction based on enzyme activity, quantitative RT-PCR and reporter gene systems are being developed. We will review the utility and limitations of these HTS approaches and highlight on-going developments and emerging technologies to answer metabolism questions at the different stages of the drug discovery process.

Mata-Greenwood E, Ito A, Westenburg H, Cui B, Mehta RG, Kinghorn AD, Pezzuto JM. **Discovery of novel inducers of cellular differentiation using HL-60 promyelocytic cell**s. Anticancer Res 2001;21 (3B):1763-70.
Abstract: Non-physiological inducers of terminal differentiation have been used as novel therapies for the prevention and therapy of cancer. We have used cultured HL-60 promyelocytic cells to monitor differentiation, proliferation and cell death events as induced by a large set of extracts derived from plants. Screening of more than 1400 extracts led to the discovery of 34 with potent activity (ED50 Z8 mg/ml). Bioassay-guided fractionation led to the isolation of zapotin and 2',5,6-trimethoxyflavone as active principles from Casimiroa edulis, dibenzyltrisulfide and 2-[(phenylmethyl)dithio]ethanol as active principles from Petiveria alliacea, and desmethylrocaglamide from Aglaia ponapensis. Zapotin demonstrated the most favorable biological profile in that induction of differentiation correlated with proliferation arrest, and a lack of cytotoxicity. We conclude that the HL-60 cell model is a useful system for the discovery of novel pharmacophores with potential to suppress the process of carcinogenesis, and that flavonoids may be especially useful in this capacity.

Mauvais-Jarvis F, Virkamaki A, Michael MD, Winnay JN, Zisman A, Kulkarni RN, Kahn CR. **A model to explore the interaction between muscle insulin resistance and beta-cell dysfunction in the development of type 2 diabetes**. Diabetes 2000;49(12):2126-34.
Abstract: Type 2 diabetes is a polygenic disease characterized by defects in both insulin secretion and insulin action. We have previously reported that isolated insulin resistance in muscle by a tissue-specific insulin receptor knockout (MIRKO mouse) is not sufficient to alter glucose homeostasis, whereas beta-cell-specific insulin receptor knockout (betaIRKO) mice manifest severe progressive glucose intolerance due to loss of glucose-stimulated acute-phase insulin release. To explore the interaction between insulin resistance in muscle and altered insulin secretion, we created a double tissue-specific insulin receptor knockout in these tissues. Surprisingly, betaIRKO-MIRKO mice show an improvement rather than a deterioration of glucose tolerance when compared to betaIRKO mice. This is due to improved glucose-stimulated acute insulin release and redistribution of substrates with increased glucose uptake in adipose tissue and liver in vivo, without a significant decrease in muscle glucose uptake. Thus, insulin resistance in muscle leads to improved glucose-stimulated first-phase insulin secretion from beta-cells and shunting of substrates to nonmuscle tissues, collectively leading to improved glucose tolerance. These data suggest that muscle, either via changes in substrate availability or by acting as an endocrine tissue, communicates with and regulates insulin sensitivity in other tissues.

Mayer D, Muhlhofer A, Biesalski HK. **A modified system to evaluate the potency of anti-oxidative compounds in different cell types in vitro**. Eur J Med Res 2001;6(5):201-8.
Abstract: Common assays for evaluation of antioxidative capacity of different compounds are usually performed in cell-free systems. By this approach, cell-specific regulatory mechanisms upon distinct stimuli are not taken into account. Therefore, there is a need to measure anti-oxidative capacity in a cellular setting. - We now developed a valid method that provides monitoring of anti-oxidative capacities of compounds in different cell types. Oxidative stress, induced by 100 microM H subset2O subset2 in human microvascular endothelial cells (HMEC-1), was quantified by the generation of oxidized, fluorescent C-DCF from C-H subset2DCF-DA/AM. As DCF-production could be almost completely blocked by diethyldithiocarbamate (DEDTC), which inhibits intracellular Cu/Zn superoxide dismutase (SOD), mainly intracellular production of C-DCF was assumed. Preincubation with alpha-tocopherol resulted in a dose-dependent reduction of both spontaneous and H subset2O subset2-induced C-DCF-production (maximal inhibition by 41.6% at 75 &mgr;M). A synergistic effect was observed with co-incubation with vitamin C (maximal inhibition 46.8% at 10 &mgr;M vitamin C and 50 &mgr;M alpha-tocopherol). In this way compounds with different modes of action and subcellular localization can be evaluated concomitantly in respect of their anti-oxidative capacities. As this method was established on 24- and 48-well plates in other cell lines (Caco-2, HFP-1), too, screening of a large array of antioxidative compounds in different cell lines can be performed.

McDonald JD. **Production of mouse models for the study of human inborn errors of metabolism.** Mol Genet Metab 2000;71(1-2):240-4.

Melnick RL, Kohn MC. **Dose-response analyses of experimental cancer data.** Drug Metab Rev 2000;32(2):193-209.

Abstract: Dose-response analysis provides a powerful tool to determine causality from experimental cancer data, estimate low-dose risk, and evaluate mechanistic hypotheses. However, the interpretation of cancer dose-response data can be influenced by how the dose and response terms are characterized. Using the poly-3 quantal response method to adjust for the extensive and early development of lethal lymphomas in butadiene-exposed mice provided a means of obtaining a better representation of dose-response relationships for late-developing tumors induced by this chemical. Fitting a Weibull model to survival-adjusted tumor data for chloroprene and butadiene indicated similar carcinogenic potencies for these chemicals in mice. In conjunction with the rodent toxicity and carcinogenicity studies conducted by the National Toxicology Program, toxicokinetic studies are performed to characterize relationships between exposure and tissue concentrations of parent compound and metabolites. A physiologically based pharmacokinetic model (PBPK) of butadiene dosimetry indicated that differences in carcinogenic response between rats and mice are not simply due to differences in tissue concentrations of epoxybutene, a mutagenic metabolic intermediate. Thus, factors beyond tissue dosimetry of this metabolite must be important in butadiene-induced carcinogenesis. A PBPK model for isoprene indicated that blood concentrations of isoprene epoxides are a better indicator of kidney cancer risk than are measurements of isoprene-exposure concentrations. An evaluation of dose-response relationships for cytotoxicity, regenerative hyperplasia, and tumor induction by trihalomethanes indicates that for this family of chemicals, cell proliferation is not a reliable predictor of tumor response.

Millenbaugh NJ, Wientjes MG, Au JL. **A pharmacodynamic analysis method to determine the relative importance of drug concentration and treatment time on effect**. Cancer Chemother Pharmacol 2000;45(4):265-72.

Abstract: PURPOSES: The pharmacodynamics of most drugs follow the empirical relationship, C(n) x T = h, where C is drug concentration, T is exposure time and h is drug exposure constant. The value of n indicates the relative importance of C and T in determining the effect. An n value greater than 1.0 indicates that for two infusions that produce the same C x T, a short infusion that delivers high concentrations over a short duration will produce a greater C(n) x T and therefore a greater effect, compared to a long infusion that delivers lower concentrations. The reverse is true for an n value less than 1.0 and would support the use of a slow infusion. Hence, it is important to determine the n values and whether the n value significantly differs from 1.0. This report describes a three-step method for this purpose. METHODS: First, we obtained experimental data on the relationship between drug concentration, treatment time and effect, and analyzed the data with a three-dimensional surface response method to obtain the pharmacodynamic model parameters and the magnitude of data variability. The experiments used mitomycin C and two human cancer cell lines, i.e. bladder RT4 and pharynx FaDu cells. The n values obtained from four experiments ranged from 1.04 to 1.16 for FaDu cells and from 1.14 to 1.46 for RT4 cells. The variability in the effect data decreased from 11.9% at 0% effect to 6.14% at 100% effect. Second, these results were used with Monte Carlo simulations to generate 100 concentration-time-effect data sets, which contained randomly and normally distributed data variability comparable to the experimentally observed variability, for each experimentally determined n value. This is analogous to performing 100 experiments under the same experimental conditions. Third, we analyzed the simulated data sets to obtain 100 estimated n values. The frequency with which these estimated n values fell above or below 1.0 indicated the probability that the experimentally determined n value used in the Monte Carlo simulations was truly different from 1.0. We

defined this frequency for individual experiments as F(one), and calculated the overall probability for multiple experiments (F(multiple)). A probability of greater than 97.5% (i.e. $P < 0.05$ for a two-tailed test) was considered statistically significant. RESULTS: Analysis of the mitomycin C pharmacodynamic data yielded F(one) and F(multiple) of 99% to 100% for FaDu and RT4 cells, indicating that the n values for these cells were significantly higher than 1.0. A comparison of the statistical significance of the n value analyzed by the three-step pharmacodynamic analysis method, a conventional statistical method such as the Student's t-test and nonlinear regression analysis, indicated two advantages for the pharmacodynamic method: fewer experiments were required (theoretically only one experiment with three replicates would be sufficient) and a higher statistical significance of the n value was obtained. CONCLUSIONS: In summary, the three-step pharmacodynamic study design and analysis method can be used to define the relative importance of drug concentration and treatment time on drug effect.

Miller MA. **Gender-based differences in the toxicity of pharmaceuticals--the Food and Drug Administration's perspective.** Int J Toxicol 2001;20(3):149-52.
Abstract: Women experience more adverse reactions to treatment with therapeutic drugs than men. Theories proposed to explain this include overdosing, different pharmacokinetics and pharmacodynamics, women are more likely to report adverse events than men, or women take more medications than men. Food and Drug Administration (FDA) Office of Women's Health (OWH) funds research to promote including women in clinical trials and understanding the biology of sex-related differences in the safety of FDA-regulated products. Including women in clinical trials advances the understanding of drug efficacy and safety in women by providing information on drug dosing, pharmacokinetics, and pharmacodynamics. A Baysian statistical analysis of sex differences in adverse events showed that although about the same number of adverse events were reported for men and women, those reported for women were more serious. One example of a sex difference in the toxicity of pharmaceuticals is the drug-induced cardiac arrhythmia, torsades de point. OWH funded studies in animals and humans to investigate the mechanism behind this sex difference. These studies demonstrated that shortening the QT interval increases the risk of developing torsades and that androgens protect against torsades by slowing cardiac repolarization and prolonging the QT interval. Understanding the mechanisms behind other reported sex-related differences in adverse drug effects requires additional research. The preliminary studies conducted to date suggest that this sex-related difference is likely to be a multifactorial problem requiring information from several fields of study. Ideally, individuals at risk for developing an adverse event should be identified prior to therapeutic intervention. The OWH plans to fund more studies to investigate the role of hormonal variations on drug metabolism and drug-drug interactions. Animal and in vitro model systems are needed to fully understand the mechanism of how gender influences drug toxicity.

Monroy A, Plata C, Hebert SC, Gamba G. **Characterization of the thiazide-sensitive Na(+)-Cl(-) cotransporter: a new model for ions and diuretics interaction**. Am J Physiol Renal Physiol 2000;279(1):F161-9.
Abstract: The thiazide-sensitive Na(+)-Cl(-) cotransporter (TSC) is the major pathway for salt reabsorption in the apical membrane of the mammalian distal convoluted tubule. When expressed in Xenopus laevis oocytes, rat TSC exhibits high affinity for both cotransported ions, with the Michaelis-Menten constant (K(m)) for Na(+) of 7.6 +/- 1.6 mM and for Cl(-) of 6.3 +/- 1.1 mM, and Hill

coefficients for Na(+) and Cl(-) consistent with electroneutrality. The affinities of both Na(+) and Cl(-) were increased by increasing concentration of the counterion. The IC(50) values for thiazides were affected by both extracellular Na(+) and Cl(-). The higher the Na(+) or Cl(-) concentration, the lower the inhibitory effect of thiazides. Finally, rTSC function is affected by extracellular osmolarity. We propose a transport model featuring a random order of binding in which the binding of each ion facilitates the binding of the counterion. Both ion binding sites alter thiazide-mediated inhibition of transport, indicating that the thiazide-binding site is either shared or modified by both Na(+) and Cl(-).

Mucci N, Ianni A, Ursini CL, Orsini M, Arzani D, Romano-Spica V. **Cytostatic drugs and health risks for exposed personnel: search for new biomarkers.** Anticancer Res 2000;20(5A):2995-3000.
Abstract: The use of antiblastic drugs has opened up new perspectives in improvement of therapy and life quality for cancer patients. The widespread clinical application of cytostatic drugs implies risks for exposed hospital personnel, due to genotoxic and toxic-reproductive effects. Biological monitoring is fundamental to identify individuals at risk but is limited by the long latency of chronic effects, absence of unique cellular targets and low sensitivity of available laboratory tests. The objective of this study was to investigate toxic mechanisms by a molecular biology approach, searching for biomarkers potentially useful in monitoring programs. The proposed experimental model consisted of cell line exposure to cyclophosphamide, an alkylating agent of wide clinical use. Cellular response has been investigated focusing on potential targets at RNA level, through reverse transcription polymerase chain reaction (RT-PCR) and differential display analysis. We studied the expression of several genes involved in differentiation, apoptosis and chemoresistance: ets1, bax, bcl-2, bag-1, bcl-X, mdr1 and mrp. Specific patterns of mRNA modulations were observed. Differential display analysis revealed candidate genes induced or repressed following exposure: their characterization is in progress. Besides improving the understanding of toxic mechanisms, identification of modulated molecular targets opens up new perspectives in exposure risk assessment, biomonitoring and preventive strategies at occupational level.

Negro GD, Bonato M, Gribaldo L. **In vitro bone marrow granulocyte-macrophage progenitor cultures in the assessment of hematotoxic potential of the new drugs.** Cell Biol Toxicol 2001;17 (2):95-105.
Abstract: In pharmaceutical research, in vitro toxicity tests, for assessing the potential toxicity of new chemical entities are necessary in the early stages of the developmental process, when no information is available about the metabolism or even the target organ toxicity of the compounds to be tested. In vitro specific organ toxicity tests, such as the granulocyte-macrophage colony-forming unit (CFU-GM) clonogenic assay, are useful tools for predicting the adverse effects of new compounds on the blood-forming system, provided that some reference points are available, e.g., toxicological information about compounds belonging to the same chemical class and structure-activity relationship data. Furthermore, when no information is available about metabolism, the in vitro system should cover as many possibilities as possible, to avoid false positive or false negative results. In fact, while many compounds are metabolized to a variety of inactive chemical species, some undergo bioactivation to form more active metabolites. The addition of a metabolic activation system to the CFU-GM assay enables assessment of direct and metabolism-mediated toxicity. The regulatory agencies and industry value the concept of assays performed with and without metabolic activation, since they often have to take decisions about compounds with unknown mechanisms of action. CFU-GM assay, designed in this way,

is an example of such a mechanism-naive assay. It has been suggested that, for new compounds, metabolites should be generated and tested both in the presence and in the absence of the parent compound itself, to identify the possible contribution of metabolites to the hematotoxicity observed, and to determine whether there is any synergistic or antagonistic effect between metabolites and the parent compound that might affect hematotoxicity in vivo. Various approaches can be used to obtain such information.

Nestorov I. **Modelling and simulation of variability and uncertainty in toxicokinetics and pharmacokinetics**. Toxicol Lett 2001;120(1-3):411-20.
Abstract: Two important methodological issues within the framework of the variability and uncertainty analysis of toxicokinetic and pharmacokinetic systems are discussed: (i) modelling and simulation of the existing physiologic variability in a population; and (ii) modelling and simulation of variability and uncertainty when there is insufficient or not well defined (e.g. small sample, semiquantitative, qualitative and vague) information available. Physiologically based pharmacokinetic models are especially suited for separating and characterising the physiologic variability from the overall variability and uncertainty in the system. Monte Carlo sampling should draw from multivariate distributions, which reflect all levels of existing dependencies in the intact organism. The population characteristics should be taken into account. A fuzzy simulation approach is proposed to model variability and uncertainty when there is semiquantitative, qualitative and vague information about the model parameters and their statistical distributions cannot be defined reliably.

Newman JW, Denton DL, Morisseau C, Koger CS, Wheelock CE, Hinton DE, Hammock BD. **Evaluation of fish models of soluble epoxide hydrolase inhibition**. Environ Health Perspect 2001;109 (1):61-6.
Abstract: Substituted ureas and carbamates are mechanistic inhibitors of the soluble epoxide hydrolase (sEH). We screened a set of chemicals containing these functionalities in larval fathead minnow (Pimphales promelas) and embryo/larval golden medaka (Oryzias latipes) models to evaluate the utility of these systems for investigating sEH inhibition in vivo. Both fathead minnow and medaka sEHs were functionally similar to the tested mammalian orthologs (murine and human) with respect to substrate hydrolysis and inhibitor susceptibility. Low lethality was observed in either larval or embryonic fish exposed to diuron [N-(3,4-dichlorophenyl), N'-dimethyl urea], desmethyl diuron [N-(3,4-dichlorophenyl), N'-methyl urea], or siduron [N-(1-methylcyclohexyl), N'-phenyl urea]. Dose-dependent inhibition of sEH was a sublethal effect of substituted urea exposure with the potency of siduron < desmethyl diuron = diuron, differing from the observed in vitro sEH inhibition potency of siduron > desmethyl diuron > diuron. Further, siduron exposure synergized the toxicity of trans-stilbene oxide in fathead minnows. Medaka embryos exposed to diuron, desmethyl diuron, or siduron displayed dose-dependent delays in hatch, and elevated concentrations of diuron and desmethyl diuron produced developmental toxicity. The dose-dependent toxicity and in vivo sEH inhibition correlated, suggesting a potential, albeit undefined, relationship between these factors. Additionally, the observed inversion of in vitro to in vivo potency suggests that these fish models may provide tools for investigating the in vivo stability of in vitro inhibitors while screening for untoward effects.

Nigg HN, Knaak JB. **Blood cholinesterases as human biomarkers of organophosphorus pesticide**

exposure. Rev Environ Contam Toxicol 2000;163:29-111.
Abstract: The organophosphorus pesticides of this review were discovered in 1936 during the search for a replacement for nicotine for cockroach control. The basic biochemical characteristics of RBC AChE and BChE were determined in the 1940s. The mechanism of inhibition of both enzymes and other serine esterases was known in the 1940s and, in general, defined in the 1950s. In 1949, the death of a parathion mixer-loader dictated blood enzyme monitoring to prevent acute illness from organophosphorus pesticide intoxication. However, many of the chemical and biochemical steps for serine enzyme inhibition by OP compounds remain unknown today. The possible mechanisms of this inhibition are presented kinetically beginning with simple (by comparison) Michaelis-Menten substrate enzyme interaction kinetics. As complicated as the inhibition kinetics appear here, PBPK model kinetics will be more complex. The determination of inter- and intraindividual variation in RBC ChE and BChE was recognized early as critical knowledge for a blood esterase monitoring program. Because of the relatively constant production of RBCs, variation in RBC AChE was determined by about 1970. The source of plasma (or serum) BChE was shown to be the liver in the 1960s with the change in BChE phenotype to the donor in liver transplant patients. BChE activity was more variable than RBC AChE, and only in the 1990s have BChE individual variation questions been answered. We have reviewed the chemistry, metabolism, and toxicity of organophosphorus insecticides along with their inhibitory action toward tissue acetyl- and butyrylcholinesterases. On the basis of the review, a monitoring program for individuals mixing-loading and applying OP pesticides for commercial applicators was recommended. Approximately 41 OPs are currently registered for use by USEPA in the United States. Under agricultural working conditions, OPs primarily are absorbed through the skin. Liver P-450 isozymes catalyze the desulfurization of phosphorothioates and phosphorodithioates (e.g., parathion and azinphosmethyl, respectively) to the more toxic oxons (P = O(S to O)). In some cases, P-450 isozymes catalyze the oxidative cleavage of P-O-aryl bonds (e.g., parathion, methyl parathion, fenitrothion, and diazinon) to form inactive water-soluble alkyl phosphates and aryl leaving groups that are readily conjugated with glucuronic or sulfuric acids and excreted. In addition to the P-450 isozymes, mammalian tissues contain ('A' and 'B') esterases capable of reacting with OPs to produce hydrolysis products or phosphorylated enzymes. 'A'-esterases hydrolyze OPs (i.e., oxons), while 'B'-esterases with serine at the active center are inhibited by OPs. OPs possessing carboxylesters, such as malathion and isofenphos, are hydrolyzed by the direct action of 'B'-esterases (i.e., carboxylesterase, CaE). Metabolic pathways shown for isofenphos, parathion, and malathion define the order in which these reactions occur, while Michaelis-Menten kinetics define reaction parameters (Vmax, K(m)) for the enzymes and substrates involved, and rates of inhibition of 'B'-esterases (kis, bimolecular rate constants) by OPs and their oxons. OPs exert their insecticidal action by their ability to inhibit AChE at the cholinergic synapse, resulting in the accumulation of acetylcholine. The extent to which AChE or other 'B'-esterases are inhibited in workers is dependent upon the rate the OP pesticide is activated (i.e., oxon formation), metabolized to nontoxic products by tissue enzymes, its affinity for AChE and other 'B'-esterases, and esterase concentrations in tissues. Rapid recovery of OP BChE inhibition may be related to reactivation of inhibited forms. AChE, BChE, and CaE appear to function in vivo as scavengers, protecting workers against the inhibition of AChE at synapses. Species sensitivity to OPs varies widely and results in part from binding affinities (Ka) and rates of phosphorylation (kp) rather than rates of activation and detoxif.

Nihlen A, Droz PO. **Toxicokinetic modelling of methyl formate exposure and implications for**

biological monitoring. Int Arch Occup Environ Health 2000;73(7):479-87.
Abstract: A toxicokinetic (TK) model was developed to describe the inhalation exposure in humans to methyl formate (MF), a catalyst used in foundries, and to discuss biological monitoring. The TK model consisted of four compartments: MF, the metabolites--methanol (MeOH) and formic acid (FA)--and, in addition, a urinary compartment describing the saturable reabsorption of FA. Levels of MeOH and FA in urine, from an experimental study (100 ppm MF, 8 h at rest), validated the present model. The TK model describes well the general behaviour of MeOH and FA in urine after MF exposure. A nonlinear and a linear relationship respectively, was predicted between MF exposure and FA or MeOH excretion in urine, and this has previously been seen after occupational MF exposure. The present model has been modified to simulate MeOH exposure as well. Generally low exposures (concentration or exercise) produce only marginal increases in FA urinary excretions, but when exposure is elevated, urinary FA excretion increases because of saturation in the mechanism of reabsorption. Using FA urinary excretion as the critical indicator, because of its link to health effects, an occupational exposure limit value for MF of no greater than 50 ppm should be selected (based on predictions with the TK model). MeOH in urine can be considered as a biomarker for MF at low exposure, because of lower background values and of a linear relationship with exposure. At higher exposures, however, FA could be used as a biomarker as it becomes progressively more sensitive. But the use of biological monitoring for MF is difficult because of individual variations in background values. Under the present state of knowledge both FA and MeOH should be used to estimate only group exposures, rather than individual exposures.

Nozaki I, Tsuji T, Sakaguchi M, Inoue Y, Hirai R, Andou A, Miyazaki M, Shimizu N, Namba M. **Establishment of a human hepatoma cell line, HLE/2E1, suitable for detection of p450 2E1-related cytotoxicity.** In Vitro Cell Dev Biol Anim 2000;36(9):566-70.
Abstract: By transfection of an expression vector of human cytochrome P450 2E1 (CYP2E1) into a human hepatoma cell line (HLE), a new cell line (HLE/2E1) that stably expresses activity of CYP2E1 has been established. The HLE/2E1 cell line expressed a higher level of CYP2E1 messenger ribonucleic acid than did the mother HLE cell line. CYP2E1 enzyme activity determined by a p-nitrophenol oxidation assay was also higher in HLE/2E1 cells than in HLE cells. In addition, the enzyme activity of the HLE/2E1 cells was increased by ethanol treatment. Exposure to acetaminophen (APAP) or buthionine sulfoximine (BSO) caused a greater decrease in viability of the HLE/2E1 cells than that of the HLE cells, as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. The cytotoxicity of APAP or BSO to HLE/2EI cells was inhibited by the addition of ethanol or vitamin E. However, the cytotoxicity of both APAP and BSO was enhanced by 24-h preincubation of HLE/2E1 cells with ethanol. These results show that this cell line provides a useful model for studying catalytic properties of CYP2E1 and cytotoxic mechanisms of chemicals metabolized by CYP2E1.

O'Brien NM, Woods JA, Aherne SA, O'Callaghan YC. **Cytotoxicity, genotoxicity and oxidative reactions in cell-culture models: modulatory effects of phytochemicals.** Biochem Soc Trans 2000;28 (2):22-6.
Abstract: Much research effort has focused on the identification of phytochemicals in fruit and vegetables which exert beneficial effects. Our research examines modulatory effects of phytochemicals on cytotoxicity, genotoxicity and oxidative reactions in cell systems. Two examples of our studies are discussed. First, the potential beneficial effects of flavonoids are demonstrated. Flavonoids are reported

to exhibit a wide variety of biological effects, including antioxidant and free-radical-scavenging activities. The aim of the study was to determine if flavonoids could protect against H2O2-induced DNA damage, as measured by the comet assay, in Caco-2 and HepG2 cells. Both cell lines were supplemented with increasing concentrations of myricetin, quercetin and rutin for 24 h followed by exposure to H2O2 (50 microM) for 30 min. Exposure to H2O2 for 30 min at 37 degrees C resulted in significant DNA damage and pre-incubation with the flavonoids before H2O2 exposure significantly (P <0.05) protected Caco-2 and HepG2 cells against H2O2-induced DNA damage. Secondly, we illustrate the use of cellular models to study oxysterol-induced toxicity. Oxysterols are generated during the cooking and processing of foods and may be produced endogenously by the oxidation of membrane lipids. Recent findings suggest that oxysterols may modulate cytotoxicity by exerting effects on the induction of apoptosis. 7beta-Hydroxycholesterol (7beta-OHC) and 25-hydroxycholesterol, both of which are commonly found in foods, were investigated for their abilities to induce apoptosis in a human monocytic blood cell line, U937, and in the human hepatoma cell line, HepG2 cells. U937 and HepG2 cells were incubated for up to 48 h with 30 microM oxysterol. 7beta-OHC induced apoptosis in U937 cells as measured by non-random DNA fragmentation, condensed and fragmented nuclei, and the generation of hypodiploid cells. In contrast, oxysterols may induce cell death by a different mechanism in the hepatoma cells, possibly by necrosis.

Oakes DJ, Pollak JK. **The in vitro evaluation of the toxicities of three related herbicide formulations containing ester derivatives of 2,4,5-T and 2,4-D using sub-mitochondrial particles.** Toxicology 2000;151(1-3):1-9.
Abstract: The aim of this investigation was to determine the contribution made by the different components of herbicide formulations to the overall toxicity of the formulations. Three related herbicide formulations were chosen. The first, Agent Orange, consisted only of the butyl esters of 2,4,5-T and 2,4-D. The second was Agent Orange diluted with diesel fuel and the third formulation tested was a tree and blackberry killer, which consisted of the butyl ester of 2,4,5-T, the ethyl ester of 2,4-D, diesel fuel and two surfactants. The potential toxic effects of these three formulations were evaluated by determining their inhibitory effects on the oxidative functions of submitochondrial particles prepared from beef heart mitochondria. The effective concentration that caused a 50% inhibition of the activities of the submitochondrial particles was determined for all three formulations. When the toxicity of the individual components of these formulations was evaluated, it was established that the so-called 'inert' components i.e. diesel fuel and surfactants contributed approximately 50% of the overall toxicity of the complete formulations. Hence the results confirm the importance of evaluating the toxicity of complete formulations, rather than only focussing on the active components. While cellular and sub-cellular assays cannot account for pharmacokinetic and pharmacodynamic changes that may affect the toxicity of xenobiotics, the sub-mitochondrial particle test is useful as an initial screening assay.

Ou YC, Conolly RB, Thomas RS, Xu Y, Andersen ME, Chubb LS, Pitot HC, Yang RS. **A clonal growth model: time-course simulations of liver foci growth following penta- or hexachlorobenzene treatment in a medium-term bioassay.** Cancer Res 2001;61(5):1879-89.
Abstract: A combination of experimental and simulation approaches were used to analyze the clonal growth of preneoplastic, enzyme-altered foci during liver carcinogenesis in an initiation-promotion regimen. Male Fisher 344 rats, 8 weeks of age, were initiated with a single dose (200 mg/kg, i.p.) of

diethylnitrosamine (DEN). Beginning 2 weeks later, animals were exposed to daily gavage consisting of 0.1 mmol/kg pentachlorobenzene (PECB) or hexachlorobenzene (HCB) in corn oil vehicle for 6 weeks. Partial hepatectomy was performed 3 weeks after initiation. Experimental data including liver weight, hepatocyte density (number of hepatocytes/unit volume), 5-bromo-2'-deoxyuridine-labeling index for analysis of cell division rate, and number and volume of glutathione-S-transferase pi-positive foci were collected 23, 26, 28, 47, or 56 days after initiation. Model parameters describing liver growth were obtained directly from the experimental data. The probability of mutation/division of normal cells and the growth rate of initiated cells were inferred by a comparison of model outcomes with the observed time courses of foci development. To describe the time-dependent increases in foci volume and the concomitant reduction of foci number observed in all treatment groups, the calibrated model for the DEN controls incorporated the hypothesis of two initiated cell populations (referred to as A and B cells) within the framework of the two-stage model. The B cells are initiated cells that have a selective growth advantage under conditions that inhibit the growth of A cells and normal hepatocytes. The parameter values defined in the DEN controls were used to evaluate experiments involving the administration of PECB or HCB. Both PECB and HCB caused a significant increase in foci volume compared with the DEN controls. HCB treatments resulted in increased proliferation of normal hepatocytes, which was not observed for PECB under the same treatment regimen. The best description of the data resulted from the model incorporating the hypothesis that PECB and HCB promoted the growth of foci via increased net growth rates of B cells. We present here a biologically based clonal growth simulation platform to describe the growth of preneoplastic foci under experimental manipulations of initiation-promotion studies. This simulation work is an example of quantitative approaches that could be useful for the analysis of other initiation-promotion studies.

Pani G, Colavitti R, Bedogni B, Anzevino R, Borrello S, Galeotti T. **A redox signaling mechanism for density-dependent inhibition of cell growth.** J Biol Chem 2000;275(49):38891-9.
Abstract: Reactive oxygen species (ROS) have recently drawn significant attention as putative mitogenic mediators downstream of activated growth factor receptors and oncogenic Ras; however, the possibility that a redox-related mechanism also operates in the negative control of cell proliferation by inhibitory signals has not been investigated thus far. Here we show that the arrest of growth induced by cell confluence ("contact inhibition") is due, at least in part, to a decrease in the steady-state levels of intracellular ROS and the consequent impairment of mitogenic redox signaling. In confluent fibroblast cultures, the decrease in the concentration of oxygen species was associated with diminished activity of the small GTPase Rac-1, a signal transducer directly involved in the ligand-dependent generation of oxygen-derived molecules, and was effectively mimicked by exposure of sparse cultures to dithiothreitol (DTT) and inhibitors of enzymes (phospholipase A2 and lipoxygenase) acting in the arachidonic acid cascade downstream of growth factor receptors and Rac-1. Sparse fibroblasts treated with nontoxic amounts of DTT underwent growth arrest, whereas a low concentration of hydrogen peroxide significantly increased thymidine incorporation in confluent cultures, demonstrating a causal link between redox changes and growth control by cell density. Removal of oxygen species from sparse cultures was accompanied by a drastic decrease of protein tyrosine phosphorylation after epidermal growth factor stimulation, which, at a biochemical level, reproduced the signaling hallmarks of contact inhibition. Moreover, the cytosolic tyrosine phosphatase SHP-2 was identified as a putative target for redox signaling by cell density because the enzyme itself and the associated substrates appear markedly

dephosphorylated in both confluent and reductant-treated cells after exposure to epidermal growth factor, and SHP-2 enzymatic activity is strongly activated by DTT in vitro. Taken together, these data support a model in which impaired generation of ROS and increased protein tyrosine phosphatase activity impede mitogenic signaling in contact-inhibited cells.

Pani G, Colavitti R, Borrello S, Galeotti T. **Redox regulation of lymphocyte signaling.** IUBMB Life 2000;49(5):381-9.
Abstract: Compelling evidence exists that reactive oxygen species can deliver intracellular signals in mammalian cells, and elicit a broad array of physiological responses according to the cell type, the oxidative burden and the cellular compartment where radicals are generated. When applied to immune cells, these concepts gain a particular relevance, in relation to the plasticity of immune functions and the biological complexity of lymphocyte response to antigens. Here we review some recent and somehow conflicting observations on the involvement of oxygen radicals and redox balance in lymphocyte activation, and propose models for how radical species could contribute to normal and pathological immunity.

Parrill AL, Wang D, Bautista DL, Van Brocklyn JR, Lorincz Z, Fischer DJ, Baker DL, Liliom K, Spiegel S, Tigyi G. **Identification of Edg1 receptor residues that recognize sphingosine 1-phosphate.** J Biol Chem 2000;275(50):39379-84.
Abstract: Originating from its DNA sequence, a computational model of the Edg1 receptor has been developed that predicts critical interactions with its ligand, sphingosine 1-phosphate. The basic amino acids Arg(120) and Arg(292) ion pair with the phosphate, whereas the acidic Glu(121) residue ion pairs with the ammonium moiety of sphingosine 1-phosphate. The requirement of these interactions for specific ligand recognition has been confirmed through examination of site-directed mutants by radioligand binding, ligand-induced [(35)S]GTPgammaS binding, and receptor internalization assays. These ion-pairing interactions explain the ligand specificity of the Edg1 receptor and provide insight into ligand specificity differences within the Edg receptor family. This computational map of the ligand binding pocket provides information necessary for understanding the molecular pharmacology of this receptor, thus underlining the potential of the computational method in predicting ligand-receptor interactions.

Pastino GM, Flynn EJ, Sultatos LG. **Genetic polymorphisms in ethanol metabolism: issues and goals for physiologically based pharmacokinetic modeling.** Drug Chem Toxicol 2000;23(1):179-201.
Abstract: Chronic exposure to excessive ethanol consumption has adverse effects on virtually all organs and tissues in the body, including but not limited to the liver, pancreas, reproductive organs, central nervous system, and the fetus. Exposure to ethanol can also enhance the toxicity of other chemicals. Not all persons exposed to the same amount of ethanol experience the same degree of adverse effects. For example, only 12% to 13% of alcohol abusers develop cirrhosis. Possible factors which may alter susceptibility include age, sex, nutritional status, health status (i.e., smokers) and race. Some of these factors affect susceptibility because they alter ethanol metabolism, which occurs primarily in the liver by alcohol dehydrogenase (ADH). Genetic polymorphisms for ADH partially account for the observed differences in ethanol elimination rates among various populations but the relative contribution to susceptibility is not completely understood. Incorporation of the kinetic parameters associated with

ADH polymorphisms into a physiologically based pharmacokinetic (PBPK) model for ethanol will aid in assessing the relative contribution to susceptibility. The specific information required to develop this model includes Km and Kcat values for each ADH isoform and the amount of each isoform present in the liver. Blood ethanol concentrations (BEC) from various populations with known ADH phenotypes are also necessary to validate the model. The impact of inclusion of these data on PBPK model predictions was examined using available information from adult white and African American males.

Patten GS, Head RJ, Abeywardena MY, McMurchie EJ. **An apparatus to assay opioid activity in the infused lumen of the intact isolated guinea pig ileum.** J Pharmacol Toxicol Methods 2001;45(1):39-46.
Abstract: A modified apparatus is described that provides for the simultaneous bathing of the serosa of an intact piece of isolated guinea pig ileum while allowing infusion of the isolated lumen. The comparative compartmental potency of the opioid agonists morphine, casomorphins, and enkephalins to inhibit electrically driven contractions are described in this system. The rank-order potency for serosally applied opioid agonists was (IC(50) values, nM): [D-Ala(2),N-Me-Phe(4),Gly-ol(5)]-enkephalin (DAMGO) (15)>[D-Ala(2),D-Leu(5)]-enkephalin (DADLE) (35)> or =morphine (46)> or =[D-Ala(2)]-met-enkephalinamide (55)>[D-Ala(2)]-beta-casomorphin[1--4] amide (122)>beta-casomorphin[1--4] amide (940)>met- and leu-enkephalin (>6000). This contrasted to the rank-order potency for the luminally applied opioid agonists: DADLE (63)>DAMGO (135)>[D-Ala(2)]-met-enkephalinamide=morphine (4700)>[D-Ala(2)]-beta-casomorphin[1--4] amide (29000). beta-Casomorphin[1--4] amide, leu-enkephalin and met-enkephalin are mostly inactive when applied luminally. Furthermore, the opioid antagonists, casoxin 4 and [D-Ala(2)]-casoxin 4, when infused into the lumen, significantly overcame the inhibitory effect of morphine added to the serosal side. This model provides an assay and screening system to differentiate between the effects of chemical agents applied via the blood stream (serosa) or food side (lumen) on quiescent or electrically driven gut activity of the nervous plexi or receptor systems of the ileum.

Pelekis M, Gephart LA, Lerman SE. **Physiological-model-based derivation of the adult and child pharmacokinetic intraspecies uncertainty factors for volatile organic compounds.** Regul Toxicol Pharmacol 2001;33(1):12-20.
Abstract: The intraspecies uncertainty factor (UF(HH)=10x) is used in the determination of the reference dose or reference concentration and accounts for the pharmacokinetic and pharmacodynamic heterogeneity within the human population. The Food Quality Protection Act of 1996 mandated the use of an additional uncertainty factor (UF(HC)=10x) to take into account potential pre- and postnatal toxicity and lack of completeness of the data with respect to exposure and toxicity to children. There is no conclusive experimental or theoretical justification to support or refute the magnitude of the UF(HH) and UF(HC) nor any conclusive evidence to suggest that a factor of 100 is needed to account for intrahuman variability. This study presents a new chemical-specific method for estimating the pharmacokinetic (PK) component of the interspecies uncertainty factor (UF(HH-PK) and UF(HC-PK)) for volatile organic compounds (VOCs). The approach utilizes validated physiological-based pharmacokinetic (PBPK) models and simplified physiological-model-based algebraic equations to translate ambient exposure concentration to tissue dose in adults and children the ratio of which is the UF (HH-PK) and UF(HC-PK). The results suggest that: (i) the UF(HH-PK) and UF(HC-PK) are chemical

specific; (ii) for the chemicals used in this study there is no significant difference between UF(HH-PK) and UF(HC-PK); (iii) the magnitude of UF(HH-PK) and UF(HC-PK) varies between 0.033 and 2.85 with respect to tissue and blood concentrations; (iv) the body weight, the rate of ventilation, the fraction of cardiac output flowing to the liver, the blood : air partition coefficient, and the hepatic extraction ratio are the only parameters that play a critical role in the variability of tissue and blood doses within species; and (v) the magnitude of the UF(HH-PK) and UF(HC-PK) obtained with the simplified steady-state equations is essentially the same with that obtained with PBPK models. Overall, this study suggests that no adult-children differences in the parent chemical concentrations of the VOCs are likely to be observed during inhalation exposures. The physiological-model-based approaches used in the present study to estimate the UF(HH-PK) and UF(HC-PK) provide a scientific basis for their magnitude. They can replace the currently used empirical default approaches to provide chemical-specific UF(HH-PK) in future risk assessments. Copyright 2001 Academic Press.

Pelletier M, Roberge CJ, Gauthier M, Vandal K, Tessier PA, Girard D. **Activation of human neutrophils in vitro and dieldrin-induced neutrophilic inflammation in vivo**. J Leukoc Biol 2001;70 (3):367-73.
Abstract: Many chemicals of environmental concern are known to alter the immune system and are considered toxic molecules because they affect immune cell functions. Inflammation related to environmental chemical exposure, however, is poorly documented, except that from air pollutants. In this study, we found that the organochlorine insecticide dieldrin could not alter the ability of human neutrophils to phagocytose opsonized sheep red blood cells at nonnecrotic concentrations (0.1, 1, 10, and 50 microM). However, dieldrin was found to increase human neutrophil superoxide production, RNA synthesis, and proinflammatory cytokine interleukin-8 production. The normal apoptotic rate of neutrophils evaluated by both cytology and flow cytometry (CD-16 staining) was not altered by dieldrin treatments, and this was correlated with its inability to inhibit spreading of neutrophils onto glass. Using the murine air pouch model, we found that dieldrin induces a neutrophilic inflammation. Taken together, these results demonstrated that dieldrin is a proinflammatory contaminant. To our knowledge, this is the first report establishing that dieldrin is a contaminant exhibiting proinflammatory properties. In addition, it is the first time that the murine air pouch model has been successfully used to confirm that a chemical of environmental concern can induce an inflammatory response in vivo.

phando D, Pongratz I, Poellinger L, Whitelaw ML. **A redox mechanism controls differential DNA binding activities of hypoxia-inducible factor (HIF) 1alpha and the HIF-like factor.** J Biol Chem 2000;275(7):4618-27.
Abstract: Hypoxia-inducible factor 1alpha (HIF-1alpha) and the HIF-like factor (HLF) are two highly related basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) homology transcription factors that undergo dramatically increased function at low oxygen levels. Despite strong similarities in their activation mechanisms (e.g. they both undergo rapid hypoxia-induced protein stabilization, bind identical target DNA sequences, and induce synthetic reporter genes to similar degrees), they are both essential for embryo survival via distinct functions during vascularization (HIF-1alpha) or catecholamine production (HLF). It is currently unknown how such specificity of action is achieved. We report here that DNA binding by HLF, but not by HIF-1alpha, is dependent upon reducing redox conditions. In vitro DNA binding and mammalian two-hybrid assays showed that a unique cysteine in the DNA-binding basic

region of HLF is a target for the reducing activity of redox factor Ref-1. Although the N-terminal DNA-binding domain of HIF-1alpha can function in the absence of Ref-1, we found that the C-terminal region containing the transactivation domain requires Ref-1 for full activity. Our data reveal that the hypoxia-inducible factors are subject to complex redox control mechanisms that can target discrete regions of the proteins and are the first to establish a discriminating control mechanism for differential regulation of HIF-1alpha and HLF activity.

Phillips JD, Jackson LK, Bunting M, Franklin MR, Thomas KR, Levy JE, Andrews NC, Kushner JP. **A mouse model of familial porphyria cutanea tarda.** Proc Natl Acad Sci U S A 2001;98(1):259-64.
Abstract: Approximately one-third of patients with porphyria cutanea tarda (PCT), the most common porphyria in humans, inherit a single mutant allele of the uroporphyrinogen decarboxylase (URO-D) gene. PCT associated with URO-D mutations is designated familial PCT. The phenotype is characterized by a photosensitive dermatosis with hepatic accumulation and urinary excretion of uroporphyrin and hepta-carboxylic porphyrins. Most heterozygotes for URO-D mutations do not express a porphyric phenotype unless hepatic siderosis is present. Hemochromatosis gene (HFE) mutations are frequently found when the phenotype is expressed. We used homologous recombination to disrupt one allele of murine URO-D. URO-D(+/-) mice had half-wild type (wt) URO-D protein and enzymatic activity in all tissues but did not accumulate hepatic porphyrins, indicating that half-normal URO-D activity is not rate limiting. When URO-D(+/-) mice were injected with iron-dextran and given drinking water containing delta-aminolevulinic acid for 21 days, hepatic porphyrins accumulated, and hepatic URO-D activity was reduced to 20% of wt. We bred mice homozygous for an HFE gene disruption (HFE(-/-)) to URO-D(+/-) mice, generating mice with the URO-D(+/-)/HFE(-/-) genotype. These animals developed a porphyric phenotype by 14 weeks of age without ALA supplementation, and URO-D activity was reduced to 14% of wt. These data indicate that iron overload alone is sufficient to reduce URO-D activity to rate-limiting levels in URO-D(+/-) mice. The URO-D(+/-) mouse serves as an excellent model of familial PCT and affords the opportunity to define the mechanism by which iron influences URO-D activity.

Podrez EA, Hoppe G, O'Neil J, Sayre LM, Sheibani N, Hoff HF. **Macrophage receptors responsible for distinct recognition of low density lipoprotein containing pyrrole or pyridinium adducts: models of oxidized low density lipoprotein.** J Lipid Res 2000;41(9):1455-63.
Abstract: Oxidation of low density lipoproteins (LDL) induced by incubation with Cu(2+) ions results in the formation of a heterogeneous group of aldehydic adducts on lysyl residues (Lys) of apolipoprotein B (apoB) that are thought to be responsible for the uptake of oxidized LDL (oxLDL) by macrophages. To define the structural and chemical criteria governing such cell recognition, we induced two modifications of lysines in LDL that mimic prototypic adducts present in oxLDL; namely, epsilon-amino charge-neutralizing pyrrolation by treatment with 2,5-hexanedione (hdLDL), and epsilon-amino charge-retaining pyridinium formation via treatment with 2,4,6-trimethylpyrylium (tmpLDL). Both modifications led to recognition by receptors on mouse peritoneal macrophages (MPM). To assess whether the murine scavenger receptor class A-I (mSR-A) was responsible for recognition of hdLDL or tmpLDL in MPM, we measured binding at 4 degrees C and degradation at 37 degrees C of these modified forms of (125)I-labeled LDL by mSR-A-transfected CHO cells. Although uptake and degradation of hdLDL by mSR-A-transfected CHO cells was quantitatively similar to that of the

positive control, acLDL, tmpLDL was not recognized by these cells. However, both tmpLDL and hdLDL were recognized by 293 cells that had been transfected with CD36. In the human monocytic cell line THP-1 that had been activated with PMA, uptake of tmpLDL was significantly inhibited by blocking monoclonal antibodies to CD36, further suggesting recognition of tmpLDL by this receptor. Macrophage uptake and degradation of LDL oxidized by brief exposure to Cu(2+) was inhibited more effectively by excess tmpLDL and hdLDL than was more extensively oxidized LDL, consistent with the recognition of the former by CD36 and the latter primarily by SR-A.Collectively, these studies suggest that formation of specific pyrrole adducts on LDL leads to recognition by both the mSR-A and mouse homolog of CD36 expressed on MPM, while formation of specific pyridinium adducts on LDL leads to recognition by the mouse homolog of CD 36 but not by mSR-A. As such, these two modifications of LDL may represent useful models for dissecting the relative contributions of specific modifications on LDL produced during oxidation, to the cellular uptake of this heterogeneous ligand.

Portier CJ. **Linking toxicology and epidemiology: the role of mechanistic modelling.** Stat Med 2001;20(9-10):1387-93.

Radivoyevitch T, Hoel DG. **Biologically-based risk estimation for radiation-induced chronic myeloid leukemia.** Radiat Environ Biophys 2000;39(3):153-9.
Abstract: Radiation cancer risks are typically determined by the use of simple statistical descriptions of epidemiological data. It is important in risk assessment in general, however, to attempt to incorporate as much biological information into the risk models as possible. We illustrate this by presenting a biologically-based linear-quadratic-exponential (LQE) incidence rate model for radiation-induced chronic myeloid leukemia (CML). The model consists of a linear-quadratic dose-response for the induction of BCR-ABL, a waiting time distribution between BCR-ABL formation and detection of CML, and an exponential cell-killing term that multiplies both the background and induced incidence rates. Using data exclusive of the A-bomb survivor cohort, Bayesian priors are defined for each of the nine parameters in this LQE model. The priors are based on chromosomal translocations in lymphocytes, hematopoietic stem cell survival experiments, CML waiting times in women irradiated for benign disease, the background CML incidence rate in the U.S. population, and genomic DNA target sizes of BCR and ABL. Fixing three of the LQE model parameters to the means of their priors, maximum likelihood estimates of the remaining six parameters were obtained using A-bomb survivor incidence data for Hiroshima males. The likelihood estimates and the corresponding six prior distributions, both approximated as multivariate normal, were then used to form Bayesian posteriors for the six parameters not fixed. With these posteriors the LQE model yields Qgamma*=0.0042 Gy(-1) where Qgamma* is the upper 95% confidence bound of the lifetime CML risk per person-gray in the limit of low doses of gamma-rays. This value is slightly less than Qgamma*=0.0049 Gy(-1) obtained from likelihood estimates of the LQE parameters, and substantially less than Qgamma*=0.0158 Gy(-1) obtained for a simple statistical model linear in dose for kermas less than 4 Gy.

Radivoyevitch T, Kozubek S, Sachs RK. **Biologically based risk estimation for radiation-induced CML. Inferences from BCR and ABL geometric distributions.** Radiat Environ Biophys 2001;40 (1):1-9.
Abstract: Chronic myeloid leukemia (CML) invites biologically based radiation risk modeling because

CML is simultaneously well-understood, homogeneous and prevalent. CML is known to be caused by a translocation involving the ABL and BCR genes, almost all CML patients have the BCR-ABL translocation, and CML is prevalent enough that its induction is unequivocally detected among Hiroshima A-bomb survivors. In a previous paper, a linear-quadratic-exponential (LQE) dose-response model was used to estimate the lifetime excess risk of CML in the limit of low doses of gamma-rays, R gamma. This estimate assumed that BCR-ABL translocation dose-response curves in stem cells for both neutrons and gamma-rays, differ only by a common proportionality constant from dicentric aberration dose-response curves in lymphocytes. In the present paper we challenge this assumption by predicting the BCR-ABL dose response. The predictions are based on the biophysical theory of dual radiation action (TDRA) as it applies to recent BCR-to-ABL distance data in G0 human lymphocytes; this data shows BCR and ABL geometric distributions that are not uniform and not independent, with close association of the two genes in some cells. The analysis speaks against the previous proportionality assumption. We compute 11 plausible LQE estimates of R gamma, 2 based on the proportionality assumption and 9 based on TDRA predictions. For each estimate of R gamma we also compute an associated estimate of the number of CML target cells, N; the biological basis of the LQE model allows us to form such estimates. Consistency between N and hematological considerations provides a plausibility check of the risk estimates. Within the group of estimates investigated, the most plausible lifetime excess risk estimates tend to lie near R gamma = 0.01 Gy-1, substantially higher than risk estimates based on the proportionality assumption.

Renwick AG, Walton K. **The use of surrogate endpoints to assess potential toxicity in humans.** Toxicol Lett 2001;120(1-3):97-110.
Abstract: Data on toxic effects in humans may come from epidemiology studies, accidental poisonings, surveillance schemes or following intentional exposures. In many cases, a surrogate endpoint related to the adverse effect is investigated. Effects produced following intentional exposures are usually restricted to readily reversible, mild surrogate endpoints of the adverse effect of concern. Not all initial interactions within the target organ are related to the toxic effect, and many measurements are biomarkers of exposure not response. Biomarkers of response represent surrogate endpoints of response only if they are critical to the mode of action. The use of biomarkers and the possible problems with using surrogate endpoints are illustrated with data on aniline, cadmium, carbon monoxide, erythrosine, paracetamol (acetaminophen) and styrene. In vivo surrogate endpoints are normally used in risk assessment directly, whereas in vitro surrogate endpoints can be incorporated by the development of a biologically based dose-response model, or used to replace a default uncertainty factor by a chemical-specific adjustment factor.

Rimbach G, Saliou C, Canali R, Virgili F. **Interaction between cultured endothelial cells and macrophages: in vitro model for studying flavonoids in redox-dependent gene expression**. Methods Enzymol 2001;335:387-97.
Abstract: This article focused on two methods to measure the activity of NF-kB. Both methods evalute "post-IkB phosphorylation" stages in the NF-kB activation cascade. In fact, EMSA performed with nuclear extracts provides an information only on NF-kB nuclear translocation and its ability to bind kB-DNA sequences. Likewise, the reporter gene assay is limited to assessing NF-kB-dependent gene expression no matter the mechanism that originally activated NF-kB. Nevertheless, the latter assay

represents a more physiological and more reproducible way of measuring NF-kB activity in mammalian cells than the EMSA does. In order to obtain further insights into NF-kB signal transduction pathways, investigating IkB degradation and phosphorylation are recommended. The cloning and characterization of IkB kinases provided new testing possibilities based on measure of their activity.

Rodrigues AD, Lin JH. **Screening of drug candidates for their drug--drug interaction potential.** Curr Opin Chem Biol 2001;5(4):396-401.
Abstract: Within the past year, additional papers have been published that focus on higher-throughput drug-interaction screening. Some papers have described enzyme assays that can be used to evaluate inhibition or induction of the human cytochrome P450s. At the same time, numerous investigators have developed computational (in silico) methods to predict interactions and have validated the approach using in vitro (assay-derived) data. These so called 'in silico--in vitro' correlations have great potential and may complement existing 'in vitro--in vivo' correlations.

Rondard P, Iiri T, Srinivasan S, Meng E, Fujita T, Bourne HR. **Mutant G protein alpha subunit activated by Gbeta gamma: a model for receptor activation?** Proc Natl Acad Sci U S A 2001;98 (11):6150-5.
Abstract: How receptors catalyze exchange of GTP for GDP bound to the Galpha subunit of trimeric G proteins is not known. One proposal is that the receptor uses the G protein's betagamma heterodimer as a lever, tilting it to pull open the guanine nucleotide binding pocket of Galpha. To test this possibility, we designed a mutant Galpha that would bind to betagamma in the tilted conformation. To do so, we excised a helical turn (four residues) from the N-terminal region of alpha(s), the alpha subunit of G(S), the stimulatory regulator of adenylyl cyclase. In the presence, but not in the absence, of transiently expressed beta(1) and gamma(2), this mutant (alpha(s)Delta), markedly stimulated cAMP accumulation. This effect depended on the ability of the coexpressed beta protein to interact normally with the lip of the nucleotide binding pocket of alpha(s)Delta. We substituted alanine for an aspartate in beta(1) that binds to a lysine (K206) in the lip of the alpha subunit's nucleotide binding pocket. Coexpressed with alpha(s)Delta and gamma(2), this mutant, beta(1)-D228A, elevated cAMP much less than did beta(1)-wild type; it did bind to alpha(s)Delta normally, however, as indicated by its unimpaired ability to target alpha(s)Delta to the plasma membrane. We conclude that betagamma can activate alpha(s) and that this effect probably involves both a tilt of betagamma relative to alpha(s) and interaction of beta with the lip of the nucleotide binding pocket. We speculate that receptors use a similar mechanism to activate trimeric G proteins.

Rossi J 3rd, Ritchie GD, Nordholm AF, Knechtges PL, Wilson CL, Lin J, Alexander WK, Still KR. **Application of neurobehavioral toxicology methods to the military deployment toxicology assessment program**. Drug Chem Toxicol 2000;23(1):113-38.
Abstract: The military Tri-Service (Army, Navy & Marines, Air Force) Deployment Toxicology Assessment Program (DTAP) represents a 30-year (1996-2026) planning effort to implement comprehensive systems for the protection of internationally deployed troops against toxicant exposures. A major objective of DTAP is the implementation of a global surveillance system to identify chemicals with the potential to reduce human performance capacity. Implementation requires prior development of complex human risk assessment models, known collectively as the Neurobehavioral Toxicity Evaluation

Instrument (NTEI), based on mathematical interpolation of results from tissue-based and in vivo animal studies validated by human performance assessment research. The Neurobehavioral Toxicity Assessment Group (NTAG) at the Naval Health Research Center Detachment-Toxicology (NHRC-TD), Dayton, OH, and associated academic institutions are developing and cross-validating cellular-level (NTAS), laboratory small animal (NTAB), nonhuman primate (GASP), and human-based (GASH) toxicity assessment batteries. These batteries will be utilized to develop and evaluate mathematical predictors of human neurobehavioral toxicity, as a function of laboratory performance deficits predicted by quantitative structural analysis relationship (QSAR-like) properties of potential toxicants identified by international surveillance systems. Finally, physiologically-based pharmacokinetic (PBPK) and pharmacodynamic (PBPD) modeling of NTAS, NTAB, GASP, GASH data will support multi-organizational development and validation of the NTEI. The validated NTEI tool will represent a complex database management system, integrating global satellite surveillance input to provide real-time decision-making support for deployed military personnel.

Schlosser SF, Azzaroli F, Dao T, Hingorani R, Nicholas Crispe I, Boyer JL. **Induction of murine hepatocyte death by membrane-bound CD95 (Fas/APO-1)-ligand: characterization of an in vitro system**. Hepatology 2000;32(4 Pt 1):779-85.
Abstract: Hepatocytes constitutively express CD95 (also called Fas/APO-1) and are therefore potential targets for CD95-ligand (CD95L)-mediated injury. To study this mechanism of cell death in hepatocytes we developed an in vitro model of liver cell apoptosis using membrane-bound CD95L as the inducing agent. Primary mouse hepatocytes were cocultured with NIH 3T3 fibroblasts, stably transfected with mouse CD95L (F(CD95L+)). Fibroblasts stably transfected with vector only (F(CD95L-)) served as controls. Hepatocytes from mice expressing low levels of CD95 (Fas(lpr) mice) served as controls for effects unrelated to CD95. Morphologic and biochemical studies indicate that CD95 is expressed in cultured mouse hepatocytes. Membrane-bound CD95 from transfected fibroblasts destroyed all cocultured hepatocytes within 24 hours in the absence of protein synthesis inhibitors. Characteristic features of apoptosis were observed in dying hepatocytes and occurred in the following sequence: formation of cytoplasmic blebs and nuclear condensation after 3 hours; nuclear fragmentation and DNA strand breaks after 4 hours. These changes were observed only when normal hepatocytes were cocultured with F(CD95L+) and were not observed with F(CD95L-) or in hepatocytes from Fas(lpr) mice. Anti-CD95 antibody (Jo2) evoked similar changes in hepatocytes, although to a much lesser extent. We conclude that coculture of mouse hepatocytes with F(CD95L+) is a useful in vitro model for CD95-mediated apoptosis induced by CD95L. The high incidence of apoptosis caused by membrane-bound CD95L differs from the much smaller effects induced by the Jo2 antibody. In view of the high sensitivity of hepatocytes towards CD95L we speculate that CD95L-induced liver damage in vivo may be minimized by restricting exposure of hepatocytes to CD95L.

Schonberger J, Bauer J, Spruss T, Weber G, Chahoud I, Eilles C, Grimm D. **Establishment and characterization of the follicular thyroid carcinoma cell line ML-1.** J Mol Med 2000;78(2):102-10.
Abstract: The present study focuses on the establishment and characterization of a new follicular thyroid carcinoma cell line. The human cell line ML-1 was derived from a dedifferentiated follicular thyroid carcinoma relapse, which progressed despite preceding surgery followed by two radioiodine therapies. More than 90% of the cells of this line express thyroglobulin, chondroitin sulfate, and vimentin antigens,

but only about 70% show cytokeratin filaments and a negative surface charge density such as human erythrocytes. More importantly, cells of this line are able to take up iodine and/or glucose both in vitro and in vivo and to secrete thyroglobulin, chondroitin sulfate, and fibronectin into the interstitial space. In addition, triiodothyronine is released constitutively into culture supernatants. Moreover, it is also suitable for xenotransplantation studies because it is tumorigenic in NMRI nude mice in vivo. The cell line forms tumors with follicular structures when transplanted to nude mice. Due to these unique features the ML-1 cell line can be considered as a very suitable test model for pharmacological and cell biological studies. Since chemicals may interfere with the production of thyroid hormones, this cell line represents also a tool for toxicological investigations.

Schwab AJ, Pang KS. **The multiple indicator dilution method and its utility in risk assessment.** Environ Health Perspect 2000;108 Suppl 5:861-72.
Abstract: The multiple-indicator dilution (MID) technique entails the injection of a mixture of labeled indicators into the blood vessel immediately at the entrance of an organ, e.g., the liver, kidney, heart, or lung, and characterization of outflow dilution profiles from timed venous samples. The mathematical basis of the method encompasses linear systems of partial differential equations that are formulated for flow- or barrier-limited transport combined with intracellular metabolism/excretion. The concept can be generalized to include metabolites. MID experiments are useful for determining tissue partition coefficients as well as kinetic parameters such as membrane permeabilities or metabolic/excretory intrinsic clearances, factors that affect the mean residence times or exposure of solutes to the organ. The main utility of the MID method lies in its role in identifying the basic mechanisms of the interaction of organs with vascular components. The concentration dependence in transport and removal is revealed by the rate coefficients upon varying the input concentrations of unlabeled substances into the organ at steady state. The data obtained with MID experiments can be incorporated into physiologically based pharmacokinetic (PBPK) models such as those used for biological risk assessment. This is especially pertinent in the case where diffusional barriers appear within organs. The insight gained from the MID organ approach may be useful for PBPK models with more realistic representation of organ kinetics.

Seidel SD, Li V, Winter GM, Rogers WJ, Martinez EI, Denison MS. **Ah receptor-based chemical screening bioassays: application and limitations for the detection of Ah receptor agonists.** Toxicol Sci 2000;55(1):107-15.
Abstract: The aromatic hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates many of the biologic and toxicologic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related chemicals. Here we utilized two AhR-dependent bioassay systems as screening tools to identify novel AhR agonists and to detect the presence of AhR agonists in sample extracts. These assays measure the ability of a chemical to activate AhR DNA binding in vitro (GRAB bioassay) or AhR-dependent (luciferase) gene expression in cultured cells (CALUX bioassay). Known AhR agonists (halogenated and nonhalogenated aromatic hydrocarbons) were positive in both assays, whereas the AhR antagonist alpha-naphthoflavone exhibited agonist activity only in the GRAB assay. In vitro GRAB analysis has identified several imidazoline receptor ligands and beta-carbolines as AhR agonists and also revealed the presence of AhR agonist activity in crude DMSO extracts of commercial newspapers. In contrast to their positive activity in the GRAB assay, the majority of these chemicals/ extracts were only weakly active or inactive in the cell-based CALUX assay. Our results not only reveal

that the ability of a chemical to activate the AhR in vitro does not necessarily correlate with its ability to induce gene expression in intact cells, but the high level of false positives obtained with the GRAB assay clearly demonstrates its inability to accurately identify AhR agonists or agonist activity. Screening of unknown chemicals, chemical classes, and samples for AhR agonist activity will require the use of intact cell bioassays.

Seralini G, Moslemi S. **Aromatase inhibitors: past, present and future.** Mol Cell Endocrinol 2001;178 (1-2):117-31.
Abstract: For the cellular physiology of sex steroid sensitive cells, the androgen/estrogen ratio may be more important than only one hormone action per se, in both sexes. This ratio is controlled in vertebrates by aromatase; its gene expression can be inhibited in different ways, and this is crucial for the treatment of estrogen-dependent diseases such as breast cancer, or gynecomastia in males for instance. To reach this goal, new steroidal and non-steroidal inhibitors are continuously being developed, and some of them are used as first or second line agents. Aromatase inhibition is also an essential tool for studying the role of estrogens in the adult, or during development. Aromatase inhibitors have shown in particular that estrogens are essential also in males for skeletal maturation and bone mineralization, development of masculine dendritic morphology in male brain linked to mating behaviour, and testicular function. Testosterone is often the prohormone converted in situ in active estrogens, at these levels. Several strategies can be used for aromatase inhibition. The first ones employed were blind screening or deductions from in vivo observations, which led for instance to the discovery of the role of aminoglutethimide in aromatase inhibition. Subsequently, in the years 1975-1990, the molecular modeling of compounds to mimic the substrate shape of the enzyme constituted the major idea. Hundreds of chemicals were synthesized by numerous authors, ranging from the well-known and very efficient 4-OHA to complicated imidazole or indane derivatives tested by sophisticated comparative molecular field analyses. Reticulum-bound active aromatase has not as yet been X-ray analyzed. Thus, aromatase inhibitors were also used more recently to probe and understand the active site conformation of the enzyme and its modelization was obtained from comparisons with bacterial-related cytochromes. We developed a mammalian model considerably closer to human aromatase in order to study the active site shape with new potent aromatase non-steroidal inhibitors. This model is equine aromatase. This enzyme was biochemically characterized, purified, and cloned by our group. It allowed testing, by site-directed mutagenesis, predictive hypotheses in human aromatase which contributed to designing of new inhibitors. The understanding of the functioning of an essential member of the cytochrome P450 family, which is necessary for cellular detoxification, was also facilitated. Inhibition of aromatase activity has also been carried out with antibodies directed to the catalytic site and at the gene level by knock-out or by control of factor-specific promoters. This may result in different mRNA synthesized by alternative splicing. We have also obtained specific inhibition of aromatase activity in human cells with antisense stable phosphorothioate oligodeoxynucleotides directed against aromatase mRNA tertiary structures. Besides known steroidal and non-steroidal inhibitors, the antiaromatase effects of compounds found in our daily environment such as dietary flavonoids or xenobiotic pollutants have also been described. Finally, we underline that all these aromatase inhibitors, or methods of aromatase inhibition, can modulate the estrogenic balance essential not only for female, but also for male physiology, including gonadal function.

Setzer RW, Lau C, Mole ML, Copeland MF, Rogers JM, Kavlock RJ. **Toward a biologically based dose-response model for developmental toxicity of 5-fluorouracil in the rat: a mathematical construct.** Toxicol Sci 2001;59(1):49-58.
Abstract: Biologically based dose-response (BBDR) models comprise one way to incorporate mechanistic information into a dose-response assessment to be used for risk assessments. The chemotherapeutic drug 5-fluorouracil (5-FU) has been used as a prototypic compound for the construction of a BBDR model for developmental toxicity. Previous work has provided data and a general mechanistic framework for the developmental toxicity of 5-FU when it was administered to pregnant rats subcutaneously on gestation day 14. In this paper, a mathematical model relating maternally administered treatment with 5-FU to embryonal thymidylate synthetase inhibition and thymidylate synthetase inhibition to various measures of deoxyribonucleotide triphosphate (dNTP) pool perturbation is developed, and parameters are estimated using the data collected. The strategy used was to develop semi-empirical submodels for each of the intervening steps, and to estimate model parameters from previously described data. The models developed predict that there is no practical threshold for dNTP pool perturbation; that is, even minimal doses of 5-FU should result in some perturbation of dNTP pools. In particular, the relationship between dNTP pool perturbation and fetal weight deficit suggests that if there is a biological threshold for the effect of 5-FU on fetal weight, the responsible repair or compensation mechanism must be downstream of dNTP pool perturbation, and saturable at 5-FU doses lower than 10 mg/kg (the lowest dose examined for developmental effects in these studies).

Shaik MS, Ikediobi O, Turnage VD, McSween J, Kanikkannan N, Singh M. **Long-circulating monensin nanoparticles for the potentiation of immunotoxin and anticancer drugs**. J Pharm Pharmacol 2001;53(5):617-27.
Abstract: The carboxylic ionophore monensin was formulated into long-circulating nanoparticles with the help of polyethylene glycol/poly (DL-lactide-co-glycolide) diblock copolymers, in an attempt to enhance the cytotoxicity of a ricin-based immunotoxin, anti-My9, and anticancer drugs like adriamycin and tamoxifen. This study looked into various aspects involving the preparation (using a homogenizer and an EmulsiFlex homogenizer-extrusion device) and lyophilization of long-circulating monensin nanoparticles (LMNP) of particle size < 200 nm in diameter. The particle size of LMNP was reduced from 194 nm to 160 nm by passing the nanoparticles through an EmulsiFlex, before freeze-drying. There was a 4.8-83.7% increase in the particle size of LMNP after freeze-drying, which was dependent upon the manufacturing conditions such as use of the EmulsiFlex for size reduction before freeze-drying, the freezing method (rapid/slow) and the concentration of lyoprotectant (mannitol or trehalose) employed for freeze-drying. LMNP freeze-dried with 2.4% of trehalose showed minimal size change (< 9%) after freeze-drying. Further, the freezing method was found to have negligible effect on the particle size of LMNP freeze-dried with trehalose in comparison with mannitol. The entrapment efficiency of monensin in LMNP was found to be 14.2 +/- 0.3%. The LMNP were found to be spherical in shape and smooth in surface texture as observed by atomic force microscopy. In-vitro release of monensin from LMNP in phosphate buffered saline (PBS) pH 7.4 or PBS supplemented with 10% human serum indicated that there was an initial rapid release of about 40% in the first 8 h followed by a fairly slow release (about 20%) in the next 88 h. In-vivo studies conducted with Sprague-Dawley rats showed that 20% of monensin remained in circulation 4-8 h after the intravenous administration of LMNP. An in-vitro dye-based cytotoxicity assay (MTS/PMS method) showed that there was 500 times and 5 times potentiation

of the cytotoxicity of anti-My9 immunotoxin by LMNP (5 x 10(-8) M of monensin) in HL-60 sensitive and resistant human tumour cell lines, respectively. Further, LMNP (5 x 10(-8) M of monensin) potentiated the cytotoxicity of adriamycin in MCF 7 and SW 620 cell lines by 100 fold and 10 fold, respectively, and that of tamoxifen by 44 fold in MCF 7 cell line as assessed by crystal violet dye uptake assay. Our results suggest that it is possible to prepare LMNP possessing appropriate particle size (< 200 nm), monensin content and in-vitro and in-vivo release characteristics with the help of a homogenizer and an EmulsiFlex homogenizer-extrusion device. LMNP can be freeze-dried with minimal increase in particle size by using a suitable concentration of a lyoprotectant like trehalose. Furthermore, LMNP could potentiate the cytotoxicity of immunotoxin, adriamycin and tamoxifen by 5-500 fold in-vitro, which will be further investigated in-vivo in a suitable animal model.

Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. **Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription.** Cell 2000;103(6):843-52.
Abstract: Many cofactors bind the hormone-activated estrogen receptor (ER), yet the specific regulators of endogenous ER-mediated gene transcription are unknown. Using chromatin immunoprecipitation (ChIP), we find that ER and a number of coactivators rapidly associate with estrogen responsive promoters following estrogen treatment in a cyclic fashion that is not predicted by current models of hormone activation. Cycles of ER complex assembly are followed by transcription. In contrast, the anti-estrogen tamoxifen (TAM) recruits corepressors but not coactivators. Using a genetic approach, we show that recruitment of the p160 class of coactivators is sufficient for gene activation and for the growth stimulatory actions of estrogen in breast cancer supporting a model in which ER cofactors play unique roles in estrogen signaling.

Sharma RP, Bhandari N, Tsunoda M, Riley RT, Voss KA, Meredith FI. **Fumonisin toxicity in a transgenic mouse model lacking the mdr1a/1b P-glycoprotein genes.** 2000;8(3):173-82.
Abstract: The toxicity of fumonisin B(1) (FB(1)) was investigated in male mdr1a/1b double knockout (MDRK) mice, lacking the drug-transporting P-glycoproteins. These transgenic animals are deficient in their blood:brain barrier and accumulate different drugs in brain and other tissues. The MDRK and their wild-type counterparts, FVB mice, were injected subcutaneously with 2.25 mg/kg per day of FB(1) for 5 days and sampled one day after the last treatment in a protocol that has resulted in marked hepatic and renal damage in other strains. FB(1) caused liver enlargement in both FVB and MDRK. Hematological parameters were not affected in either strain. Plasma levels of alanine aminotransferase and aspartate aminotransferase, measures of liver damage, were increased by FB(1) in both FVB and MDRK mice. Histopathological evaluation of liver corroborated this finding. Kidney lesions were induced by FB(1) in both types of mice. Concentrations of free sphingosine and sphinganine increased in liver and kidney of both strains after the FB(1) treatment, although the increase in liver sphingoid bases was half as much in MDRK as compared to FVB. The levels of sphinganine-containing complex sphingolipids were increased in kidney. The levels of sphingosine-containing complex sphingolipids in kidney were unaffected by FB(1) treatment but were significantly lower in control MDRK than in FVB mice. The levels of neurotransmitters and their metabolites were similarly affected in both strains by FB(1), suggesting no influence of disrupted blood:brain barrier on FB(1)-induced neurotoxicity. In both strains, the liver mRNA for tumor necrosis factor alpha was increased; however, the increase was statistically significant only in FVB. It was apparent that mice deficient in P-glycoprotein do not exhibit greater

sensitivity to FB(1), the cellular or brain transport of FB(1) appears to be independent of this multidrug transporting system.

Sharma SC, Richards JS. **Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation.** J Biol Chem 2000;275 (43):33718-28.
Abstract: AP1 transcription factors control rapid responses of mammalian cells to stimuli that impact proliferation, differentiation, and transformation. To determine which AP1 factors are present in and regulated by hormones in ovarian cells during specific stages of proliferation and differentiation, we used both in vitro and in vivo models, Western blotting, immunohistochemistry, DNA binding assays, and transfections of AP1 promoter-reporter constructs. The expression patterns of Jun and Fos family members in response to hormones (follicle-stimulating hormone (FSH), luteinizing hormone (LH), and cAMP) were distinct. JunB, c-Jun, c-Fos, and Fra2 were rapidly but transiently induced by FSH in immature granulosa cells. JunD and Fra2 were induced by LH and maintained as granulosa cells terminally differentiated into luteal cells. Forskolin and phorbol myristate acetate acted synergistically to enhance transcription of an AP1(-73COL)-luciferase construct. JunD appears to be one mediator of this effect, since JunD was a major component of the AP1-DNA binding complex in granulosa cells, and menin, a selective inhibitor of JunD, blocked transcription of -73COL-luciferase. Thus, FSH and LH via cAMP induce specific AP1 factors, the AP1 expression patterns are distinct, and that of JunD and Fra2 correlates with the transition of proliferating granulosa cells to terminally differentiated, non-dividing luteal cells.

Sharp SY, Kelland LR, Valenti MR, Brunton LA, Hobbs S, Workman P. **Establishment of an isogenic human colon tumor model for NQO1 gene expression: application to investigate the role of DT-diaphorase in bioreductive drug activation in vitro and in vivo.** Mol Pharmacol 2000;58(5):1146-55.
Abstract: Many tumors overexpress the NQO1 gene, which encodes DT-diaphorase (NADPH:quinone oxidoreductase; EC 1.6.99.2). This obligate two-electron reductase deactivates toxins and activates bioreductive anticancer drugs. We describe the establishment of an isogenic human tumor cell model for DT-diaphorase expression. An expression vector was used in which the human elongation factor 1alpha promoter produces a bicistronic message containing the genes for human NQO1 and puromycin resistance. This was transfected into the human colon BE tumor line, which has a disabling point mutation in NQO1. Two clones, BE2 and BE5, were selected that were shown by immunoblotting and enzyme activity to stably express high levels of DT-diaphorase. Drug response was determined using 96-h exposures compared with the BE vector control. Functional validation of the isogenic model was provided by the much greater sensitivity of the NQO1-transfected cells to the known DT-diaphorase substrates and bioreductive agents streptonigrin (113- to 132-fold) and indoloquinone EO9 (17- to 25-fold) and the inhibition of this potentiation by the DT-diaphorase inhibitor dicoumarol. A lower degree of potentiation was seen with the clinically used agent mitomycin C (6- to 7-fold) and the EO9 analogs, EO7 and EO2, that are poorer substrates for DT-diaphorase (5- to 8-fold and 2- to 3-fold potentiation, respectively), and there was no potentiation or protection with menadione and tirapazamine. Exposure time-dependent potentiation was seen with the diaziquone analogs methyl-diaziquone and RH1 [2, 5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone], the latter being an agent in preclinical development. In contrast to the in vitro potentiation, there was no difference in the response to

mitomycin C when BE2 and BE vector control were treated as tumor xenografts in vivo. This isogenic model should be valuable for mechanistic studies and bioreductive drug development.

Shea MA, Sorensen BR, Pedigo S, Verhoeven AS. **Proteolytic footprinting titrations for estimating ligand-binding constants and detecting pathways of conformational switching of calmodulin.** Methods Enzymol 2000;323:254-301.
Abstract: To dissect the chemical basis for interactions controlling regulatory properties of macromolecular assemblies, it is essential to explore experimentally the linkage between ligand binding, conformational change, and subunit assembly. There are many advantages to using techniques that will probe the occupancy of individual binding sites or monitor conformational responses of individual residues, as described here. Proteolytic footprinting titrations may be used to infer binding free energies for ligands interacting with multiple sites or domains and to detect otherwise unrecognized "silent" interdomain interactions. Microgram quantities of pure protein are required, which is low relative to the hundreds of milligrams needed for comparable discontinuous equilibirum titrations monitored by NMR. By running comparative studies with several proteases, it is easy to determine whether resulting titration curves are consistent, independent of the protease used and therefore representative of the structural response of the protein to ligand binding or other differences in solution conditions (pH, salt, temperature). The results from multiple techniques (e.g., NMR, fluorescence, and footprinting) applied to aliquots from the same discontinuous titration may be compared easily to test for consistency. Classic methods for determining thermodynamic and kinetic properties of calcium binding to calmodulin include filter binding and equilibrium or flow dialysis (employing the isotope 45Ca), spectroscopic studies of stopped-flow fluorescence, calorimetry, and direct ion titrations. A cautionary note is that many different sets of microscopic data would be consistent with a single set of macroscopic constants determined by classic methods. This was well illustrated in Fig. 9. Thus, while it is important to compare results with those obtained by classic binding methods, they are, by definition, incapable of resolving the microscopic constants of interest. Thus, there is only one "direction" for comparison. Quantitative proteolytic footprinting titrations applied to studying calmodulin provided the first direct quantitative estimate of negative interactions between domains. Although studies of site-knockout mutants had suggested interactions between domains, this approach gave the first evidence for the pathway of anticooperative interactions between domains by showing that helix B responds structurally to calcium binding to sites III and IV in the C-domain. Despite two decades of study of calmodulin and the application of limited proteolysis studies to the apo and fully saturated forms, this finding emerged only when titration studies were undertaken as described. This highlights the general observation that while the behavior of the intermediate states in a cooperative switch are the key elements of the transition mechanism, they are the most difficult to observe. The unexpected finding that the isolated domains are nearly equivalent in their calcium-binding properties (Fig. 23 B) leaves us with many of the questions we had at the start: How does the sum of two nearly equivalent domains result in a molecule that switches sequentially rather than simultaneously? But it underscores why it is not yet possible to understand similar proteins by sequence gazing alone.

Spencer TA, Li D, Russel JS, Collins JL, Bledsoe RK, Consler TG, Moore LB, Galardi CM, McKee DD, Moore JT, et al. **Pharmacophore analysis of the nuclear oxysterol receptor LXRalpha.** J Med Chem 2001;44(6):886-97.

Abstract: A cell-free assay was developed for the orphan nuclear receptor LXRalpha that measures the ligand-dependent recruitment of a peptide from the steroid receptor coactivator 1 (SRC1) to the nuclear receptor. Using this ligand-sensing assay (LiSA), the structural requirements for activation of the receptor by oxysterols and related compounds were studied. The minimal pharmacophore for receptor activation was shown to be a sterol with a hydrogen bond acceptor at C24. 24(S),25-Epoxycholesterol (1), which meets this criterion, is among the most efficacious of the oxysterols and is an attractive candidate as the LXRalpha natural hormone. Cholenic acid dimethylamide (14) showed increased efficacy compared to 1, whereas the unnatural oxysterol 22(S)-hydroxycholesterol (4) was shown to be an antagonist of 1 in the LiSA. The structural requirements for SRC1 recruitment in the LiSA correlated with the transcriptional activity of compounds in a cell-based reporter assay employing LXRalpha-GAL4 chimeric receptors. Site-directed mutagenesis identified Trp(443) as an amino acid critical for activation of LXRalpha by oxysterol ligands. This information was combined with the structure-activity relationship developed from the LiSA to develop a 3D homology model of LXRalpha. This model may aid the design of synthetic drugs targeted at this transcriptional regulator of cholesterol homeostasis.

Stein RA, Wilkinson JC, Guyer CA, Staros JV. **An analytical approach to the measurement of equilibrium binding constants: application to EGF binding to EGF receptors in intact cells measured by flow cytometry.** Biochemistry 2001;40(20):6142-54.
Abstract: In ligand binding studies, ligand depletion often limits the accuracy of the results obtained. This problem is approached by employing the simple observation that as the concentration of receptor in the assay is reduced, ligand depletion is also reduced. Measuring apparent K(D)'s of a ligand at multiple concentrations of receptor with extrapolation to infinitely low receptor concentration takes ligand depletion into account and, depending on the binding model employed, yields a K(D) within the defined limits of accuracy. We apply this analysis to the binding of epidermal growth factor (EGF) to the EGF receptor expressed in intact 32D cells, using a homogeneous fluorescein-labeled preparation of EGF and measuring binding by flow cytometry. Binding isotherms were carried out at varying cell densities with each isotherm fit to the generally applied model with two independent binding sites. Examination of the variation in the K(D)'s versus cell density yields a high-affinity site that accounts for 18% of the sites and a lower affinity site that accounts for the remainder. However, further examination of these data suggests that while consistent with each individual isotherm, the simple model of two independent binding sites that is generally applied to EGF binding to the EGF receptor is inconsistent with the changes in the apparent K(D)'s seen across varying cell densities.

Steiner S, Anderson NL. **Pharmaceutical proteomics.** Ann N Y Acad Sci 2000;919:48-51.
Abstract: Genomics and proteomics are today well established in drug discovery and, in combination with combinatorial chemistry and high-throughput screening, are helping to bring forward an unprecedented number of potential lead compounds. To avoid the generation of bottlenecks downstream in drug development, increasing pressure is arising to integrate these technologies into the development environment. Proteomics has demonstrated proof-of-concept in toxicology as shown by a number of successful applications in mechanistic toxicology and lead selection. The "technology wave" is now starting to impact the clinical phase of drug development. Expected benefits are optimized clinical trials based on the availability of biologically relevant markers of drug efficacy and safety.

Strunck E, Frank K, Tan MI, Vollmer G. **Expression of l-3-phosphoserine phosphatase is regulated by reconstituted basement membrane.** Biochem Biophys Res Commun 2001;281(3):747-53.
Abstract: Reconstituted basement membrane (Matrigel) promotes differentiation of endometrial adenocarcinoma cells in vitro. However, little is known about the molecular basis of these in vitro differentiation processes. Using differential display RT-PCR to search for potential molecular markers we screened for genes which respond to contact to basement membrane by alteration of expression levels. Here we report that the cDNA MT32 represents an mRNA with a time dependent biphasic response pattern to contact to basement membrane. Characterizing MT32 revealed that the sequence of MT32 is identical to l-3-phosphoserine phosphatase. PCR analysis of l-3-phosphoserine phosphatase expression surprisingly revealed at least three variants of this enzyme. In summary, and in view of the literature, l-3-phosphoserine phosphatase and potential variants or family members represent molecular markers to study regulation of gene expression by components of the extracellular matrix. In conclusion, l-3-phosphoserine phosphatase(s) may be important in endometrial carcinogenesis since this enzyme synthesizes important metabolic intermediates which serve both as building blocks for peptide synthesis and for signal transducing molecules. Copyright 2001 Academic Press.

Sweeney LM, Himmelstein MW, Gargas ML. **Development of a preliminary physiologically based toxicokinetic (PBTK) model for 1,3-butadiene risk assessment.** Chem Biol Interact 2001;135-136:303-22.
Abstract: Potential health effects of human exposure to 1,3-butadiene (BD) are of concern due to the use of BD in industry and its low-level presence throughout the environment. Physiologically based toxicokinetic (PBTK) models of BD in rodents have been developed by multiple research groups in an effort to explain species differences in toxicity (especially carcinogenic potency) through toxicokinetics. PBTK modeling of dose metrics related to a non-cancer endpoint, ovotoxicity in experimental animals, was conducted. The cumulative area under the blood concentration vs. time curve (AUC) for the metabolite diepoxybutane (butadiene diepoxide, DEB) was found to be consistent with ovotoxicity in mice and rats exposed to BD by inhalation or epoxybutene (butadiene monoepoxide, EB) or DEB by intraperitoneal injection. This suggests that cumulative DEB AUC may also be an appropriate metric for possible human risk. A preliminary human PBTK model was assembled for the eventual assessment of reproductive risk to humans and for prioritizing the determination of model parameters. The preliminary model accurately predicted published data on exhaled breath BD concentrations in a human volunteer exposed to BD by inhalation. The fit was relatively insensitive to the rate constant for BD epoxidation. Sensitivity analyses were conducted on this human PBTK model. Using a range of published rate constants, human blood DEB was found to be sensitive to rates of epoxidation of EB to DEB and hydrolysis of EB and DEB, but not BD epoxidation. Because of the large ranges of rates measured in vitro for these reactions, different combinations of in-vitro rates produce varying predictions of blood DEB concentration. Thus, validation of a human PBTK model with human biomonitoring data will be essential to produce a PBTK model that can be applied to risk assessment.

Sweeney LM, Tyler TR, Kirman CR, Corley RA, Reitz RH, Paustenbach DJ, Holson JF, Whorton MD, Thompson KM, Gargas ML. **Proposed occupational exposure limits for select ethylene glycol ethers using PBPK models and Monte Carlo simulations.** Toxicol Sci 2001;62(1):124-39.
Abstract: Methoxyethanol (ethylene glycol monomethyl ether, EGME), ethoxyethanol (ethylene glycol

monoethyl ether, EGEE), and ethoxyethyl acetate (ethylene glycol monoethyl ether acetate, EGEEA) are all developmental toxicants in laboratory animals. Due to the imprecise nature of the exposure data in epidemiology studies of these chemicals, we relied on human and animal pharmacokinetic data, as well as animal toxicity data, to derive 3 occupational exposure limits (OELs). Physiologically based pharmacokinetic (PBPK) models for EGME, EGEE, and EGEEA in pregnant rats and humans have been developed (M. L. Gargas et al., 2000, Toxicol. Appl. Pharmacol. 165, 53-62; M. L. Gargas et al., 2000, Toxicol. Appl. Pharmacol. 165, 63-73). These models were used to calculate estimated human-equivalent no adverse effect levels (NAELs), based upon internal concentrations in rats exposed to no observed effect levels (NOELs) for developmental toxicity. Estimated NAEL values of 25 ppm for EGEEA and EGEE and 12 ppm for EGME were derived using average values for physiological, thermodynamic, and metabolic parameters in the PBPK model. The uncertainties in the point estimates for the NOELs and NAELs were estimated from the distribution of internal dose estimates obtained by varying key parameter values over expected ranges and probability distributions. Key parameters were identified through sensitivity analysis. Distributions of the values of these parameters were sampled using Monte Carlo techniques and appropriate dose metrics calculated for 1600 parameter sets. The 95th percentile values were used to calculate interindividual pharmacokinetic uncertainty factors (UFs) to account for variability among humans (UF(h,pk)). These values of 1.8 for EGEEA/EGEE and 1.7 for EGME are less than the default value of 3 for this area of uncertainty. The estimated human equivalent NAELs were divided by UF(h,pk) and the default UFs for pharmacodynamic variability among animals and among humans to calculate the proposed OELs. This methodology indicates that OELs (8-h time-weighted average) that should protect workers from the most sensitive adverse effects of these chemicals are 2 ppm EGEEA and EGEE (11 mg/m(3) EGEEA, 7 mg/m(3) EGEE) and 0.9 ppm (3 mg/m(3)) EGME. These recommendations assume that dermal exposure will be minimal or nonexistent.

Teeguarden JG, Dragan Y, Pitot HC. **Hazard assessment of chemical carcinogens: the impact of hormesis.** J Appl Toxicol 2000;20(2):113-20.
Abstract: The recent report of reductions in the number and area of preneoplastic hepatic lesions in response to low doses of the tumor promoter phenobarbital provides important new support for the existence of hormetic responses to carcinogens. The presence of hormetic responses to carcinogenic agents and the corollary that beneficial doses of these compounds can be determined have several implications for the bioassay and hazard assessment of carcinogens as well as for public policy regulating exposure to these agents. To be adequately sensitive to detect and quantify hormetic or other non-linear dose-response functions, current study designs must be modified to include lower doses and sufficiently large numbers of animals. Short- or medium-term animal studies are a cost-effective means of addressing these needs and have been used recently to describe a classical hormetic response to the non-genotoxic carcinogen phenobarbital. These basic changes should be supported by a continuing emphasis on mechanistic research and the development of biologically based quantitative models of toxicant action. Linking these models with physiologically based pharmacokinetic model descriptions of target dose holds the greatest promise for improving the description of the dose-response curve at low doses. These approaches are generally encouraged by the USEPA in the form of The 1996 Proposed Carcinogen Risk Assessment Guidelines. However, there remain substantial questions regarding integration of the concept of hormesis into hazard testing and public policy that require careful consideration. Herein, we explore the issues that surround testing for hormetic responses and the

implications for public policy.

Thrall KD, Callahan PJ, Weitz KK, Edwards JA, Brinkman MC, Kenny DV. **Design and evaluation of a breath-analysis system for biological monitoring of volatile compound.** AIHAJ 2001;62(1):28-35.
Abstract: To ensure the health and safety of workers, integrated industrial hygiene methodologies often include biological monitoring of the workers to help understand their exposure to chemicals. To this end, a field-portable breath-analysis system was developed and tested to measure selected solvents in exhaled air. The exhaled breath data were evaluated using a physiologically based pharmacokinetic (PBPK) model to relate exposure to tissue dose. The system was designed to monitor workers every time they entered or left a work environment--a vast improvement over current 8-hour integrated monitoring strategies. The system combines (1) chemical dosimeters to measure airborne contaminant levels (analyzed in the field/ workplace); (2) real-time breath analysis to quantitate exposure; and 3) PBPK models to estimate internal target tissue dose. To evaluate the system, field tests were conducted at two locations: (1) at an incinerator in Tennessee monitoring benzene and toluene exposures; and (2) a waste repackaging facility in Washington State where hexane, trimethylbenzene, and methylene chloride was monitored. Exhaled breath was sampled and analyzed before and after each specific job task, which ranged from 15 min to 8 hours in duration. In both field studies several volunteers had posttask breath levels higher than pretask levels. The greatest increase corresponded to 573 ppb for methylene chloride and 60 ppb for toluene. Compared with breath analysis, the chemical dosimeters underpredicted the dosimetry, particularly for longer sampling intervals when the volume of air sampled may have diluted exposures. The results of the field studies illustrate the utility of monitoring workers for exposures throughout the day, particularly when job-specific tasks may indicate a potential for exposure.

Thrall KD, Poet TS. **Determination of biokinetic interactions in chemical mixtures using real-time breath analysis and physiologically based pharmacokinetic modeling.** J Toxicol Environ Health A 2000;59(8):653-70.
Abstract: Regulatory agencies are challenged to conduct risk assessments on chemical mixtures without full information on toxicological interactions that may occur at real-world, low-dose exposure levels. The present study was undertaken to investigate the pharmacokinetic impact of low-dose coexposures to toluene and trichloroethylene in vivo in male F344 rats using a real-time breath analysis system coupled with physiologically based pharmacokinetic (PBPK) modeling. Rats were exposed to compounds alone or as a binary mixture, at low (5 to 25 mg/kg) or high (240 to 800 mg/kg) dose levels. Exhaled breath from the exposed animals was monitored for the parent compounds and a PBPK model was used to analyze the data. At low doses, exhaled breath kinetics from the binary mixture exposure compared with those obtained during single exposures, thus indicating that no metabolic interaction occurred with these low doses. In contract, at higher doses the binary PBPK model simulating independent metabolism was found to underpredict the exhaled breath concentration, suggesting an inhibition of metabolism. Therefore the binary mixture PBPK model was used to compare the measured exhaled breath levels from high- and low-dose exposures with the predicted levels under various metabolic interaction simulations (competitive, noncompetitive, or uncompetitive inhibition). Of these simulations, the optimized competitive metabolic interaction description yielded a Ki value closest to the Km of the inhibitor solvent, indicating that competitive inhibition is the most plausible type of metabolic interaction between these two solvents.

Thrall KD, Poet TS, Corley RA, Tanojo H, Edwards JA, Weitz KK, Hui X, Maibach HI, Wester RC. **A real-time in-vivo method for studying the percutaneous absorption of volatile chemicals.** Int J Occup Environ Health 2000;6(2):96-103.
Abstract: Realistic estimates of percutaneous absorption following exposures to solvents in the workplace, or through contaminated soil and water, are critical to understanding human health risks. A method was developed to determine dermal uptake of solvents under non-steady-state conditions using real-time breath analysis in rats, monkeys, and humans. The exhaled breath was analyzed using an ion-trap mass spectrometer, which can quantitate chemicals in the exhaled breath stream in the 1-5 ppb range. The resulting data were evaluated using physiologically-based pharmacokinetic (PBPK) models to estimate dermal permeability constants (Kp) under various exposure conditions. The effects of exposure matrix (soil versus water), occlusion versus non-occlusion, and species differences on the absorption of methyl chloroform, trichloroethylene, and benzene were compared. Exposure concentrations were analyzed before and at 0.5-hour intervals throughout the exposures. The percentage of each chemical absorbed and the corresponding Kp were estimated by optimization of the PBPK model to the medium concentration and the exhaled-breath data. The method was found to be sufficiently sensitive for animal and human dermal studies at low exposure concentrations over small body surface areas, for short periods, using non-steady-state exposure conditions.

Thrall KD, Vucelick ME, Gies RA, Zangar RC, Weitz KK, Poet TS, Springer DL, Grant DM, Benson JM. **Comparative metabolism of carbon tetrachloride in rats, mice, and hamsters using gas uptake and PBPK modeling**. J Toxicol Environ Health A 2000;60(8):531-48.
Abstract: No study has comprehensively compared the rate of metabolism of carbon tetrachloride (CCl4) across species. Therefore, the in vivo metabolism of CCl4 was evaluated using groups of male animals (F344 rats, B6C3F1 mice, and Syrian hamsters) exposed to 40-1800 ppm CCl4 in a closed, recirculating gas-uptake system. For each species, an optimal fit of the family of uptake curves was obtained by adjusting Michaelis-Menten metabolic constants Km (affinity) and Vmax (capacity) using a physiologically based pharmacokinetic (PBPK) model. The results show that the mouse has a slightly higher capacity and lower affinity for metabolizing CCl4 compared to the rat, while the hamster has a higher capacity and lower affinity than either rat or mouse. A comparison of the Vmax to Km ratio, normalized for milligrams of liver protein (L/h/mg) across species, indicates that hamsters metabolize more CCl4 than either rats or mice, and should be more susceptible to CCl4-induced hepatotoxicity. These species comparisons were evaluated against toxicokinetic studies conducted in animals exposed by nose-only inhalation to 20 ppm 14C-labeled CCl4 for 4 h. The toxicokinetic study results are consistent with the in vivo rates of metabolism, with rats eliminating less radioactivity associated with metabolism (14CO2 and urine/feces) and more radioactivity associated with the parent compound (radioactivity trapped on charcoal) compared to either hamsters or mice. The in vivo metabolic constants determined here, together with in vitro constants determined using rat, mouse, hamster, and human liver microsomes, were used to estimate human in vivo metabolic rates of 1.49 mg/h/kg body weight and 0.25 mg/L for Vmax and Km, respectively. Normalizing the rate of metabolism (Vmax/Km) by milligrams liver protein, the rate of metabolism of CCl4 differs across species, with hamster > mouse > rat > human.

Tiffert T, Daw N, Perdomo D, Lew VL. **A fast and simple screening test to search for specific**

inhibitors of the plasma membrane calcium pump. J Lab Clin Med 2001;137(3):199-207.
Abstract: No specific inhibitors of the plasma membrane Ca(2+) pump have been found to date, limiting research on the particular contribution of this pump to the Ca(2+) homeostasis of animal cells. The search for Ca(2+) pump inhibitors may have been hampered by the lack of an efficient screening method to measure pump activity that would provide an alternative to the lengthy and costly adenosine triphosphatase or Ca(2+)-flux measurements. We propose here a novel screening method in which Ca(2+) pump inhibition is translated into easily measurable cell dehydration. Intact human red cells, suspended in Ca(2+)-containing, low-K(+) buffers were exposed to sequential additions of (1) ionophore A23187 (t = 0) to load the cells with Ca(2+); (2) CoCl(2) (t = 1 minute) to block ionophore-mediated Ca (2+) transport and to allow complete extrusion of the Ca(2+) load by the pump in less than 5 minutes; and (3) NaSCN (t = 6 minutes) to accelerate cell dehydration via Ca(2+)-sensitive K(+) channels when the Ca(2+) load is retained as a result of Ca(2+) pump inhibition. Samples were taken at 10 to 25 minutes after ionophore addition and delivered into hypotonic media containing about 45 mmol/L NaCl. Non-dehydrated cells-with normal, uninhibited pumps-instantly underwent lysis, whereas dehydrated cells-with inhibited pumps-resisted lysis, resulting in translucent or opaque samples, respectively, which were quantifiable by light-absorption measurements. Vanadate was used as a test substance to assess the effect of putative pump inhibitors. This method offers a cost-efficient and easily automated alternative for testing large numbers of natural or synthetic agents.

Tsuzuki T, Egashira A, Igarashi H, Iwakuma T, Nakatsuru Y, Tominaga Y, Kawate H, Nakao K, Nakamura K, Ide F, et al. **Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase.** Proc Natl Acad Sci U S A 2001;98(20):11456-61.
Abstract: Oxygen radicals, which can be produced through normal cellular metabolism, are thought to play an important role in mutagenesis and tumorigenesis. Among various classes of oxidative DNA damage, 8-oxo-7,8-dihydroguanine (8-oxoG) is most important because of its abundance and mutagenicity. The MTH1 gene encodes an enzyme that hydrolyzes 8-oxo-dGTP to monophosphate in the nucleotide pool, thereby preventing occurrence of transversion mutations. By means of gene targeting, we have established MTH1 gene-knockout cell lines and mice. When examined 18 months after birth, a greater number of tumors were formed in the lungs, livers, and stomachs of MTH1-deficient mice, as compared with wild-type mice. The MTH1-deficient mouse will provide a useful model for investigating the role of the MTH1 protein in normal conditions and under oxidative stress.

van Birgelen AP, van den Berg M. **Toxicokinetics.** Food Addit Contam 2000;17(4):267-73.
Abstract: The toxicokinetic determinants of dioxin and related chemicals depend on three major properties: lipophilicity, metabolism, and binding to CYP1A2 in the liver. The induction of CYP1A2 is partially under the control of the aryl hydrocarbon receptor (AhR). Lipophilicity increases with more chlorination and controls absorption and tissue partitioning. Metabolism is the rate-limiting step for elimination. Induction of CYP1A2 leads to hepatic sequestration of TCDD. Binding to this inducible hepatic protein results in non-linear dose-dependent tissue distribution: with increasing doses, the relative concentration in extra-hepatic tissues decreases while that in liver increases. The induction of this protein occurs in both animals and humans and results in an increase in the liver to fat ratio of these compounds. Humans have similar sensitivities to rodents for dioxin-like compounds when using tissue concentration (from in vitro studies), body burden, average lifetime serum lipid concentration, or

lifetime area-under-the-curve concentration based on both low dose (biochemical) and high dose (cancer) driven endpoints. To reach the same tissue concentration in humans as rodents however, humans need a lower daily intake than rodents based on differences in pharmacokinetic behaviour. This clearly indicates that physiologically based pharmacokinetic models should be explored for the estimation of the daily intake of dioxin-like compounds in humans based on tissue dose levels or derivatives of those.

Van Ostade X, Schauvliege L, Pattyn E, Verhee A, Vandekerckhove J, Tavernier J. **A sensitive and versatile bioassay for ligands that signal through receptor clustering.** J Interferon Cytokine Res 2000;20(1):79-87.
Abstract: The induced expression of xanthine-guanine phosphoribosyl transferase (XGPRT) by low concentrations (-2 pg/ml) of interferon-alpha (IFN-alpha) or IFN-beta, in the 2fTPGH cell line caused a 50% cytotoxicity when these cells were grown in medium containing 6-thioguanine. We extended the application of this sensitive, reliable, and easy bioassay to other members of the cytokine family. To activate the IFN signaling pathway, we made receptor chimeras, consisting of the IFN type I receptor intracellular and transmembrane domains, fused to either the interleukin-5 (IL-5) receptors or erythropoietin (Epo) receptor extracellular domains as model systems. 2fTGH cells, stably transfected with these receptor chimeras, responded to very low concentrations of IL-5 or Epo (IC50 values of approximately 15 pg and 3 pg/ml, respectively) and thus can be used as a very sensitive bioassay for both ligands. Background activity of IL-5, Epo, tumor necrosis factor (TNF), IL-6, or leptin on cells that did not carry the receptor chimeras was very low. This methodology can in principle be extended to any ligand that acts via clustering of its receptors.

Verkman AS, Mitra AK. **Structure and function of aquaporin water channels.** Am J Physiol Renal Physiol 2000;278(1):F13-28.
Abstract: The aquaporins (AQPs) are a family of small membrane-spanning proteins (monomer size approximately 30 kDa) that are expressed at plasma membranes in many cells types involved in fluid transport. This review is focused on the molecular structure and function of mammalian aquaporins. Basic features of aquaporin structure have been defined using mutagenesis, epitope tagging, and spectroscopic and freeze-fracture electron microscopy methods. Aquaporins appear to assemble in membranes as homotetramers in which each monomer, consisting of six membrane-spanning alpha-helical domains with cytoplasmically oriented amino and carboxy termini, contains a distinct water pore. Medium-resolution structural analysis by electron cryocrystallography indicated that the six tilted helical segments form a barrel surrounding a central pore-like region that contains additional protein density. Several of the mammalian aquaporins (e.g., AQP1, AQP2, AQP4, and AQP5) appear to be highly selective for the passage of water, whereas others (recently termed aquaglyceroporins) also transport glycerol (e.g., AQP3 and AQP8) and even larger solutes (AQP9). Evidence for possible movement of ions and carbon dioxide through the aquaporins is reviewed here, as well as evidence for direct regulation of aquaporin function by posttranslational modification such as phosphorylation. Important unresolved issues include definition of the molecular pathway through which water and solutes move, the nature of monomer-monomer interactions, and the physiological significance of aquaporin-mediated solute movement. Recent results from knockout mice implicating multiple physiological roles of aquaporins suggest that the aquaporins may be suitable targets for drug discovery by structure-based and/

or high-throughput screening strategies.

Villeneuve DL, Khim JS, Kannan K, Giesy JP. **In vitro response of fish and mammalian cells to complex mixtures of polychlorinated naphthalenes, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons.** Aquat Toxicol 2001;54(1-2):125-41.
Abstract: In vitro characterization and comparison of responses to different classes of biologically active compounds can increase the utility of bioassays. In this study, the relative potencies (REPs) of mixtures of polychlorinated naphthalenes (PCNs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs), to induce in vitro ethoxyresorufin-O-deethylase (EROD) in PLHC-1 fish hepatoma cells, H4IIE wild type (H4IIE-wt) rat hepatoma cells, and recombinant H4IIE cells (H4IIE-EROD) were determined. The mixtures were also analyzed by in vitro luciferase assay with recombinant H4IIE cells (H4IIE-luc). Halowaxes 1051, 1014, and 1013 caused significant induction in all three H4IIE assays at concentrations less than 10 mg/l, but did not elicit a significant response in the PLHC-1 assay. Based on H4IIE results, the Halowaxes were estimated to have relative potencies (REPs) of approximately 10(-6)-10(-8) relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Less than 5 mg/l of Aroclors 1242, 1248, 1254; Clophens A60, T64; and Chlorofen induced significant responses in the H4IIE assays, while only Clophens A60 and T64 caused a significant response in the PLHC-1 assay. The efficacy of the Aroclor mixtures was generally insufficient to allow for quantitative REP estimates, but, based on their responses in the H4IIE assays, Clophen A60 and Chlorofen were estimated to have REPs of approximately 10(-6) and 10(-7), respectively. A mixture of 16 priority PAHs caused significant induction in all four cell types and was estimated to have a REP of approximately 10(-4). Overall, the results of this study add to a growing database on the dioxin-like potency of complex mixtures of xenobiotics, and suggested that H4IIE-based in vitro bioassays were more sensitive than PLHC-1 cells for detecting dioxin-like activity in complex mixtures.

Vinegar A, Jepson GW, Cisneros M, Rubenstein R, Brock WJ. **Setting safe acute exposure limits for halon replacement chemicals using physiologically based pharmacokinetic modeling.** Inhal Toxicol 2000;12(8):751-63.
Abstract: Most proposed replacements for Halon 1301 as a fire suppressant are halogenated hydrocarbons. The acute toxic endpoint of concern for these agents is cardiac sensitization. An approach is described that links the cardiac endpoint as assessed in dogs to a target arterial concentration in humans. Linkage was made using a physiologically based pharmacokinetic (PBPK) model. Monte Carlo simulations, which account for population variability, were used to establish safe exposure times at different exposure concentrations for Halon 1301 (bromotrifluoromethane), CF(3)I (trifluoroiodomethane), HFC-125 (pentafluoroethane), HFC-227ea (1,1,1,2,3,3,3-heptafluoropropane), and HFC-236fa (1,1,1,3,3,3-hexafluoropropane). Application of the modeling technique described here not only makes use of the conservative cardiac sensitization endpoint, but also uses an understanding of the pharmacokinetics of the chemical agents to better establish standards for safe exposure. The combined application of cardiac sensitization data and physiologically based modeling provides a quantitative approach, which can facilitate the selection and effective use of halon replacement candidates.

Wade M, Allday MJ. **Epstein-Barr virus suppresses a G(2)/M checkpoint activated by genotoxins.**

Mol Cell Biol 2000;20(4):1344-60.
Abstract: Several Epstein-Barr virus (EBV)-negative Burkitt lymphoma-derived cell lines (for example, BL41 and Ramos) are extremely sensitive to genotoxic drugs despite being functionally null for the tumor suppressor p53. They rapidly undergo apoptosis, largely from G(2)/M of the cell cycle. 5-bromo-2'-deoxyuridine labeling experiments showed that although the treated cells can pass through S phase, they are unable to complete cell division, suggesting that a G(2)/M checkpoint is activated. Surprisingly, latent infection of these genotoxin-sensitive cells with EBV protects them from both apoptosis and cell cycle arrest, allowing them to complete the division cycle. However, a comparison with EBV-immortalized B-lymphoblastoid cell lines (which have functional p53) showed that EBV does not block apoptosis per se but rather abrogates the activation of, or signalling from, the checkpoint in G(2)/M. Furthermore, analyses of BL41 and Ramos cells latently infected with P3HR1 mutant virus, which expresses only a subset of the latent viral genes, showed that LMP-1, the main antiapoptotic latent protein encoded by EBV, is not involved in the protection afforded here by viral infection. This conclusion was confirmed by analysis of clones of BL41 stably expressing LMP-1 from a transfected plasmid, which respond like the parental cell line. Although steady-state levels of Bcl-2 and related proteins varied between BL41 lines and clones, they did not change significantly during apoptosis, nor was the level of any of these anti- or proapoptotic proteins predictive of the outcome of treatment. We have demonstrated that a subset of EBV latent gene products can inactivate a cell cycle checkpoint for monitoring the fidelity and timing of cell division and therefore genomic integrity. This is likely to be important in EBV-associated growth transformation of B cells and perhaps tumorigenesis. Furthermore, this study suggests that EBV will be a unique tool for investigating the intimate relationship between cell cycle regulation and apoptosis.

Walker NJ. **Real-time and quantitative PCR: applications to mechanism-based toxicology.** J Biochem Mol Toxicol 2001;15(3):121-7.
Abstract: There is increasing awareness that quantitative analysis of changes in molecular targets plays a key role in addressing scientific questions in molecular toxicology, molecular epidemiology, and human risk assessment. One of the emerging technologies that is being used to analyze these molecular targets is real-time and quantitative (RTAQ) polymerase chain reaction (PCR). The aim of this review is to provide the reader with an overview of this technology and to highlight specific applications of this technology to some key areas of molecular toxicology.

Wang J, Chen W, Tsai S, Sung P, Huang R. **An in vitro model for evaluation of vaporous toxicity of trichloroethylene and tetrachloroethylene to CHO-K1 cells.** Chem Biol Interact 2001;137(2):139-54.
Abstract: Toxicokinetics of trichloroethylene (TCE) and tetrachloroethylene (PER) in culture medium and their toxicity to CHO-K1 cells were investigated by employing an in vitro vapor exposure system. Cells were cultured in a 60 mm petri dish with a 25 mm glass dish glued in the central area. TCE or PER was added to the central glass dish so that it would evaporate and dissolve in the surrounding medium in which cells were growing. The results showed that the concentration of TCE or PER in medium increased significantly within 20 min and then decreased very rapidly with time. After a 24 h incubation, the residual of TCE or PER in the medium was very low, but was displayed in a dose-dependent manner. Treatment of cells with either TCE or PER resulted in a dose- and time-dependent inhibition of cell growth. A significantly increase in the frequency of micronuclei (MN) was also observed with either

TCE or PER treatment. Low doses of TCE (5-20 &mgr;l) or PER (1-5 &mgr;l) significantly enhanced the intracellular glutathione (GSH) level. However, the level of GSH rapidly decreased with higher doses of TCE (40-80 &mgr;l) or PER (10-20 &mgr;l). Depletion of cellular GSH showed no effect on the sensitivity of cells to TCE or PER treatment. GSH-conjugation has been proposed as an activation mechanism to account for the nephrotoxicity of TCE and PER, however the toxicity of TCE and PER to CHO-K1 cells is probably mediated through a distinct mechanism.

Wang X, Santostefano MJ, DeVito MJ, Birnbaum LS. **Extrapolation of a PBPK model for dioxins across dosage regimen, gender, strain, and species.** Toxicol Sci 2000;56(1):49-60.
Abstract: A physiologically based pharmacodynamic (PBPK) model for 2,3,7, 8-tetrachlorodibenzo-p-dioxin (TCDD) was developed based on pharmacokinetic data from acute oral exposures of TCDD to female Sprague-Dawley rats (Wang et al., 1997, Toxicol Appl. Pharmacol 147, 151-168). In the present study, the utility of this model to predict the disposition of TCDD in male and female Sprague-Dawley and female Wistar rats exposed to TCDD through different dosage regimens was examined. The ability of the model to predict the disposition of 2-iodo-3,7,8-trichlorodibenzo-p-dioxin (ITrCDD) in mice (Leung, et al., 1990, Toxicol. Appl. Pharmacol. 103, 399-410) was also examined. The ability of the model to predict across routes of exposure was assessed with intravenous injection data (5.6 microg/kg bw) (Li et al., 1995, Fundam. Appl. Toxicol. 27, 70-76) in female rats. Analysis across gender extrapolations used data for male Sprague-Dawley rats exposed intravenously to 9.25 microg TCDD/kg bw (Weber et al., 1993, Fundam. Appl. Toxicol. 21, 523-534). The analysis of across-dosage regimen and stains of rats extrapolations were assessed using data from rats exposed to TCDD through a loading/ maintenance dosage regimen (Krowke et al., 1989, Arch. Toxicol. 63, 356-360). The physiological differences between gender, strain, and species were taken into account when fitting the PBPK model to these data sets. The results demonstrate that the PBPK model for TCDD developed for female Sprague-Dawley rats exposed by acute oral dosing accurately predicts the disposition of TCDD, for different gender and strain of rats across varying dosage regimens, as well as in a strain of mice. Minimal changes in fitted parameters were required to provide accurate predictions of these data sets. This study provides further confirmation of the potential use of physiological modeling in understanding pharmacokinetics and pharmacodynamics.

Wardman P. **Electron transfer and oxidative stress as key factors in the design of drugs selectively active in hypoxia.** Curr Med Chem 2001;8(7):739-61.
Abstract: Hypoxia is a feature of some regions of many tumours, ischaemic events, and arthritis. Drugs activated in hypoxia have wide potential application, particularly in overcoming the resistance of hypoxic tumour cells to radiotherapy. Key features of such drugs include redox properties appropriate for activation by reductase enzymes (typically flavoproteins), and oxygen-sensitive reduction chemistry such that normal levels of oxygen inhibit or reverse reduction. In many cases this selectivity is achieved by a fast, free-radical reaction in which the drug radical (often an obligate intermediate in drug reduction) reduces oxygen to form superoxide radicals and thus 'futile cycles' the drug in normoxic tissues. However, this enhances cellular oxidative stress, which may be linked to normal tissue toxicity. Appropriate redox properties are found with nitroarene, quinone, or aromatic N-oxide moieties. A particularly promising and versatile exploitation of bioreductive activation is for reduction of such 'triggers' to activate release of an 'effector', an agent that can obviously be active against diverse

conditions associated with hypoxia. The same approach can also be used in diagnosis of hypoxia. Much information concerning the reactions of intermediates in drug action and the quantitative prediction of redox properties of analogues has been accrued. Drug design can be mechanism-led, with the wealth of literature quantifying redox properties of drug candidates a rich source of potential new leads. There is a clear appreciation of the kinetic factors that limit drug efficacy or selectivity. Thus the potential for rapid expansion of these concepts to diverse diseases is considerable.

Wen YH, Chan HM. **A pharmacokinetic model for predicting absorption, elimination, and tissue burden of toxaphene in rats.** Toxicol Appl Pharmacol 2000;168(3):235-43.
Abstract: A two-compartment pharmacokinetic model was formulated to predict absorption, elimination, and tissue burden of toxaphene in rats. The model was constructed based on the database of Crowder and Dindal (Bull. Environ. Contam. Toxicol. 12, 320-327, 1974) and included six tissue compartments: blood, brain, liver, muscle, fat, and carcass. The pharmacokinetically based dosimetry indicated that absorption of toxaphene was fast in fat, whole body, carcass, and blood, relatively slow in liver and muscle, and slow in brain. In contrast, the elimination rate was rapid in whole body, muscle, and blood, moderate in carcass and brain, and slow in liver and fat. Tissue burden was highest in fat, whole body, and blood, intermediate in liver, and lowest in brain. The model performance was evaluated by the data set of Pollock and Hillstrand (J. Environ. Sci. Health B 17, 635-648, 1982) on toxaphene absorption and elimination in pregnant rats. Validity of the model was confirmed by the close agreement between the predicted and observed tissue burdens of toxaphene in target tissues. Disposition of toxaphene via feces was a dominant excretory pathway while urinary excretion was a minor elimination route in male rats. However, for pregnant rats, excretion of toxaphene both in urine and feces were of similar magnitude. These characteristics of elimination are valuable for understanding the metabolism of toxaphene in pregnant rats. The model serves as a starting point for a quantitative, mechanism-based understanding of the processes that influence the pharmacokinetics of toxaphene in mammalian systems.

Westerhoff HV, Riethorst A, Jongsma AP. **Relating multidrug resistance phenotypes to the kinetic properties of their drug-efflux pumps.** Eur J Biochem 2000;267(17):5355-68.
Abstract: The simplest model for pump-mediated multidrug resistance is elaborated quantitatively. The way in which toxicity data should be evaluated to characterize most effectively the drug-efflux pump is then examined. The isotoxic drug dose (D10) depends on too many unrelated properties. The D10 of a cell line taken relative to that of the parental (nonresistant) cell line has been called the relative resistance (RR). This is inappropriate for characterizing the drug pump, as it depends on the extent of amplification of the latter. The reduced RR (RRR) is newly defined as the ratio of the (RR - 1) for one drug to the (RR - 1) for a different drug. This RRR should be independent of both the drug-target affinity and the extent of amplification of the drug pump in cell lines belonging to a family. The RRR depends on the avidities with which the pump extrudes the drugs relative to the passive membrane permeabilities of the latter. In plots of RRR for one drug combination vs. that for a second drug combination, cell lines that have the same pump amplified should cluster, whereas those with amplification of (functionally) different drug-efflux pumps should segregate. Both a set of new experimental data and literature results are discussed in terms of RRR. RRRs discriminate between human MDR1 and mouse mdr1a and mdr1b, between hamster pgp1 and a mutant thereof, as well as between human MDR1 and a mutant thereof. RRRs are not affected by changes in membrane surface

area. Our results indicate that RRR may be used to (a) characterize drug-resistance mechanisms and (b) determine which drug-resistance mechanism is operative. Moreover, our analysis suggests that some of the reported phenotypic diversity among multidrug-resistant cell lines may not be due to diversity in the resistance mechanism.

Wiegand C, Pflugmacher S, Giese M, Frank H, Steinberg C. **Uptake, toxicity, and effects on detoxication enzymes of atrazine and trifluoroacetate in embryos of zebrafish**. Ecotoxicol Environ Saf 2000;45(2):122-31.
Abstract: The uptake, toxicity, and elimination of atrazine and trifluoroacetate (TFA) were studied in early life stages of the zebrafish (Danio rerio). Furthermore, the effects of these xenobiotics on soluble (s) and microsomal (m) glutathione S-transferases (GST) of zebrafish embryos were investigated using 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and [(14)C]atrazine. [(14)C] Atrazine was taken up by the embryos within seconds, unhindered by the chorions. It accumulated in the embryos by a factor of 19 after 24 h of exposure time. LC(50) (48 h) was determined at 36.8 mg/L. At a level of 5 mg/L atrazine, activities of s and m GSTs were elevated in most stages, especially in prim 6 and long pec stage (24, 48 h after fertilization, respectively). GST activity toward atrazine was detectable only in untreated D. rerio eggs, increasing with developmental time. [(14)C]Atrazine was eliminated from the embryos between 24 and 48 h, indicating a possible metabolism to a more hydrophilic GSH conjugate. [(14)C]TFA was taken up by embryos, reaching at maximum fivefold the concentration of the incubation medium after 10 h. The chorions served no physiological protection. TFA (1 g/L) caused low elevation of the GST activity. No acute toxic effects (48 h) were observed up to 4 g/L TFA.

Wilkinson CF, Christoph GR, Julien E, Kelley JM, Kronenberg J, McCarthy J, Reiss R. **Assessing the risks of exposures to multiple chemicals with a common mechanism of toxicity: how to cumulate?** Regul Toxicol Pharmacol 2000;31(1):30-43.
Abstract: The Food Quality Protection Act (FQPA) of 1996 requires the U.S. EPA to consider the "cumulative effects" of pesticides and other substances that have a "common mechanism of toxicity." Several different methods for combining the exposures to estimate the risk of groups of common mechanism chemicals with different potencies and exposure characteristics are critically evaluated. These are the hazard index (HI), toxicity equivalence factor (TEF), and combined margin of exposure (MOE(T)) procedures as well as the point of departure index (PODI) and cumulative risk index (CRI) methods that are the reciprocals of the HI and MOE(T) approaches, respectively. Each of these methods ideally requires, at a minimum, the availability of in vivo toxicology data for the same toxicological endpoint in the same animal species. Furthermore, all assume that the effects of the individual components in the mixture are independent in nature (i.e., are additive rather than synergistic or antagonistic) and that the dose-response functions for all compounds have a similar slope. The point of departure (POD), preferably the dose corresponding to a given effect level (e.g., the ED(10)), can be used as a measure of the relative potency of the different chemicals in the group. If appropriate exposure and toxicology data are available, and the chemicals in the group have a common uncertainty factor (UF), all the procedures yield a numerically identical result. The fact that different chemicals in the group often have different UFs raises issues for all summation procedures and, in the case of the TEF approach, the UF of the index chemical selected dictates the final result of the assessment. A major

distinction between the different methods for addition is the point in the process at which uncertainty is considered. The HI and CRI approaches are problematic because they require application of policy-driven UFs (in the form of RfDs) at that stage of the process where exposure should be expressed in terms of potency. In contrast, the PODI and MOE(T) approaches require application of a single group UF (G) at the end of the risk assessment process although they will also accommodate the application of data-based adjustments earlier in the analysis. Importantly, both the PODI and the MOE(T) approaches allow policy- and data-driven UFs to be separated and thus make the process more transparent; these should be considered the methods of choice for cumulative risk assessment. Assignment of a single group UF is somewhat different from developing an UF for a single chemical and the total weight of evidence available in the group database can be used to advantage to reduce the UFs that need to be applied to the group. This larger database can also be used to refine the PODs for individual members of the group. It is important to emphasize that there remains a great deal of scientific uncertainty about how to proceed with cumulative risk assessment as described in the FQPA. The serious difficulties associated with defining "common mechanism of toxicity" and "concurrent exposure" combined with the current paucity of data and methodology required to conduct cumulative risk assessment suggest that the procedure is not yet ready for use in pesticide regulation.

Willems BA, Melnick RL, Kohn MC, Portier CJ. **A physiologically based pharmacokinetic model for inhalation and intravenous administration of naphthalene in rats and mice.** Toxicol Appl Pharmacol 2001;176(2):81-91.
Abstract: A diffusion limited physiologically based pharmacokinetic model for rats and mice was developed to characterize the absorption, distribution, metabolism, and elimination of naphthalene after inhalation exposure. This model includes compartments for arterial and venous blood, lung, liver, kidney, fat, and other organs. Primary sites for naphthalene metabolism to naphthalene oxide are the lung and the liver. The data used to create this model were generated from National Toxicology Program inhalation and iv studies on naphthalene and consisted of blood time-course data of the parent compound in both rats and mice. To examine the basis for possible interspecies differences in response to naphthalene, the model was extended to describe the distribution and metabolism of naphthalene oxide and the depletion and resynthesis of glutathione. After testing several alternative models, the one presented in this paper shows the best fit to the data with the fewest assumptions possible. The model indicates that tissue dosimetry of the parent compound alone does not explain why this chemical was carcinogenic to the female mouse lung but not to the rat lung. The species difference may be due to a combination of higher levels of naphthalene oxide in the mouse lung and a greater susceptibility of the mouse lung to epoxide-induced carcinogenesis. However, conclusions regarding which metabolite(s) may be responsible for the lung toxicity could not be reached.

Wrisch A, Grissmer S. **Structural differences of bacterial and mammalian K+ channels.** J Biol Chem 2000;275(50):39345-53.
Abstract: Using a peptide toxin, kaliotoxin (KTX), we gained new insight into the topology of the pore region of a voltage-gated potassium channel, mKv1.1. In order to find new interactions between mKv1.1 and KTX, we investigated the pH dependence of KTX block which was stronger at pH(o) 6.2 compared with pH(o) 7.4. Using site-directed mutagenesis on the channel and the toxin, we found that protonation of His(34) in KTX caused the pH(o) dependence of KTX block. Glu(350) and Glu(353) in mKv1.1,

which interact with His(34) in KTX, were calculated to be 4 and 7 A away from His(34)/KTX, respectively. Docking of KTX into a homology model of mKv1.1 based on the KcsA crystal structure using this and other known interactions as constraints showed structural differences between mKv1.1 and KcsA within the turret (amino acids 348-357). To satisfy our data, we would have to modify the KcsA crystal structure for the mKv1.1 channel orienting Glu(350) 7 A and Glu(353) 4 A more toward the center of the pore compared with KcsA. This would place Glu(350) 15 A and Glu(353) 11 A away from the center of the pore instead of the distances for the equivalent KcsA residues with 22 A for Gly (53) and 15 A for Gly(56), respectively. Bacterial and mammalian potassium channels may have structural differences regarding the turret of the outer pore vestibule. This topological difference between both channel types may have substantial influence on structure-guided development of new drugs for mammalian potassium channels by rational drug design.

Yang Y, Griffiths WJ, Nordling M, Nygren J, Moller L, Bergman J, Liepinsh E, Otting G, Gustafsson JA, Rafter J, et al. **Ring opening of benzo[a]pyrene in the germ-free rat is a novel pathway for formation of potentially genotoxic metabolites.** Biochemistry 2000;39(50):15585-91.
Abstract: The metabolism of benzo[a]pyrene (BP) is known to lead to a large number of oxygenated compounds, some of which can bind covalently to DNA. We have studied the integrated metabolism of BP in vivo in germ-free rats given (14)C-labeled BP. Urinary metabolites were separated into groups according to acidity using lipophilic ion exchangers. The groups were analyzed by mass spectrometry and were further fractionated by high-performance liquid chromatography. The fraction of urinary metabolites previously shown to contain N-acetylcysteine and glucuronic acid conjugates was found to contain derivatives of 7-oxo-benz[d]anthracene-3,4-dicarboxylic acid as major components. These compounds, which were identified by mass spectrometry and NMR, accounted for about 30% of the total metabolites in urine, demonstrating that, surprisingly, ring opening is a major pathway for metabolism of BP in the germ-free rat. The dicarboxylic acid may be excreted in urine as an ester glucuronide. By using the single cell gel electrophoresis or COMET assay, we were able to demonstrate that the anhydride of 7-oxo-benz[d]anthracene-3, 4-dicarboxylic acid was an efficient inducer of DNA damage. Taken together, these results indicate that the novel ring opening metabolic pathway may provide alternative mechanisms for the toxicity of BP.

Yang Y, Yan J, Churchwell M, Beger R, Chan P, Doerge DR, Fu PP, Chou MW. **Development of a (32) P-postlabeling/HPLC method for detection of dehydroretronecine-derived DNA adducts in vivo and in vitro.** Chem Res Toxicol 2001;14(1):91-100.
Abstract: Pyrrolizidine alkaloids are naturally occurring genotoxic chemicals produced by a large number of plants. Metabolism of pyrrolizidine alkaloids in vivo and in vitro generates dehydroretronecine (DHR) as a common reactive metabolite. In this study, we report the development of a (32)P-postlabeling/HPLC method for detection of (i) two DHR-3'-dGMP and four DHR-3'-dAMP adducts and (ii) a set of eight DHR-derived DNA adducts in vitro and in vivo. The approach involves (1) synthesis of DHR-3'-dGMP, DHR-3'-dAMP, and DHR-3',5'-dG-bisphosphate standards and characterization of their structures by mass and (1)H NMR spectral analyses, (2) development of optimal conditions for enzymatic DNA digestion, adduct enrichment, and (32)P-postlabeling, and (3) development of optimal HPLC conditions. Using this methodology, we have detected eight DHR-derived DNA adducts, including the two epimeric DHR-3',5'-dG-bisphosphate adducts both in vitro and

in vivo.

Yin M, Gabele E, Wheeler MD, Connor H, Bradford BU, Dikalova A, Rusyn I, Mason R, Thurman RG. **Alcohol-induced free radicals in mice: Direct toxicants or signaling molecules**? Hepatology 2001;34 (5):935-42.
Abstract: Tumor necrosis factor alpha (TNF-alpha) and free radicals are produced in early alcohol-induced liver injury. Recently, pathology caused by alcohol was blocked nearly completely in tumor necrosis factor alpha receptor 1 (TNF-R1) knockout mice. With this model, it is now possible to evaluate whether free radicals are directly toxic or act as redox regulators of TNF-alpha production. Specifically, if free radicals were directly toxic, a parallel decrease in free radicals and pathology in TNF-R1 knockout mice would be predicted. If they only affect TNF-alpha production, radicals would be expected to remain high while pathology is diminished. Accordingly, free radical production in TNF-R1 knockout mice was studied here. The enteral alcohol delivery model used mice lacking TNF-R1 (p55) and wild-type control C57Bl/6J mice. Animals received a liquid diet continuously with either ethanol or isocaloric maltose-dextrin as control for 4 weeks. Urine ethanol levels fluctuated from 10 to 500 mg/dL in a cyclic pattern in mice receiving ethanol. Ethanol elevated liver:body weight ratios, serum alanine transaminase (ALT) levels, and pathology scores in wild-type mice. These parameters were blunted nearly completely in TNF-R1 knockout mice. Ethanol treatment increased free radical production in wild-type mice compared with animals fed a high-fat control diet. There were no differences in intensity of free radical signals regardless of the presence or absence of TNF-R1; however, pathology differed markedly between these groups. These findings are consistent with the hypothesis that free radicals act as redox signals for TNF-alpha production and do not directly damage cells in early alcohol-induced hepatic injury.

Young JF, Wosilait WD, Luecke RH. **Analysis of methylmercury disposition in humans utilizing a PBPK model and animal pharmacokinetic data.** J Toxicol Environ Health A 2001;63(1):19-52.
Abstract: Physiologically based pharmacokinetic (PBPK) models are excellent tools to aid in the extrapolation of animal data to humans. When the fate of the chemical is the same among species being compared, animal data can appropriately be considered as a model for human exposure. For methylmercury exposure, sufficient data exist to allow comparison of numerous mammalian species to humans. PBPK model validation entails obtaining blood and tissue concentrations of the parent chemical and metabolite(s) at various times following a known exposure. From ethical and practical considerations, human tissue concentrations following a known exposure to an environmental toxicant are scarce. While animal-to-human extrapolation demands that sufficient human data exist to validate the model, the validation requirements are less stringent if multiple animal models are utilized within a single model template. A versatile PBPK model was used to analyze the distribution and elimination of methylmercury and its metabolite, inorganic mercury. Uniquely, the model is formed in a generic way from a single basic template during the initial program compilation. Basic parameters are defined for diffferent PBPK models for mammalian species that span a relatively large range of sizes. In this article, the analyses include 12 species (mouse, hamster, rat, guinea pig, cat, rabbit, monkey, sheep, pig, goat, cow, and human). Allometric (weight-based) correlations of tissue binding coefficients, metabolism rate constants, and elimination parameters for both methylmercury and inorganic mercury are presented for species for which sufficient data are available. The resulting human model, in accord with the animal

models, predicts relatively high inorganic mercury levels in the kidneys long after the disappearance of methylmercury from the blood.

Zaccolo M, De Giorgi F, Cho CY, Feng L, Knapp T, Negulescu PA, Taylor SS, Tsien RY, Pozzan T. **A genetically encoded, fluorescent indicator for cyclic AMP in living cells.** Nat Cell Biol 2000;2(1):25-9.
Abstract: Cyclic AMP controls several signalling cascades within cells, and changes in the amounts of this second messenger have an essential role in many cellular events. Here we describe a new methodology for monitoring the fluctuations of cAMP in living cells. By tagging the cAMP effector protein kinase A with two suitable green fluorescent protein mutants, we have generated a probe in which the fluorescence resonance energy transfer between the two fluorescent moieties is dependent on the levels of cAMP. This new methodology opens the way to the elucidation of the biochemistry of cAMP in vivo.

Arafa HM, Elmazar MM, Hamada FM, Reichert U, Shroot B, Nau H. **Selective agonists of retinoic acid receptors: comparative toxicokinetics and embryonic exposure.** Arch Toxicol 2000;73(10-11):547-56.
Abstract: Three biologically active synthetic retinoids were investigated that bind selectively to retinoic acid receptors RARs (alpha, beta and gamma). The retinoids were previously demonstrated to have different teratogenic effects in the mouse in terms of potency and regioselectivity. The teratogenic potency rank order (alpha >beta >gamma) was found to be more or less compatible with the receptor binding affinities and transactivation potencies of the retinoid ligands to their respective receptors. The RARalpha agonist (Am580; CD336) induced a wide spectrum of malformations; CD2019 (RARbeta agonist) and especially CD437 (RARgamma agonist) produced more restricted defects. In the current study we tried to address whether the differences in teratogenic effects are solely related to binding affinity and transactivation differences or also due to differences in embryonic exposure. Therefore, transplacental kinetics of the ligands were assessed following administration of a single oral dose of 15 mg/kg of either retinoid given to NMRI mice on day 11 of gestation. Am580 was rapidly transferred to the embryo resulting in the highest embryonic exposure [embryo to maternal plasma area under the time vs concentration curve (AUC)(0-24 h)ratio (E/M) was 1.7], in accordance with its highest teratogenic potency. The low placental transfer of CD2019 (E/M of 0.3) was compatible with its lower teratogenic potential. Of major interest was the finding that the CD437, though being least teratogenic, exhibited considerable embryonic exposure (E/M of 0.6). These findings suggest that both the embryonic exposure and receptor binding transactivation selectivity are crucial determinants of the teratogenicity of these retinoid ligands.

Barouki R, Morel Y. **Repression of cytochrome P450 1A1 gene expression by oxidative stress: mechanisms and biological implications.** Biochem Pharmacol 2001;61(5):511-6.
Abstract: Cytochrome P450 1A1 (CYP1A1) is a member of a multigenic family of xenobiotic-metabolizing enzymes. Beyond its usual role in the detoxification of polycyclic aromatic compounds, the activity of this enzyme can be deleterious since it can generate mutagenic metabolites and oxidative stress. The CYP1A1 gene is highly inducible by the environmental contaminants dioxin and benzo[a] pyrene. We discuss here the regulatory mechanisms that limit this induction. Several feedback loops

control the activation of this gene and the subsequent potential toxicity. The oxidative repression of the CYP1A1 gene seems to play a central role in these regulations. The transcription factor Nuclear Factor I/ CCAAT Transcription Factor (NFI/CTF), which is important for the transactivation of the CYP1A1 gene promoter, is particularly sensitive to oxidative stress. A critical cysteine within the transactivating domain of NFI/CTF appears to be the target of H(2)O(2). The DNA-binding domains of several transcription factors have been described as targets of oxidative stress. However, recent studies described here suggest that more attention should be given to transactivating domains that may represent biologically relevant redox targets of cellular signaling.

Brewer SK, Little EE, DeLonay AJ, Beauvais SL, Jones SB, Ellersieck MR. **Behavioral dysfunctions correlate to altered physiology in rainbow trout (Oncorynchus mykiss) exposed to cholinesterase-inhibiting chemicals.** Arch Environ Contam Toxicol 2001;40(1):70-6.
Abstract: We selected four metrics of swimming behavior (distance swam, speed, rate of turning, and tortuosity of path) and the commonly used biochemical marker, brain cholinesterase (ChE) activity, to assess (1) the sensitivity and reliability of behavior as a potential biomarker in monitoring work, (2) the potential for these endpoints to be used in automated monitoring, and (3) the linkage between behavior and its underlying biochemistry. Malathion-exposed fish exhibited large decreases in distance and speed and swam in a more linear path than control fish after 24 h exposure. By 96 h exposure, fish still swam slower and traveled less distance; fish fully recovered after 48 h in clean water. Diazinon-exposed fish exhibited decreases in distance, speed, and turning rate compared to controls. After 48 h recovery in clean water, fish exposed to diazinon had not recovered to control levels. The behavioral responses provided measures of neurotoxicity that were easily quantifiable by automated means, implying that the inclusion of behavior in monitoring programs can be successful. Furthermore, correlations between behavior and biochemical endpoints, such as ChE inhibition, suggest that this approach can provide a meaningful link between biochemistry and behavior and can provide useful information on toxicant impacts.

Bridges B. **DNA polymerases and SOS mutagenesis: can one reconcile the biochemical and genetic data?** Bioessays 2000;22(10):933-7.
Abstract: Until recently, it had been concluded from genetic evidence that DNA polymerase III (Pol III, the main replicative polymerase in E. coli) was also responsible for mutagenic translesion synthesis on damaged templates, albeit under the influence of inducible proteins UmuD' and UmuC. Now it appears that these proteins themselves have polymerase activity (and are now known as Pol V) and can carry out translesion synthesis in vitro in the absence of Pol III. Here I discuss the apparent contradictions between genetics and biochemistry with regard to the role of Pol III in translesion synthesis. Does Pol V interact with Pol III and constitute an alternative component of the replication factory (replisome)? Where do the other three known polymerases fit in? What devices does the cell have to ensure that the "right" polymerase is used in a given situation? The debate about the role of Pol III in translesion synthesis reveals a deeper divide between models that interpret everything in terms of mass action effects and those that embrace a replisome held together by protein-protein interactions and located as a structural entity within the cell.

Burczynski ME, Penning TM. **Genotoxic polycyclic aromatic hydrocarbon ortho-quinones**

generated by aldo-keto reductases induce CYP1A1 via nuclear translocation of the aryl hydrocarbon receptor. Cancer Res 2000;60(4):908-15.
Abstract: Procarcinogenic polycyclic aromatic hydrocarbons (PAHs) induce their own metabolism and activation by binding to the cytosolic aryl hydrocarbon receptor (AhR), which then translocates to the nucleus and activates CYP1A1 gene transcription via xenobiotic response elements (XREs). Although the AhR demonstrates a strict specificity for planar aromatics, nonplanar (+/-)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene also induced CYP1A1 expression in HepG2 cells over a delayed timecourse (approximately 6-12 h), suggesting a requirement for (+/-)trans-7,8-dihydrobenzo(a)pyrene metabolism. Aldo-keto reductase (AKR) inhibitors blocked this effect, suggesting that benzo(a)pyrene-7,8-dione (BPQ), a planar PAH o-quinone generated by AKRs, was the downstream inducer. BPQ was found to be a potent and rapid inducer of CYP1A1, with an EC50 value in HepG2 cells identical to that of the parent benzo(a)pyrene. BPQ was a more potent inducer of CYP1A1 when compared with the 1,6-, 3,6-, and 6,12-benzo(a)pyrene-diones. Multiple PAH o-quinones caused induction of CYP1A1, demonstrating that this was a general property of AKR-generated PAH o-quinones. HepG2-101L cells stably transfected with a XRE-luciferase construct showed that BPQ activated CYP1A1 transcription via a XRE-dependent mechanism. BPQ failed to induce CYP1A1 in AhR-deficient and AhR nuclear translocator-deficient murine hepatoma cell lines and confirmed that induction of CYP1A1 was AhR and AhR nuclear translocator-dependent. Electrophoretic mobility shift assays demonstrated the specific appearance of BPQ-activated AhR in the nucleus, and immunofluorescence studies confirmed that BPQ mediated nuclear translocation of the AhR. Classical bifunctional inducers elevate CYP1A1 expression via a XRE and are subsequently converted by CYP1A1 to electrophiles that induce phase II enzymes via an electrophilic response element/antioxidant response element PAH o-quinones represent a novel class of bifunctional inducer because they are electrophiles produced by phase II enzymes that simultaneously induce phase I enzymes via a XRE and phase II enzymes via a electrophilic response element/ antioxidant response element (see also M. E. Burczynski et al., Cancer Res., 59: 607-614, 1999). This study shows that the AhR provides the only known mechanism by which genotoxic PAH o-quinones generated in the cytosol can be targeted to the nucleus with specificity.

Carmichael AB, Wong LL. **Protein engineering of Bacillus megaterium CYP102.The oxidation of polycyclic aromatic hydrocarbons.** Eur J Biochem 2001;268(10):3117-25.
Abstract: Cytochrome P450 (CYP) enzymes are involved in activating the carcinogenicity of polycyclic aromatic hydrocarbons (PAHs) in mammals, but they are also utilized by microorganisms for the degradation of these hazardous environmental contaminants. Wild-type CYP102 (P450(BM-3)) from Bacillus megaterium has low activity for the oxidation of the PAHs phenanthrene, fluoranthene and pyrene. The double hydrophobic substitution R47L/Y51F at the entrance of the substrate access channel increased the PAH oxidation activity by up to 40-fold. Combining these mutations with the active site mutations F87A and A264G lead to order of magnitude increases in activity. Both these mutations increased the NADPH turnover rate, but the A264G mutation increased the coupling efficiency while the F87A mutation had dominant effects in product selectivity. Fast NADPH oxidation rates were observed (2250 min-1 for the R47L/Y51F/F87A mutant with phenanthrene) but the coupling efficiencies were relatively low (< 13%), resulting in a highest substrate oxidation rate of 110 min-1 for fluoranthene oxidation by the R47L/Y51F/A264G mutant. Mutation of M354 and L437 inside the substrate access channel reduced PAH oxidation activity. The PAHs were oxidized to a mixture of phenols and quinones.

Notably mutants containing the A264G mutation showed some similarity to mammalian CYP enzymes in that some 9,10-phenanthrenequinone, the K-region oxidation product from phenanthrene, was formed. The results suggest that CYP102 mutants could be useful models for PAH oxidation by mammalian CYP enzymes, and also potentially for the preparation of novel PAH bioremediation systems.

Csanady GA, Denk B, Putz C, Kreuzer PE, Kessler W, Baur C, Gargas ML, Filser JG. **A physiological toxicokinetic model for exogenous and endogenous ethylene and ethylene oxide in rat, mouse, and human: formation of 2-hydroxyethyl adducts with hemoglobin and DNA.** Toxicol Appl Pharmacol 2000;165(1):1-26.
Abstract: Ethylene (ET) is a gaseous olefin of considerable industrial importance. It is also ubiquitous in the environment and is produced in plants, mammals, and humans. Uptake of exogenous ET occurs via inhalation. ET is biotransformed to ethylene oxide (EO), which is also an important volatile industrial chemical. This epoxide forms hydroxyethyl adducts with macromolecules such as hemoglobin and DNA and is mutagenic in vivo and in vitro and carcinogenic in experimental animals. It is metabolically eliminated by epoxide hydrolase and glutathione S-transferase and a small fraction is exhaled unchanged. To estimate the body burden of EO in rodents and human resulting from exposures to EO and ET, we developed a physiological toxicokinetic model. It describes uptake of ET and EO following inhalation and intraperitoneal administration, endogenous production of ET, enzyme-mediated oxidation of ET to EO, bioavailability of EO, EO metabolism, and formation of 2-hydroxyethyl adducts of hemoglobin and DNA. The model includes compartments representing arterial, venous, and pulmonary blood, liver, muscle, fat, and richly perfused tissues. Partition coefficients and metabolic parameters were derived from experimental data or published values. Model simulations were compared with a series of data collected in rodents or humans. The model describes well the uptake, elimination, and endogenous production of ET in all three species. Simulations of EO concentrations in blood and exhaled air of rodents and humans exposed to EO or ET were in good agreement with measured data. Using published rate constants for the formation of 2-hydroxyethyl adducts with hemoglobin and DNA, adduct levels were predicted and compared with values reported. In humans, predicted hemoglobin adducts resulting from exposure to EO or ET are in agreement with measured values. In rodents, simulated and measured DNA adduct levels agreed generally well, but hemoglobin adducts were underpredicted by a factor of 2 to 3. Obviously, there are inconsistencies between measured DNA and hemoglobin adduct levels.

Fantel AG, Stamps LD, Tran TT, Mackler B, Person RE, Nekahi N. **Free radical formation and toxicity in the limb teratogenicity of L-NAME: a new mechanistic model of vascular disruption.** Teratology 2000;62(4):237

Helleberg H, Tornqvist M. **A new approach for measuring protein adducts from benzo[a]pyrene diolepoxide by high performance liquid chromatography/tandem mass spectrometry.** Rapid Commun Mass Spectrom 2000;14(18):1644-53.
Abstract: The long-range goal of the present study is the development of a general approach for in vivo dosimetry of reactive metabolites of polycyclic aromatic hydrocarbons (PAHs), to be used as a tool in cancer risk assessment. With benzo[a]pyrene (BaP) chosen as indicator and a model of PAHs this study aims at the development of a method for the determination of adducts to histidine (His) in hemoglobin

(Hb) and serum albumin (SA) of reactive metabolites of BaP. The predominantly mutagenic metabolite of BaP has been shown to be a diolepoxide isomer, +(anti)r-7, t-8-dihydroxy-t-9,10-epoxy-7,8, 9,10-tetrahydrobenzo[a]pyrene (+BPDE). In comparison with other methods for protein degradation, hydrazinolysis was found to be sufficiently effective and mild. The His adduct isolated after protein hydrazinolysis, with protection by tert-butyloxycarbonyl (Boc) of the hydrazide and alpha-amino groups, was shown to be N(im)- +/- (r-7, t-8, t-9-trihydroxy-7, 8, 9, 10-tetrahydrobenzo[a]pyren-c-10-yl)-N(alpha), N(2)-bis(tert-butyloxycarbonyl)-L-histidinehydrazide. Isomers of this compound, used as references, were synthesized and characterized by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Adducts in Hb and SA from in vitro treatment with BPDE were characterized after hydrazinolysis by HPLC-UV/MS, muHPLC/MS/MS and gas chromatography/mass spectrometry (GC/MS). Approximately 70 and 10% of the isolated BPDE adducts from SA and Hb, respectively, were His adducts. Other products were released as BaP tetrols and BaP triols. For the purpose of enrichment/purification of BPDE-His adducts, C(18) and cation exchange solid phase extraction (SPE) were utilized. The sensitivity obtained by this new approach, based on hydrazinolysis of protein, enrichment by SPE and analysis with muHPLC/MS/MS (APCI), is in the low-fmole range.

Li P, Rossman TG. **Genes upregulated in lead-resistant glioma cells reveal possible targets for lead-induced developmental neurotoxicity.** Toxicol Sci 2001;64(1):90-9.
Abstract: Identifying genes upregulated in lead-resistant cells should give insight into lead toxicity and cellular protective mechanisms and may also result in identification of proteins that may be useful as biomarkers. Glial cells are thought to protect neurons against heavy metals. Rat glioma C6 cells share many properties of normal glial cells. To identify and analyze genes upregulated in a lead-resistant variant, PbR11, suppression subtractive hybridization (SSH) between mRNAs of wild-type and PbR11 cells was performed. Sequencing and database searches identified three genes, thrombospondin-1, heparin sulfate 6-sulfotransferase, and neuropilin-1, which play important roles in angiogenesis and axon growth during development. Two genes, HSP90 and UBA3, are involved in the ubiquitin-proteosome system. One gene was identified as that of a rat endogenous retrovirus and another, 2C9, is a transcript expressed in fos-transformed cells. PbR11 also overexpresses c-fos. Expression of these genes and effects of short-term lead exposure (24 h, up to 600 &mgr;M) on their expression in C6 cells was examined. The rat endogenous retrovirus and 2C9 are expressed only in PbR11 cells, and show no expression, either constitutive or lead-induced, in wild-type C6 cells. HSP90 is expressed at low level constitutively in C6 cells, but can be induced in a dose-dependent manner by lead. In contrast, thrombospondin-1 is repressed in a dose-dependent manner by lead. The other genes (HS6ST, neuropilin, and UBA3) show low constitutive expression and are neither upregulated nor downregulated by exposure to lead. We suggest that neuropilin-1, heparin sulfate 6-sulfotransferase, and thrombospondin-1 may be important targets for lead-induced developmental neurotoxicity.

Wise AA, Kuske CR. **Generation of novel bacterial regulatory proteins that detect priority pollutant phenols.** Appl Environ Microbiol 2000;66(1):163-9.
Abstract: The genetic systems of bacteria that have the ability to use organic pollutants as carbon and energy sources can be adapted to create bacterial biosensors for the detection of industrial pollution. The creation of bacterial biosensors is hampered by a lack of information about the genetic systems that control production of bacterial enzymes that metabolize pollutants. We have attempted to overcome this

problem through modification of DmpR, a regulatory protein for the phenol degradation pathway of Pseudomonas sp. strain CF600. The phenol detection capacity of DmpR was altered by using mutagenic PCR targeted to the DmpR sensor domain. DmpR mutants were identified that both increased sensitivity to the phenolic effectors of wild-type DmpR and increased the range of molecules detected. The phenol detection characteristics of seven DmpR mutants were demonstrated through their ability to activate transcription of a lacZ reporter gene. Effectors of the DmpR derivatives included phenol, 2-chlorophenol, 2,4-dichlorophenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol, 2-nitrophenol, and 4-nitrophenol.

Yonezawa Y, Kawamura S, Yamato M, Nishioka H. **Mut-Test to detect substances suppressing spontaneous mutation due to oxidative damage.** Mutat Res 2001;490(1):21-6.
Abstract: Since it has been considered that suppression of spontaneous mutation in cells is related to suppression of spontaneous carcinogenesis, it is significant to detect substances which suppress spontaneous mutation in bacterial cells such as Escherichia coli and Salmonella typhimurium in the environment. However, since the frequency of spontaneous mutation in bacteria is usually very low, generally 10(-8)-10(-10),it is difficult to determine significant suppressive ability of such substances on spontaneous mutation. A new method, Mut-Test, was developed by us, applying Luria & Delbruck fluctuation test, to detect substances which suppress spontaneous mutation using E. coli mutT mutant in which spontaneous mutation frequency due to oxidative damage is enhanced to approximately 500-1000 times of the wild type strain. Suppressive abilities of two hydroxyl radical scavengers: D(-)-mannitol and thiourea, were examined and clear positive results were obtained, suggesting that the radical scavengers are suitable as the positive control for the test. Using Mut-Test, suppressive abilities of four vitamins: L-ascorbic acid, beta-carotene, folic acid and riboflavin; 10 polyphenols: caffeic acid, ellagic acid, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, gallic acid, pyrocatechol, pyrogallol, quercetin and tannic acid which are recognized as antimutagens, were examined. Furthermore, the concentrations for 50% of suppressive abilities of five positive samples, L-ascorbic acid, folic acid, caffeic acid, pyrocatechol and pyrogallol were compared. Negative results were obtained in nine samples, riboflavin, tannic acid, etc. suggesting that their antimutagenic effect on cells may not be related to oxidative damage in cells.

PULMONARY TOXICITY

Aoki Y, Sato H, Nishimura N, Takahashi S, Itoh K, Yamamoto M. **Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust.** Toxicol Appl Pharmacol 2001;173(3):154-60.
Abstract: Diesel exhaust (DE) has been recognized as a noxious mutagen and/or carcinogen, because its components can form DNA adducts. Mechanisms governing the susceptibility to DE and the efficiency of such DNA adduct formation require clarification. The transcription factor Nrf2 is essential for inducible and/or constitutive expression of a group of detoxification and antioxidant enzymes, and we hypothesized that the nrf2 gene knockout mouse might serve as an excellent model system for analyzing DE toxicity. To address this hypothesis, lungs from nrf2(-/-) and nrf2(+/-) mice were examined for the production of xenobiotic-DNA adducts after exposure to DE (3 mg/m(3) suspended particulate matter) for 4 weeks. Whereas the relative adduct levels (RAL) were significantly increased in the lungs of both

nrf2(+/-) and nrf2(-/-) mice upon exposure to DE, the increase of RAL in the lungs from nrf2(-/-) mice exposed to DE were approximately 2.3-fold higher than that of nrf2(+/-) mice exposed to DE. In contrast, cytochrome P4501A1 mRNA levels in the nrf2(-/-) mouse lungs were similar to those in the nrf2(+/-) mouse lungs even after exposure to DE, suggesting that suppressed activity of phase II drug-metabolizing enzymes is important in giving rise to the increased level of DNA adducts in the Nrf2-null mutant mouse subjected to DE. Importantly, severe hyperplasia and accumulation of the oxidative DNA adduct 8-hydroxydeoxyguanosine were observed in the bronchial epidermis of nrf2(-/-) mice following DE exposure. These results demonstrate the increased susceptibility of the nrf2 germ line mutant mouse to DE exposure and indicate the nrf2 gene knockout mouse may represent a valuable model for the assessment of respiratory DE toxicity. Copyright 2001 Academic Press.

Aufderheide M, Mohr U. **CULTEX--an alternative technique for cultivation and exposure of cells of the respiratory tract to airborne pollutants at the air/liquid interface.** Exp Toxicol Pathol 2000;52 (3):265-70.
Abstract: The assessment of cytotoxicity of air contaminants such as gaseous or particulate compounds and complex mixtures has traditionally involved animal experiments, due to the difficulties in exposing cell cultures directly to these substances. New cultivation and exposure techniques enhance the efficiency of in vitro studies, as demonstrated by a new experimental system called CULTEX which allows direct exposure of cells at the air/liquid interface. In this case, human bronchial epithelial cells are cultivated on porous transwell membranes in a device allowing intermittent medium supply. The medium is pumped into a special modular culture unit through the transwell membrane supporting the cells. At certain time intervals, the medium is completely removed and the cells can be maintained and exposed at the air/liquid interface until the next medium supply without loss of viability. In comparison to conventional submersed culture conditions, the cells have been grown on transwell membranes using the new pulse submersion technique. There are no deleterious effects on cell viability due to the direct exposure to airborne pollutants. Thus, the introduction of these new cultivation and exposure techniques offers new testing strategies for the toxicological evaluation of inhalable soluble and inert substances as well as complex mixtures.

Aufderheide M, Ritter D, Knebel JW, Scherer G. **A method for in vitro analysis of the biological activity of complex mixtures such as sidestream cigarette smoke.** Exp Toxicol Pathol 2001;53 (2-):141-52.
Abstract: Studies of the cytotoxicity of air contaminants such as gaseous or particulate compounds and complex mixtures have traditionally used in animal experiments because of the difficulties in exposing cell cultures directly to these substances. New cultivation and exposure techniques enhance the efficiency of in vitro methods, as demonstrated by a new system called CULTEX* which uses a transwell membrane technique for direct exposure of complex mixtures like sidestream cigarette smoke at the air/liquid interface. The factors influencing the susceptibility of human bronchial epithelial cells (e. g. gas flow rate or duration of exposure) were studied and the cells were finally exposed for one hour to clean air or different concentrations of sidestream smoke. The biological parameters estimated were number of cells, metabolic activity and glutathione concentration. After exposure of the cells to sidestream cigarette smoke, dose-dependent effects were measured. Thus, the introduction of these cultivation and exposure techniques offers new testing strategies for the toxicological evaluation of a

broad range of airborne and inhalable compounds.

Conolly RB, Lilly PD, Kimbell JS. **Simulation modeling of the tissue disposition of formaldehyde to predict nasal DNA-protein cross-links in Fischer 344 rats, rhesus monkeys, and humans.** Environ Health Perspect 2000;108 Suppl 5:919-24.
Abstract: Formaldehyde inhalation causes formation of DNA-protein cross-links (DPX) in the nasal mucosa of Fischer 344 (F344) rats and rhesus monkeys. DPX are considered to be part of the mechanism by which cytotoxic and carcinogenic effects of formaldehyde in laboratory animals are exerted, and DPX data have been used as a measure of tissue dose in cancer risk assessments for formaldehyde. Accurate prediction of DPX concentrations in humans is therefore desirable. The goal of this work was to increase confidence in the prediction of human DPX by refining earlier models of formaldehyde disposition and DPX kinetics in the nasal mucosa. Anatomically accurate, computational fluid dynamics models of the nasal airways of F344 rats, rhesus monkeys, and humans were used to predict the regional flux of formaldehyde to the respiratory and olfactory mucosa. A previously developed model of the tissue disposition of formaldehyde and of DPX kinetics was implemented in the graphical simulation tool SIMULINK and linked to the regional flux predictions. Statistical optimization was used to identify parameter values, and good simulations of the data were obtained. The parameter estimates for rats and monkeys were used to guide allometric scale-up to the human case. The relative levels of nasal mucosal DPX in rats, rhesus monkeys, and humans for a given inhaled concentration of formaldehyde were predicted by the model to vary with concentration. This modeling approach reduces uncertainty in the prediction of human nasal mucosal DPX resulting from formaldehyde inhalation.

Kilgour JD, Simpson SA, Alexander DJ, Reed CJ. **A rat nasal epithelial model for predicting upper respiratory tract toxicity: in vivo-in vitro correlations.** Toxicology 2000;145(1):39-49.
Abstract: An in vitro model of the rat nasal cavity has been used to compare the responses of nasal tissues in vitro, using loss of intracellular ATP and potassium as indices of toxicity, with the pathological changes occurring following in vivo exposure to four test compounds. Turbinates were incubated in vitro with the test compounds for 4 h, for 24 h or for 4 h followed by 20 h in fresh medium. Titanium dioxide caused little or no loss of ATP in either olfactory epithelium (OE) or respiratory epithelium (RE). Sodium carbonate decreased olfactory, but not respiratory ATP, while acetic acid and 3-methylindole markedly decreased ATP in both tissues. Intracellular potassium concentrations were generally affected to a lesser degree. In vivo, no morphological changes were observed in the nasal cavity following inhalation exposure to either titanium dioxide or sodium carbonate. Inhalation of acetic acid resulted in a very focal lesion in the RE of the dorsal meatus of level 1, while administration of 3-methylindole by intraperitoneal injection caused severe degeneration of OE. In further experiments olfactory turbinates were exposed to a range of concentrations (0-100 mM) of sodium carbonate, acetic acid and 3-methylindole for 4 h and ATP concentrations determined. Concentration-dependent decreases in ATP were observed for sodium carbonate and 3-methylindole, with EC(50) values estimated as 2.57 and 0.91 mM, respectively. Acetic acid only decreased ATP significantly at the 100-mM concentration. In summary, this in vitro model has predicted the nasal toxicity of several compounds, including both direct-acting agents (sodium carbonate, acetic acid) and one requiring metabolic activation (3-methylindole). However, the lack of airflow-dependent dosimetry, results in some lack of discrimination between the different regions of the nasal cavity and may make this model overly sensitive.

Kuempel ED. **Comparison of human and rodent lung dosimetry models for particle clearance and retention.** Drug Chem Toxicol 2000;23(1):203-22.
Abstract: Interspecies differences in the kinetics and/or mechanisms of particle retention can influence the amount and location of particle retention in the lungs, which can also influence the tissue response to a given particle burden. Dosimetric models may be used to adjust for differences in the exposure-dose relationships in different species, thus allowing for comparison of lung responses at equivalent doses. Although the rat is one of the most frequently used animal models for assessing the risk of exposures to hazardous substances in humans, few data are available for comparison of human and animal responses to inhaled particles. A biologically-based human dosimetric lung model was developed to describe the fate of respirable particles in the lungs of humans, using data from U.S. coal miners and assumptions about the overloading of alveolar clearance from studies in rats. This model includes alveolar, interstitial, and hilar lymph node compartments. The form of the model that provides the best fit to the lung dust burden data in these coal miners includes a first-order interstitialization process and either no dose-dependent decline in alveolar clearance or much less decline than expected from rodent studies. These findings are consistent with the particle retention patterns observed previously in the lungs of primates. This human lung dosimetry model is useful for investigating the factors that may influence the relationships between the airborne particle exposure, lung dust burden, and fibrotic lung disease.

Kuempel ED, O'Flaherty EJ, Stayner LT, Smith RJ, Green FH, Vallyathan V. **A biomathematical model of particle clearance and retention in the lungs of coal miners.** Regul Toxicol Pharmacol 2001;34(1):69-87.
Abstract: To understand better the factors influencing the relationships among airborne particle exposure, lung burden, and fibrotic lung disease, we developed a biologically based kinetic model to predict the long-term retention of particles in the lungs of coal miners. This model includes alveolar, interstitial, and hilar lymph node compartments. The 131 miners in this study had worked in the Beckley, West Virginia, area and died during the 1960s. The data used to develop this model include exposure to respirable coal mine dust by intensity and duration within each job, lung and lymph node dust burdens at autopsy, pathological classification of fibrotic lung disease, and smoking history. Initial parameter estimates for this model were based on both human and animal data of particle deposition and clearance and on the biological and physical factors influencing these processes. Parameter estimation and model fit to the data were determined using least squares. Results show that the end-of-life lung dust burdens in these coal miners were substantially higher than expected from first-order clearance kinetics, yet lower than expected from the overloading of alveolar clearance predicted from rodent studies. The best-fitting and most parsimonious model includes processes for first-order alveolar-macrophage-mediated clearance and transfer of particles to the lung interstitium. These results are consistent with the particle retention patterns observed previously in the lungs of primates. The findings indicate that rodent models extrapolated to humans, without adjustment for the kinetic differences in particle clearance and retention, would be inadequate for predicting lung dust burdens in humans. Also, this human lung kinetic model predicts greater retained lung dust burdens from occupational exposure than predicted from current human models based on lower exposure data. This model is useful for risk assessment of particle-induced lung diseases, by estimating equivalent internal doses in rodents and humans and predicting lung burdens in humans with occupational dust exposures.

Kuempel ED, Tran CL, Bailer AJ, Smith RJ, Dankovic DA, Stayner LT. **Methodological issues of using observational human data in lung dosimetry models for particulates.** Sci Total Environ 2001;274(1-3):67-77.
Abstract: INTRODUCTION: The use of human data to calibrate and validate a physiologically based pharmacokinetic (PBPK) model has the clear advantage of pertaining to the species of interest, namely humans. A challenge in using these data is their often sparse, heterogeneous nature, which may require special methods. Approaches for evaluating sources of variability and uncertainty in a human lung dosimetry model are described in this study. METHODS: A multivariate optimization procedure was used to fit a dosimetry model to data of 131 U.S. coal miners. These data include workplace exposures and end-of-life particle burdens in the lungs and hilar lymph nodes. Uncertainty in model structure was investigated by fitting various model forms for particle clearance and sequestration of particles in the lung interstitium. A sensitivity analysis was performed to determine which model parameters had the most influence on model output. Distributions of clearance parameters were estimated by fitting the model to each individual's data, and this information was used to predict inter-individual differences in lung particle burdens at given exposures. The influence of smoking history, race and pulmonary fibrosis on the individual's estimated clearance parameters was also evaluated. RESULTS: The model structure that provided the best fit to these coal miner data includes a first-order interstitialization process and no dose-dependent decline in alveolar clearance. The parameter that had the largest influence on model output is fractional deposition. Race and fibrosis severity category were statistically significant predictors of individual's estimated alveolar clearance rate coefficients ($P < 0.03$ and $P < 0.01$-0.06, respectively), but smoking history (ever, never) was not ($P < 0.4$). Adjustments for these group differences provided some improvement in the dosimetry model fit (up to 25% reduction in the mean squared error), although unexplained inter-individual differences made up the largest source of variability. Lung burdens were inversely associated with the miners' estimated clearance parameters, e.g. individuals with slower estimated clearance had higher observed lung burdens. CONCLUSIONS: The methods described in this study were used to examine issues of uncertainty in the model structure and variability of the miners' estimated clearance parameters. Estimated individual clearance had a large influence on predicted lung burden, which would also affect disease risk. These findings are useful for risk assessment, by providing estimates of the distribution of lung burdens expected under given exposure conditions.

Lame MW, Jones AD, Wilson DW, Dunston SK, Segall HJ. **Protein targets of monocrotaline pyrrole in pulmonary artery endothelial cells.** J Biol Chem 2000;275(37):29091-9.
Abstract: A single administration of monocrotaline to rats results in pathologic alterations in the lung and heart similar to human pulmonary hypertension. In order to produce these lesions, monocrotaline is oxidized to monocrotaline pyrrole in the liver followed by hematogenous transport to the lung where it injures pulmonary endothelium. In this study, we determined specific endothelial targets for (14)C-monocrotaline pyrrole using two-dimensional gel electrophoresis and autoradiographic detection of protein metabolite adducts. Selective labeling of specific proteins was observed. Labeled proteins were digested with trypsin, and the resulting peptides were analyzed using matrix-assisted laser desorption ionization mass spectrometry. The results were searched against sequence data bases to identify the adducted proteins. Five abundant adducted proteins were identified as galectin-1, protein-disulfide

isomerase, probable protein-disulfide isomerase (ER60), beta- or gamma-cytoplasmic actin, and cytoskeletal tropomyosin (TM30-NM). With the exception of actin, the proteins identified in this study have never been identified as potential targets for pyrroles, and the majority of these proteins have either received no or minimal attention as targets for other electrophilic compounds. The known functions of these proteins are discussed in terms of their potential for explaining the pulmonary toxicity of monocrotaline.

Pauluhn J, Mohr U. **Inhalation studies in laboratory animals--current concepts and alternatives.** Toxicol Pathol 2000;28(5):734-53.
Abstract: Highly standardized and controlled inhalation studies are required for hazard identification to make test results reproducible and comparable and to fulfill general regulatory requirements for the registration of new drugs, pesticides, or chemicals. Despite significant efforts, the results of inhalation studies have to be analyzed judiciously due to the great number of variables. These variables may be related to technical issues or to the specific features of the animal model. Although inhalation exposure of animals mimics human exposure best, ie, error-prone route-to-route extrapolations are not necessary, not all results obtained under such very rigorous test conditions may necessarily also occur under real-life exposure conditions. Attempts are often made to duplicate as closely as possible these real-life exposure conditions of humans in appropriate bioassays. However, this in turn might affect established baseline data, rendering the interpretation of new findings difficult. In addition, specific use patterns, eg, of inhalation pharmaceuticals or pesticide-containing consumer products, may impose test agent-specific constraints that challenge traditional approaches. Moreover, specific modes of action of the substance under investigation, the evaluation of specific endpoints, or the clarification of equivocal findings in common rodent species may require exposure paradigms or the use of animal species not commonly used in inhalation toxicology. However, particularly in inhalation toxicology, the choice of animal models for inhalation toxicity testing is usually based on guideline requirements and practical considerations, such as exposure technology, expediency, and previous experience rather than validity for use in human beings. Larger animal species, apart from the welfare aspects, may require larger inhalation chambers to accommodate the animals, but for technical reasons and the difficulty of generating homogeneous exposure atmospheres in such inhalation chambers, this may jeopardize the outcome of the study. Some of the many variables and possible artifacts likely to occur in animal inhalation studies are addressed in this paper.

Sugawara I, Yamada H, Otomo K, Aoki T, Mizuno S, Udagawa T. **[Optimal conditions for establishment of experimental tuberculosis model using an automated inhalation exposure apparatus and its application].** Kekkaku 2000;75(7):463-9 [Jpn].
Abstract: Animal (mouse and guinea pig) pulmonary tuberculosis models were established, using an automated inhalation exposure apparatus (Glas-Col Corp., USA, Model 099CA-4212). This apparatus includes four steps--preheating, nebulization, cloud decay and decontamination. The optimal conditions for M. tuberculosis H37Rv strain infection experiments were as follows: 10(5-6) colony forming unit (cfu) tubercle bacilli; preheating for 15 min.; nebulization for 90 min.; cloud decay for 15 min. and decontamination for 5 min. When 10(4) cfu M. tuberculosis H37Rv strain were introduced into the lungs of interferon (IFN)-gamma knockout mice, using the inhalation exposure apparatus and were followed up for 9 months, the primitive cavitary lesions were observed. This apparatus was also useful for

inhalation exposure experiments of guinea pigs. This apparatus can also be utilized for animal inhalation experiments of allergens.

Vertrees RA, Deyo DJ, Quast M, Lightfoot KM, Boor PJ, Zwischenberger JB. **Development of a human to murine orthotopic xenotransplanted lung cancer model.** J Invest Surg 2000;13(6):349-58.
Abstract: The goal was to develop a clinically relevant animal model that could be used to assess the efficacy of therapeutic interventions in lung cancer. Two cell lines, noncancerous control (BEAS2-B, immortalized human bronchial-epithelial cell line) and cancerous (BZR-T33, H-ras transformed BEAS2-B) were implanted into nude (athymic) mice. Two groups (n = 10 each) received dorsoscapular subcutaneous injection of 10(6) cells from either cell line. BEAS2-B cells were nontumorigenic, whereas mice with BZR-T33 cells had tumors (9,510 +/- 4,307 mm3) confirmed by histology, and a significantly smaller body weight (BZR-T33, 28.5 +/- 0.49 vs. BEAS2-B, 30.7 +/- 0.75 g, p < .05). The next phase evaluated invasion/metastasis. Two groups (n = 10 each) received 10(6) cells from either cell line injected into tail veins. Animals receiving BZR-T33 cells had a smaller body weight, palpable lung masses (67%), obvious tail masses (44%), and average tumor burden (1,120 +/- 115 mm3), and histology revealed invasion of lung tissue and interstitial hemorrhage. In development of the orthotopic xenotransplanted model, mice (2 groups, n = 10 each) received 10(6) cells from either cell line implanted into the lungs through a tracheotomy. Animals with BZR-T33 cells did not survive past 59 days and had a smaller body weight, increased lung weight, lung masses (100%), and metastatic loci (30%). Magnetic resonance imaging (MRI) confirmed the presence of masses in intubated live mice, later confirmed by histology. In summary, the H-ras transfected cell line developed lung masses following tail-vein injection and endotracheal seeding. Evaluation by MRI allows for a comprehensive model with significant potential in the study of lung cancer.

Wallaert B, Fahy O, Tsicopoulos A, Gosset P, Tonnel AB. **Experimental systems for mechanistic studies of toxicant induced lung inflammation.** Toxicol Lett 2000;112-113:157-63.
Abstract: Human breath contains a large array of complex and poorly characterized mixtures. We can measure the potential risk of these exposures at molecular, cell, organ, organismic levels or in population. This paper emphasizes the characteristics of in vitro tests of lung cells and discusses the use of in vitro systems to determine the health effects of inhaled pollutants. Exposure to gases can be performed with roller bottles fitted with modified rotating caps with tubing connections, or by using dishes on rocker platforms, which tilt back and forth to expose the cell culture to gases. Exposure of cells may also be obtained by using very thin gas-permable membrane on which cells grow. However, it is clear that in using these systems, the culture medium constitutes a barrier between the gas and the target cells and thus does not permit a physiological approach of the toxic effects of gases. This is the reason why an experimental model, using a biphasic cell culture technique in gas phase, was developed. We report the value and the limits of this method using bronchial cells or alveolar macrophages. Exposure of lung cells to gas pollutants or particles may be responsible for either cell injury or cell activation associated with the overexpression of mRNA and the release of various bioactive mediators. In vitro assays have some limitations, particularly because the human pulmonary response to inhaled pollutants is the result of complex interactions involving many different cell types within the lungs. However, cell culture using biphasic systems in aerobiosis opens new ways for the research on the biological effects of gas pollutants.

Urtreger AJ, Diament MJ, Ranuncolo SM, Del C Vidal M, Puricelli LI, Klein SM, De Kier Joffe ED. **New murine cell line derived from a spontaneous lung tumor induces paraneoplastic syndromes.** Int J Oncol 2001;18(3):639-47.
Abstract: LP07 is a new cell line derived from P07 lung tumor, spontaneously arisen in a BALB/c mouse. LP07 is composed of heterogeneous epithelioid polyhedric cells that proliferate at a slow rate, have low plating efficiency and are unable to grow in soft agar. Only some LP07 cells expressed cytokeratins while most of them were positive for vimentin. Ultrastructure studies showed that LP07 cells established rudimentary intercellular unions, formed glandular-like conducts and presented conspicuous secretory granules, suggesting an epithelial-glandular origin, with neuroendocrine components. Upon injection LP07 cells formed poorly differentiated non-invasive adenocarcinomas, and tumor bearing mice developed leukocytosis, hypercalcemia and cachexia. This tumor cell line constitutes a useful tool to study lung tumor biology and paraneoplastic syndromes.

QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIPS

Afzelius L, Zamora I, Ridderstrom M, Andersson TB, Karlen A, Masimirembwa CM. **Competitive CYP2C9 inhibitors: enzyme inhibition studies, protein homology modeling, and three-dimensional quantitative structure-activity relationship analysis.** Mol Pharmacol 2001;59(4):909-19.
Abstract: This study describes the generation of a three-dimensional quantitative structure activity relationship (3D-QSAR) model for 29 structurally diverse, competitive CYP2C9 inhibitors defined experimentally from an initial data set of 73 compounds. In parallel, a homology model for CYP2C9 using the rabbit CYP2C5 coordinates was built. For molecules with a known interaction mode with CYP2C9, this homology model, in combination with the docking program GOLD, was used to select conformers to use in the 3D-QSAR analysis. The remaining molecules were docked, and the GRID interaction energies for all conformers proposed by GOLD were calculated. This was followed by a principal component analysis (PCA) of the GRID energies for all conformers of all compounds. Based on the similarity in the PCA plot to the inhibitors with a known interaction mode, the conformer to be used in the 3D-QSAR analysis was selected. The compounds were randomly divided into two groups, the training data set (n = 21) to build the model and the external validation set (n = 8). The PLS (partial least-squares) analysis of the interaction energies against the K(i) values generated a model with r(2) = 0.947 and a cross-validation of q(2) = 0.730. The model was able to predict the entire external data set within 0.5 log units of the experimental K(i) values. The amino acids in the active site showed complementary features to the grid interaction energies in the 3D-QSAR model and were also in agreement with mutagenesis studies.

Altomare C, Perrone G, Zonno MC, Evidente A, Pengue R, Fanti F, Polonelli L. **Biological characterization of fusapyrone and deoxyfusapyrone, two bioactive secondary metabolites of Fusarium semitectum.** J Nat Prod 2000;63(8):1131-5.
Abstract: Fusapyrone (1) and deoxyfusapyrone (2), two alpha-pyrones originally isolated from rice cultures of Fusarium semitectum, were tested in several biological assays. Compounds 1 and 2 showed considerable antifungal activity against several plant pathogenic and/or mycotoxigenic filamentous fungi, although they were inactive toward yeasts isolated from plants and the Gram-positive bacterium

Bacillus megaterium in disk diffusion assays. Compound 1 was consistently more active than 2. Among the tested fungi, Fusarium species were the least sensitive to the two pyrones, while Alternaria alternata, Ascochyta rabiei, Aspergillusflavus, Botrytis cinerea, Cladosporium cucumerinum, Phoma tracheiphila, and Penicillium verrucosum were the most sensitive. Compounds 1 and 2 also showed good inhibitory activity toward agents of human mycoses. Aspergilli were the most sensitive, while some species-specific variability was found among the Candida spp. In an Artemia salina larvae bioassay, 1 was not toxic at the highest concentration tested (500 microM), whereas the LC(50) of 2 was 37.1 microM (21.8 microg/mL). Neither 1 nor 2 was phytotoxic in a panel of assays that monitored plant-cell toxicity, as well as wilt-, chlorosis-, and necrosis-inducing activity. Moreover, 2 stimulated the root elongation of tomato seedlings at doses of 10 and 100 microM. In consideration of the biological activities evidenced in this study, 1 and 2 appear to be potential candidates for biotechnological applications, as well as good models for studies on mechanism(s) of action and structure-activity relationships.

Barratt MD. **Prediction of toxicity from chemical structure.** Cell Biol Toxicol 2000;16(1):1-13.
Abstract: The basis for the prediction of toxicity from chemical structure is that the properties of a chemical are implicit in its molecular structure. Biological activity can be expressed as a function of partition and reactivity, that is, for a chemical to be able to express its toxicity, it must be transported from its site of administration to its site of action and then it must bind to or react with its receptor or target. This process may also involve metabolic transformation of the chemical. The application of these principles to the prediction of the toxicity of new or untested chemicals has been achieved in a number of different ways covering a wide range of complexity, from computer systems containing databases of hundreds of chemicals, to simple "reading across" between chemicals with similar chemical/toxicological functionality. The common feature of the approaches described in this article is that their starting point is a mechanistic hypothesis linking chemical structure and/or functionality with the toxicological endpoint of interest. The prediction of toxicity from chemical structure can make a valuable contribution to the reduction of animal usage in the screening out of potentially toxic chemicals at an early stage and in providing data for making positive classifications of toxicity.

Barratt MD, Rodford RA. **The computational prediction of toxicity.** Curr Opin Chem Biol 2001;5(4):383-8.
Abstract: Recent developments in the prediction of toxicity from chemical structure have been reviewed. Attention has been drawn to some of the problems that can be encountered in the area of predictive toxicology, including the need for a multi-disciplinary approach and the need to address mechanisms of action. Progress has been hampered by the sparseness of good quality toxicological data. Perhaps too much effort has been devoted to exploring new statistical methods rather than to the creation of data sets for hitherto uninvestigated toxicological endpoints and/or classes of chemicals.

Bartulewicz D, Bielawski K, Bielawska A, Rozanski A. **Synthesis, molecular modelling, and antiproliferative and cytotoxic effects of carbocyclic derivatives of distamycin with chlorambucil moiety.** Eur J Med Chem 2001;36(5):461-7.
Abstract: New carbocylic analogues of distamycin and netropsin with chlorambucil moieties 5-8 have been synthesised. Data from the ethidium displacement assay showed that these compounds bind in the minor groove of DNA. The observed reduced affinity to AT pairs and increased affinity towards GC

sequences of the carbocyclic lexitropsins with chlorambucil moiety 5-8 in comparison with netropsin and distamycin was observed and rationalised by means of molecular modelling techniques. All of the compounds 5-8 showed antiproliferative and cytotoxic effects in the standard cell line of the mammalian tumour MCF-7.

Bartulewicz D, Markowska A, Wolczynski S, Dabrowska M, Rozanski A. **Molecular modelling, synthesis and antitumour activity of carbocyclic analogues of netropsin and distamycin--new carriers of alkylating elements.** Acta Biochim Pol 2000;47(1):23-35.
Abstract: A series of netropsin and distamycin analogues was synthesised and investigated by molecular modelling. The lowest-energy conformations of four carbocyclic lexitropsins, potential carriers of alkylating elements, were obtained using the HyperChem 4.0 program, and compared with the DNA-lexitropsin crystal structures from the Brookhaven National Laboratory Protein Data Bank. A method for synthesis of carbocyclic lexitropsins was elaborated, with the use of a nitro group or azobenzene as precursors for the aromatic amino group. The influence of methoxy group in ortho position with respect to amide groups on the activity of the new compounds was investigated. All of the compounds tested showed high antitumour activity in the standard cell line of mammalian tumour MCF-7.

Basak SC, Grunwald GD, Gute BD, Balasubramanian K, Opitz D. **Use of statistical and neural net approaches in predicting toxicity of chemicals.** J Chem Inf Comput Sci 2000;40(4):885-90.
Abstract: Hierarchical quantitative structure-activity relationships (H-QSAR) have been developed as a new approach in constructing models for estimating physicochemical, biomedicinal, and toxicological properties of interest. This approach uses increasingly more complex molecular descriptors in a graduated approach to model building. In this study, statistical and neural network methods have been applied to the development of H-QSAR models for estimating the acute aquatic toxicity (LC50) of 69 benzene derivatives to Pimephales promelas (fathead minnow). Topostructural, topochemical, geometrical, and quantum chemical indices were used as the four levels of the hierarchical method. It is clear from both the statistical and neural network models that topostructural indices alone cannot adequately model this set of congeneric chemicals. Not surprisingly, topochemical indices greatly increase the predictive power of both statistical and neural network models. Quantum chemical indices also add significantly to the modeling of this set of acute aquatic toxicity data.

Basak SC, Gute BD, Lucic B, Nikolic S, Trinajstic N. **A comparative QSAR study of benzamidines complement-inhibitory activity and benzene derivatives acute toxicity.** Comput Chem 2000;24 (2):181-91.
Abstract: A novel QSAR study of benzamidines complement-inhibitory activity and benzene derivatives acute toxicity is reported and a new efficient method for selecting descriptors is used. Complement-inhibitory activity QSAR models of benzamidines contain from one to five descriptors. The best, according to fitted and cross-validated statistical parameters, is shown to be the five-descriptor model. Models with a higher number of indices did not improve over the five-descriptor model. The benzene derivatives structure-toxicity models involve up to seven linear descriptors. Multiregression models, containing up to ten nonlinear descriptors, are also reported for the sake of comparison with previously obtained additivity models. Comparison with benzamidine complement-inhibitory activity models and with benzene derivatives toxicity models from the literature favors our novel approach.

Basak SC, Mills DR, Balaban AT, Gute BD. **Prediction of mutagenicity of aromatic and heteroaromatic amines from structure: a hierarchical QSAR approach.** J Chem Inf Comput Sci 2001;41(3):671-8.
Abstract: Due to the lack of experimental data, there has been increasing use of theoretical structural descriptors in the hazard assessment of chemicals. We have used a hierarchical approach to develop class-specific quantitative structure-activity relationship (QSAR) models for the prediction of mutagenicity of a set of 95 aromatic and heteroaromatic amines. The hierarchical approach begins with the simplest molecular descriptors, the topostructural, which encode limited chemical information. The complexity is then increased, adding topochemical, geometric, and finally quantum chemical parameters. We have also added log P to the set of independent variables. The results indicate that the topological parameters, i.e., the topostructural and topochemical indices, explain the majority of the variance, and that the inclusion of log P, geometric, and quantum chemical parameters does not result in significantly improved predictive models.

Beger RD, Freeman JP, Lay JO Jr, Wilkes JG, Miller DW. **(13)C NMR and electron ionization mass spectrometric data-activity relationship model of estrogen receptor binding.** Toxicol Appl Pharmacol 2000;169(1):17-25.
Abstract: Two Spectroscopic Data-Activity Relationship (SDAR) models based on (13)C nuclear magnetic resonance (NMR) and electron ionization mass spectra (EI MS) data were developed for 108 compounds whose relative binding affinities (RBA) to the estrogen receptor are known. The (13)C NMR and EI MS data were used as spectrometric digital fingerprints to reflect the electronic and structural characteristics of the compounds. Both SDAR models segregated the 108 compounds into 20 strong, 15 medium, and 73 weak relative binding classifications. The first SDAR model, based on (13)C NMR data alone, gave a leave-one-out (LOO) cross-validation of 75.0%. The second SDAR model, based on a composite of (13)C NMR and EI MS data, gave a LOO cross-validation of 82.4%. Many of the misidentifications from the cross-validations were between medium and weak classifications, where there were fewer specific spectrometric characteristics to identify the relationship of spectra to estrogen receptor binding. Real and predicted (13)C NMR chemical shifts were used to test the predictive behavior of both SDAR models. The ease of use and speed of SDAR modeling may facilitate their use with other toxicological endpoints.

Bogaerts P, Bohatier J, Bonnemoy F. **Use of the ciliated protozoan Tetrahymena pyriformis for the assessment of toxicity and quantitative structure--activity relationships of xenobiotics: comparison with the Microtox test.** Ecotoxicol Environ Saf 2001;49(3):293-301.
Abstract: Cytotoxicity and quantitative structure-activity relationships of 13 inorganic and 21 organic substances were determined using three bioassays performed on the ciliated protozoan Tetrahymena pyriformis and the luminescent bacterium Vibrio fischeri. The best concordance of toxicity results was observed between the T. pyriformis FDA--esterase activity and population growth inhibition tests for the organic compounds. The sensitivity of these two assays is compared with that of the Microtox test. The T. pyriformis FDA test showed a high sensitivity is most cases. The aim of the current research was to determine whether the relative toxicity of metal ions and organic molecules, with these three bioassays, was predictable using three ion characteristics and hydrophobicity, respectively. For metal ions, the

variable that best modeled the toxicity data obtained with the two T. pyriformis tests was the softness index [sigma(p), i.e., (coordinate bond energy of the metal fluoride--coordinate bond energy of the metal iodide)/(coordinate bond energy of the metal fluoride)]. No correlation was found with the Microtox test. For organic compounds, a significant correlation was observed between the hydrophobicity coefficient and the toxicity data. This correlation is closer with the two tests using Tetrahymena. Copyright 2001 Academic Press.

Bradbury S, Kamenska V, Schmieder P, Ankley G, Mekenyan O. **A computationally based identification algorithm for estrogen receptor ligands: part 1. Predicting hERalpha binding affinity.** Toxicol Sci 2000;58(2):253-69.
Abstract: The common reactivity pattern (COREPA) approach is a 3-dimensional, quantitative structure activity relationship (3-D QSAR) technique that permits identification and quantification of specific global and local stereoelectronic characteristics associated with a chemical's biological activity. It goes beyond conventional 3-D QSAR approaches by incorporating dynamic chemical conformational flexibility in ligand-receptor interactions. The approach provides flexibility in screening chemical data sets in that it helps establish criteria for identifying false positives and false negatives, and is not dependent upon a predetermined and specified toxicophore or an alignment of conformers to a lead compound. The algorithm was recently used to screen chemical data sets for rat androgen receptor binding affinity. To further explore the potential application of the algorithm in establishing reactivity patterns for human estrogen receptor alpha (hERalpha) binding affinity, the stereoelectronic requirements associated with the binding affinity of 45 steroidal and nonsteroidal ligands to the receptor were defined. Reactivity patterns for relative hERalpha binding affinity (RBA; 17ss-estradiol = 100%) were established based on global nucleophilicity, interatomic distances between electronegative heteroatoms, and electron donor capability of heteroatoms. These reactivity patterns were used to establish descriptor profiles for identifying and ranking compounds with RBA of > 150%, 100-10%, 10-1%, and 1-0.1%. Increasing specificity of reactivity patterns was detected for ligand data sets with RBAs above 10%. Using the results of this analysis, an exploratory expert system was developed for use in ranking relative ER binding affinity potential for large chemical data sets.

Breier A, Drobna Z, Docolomansky P, Barancik M. **Cytotoxic activity of several unrelated drugs on L1210 mouse leukemic cell sublines with P-glycoprotein (PGP) mediated multidrug resistance (MDR) phenotype**. A QSAR study. Neoplasma 2000;47(2):100-6.
Abstract: L1210/VCR-1 and L1210/VCR-2 cell lines are multidrug resistant (MDR) sublines obtained by adaptation of mouse leukemic cell line L1210 to vincristine and, the development of MDR in these cell lines has been found to be associated with an overexpression of P-glycoprotein (PGP). In the present work we studied the relationship between the structure of 15 cytotoxic active substances (drugs) and their cytotoxicities on L1210/VCR-1 and L1210/VCR-2 resistant cell lines. The resistance of these MDR cells to the respective drugs was expressed as the ratio of IC50 values obtained for resistant and sensitive cells. These values of resistance were correlated with the following physico-chemical constants of the test substances: binding energy, Ebind; total energy of the molecule, Esum; aromaticity, Kpi; molecular weight, Mw; acidobasic constant, pKa; partition coefficient in water/octanol two phase system, log(p). It has been found that according to the cytotoxic effects the tested drugs may be divided into three groups: (i) drugs with higher cytotoxicity to the resistant cell lines as to sensitive cells

(collateral hypersensitivity); (ii) drugs exhibiting approximately similar effects on sensitive and resistant cell lines; (iii) drugs with weaker cytotoxicity to resistant cells than to sensitive cells. No direct correlations with any physico-chemical constant described above could be established for cell resistance to the drug studied. However, resistance values could be fitted by multiple exponential regression with all described physico-chemical constants implied as six independent variables. The latter procedure made us to conclude that the ability of a drug to be a substrate for PGP is connected with its fulfilling the following criteria: (i) flexible structure of its molecule; (ii) molecular weight lower than approximately 1,300 g/mol; (iii) nonprotonized character at pH 7.0.

Bruggemann R, Pudenz S, Carlsen L, Sorensen PB, Thomsen M, Mishra RK. **The use of Hasse diagrams as a potential approach for inverse QSAR.** SAR QSAR Environ Res 2001;11(5-6):473-87.
Abstract: Quantitative structure-activity relationships are often based on standard multidimensional statistical analyses and sophisticated local and global molecular descriptors. Here, the aim is to develop a tool helpful to define a molecule or a class of molecules which fulfills pre-described properties, i.e., an Inverse QSAR approach. If highly sophisticated descriptors are used in QSAR, the structure and then the synthesis recipe may be hard to derive. Thus, descriptors, from which the synthesis recipe can be easily derived, seem appropriate to be included within this study. However, if descriptors simple enough to be useful for defining syntheses recipes of chemicals were used, the accuracy of a numeric expression may fail. This paper suggests a method, based on very simple elements of the theory of partially ordered sets, to find a qualitative basis for the relationship between such fairly simple descriptors on the one side and a series of ecotoxicological properties, on the other side. The partial order ranking method assumes neither linearity nor certain statistical distribution properties. Therefore the method may be more general compared to many standard statistical techniques. A series of chlorinated aliphatic compounds has been used as an illustrative example and a comparison with more sophisticated descriptors derived from quantum chemistry and graph theory is given. Among the results, it was disclosed that only for algae lethal concentration, as one of the four ecotoxicological properties, the synthesis specific predictors seem to be good estimators. For all other ecotoxicological properties quantum chemical descriptors appear as the more suitable estimators.

Burden FR. **Quantitative structure-activity relationship studies using Gaussian processes.** J Chem Inf Comput Sci 2001;41(3):830-5.
Abstract: A Gaussian process method (GPM) is described and applied to the production of some QSAR models. These models have the potential to solve a number of problems which arise in QSAR modeling in that no parameters have to be supplied and only one hyperparameter is used in finding the optimal solution. The application of the method to QSAR is illustrated using data sets of compounds active at the benzodiazepine and muscarinic receptors as well as the data set of the toxicity of substituted benzenes to the ciliate, Tetrahymena Pyriformis.

Burden FR, Ford MG, Whitley DC, Winkler DA. **Use of automatic relevance determination in QSAR studies using Bayesian neural networks.** J Chem Inf Comput Sci 2000;40(6):1423-30.
Abstract: We describe the use of Bayesian regularized artificial neural networks (BRANNs) coupled with automatic relevance determination (ARD) in the development of quantitative structure-activity relationship (QSAR) models. These BRANN-ARD networks have the potential to solve a number of

problems which arise in QSAR modeling such as the following: choice of model; robustness of model; choice of validation set; size of validation effort; and optimization of network architecture. The ARD method ensures that irrelevant or highly correlated indices used in the modeling are neglected as well as showing which are the most important variables in modeling the activity data. The application of the methods to QSAR of compounds active at the benzodiazepine and muscarinic receptors as well as some toxicological data of the effect of substituted benzenes on Tetetrahymena pyriformis is illustrated.

Burden FR, Winkler DA. **A quantitative structure--activity relationships model for the acute toxicity of substituted benzenes to Tetrahymena pyriformis using Bayesian-regularized neural networks.** Chem Res Toxicol 2000;13(6):436-40.
Abstract: We have used a new, robust structure-activity mapping technique, a Bayesian-regularized neural network, to develop a quantitative structure-activity relationships (QSAR) model for the toxicity of 278 substituted benzenes toward Tetrahymena pyriformis. The independent variables used in the modeling were derived solely from the molecular structure, and the model was tested on 20% of the data set selected from the whole set by cluster analysis and which had not been used in training the network. The results show that the method is robust and reliable and give results for mixed class compounds which are comparable to earlier QSAR work on single-chemical class subsets of the 278 compounds and which employed measured physicochemical parameters as independent variables. Comparisons of Bayesian neural net models with those derived by classical PLS analysis showed the superiority of our method. The method appears to be able to model more diverse chemical classes and more than one mechanism of toxicity.

Cao Z, Pantazis P, Mendoza J, Early J, Kozielski A, Harris N, Vardeman D, Liehr J, Stehlin JS, Giovanella B. **Structure-activity relationship of alkyl camptothecin esters.** Ann N Y Acad Sci 2000;922:122-35.
Abstract: The cytotoxicity of camptothecin (CPT) esters 1-6 was measured. Like parental camptothecin, esters 2 and 3, but not 1, 4, 5, and 6, inhibited proliferation of human leukemia cells in culture and induced programmed cell death as assessed by flow cytometry studies. Exhibition of similar levels of antiproliferative activities of CPT 2 and 3 required different incubation time periods in cell cultures, with CPT and 3 requiring the shortest and longest periods, respectively. Both 2 and 3 were inactive against cells resistant to the semisynthetic CPT derivative 9-nitrocamptothecin and unable to stabilize DNA-topoisomerase I (Topo I) "cleavable complexes" in a cell-free system, suggesting that Topo I activity was required but insufficient for the mechanism of action of 2 and 3. Mouse liver homogenate converted esters to parental CPT, but the conversion rates were different with different esters. Of four tested esters in this experiment, ester 2 had the fastest conversion rate. In vivo studies showed that ester 2 had an exceptional lack of toxicity in nude mice, even at enormous doses, and demonstrated extensive activity against human breast and colon tumors grown as xenografts in immunodeficient nude mice, whereas no antitumor activity was observed for the other esters. In conclusion, ester 2 is a prodrug of the antitumor compound CPT, and it can be administered at very high doses in mice with no appearance of toxicity. This study provides a basis for further evaluation of CPT ester 2 as an investigational anticancer agent.

Cash GG. **Prediction of the genotoxicity of aromatic and heteroaromatic amines using**

electrotopological state indices. Mutat Res 2001;491(1-2):31-7.
Abstract: In the past decade, electrotopological state (E-state) indices have come into their own as useful descriptors for correlating a variety of physicochemical and biological properties of chemical compounds. Genotoxicity and mutagenicity, however, appear not to have been previously considered. In the present study, the genotoxicity of a set of 95 aromatic and heteroaromatic amines, which has been modeled previously using several sets of parameters, is modeled using E-state indices, both with and without principal components analysis. Parallels are drawn between E-state indices that were important in these models and other types of descriptors found significant in previous studies, thus, shedding light on connections to the molecular mechanism of activity. The best result had a correlation coefficient r = 0.876 and a standard error s< or = 1 log unit. These values are comparable to those in previously published models that were based on topological/geometric or on physicochemical parameters. They are not as good as those for a model based on descriptors derived from extensive quantum mechanical analysis, but E-state indices are much easier to compute.

Cenas N, Nemeikaite-Ceniene A, Sergediene E, Nivinskas H, Anusevicius Z, Sarlauskas J. **Quantitative structure-activity relationships in enzymatic single-electron reduction of nitroaromatic explosives: implications for their cytotoxicity.** Biochim Biophys Acta 2001;1528(1):31-8.
Abstract: The mechanisms of cytotoxicity of polynitroaromatic explosives, an important group of environmental pollutants, remain insufficiently studied so far. We have found that the rate constants of single-electron enzymatic reduction, and the enthalpies of single-electron reduction of nitroaromatic compounds (DeltaHf(ArNO(2)(-*)), obtained by quantum mechanical calculation, may serve as useful tools for the analysis of cytotoxicity of nitroaromatic explosives with respect to the possible involvement of oxidative stress. The single-electron reduction rate constants of a number of explosives including 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenyl-N-methylnitramine (tetryl), and model nitroaromatic compounds by ferredoxin:NADP(+) reductase (FNR, EC 1.18.1.2) and NADPH: cytochrome P-450 reductase (P-450R, EC 1.6.2.4) increased with a decrease in DeltaHf(ArNO(2)(-*)). This indicates that the reduction rates are determined by the electron transfer energetics, but not by the particular structure of the explosives. The cytotoxicity of explosives to bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) increased with a corresponding increase in their reduction rate constant by P-450R and FNR, or with a decrease in their DeltaHf(ArNO(2)(-*)). This points to an importance of oxidative stress in the toxicity of explosives in this cell line, which was further evidenced by the protective effects of desferrioxamine and the antioxidant N,N'-diphenyl-p-phenylene diamine, and an increase in lipid peroxidation. DT-diaphorase (EC 1.6.99.2) exerted a minor and equivocal role in the cytotoxicity of explosives to FLK cells.

Combes RD. **The use of structure-activity relationships and markers of cell toxicity to detect non-genotoxic carcinogens.** Toxicol In Vitro 2000;14(4):387-99.
Abstract: In contrast to the situation for genotoxic carcinogens, few in vitro tests exist that can detect early markers of the events thought to be associated with non-genotoxic carcinogenesis. Also, comparatively little is known about the quantitative structure-activity relationships (Q)SARs of these agents. This review discusses published SAR studies conducted on non-genotoxic carcinogens, in relation to the use of several markers of in vitro cell toxicity (inhibition of gap-junctional intercellular communication, inhibition of tubulin polymerization, modulation of apoptosis and induction of cell

proliferation), which are used as endpoints for screening this class of carcinogen. Much of the work has involved the identification of new biophores (substructural features of molecules associated with toxicity), as well as other structural features, which are thought to predispose the chemicals to ligand binding with specific target molecules acting as possible receptors (e.g. protein kinase C, the oestrogen, peroxisome-proliferator and tubulin protein receptors), implicated in the mechanism of toxicity involved. It is concluded that (a) there is an urgent need for more information on (Q)SARs for non-genotoxic carcinogens; (b) this information should be acquired by using several different approaches in a variety of laboratories; and (c) such research should proceed together with more studies on the mechanisms of cell toxicity caused by these chemicals, including the identification and characterisation of further specific receptors involved in mediating the various types of cell toxicity associated with this type of carcinogenesis.

Constantine KL. **Evaluation of site-directed spin labeling for characterizing protein-ligand complexes using simulated restraints.** Biophys J 2001;81(3):1275-84.
Abstract: Simulation studies have been performed to evaluate the utility of site-directed spin labeling for determining the structures of protein-ligand complexes, given a known protein structure. Two protein-ligand complexes were used as model systems for these studies: a 1.9-A-resolution x-ray structure of a dihydrofolate reductase mutant complexed with methotrexate, and a 1.5-A-resolution x-ray structure of the V-Src tyrosine kinase SH2 domain complexed with a five-residue phosphopeptide. Nitroxide spin labels were modeled at five dihydrofolate reductase residue positions and at four SH2 domain residue positions. For both systems, after energy minimization, conformational ensembles of the spin-labeled residues were generated by simulated annealing while holding the remainder of the protein-ligand complex fixed. Effective distances, simulating those that could be obtained from (1)H-NMR relaxation measurements, were calculated between ligand protons and the spin labels. These were converted to restraints with several different levels of precision. Restrained simulated annealing calculations were then performed with the aim of reproducing target ligand-binding modes. The effects of incorporating a few supplementary short-range (< or =5.0 A) distance restraints were also examined. For the dihydrofolate reductase-methotrexate complex, the ligand-binding mode was reproduced reasonably well using relatively tight spin-label restraints, but methotrexate was poorly localized using loose spin-label restraints. Short-range and spin-label restraints proved to be complementary. For the SH2 domain-phosphopeptide complex without the short-range restraints, the peptide did not localize to the correct depth in the binding groove; nevertheless, the orientation and internal conformation of the peptide was reproduced moderately well. Use of the spin-label restraints in conjunction with the short-range restraints resulted in relatively well defined structural ensembles. These results indicate that restraints derived from site-directed spin labeling can contribute significantly to defining the orientations and conformations of bound ligands. Accurate ligand localization appears to require either a few supplementary short-range distance restraints, or relatively tight spin-label restraints, with at least one spin label positioned so that some of the restraints draw the ligand into the binding pocket in the latter case.

Costantino G, Macchiarulo A, Entrena Guadix A, Pellicciari R. **QSAR and molecular modeling studies of baclofen analogues as GABA(B) agonists. Insights into the role of the aromatic moiety in GABA(B) binding and activation.** J Med Chem 2001;44(11):1827-32.

Abstract: An integrated QSAR/molecular modeling study is carried out on a series of baclofen analogues with the aim of addressing the role of their aromatic moiety in GABA(B) receptor binding and activation. The strong correlation found between electronic descriptors (HOMO and LUMO orbital energies) and the biological activity expressed as receptor binding is discussed also on the basis of available experimental mutagenesis data and of the results obtained from homology modeling of GABA (B) receptor. In particular, it can be inferred from the QSAR analysis that the ability of being involved in aromatic-aromatic pi interaction is the distinctive feature of the p-chlorophenyl moiety of baclofen. This conclusion is confirmed by homology modeling and docking studies which indicate that the p-chlorophenyl moiety of baclofen is disposed into a pocket formed by Tyr366 and Tyr395. These results are discussed in terms of mechanism of GABA(B) activation promoted by baclofen or GABA.

Cotecchia S, Rossier O, Fanelli F, Leonardi A, De Benedetti PG. **The alpha 1a and alpha 1b-adrenergic receptor subtypes: molecular mechanisms of receptor activation and of drug action.** Pharm Acta Helv 2000;74(2-3):173-9.
Abstract: In this chapter we summarize some aspects of the structure-functional relationship of the alpha 1a and alpha 1b-adrenergic receptor subtypes related to the receptor activation process as well as the effect of different alpha-blockers on the constitutive activity of the receptor. Molecular modeling of the alpha 1a and alpha 1b-adrenergic receptor subtypes and computational simulation of receptor dynamics were useful to interpret the experimental findings derived from site directed mutagenesis studies.

Croni MT, Bowers GS, Sinks GD, Schultz TW. **Structure-toxicity relationships for aliphatic compounds encompassing a variety of mechanisms of toxic action to Vibrio fischeri.** SAR QSAR Environ Res 2000;11(3-4):301-12.
Abstract: QSARs based upon the logarithm of the octanol-water partition coefficient, log P, and energy of the lowest unoccupied molecular orbital, ELUMO were developed to model the toxicity of aliphatic compounds to the marine bacterium Vibrio fischeri. Statistically robust, hydrophobic-dependent QSARs were found for chloroalcohols and haloacetonitriles. Modelling of the toxicity of the haloesters and the diones required the use of terms to describe both hydrophobicity and electrophilicity. The differences in intercepts, slopes, and fit of these models suggest different electrophilic mechanisms occur between classes, as well as within the diones and haloesters. In order to model globally the toxicity of aliphatic compounds to V. fischeri, all the data determined in this study were combined with those determined previously for alkanones, alkanals, and alkenals. A highly predictive two-parameter QSAR [pT15 = 0.760(log P) - 0.625(ELUMO) - 0.466; n = 63, s = 0.462, r2 = 0.846, F = 171, Pr > F = 0.0001] was developed for the combined data that models across classes and is independent of mechanisms of action. The toxicity of these compounds to V. fischeri compares well to the toxicity (50% population growth inhibition) to the ciliate Tetrahymena pyriformis (r2 = 0.850).

Cronin MT. **Prediction of drug toxicity.** Farmaco 2001;56(1-2):149-51.
Abstract: The use of computer-aided methods to predict the toxicity of drugs is described. These methods can assist in the identification of toxic compounds early in the drug development process. Thus, there is potential for these methods to be combined with combinatorial synthesis and library design. Quantitative structure-activity relationships allow for the prediction of individual endpoints, usually for restricted groups of compounds. Expert systems for toxicity prediction are based on a number of

methodologies, each with its own strengths and weaknesses. The relative merit of each individual technique and methodology is described. However, more toxicity data are required, both to produce and to validate expert systems. Potential sources of new data include the use of high-throughput screening and microarrays for toxicology.

Dai J, Jin L, Yao S, Wang L. **Prediction of partition coefficient and toxicity for benzaldehyde compounds by their capacity factors and various molecular descriptors.** Chemosphere 2001;42 (8):899-907.
Abstract: The log Kow and log Sw values of 14 substituted benzaldehyde compounds were determined by the shake-flask method. Acute toxicities of 14 substituted benzaldehyde compounds to Daphnia magna were recorded. Their capacity factors (k') were determined by reversed phased high-performance liquid chromatography (RP-HPLC) on C18 column and methanol-water eluent. Molecular connectivity indices, the linear solvation energy relationships (LSER) parameters, and quantum chemical parameters were calculated for the tested chemicals and used to develop quantitative structure-retention relationship (QSRR) and quantitative structure-property/activity relationship (QSPR/QSAR). Results demonstrated that the molecular connectivity indices, LSER parameters, and quantum chemical parameters could be used to predict the k' for compounds studied, LSER method was more accurate. The results also show that chromatographic retention data, log k', can be used to predict log Kow and log Sw for tested compounds. The log k'w can be directly utilized as hydrophobic descriptors to predict the toxicity to D. Magna for benzaldehyde compounds.

Del Fiol G, Rocha BH, Kuperman GJ, Bates DW, Nohama P. **Comparison of two knowledge bases on the detection of drug-drug interactions.** Proc AMIA Symp 2000;171-5.
Abstract: This paper describes a drug ordering decision support system that helps with the prevention of adverse drug events by detecting drug-drug interactions in drug orders. The architecture of the system was devised in order to facilitate its use attached to physician order entry systems. The described model focuses in issues related to knowledge base maintenance and integration with external systems. Finally, a retrospective study was performed. Two knowledge bases, developed by different academic centers, were used to detect drug-drug interactions in a dataset with 37,237 drug prescriptions. The study concludes that the proposed knowledge base architecture enables content from other knowledge sources to be easily transferred and adapted to its structure. The study also suggests a method that can be used on the evaluation and refinement of the content of drug knowledge bases.

Devillers J. **A general QSAR model for predicting the acute toxicity of pesticides to Lepomis macrochirus.** SAR QSAR Environ Res 2001;11(5-6):397-417.
Abstract: A Quantitative Structure-Activity Relationship (QSAR) model was derived for estimating the acute toxicity of pesticides against Lepomis macrochirus under varying experimental conditions. Chemicals were described by means of autocorrelation descriptors encoding lipophilicity (H(0) to H(5)) and the H-bonding acceptor ability (HBA(0)) and H-bonding donor ability (HBD(0)) of the pesticides. A three-layer feedforward neural network trained by the back-propagation algorithm was used as statistical engine for deriving a powerful QSAR model accounting for the weight of the fish, time of exposure, temperature, pH, and water hardness.

Devillers J, Flatin J. **A general QSAR model for predicting the acute toxicity of pesticides to Oncorhynchus mykiss.** SAR QSAR Environ Res 2000;11(1):25-43.
Abstract: A Quantitative Structure-Activity Relationship (QSAR) model was derived for estimating the acute toxicity of pesticides against Oncorhynchus mykiss under varying experimental conditions. Chemicals were described by means of autocorrelation descriptors encoding lipophilicity (H0 to H5) and the H-bonding acceptor ability (HBA0) and H-bonding donor ability (HBD0) of the pesticides. A three-layer feedforward neural network trained by the back-propagation algorithm was used as statistical engine for deriving a powerful QSAR model accounting for the weight of the fish, time of exposure, temperature, pH, and hardness.

Ekins S, Wrighton SA. **Application of in silico approaches to predicting drug--drug interactions.** J Pharmacol Toxicol Methods 2001;45(1):65-9.
Abstract: In an environment driven to find the next blockbuster drug, failure years into a project should not be an option. Recent studies have shown that poor absorption, distribution, metabolism, and excretion (ADME), and the related properties of toxicity and pharmacokinetics are responsible for a large proportion of failures. One way to understand and potentially predict molecules likely to be successful in humans as drugs from an ADME point of view is to use simulations. Such simulations may include simple rule-based approaches, structure--activity relationships, three-dimensional quantitative structure--activity relationships (3D-QSAR), and pharmacophores. All of these represent useful tools in understanding metabolism by the cytochromes P450, predicting drug--drug interactions (DDIs), and other pharmacokinetic parameters. The present paper briefly reviews the application of some computational tools applied to predicting DDIs and will provide the reader with an idea of their utility.

Fan Y, Shi LM, Kohn KW, Pommier Y, Weinstein JN. **Quantitative structure-antitumor activity relationships of camptothecin analogues: cluster analysis and genetic algorithm-based studies.** J Med Chem 2001;44(20):3254-63.
Abstract: Topoisomerase 1 (top1) inhibitors are proving useful against a range of refractory tumors, and there is considerable interest in the development of additional top1 agents. Despite crystallographic studies, the binding site and ligand properties that lead to activity are poorly understood. Here we report a unique approach to quantitative structure-activity relationship (QSAR) analysis based on the National Cancer Institute's (NCI) drug databases. In 1990, the NCI established a drug discovery program in which compounds are tested for their ability to inhibit the growth of 60 different human cancer cell lines in culture. More than 70 000 compounds have been screened, and patterns of activity against the 60 cell lines have been found to encode rich information on mechanisms of drug action and drug resistance. Here, we use hierarchical clustering to define antitumor activity patterns in a data set of 167 tested camptothecins (CPTs) in the NCI drug database. The average pairwise Pearson correlation coefficient between activity patterns for the CPT set was 0.70. Coherence between chemical structures and their activity patterns was observed. QSAR studies were carried out using the mean 50% growth inhibitory concentrations (GI(50)) for 60 cell lines as the dependent variables. Different statistical methods, including stepwise linear regression, principal component regression (PCR), partial least-squares regression (PLS), and fully cross-validated genetic function approximation (GFA) were applied to construct quantitative structure-antitumor relationship models. For our data set, the GFA method performed better in terms of correlation coefficients and cross-validation analysis. A number of

molecular descriptors were identified as being correlated with antitumor activity. Included were partial atomic charges and three interatomic distances that define the relative spatial dispositions of three significant atoms (the hydroxyl hydrogen of the E-ring, the lactone carbonyl oxygen of the E-ring, and the carbonyl oxygen of the D-ring). The cross-validated r(2) for the final GFA model was 0.783, indicating a predictive QSAR model.

Franke R, Gruska A, Giuliani A, Benigni R. **Prediction of rodent carcinogenicity of aromatic amines: a quantitative structure-activity relationships model.** Carcinogenesis 2001;22(9):1561-71.
Abstract: The aromatic amines are widely used industrial chemicals and can be found in tobacco smoke as well as in products generated during cooking. In a previous study, we established quantitative structure-activity relationship (QSAR) models linking the carcinogenic potency of non-heterocyclic carcinogenic aromatic amines to a series of molecular determinants. We also found that QSAR models for carcinogenic potency were inadequate in describing the difference between carcinogenic and non-carcinogenic amines [Benigni,R., Giuliani,A., Franke,R. and Gruska,A. (2000) CHEM: Rev., 100, 3697-3714]. In this paper, we derived specific QSAR models for separating active from inactive amines. It appeared that hydrophobicity (as measured by the octanol/water partition coefficient, logP) played a major role in modulating the potency of the carcinogens, whereas mainly electronic (reactivity) and steric characteristics separated the carcinogens from the non-carcinogens. Interestingly, a similar pattern was previously demonstrated by us regarding their mutagenic activity [Benigni,R., Passerini,L., Gallo, G., Giorgi,F. and Cotta-Ramusino,M. (1998) ENVIRON: Mol. Mutagen., 32, 75-83]. Based on the QSAR models found, the molecular determinants of the mechanisms of action of aromatic amines are discussed in detail. The QSAR models obtained can be used directly for estimating the carcinogenicity of other non-heterocyclic aromatic amines for which experimental data are not available. With the QSARs in Benigni et al. (2000) and the present results, a two-step prediction of carcinogenicity of aromatic amines is possible: (i) step 1, yes/no activity from the discriminant functions; and (ii) step 2, if the answer from step 1 is yes then prediction of the degree of potency from the equations in Benigni et al. (2000). Thus, QSAR models can contribute to the following: the direct synthesis of safer chemicals; the estimation of the risk posed by amines present in the environment; setting priorities for further experimentation, thus also reducing the use of experimental animals. Whereas the quality of in vivo experimental data is often questioned, the robustness and interpretability of the present results strongly support the reliability of the rodent carcinogenicity assay.

Gallegos A, Robert D, Girones X, Carbo-Dorca R. **Structure-toxicity relationships of polycyclic aromatic hydrocarbons using molecular quantum similarity.** J Comput Aided Mol Des 2001;15 (1):67-80.
Abstract: The establishment of quantitative structure-activity relationship (QSAR) models for the toxicity of polycylic aromatic hydrocarbons (PAHs) is described. Two properties, in vitro percutaneous absorption in rat skin and discrete levels of carcinogenic activity, are examined using molecular quantum similarity measures (MQSM). The results show that MQSM produces comparable, or even better, results than other approaches using physicochemical, topological and quantum-chemical molecular descriptors. Furthermore, a careful analysis puts into evidence that most of the information characterized by the original descriptors is in fact contained in the molecular density functions, the basis of MQSM. The present paper, together with several other reported by our laboratory, proves that MQSM

might be appropriate theoretical tools for QSAR and computer-aided drug design, comparable to other highly predictive QSAR methodologies.

Garg R, Denny WA, Hansch C. **Comparative QSAR studies on substituted bis-(acridines) and bis-(phenazines)-carboxamides: a new class of anticancer agents.** Bioorg Med Chem 2000;8(7):1835-9.
Abstract: Quantitative structure-activity relationships have been formulated for two sets of DNA binding topoisomerase agents (bis-acridines and bis-phenazines) acting on murine P388 leukemia cells, murine Lewis lung carcinoma (LL(C)) cells and human Jurkat leukemia wild-type (JL(C)) cells. For the acridines, all three QSARs (1-3) show only a (small negative) hydrophobic effect. In sharp contrast, the phenazines in all three studies (4-6) show a strong hydrophobic effect, with the optimum ClogP being near 7.3 for all examples. This suggests that, despite the structural similarity of the compounds, different modes of enzyme and/or DNA binding may be involved.

Garg R, Kapur S, Hansch C. **Radical toxicity of phenols: a reference point for obtaining perspective in the formulation of QSAR.** Med Res Rev 2001;21(1):73-82.
Abstract: In this report we discuss some of the surprising ways phenols interact in vivo and how some of their toxic activity can be understood in terms of QSAR and in fact can be related via electronic terms to be similar to processes of simple chemical reactions. A simple two-term QSAR is found to be a good predictor of estrogenic toxicity. However, it is also shown that even the simplest of phenols can yield quite unexpected results than can be elucidated via QSAR. We still have a long way to go before we can predict under what conditions a phenol will produce toxic effects such as cancer and how much phytophenols one can consume before reaping a toxic reaction.

Garg R, Kurup A, Hansch C. **Comparative QSAR: on the toxicology of the phenolic OH moiety.** Crit Rev Toxicol 2001;31(2):223-45.
Abstract: In this report we consider the effect of substituents on phenol toxicity and show how the parameters used in Quantitative Structure-Activity Relationships (QSAR) can be used to draw mechanistic inferences of value in understanding the reasons behind the various types of toxicity. In particular, we are interested in gaining clearer insight into mechanisms via the Hammett-type parameters sigma, sigma(-), sigma(+) and octanol/water parti tion coefficients. Particular attention is given to the role of radical reactions and their role in attacking DNA to cause cancer or estrogenic toxicity.

Garg S, Achenie LE. **Mathematical programming assisted drug design for nonclassical antifolates.** Biotechnol Prog 2001;17(3):412-8.
Abstract: A concept from optimization theory, specifically, mathematical programming, is proposed for designing drugs with desired properties. The mathematical programming formulation is solved to obtain the optimal descriptor values, which are employed in the Cerius(2) modeling environment to infer the optimal lead candidates, in the sense that they exhibit both high selectivity and activity while ensuring low toxicity. It has been observed that unique substituent groups and their molecular conformations are responsible for attaining the goal of simultaneous high selectivity and activity. Both linear and nonlinear quantitative structure activity relationships (QSARs) have been developed for use in the proposed approach. A comparative study of these models is done, and it is shown that the QSARs are well represented by nonlinear models. The proposed mathematical programming strategy has been

demonstrated for a class of nonclassical antifolates for Pneumocistis carinii and Toxoplasma gondii dihydofolate reductase. Some of the potential leads found in this study have biological properties similar to those in the open literature. We believe the technique proposed is general and can be applied to other structure based drug design.

gese E, Bettiol C, Agnoli F, Zambon A, Mazzola M, Ghirardini AV. **Assessment of chloroaniline toxicity by the submitochondrial particle assay.** Environ Toxicol Chem 2001;20(4):826-32.
Abstract: The effects on mitochondrial respiration of 15 chloroanilines were recorded by using the in vitro response of submitochondrial particles (SMP) from beef heart mitochondria. The bioassay procedure for SMP is based on the process of reverse electron transfer, which can be negatively affected by inhibitors of electron transport, by uncouplers, and by chemicals that impair membrane integrity. The EC50 values, determined for the tested chloroanilines, indicate a general tendency of increasing toxicity with increasing chlorine substitution. In order to validate the results obtained and to evaluate the capability of the SMP assay to reproduce the toxic effects of the examined compounds on different freshwater species, the EC50 values were compared with literature data from other biological assays regarding both in vitro systems and whole organisms. A good correlation was found in particular with two widely used testing systems, the Microtox and the Tetrahymena assays. In addition, quantitative structure-activity relationships (QSARs) were established between the EC50 values and various molecular descriptors for hydrophobic, steric, and electronic interactions. The results obtained were utilized to elucidate the mechanism of toxic action of chloroanilines, which are commonly reported to act by the polar narcosis mode of action. Moreover, they confirmed that the SMP assay can be a useful tool for studying the toxicity of chemicals that act nonspecifically by impairing membrane structure and functions.

Girones X, Amat L, Robert D, Carbo-Dorca R. **Use of electron-electron repulsion energy as a molecular descriptor in QSAR and QSPR studies**. J Comput Aided Mol Des 2000;14(5):477-85.
Abstract: Electron-electron repulsion energy (<Vee>) is presented as a new molecular descriptor to be employed in QSAR and QSPR studies. Here it is shown that this electronic energy parameter is connected to molecular quantum similarity measures (MQSM), and as a consequence can be considered as a complement to steric and electronic parameters in description of molecular properties and biological responses of organic compounds. The present strategy considers the molecule as a whole, thus there is no need to employ contributions of isolated fragments as in many calculations of molecular descriptors, like log P or the Free-Wilson analysis. The procedure has been tested in a widespread set of molecules: alcohols, alkanamides, indole derivatives and 1-alkylimidazoles. Molecular properties, as well as toxicity, are correlated using <Vee> as a parameter, and extensions to the method are given for handling difficult systems. In almost all studied cases, satisfactory linear relationships were finally obtained.

Gottmann E, Kramer S, Pfahringer B, Helma C. **Data quality in predictive toxicology: reproducibility of rodent carcinogenicity experiments.** Environ Health Perspect 2001;109(5):509-14.
Abstract: We compared 121 replicate rodent carcinogenicity assays from the two parts (National Cancer Institute/National Toxicology Program and literature) of the Carcinogenic Potency Database (CPDB) to estimate the reliability of these experiments. We estimated a concordance of 57% between the overall rodent carcinogenicity classifications from both sources. This value did not improve substantially when

additional biologic information (species, sex, strain, target organs) was considered. These results indicate that rodent carcinogenicity assays are much less reproducible than previously expected, an effect that should be considered in the development of structure-activity relationship models and the risk assessment process.

Gramatica P, Consolaro F, Pozzi S. **QSAR approach to POPs screening for atmospheric persistence.** Chemosphere 2001;43(4-7):655-64.
Abstract: The environmental behaviour of a persistent organic pollutant (POP) is mainly controlled by its persistence, its tendency to undergo long-range transport (LRT) and its physicochemical properties. Atmospheric half-life is one of the criteria commonly used to study air persistence and LRT potential. For the 12 UNEP POPs and another 48 possible POPs, the mean and maximum half-life estimations for degradation in air are modelled using different molecular structure descriptors (atom and fragment counts, topological and WHIM descriptors), selected by Genetic Algorithm, in QSAR regression models. Both values are modelled to obtain an average estimate and a precautionary value for ranking and screening purposes. The models, validated for their predictivity, could be applied to predict unavailable data. Principal component analysis (PCA) was then used to explore the half-life data in addition to the physicochemical properties that are most relevant to atmospheric mobility; the aim has been to screen and rank POPs with regard to their tendency towards atmospheric persistence and mobility, and to obtain a persistence index in air and an LRT index. These indexes were also modelled by molecular descriptors, thus allowing a preliminary screening of new compounds.

Gramatica P, Vighi M, Consolaro F, Todeschini R, Finizio A, Faust M. **QSAR approach for the selection of congeneric compounds with a similar toxicological mode of action.** Chemosphere 2001;42(8):873-83.
Abstract: The selection of compounds with a similar toxicological mode of action is a key problem in the study of chemical mixtures. In this paper, an approach for the selection of chemicals with similar mode of action, based on the analysis of structural similarities by means of QSAR and chemometric methods, is described. As a first step, a complete representation of chemical structures for examined chemicals (phenylureas and triazines) by different sets of molecular descriptors allows a preliminary exploration of similarity using multi-dimensional scaling (MDS). The use of genetic algorithm (GA) to select the most relevant molecular descriptors in modeling toxicity data makes it possible to develop predictive toxicity models. The final step is a similarity analysis, based again on MDS, using selected molecular descriptors, really relevant in describing the toxicological effect.

Hansch C, Garg R, Kurup A. **Searching for allosteric effects via QSARs.** Bioorg Med Chem 2001;9 (2):283-9.
Abstract: A study of our database of 7,000 QSARs involving chemical-biological interaction uncovered 11 examples where the QSARs all contain inverted parabolas based on molecular refractivity. That is, biological activity first decreases with increase in MR and then increases. Two of the examples are for enzymes: cyclooxygenase and trypsin. The others are for various receptors. The results seem to be best rationalized by the larger compounds inducing a change in a receptor unit that allows for a new mode of interaction.

Hau KM, Connell DW, Richardson BJ. **Use of partition models to evaluate guidelines for mixtures of volatile organic compounds.** Regul Toxicol Pharmacol 2000;32(1):36-41.
Abstract: Partition models based on the octanol-air parition coefficients and associated quantitative structure-activity relationships (QSARs) have been developed to describe the triggering of odor response and nasal irritation by common volatile organic compounds (VOCs). This study made use of the QSARs developed by Hau and Connell (1998, Indoor Air 8, 23-33) and Hau et al. (1999, Toxicol. Sci. 47, 93-98) to evaluate risk-based guidelines on the airborne concentrations of common VOCs in the nonindustrial environment. A new concept referred to as the "apparent internal threshold concentration" was developed for evaluating the odor and nasal pungency responses to a typical low-concentration VOC mixture described by Otto et al. (1990, Neurotoxicol. Teratol. 12, 649-652). The assessment indicated that odor can be detected at a total VOC concentration of about 3 mg/m(3), consistent with the findings of Molhave et al. (1991, Atmos. Environ. 25, 1283-1293). Nasal pungency, according to our assessment, should not ocur at a total concentration of 25 mg/m(3), which is apparently in conflict with the findings of Molhave (1986, ASHRAE Trans. 92(1A), 306-316). It can be inferred from this investigation that pure nasal pungency without the influence of odor is unlikely to result from exposure to low-concentration VOC mixtures typically found in the nonindustrial environment.

Helma C, Gottmann E, Kramer S. **Knowledge discovery and data mining in toxicology.** Stat Methods Med Res 2000;9(4):329-58.
Abstract: Knowledge discovery and data mining tools are gaining increasing importance for the analysis of toxicological databases. This paper gives a survey of algorithms, capable to derive interpretable models from toxicological data, and presents the most important application areas. The majority of techniques in this area were derived from symbolic machine learning, one commercial product was developed especially for toxicological applications. The main application area is presently the detection of structure-activity relationships, very few authors have used these techniques to solve problems in epidemiological and clinical toxicology. Although the discussed algorithms are very flexible and powerful, further research is required to adopt the algorithms to the specific learning problems in this area, to develop improved representations of chemical and biological data and to enhance the interpretability of the derived models for toxicological experts.

Helma C, Kramer S, Pfahringer B, Gottmann E. **Data quality in predictive toxicology: identification of chemical structures and calculation of chemical properties**. Environ Health Perspect 2000;108 (11):1029-33.
Abstract: Every technique for toxicity prediction and for the detection of structure-activity relationships relies on the accurate estimation and representation of chemical and toxicologic properties. In this paper we discuss the potential sources of errors associated with the identification of compounds, the representation of their structures, and the calculation of chemical descriptors. It is based on a case study where machine learning techniques were applied to data from noncongeneric compounds and a complex toxicologic end point (carcinogenicity). We propose methods applicable to the routine quality control of large chemical datasets, but our main intention is to raise awareness about this topic and to open a discussion about quality assurance in predictive toxicology. The accuracy and reproducibility of toxicity data will be reported in another paper.

Inazu N, Kato J. **[Identification of environmental estrogens with a three-dimensional quantitative structure-activity relationship(3D-QSAR) analysis].** Nippon Rinsho 2000;58(12):2469-76 [Jpn].
Abstract: The quantitative structure-activity relationship(QSAR) technique has been used by computing the structural data from many chemical compounds. In order to predict the estrogenic actions of chemical compounds, various steric(van der Waals) and electrostatic(Coulombic) interaction energy from those compounds are calculated at the standard with a comparative molecular field analysis (CoMFA) and the comparison of these results with template estradiol is drawn as a three-dimensional image. The three-dimensional structural features of various chemical compounds are compared with those of estradiol. The binding affinities of various chemical compounds with estrogen receptor could be predicted. Steric bulk of a chemical compound molecule and electric potential of substituents bound to the molecule can be also drawn on the basis of the three-dimensional data obtained. The results from CoMFA/QSAR analysis correlates well with ones from the presently widely-used bioassay, indicating compatibility of two methods. The 3D-QSAR technique with CoMFA/QSAR analysis seems to be useful for selection of the target disruptors prior to the time-consuming and tedious bioassays.

Ivanciuc O. **QSAR comparative study of Wiener descriptors for weighted molecular graphs.** J Chem Inf Comput Sci 2000;40(6):1412-22.
Abstract: Quantitative structure-property relationship (QSPR) and quantitative structure-activity relationship (QSAR) studies use statistical models to compute physical, chemical, or biological properties of a chemical substance from its molecular structure, encoded in a numerical form with the aid of various descriptors. Structural indices derived from molecular graph matrices represent an important group of descriptors used in QSPR and QSAR models; recently, their utilization was extended to molecular similarity and diversity, in database mining and virtual screening of combinatorial libraries. Initially defined from the distance matrix, the Wiener index W was the source of novel graph descriptors derived from recently proposed molecular matrices and of the Wiener graph operator. In this work we present a comparative study of several Wiener-type descriptors computed for vertex- and edge-weighted molecular graphs, corresponding to organic compounds with heteroatoms and multiple bonds. The acute toxicities toward Tetrahymena pyriformis of 47 nitrobenzenes are modeled with multilinear regression equations, using as structural descriptors the hydrophobicity (corrected for ionization) and various Wiener-type indices, with better results than a comparative molecular field analysis model.

Kaiser KL, Niculescu SP. **Modeling acute toxicity of chemicals to Daphnia magna: a probabilistic neural network approach.** Environ Toxicol Chem 2001;20(2):420-31.
Abstract: A methodology based on probabilistic neural networks (PNNs) is applied to model the acute toxicity (48-h LC50) of a set of 700 highly diverse chemicals to Daphnia magna. First, cross-validation experiments confirming the potential use of the PNN as modeling tool for the problem at hand were performed. Next, various approaches to construct-improved models are presented. The resulting four models are then validated using an external test set of 76 additional compounds. Input to the PNNs is derived solely from simple molecular descriptors and structural fragments and excludes bulk property parameters, such as the water solubility or the octanol/water partition coefficient.

Katritzky AR, Petrukhin R, Tatham D, Basak S, Benfenati E, Karelson M, Maran U. **Interpretation of quantitative structure-property and -activity relationships.** J Chem Inf Comput Sci 2001;41(3):679-

85.
Abstract: The potential utility of data reduction methods (e.g. principal component analysis) for the analysis of matrices assembled from the related properties of large sets of compounds is discussed by reference to results obtained from solvent polarity scales, ongoing work on solubilities and sweetness properties, and proposed general treatments of toxicities and gas chromatographic retention indices.

Khadikar PV, Karmarkar S, Agrawal VK. **A novel PI index and its applications to QSPR/QSAR studies.** J Chem Inf Comput Sci 2001;41(4):934-49.
Abstract: A novel topological index, PI (Padmakar-Ivan index), is derived in this paper. The index is very simple to calculate and has disseminating power similar to that of the Wiener (W) and the Szeged (Sz) indices. The comprehensive studies show that the proposed PI index correlates highly with W and Sz as well as with physicochemical properties and biological activities of a large number of diversified and complex compounds. The proposed PI index promises to be a useful parameter in the QSPR/QSAR studies. The stability of each model is demonstrated by applying cross-validation test. Furthermore, more favorable comparison with other representative indices such as the Randic index is also made in order to establish the predictive ability of the PI index. The results have shown that in several cases the PI index gave better results.

Krop HB, van Noort PC, Govers HA. **Determination and theoretical aspects of the equilibrium between dissolved organic matter and hydrophobic organic micropollutants in water (Kdoc).** Rev Environ Contam Toxicol 2001;169:1-122.
Abstract: Literature on the equilibrium constant for distribution between dissolved organic carbon (DOC) (Kdoc) data of strongly hydrophobic organic contaminants were collected and critically analyzed. About 900 Kdoc entries for experimental values were retrieved and tabulated, including those factors that can influence them. In addition, quantitative structure-activity relationship (QSAR) prediction equations were retrieved and tabulated. Whether a partition or association process between the contaminant and DOC takes place could not be fully established, but indications toward an association process are strong in several cases. Equilibrium between a contaminant and DOC in solution was shown to be achieved within a minute. When the equilibrium shifts in time, this was caused by either a physical or chemical change of the DOC, affecting the lighter fractions most. Adsorption isotherms turned out to be linear in the contaminant concentration for the relevant DOC concentration up to 100 mg of C/L. Eighteen experimental methods have been developed for the determination of the pertinent distribution constant. Experimental Kdoc values revealed the expected high correlation with partition coefficients over n-octanol and water (Kow) for all experimental methods, except for the HPLC and apparent solubility (AS) method. Only fluorescence quenching (FQ) and solid-phase microextraction (SPME) methods could quantify fast equilibration. Only 21% of the experimental values had a 95% confidence interval, which was statistically significantly different from zero. Variation in Kdoc values was shown to be high, caused mainly by the large variation of DOC in water samples. Even DOC from one sample gave different equilibrium constants for different DOC fractions. Measured Kdoc values should, therefore, be regarded as average values. Kdoc was shown to increase on increasing molecular mass, indicating that the molecular mass distribution is a proper normalization function for the average Kdoc at the current state of knowledge. The weakly bound fraction could easily be desorbed when other adsorbing media, such as a SepPak column or living organism, are present. The amount that

moves from the DOC to the other medium will depend, among other reasons, on the size of the labile DOC fraction and the equilibrium constant of the other medium. Variation of Kdoc with temperature turned out to be small, probably caused by a small enthalpy of transfer from water to DOC. Ionic strength turned out to be more important, leading to changes of a factor of 2-5. The direction of this effect depends on the type of ion. With respect to QSAR relationships between Kdoc and macroscopic or molecular descriptors, it was concluded that only a small number of equations are available in the literature, for apolar compounds only, and with poor statistics and predictive power. Therefore, a first requirement is the improvement of the availability and quality of experimental data. Along with this, theoretical (mechanistic) models for the relationship between DOC plus contaminant descriptors on the one side and Kdoc on the other should be further developed. Correlations between Kdoc and Kow and those between the soil-water partition constant (Koc) and Kow were significantly different only in the case of natural aquatic DOC, pointing at substantial differences between these two types of organic material and at a high correspondence for other types of commercial and natural DOC.

Lee JH, Landrum PF, Field LJ, Koh CH. **Application of a sigmapolycyclic aromatic hydrocarbon model and a logistic regression model to sediment toxicity data based on a species-specific, water-only LC50 toxic unit for Hyalella azteca.** Environ Toxicol Chem 2001;20(9):2102-13.
Abstract: Two models, a sigmapolycyclic aromatic hydrocarbon (PAH) model based on equilibrium partitioning theory and a logistic-regression model, were developed and evaluated to predict sediment-associated PAH toxicity to Hyalella azteca. A sigmaPAH model was applied to freshwater sediments. This study is the first attempt to use a sigmaPAH model based on water-only, median lethal concentration (LC50) toxic unit (TU) values for sediment-associated PAH mixtures and its application to freshwater sediments. To predict the toxicity (i.e., mortality) from contaminated sediments to H. azteca, an interstitial water TU, calculated as the ambient interstitial water concentration divided by the water-only LC50 in which the interstitial water concentrations were predicted by equilibrium partitioning theory, was used. Assuming additive toxicity for PAH, the sum of TUs was calculated to predict the total toxicity of PAH mixtures in sediments. The sigmaPAH model was developed from 10- and 14-d H. azteca water-only LC50 values. To obtain estimates of LC50 values for a wide range of PAHs, a quantitative structure-activity relationship (QSAR) model (log LC50 - log Kow) with a constant slope was derived using the time-variable LC50 values for four PAH congeners. The logistic-regression model was derived to assess the concentration-response relationship for field sediments, which showed that 1.3 (0.6-3.9) TU were required for a 50% probability that a sediment was toxic. The logistic-regression model reflects both the effects of co-occurring contaminants (i.e., nonmeasured PAH and unknown pollutants) and the overestimation of exposure to sediment-associated PAH. An apparent site-specific bioavailability limitation of sediment-associated PAH was found for a site contaminated by creosote. At this site, no toxic samples were less than 3.9 TU. Finally, the predictability of the sigmaPAH model can be affected by species-specific responses (Hyalella vs Rhepoxynius); chemical specific (PAH vs DDT in H. azteca) biases, which are not incorporated in the equilibrium partitioning model; and the uncertainty from site-specific effects (creosote vs other sources of PAH contamination) on the bioavailability of sediment-associated PAH mixtures.

Liubimova IK, Abilev SK, Gal'berstam NM, Baskin II, Paliulin VA, Zefirov NS. **[Computer-aided prediction of the mutagenic activity substituted polycyclic compounds].** Izv Akad Nauk Ser Biol

2001;(2):180-6 [Rus].
Abstract: The relationship between mutagenic activity and chemical structure was studied for 54 polycyclic compounds using two approaches: multiple linear regression analysis and artificial neural networks. Structural fragments, quantum chemical indices, and hydrophobicity (octanol-water partition coefficient) were used as descriptors (properties of the molecules introduced in the model). Both linear regression equations and nonlinear relationships obtained with the help of a neural network were shown to accurately predict mutagenic activity for the compounds structurally similar to those in the training sample. The introduction of experimentally selected descriptors is substantiated to verify the proposed mechanism of related compounds mutagenic activity.

Llorens O, Perez JJ, Villar HO. **Toward the design of chemical libraries for mass screening biased against mutagenic compounds.** J Med Chem 2001;44(17):2793-804.
Abstract: The ability to develop a chemical into a drug depends on multiple factors. Beyond potency and selectivity, ADME/PK and the toxicological profile of the compound play a significant role in its evaluation as a candidate for development. Those factors are being brought into bear earlier in the discovery process and even into the design of libraries for screening. The purpose of our study is the comparative analysis of simple physical characteristics of compounds that have been reported to be mutagens and nonmutagenic ones. The analysis of differences can lead to the development of knowledge-based biases in the libraries designed for massive screening. For each of four Salmonella strains, TA-98, TA-100, TA-1535, and TA-1537, an analysis of the statistical significance of the deviance of the averages for a number of global properties was carried out. The properties studied included parameters, such as topological indices, and bit strings representing the presence or absence of certain chemical moieties. The results suggest that mutagens display a larger number of hydrogen bond acceptor centers for most strains. Moreover, the use of bit strings points to the importance of certain molecular fragments, such a nitro groups, for the outcome of a mutagenicity study. Development of multivariate models based on global molecular properties or bit strings point to a small advantage of the latter for the prediction of mutagenicity. The benefits of the bit strings are in accord with the use of fragment-based approaches for the prediction of carcinogenicity and mutagenicity in methods described in the literature.

Lu X, Tao S, Hu H, Dawson RW. **Estimation of bioconcentration factors of nonionic organic compounds in fish by molecular connectivity indices and polarity correction factors.** Chemosphere 2000;41(10):1675-88.
Abstract: A bioconcentration factor (BCF) estimation model for a wide range of nonionic organic compounds was developed on the basis of molecular connectivity indices and polarity correction factors. The nonlinear topological modeling using polarity correction factors resulted in the best BCF estimation quality for all of the 239 compounds studied, with a mean absolute estimation error of 0.478 log units. Residual analysis indicated that the estimation errors came from many sources including BCF measurement, test species, and selection of descriptors. Statistical robustness of the developed model was validated by modified jackknifed tests where random deletion of a set of compounds and specific deletion of a class of compounds were both performed. Comparison between the MCI-based (molecular connectivity indices) model and a Kow-based (octanol/water partition coefficient) model revealed that the BCF estimation based on topological parameters was as good as that achieved by Kow.

Luong TN, Kirsch JF. **A general method for the quantitative analysis of functional chimeras: applications from site-directed mutagenesis and macromolecular association.** Protein Sci 2001;10 (3):581-91.
Abstract: Two new parameters, I: and C:, are introduced for the quantitative evaluation of functional chimeras: I: (impact) and C: (context dependence) are the free energy difference and sum, respectively, of the effects on a given property measured in forward and retro chimeras. The forward chimera is made by substitution of a part "a" from ensemble A into the analogous position of homologous ensemble B (S: (B --> A)). The C: value is a measure of the interaction of the interrogated position with its surroundings, whereas I: is an expression of the quantitative importance of the probed position. Both I: and C: vary with the evaluated property, for example, kinetics, binding, thermostability, and so forth. The retro chimera is the reverse substitution of the analogous part "b" from B into A, S:(A --> B). The I: and C: values derived from original data for forward and retro mutations in aspartate and tyrosine aminotransferase, from literature data for quasi domain exchange in oncomodulin and for the interaction of Tat with bovine and human TAR are evaluated. The most salient derived conclusions are, first, that Thr 109 (AATase) or Ser 109 (TATase) is an important discriminator for dicarboxylic acid selectivity by these two enzymes (I: < -2.9 kcal/mol). The T109S mutation in AATase produces a nearly equal and opposite effect to S109T in TATase (C: < 0.4 kcal/mol). Second, an I: value of 5.5 kcal/mol describes the effects of mirror mutations D94S (site 1) and S55D (site 2) in the Ca(2+) binding sites of oncomodulin on Ca(2+) affinity. The second mirror set, G98D (site 1) and D59G (site 2), yields a smaller impact (I: = -3.4 kcal/mol) on Ca(2+) binding; however, the effect is significantly more nearly context independent (C: = -0.6 versus C: = -2.7 kcal/mol). Third, the stem and loop regions of HIV and BIV TAR are predominantly responsible for the species specific interaction with BIV Tat(65-81) (I: = -1.5 to -1.6 kcal/mol), whereas I: = 0.1 kcal/mol for bulge TAR chimeras. The C: values are from -0.3 to -1.2 kcal/mol. The analysis described should have important applications to protein design.

Macina OT, Sussman NB, Claycamp HG, Grant SG. **Physicochemical and graph theoretical descriptors in developmental toxicity SAR: a comparative study.** SAR QSAR Environ Res 2001;11 (5-6):345-62.
Abstract: Chemical insults to the developing fetus can lead to growth retardation, malformation, death, and functional deficits. The present study seeks to determine if physicochemical and/or graph theoretical parameters can be used to determine a structure-activity relationship (SAR) for developmental toxicity, and if consistency is observed among the selected features. The biological data utilized consists of a diverse series of compounds evaluated within the Chernoff-Kavlock in vivo mouse assay. Physicochemical parameters calculated correspond to electronic, steric, and transport properties. Graph theoretical parameters calculated include the simple, valence, and kappa indices. Both sets of parameters were independently applied to derive SARs in order to compare the quality of the respective models. Multiple random sampling, without replacement, was utilized to obtain ten training/test partitions. Models were built by linear discriminant analysis, decision trees, and neural networks respectively. Comparisons on identical sets of data were carried out to determine if any of the model building procedures had a significant advantage in terms of predictive performance. Furthermore, comparison of the features selected within and across the model building processes led to the determination of model consistency. Our results indicate that consistent features related to developmental toxicity are observed

and that both physicochemical and graph theoretical parameters have equal utility.

Marder M, Estiu G, Blanch LB, Viola H, Wasowski C, Medina JH, Paladini AC. **Molecular modeling and QSAR analysis of the interaction of flavone derivatives with the benzodiazepine binding site of the GABA(A) receptor complex.** Bioorg Med Chem 2001;9(2):323-35.
Abstract: A large number of structurally different classes of ligands, many of them sharing the main characteristics of the benzodiazepine (BDZ) nucleus, are active in the modulation of anxiety, sedation, convulsion, myorelaxation, hypnotic and amnesic states in mammals. These compounds have high affinity for the benzodiazepine binding site (BDZ-bs) of the GABA(A) receptor complex. Since 1989 onwards our laboratories established that some natural flavonoids were ligands for the BDZ-bs which exhibit medium to high affinity in vitro and anxiolytic activity in vivo. Further research resulted in the production of synthetic flavonoid derivatives with increased biochemical and pharmacological activities. The currently accepted receptor/pharmacophore model of the BDZ-bs (Zhang, W.; Koeler, K. F.; Zhang, P.; Cook, J. M. Drug Des. Dev. 1995, 12, 193) accounts for the general requirements that should be met by this receptor for ligand recognition. In this paper we present a model pharmacophore which defines the characteristics for a ligand to be able to interact and bind to a flavone site, in the GABA(A) receptor. closely related to the BDZ-bs. A model of a flavone binding site has already been described (Dekermendjian, K.; Kahnberg, P.; Witt, M. R.; Sterner, O.; Nielsen, M.; Liljerfors, T. J. Med. Chem. 1999, 42, 4343). However, this alternative model is based only on graphic superposition techniques using as template a non-BDZ agonist. In this investigation all the natural and synthetic flavonoids found to be ligands for the BDZ-bs have been compared with the classical BDZ diazepam. A QSAR regression analysis of the parameters that describe the interaction demonstrates the relevance of the electronic effects for the ligand binding, and shows that they are associated with the negatively charged oxygen atom of the carbonyl group of the flavonoids and with the nature of the substituent in position 3'.

Matthews EJ, Benz RD, Contrera JF. **Use of toxicological information in drug design.** J Mol Graph Model 2000;18(6):605-15.
Abstract: This paper is an extension of the keynote address and another talk at the Symposium on the Use of Toxiciological Information in Drug Design. The symposium was organized by American Chemical Society's Chemical Information Division at the 220th National Meeting of the American Chemical Society in Washington, DC, August 20-24, 2000. We outline an approach for meeting the scientific information needs of the U.S. Food and Drug Administration (FDA). Ready access to scientific information is critical to support safety-related regulatory decisions and is especially valuable in situations where available experimental information from in vivo/in vitro studies are inadequate or unavailable. This approach also has applications for lead selection in drug discovery. A pilot electronic toxicology/safety knowledge base and computational toxicology initiative is underway in the FDA Center for Drug Evaluation and Research (CDER) that may be a prototype for an FDA knowledge base. The objectives of this effort are: (i) to strengthen and broaden the scientific basis of regulatory decisions, (ii) to provide the Agency with an electronic scientific institutional memory, (iii) to create a scientific resource for regulatory and applied research, and (iv) to establish an internal Web-based support service that can provide decision support information for regulators that will facilitate the review process and improve consistency and uniformity. An essential component of this scientific knowledge base is the creation of a comprehensive electronic inventory of CDER-regulated substances that permit

identification of clusters of substances having similar chemical, pharmacological or toxicological activities, and molecular structure/substructures. Furthermore, the inventory acts as a pointer and link to other databases and critical non-clinical and clinical pharmacology/toxicology studies and reviews in FDA archives. Clusters of related substances are identified through the use of: (i) an extensive index of alternative names for each substance, (ii) a molecular structure key field consisting of a rudimentary or core structure represented as an ISIS.mol-file, (iii) global search terms (molecular group, chemical class, clinical indication, or pharmacologic activity), and (iv) molecular clustering using structure/sub-structure similarity indices. The information contained in a toxicology knowledge database has limited value unless means are available to extract information, identify relationships, and create and test hypotheses. One such means is computational toxicology, also called in silico toxicology, ComTox, or e-TOX. Computational toxicology is the application of computer technology and information processing (informatics) to analyze, model, and estimate chemical toxicity based upon structure activity relationships (SAR). A computational toxicology software package, MCASE, has been evaluated and successfully improved by CDER through the incorporation of data from FDA archives and concomitant alterations of the logic used in the interpretation of the results to reflect the data analysis and hazard identification practices and priorities of the Center. Our modifications and uses of the MCASE program are discussed in detail.

McIntosh JM, Heffron JJ. **Modelling alterations in the partition coefficient in in vitro biological systems using headspace gas chromatography.** J Chromatogr B Biomed Sci Appl 2000;738(2):207-16.
Abstract: Headspace gas chromatography was used to determine the physiological media-air partition coefficient (K) of four volatile organic solvents of industrial importance. The experimental conditions were those likely to be used in in vitro metabolic and toxicological studies on volatile compounds. The addition of solvent to the liquid phase from a stock solution in ethanol, or the presence of organic material at concentrations normally seen in in vitro studies, did not significantly alter the K value. Binary solvent addition resulted in a dose-dependent decrease in K for each solvent that was also influenced by the solvent solubility and the constituents of the liquid matrix. The aromatic solvents exerted the greatest effect and showed the greatest change in K value.

cKinney JD, Richard A, Waller C, Newman MC, Gerberick F. **The practice of structure activity relationships (SAR) in toxicology.** Toxicol Sci 2000;56(1):8-17.
Abstract: Both qualitative and quantitative modeling methods relating chemical structure to biological activity, called structure-activity relationship analyses or SAR, are applied to the prediction and characterization of chemical toxicity. This minireview will discuss some generic issues and modeling approaches that are tailored to problems in toxicology. Different approaches to, and some facets and limitations of the practice and science of, SAR as they pertain to current toxicology analyses, and the basic elements of SAR and SAR-model development and prediction systems are discussed. Other topics include application of 3-D SAR to understanding of the propensity of chemicals to cause endocrine disruption, and the use of models to analyze biological activity of metal ions in toxicology. An example of integration of knowledge pertaining to mechanisms into an expert system for prediction of skin sensitization to chemicals is also discussed. This minireview will consider the utility of modeling approaches as one component for better integration of physicochemical and biological properties into risk assessment, and also consider the potential for both environmental and human health effects of

chemicals and their interactions.

Mekenyan OG, Kamenska V, Schmieder PK, Ankley GT, Bradbury SP. **A computationally based identification algorithm for estrogen receptor ligands: part 2. Evaluation of a hERalpha binding affinity model.** Toxicol Sci 2000;58(2):270-81.
Abstract: The objective of this study was to evaluate the capability of an expert system described in the previous paper (S. Bradbury et al., Toxicol. Sci. 58, 253-269) to identify the potential for chemicals to act as ligands of mammalian estrogen receptors (ERs). The basis of the expert system was a structure activity relationship (SAR) model, based on relative binding affinity (RBA) values for steroidal and nonsteroidal chemicals derived from human ERalpha (hERalpha) competitive binding assays. The expert system enables categorization of chemicals into (RBA ranges of < 0.1, 0.1 to 1, 1 to 10, 10 to 100, and >150% relative to 17ss-estradiol. In the current analysis, the algorithm was evaluated with respect to predicting RBAs of chemicals assayed with ERs from MCF7 cells, and mouse and rat uterine preparations. The best correspondence between predicted and observed RBA ranges was obtained with MCF7 cells. The agreement between predictions from the expert system and data from binding assays with mouse and rat ER(s) were less reliable, especially for chemicals with RBAs less than 10%. Prediction errors often were false positives, i.e., predictions of greater than observed RBA values. While discrepancies were likely due, in part, to species-specific variations in ER structure and ligand binding affinity, a systematic bias in structural characteristics of chemicals in the hERalpha training set, compared to the rodent evaluation data sets, also contributed to prediction errors. False-positive predictions were typically associated with ligands that had shielded electronegative sites. Ligands with these structural characteristics were not well represented in the training set used to derive the expert system. Inclusion of a shielding criterion into the original expert system significantly increased the accuracy of RBA predictions. With this additional structural requirement, 38 of 46 compounds with measured RBA values greater than 10% in hERalpha, MCF7, and rodent uterine preparations were correctly categorized. Of the remaining 129 compounds in the combined data sets, RBA values for 65 compounds were correctly predicted, with 47 of the incorrect predictions being false positives. Based upon this exploratory analysis, the modeling approach, combined with a high-quality training set of RBA values derived from a diverse set of chemical structures, could provide a credible tool for prioritizing chemicals with moderate to high ER binding affinity for subsequent in vitro or in vivo assessments.

Motohashi N, Yamagami C, Tokuda H, Okuda Y, Ichiishi E, Mukainaka T, Nishino H, Saito Y. **Structure-activity relationship in potentially anti-tumor promoting benzalacetone derivatives, as assayed by the epstein-barr virus early antigen activation.** Mutat Res 2000;464(2):247-54.
Abstract: The in vitro anti-tumor promoting activities of antimutagenic benzalacetone (4-phenyl-3-buten-2-one), its monosubstituted derivatives and related compounds, cinnamaldehydes and cinnamic acids, were evaluated by determining the inhibitory effect on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cells. In this short-term assay, benzalacetone, which is the basic structure of dehydrozingerone (one-half analog of curcumin) inhibited the EBV-EA activation; the IC(50) value, the molar ratio of benzalacetone to TPA needed for inhibiting 50% of positive cells activated with 32 pmol TPA, was 129. IC(50) values of 2- and 4-methoxybenzalacetones were about one-half of that of benzalacetone and the methoxy compounds were

more effective than hydroxybenzalacetones. IC(50) values of chloro- and trifluoromethyl-benzalacetones were higher than that of benzalacetone, indicating that these compounds are weaker inhibitors. In addition, the position of a substituent on the benzene ring affected the inhibitory effect. In benzalacetone derivatives substituted by a hydroxy-, methoxy-, chloro- or trifluoromethyl group, the 2-substituted derivatives exhibited the strongest inhibitory effect, followed by the 3- and the 4-substituents. Cinnamic acid derivatives also decreased the inhibitory effects in the same order. In the side chain of benzalacetone, the terminal group adjacent to the carbon-carbon double bond also affected the inhibitory effect. The conversions of the methylketone to aldehyde and carboxyl groups, i.e., cinnamaldehyde and cinnamic acid, increased the inhibitory effect: the IC(50) values were about one-third of that of benzalacetone. beta-Methyl styrene, which in the side chain has no carbonyl group adjacent to the double bond, inhibited the EBV-EA activation at the concentration of about one-third of that of benzalacetone, indicating that the carbonyl group negatively affects the inhibitory effect. This agreed with the previous observation between isoeugenol and dehydrozingerone, 4-hydroxy-3-methoxy derivatives of beta-methyl styrene and benzalacetone, respectively. The mechanism of the EBV-EA activation inhibition was discussed by being compared with the inhibition of mutagenesis for which the unsaturated bonded-carbonyl system is necessary.

Niculescu SP, Kaiser KL, Schultz TW. **Modeling the toxicity of chemicals to Tetrahymena pyriformis using molecular fragment descriptors and probabilistic neural networks.** Arch Environ Contam Toxicol 2000;39(3):289-98.
Abstract: The results of an investigation into the use of a probabilistic neural network (PNN)-based methodology to model the 48-h ICG50 (inhibitory concentration for population growth) sublethal toxicity of 825 chemicals to the ciliate Tetrahymena pyriformis are presented. The information fed into the neural networks is solely based on simple molecular descriptors as can be derived from the chemical structure. In contrast to most other toxicological models, the octanol/water partition coefficient is not used as an input parameter, and no rules of thumb or other substance selection criteria are employed. The cross-validation and external validation experiments confirmed excellent recognitive and predictive capabilities of the resulting models and recommend their future use in evaluating the potential of most organic molecules to be toxic to Tetrahymena.

Parkerton TF, Konkel WJ. **Application of quantitative structure--activity relationships for assessing the aquatic toxicity of phthalate esters.** Ecotoxicol Environ Saf 2000;45(1):61-78.
Abstract: Phthalate esters (PEs) are an important class of industrial chemicals for which an extensive aquatic toxicity database is available. The objectives of this study were to use these data to develop quantitative structure-activity relationships (QSARs) that describe aquatic toxicity for different freshwater and marine species, gain insights into toxicity mechanisms, and calculate PE water quality criteria using statistical extrapolation procedures. Results for low-molecular-weight PEs with log Kow<6 indicate that toxicity data conform to a simple log Kow-dependent QSAR. Fish were found to be more sensitive than algae while invertebrates spanned a wide range in toxicological response. Freshwater and marine species demonstrated a similar distribution of sensitivities. Comparison of species-dependent QSARs supports the hypothesis that biotransformation plays an important role in explaining toxicity differences observed between species. Estimated critical body residues (CBRs) for parent PE in fish were in the range reported for other polar organic chemicals while CBRs for parent PE

plus associated metabolites were in the range reported for nonpolar narcotics (i.e., baseline toxicity) suggesting a possible putative role of PE metabolites. Depending on extrapolation procedure and assumptions, predicted no-effect concentrations (PNECs) for dimethyl, diethyl, dibutyl, and butybenzyl phthalate ranged from 3109 to 4780, 865 to 1173, 43 to 62, and 38 to 60 microg l(-1), respectively. PNECs derived using this approach provide a transparent technical basis to support aquatic risk assessment for low-molecular-weight PEs. Results for high-molecular-weight PEs (log Kow>6) indicate that these chemicals are not acutely or chronically toxic to freshwater or marine organisms due to the combined role of low water solubility and limited bioconcentration potential which precludes attainment of internal concentrations that are required to elicit adverse effects. It is concluded that attempts to establish aquatic PNECs for high-molecular-weight PEs are not scientifically defensible.

Prival MJ. **Evaluation of the TOPKAT system for predicting the carcinogenicity of chemicals.** Environ Mol Mutagen 2001;37(1):55-69.
Abstract: The TOPKAT computer-based system for predicting chemical carcinogens was evaluated by determining its ability to predict the carcinogenicity of chemicals tested by the National Toxicology Program. TOPKAT was not effective in identifying potential rodent carcinogens and noncarcinogens in the data set analyzed. The chemicals in the TOPKAT database of known carcinogens and noncarcinogens that the software identifies as most "similar" to unknown chemicals are illustrated using six examples. These "similar" chemicals generally bear no apparent relationship to the chemical of interest with regard to metabolism or potential mechanism of carcinogenicity. Environ. Mol. Mutagen. 37:55-69, 2001 Published 2001 Wiley-Liss, Inc.

Rao S, Aoyama R, Schrag M, Trager WF, Rettie A, Jones JP. **A refined 3-dimensional QSAR of cytochrome P450 2C9: computational predictions of drug interactions.** J Med Chem 2000;43 (15):2789-96.
Abstract: A ligand-based model is reported that predicts the Ki values for cytochrome P450 2C9 (CYP2C9) inhibitors. This CoMFA model was used to predict the affinity of 14 structurally diverse compounds not in the training set and appears to be robust. The mean error of the predictions is 6 microM. The experimentally measured Ki values of the 14 compounds range from 0.1 to 48 microM. Leave-one-out cross-validated partial least-squares gives a q2 value of between 0.6 and 0.8 for the various models which indicates internal consistency. Random assignment of biological data to structure leads to negative q2 values. These models are useful in that they establish a pharmacophore for binding to CYP2C9 that can be tested with site-directed mutagenesis. These models can also be used to screen for potential drug interactions and to design compounds that will not bind to this enzyme with high affinity.

Raymond JW, Rogers TN, Shonnard DR, Kline AA. **A review of structure-based biodegradation estimation methods.** J Hazard Mater 2001;84(2-3):189-215.
Abstract: Biodegradation, being the principal abatement process in the environment, is the most important parameter influencing the toxicity, persistence, and ultimate fate in aquatic and terrestrial ecosystems. Biodegradation of an organic chemical in natural systems may be classified as primary (alteration of molecular integrity), ultimate (complete mineralization; i.e. conversion to inorganic compounds and/or normal metabolic processes), or acceptable (toxicity ameliorated). Most of the

biodegradation correlations presented in the literature focus on the characterization of primary or ultimate, aerobic degradation. The US Environmental Protection Agency (USEPA) is charged with determining the risks associated with the thousands of chemicals employed in commerce, an effort that is being facilitated through much research aimed at reliable structure-activity relationships (SAR) to predict biodegradation of chemicals in natural systems. To this end, models are needed to understand the mechanisms of biodegradation, to classify chemicals according to relative biodegradability, and to develop reliable biodegradation estimation methods for new chemicals. Frequently, published correlations associating molecular structure to biodegradation will attempt to quantify the degradability of a limited set of homologous chemicals. These correlations have been dubbed quantitative structure biodegradability relationships (QSBRs). More scarce and valuable to researchers are those models that predict the biodegradability of compounds possessing a wide variety of chemical structures. The latter may use any of several techniques and molecular descriptors to correlate biodegradability: QSBRs, pattern recognition, discriminant analysis, and principle component analysis (PCA), to name several. Generally, models either predict the propensity of a chemical to biodegrade using Boolean-type logic (i. e. whether a chemical will "readily biodegrade" or not), or else they quantify the degree of biodegradation by providing information such as rate constants. Such quantitative predictions of biodegradability come in a diversity of parameters, including half-lives, various biodegradation rates and rates constants, theoretical oxygen demand (ThOD), biological oxygen demand (BOD), and others.In this paper, after describing the advantages and disadvantages of the various biodegradation estimation methods found in the literature, the best models are compared to conclude which provide the greatest utility for determining the biodegradability of chemicals with widely varying structures. The group contribution technique presented by Boethling et al. [Environmen. Sci. Technol. 28 (1994) 459] appears to be the most advantageous for use in broad screening for tendency to biodegrade. The model is simple to use, calculating a probability of biodegrading ranging from 0 (none) to 1 (certain), and has proven to be accurate for a wide range of chemical structures, as established by the large, high-quality data set (BIODEG evaluated biodegradation database, Syracuse Research Corporation, Merrill Lane, Syracuse, NY 13210) used to develop this correlation. The authors, therefore, recommend the method of Boethling et al. [Environ. Sci. Technol. 28 (1994) 459] for the initial screening of chemicals to aid in determining whether additional information is necessary to establish relative biodegradability. For readers with applications requiring more quantitative results, such as biodegradation rate constants, enough model details are presented in this paper to allow the reader to pick a suitable correlation, although the reader is cautioned to consult the original, primary reference for the complete method description, equations, and limitations.

Robertson DG, Reily MD, Sigler RE, Wells DF, Paterson DA, Braden TK. **Metabonomics: evaluation of nuclear magnetic resonance (NMR) and pattern recognition technology for rapid in vivo screening of liver and kidney toxicants.** Toxicol Sci 2000;57(2):326-37.

Abstract: The purpose of this study was to evaluate the feasibility of metabonomics technology for developing a rapid-throughput toxicity screen using 2 known hepatotoxicants: carbon tetrachloride (CCl (4)) and alpha-naphthylisothiocyanate (ANIT) and 2 known nephrotoxicants: 2-bromoethylamine (BEA) and 4-aminophenol (PAP). In addition, the diuretic furosemide (FURO) was also studied. Single doses of CCl(4) (0.1 and 0.5 ml/kg), ANIT (10 and 100 mg/kg), BEA (15 and 150 mg/kg), PAP (15 and 150 mg/kg) and FURO (1 and 5 mg) were administered as single IP or oral doses to groups of 4 male Wistar

rats/dose. Twenty-four-h urine samples were collected pretest, daily through Day 4, and on Day 10 (high dose CCl(4) and BEA only). Blood samples were taken on Days 1, 2, and 4 or 1, 4, and 10 for clinical chemistry assessment, and the appropriate target organ was examined microscopically. NMR spectra of urine were acquired and the data processed and subjected to principal component analyses (PCA). The results demonstrated that the metabonomic approach could readily distinguish the onset and reversal of toxicity with good agreement between clinical chemistry and PCA data. In at least 2 instances (ANIT and BEA), PCA analysis suggested effects at low doses, which were not as evident by clinical chemistry or microscopic analysis. Furosemide, which had no effect at the doses employed, did not produce any changes in PCA patterns. These data support the contention that the metabonomic approach represents a promising new technology for the development of a rapid throughput in vivo toxicity screen.

Rosenkranz HS, Cunningham AR. **Chemical categories for health hazard identification: a feasibility study.** Regul Toxicol Pharmacol 2001;33(3):313-8.
Abstract: The use of chemical categories has been suggested in order to lower the number of chemicals tested in the High Production Volume (HPV) Chemical Challenge Program. In this investigation we examined the reliability of using organic chemical categories to classify chemicals as either toxic or nontoxic for individual toxicological effects as well as for panels of such endpoints. The analyses indicate that chemical categories are unable to consistently identify groups of chemicals with similar toxic responses either for a multiplicity of endpoints or for single effects. Our analyses suggest that if chemical categories are to be used to identify health hazards, that computer-based SAR approaches appear to be superior to arbitrary chemical categories for predicting specific toxicological effects but they are not, at this time, useful for defining the overall toxicity. Copyright 2001 Academic Press.

Sabljic A. **QSAR models for estimating properties of persistent organic pollutants required in evaluation of their environmental fate and risk.** Chemosphere 2001;43(3):363-75.
Abstract: The molecular connectivity indices (MCIs) have been successfully used for over 20 years in quantitative structure activity relationships (QSAR) modelling in various areas of physics, chemistry, biology, drug design, and environmental sciences. With this review, we hope to assist present and future QSAR practitioners to apply MCIs more wisely and more critically. First, we have described the methods of calculation and systematics of MCIs. This section should be helpful in rational selection of MCIs for QSAR modelling. Then we have presented our long-term experience in the application of MCIs through several characteristic and successful QSAR models for estimating partitioning and chromatographic properties of persistent organic pollutants (POPs). We have also analysed the trends in calculated MCIs and discussed their physical interpretation. In conclusion, several practical recommendations and warnings, based on our research experience, have been given for the application of MCIs in the QSAR modelling.

Schmitt H, Altenburger R, Jastorff B, Schuurmann G. **Quantitative structure-activity analysis of the algae toxicity of nitroaromatic compounds.** Chem Res Toxicol 2000;13(6):441-50.
Abstract: Proliferation toxicity toward the algae Scenedesmus vacuolatus in a 24 h one-generation reproduction assay was determined for nitrobenzene and 18 derivatives, including two phenols. The resultant EC(50) values covering more than 4 orders of magnitude were subjected to a quantitative structure-activity analysis (QSAR) using hydrophobicity in terms of the octanol/water partition

coefficient in logarithmic form, log K(ow), and 16 quantum chemical descriptors of molecular reactivity that were calculated with the AM1 scheme. For 13 mononitro derivatives and the highly hydrophobic trifluralin, a narcotic-type mode of action can explain most of the toxicity variation. Correction of log K (ow) for ionization for the phenols and quantification of the molecular susceptibility for one-electron reduction as apparently rate-determining biotransformation step by the energy of the lowest unoccupied molecular orbital, E(LUMO), yields a highly significant QSAR for all 19 compounds (r(adj)(2) = 0.90), which can be further improved when adding the maximum net atomic charge at the nitro nitrogen, q (nitro)(-)(N), as the third descriptor (r(adj)(2) = 0.93). Comparison of the energy of the singly occupied molecular orbital, E(SOMO), of the radical anions as initial metabolites with the E(SOMO) of known redox cyclers suggests that dinitrobenzenes and TFM as well as multiply chlorinated nitrobenzenes may also exert oxidative stress. This is based on an E(SOMO) window of -0.30 to 0. 55 eV as a tentative criterion for molecular structures to have the potential for redox cycling, derived from a set of eight known redox cyclers. The discussion includes a detailed analysis of apparently relevant metabolic pathways and associated modes of toxic action of nitroaromatics.

Sello G, Sala L, Benfenati E. **Predicting toxicity: a mechanism of action model of chemical mutagenicity.** Mutat Res 2001;479(1-2):141-71.
Abstract: The increasing importance of theoretical studies for predicting toxicology has aroused the interest of many computational chemists. A new approach has been developed, based on studying at the molecular level two potential mechanisms of action that are related to compound mutagenicity. This approach is the first example that considers both the toxicant and the biological target molecules involved in the interaction. Using some calculated descriptors and a simulation of the interaction chemical, compounds can be classified. More important, the approach helps in understanding and explaining both the correct and the incorrect results, and gives a deeper understanding of the toxic mechanisms. The model has been applied to many compounds and the results are compared with experimental results reported for the corresponding Salmonella tests.

Silvestri R, Artico M, De Martino G, Novellino E, Greco G, Lavecchia A, Massa S, Loi AG, Doratiotto S, La Colla P. **Computer-assisted design, synthesis and biological evaluation of novel pyrrolyl heteroaryl sulfones targeted at HIV-1 reverse transcriptase as non-nucleoside inhibitors.** Bioorg Med Chem 2000;8(9):2305-9.
Abstract: Three pyrrolyl heteroaryl sulfones (ethyl 1-[(1H-benzimidazol-2(3H)one-5-yl)sulfonyl]-1H-pyrrole-2-carboxyla te, ethyl 1-[(1H-benzimidazol-5(6)-yl)sulfonyl]-1H-pyrrole-2-carboxylate and ethyl 1-[(1H-benzotriazol-5(6)-yl)sulfonyl]-1H-pyrrole-2-carboxylate) were designed as novel HIV-1 reverse transcriptase non-nucleoside inhibitors using structure-based computational methods. Although these compounds were inactive in the cell-based assay, they inhibited the target enzyme with micromolar potency (IC50s = 2 microM, 3 microM and 9 microM, respectively).

Singh AK. **Development of QSAR models to predict estrogenic, carcinogenic, and cancer protective effects of phytoestrogens.** Cancer Invest 2001;19(2):201-16.
Abstract: An integrated QSAR model has been formulated to predict estrogenic, carcinogenic, and cancer protective effects of phytoestrogens (PE). Relative binding of PEs to estrogen receptors ER alpha and ER beta exhibited a parabolic relationship with dipole moment (mu). The high-affinity binding of

PEs to ER alpha correlated with Dif0 (0 chi-0 chi v difference index encoding nonsigma electronic charge), while the low-affinity binding of PEs to ER alpha correlated with H bonding (positive coefficient) and % hydrophilic surface (negative coefficient). The high-affinity binding of PEs to ER beta correlated with molecular with (MWd) and Dif0, while the low-affinity binding of PEs to ER beta correlated with H bonding (positive coefficient) and hydrophilic-lipophilic balance (negative coefficient). Thus an increase in electronic or ionic charge, formation of H bonds, or a decrease in hydrophilic property of PEs may increase their binding to ER. The relative transcription activity (RTA) of ER alpha correlated with Dif0-Dif1, while RTA of ER beta correlated with H bonding and polarity. The PE-induced stimulation of DNA synthesis in estrogen-sensitive breast cancer (BC) cells correlated positively with (MD*4 chi v) where MD is molecular depth and 4 chi v is the valence of a 4th order fragment. IC50 for PE-induced inhibition of DNA synthesis in estrogen-sensitive BC cells correlated with (MD*Log P) and Dif3 (3 chi-3 chi v difference index encoding nonsigma electronic charge of fragments consisting of four atoms and three bonds) and Dif3(2). IC50 for PE-induced inhibition of DNA synthesis in estrogen-independent cancer cell lines correlated with (MD*Log P) and 1/water solubility. Thus molecular shape and molecular connectivity of PEs play a key role in modulating estrogen-induced transactivation activity and DNA synthesis in BC cells.

Singh AK. **Development of quantitative structure-activity relationship (QSAR) models for predicting risk of exposure from carcinogens in animals.** Cancer Invest 2001;19(6):611-20.
Abstract: Quantitative structure-activity relationship (QSAR) models capable of predicting acute toxicity and carcinogen potency of polychlorinated dibenzo-p-dioxin (PCDD), polychlorinated hydrocarbons, and chlorinated insecticides have been formulated. Median lethal dose (LD50) for PCDD-exposed mice correlated negatively with polarity and positively with (H acceptor x 2 chi), whereas LD50 for PCDD-exposed guinea pigs correlated with (H acceptor x density). Both (H acceptor x 2 chi) and (H acceptor x density) exhibited parabolic relationship with log P (partition coefficient). Carcinogenic potency, determined from order of magnitude (OM) values, correlated negatively with log P and positively with (length x width). Thus, a hydrophobic mechanism plays a key role in the lethal effects of PCDD in mice, whereas both hydrophobic and electronic mechanisms are involved in the lethal effects of PCDD in guinea pigs. However, the molecule's lipophilicity, length, and width may play important roles in the carcinogenic effects of chlorinated compounds.

Skibo EB, Xing C, Dorr RT. **Aziridinyl quinone antitumor agents based on indoles and cyclopent[b] indoles: structure-activity relationships for cytotoxicity and antitumor activity.** J Med Chem 2001;44(22):3545-62.
Abstract: A large number of aziridinyl quinones represented by series 1-9 were studied with respect to their DT-diaphorase substrate activity, DNA reductive alkylation, cytostatic/cytotoxic activity, and in vivo activity. As a result, generalizations have been made with respect with respect to the following: DT-diaphorase substrate design, DT-diaphorase-cytotoxicity quantitative structure-activity relationship (QSAR), and DNA reductive alkylating agent design. A saturating relationship exists between the substrate specificity for human recombinant DT-diaphorase and the cytotoxicity in the human H460 non-small-cell lung cancer cell line. The interpretation of this relationship is that reductive activation is no longer rate-limiting for substrates with high DT-diaphorase substrate specificities. High DT-diaphorase substrate specificity is not desirable in the indole and cylopent[b]indole systems because of the result is

the loss of cancer selectivity along with increased toxicity. We conclude that aziridinyl quinones of this type should possess a substrate specificity (V(max)/K(M)) < 10 x 10(-4) s(-1) for DT-diaphorase in order not to be too toxic or nonselective. While some DNA alkylation was required for cytostatic and cytotoxic activity by series 1-9, too much alkylation results in loss of cancer selectivity as well as increased in vivo toxicity. Indeed, the most lethal compounds are the indole systems with a leaving group in the 3alpha-position (like the antitumor agent EO9). We conclude that relatively poor DNA alkylating agents (according to our assay) show the lowest toxicity with the highest antitumor activity.

Smith JS, Macina OT, Sussman NB, Luster MI, Karol MH. **A robust structure-activity relationship (SAR) model for esters that cause skin irritation in humans.** Toxicol Sci 2000;55(1):215-22.
Abstract: A structure-activity relationship (SAR) model has been developed to discriminate skin irritant from nonirritant esters. The model is based on the physicochemical properties of 42 esters that were tested in humans for skin irritation. Nineteen physicochemical parameters that represent transport, electronic, and steric properties were calculated for each chemical. Best subsets regression analysis indicated candidate models for further analysis. Regression analyses identified significant models ($p < 0.05$) that had variables that were also significant ($p < 0.05$). These candidate models were evaluated using linear discriminant analysis to determine if the irritant esters could be discriminated from nonirritant esters. The stability of the model was evident from the consistency of parameters among ten submodels generated using multiple random sampling of the database. The sensitivity of the ten models, evaluated by "leave-one-out" cross-validation, ranged from 0. 846 to 0.923, with a mean of 0.885 +/- 0.025 (95% CI). The specificity ranged from 0.615 to 0.923, with a mean of 0.738 +/- 0.06 (CI). Compared with nonirritant esters, irritant esters had lower density, lower water solubility, lower sum of partial positive charges, higher Hansen hydrogen bonding parameter, and higher Hansen dispersion parameter. The results indicate that physicochemical features of esters contribute to their ability to cause skin irritation in humans, and that chemical partitioning into the epidermis and intermolecular reactions are likely important components of the response. This model is applicable for prediction of human irritation of esters yet untested.

Soffers AE, Boersma MG, Vaes WH, Vervoort J, Tyrakowska B, Hermens JL, Rietjens IM. **Computer-modeling-based QSARs for analyzing experimental data on biotransformation and toxicity.** Toxicol In Vitro 2001;15(4-5):539-51.
Abstract: Over the past decades the description of quantitative structure-activity relationships (QSARs) has been undertaken in order to find predictive models and/or mechanistic explanations for chemical as well as biological activities. This includes QSAR studies in toxicology. In an approach beyond the classical QSAR approaches, attempts have been made to define parameters for the QSAR studies on the basis of quantum mechanical computer calculations. The conversion of relatively small xenobiotics within the active sites of biotransformation enzymes can be expected to follow the general rules of chemistry. This makes the description of QSARs on the basis of only one parameter, chosen on the basis of insight in the mechanism, feasible. In contrast, toxicological endpoints can very often be the result of more than one physico-chemical interaction of the compound with the model system of interest. Therefore the description of quantitative structure-toxicity relationships often does not follow a one-descriptor mechanistic approach but starts from the other end, describing QSARs by multi-parameter approaches. The present paper focuses on the possibilities and restrictions of using computer-based

QSAR modeling for analyzing experimental toxicological data, with emphasis on examples from the field of biotransformation and toxicity.

Suzuki T, Ide K, Ishida M, Shapiro S. **Classification of environmental estrogens by physicochemical properties using principal component analysis and hierarchical cluster analysis.** J Chem Inf Comput Sci 2001;41(3):718-26.
Abstract: A structurally diverse assortment of 60 environmental estrogens was divided into two main clusters ("A", "B") and a pair of subclusters ("C1", "C2") by applying principal component analysis to selected 1D and 2D molecular descriptors and subjecting the PCs to hierarchical cluster analysis. Although clustering was predicated solely on physicochemical properties, the dependence on particular physicochemical parameters of xenoestrogen binding affinities (pK(i)) to murine uterine cytosolic estrogen receptor (ER) proved greater for compounds within (sub)clusters than for compounds between (sub)clusters. Quantitative structure-binding affinity relationships derived using molecular descriptors and PCs suggested differences in the driving forces for xenoestrogen-ER binding for different (sub) clusters. The modeling power for xenoestrogen-ER binding affinities of a combination of TLSER and WHIM 3D indices was much greater than that of combinations of 1D and 2D molecular descriptors or the PCs derived therefrom. The clusterings obtained using PCs also proved applicable to the 3D-QSARs.

Trohalaki S, Gifford E, Pachter R. **Improved QSARs for predictive toxicology of halogenated hydrocarbons**. Comput Chem 2000;24(3-4):421-7.
Abstract: In our continuing efforts to provide a predictive toxicology capability, we seek to improve QSARs (quantitative structure-activity relationships) for chemicals of interest. Currently, although semi-empirical molecular orbital methods are hardly the state of the art for studying small molecules, AM1 calculations appear to be the method of choice when calculating quantum-chemical descriptors. However, with the advent of modern computational capabilities and the development of fast algorithms, ab initio molecular orbital and first principles density functional methods can be expeditiously applied in current QSAR studies. We present a study on halogenated alkanes to assess whether more accurate quantum methods result in QSARs that correlate better with experimental data. Furthermore, improved QSARs can also be obtained through development of new descriptors with explicit physical interpretations that should lead to better understanding of the mechanisms involved in the toxic response. We show that descriptors calculated from chemical intermediates may be useful in future QSARs.

Tuppurainen K, Ruuskanen J. **Electronic eigenvalue (EEVA): a new QSAR/QSPR descriptor for electronic substituent effects based on molecular orbital energies.** A QSAR approach to the Ah receptor binding affinity of polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs). Chemosphere 2000;41(6):843-8.
Abstract: A new descriptor of molecular structure for use in the derivation of predictive QSAR and QSPR models, electronic eigenvalue (EEVA), is described. This is a modification of the recently proposed EVA approach, but is based on computationally-derived molecular orbital energies instead of vibrational frequencies. Like EVA, it is also invariant as to the alignment of the structures concerned. Its performance has been tested with respect to the Ah receptor binding of PCBs, PCDDs and PCDFs, and its predictive ability has been clearly demonstrated. In particular, it seems to be suitable for 'pure'

electronic substituent effects. i.e., for cases in which both hydrophobic and steric factors are of minor importance.

Vajragupta O, Boonchoong P, Wongkrajang Y. **Comparative quantitative structure-activity study of radical scavengers.** Bioorg Med Chem 2000;8(11):2617-28.
Abstract: Classic and three-dimensional (3-D) QSAR analyses of 13 radical scavengers (1-13) were performed to derive two classic, two Apex-3-D and one comparative field analysis (CoMFA) models. Two classical models with predictive cross-validated r2 (Q2) over 0.96 indicated that the activity was attributed to the electronic COH and ELUMO, steric molar refractivity (MR) and lipophilic log P. Three-dimensional quantitative structure-activity relationship (3-D-QSAR) studies were performed by 3-D pharmacophore generation (Apex-3-D) and CoMFA techniques. For Apex-3-D studies, two best models with high Q2 (0.94 and 0.97) were yielded. Structural properties contributing to the activity were not only lipophilic but also the optimum steric property and geometry of side-chain composition. For CoMFA studies, the sp3 C(+1) probe provided the best Q2 of 0.79 with steric and electrostatic contributions of 42.3 and 57.7%, respectively. The activity of four new compounds (14-17) not included in the derivation were predicted with these models. Although the derived models were from limited data, the statistic relation was predictive. The linear correlations between the experimental IC50 values and the predicted values from classical and Apex-3-D models were found to be high and significant. The predicted activity of 17 from CoMFA was much lower than the experimental value; this deviation occurred according to the missing of hydrophobic field in standard CoMFA study. In vitro and ex vivo antilipid peroxidation in mouse brain and ESR studies of 14-17 were investigated for the radical-scavenging ability. The difference between the in vitro results, antilipid peroxidation and electron spin resonance (ESR) and ex vivo results in coumarin series was found. Thus, other properties for good bioavailability besides log P should also be taken into consideration.

Vedani A, Dobler M. **Internet laboratory for predicting harmful effects triggered by drugs and chemicals.** Concept and call for co-operation. ALTEX 2001;18(2):110-4.
Abstract: It is our objective to establish a virtual laboratory on the Internet to allow for an in silico estimation of harmful effects triggered by drugs, chemicals and their metabolites. Presently, our database includes validated models for five biological targets -- the Aryl hydrocarbon, serotonin 5HT-2A, cannabinoid, GABA (gamma-amino butter acid), and steroid receptors. It shall be continuously extended to include surrogates for any bioregulator known or presumed to mediate harmful effects. Free access to this virtual laboratory shall allow any interested party to estimate the harmful potential of a given substance prior to its synthesis. This is achieved by generating the three-dimensional structure of the compound and its possible metabolites in the computer, followed by calculating their binding affinity towards each receptor surrogate in the database. Only compounds/metabolites passing through this surrogate battery without displaying a significant affinity towards any member may be cleared for synthesis and preclinical studies. This way, potentially harmful compounds can be withdrawn from the evaluation pipeline before in vivo test are conducted, hence contributing to the reduction of animal testing in chemical and pharmaceutical research and development.

Vracko M. **A study of structure-carcinogenicity relationship for 86 compounds from NTP data base using topological indices as descriptors.** SAR QSAR Environ Res 2000;11(2):103-15.

Abstract: 86 compounds from NTP carcinogenic potency data base have been used to derive neural network models. Compounds were described with topological indices. Carcinogenicity has been given as a binary quantity--a compound is carcinogenic or non carcinogenic. Several models have been tested with a recognition ability test and with the leave-one-out cross validation method. For the best model the ratio between correct and wrong classifications was 70/30. Furthermore, the model has been used to classify 17 compounds not used for setting of the models. The predicted carcinogenic classes and the neighbors in the neural network influencing the predictions have been discussed.

Wang X, Dong Y, Wang L, Han S. **Acute toxicity of substituted phenols to Rana japonica tadpoles and mechanism-based quantitative structure-activity relationship (QSAR) study.** Chemosphere 2001;44(3):447-55.
Abstract: Acute 12 h and 24 h lethal toxicity (12 h-LC50 and 24 h-LC50) of 31 substituted phenols to Rana japonica tadpoles was determined. Results indicate that toxicity of phenols to tadpoles varied only slightly with length of exposure and the 12-h test could serve as surrogate of the 24-h test. A mechanism-based quantitative structure-activity relationship (QSAR) method was employed and 1-octanol/water partition coefficient (log K(ow))-dependent models were developed to study different modes of toxic action. Most phenols elicited their response via a polar narcotic mechanism and an excellent logK(ow)-dependent model was obtained. Soft electrophilicity and pro-electrophilicity were observed for some phenols and a good log K(ow)-dependent model was also achieved. Additionally, the significant dissociation of carboxyl on benzoic acid derivatives sharply reduced their toxicity. A statistically robust QSAR model was developed for all studied compounds with the combined application of log K(ow), energy of lowest unoccupied orbital (E(lumo)), heat of formation (HOF) and the first-order path molecular connectivity dices (1chi(p)).

Wang X, Sun C, Wang L, Han S. **Quantitative structure-activity relationships: comparative inhibition of nitrogen-containing aromatics on germination of Cucumis sativus.** Environ Toxicol Chem 2001;20(4):913-6.
Abstract: Comparative inhibition effect of selected nitrogen-containing aromatics on germination rate of Cucumis sativus was investigated, and quantitative structure-activity relationships were developed and mechanistically interpreted. Energy of lowest unoccupied orbital was found to successfully model the toxicity of the test chemicals. It was discovered that inhibition of nitrogen-containing aromatics to germination of Cucumis sativus was caused mainly by their in vivo redox reaction.

Xi X, Xu F, Cao J, Tao S. **[Estimation of LC50 of chemicals to rainbow trout by fragment constant method].** Huan Jing Ke Xue 2001;22(4):29-32 [Chi].
Abstract: A fragment constant model for prediction of 96 h LC50 of chemicals to rainbow trout was developed based on measured experimental data of 258 chemicals collected from the literature. The accuracy and the robustness of the model were discussed. The coefficient of determination of the model is 0.9495 and the mean residual is 0.42 log-unit. The model is robust for both individual chemical or chemical class.

Xu S, Li L, Tan Y, Feng J, Wei Z, Wang L. **Prediction and QSAR analysis of toxicity to Photobacterium phosphoreum for a group of heterocyclic nitrogen compounds.** Bull Environ

Contam Toxicol 2000;64(3):316-22.

DeMarini DM, Landi S, Ohe T, Shaughnessy DT, Franzen R, Richard AM. **Mutation spectra in Salmonella of analogues of MX: implications of chemical structure for mutational mechanisms.** Mutat Res 2000;453(1):51-65.
Abstract: We determined the mutation spectra in Salmonella of four chlorinated butenoic acid analogues (BA-1 through BA-4) of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) and compared the results with those generated previously by us for MX and a related compound, MCF. We then considered relationships between the properties of mutagenic potency and mutational specificity for these six chlorinated butenoic acid analogues. In TA98, the three most potent mutagens, BA-3, BA-4, MX, and the organic extract, all induced large percentages of complex frameshifts (33-67%), which distinguish these agents from any other class of compound studied previously. In TA100, which has only GC sites for mutation recovery, >71% of the mutations induced by all of the agents were GC-->TA transversions. The availability of both GC and TA sites for mutation in TA104 resulted in greater distinctions in mutational specificity than in TA100. MX targeted GC sites almost exclusively (98%); the structurally similar BA-4 and BA-2 produced mutations at similar frequencies at both GC and AT sites; and the structurally similar BA-3 and BA-1 induced most mutations at AT sites (69%). Thus, large variations in structural properties influencing relative mutagenic potency appeared to be distinct from the more localized similar structural features influencing mutagenic specificity in TA104. Among a set of physicochemical properties examined for the six butenoic acids, a significant correlation was found between pK(a) and mutagenic potency in TA100, even when the unionized fraction of the activity dose was considered. In addition, a correlation in CLOGP for BA-1 to BA-4 suggested a role for bioavailability in determining mutagenic potency. These results illustrate the potential value of structural analyses for exploring the relationship between chemical structure and mutational mechanisms. To our knowledge, this is the first study in which such analyses have been applied to structural analogues for which both mutagenic potency and mutation spectra date were available.

Galatin PS, Abraham DJ. **QSAR: Hydropathic analysis of inhibitors of the p53-mdm2 interaction.** Proteins 2001;45(3):169-75.
Abstract: To date, a number of p53-derived peptides have been evaluated in vitro for their ability to inhibit the carcinogenic p53-mdm2 interaction. Design of second-generation nonpeptidic compounds requires the reduction of large peptide structures down to small molecules maintaining the proper spatial arrangement of key functional groups. Molecular modeling software exists that can predict and rank intermolecular interactions from the p53-mdm2 complex crystal structure. Such analyses can yield a pharmacophore model suitable as a search query for a 3D chemical database to generate new lead compounds. As preliminary validation of this methodology, the Hydropathic INTeractions (HINT) program has been used to generate noncovalent interaction measurements between reported peptide inhibitors and mdm2. Quantitative structure-activity relationships were developed expressing peptide activity as a linear combination of hydropathic descriptors. In general, HINT measurements accurately modeled the effects of even single-atom alterations of the p53-peptide structure on activity, accounting for 70-90% of variation in experimental inhibition constants. These results surpassed those of a recently described molecular dynamics-based approach and required significantly less computation time. In

conclusion, the HINT program can be integrated into the drug design cycle for next-generation p53-mdm2 complex inhibitors with confidence in its ability to simulate this noteworthy protein-protein interaction. Proteins 2001;45:169-175.

Hong K, Mano I, Driscoll M. **In vivo structure-function analyses of Caenorhabditis elegans MEC-4, a candidate mechanosensory ion channel subunit.** J Neurosci 2000;20(7):2575-88.
Abstract: Mechanosensory signaling mediated by mechanically gated ion channels constitutes the basis for the senses of touch and hearing and contributes fundamentally to the development and homeostasis of all organisms. Despite this profound importance in biology, little is known of the molecular identities or functional requirements of mechanically gated ion channels. We report a genetically based structure-function analysis of the candidate mechanotransducing channel subunit MEC-4, a core component of a touch-sensing complex in Caenorhabditis elegans and a member of the DEG/ENaC superfamily. We identify molecular lesions in 40 EMS-induced mec-4 alleles and further probe residue and domain function using site-directed approaches. Our analysis highlights residues and subdomains critical for MEC-4 activity and suggests possible roles of these in channel assembly and/or function. We describe a class of substitutions that disrupt normal channel activity in touch transduction but remain permissive for neurotoxic channel hyperactivation, and we show that expression of an N-terminal MEC-4 fragment interferes with in vivo channel function. These data advance working models for the MEC-4 mechanotransducing channel and identify residues, unique to MEC-4 or the MEC-4 degenerin subfamily, that might be specifically required for mechanotransducing function. Because many other substitutions identified by our study affect residues conserved within the DEG/ENaC channel superfamily, this work also provides a broad view of structure-function relations in the superfamily as a whole. Because the C. elegans genome encodes representatives of a large number of eukaryotic channel classes, we suggest that similar genetic-based structure-activity studies might be generally applied to generate insight into the in vivo function of diverse channel types.

Yourtee D, Holder AJ, Smith R, Morrill JA, Kostoryz E, Brockmann W, Glaros A, Chappelow C, Eick D. **Quantum mechanical quantitative structure activity relationships to avoid mutagenicity in dental monomers.** J Biomater Sci Polym Ed 2001;12(1):89-105.
Abstract: The objective of this study was to identify through quantum mechanical quantitative structure activity relationships (Q-QSARs) chemical structures in dental monomers that influence their mutagenicity. AMPAC, a semiempirical computer program that provides quantum mechanical information for chemical structures, was applied to three series of reference chemicals: a set of methacrylates, a set of aromatic and a set of aliphatic epoxy compounds. QSAR models were developed using this chemical information together with mutagenicity data (Salmonella TA 100, Ames Test). CODESSA, a QSAR program that calculates quantum chemical descriptors from information generated by AMPAC and statistically matches these descriptors with observed biological properties was used. QSARs were developed which had r2 values exceeding 0.90 for each study series. These QSARs were used to accurately predict the mutagenicity of BISGMA. a monomer commonly used in dentistry, and two epoxy monomers with developing use in dentistry, GY-281 and UVR-6105. The Q-QSAR quantum mechanical descriptors correctly predicted the level of mutagenicity for all three compounds. The descriptors in the correlation equation pointed to components of structure that may contribute to mutagenesis. The QSARs also provided 'dose windows' for testing mutagenicity, circumventing the need

for extensive dose exploration in the laboratory. The Q-QSAR method promises an approach for biomaterials scientists to predict and avoid mutagenicity from the chemicals used in new biomaterial designs.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Ando H, Furuta T, Tsien RY, Okamoto H. **Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos.** Nat Genet 2001;28(4):317-25.
Abstract: We report a new and simple technique for photo-mediated temporal and spatial control of gene activation in zebrafish embryos as an alternative to the gene 'knockdown' approach using antisense, morpholino-modified oligonucleotides (morpholinos). The synthetic compound 6-bromo-4-diazomethyl-7-hydroxycoumarin (Bhc-diazo) forms a covalent bond with the phosphate moiety of the sugar-phosphate backbone of RNA, a process known as caging. The 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) group binds to approximately 30 sites on the phosphate moieties per 1 kb of RNA sequence. Bhc-caged mRNA undergoes photolysis (uncaging) when exposed to long-wave ultraviolet light (350 to 365 nm). We show that Bhc-caged green fluorescent protein (Gfp) mRNA has severely reduced translational activity in vitro, whereas illumination of Bhc-caged mRNA with ultraviolet light leads to partial recovery of translational activity. Bhc-caged mRNA is highly stable in zebrafish embryos. In embryos injected with Bhc-caged Gfp mRNA at the one-cell stage, GFP protein expression and fluorescence is specifically induced by ultraviolet light. We also show that, consistent with results obtained using other methods, uncaging eng2a (which encodes the transcription factor Engrailed2a) in the head region during early development causes a severe reduction in the size of the eye and enhanced development of the midbrain and the midbrain-hindbrain boundary at the expense of the forebrain.

Araki K, Okamoto H, Graveson AC, Nakayama I, Nagoya H. **Analysis of haploid development based on expression patterns of developmental genes in the medaka Oryzias latipes.** Dev Growth Differ 2001;43(5):591-9.
Abstract: The abnormalities of haploid medaka embryos were characterized by comparative analysis of histologic sections and expression patterns of some developmental marker genes between haploids and diploids to clarify whether medaka haploids are useful for identifying mutants. During gastrulation, an obvious defect was first observed as a delay of epiboly and involution. This delay was shown to be caused not by the perturbation of mesoderm induction, but by widespread cell death and disorganization of cell arrangement in the blastoderm. This disorganization of cell arrangement was also detected in various organs, such as the brain, somite and notochord, at a late developmental stage. Ten days after fertilization, a small head and a short body axis were formed; these changes were also observed in haploid embryos in other species, but their cause is unknown. Based on the expression patterns of HNF3beta and goosecoid, it was demonstrated that a short and impotent prechordal plate induced near the marginal zone in haploid embryos was responsible for this defect. However, in these experiments it was also demonstrated that many major organs in haploids, such as the somite and notochord, differentiated incompletely but were present. Therefore, it was concluded that haploid screening is suitable for identifying mutations revealed by an obvious phenotype, such as dorsoventral polarity.

Ashby J. **Validation of in vitro and in vivo methods for assessing endocrine disrupting chemicals.**

Toxicol Pathol 2000;28(3):432-7.
Abstract: The concepts that require validation in terms of the subject of endocrine disruption are listed and discussed. The main mechanisms by which endocrine disruption can occur are identified, and the assays required for the detection of adverse endocrine disruption toxicities associated with these mechanisms are discussed. The process of assay validation is considered. The validation of structure-activity relationships, the need for reference chemicals, and the problems recently encountered when attempting to reproduce endocrine disruption data are also explored. The most important conclusions derived from this analysis are that given the immature state of research into endocrine disruption toxicity, testing strategies and the types of assay employed should be kept under constant review; inevitably researchers need to accept the fact that future revision of each assay will be required. Second, given the current absence of any chemical that is universally accepted to be devoid of endocrine toxicity, assay specificity will be difficult to assess, and that imposes the need for alternative objective criteria for assessing the value of individual assays.

Bauer H, Lele Z, Rauch GJ, Geisler R, Hammerschmidt M. **The type I serine/threonine kinase receptor Alk8/Lost-a-fin is required for Bmp2b/7 signal transduction during dorsoventral patterning of the zebrafish embryo.** Development 2001;128(6):849-58.
Abstract: Ventral specification of mesoderm and ectoderm depends on signaling by members of the bone morphogenetic protein (Bmp) family. Bmp signals are transmitted by a complex of type I and type II serine/threonine kinase transmembrane receptors. Here, we show that Alk8, a novel member of the Alk1 subgroup of type I receptors, is disrupted in zebrafish lost-a-fin (laf) mutants. Two alk8/laf null alleles are described. In laf(tm110), a conserved extracellular cysteine residue is replaced by an arginine, while in laf(m100), Alk8 is prematurely terminated directly after the transmembrane domain. The zygotic effect of both mutations leads to dorsalization of intermediate strength. A much stronger dorsalization, similar to that of bmp2b/swirl and bmp7/snailhouse mutants, however, is obtained by inhibiting both maternally and zygotically supplied alk8 gene products with morpholino antisense oligonucleotides. The phenotype of laf mutants and alk8 morphants can be rescued by injected mRNA encoding Alk8 or the Bmp-regulated transcription factor Smad5, but not by mRNA encoding Bmp2b or Bmp7. Conversely, injected mRNA encoding a constitutively active version of Alk8 can rescue the strong dorsalization of bmp2b/swirl and bmp7/snailhouse mutants, whereas smad5/somitabun mutant embryos do not respond. Altogether, the data suggest that Alk8 acts as a Bmp2b/7 receptor upstream of Smad5.

Bauer MP, Goetz FW. **Isolation of gonadal mutations in adult zebrafish from a chemical mutagenesis screen.** Biol Reprod 2001;64(2):548-54.
Abstract: A mutagenesis screen was conducted on zebrafish using N:-ethyl N:-nitrosourea as a mutagen and an F2 crossing scheme to obtain homozygous mutants in the F3 generation. Whole abdomens of 3-mo-old F3 zebrafish progeny were fixed and mass-embedded in paraffin blocks. Blocks were cut with a microtome to obtain cross-sections of the entire body cavity that included the ovaries and testes. Slides of the cross-sections were analyzed for alterations in gonadal structure and gametogenesis and were compared with gonads of wild-type fish. A total of 125 mutagenized genomes in 81 families were screened and 11 mutations were observed that produced visible phenotypes in only one sex per family. Male mutations included testes without mature sperm that contained either predominantly spermatocytes

or spermatogonia. Female mutations included ovaries containing 1) degenerating oocytes surrounded by hypertrophied follicle walls or stroma, 2) extrafollicular tissue proliferation, 3) proliferating postovulatory follicle walls, and 4) large numbers of degenerating preovulatory and postovulatory oocytes. While past screens on zebrafish have concentrated on early developmental mutations, the results of this study demonstrate for the first time that mutagenesis can be used with zebrafish to study reproduction in adult animals.

Boomsma RA, Scott H, Walters K. **Immunocytochemical localization of epidermal growth factor receptor in early embryos of the Japanese medaka fish (Oryzias latipes).** Histochem J 2001;33 (1):37-42.
Abstract: This study was undertaken to localize epidermal growth factor receptor (EGFR) during early development of Japanese medaka embryos using immunocytochemistry. Specific staining was observed in all stages studied. All of the cells of the embryonic disc from the germinal disc (1 cell) through the late high blastula stages stained moderately for EGFR. Beginning with the flat blastula stage, the surface and lateral cells of the embryonic disc and the cells migrating around the yolk stained intensely for EGFR, and this continued throughout the study period. The presence of the keel at the late gastrula stage did not affect the moderate staining of the majority of the embryonic disc cells. When somites first appeared, the keel region stained less intensely than before, but scattered individual cells stained intensely for EGFR. Embryos with 12 somites had a neural tube that was lightly stained except for a few intensely stained individual cells. The neural tube, notochord and somites in 24-somite embryos lacked immunostaining. However, the surface epithelium, aorta, intestinal epithelium and pronephric duct demonstrated EGFR immunostaining. This study demonstrates that EGFR is present during medaka development and supports the hypothesis that EGFR ligands are important during cleavage, gastrulation and early organogenesis.

Bremer S, Worth AP, Paparella M, Bigot K, Kolossov E, Fleischmann BK, Hescheler J, Balls M. **Establishment of an in vitro reporter gene assay for developmental cardiac toxicity.** Toxicol In Vitro 2001;15(3):215-23.
Abstract: This study is based on the unique potential of pluripotent embryonic stem (ES) cells to differentiate in vitro into embryoid bodies containing cell lineages representative of most cell types found in the mammalian fetus. However, the use of wild type ES cells as an in vitro assay for embryotoxicological studies is complicated by the simultaneous development of various cellular phenotypes. This prevents a quantitative assessment of drug effects on one specific cell type. Here we report the effects of 15 chemicals on cardiac differentiation as determined by various specific toxicological endpoints such as morphological inspection (contractile activity), quantitative mRNA analysis and cardiac-specific expression of green fluorescent protein (GFP), used as a quantitative reporter. The data from the different endpoints have been subjected to a statistical analysis, and a preliminary prediction model is proposed. The results demonstrate that genetically-engineered ES cells could provide a valuable tool for estimating the developmental cardiotoxic potential of compounds in vitro and form the basis for automated analysis in a high-throughput system.

Brunner B, Hornung U, Shan Z, Nanda I, Kondo M, Zend-Ajusch E, Haaf T, Ropers HH, Shima A, Schmid M, et al. **Genomic organization and expression of the doublesex-related gene cluster in**

vertebrates and detection of putative regulatory regions for dmrt1. Genomics 2001;77(1/2):8-17.
Abstract: Genes related to the Drosophila melanogaster doublesex and Caenorhabditis elegans mab-3 genes are conserved in human. They are identified by a DNA-binding homology motif, the DM domain, and constitute a gene family (DMRTs). Unlike the invertebrate genes, whose role in the sex-determination process is essentially understood, the function of the different vertebrate DMRT genes is not as clear. Evidence has accumulated for the involvement of DMRT1 in male sex determination and differentiation. DMRT2 (known as terra in zebrafish) seems to be a critical factor for somitogenesis. To contribute to a better understanding of the function of this important gene family, we have analyzed DMRT1, DMRT2, and DMRT3 from the genome model organism Fugu rubripes and the medakafish, a complementary model organism for genetics and functional studies. We found conservation of synteny of human chromosome 9 in F. rubripes and an identical gene cluster organization of the DMRTs in both fish. Although expression analysis and gene linkage mapping in medaka exclude a function for any of the three genes in the primary step of male sex determination, comparison of F. rubripes and human sequences uncovered three putative regulatory regions that might have a role in more downstream events of sex determination and human XY sex reversal.

Buxton P, Edwards C, Archer CW, Francis-West P. **Growth/differentiation factor-5 (GDF-5) and skeletal development.** J Bone Joint Surg Am 2001;83-A Suppl 1(Pt 1):S23-30.
Abstract: BACKGROUND: Growth/differentiation factor-5 (GDF-5) has been shown to be essential for normal appendicular skeletal and joint development in humans and mice. In brachypod, a Gdf-5 gene mouse mutant, the defect is first apparent during early chondrogenesis, with the cartilage blastema already reduced in size by E12.5. This defect is associated with changes in the expression of cell surface molecules. METHODS: To understand further how GDF-5 controls cartilage formation, we first mapped the expression of the Gdf-5 gene during skeletal development (please note that the abbreviation for the gene is given in italics and the abbreviation for the protein expressed by the gene is given in capital letters). Subsequently, we over-expressed GDF-5 in the developing chick embryo using a replication competent retrovirus, RCAS(BP). We determined its effects on skeletal development by histological examination and its effects on early growth by autoradiography of proliferating cells. In addition, we examined the effect of GDF-5 on chondrogenic differentiation using micromass and single cell suspension cultures of limb mesenchymal cells, RESULTS: These studies show that the Gdf-5 gene is expressed in the early cartilage condensation, the perichondrium, and the joint interzone. Over-expresSion of GDF-5 in chick limb buds, during the condensation stage or later when the skeletal elements have formed, increased the size of the affected elements. In both cases, the increase in size was associated with an increase in cell number and, at later stages, this was correlated with an increase in S-phase cells. In vitro studies showed that GDF-5 could increase cell adhesiveness, and this may be a mechanism through which GDF-5 initiates condensation formation. CONCLUSION: These studies show that GDF-5 acts at two stages of skeletal development and by two distinct mechanisms. First, GDF-5 promotes the initial stages of chondrogenesis by promoting cell adhesion, which is consistent with the expression of Gdf-5 in the cartilage condensation. Second, GDF-5 can increase the size of the skeletal elements by increasing proliferation within the epiphyseal cartilage adjacent to its expression within the joint interzone.

Buznikov GA, Nikitina LA, Bezuglov VV, Lauder JM, Padilla S, Slotkin TA. **An invertebrate model**

of the developmental neurotoxicity of insecticides: effects of chlorpyrifos and dieldrin in sea urchin embryos and larvae. Environ Health Perspect 2001;109(7):651-61.
Abstract: Chlorpyrifos targets mammalian brain development through a combination of effects directed at cholinergic receptors and intracellular signaling cascades that are involved in cell differentiation. We used sea urchin embryos as an invertebrate model system to explore the cellular mechanisms underlying the actions of chlorpyrifos and to delineate the critical period of developmental vulnerability. Sea urchin embryos and larvae were exposed to chlorpyrifos at different stages of development ranging from early cell cleavages through the prism stage. Although early cleavages were unaffected even at high chlorpyrifos concentrations, micromolar concentrations added at the mid-blastula stage evoked a prominent change in cell phenotype and overall larval structure, with appearance of pigmented cells followed by their accumulation in an extralarval cap that was extruded from the animal pole. At higher concentrations (20-40 microM), these abnormal cells constituted over 90% of the total cell number. Studies with cholinergic receptor blocking agents and protein kinase C inhibitors indicated two distinct types of effects, one mediated through stimulation of nicotinic cholinergic receptors and the other targeting intracellular signaling. The effects of chlorpyrifos were not mimicked by chlorpyrifos oxon, the active metabolite that inhibits cholinesterase, nor by nonorganophosphate cholinesterase inhibitors. Dieldrin, an organochlorine that targets GABA(A)receptors, was similarly ineffective. The effects of chlorpyrifos and its underlying cholinergic and signaling-related mechanisms parallel prior findings in mammalian embryonic central nervous system. Invertebrate test systems may thus provide both a screening procedure for potential neuroteratogenesis by organophosphate-related compounds, as well as a system with which to uncover novel mechanisms underlying developmental vulnerability.

Campagna C, Sirard MA, Ayotte P, Bailey JL. **Impaired maturation, fertilization, and embryonic development of porcine oocytes following exposure to an environmentally relevant organochlorine mixture.** Biol Reprod 2001;65(2):554-60.
Abstract: The reproductive health risks related to exposure to persistent organic pollutants in the environment remain controversial. This debate is partly because most studies have investigated only one or two chemicals at a time, whereas populations are exposed to a large spectrum of persistent chemicals in their environment. Using the pig as a toxicological model, we hypothesized that exposing immature cumulus-oocyte complexes to an organochlorine mixture during in vitro maturation (IVM) would adversely affect oocyte maturation, fertilization, and subsequent embryo development. This organochlorine mixture mimics that which contaminates the arctic marine food chain. Cumulus-oocyte complexes were cultured in IVM medium containing increasing concentrations of the organochlorine mixture, similar to that found in women of highly exposed populations. Organochlorines reduced the quality of cumulus expansion and the viability of cumulus cells in a dose-response manner. The proportion of apoptotic cumulus cells also increased due to organochlorine exposure. Half of the oocytes were fixed after insemination, and the remainders were cultured for 8 days. Concentrations of organochlorines did not affect the rates of oocyte degeneration, sperm penetration, and development to morula. However, incidence of incompletely matured oocytes increased and polyspermy rate decreased, both in a dose-response manner with increasing organochlorine concentrations. Blastocyst formation and number of cells per blastocyst declined with organochlorine concentration. Exposing porcine cumulus-oocyte complexes to an environmentally pertinent organochlorine mixture during IVM disturbs oocyte development, supporting recent concerns that such pollutants harm reproductive health in humans and

other mammalian species.

Capehart AA. **In vitro production of monoclonal antibodies to cultured embryonic chick limb mesenchyme.** Methods Cell Sci 2000;22(4):319-27.
Abstract: A simple, rapid protocol for the in vitro production of monoclonal antibodies (MAbs) that recognize native antigens in cultured chick limb mesenchyme during chondrogenic differentiation is described. Murine lymphocytes were stimulated by direct exposure to methanol-fixed micromass cultures of limb mesenchyme derived from the distal tip of stage 25 chick limb buds. Initial immunohistochemical characterization of two antibodies (DIDI and DIIA5) produced by this method showed preferential localization of reactivity with antigens in developing cartilage nodules during chondrogenesis in cultured chick limb mesenchyme. This study demonstrates the utility of in vitro immunization of lymphocytes for the production of MAbs to native antigens expressed by differentiating embryonic limb cells in culture. Immunohistochemical data provided by DIDI and DIIA5 suggest that antigens bearing these epitopes may be important in early morphogenetic events during limb skeletal development.

Celius T, Matthews JB, Giesy JP, Zacharewski TR. **Quantification of rainbow trout (Oncorhynchus mykiss) zona radiata and vitellogenin mRNA levels using real-time PCR after in vivo treatment with estradiol-17 beta or alpha-zearalenol.** J Steroid Biochem Mol Biol 2000;75(2-3):109-19.
Abstract: Estrogen receptor-mediated induction of zona radiata (ZR) and vitellogenin (VTG) mRNA and protein in rainbow trout (Oncorhynchus mykiss) was compared to assess their utility as biomarkers for exposure to estrogenic compounds. Partial sequences of rainbow trout ZR and beta-actin were cloned by reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers based on conserved regions across a number of species. A 549 bp fragment of the rainbow trout ZR-gene showed a high degree of amino acid sequence identity to that of salmon (77%), winter flounder (64%), carp ZP2 (63%) and medaka (61%) ZR-proteins. The 1020 bp beta-actin fragment was approximately 100% identical to sequences from several species. Real-time PCR was used to quantify the induction of ZR-gene and VTG in rainbow trout liver after in vivo exposure to estradiol-17 beta (E(2)) (0.01, 0.1, 1.0 or 10 mg/kg body weight (bw) fish) or alpha-zearalenol (alpha-ZEA) (0.1, 1.0 or 10 mg/kg bw). Real-time PCR and indirect enzyme-linked immunosorbent assay (ELISA) showed that ZR and VTG were induced in both the liver and the plasma after a single injection of E(2) or alpha-ZEA. ZR was more responsive to low levels of E(2) and alpha-ZEA than VTG, and real-time PCR was shown to be more sensitive than the ELISA. Rainbow trout ZR-gene and proteins provide a sensitive biomarker for assessing estrogenic activity.

Chan PJ, Calinisan JH, Corselli JU, Patton WC, King A. **Updating quality control assays in the assisted reproductive technologies laboratory with a cryopreserved hamster oocyte DNA cytogenotoxic assay**. J Assist Reprod Genet 2001;18(3):129-34.
Abstract: PURPOSE: Despite advances in assisted reproduction, there is no progress in quality control bioassays. The objectives were to develop a comet assay to measure DNA fragmentation in thawed cryopreserved oocytes and compare this assay with one-cell mouse embryo bioassay. METHODS: Thawed hamster oocytes from a commercial source were incubated in culture media with either 0-, 50-, or 100-microM hydrogen peroxide, or, in media exposed to different contact materials and unknown

proficiency analytes. Incubation time was 1.5 h at 37 degrees C. The oocytes were dried, fixed, stained with acridine orange, embedded in a mini-agarose layer and electrophoresis was carried out. Fluorescent images were analyzed. The results were compared with standard one-cell mouse assay data. RESULTS: The 100-microM hydrogen peroxide treatment caused greatest DNA fragmentation in the hamster oocytes at Hours 1 and 2. A dose response was observed. Intraassay coefficient of variation was 5.7%. Only one of the five materials tested passed both assays. The data for the unknown proficiency analytes were similar for both assays. CONCLUSIONS: The oocyte comet assay demonstrated DNA fragmentation in the presence of toxic substances. The detection of toxicity in two materials that passed the mouse bioassay suggested increased sensitivity in the new assay. The oocyte comet assay and the mouse bioassay results matched in the proficiency test. However, more studies are still needed to determine optimal sensitivity.

Chan PJ, Corselli JU, Patton WC, Jacobson JD, Chan SR, King A. **A simple comet assay for archived sperm correlates DNA fragmentation to reduced hyperactivation and penetration of zona-free hamster oocytes.** Fertil Steril 2001;75(1):186-92.
Abstract: OBJECTIVE: To correlate sperm variables with sperm DNA fragmentation, as assessed by using a modified alkaline comet assay for sperm smears. DESIGN: The comet assay was adapted for fixed sperm smears (59 cases), and the level of DNA fragmentation was determined. SETTING: Clinical and academic research environment. PATIENT(S): 59 patients undergoing fertility treatment. INTERVENTION(S): Sperm samples leftover from IVF procedures were fixed and processed for the comet assay. MAIN OUTCOME MEASURE(S): Sperm head DNA density and sperm variables. RESULT(S): A correlation was observed between increased sperm head DNA fragmentation and decreased penetration of zona-free hamster oocytes. Heat-induced hyperactive motility decreased as DNA fragmentation increased. The DNA fragmentation did not correlate with percentages of intact acrosome, normality, maturity, and strict normal morphology. CONCLUSION(S): The advantages of the comet assay for archived cells include simplicity, low intraassay coefficient of variation, and low performance cost; in addition, DNA analysis can be carried out at leisure. Low DNA damage was associated with higher hyperactivation and oocyte penetration, suggesting that failed fertilization was linked to compromised DNA integrity in the sperm. Exploration of compounds to repair damaged DNA is warranted.

Chung NP, Cheng CY. **Is cadmium chloride-induced inter-sertoli tight junction permeability barrier disruption a suitable in vitro model to study the events of junction disassembly during spermatogenesis in the rat testis?** Endocrinology 2001;142(5):1878-88.
Abstract: The events of germ cell movement during spermatogenesis are composed of intermittent phases of junction disassembly and reassembly. Although primary Sertoli cells cultured in vitro can be used to study junction reassembly, an in vitro model to study the events of junction disassembly is still lacking. We have assessed whether the CdCl(2)-induced inter-Sertoli tight junction (TJ) permeability barrier disruption in vitro can fill this gap. When Sertoli cells (1.2 x 10(6) cells/cm(2)) were cultured on Matrigel-coated bicameral units to allow the assembly of inter-Sertoli TJs, it was manifested by a steady rise in transepithelial electrical resistance across the Sertoli cell epithelia. Exposure of these cells on day 1 (i.e. 24 h after their isolation) to CdCl(2) at 5-10 microM for 8 h could perturb the inter-Sertoli TJ assembly dose dependently without any apparent cytotoxicity. Likewise, when cells were exposed to

CdCl(2) (0.1-5 microM) on day 4 for 8 h after inter-Sertoli TJs were already assembled, CdCl(2) also perturbed the maintenance of inter-Sertoli TJ permeability barrier dose dependently without signs of cell cytotoxicity. Although the perturbed inter-Sertoli TJs were not capable of resealing even after the removal of CdCl(2), the presence of testosterone (T) at 1 x 10(-9) M allowed resealing of the inter-Sertoli TJ barrier after CdCl(2) was removed, whereas the presence of 2 x 10(-7) M testosterone even protected Sertoli cells from CdCl(2)-induced damage. More important, the reassembly of inter-Sertoli TJs after CdCl(2)-induced TJ disruption was accompanied by changes in cellular gene expression of occludin and urokinase plasminogen activator, which mimicked their patterns during inter- Sertoli TJ assembly in vitro without CdCl(2) treatment. Based on these results, it is apparent that CdCl(2)-induced inter-Sertoli TJ disassembly is a potential in vitro model to study the events of junction disassembly.

DeLise AM, Stringa E, Woodward WA, Mello MA, Tuan RS. **Embryonic limb mesenchyme micromass culture as an in vitro model for chondrogenesis and cartilage maturation.** Methods Mol Biol 2000;137:359-75.

Fang H, Tong W, Perkins R, Soto AM, Prechtl NV, Sheehan DM. **Quantitative comparisons of in vitro assays for estrogenic activities.** Environ Health Perspect 2000;108(8):723-9.
Abstract: Substances that may act as estrogens show a broad chemical structural diversity. To thoroughly address the question of possible adverse estrogenic effects, reliable methods are needed to detect and identify the chemicals of these diverse structural classes. We compared three assays--in vitro estrogen receptor competitive binding assays (ER binding assays), yeast-based reporter gene assays (yeast assays), and the MCF-7 cell proliferation assay (E-SCREEN assay)--to determine their quantitative agreement in identifying structurally diverse estrogens. We examined assay performance for relative sensitivity, detection of active/inactive chemicals, and estrogen/antiestrogen activities. In this examination, we combined individual data sets in a specific, quantitative data mining exercise. Data sets for at least 29 chemicals from five laboratories were analyzed pair-wise by X-Y plots. The ER binding assay was a good predictor for the other two assay results when the antiestrogens were excluded (r(2) is 0.78 for the yeast assays and 0.85 for the E-SCREEN assays). Additionally, the examination strongly suggests that biologic information that is not apparent from any of the individual assays can be discovered by quantitative pair-wise comparisons among assays. Antiestrogens are identified as outliers in the ER binding/yeast assay, while complete antagonists are identified in the ER binding and E-SCREEN assays. Furthermore, the presence of outliers may be explained by different mechanisms that induce an endocrine response, different impurities in different batches of chemicals, different species sensitivity, or limitations of the assay techniques. Although these assays involve different levels of biologic complexity, the major conclusion is that they generally provided consistent information in quantitatively determining estrogenic activity for the five data sets examined. The results should provide guidance for expanded data mining examinations and the selection of appropriate assays to screen estrogenic endocrine disruptors.

Fenske M, van Aerle R, Brack S, Tyler CR, Segner H. **Development and validation of a homologous zebrafish (Danio rerio Hamilton-Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals.** Comp Biochem Physiol C Toxicol Pharmacol 2001;129(3):217-32.

Abstract: Vitellogenin (VTG) was isolated by anion exchange chromatography from plasma of female zebrafish (Danio rerio) induced with 17alpha-ethinylestradiol (EE2). The purity of the VTG isolate was confirmed by polyacrylamide gel electrophoresis (SDS-PAGE). Purified VTG was used to raise polyclonal antibodies in rabbits and the specificity of the antisera for VTG confirmed by Western blot analysis of plasma proteins separated by SDS-PAGE. The antibodies cross-reacted with two proteins in the plasma of female zebrafish, with molecular masses of approximately 142 and 171 kDa. No cross-reactivity was observed with any other plasma proteins. A competitive enzyme-linked immunosorbent assay (ELISA) was developed using the polyclonal zebrafish VTG (z-VTG) antibodies and purified z-VTG as ligand and standard, respectively. The z-VTG ELISA was sensitive with a detection limit of between 2.0 and 3.0 ng purified VTG/ml, and a working range between 3 and 500 ng/ml (30-85% binding). The ELISA demonstrated precision, with inter- and intra-assay variations of 7.5+/-2.7 and 4.9 +/-1.4%, respectively. Plasma from adult zebrafish and whole body homogenates from juvenile zebrafish diluted parallel with the z-VTG standard in the ELISA, validating the assay for quantifying z-VTG in both of these tissues. Exposure of adult male zebrafish to EE2 via water induced a concentration-dependent induction of VTG with a lowest observed effect concentration (LOEC) < or =1.67 ng EE2/l (for a 21-day exposure). The homologous z-VTG ELISA provides a valuable tool for the study of environmental estrogens in zebrafish.

Fort DJ, Rogers RL, Paul RR, Stover EL, Finch RA. **Optimization of an exogenous metabolic activation system for FETAX.** II. Preliminary evaluation. Drug Chem Toxicol 2001;24(2):117-27.
Abstract: The developmental toxicities of five test compounds including carbon tetrachloride, urethane, phenacetin, parathion, and chloroform, were evaluated using Frog Embryo Teratogenesis Assay--Xenopus (FETAX), with minor modification. Post-isolation mixtures of differently-induced rat liver microsomes (phenobarbital- (PB), beta-naphthoflavone- (beta-NF), and isoniazid- (INH)-induced preparations) were co-cultured directly with X. laevis embryos. Results from these studies suggest that the Aroclor 1254-induced MAS could effectively be replaced by a mixed lot of PB-, beta-NF-, and INH-induced rat liver microsomes. Each of the test materials were found to be developmentally toxic when bioactivated by the mixed MAS.

Fort DJ, Rogers RL, Stover EL, Finch RA. **Optimization of an exogenous metabolic activation system for FETAX**. I. Post-isolation rat liver microsome mixtures. Drug Chem Toxicol 2001;24(2):103-15.
Abstract: The developmental toxicity of cyclophosphamide, coumarin, 2-acetyl-aminofluorine (2-AAF), and trichloroethylene (TCE) was assessed with Frog Embryo Teratogenesis Assay: Xenopus (FETAX). Late Xenopus laevis blastulae were exposed to each test material for 96-h in two separate static-renewal tests with and without the presence of five differently induced exogenous metabolic activation systems (MAS). The MAS consisted of Aroclor 1254- (Aroclor 1254 MAS), isoniazid- (INH MAS), phenobarbital- (PB MAS), or beta-naphthoflavone- (beta-NF MAS), or a post-isolation mixture (mixed MAS) of INH-, PB-, and beta-NF-induced rat liver microsomes. Addition of the Aroclor 1254 MAS bioactivated cyclophosphamide, coumarin, 2-AAF, but not TCE. Addition of the PB MAS bioactivated cyclophosphamide, weakly bioactivated coumarin and 2-AAF, but had no effect on TCE developmental toxicity. The beta-NF MAS bioactivated coumarin and 2-AAF, weakly bioactivated cyclophosphamide, but did not alter the developmental toxicity of TCE. Addition of the INH-induced MAS only

bioactivated TCE, whereas the post-isolation mixed MAS bioactivated each test material. Based on LC50 and EC50 (malformation) values, embryo growth, and types and severity of induced malformations, each test material was developmentally toxic. Use of post-microsome isolation mixtures from differentially induced rat livers increased the efficacy of the exogenous MAS routinely used by FETAX.

Fort DJ, Stover EL, Bantle JA, Dumont JN, Finch RA. **Evaluation of a reproductive toxicity assay using Xenopus laevis: boric acid, cadmium and ethylene glycol monomethyl ether.** J Appl Toxicol 2001;21(1):41-52.
Abstract: Cadmium (Cd), boric acid (BA) and ethylene glycol monomethyl ether (EGME) were evaluated for reproductive and developmental toxicity in Xenopus laevis. Eight reproductively mature adult male and eight superovulated female Xenopus laevis were exposed to at least five separate sublethal concentrations of each material via the culture water for a period of 30 days. Four respective pairs were mated and the offspring evaluated for developmental effects; an evaluation of reproductive status was performed on the remaining four specimens. Ovary pathology, oocyte count, oocyte maturity and maturation capacity (germinal vesicle breakdown, GVBD) and necrosis were evaluated in the female, whereas testis pathology, sperm count, dysmorphology and motility were studied in the male. Based on this assessment, each test material exerted reproductive toxicity in Xenopus laevis, but with varying potencies. Adult female exposure to Cd and EGME particularly, and to a lesser extent to BA, resulted in transgenerational toxicity to the developing progeny. Further, this model appears to be a useful tool in the initial assessment and prioritization of potential reproductive toxicants for further testing. Copyright 2001 John Wiley & Sons, Ltd.

Fort DJ, Stover EL, Bantle JA, Finch RA. **Evaluation of the developmental toxicity of thalidomide using frog embryo teratogenesis assay-xenopus (FETAX): biotransformation and detoxification.** Teratog Carcinog Mutagen 2000;20(1):35-47.
Abstract: The developmental toxicity of thalidomide was evaluated using FETAX (Frog Embryo Teratogenesis Assay - Xenopus). Young X. Laevis embryos were exposed to this compound in each of two concentration-response experiments with and without differently induced exogenous metabolic activation systems (MASs) and/or inhibited MASs. Young male Sprague-Dawley rats were treated with either isoniazid or Aroclor 1254 to induce cytochrome P-450. Several of the rats were subsequently treated with diethyl maleate (DM) to deplete glutathione reserves. Specific aliquots of rat liver microsomes were treated with 3-amino-1,2,4-triazole (ATZ) or alpha-napthoflavone (alpha-N) to selectively inhibit P-450 activity. Bioactivation was indicated by increased developmental toxicity observed in MAS tests. Results obtained indicated that thalidomide was predominantly activated by P-450 isozyne CYP2E1, although weak cross-specificity between CYP1A1/A2 may have existed. Detoxification pathways for thalidomide were investigated by treatment of the MAS with cyclohexene oxide (CHO) and DM to inhibit the epoxide hydrolase and glutathione conjugation pathways, respectively. Results indicated that epoxide hydrolase was primarily responsible for the detoxification of bioactivated thalidomide. Teratogenesis Carcinog. Mutagen. 20:35-47, 2000.

Fort DJ, Stover EL, Farmer DR, Lemen JK. **Assessing the predictive validity of frog embryo teratogenesis assay-Xenopus (FETAX).** Teratog Carcinog Mutagen 2000;20(2):87-98.

Abstract: The ability of frog embryo teratogenesis assay - Xenopus (FETAX) to identify the potential developmental toxicity of a group of diverse chemicals was evaluated by comparison with results from in vivo studies in rats. A total of 12 chemicals, three of which were shown to be teratogenic in vivo, four of which were embryolethal (but not teratogenic) in vivo, and five which did not produce any developmental toxicity in vivo in the rat were evaluated using FETAX. Results of the FETAX test with these 12 blind-coded compounds correctly predicted that three chemicals had strong teratogenic potential, four had low teratogenic hazard potential but were embryolethal, and five posed little if any developmental toxicity hazard. In addition, this study concluded that within a family of chemistry analogs could be ranked according to relative teratogenic hazard and that for the teratogenic compounds the types of malformations induced in Xenopus mimicked the abnormalities induced in vivo in rats. In summary, these results confirmed that the FETAX assay is predictive and can be useful in an integrated biological hazard assessment for the preliminary screening of chemicals. Teratogenesis Carcinog. Mutagen. 20:87-98, 2000.

Genschow E, Scholz G, Brown N, Piersma A, Brady M, Clemann N, Huuskonen H, Paillard F, Bremer S, Becker K, et al. **Development of prediction models for three in vitro embryotoxicity tests in an ECVAM validation study.** In Vitr Mol Toxicol 2000;13(1):51-66.
Abstract: Since 1997 the National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments, ZEBET, in Berlin, has been coordinating a validation study aimed at prevalidation and validation of three in vitro embryotoxicity tests, funded by the European Center for the Validation of Alternative Methods (ECVAM) at the Joint Research Center (JRC, Ispra, Italy). The tests use the cultivation of postimplantation rat whole embryos (WEC test), cultures of primary limb bud cells of rat embryos (micromass or, MM, test), and cultures of a pluripotent mouse embryonic stem cell line (embryonic stem cell test or EST). Each of the tests was performed in four laboratories under blind conditions. In the preliminary phase of the validation study 6 out of 20 test chemicals comprising different embryotoxic potential (non, weakly, and strongly embryotoxic) were tested. The results were used to define biostatistically based prediction models (PMs) to identify the embryotoxic potential of test chemicals for the WEC test and the MM test. The PMs developed with the results of the preliminary phase of the validation study (training set) will be evaluated with the results of the remaining 14 test chemicals (definitive phase) by the end of the study. In addition, the existing, improved PM (iPM) for the EST, which had been defined previously, was evaluated using the results of the preliminary phase of this study. Applying the iPM of the EST to the results of this study, in 79% of the experiments, chemicals were classified correctly according to the embryotoxic potential defined by in vivo testing. For the MM and the WEC test, the PMs developed during the preliminary phase of this validation study provided 81% (MM test) and 72% (WEC test) correct classifications. Because the PM of the WEC test took into account only parameters of growth and development, but not cytotoxicity data, a second PM (PM2) was developed for the WEC test by incorporating cytotoxicity data of the differentiated mouse fibroblast cell line 3T3, which was derived from the EST. This approach, which has previously never been used, resulted in an increase to 84% correct classifications in the WEC test.

Gutendorf B, Westendorf J. **Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens.** Toxicology 2001;166(1-2):79-89.

Abstract: Many chemicals in surface waters and sediments have recently been discovered to have estrogenic/antiestrogenic activity. Among these compounds, known as 'endocrine disrupters', are natural and synthetic hormones, phytoestrogenes and a variety of industrial chemicals, such as certain detergents and pesticides. These substances are supposed to affect the development and reproduction in wildlife and humans and may also be involved in the induction of cancer. In order to assess the estrogenic/ antiestrogenic potential of pure compounds and complex environmental samples we compared an array of in vitro test systems, (i) two luciferase reporter gene assays using transgenic human MVLN cells (derived from MCF-7 cells) and HGELN cells (derived from HeLa cells); (ii) a competitive binding assay with recombinant human estrogen receptors (ER) alpha and beta; and (iii) a proliferation assay with MCF7-cells (E-Screen). The sensitivity of the assays for 17-beta-estradiol decreased in the order: MVLN-cells=E-Screen>HGELN-cells>binding to ER-alpha>binding to ER-beta. A good correlation was obtained between the estrogenic potencies of 11 compounds (17-beta-estradiol (E(2)), estrone (E (1)), estriol (E(3)), ethinylestradiol (EE(2)), diethylstilbestrol (DES), coumestrol, beta-sitosterol, genistein, 4-nonylphenol, 4-octylphenol, bisphenol A) in the three tissue culture assays. The relative potencies of the compounds obtained by the cell free binding assays were one to two orders of magnitude higher compared with the cell culture assays. The phytoestrogens showed a preference to bind to ER-beta, but only genistein showed a much lower activity in the E-Screen (growth induction in breast cancer cells) compared with the luciferase induction in MVLN and HGELN-cells.

Hays SM, Elswick BA, Blumenthal GM, Welsch F, Conolly RB, Gargas ML. **Development of a physiologically based pharmacokinetic model of 2-methoxyethanol and 2-methoxyacetic acid disposition in pregnant rats.** Toxicol Appl Pharmacol 2000;163(1):67-74.
Abstract: An accurate description of developing embryos' exposure to a xenobiotic is a desirable component of mechanism-based risk assessments for humans exposed to potential developmental toxicants during pregnancy. 2-Methoxyethanol (2-ME), a solvent used in the manufacture of semiconductors, is embryotoxic and teratogenic in all species tested including nonhuman primates. 2-Methoxyacetic acid (2-MAA) is the primary metabolite of 2-ME and the proximate embryotoxic agent. The objective of the work described here was to adapt an existing physiologically based pharmacokinetic (PBPK) model for 2-ME and 2-MAA kinetics during midorganogenesis in mice to rats on gestation days (GD) 13 and 15. Blood and tissue data were analyzed using the extrapolated PBPK model that was modified to simulate 2-ME and 2-MAA kinetics in maternal plasma and total embryo tissues in pregnant rats. The original mouse model was simplified by combining the embryos and placenta with the richly perfused tissue compartment. The model includes a description of the growth of the developing embryo and changes in the physiology of the dam during pregnancy. Biotransformation pathways of 2-ME to either ethylene glycol (EG) or to 2-MAA were described as first-order processes based on the data collected from rats by Green et al., (Occup. Hyg. 2, 67-75, 1996). Tissue partition coefficients (PCs) for 2-ME and 2-MAA were determined for a variety of maternal tissues and the embryos. Model simulations closely reflected the biological measurement of 2-ME and 2-MAA concentrations in blood and embryo tissue following gavage or iv administration of 2-ME or 2-MAA. The PBPK model for rats as described here is well suited for extrapolation to pregnant women and for assessment of 2-MAA dosimetry under various conditions of possible human exposure to 2-ME.

Inohaya K, Kudo A. **[Medaka as a model organism of skeletal development].** Tanpakushitsu Kakusan

Koso 2000;45(17 Suppl):2745-51 [Jpn].

Ishikawa Y. Medakafish as a model system for vertebrate developmental genetics. Bioessays 2000;22 (5):487-95.
Abstract: Several teleosts, such as the zebrafish and the medakafish or medaka (Oryzias latipes), are used as vertebrate model systems in various fields of biology. The medaka is suitable for use in genomic studies because of its small genome size. Moreover, our recent results of small-scale mutagenesis in the medaka indicate that it is possible to identify mutations, the phenotypes of which could not be found in zebrafish mutants obtained by large-scale mutagenesis. An example is Oot (One-sided optic tectum), a maternal-effect mutation. In the Oot phenotype, bilateral symmetry is broken in the optic tectum in the early developmental stages, and either the left or right morphology is duplicated on both sides. Medaka inbred strains can be produced and used to study quantitative traits in vertebrate development. Data presented support the use of medaka as another important fish model for the study of vertebrate developmental genetics.

Johnson I, Harman M, Forrow D, Norris M. **An assessment of the feasibility of using image analysis in the oyster embryo-larval development test.** Environ Toxicol 2001;16(1):68-77.
Abstract: In this study the feasibility of using the latest image capture, processing, and analysis techniques in the oyster embryo-larval development (OEL) test was assessed. This initially involved determining whether the OEL test could be carried out in multiwell plates (which would assist in the application of the image analysis technique), based on data from tests with the reference toxicant zinc and industrial effluents. The study then ascertained which of the 31 image analysis parameters of the Image Pro Plus software used was most appropriate for differentiating between the D larvae and non-D larvae at the end of the test procedure in a manner similar to that of visual observations. On the basis of the zinc reference toxicant and effluent test data derived in this study, the OEL test can be effectively carried out in 24 chamber multiwell plates, which provides the opportunity to count objects with image analysis software. The use of the image analysis parameters area and size (length) in combination resulted in mean control abnormalities and EC50 values in zinc reference toxicant tests which were not significantly different statistically from corresponding values derived using visual observations. Discrimination using the area and length parameters may be improved by the inclusion of other parameters in a suite of measurements which would reduce interference from extraneous material or lighting artefacts. Furthermore, the use of multiwell plates and image analysis can eliminate the variability associated with sub-sampling and inter-operator differences in the counts of D larvae and non-D larvae which is evident with the current visual observation method.

Kaminuma T, Takai-Igarashi T, Nakano T, Nakata K. **Modeling of signaling pathways for endocrine disruptors.** Biosystems 2000;55(1-3):23-31.
Abstract: The so called endocrine disruptors have become an important working hypothesis for a wide range of toxicology researchers. This hypothesis has also attracted those who have worked on designer estrogens or selective estrogen receptor modulators. Already numbers of substances have been identified as such chemicals, but there remain a large number of chemicals waiting to be tested for their endocrine modulating capabilities. Because of the time and costs required for wet lab tests, it is unrealistic to apply these kinds of tests to all such suspicious or probable chemicals. Thus some theoretical methods must be

developed for this purpose. However the conventional QSAR (quantitative structure activity relationships) approach is of limited relevance to this problem, because these methods do not take detailed mechanisms of molecular interactions in biological systems into account. Thus we have developed a database complex system that enables one to trace molecular interactions triggered by interaction of receptors with xenobiotic chemicals. The main components of this database complex are a potential endocrine disruptor database, a receptor database, a cell signaling networks database, a transcription factor database, and an affinity binding database based on modes of actions.

Kanamori A. **[Medaka as a model for gonadal sex differentiation in vertebrates].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2949-53 [Jpn].

Kinoshita M, Kani S, Ozato K, Wakamatsu Y. **Activity of the medaka translation elongation factor 1alpha-A promoter examined using the GFP gene as a reporter.** Dev Growth Differ 2000;42(5):469-78.
Abstract: The translation elongation factor 1alpha (EF-1alpha) is known to have several isoforms, which are expressed in a tissue- and stage-specific manner. Two genes encoding EF-1alpha exist per haploid genome in the medaka. In the present study, the promoter activity of the 5'-flanking region of the medaka EF-1alpha-A gene, an isoform of EF-1alpha, was characterized using transgenic techniques. First, using CAT gene as a reporter, it was revealed that about 1.8 kbp 5'-flanking sequence from the transcription initiation site of EF-1alpha-A was sufficient for high-level promoter activity. Second, the green fluorescent protein (GFP) gene fused to this region was introduced into medaka eggs using the microinjection method. Three germline transgenic individuals (one male and two female) were mated with non-transgenic medaka to obtain F1 offspring. In the case of embryonic and adult F1 transgenic individuals, GFP fluorescence was observed in almost all the tissues examined (e.g. kidney, liver, heart, gill, ovary, and testis), except for the skeletal muscle. In the case of F2 transgenic embryos derived from F1 transgenic males and non-transgenic females, the fluorescence was observed from the early gastrula stage. On the other hand, in the case of F2 transgenic embryos derived from F1 transgenic females and non-transgenic males, the fluorescence was observed even at the 1-cell stage, suggesting that this region is transcriptionally active during oogenesis. The usefulness of the EF-1alpha-A promoter as a tool for introducing foreign proteins into oocytes is discussed.

Kledal TJ, Jorgensen M, Mengarda F, Skakkebaek NE, Leffers H. **New methods for detection of potential endocrine disruptors.** Andrologia 2000;32(4-5):271-8.
Abstract: It has been hypothesized that recent adverse trends in humans are linked to an increased exposure to potential endocrine disrupting agents. These include widely used compounds that mimic the action of sex hormones, including bisphenol A, phthalates and parabens. Since the chemical structure is not sufficient to determine whether a chemical will act as an oestrogen, there is a need for assays that can determine whether a compound interferes with the endocrine systems. The Environmental Protection Agency has recently suggested a testing scheme, composed of an initial screening followed by a more comprehensive investigation of chemicals that are positive in the screening. The screening will use several short-term assays to screen many thousands of compounds for potential endocrine disrupting properties. However, none of these tests determines compound-induced effects on the expression of endogenous genes, which is the cause of the adverse effects. We propose to use a precise quantification

of the expression levels of endogenous oestrogen-regulated genes to test whether a chemical has oestrogenic properties, and describe how an endogenous gene expression assay can be established and conducted. Furthermore, different applications of such an assay are discussed: in cell cultures; in experimental animals; or, optimally, directly in blood samples from exposed humans.

Koga A. **[Evolution of transposable elements in the Medaka fish and its related species].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2900-8 [Jpn].

Koger CS, Teh SJ, Hinton DE. **Determining the sensitive developmental stages of intersex induction in medaka (Oryzias latipes) exposed to 17 beta-estradiol or testosterone.** Mar Environ Res 2000;50 (1-5):201-6.
Abstract: Certain environmentally persistent compounds can adversely affect reproduction by acting as steroid hormone agonists or antagonists. The goal of the present study was to determine the developmental stage most susceptible to exogenous hormone (estradiol and testosterone) exposure using a small teleost model. In the first (pilot study) of two experiments, medaka (Oryzias latipes), at varying developmental stages, were bath-exposed to 5 micrograms/l 17 beta-estradiol for 24 h. At 5 months of age, fecundity, fertility and embryo and larval viability (reproductive success) were investigated in control and exposed groups. Fish at 1, 1.5, 2 and 5.5 months of age were also sampled, processed and examined histologically for gonadal alteration. No significant differences in mortality, gonadal morphology, body weight, sex-ratio or time to maturity were seen between control and exposed fish. At 5 months, however, when exposure groups were compared to controls, significant differences were seen in reproductive success and viability of offspring. A second experiment exposed embryo stage 10, and 1-, 7- and 21-day-old larvae for 6 days to 15 micrograms/l 17 beta-estradiol or 100 micrograms/l testosterone. No significant differences were seen at 5 months in mortality, body weight, or time to sexual maturity. However, sex-ratios were significantly biased toward female in the stage 10, 1- and 7-day post-hatch estradiol exposure groups. No significant changes in sex-ratio were associated with testosterone exposure at any developmental stage. Further, intersex gonads were observed in fish from all groups exposed to 15 micrograms/l estradiol. Only those fish exposed as newly hatched fry or at 1 week post-hatch displayed intersex gonads following 100 micrograms/l testosterone exposure. Data from these experiments show that newly hatched fry are that life stage most sensitive to hormone exposure and the most appropriate to use in determining effects of known endocrine-disrupting compounds.

Kondo M, Nagao E, Mitani H, Shima A. **Differences in recombination frequencies during female and male meioses of the sex chromosomes of the medaka, Oryzias latipes.** Genet Res 2001;78(1):23-30.
Abstract: In the medaka, Oryzias latipes, sex is determined chromosomally. The sex chromosomes differ from those of mammals in that the X and Y chromosomes are highly homologous. Using backcross panels for linkage analysis, we mapped 21 sequence tagged site (STS) markers on the sex chromosomes (linkage group 1). The genetic map of the sex chromosome was established using male and female meioses. The genetic length of the sex chromosome was shorter in male than in female meioses. The region where male recombination is suppressed is the region close to the sex-determining gene y, while female recombination was suppressed in both the telomeric regions. The restriction in recombination

does not occur uniformly on the sex chromosome, as the genetic map distances of the markers are not proportional in male and female recombination. Thus, this observation seems to support the hypothesis that the heterogeneous sex chromosomes were derived from suppression of recombination between autosomal chromosomes. In two of the markers, Yc-2 and Casp6, which were expressed sequence-tagged (EST) sites, polymorphisms of both X and Y chromosomes were detected. The alleles of the X and Y chromosomes were also detected in O. curvinotus, a species related to the medaka. These markers could be used for genotyping the sex chromosomes in the medaka and other species, and could be used in other studies on sex chromosomes.

Lamb JC, Brown SM. **Chemical testing strategies for predicting health hazards to children.** Reprod Toxicol 2000;14(2):83-94.
Abstract: The United States Environmental Protection Agency has proposed the development of a Children's Health Test Program under the Toxic Substances Control Act. The Environmental Protection Agency's proposal for the children's health test battery has 12 different assays including general toxicity, genotoxicity, carcinogenicity, neurotoxicity, and developmental and reproductive toxicity. The current Environmental Protection Agency testing proposal is an "all or nothing" test battery. An alternative and preferable approach would be to use a science-based, tiered testing scheme. It is proposed that the Screening Information Dataset program, currently used by the Organization for Economic Co-operation and Development (OECD) for the Screening Information Dataset-High Production Volume test battery, or equivalent, be considered for the first step. Step 1 would include acute and repeat dose toxicity testing, developmental toxicity testing (first species OECD 414 or OECD 422), reproductive toxicity screening (OECD 415 or 422), and genetic toxicity testing. For this step, the rat would be the initial and only species tested unless the mouse was used for in vivo genetic toxicity. Step 2 of the proposed children's health test battery would include developmental testing (second species OECD 414) or special mode of action studies performed for those chemicals that proved to be developmental toxicants in Step 1. Those chemicals that tested positive as reproductive toxicants in Step 1 would be tested in a two-generation reproduction study (OECD 416) or a special mode of action study. Steps 1 and 2 provide information on whether oncogenicity or developmental neurotoxicity testing is useful. Step 3 would include chronic toxicity/oncogenicity testing for those chemicals that tested positive for genetic toxicity in Step 1, and positive for developmental concerns in Step 2. In this step, chemicals would also be tested for developmental neurotoxicity if they showed evidence of neuropathy, behavioral effects, or neurotoxic potential in earlier studies. This stepwise approach would conserve resources and answer scientific questions in a logical, orderly, timely, and cost-effective manner.

Le Guevel R, Pakdel F. **Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods.** Hum Reprod 2001;16(5):1030-6.
Abstract: Three in-vitro bioassays were used to compare the oestrogenic potency of chemicals used as growth promoter in beef cattle in certain non-European Union countries (17beta-oestradiol, alpha-zearalanol, testosterone, trenbolone, trenbolone acetate, melengestrol acetate) or found as food contaminant such as the mycotoxin zearalenone and some of their metabolites (17alpha-oestradiol, oestrone, 17alpha-epitestosterone, 19-nortestosterone, androstendione, zearalanone, alpha-zearalanol, beta-zearalanol, alpha-zearalenol, beta-zearalenol). The strong oestrogens 17alpha-ethinyl oestradiol and diethylstilboestrol were used as standards. The first bioassay was based on the activation of a reporter

gene by oestrogens in recombinant yeast expressing human or rainbow trout oestrogen receptor. In the second bioassay, the vitellogenin gene induction of rainbow trout hepatocyte cultures was used as a biomarker for the exposure to oestrogens. The third bioassay was based on the alkaline phosphatase gene induction by oestrogens in the human endometrial Ishikawa cell line. The assessment of oestrogenic potency of these chemicals clearly demonstrates the strong oestrogenicity of the mycotoxin zearalenone and its metabolites and particularly alpha-zearalenol which was as potent as ethinyl oestradiol and diethylstilboestrol in the human endometrial Ishikawa cell line.

Lekven AC, Helde KA, Thorpe CJ, Rooke R, Moon RT. **Reverse genetics in zebrafish.** Physiol Genomics 2000;2(2):37-48.
Abstract: The zebrafish has become a popular model system for the study of vertebrate developmental biology because of its numerous strengths as a molecular genetic and embryological system. To determine the requirement for specific genes during embryogenesis, it is necessary to generate organisms carrying loss-of-function mutations. This can be accomplished in zebrafish through a reverse genetic approach. This review discusses the current techniques for generating mutations in known genes in zebrafish. These techniques include the generation of chromosomal deletions and the subsequent identification of complementation groups within deletions through noncomplementation assays. In addition, this review will discuss methods currently being evaluated that may improve the methods for finding mutations in a known sequence, including screening for randomly induced small deletions within genes and screening for randomly induced point mutations within specific genes.

Lin N, Garry VF. **In vitro studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota.** J Toxicol Environ Health A 2000;60(6):423-39.
Abstract: Recent epidemiologic studies showed increased frequency of birth defects in pesticide applicators and general population of the Red River Valley, Minnesota. These studies further indicated that this crop growing area used more chlorophenoxy herbicides and fungicides than elsewhere in Minnesota. Based on frequency of use and known biology, certain herbicides, pesticide additives, fungicides, and mycotoxins are suspect agents. To define whether these agents affect developmental endpoints in vitro, 16 selected agrochemicals were examined using the MCF-7 breast cancer cell line. In the flow cytometric assay, cell proliferation in this estrogen-responsive cell line indicates xenobiotic-mediated estrogenic effects. Cell viability, morphology, ploidy, and apoptosis were incorporated in this assay. Data showed that the adjuvants X-77 and Activate Plus induced significant cell proliferation at 0.1 and 1 microg/ml. The commercial-grade herbicides 2,4-D LV4 and 2,4-D amine induced cell proliferation at 1 and 10 microg/ml. The reagent-grade 2,4-D products failed to induce proliferation over the same concentration range, suggesting that other ingredients in the commercial products, presumably adjuvants, could be a factor in these results. The fungicides triphenyltin and mancozeb induced apoptosis at concentrations of 4.1 microg/ml (10(-5) M) and 50 microg/ml, respectively. Triphenyltin also induced aneuploidy (C2/M arrest) at 0.41 microg/ml (10(-6) M). Data provide a mechanistic step to understanding human reproductive and developmental effects in populations exposed to these agrochemicals, and initiative to focusing limited resources for future in vivo animal developmental toxicity studies.

Loosli F, Koster RW, Carl M, Kuhnlein R, Henrich T, Mucke M, Krone A, Wittbrodt J. **A genetic screen for mutations affecting embryonic development in medaka fish (Oryzias latipes).** Mech Dev 2000;97(1-2):133-9.
Abstract: In a pilot screen, we assayed the efficiency of ethylnitrosourea (ENU) as a chemical mutagen to induce mutations that lead to early embryonic and larval lethal phenotypes in the Japanese medaka fish, Oryzias latipes. ENU acts as a very efficient mutagen inducing mutations at high rates in germ cells. Three repeated treatments of male fish in 3 mM ENU for 1 h results in locus specific mutation rates of 1.1-1.95 x10(-3). Mutagenized males were outcrossed to wild type females and the F1 offspring was used to establish F2 families. F2 siblings were intercrossed and the F3 progeny was scored 24, 48 and 72 h after fertilization for morphological alterations affecting eye development. The presented mutant phenotypes were identified using morphological criteria and occur during early developmental stages of medaka. They are stably inherited in a Mendelian fashion. The high efficiency of ENU to induce mutations in this pilot screen indicates that chemical mutagenesis and screening for morphologically visible phenotypes in medaka fish allows the genetic analysis of specific aspects of vertebrate development complementing the screens performed in other vertebrate model systems.

Marty MS, Crissman JW, Carney EW. **Evaluation of the male pubertal onset assay to detect testosterone and steroid biosynthesis inhibitors in CD rats.** Toxicol Sci 2001;60(2):285-95.
Abstract: The male pubertal onset assay has been recommended by the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) as an alternate Tier I screening assay to detect potential endocrine-active chemicals (EACs). Recently, this assay was evaluated by several laboratories using a variety of dosing schemes. This study used a 30-day dosing period to confirm and extend previous work on the assay's ability to detect steroid biosynthesis inhibitors. Weanling male rats were dosed by gavage from 21 to 50 days of age with vehicle (0.5% methocel) or chemicals from the following EAC classes: an androgen (testosterone propionate [TP], 0.1 or 0.4 mg/kg/day), a broad-spectrum steroid biosynthesis inhibitor (ketoconazole [KETO], 24 mg/kg/day), a 5alpha-reductase inhibitor (finasteride [FIN], 20 or 80 mg/kg/day), a moderately specific aromatase inhibitor (testolactone [TL], 220 mg/kg/day), or a highly specific aromatase inhibitor (fadrozole [FAD], 0.6 or 6.0 mg/kg/day). None of these treatments altered relative thyroid weights. However, TL, KETO, and FIN were positive for endocrine activity based on decreases in one or more reproductive or accessory sex gland organ weights. Of these three inhibitors, only TL significantly increased the age at PPS, indicating that PPS was less sensitive for detecting these EACs. Based on its profile of effects, TL may have been detected as an antiandrogen. TP and FAD were negative in this assay, even at doses that caused effects in other studies. With TP, oral administration limited assay sensitivity such that higher TP doses would be needed for detection. FAD decreased body weight gains, but did not significantly alter any other assay end points; thus, the capacity of this assay to detect aromatase inhibitors remains in question.

Matsuda M, Nagahama Y. **[Construction of a Medaka BAC library].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2880-5 [Jpn].

McLachlan JA, Burow M, Chiang TC, Li SF. **Gene imprinting in developmental toxicology: a possible interface between physiology and pathology.** Toxicol Lett 2001;120(1-3):161-4.
Abstract: Gene imprinting is an epigenetic mechanism for accomplishing persistent change in gene

expression. In this brief paper, we explore the mechanisms for imprinting genes and present data showing that the synthetic estrogen, diethylstilbestrol (DES) can developmentally imprint genes by changing the pattern of DNA methylation. We further discuss the implications of this and other findings for non-mutagenic aspects of developmental toxicology, and suggest ways to use this concept in modifying in vitro screening for developmental toxicants.

Merhi RA, Guillaud L, Delouis C, Cotinot C. **Establishment and characterization of immortalized ovine sertoli cell lines.** In Vitro Cell Dev Biol Anim 2001;37(9):581-8.
Abstract: The objective of this study was to generate immortalized Sertoli cell lines from prepubertal lamb testes to facilitate investigations during the course of testicular differentiation. The Sertoli cells were enzymatically isolated and immortalized by transfection, with the sequences coding for the SV40 large T-antigen fused downstream of regulatory elements from the human vimentin gene. The different cell lines were positively stained with antibodies to vimentin and transferrin, in agreement with their Sertoli origin. Reverse transcriptase polymerase chain reaction was used to analyze the specific expression of molecular markers (clusterin/sulfated glycoprotein ISGP-2], follicle-stimulating hormone [rFSH], alpha-inhibin, anti-Mullerian hormone, Wilms' tumor gene [WT-1], steroidogenic factor 1 [SF-1], SRY-related HMG box gene g [SOX9], and sex-determining region of Y chromosome) normally expressed in this cellular type. All were shown to express messenger ribonucleic acids for SGP-2, alpha-inhibin, WT-1, SOX9, and SF-1 (except SF-1 for clone no. 1). Moreover, we performed alkaline phosphatase and receptor tyrosine kinase p145 (c-kit) detection to ensure the absence of contamination by peritubular, germ cells, and Leydig cells. Both tests were negative for all the seven cell lines. These ovine Sertoli cell lines are the first ones obtained from livestock that exhibit specific Sertoli cell characteristics resembling different stages of phenotypic development. They provide useful in vitro model systems for toxicological investigations, coculture, and transfection experiments, making it possible to study signal transduction pathways, cell-cell interactions, and gene expression in species other than rodents.

Monsees TK, Franz M, Gebhardt S, Winterstein U, Schill WB, Hayatpour J. **Sertoli cells as a target for reproductive hazards.** Andrologia 2000;32(4-5):239-46.
Abstract: Male fertility can be impaired by various toxicants. Some of them are known to target mainly Sertoli cells, which play an essential role in spermatogenesis. In this study, the in vitro response of immature rat Sertoli cells to various environmental pollutants, including pesticides, oestrogenic compounds and heavy metals, has been investigated. Mitochondrial dehydrogenase activity has been used to measure Sertoli cell viability, while production of lactate and secretion of inhibin B have been used as general and specific cell markers. Sertoli cell viability was not affected after 24-h exposure to lindane, DDT, ethinyloestradiol or bisphenol A in the concentration range analysed (up to 100, 25 or 50 microM, respectively). In contrast, mercury(II) (EC50 = 31 microM) and cisplatin (15% decrease in viability at 100 microM) induced some cytotoxic effect. With the exception of the pesticide DDT, all chemicals investigated induced a significant dose-dependent increase in lactate production after 24-h exposure to Sertoli cells. Owing to the cytotoxic effect of mercury(II), lactate levels dropped again at concentrations above 20 microM. The pesticide lindane (but not DDT) and both oestrogens significantly increased the production of the Sertoli cell specific hormone inhibin B without affecting cell viability. In contrast, the heavy metals mercury(II) and platinum(II) markedly decreased inhibin B levels. This sharp

decrease was already significant at metal concentrations that reduced Sertoli cell viability only moderately (10-15%). In conclusion, the secretion of lactate and inhibin B by immature rat Sertoli cells seems to be a useful and sensitive marker with which to explore potential Sertoli cell toxicants.

Nishi Y, Yanase T, Mu Y, Oba K, Ichino I, Saito M, Nomura M, Mukasa C, Okabe T, Goto K, et al. **Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor.** Endocrinology 2001;142(1):437-45.
Abstract: We established a steroidogenic human ovarian granulosa-like tumor cell line, designated KGN, from a patient with invasive ovarian granulosa cell carcinoma. KGN had a relatively long population doubling time of about 46.4 h and had an abnormal karyotype of 45,XX, 7q-, -22. A steroid analysis of the cultured medium by RIA performed 5 yr after the initiation of culture showed that KGN was able to secrete pregnenolone and progesterone, and both dramatically increased after stimulation with (Bu)(2)cAMP. However, little or no secretion of 17alpha-hydroxylated steroids, dehydroepiandrosterone, androstenedione, or estradiol was observed. The aromatase activity of KGN was relatively high and was further stimulated by (Bu)(2)cAMP or FSH. These findings showed a pattern similar to that of steroidogenesis in human granulosa cells, thus allowing analysis of naturally occurring steroidogenesis in human granulosa cells. Fas-mediated apoptosis of KGN was also observed, which mimicked the physiological regulation of apoptosis in normal human granulosa cells. Based on these findings, this cell line is considered to be a very useful model for understanding the regulation of steroidogenesis, cell growth, and apoptosis in human granulosa cells.

O'Connor JC, Davis LG, Frame SR, Cook JC. **Detection of dopaminergic modulators in a tier I screening battery for identifying endocrine-active compounds (EACs).** Reprod Toxicol 2000;14 (3):193-205.
Abstract: Apomorphine (APO; D(2) receptor agonist), haloperidol (HAL; D(2) receptor antagonist), and reserpine (RES; a dopamine depletor that acts to lower brain dopamine levels by depleting central nervous system monoamines via disrupting storage vesicle function) have been examined in a Tier I screening battery, which has been designed to detect endocrine-active compounds (EACs). The Tier I battery incorporates two short-term in vivo tests (a 5-day ovariectomized female battery and a 15-day intact male battery using Sprague-Dawley rats) and an in vitro yeast transactivation system (YTS). In addition, two blood collection procedures were evaluated for their utility in detecting HAL-induced increases in serum prolactin (PRL) levels (i.e., the stress associated with each procedure). In the in vivo female battery, both HAL and RES increased serum PRL concentrations as expected, although the increase caused by RES was marginal. Increases in serum PRL levels are enhanced when daily dosages are administered via multiple-daily dosing of the test compound, which results in higher sustained blood levels of the test compounds. APO failed to decrease serum PRL concentrations in the female battery. In the in vivo male battery, HAL increased serum PRL concentrations as expected. However, APO and RES failed to affect serum PRL concentrations. The blood collection comparison experiment demonstrated that possible confounding of the data can occur with serum PRL concentrations when animals are exposed to stress. Basal levels of PRL were approximately fourfold higher in animals that were bled via the tail vein procedure when compared to PRL levels from animals that were bled under CO(2) anesthesia at euthanization. As a result of the higher basal PRL levels, the HAL-induced increase in serum PRL concentrations was completely attenuated in the tail-vein bled animals (1.3-fold). In

contrast, HAL produced a fivefold increase in serum PRL in animals where blood was collected under CO(2) anesthesia at euthanization. Hence, collection of blood from animals under CO(2) anesthesia at euthanization is an acceptable approach for detection of compounds that increase PRL. In summary, HAL-like compounds would be identified in the Tier I male and female battery primarily via increased serum PRL concentrations. RES-like compounds would be identified in the Tier I male battery via decreased gonadotropins and steroids and possibly in the Tier I female battery by a minimal increase in serum PRL concentrations. Compounds that produce a marginal increase in serum PRL when administered using single daily dosing can also be confirmed in an in vivo female battery with multiple dosing because this regimen increases the magnitude of the PRL increase. APO, a D(2) receptor agonist, was not detected in the in vivo male or female batteries, but in both instances the top dosage produced minimal decreases in body weight (99 to 96% of control). Hence, the proposed Tier I battery needs to be further evaluated with higher dosages of APO and other D(2) receptor agonists to determine whether it is capable of detecting such agents.

O'Connor JC, Davis LG, Frame SR, Cook JC. **Evaluation of a Tier I screening battery for detecting endocrine-active compounds (EACs) using the positive controls testosterone, coumestrol, progesterone, and RU486.** Toxicol Sci 2000;54(2):338-54.
Abstract: After previously examining 12 compounds with known endocrine activities, we have now evaluated 4 additional compounds in a Tier I screening battery for detecting endocrine-active compounds (EACs): a weak estrogen receptor (ER) agonist (coumestrol; COUM), an androgen receptor (AR) agonist (testosterone; TEST), a progesterone receptor (PR) agonist (progesterone; PROG), and a PR antagonist (mifepristone; RU486). The Tier I battery incorporates 2 short-term in vivo tests (5-day ovariectomized female battery; 15-day intact male battery) and an in vitro yeast transactivation system (YTS). The Tier I battery is designed to identify compounds that have the potential to act as agonists or antagonists to the estrogen, androgen, progesterone, or dopamine receptors; steroid biosynthesis inhibitors (aromatase, 5alpha-reductase, and testosterone biosynthesis); or compounds that alter thyroid function. In addition to the Tier I battery, a 15-day dietary restriction experiment was performed using male rats to assess confounding due to treatment-related decreases in body weight. In the Tier I female battery, TEST administration increased uterine weight, uterine stromal cell proliferation, and altered hormonal concentrations (increased serum testosterone [T] and prolactin [PRL]; and decreased serum FSH and LH). In the male battery, TEST increased accessory sex gland weights, altered hormonal concentrations (increased serum T, dihydrotestosterone [DHT], estradiol [E2], and PRL; decreased serum FSH and LH), and produced microscopic changes of the testis (Leydig cell atrophy and spermatid retention). In the YTS, TEST activated gene transcription in the yeast containing the AR or PR. In the female battery, COUM administration increased uterine weight, uterine stromal cell proliferation, and uterine epithelial cell height, and increased serum PRL concentrations. In the male battery, COUM altered hormonal concentrations (decreased serum T, DHT, E2; increased serum PRL) and, in the YTS, COUM activated gene transcription in the yeast containing the ER. In the female battery, PROG administration increased uterine weight, uterine stromal cell proliferation, and uterine epithelial cell height and altered hormonal concentrations (increased serum progesterone and decreased serum FSH and LH). In the male battery, PROG decreased epididymis and accessory sex gland weights, altered hormonal concentrations (decreased serum T, PRL, FSH, and LH; increased serum progesterone and E2), and produced microscopic changes of the testis (Leydig cell atrophy). In the YTS, PROG activated

gene transcription in the yeast containing the AR or PR. In the female battery, RU486 administration increased uterine weight and decreased uterine stromal cell proliferation. In the male battery, RU486 decreased epididymis and accessory sex gland weights and increased serum FSH and LH concentrations. In the YTS, RU486 activated gene transcription in the yeast containing the ER, AR, or PR. Dietary restriction data demonstrate that confounding due to decrements in body weight are not observed when body weight decrements are 10% or less in the Tier I male battery. In addition, minimal confounding is observed at body decrements of 15% (relative liver weight, T3, and T4). Hence, compounds can be evaluated in this Tier I at levels that produce a 10% decrease in body weight without confounding of the selected endpoints. Using the responses obtained for all the endpoints in the Tier I battery, a distinct "fingerprint" was produced for each type of endocrine activity against which compounds with unknown activity can be compared. These data demonstrate that the described Tier I battery is useful for identifying EACs and they extend the compounds evaluated to 16.

Owens KD, Baer KN. **Modifications of the topical Japanese medaka (Oryzias latipes) embryo larval assay for assessing developmental toxicity of pentachlorophenol and p, p'-dichlorodiphenyltrichloroethane.** Ecotoxicol Environ Saf 2000;47(1):87-95.
Abstract: One method currently available for investigating developmental toxicity in teleost species is the Japanese medaka embryo larval assay (MELA). In the present study, the MELA was modified to evaluate repeated topical exposures to pentachlorophenol (PCP) and p, p'-dichlorodiphenyltrichloroethane (DDT) and to identify sensitive stages of embryonic development. A single topical exposure using embryos at 48 h postfertilization resulted in a statistically significant increase in embryo mortality at 688 and 1250 ng PCP/egg compared with controls. In contrast, the toxicity following exposure to 11, 36, 78, 120, 208, and 400 ng DDT/egg was expressed only in larvae after hatching. Results further demonstrate that the MELA can be optimized to accommodate repeated daily topical exposures starting at 48 h postfertilization and ending at 120 h postfertilization. In addition, the neurula stage (24 h postfertilization) represented the most sensitive embryonic stage following a single topical exposure of PCP. However, no differences were observed in the sensitivity of embryonic stages following DDT exposure. The modified MELA was also used to evaluate sediment extracts contaminated with DDT metabolites obtained from the Tensas River, Louisiana. Results indicate that there is a low potential for developmental toxicity using the present extraction and exposure scenario even though elevated levels of DDE and toxaphene currently exist in several adult fish species at this site. The MELA as a screen for evaluating the potential for developmental toxicity of contaminated sediments is discussed.

Paw BH, Zon LI. **Zebrafish: a genetic approach in studying hematopoiesis.** Curr Opin Hematol 2000;7(2):79-84.
Abstract: The zebrafish (Danio rerio) has emerged in recent years as an exciting animal model system for studying vertebrate organ development and, in particular, the development of the hematopoietic system. The combined advantages of developmental biology and genetic screens for mutations in zebrafish have provided insights into early events in hematopoiesis and identified several genes required for normal blood development in vertebrates. As a result of the large-scale mutagenesis screens for developmental mutants, several zebrafish mutants with defects in blood development have been recovered. This review discusses how these blood mutations in zebrafish have given new perspectives

on hematopoietic development.

Pinney E, Liu K, Sheeman B, Mansbridge J. **Human three-dimensional fibroblast cultures express angiogenic activity.** J Cell Physiol 2000;183(1):74-82.
Abstract: Human neonatal fibroblasts were cultured on a lactate-glycollate copolymer scaffold for 12-16 days to form a three-dimensional dermal equivalent tissue. The cellular content of vascular endothelial growth factor (VEGF) mRNA in these three-dimensional cultures was 22-fold greater than that observed in the same fibroblasts grown as monolayers. No induction was shown by hepatocyte growth factor (HGF) or angiopoietin 1 indicating that the effect was specific to VEGF. The predominant VEGF splice variant, detected by RT-PCR corresponded to the 121 amino acid form, with less of the 165 amino acid form. The cell-associated forms (189 and 206 amino acids) comprised less than 1% of the total VEGF mRNA. VEGF and HGF proteins, determined by ELISA, were secreted in physiologically significant amounts, 0.5-4 ng per 24 h/10(6) cells. Conditioned medium from the three-dimensional cultures stimulated proliferation of endothelial cells in a dose-dependent manner and induced cellular expression of integrin alpha(v)beta(3). Conditioned medium from the same dermal fibroblasts cultured in monolayer showed little angiogenic activity in any of these assays. Using the chorioallantoic membrane (CAM) angiogenesis assay, the cultures stimulated blood vessel production 2.8-fold over scaffold alone. VEGF-neutralizing antibody reduced the vessel development in the CAM to the level in the scaffold control. Anti-HGF antibody had no significant effect. In conclusion, three-dimensional cultures of dermal equivalent tissue express angiogenic activity to a greater extent than monolayer cultures, some of which can be assigned to VEGF.

Ponzetto C, Pante G, Prunotto' C, Ieraci A, Maina F. **Met signaling mutants as tools for developmental studies.** Int J Dev Biol 2000;44(6 Spec No):645-53.
Abstract: The Met receptor is widely expressed in embryonic and adult epithelial tissues; its ligand (hepatocyte growth factor/scatter factor, HGF/SF) is expressed in the mesenchymal component of various organs. The generation of hgf and met null mice has revealed an essential role for this ligand-receptor pair in the development of the placenta, liver, and limb muscles. However the early lethality of the null mutants has precluded analysis of Met function in late development. To extend the possible observation period, we generated mutant metalleles of different severity. This was done by impairing the ability of the receptor to transduce the HGF/SF signal, via mutation of consensus sequences in the multifunctional docking site present in the C-terminal tail of the receptor. Mice expressing a Met mutant still active as a kinase, but unable to recruit its effectors, died in mid-gestation with the same phenotype as the metknockout, proving the importance of phosphotyrosine-SH2 interactions in vivo. Mice expressing a Met receptor with partial loss of signaling function survived until birth and revealed novel aspects of HGF/SF-Met function during muscle development.

Riecke K, Stahlmann R. **Test systems to identify reproductive toxicants.** Andrologia 2000;32(4-5):209-18.
Abstract: Experience with drugs and other xenobiotics indicates that both animal testing and epidemiological studies are necessary to provide adequate data for an estimation of risks that might be associated with exposure to a chemical substance. In this review, the pros and cons of test systems for reproductive toxicity are discussed. Usually, several studies are performed to cover the different phases

of the reproductive cycle. In the preclinical development of drugs, the three so-called 'segment testing protocols' have been used for several decades now. More recently, new testing concepts have been accepted internationally which include more flexibility in implementation. Several examples of compounds with the potential for reproductive toxicity are presented in more detail in a discussion of some pitfalls of the tests for fertility (phthalates and fluoroquinolones), teratogenicity (acyclovir and protease inhibitors) and postnatal developmental toxicity (fluoroquinolones). In addition, important aspects of kinetics and metabolism as a prerequisite for a rational interpretation of results from toxicological studies are briefly discussed. In vitro assays are useful for supplementing the routinely used in vivo approaches or for studying an expected or defined effect, but they are not suitable for revealing an unknown effect of a chemical on the complex reproductive process.

Rohwedel J, Guan K, Hegert C, Wobus AM. **Embryonic stem cells as an in vitro model for mutagenicity, cytotoxicity and embryotoxicity studies: present state and future prospects.** Toxicol In Vitro 2001;15(6):741-53.
Abstract: Primary cultures or established cell lines of vertebrates are commonly used to analyse the mutagenic, embryotoxic or teratogenic potential of environmental factors, drugs and xenobiotics in vitro. However, these cellular systems do not include developmental processes from early embryonic stages up to terminally differentiated cell types. An alternative approach has been offered by permanent lines of pluripotent stem cells of embryonic origin, such as embryonic carcinoma (EC), embryonic stem (ES) and embryonic germ (EG) cells. The undifferentiated stem cell lines are characterized by nearly unlimited self-renewal capacity and have been shown to differentiate in vitro into cells of all three primary germ layers. Pluripotent embryonic stem cell lines recapitulate cellular developmental processes and gene expression patterns of early embryogenesis during in vitro differentiation, data which are summarized in this review. In addition, recent studies are presented which investigated mutagenic, cytotoxic and embryotoxic effects of chemical substances using in vitro systems of pluripotent embryonic stem cells. Furthermore, an outlook is given on future molecular technologies using embryonic stem cells in developmental toxicology and embryotoxicology.

Schlumpf M, Cotton B, Conscience M, Haller V, Steinmann B, Lichtensteiger W. **In vitro and in vivo estrogenicity of UV screens.** Environ Health Perspect 2001;109(3):239-44.
Abstract: Ultraviolet (UV) screens are increasingly used as a result of growing concern about UV radiation and skin cancer; they are also added to cosmetics and other products for light stability. Recent data on bioaccumulation in wildlife and humans point to a need for in-depth analyses of systemic toxicology, in particular with respect to reproduction and ontogeny. We examined six frequently used UVA and UVB screens for estrogenicity in vitro and in vivo. In MCF-7 breast cancer cells, five out of six chemicals, that is, benzophenone-3 (Bp-3), homosalate (HMS), 4-methyl-benzylidene camphor (4-MBC), octyl-methoxycinnamate (OMC), and octyl-dimethyl-PABA (OD-PABA), increased cell proliferation with median effective concentrations (EC(50)) values between 1.56 and 3.73 microM, whereas butyl-methoxydibenzoylmethane (B-MDM) was inactive. Further evidence for estrogenic activity was the induction of pS2 protein in MCF-7 cells and the blockade of the proliferative effect of 4-MBC by the estrogen antagonist ICI 182,780. In the uterotrophic assay using immature Long-Evans rats that received the chemicals for 4 days in powdered feed, uterine weight was dose-dependently increased by 4-MBC (ED(50)309mg/kg/day), OMC (ED(50) 935 mg/kg/day), and weakly by Bp-3 (active at

1,525 mg/kg/day). Three compounds were inactive by the oral route in the doses tested. Dermal application of 4-MBC to immature hairless (hr/hr) rats also increased uterine weight at concentrations of 5 and 7.5% in olive oil. Our findings indicate that UV screens should be tested for endocrine activity, in view of possible long-term effects in humans and wildlife.

Sharpe PT. **Fish scale development: Hair today, teeth and scales yesterday?** Curr Biol 2001;11(18): R751-2.
Abstract: A group of genes in the tumour necrosis factor signalling pathway are mutated in humans and mice with ectodermal dysplasias - a failure of hair and tooth development. A mutation has now been identified in one of these genes, ectodysplasin-A receptor, in the teleost fish Medaka, that results in a failure of scale formation.

Spielmann H, Liebsch M. **Lessons learned from validation of in vitro toxicity test: from failure to acceptance into regulatory practice.** Toxicol In Vitro 2001;15(4-5):585-90.
Abstract: As no scientific approach or regulatory guidelines existed for the experimental validation of in vitro toxicity tests, in 1990 a US/European validation workshop agreed in Amden (Switzerland) on a simple definition of the validation process. Several international validation studies failed, although they were conducted according to these recommendations. Taking into account the lessons learned from this experience, a second validation workshop was held by ECVAM in Amden in 1994 to develop a more precisely defined validation concept. Prevalidation and the development of biostatistically defined prediction models were added as essential elements to the validation process. In 1995/1996 the ECVAM validation procedure was officially accepted by EU member countries and at the international level by the US regulatory agencies and the OECD. The improved validation concept was immediately introduced into ongoing validation studies. In 1996 the ECVAM/COLIPA validation study of the in vitro phototoxicity test, which was conducted according to the ECVAM/OECD validation concept, was finished successfully and in 1998 a supporting study on UV-filter chemicals was undertaken. In 1998 the 3T3 NRU PT in vitro phototoxicity test was the first experimentally validated in vitro toxicity test that was recommended for regulatory purposes by ESAC, the ECVAM Scientific Advisory Committee, and by the DG ENV of the EU Commission. Meanwhile, two in vitro skin corrosivity tests have successfully been validated by ECVAM. Finally, in June 2000 the three experimentally validated tests were accepted by EU member states for regulatory purposes as the first in vitro toxicity tests. In addition, ECVAM has funded a successful validation study of three in vitro embryotoxicity tests, which was conducted in 12 European laboratories and finished in July 2000. The three tests validated in this study were the whole embryo culture (WEC) test applied to rat embryos, the micromass (MM) test employing primary cultures of dissociated mouse limb bud cells and the mouse embryonic stem cell test (EST). Examples will be given of successful validation studies during the past decade with particular reference to in vitro toxicity tests that were evaluated for regulatory purposes either by the US validation centre ICCVAM or ECVAM in the fields of sensitisation, phototoxicity and embryotoxicity.

Strunck E, Stemmann N, Hopert A, Wunsche W, Frank K, Vollmer G. **Relative binding affinity does not predict biological response to xenoestrogens in rat endometrial adenocarcinoma cells.** J Steroid Biochem Mol Biol 2000;74(3):73-81.
Abstract: The possible adverse effects of the so-called environmental estrogens have raised considerable

concern. Developmental, endocrine and reproductive disorders in wildlife animals have been linked to high exposure to persistent environmental chemicals with estrogen-like activity (xenoestrogens); yet, the potential impact of environmental estrogens on human health is currently under debate also due to lack of data. A battery of in vitro assays exist for identifying compounds with estrogenic activity, but only a few models are available to assess estrogenic potency in a multiparametric analysis. We have recently established the endometrial adenocarcinoma cell line RUCA-I; it enables us to compare estrogenic effects both in vitro and in vivo as these cells are estrogen responsive in vitro and grow estrogen sensitive tumors if inoculated in syngeneic animals in vivo. Here we report in vitro data concerning (a) the relative binding affinity of the selected synthetic chemicals Bisphenol A, nonylphenol, p-tert-octylphenol, and o,p-DDT to the estrogen receptor of RUCA-I cells and (b) the relative potency of these compounds in inducing increased production of complement C3, an endogenous estrogen-responsive gene. Competitive Scatchard analysis revealed that xenoestrogens bound with an at least 1000-fold lower affinity to the estrogen receptor of RUCA-I cells than estradiol itself, thereby exhibiting the following affinity ranking, estradiol>>>nonylphenol>bisphenol A approximately p-tert-octylphenol>o,p-DDT. Despite these low binding affinities, bisphenol A, nonylphenol and p-tert-octylphenol increased production of complement C3 in a dose dependent manner. Compared with estradiol, only 100-fold higher concentrations were needed for all the compounds to achieve similar levels of induction, except o, p-DDT which was by far less potent. Northern blot analyses demonstrated that the increased production of complement C3 was mediated by an increased transcription. In summary, cultured RUCA-I cells represent a valuable endometrial derived model system to assess the relative potencies and the molecular mode of action of environmental estrogens in vitro. Our results further show that no intimate correlation exists between the relative binding affinity and the biological response of these compounds. Therefore, data obtained from single-parametric analyses may result in misleading conclusions. On the other hand, the presented in vitro data will provide us with tools to study the activity of xenoestrogens in vivo and thus carry risk assessment one step further.

Sumida K, Ooe N, Nagahori H, Saito K, Isobe N, Kaneko H, Nakatsuka I. **An in vitro reporter gene assay method incorporating metabolic activation with human and rat S9 or liver microsomes.** Biochem Biophys Res Commun 2001;280(1):85-91.
Abstract: A metabolic activation system with an S9 fraction or liver microsomes was applied to a reporter gene assay in vitro for the screening of estrogenicity of chemicals. The endpoint (luciferase) was luciferase induction in cells transfected with a reporter plasmid containing an estrogen-responsive element linked to the luciferase gene. Compounds were applied to the reporter gene assay system after pretreatment or simultaneous treatment with an S9 fraction or liver microsomes. Both trans-stilbene and methoxychlor themselves showed no or little estrogenicity, but when they were treated with an S9 fraction or liver microsomes, they demonstrated strong effects, indicating their metabolites to be estrogenic. When four pyrethroid insecticides were subjected to this assay system, however, they showed no estrogenicity even with liver microsome or S9 mix treatment. Copyright 2001 Academic Press.

Tanaka M, Kinoshita M, Kobayashi D, Nagahama Y. **Establishment of medaka (Oryzias latipes) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cells in a live vertebrate.** Proc Natl Acad Sci U S A 2001;98

(5):2544-9.
Abstract: We have generated transgenic medaka (teleost, Oryzias latipes), which allow us to monitor germ cells by green fluorescent protein (GFP) fluorescence in live specimens. Two medaka strains, himedaka (orange-red variety) and inbred QurtE, were used. The transgenic lines were achieved by microinjection of a construct containing the putative promoter region and 3' region of the medaka vasa gene (olvas). The intensity of GFP fluorescence increases dramatically in primordial germ cells (PGCs) located in the ventrolateral region of the posterior intestine around stage 25 (the onset of blood circulation). Whole-mount in situ hybridization and monitoring of ectopically located cells by GFP fluorescence suggested that (i) the increase in zygotic olvas expression occurs after PGC specification and (ii) PGCs can maintain their cell characteristics ectopically after stages 20-25. Around the day of hatching, the QurtE strain clearly exhibits sexual dimorphisms in the number of GFP fluorescent germ cells, a finding consistent with the appearance of leucophores, a sex-specific marker of QurtE. The GFP expression persists throughout the later stages in the mature ovary and testis. Thus, these transgenic medaka represent a live vertebrate model to investigate how germ cells migrate to form sexually dimorphic gonads, as well as a potential assay system for environmental substances that may affect gonad development. The use of a transgenic construct as a selective marker to efficiently isolate germ-line-transmitting founders during embryogenesis is also discussed.

Thompson S, Tilton F, Schlenk D, Benson WH. **Comparative vitellogenic responses in three teleost species: extrapolation to in situ field studies.** Mar Environ Res 2000;50(1-5):185-9.
Abstract: Induction of vitellogenin (VTG) was compared among three teleostean species to determine their relative sensitivity of exposure to 17 beta-estradiol (E2). Japanese medaka (Oryzias latipes), sunshine bass (Morone saxatalis x Morone chrysops) and channel catfish (Ictalurus punctatus) were exposed to aqueous concentrations of E2 ranging from 10 to 100,000 ng/l for 21 days. Respective EC50 values for plasma VTG detected by western blot in medaka, catfish and bass were 200, 170 and 1560 ng E2/l. Since these EC50 values are based on VTG induction curves calculated relative to control values, they indicate differences in species' sensitivity to E2 exposure. Catfish and bass VTG responses obtained in laboratory exposures were compared to VTG responses previously observed with 21-day wastewater treatment plant effluent exposures. Plasma VTG induction in effluent-exposed fish ranged from 14 to 82% above reference values depending on species. Extrapolation of field responses with laboratory-exposed fish indicate catfish and bass were exposed to the equivalent of 27-240 ng E2/l in sewage effluent.

Wade CB, Robinson S, Shapiro RA, Dorsa DM. **Estrogen receptor (ER)alpha and ERbeta exhibit unique pharmacologic properties when coupled to activation of the mitogen-activated protein kinase pathway.** Endocrinology 2001;142(6):2336-42.
Abstract: The rapid, nongenomic effects of estrogen are increasingly recognized as playing an important role in several aspects of estrogen action. Rapid activation of the mitogen-activated protein kinase (MAPK) signaling pathway by estrogen is among the more recently identified of these effects. To explore the role of estrogen receptors (ERs) in mediating these effects, we have transfected ER-negative Rat-2 fibroblasts with complementary DNA clones encoding either human ERalpha or rat ERbeta and examined their ability to couple to activation of MAPK in response to 17beta-estradiol (17beta-E(2)) and other ligands. For both receptors, addition of E(2) resulted in a rapid phosphorylation of MAPK.

Activation of MAPK in ERalpha-transfected cells was partially and completely blocked by the antiestrogens tamoxifen and ICI 182,780, respectively. In ERbeta-transfected cells, MAPK activation was less sensitive to inhibition by tamoxifen and ICI 182,780. We have also observed that, in this model system, a membrane-impermeable estrogen (BSA-E(2)) and 17alpha-E(2) were both able to activate MAPK in a manner similar to E(2) alone. Here also, ICI 182,780 blocked the ability of BSA-E(2) to activate MAPK through ERalpha, but failed to block ERbeta-mediated effects. BSA-E(2) treatment, however, failed to activate nuclear estrogen-response-element-mediated gene transcription. These data show that these nuclear ERs are necessary for estrogen's effects at the membrane. This model system will be useful in identifying molecular interactions involved in the rapid effects mediated by the ERs.

Wakamatsu Y, Ju B, Pristyaznhyuk I, Niwa K, Ladygina T, Kinoshita M, Araki K, Ozato K. **Fertile and diploid nuclear transplants derived from embryonic cells of a small laboratory fish, medaka (Oryzias latipes).** Proc Natl Acad Sci U S A 2001;98(3):1071-6.
Abstract: Fertile and diploid nuclear transplants were successfully generated by using embryonic cells as donors in a small laboratory fish, medaka (Oryzias latipes). Embryonic cell nuclei from transgenic fish carrying the green fluorescent protein (GFP) gene were transplanted into unfertilized eggs enucleated by x-ray irradiation. In this study, 1 out of 588 eggs transplanted in the first experiment and 5 out of 298 eggs transplanted in the second experiment reached the adult stage. All of these nuclear transplants were fertile and diploid, and the natural and GFP markers of the donor nuclei were transmitted to the F(1) and F(2) offspring in a Mendelian fashion. This systematic study proves the feasibility of generating nuclear transplants by using embryonic cells from fish as donors, and it is supported by convincing evidence.

Wakamatsu Y, Niwa K, Kani S, Ozato K. **[Nuclear transplantation in Medaka].** Tanpakushitsu Kakusan Koso 2000;45(17 Suppl):2962-6 [Jpn].

Wakamatsu Y, Pristyazhnyuk S, Kinoshita M, Tanaka M, Ozato K. **The see-through medaka: a fish model that is transparent throughout life.** Proc Natl Acad Sci U S A 2001;98(18):10046-50.
Abstract: The see-through medaka is a vertebrate model with a transparent body in the adult stage, as well as during the embryonic stages, that was generated from a small laboratory fish, medaka (Oryzias latipes). In this fish model, most of the pigments are genetically removed from the entire body by a combination of recessive alleles at four loci. The main internal organs, namely, heart, spleen, blood vessels, liver, gut, gonads, kidney, brain, spinal cord, lens, air bladder, and gills, in living adult fish are visible to the naked eye or with a simple stereoscopic microscope. This fish is healthy and fertile. A transgenic see-through medaka was produced by using the green fluorescent protein (GFP) gene fused to the regulatory regions of the medaka vasa gene, in which germ cell-specific expression of GFP was visualized. The fluorescent tag also efficiently improved visibility of gonadal tissues. The process of oocyte maturation in the ovary was monitored by repeated observations from the outside of the body during one spawning cycle in the same living females of the transgenic see-through stock. The see-through medaka will provide an opportunity for noninvasive studies of morphological and molecular events that occur in internal organs in the later stages of life.

Whaley DA, Keyes D, Khorrami B. **Incorporation of endocrine disruption into chemical hazard scoring for pollution prevention and current list of endocrine disrupting chemicals.** Drug Chem

Toxicol 2001;24(4):359-420.
Abstract: Research continues to support the theory of endocrine disruption. Endocrine disruption is defined as the ability of a chemical contaminating the workplace or the environment to interfere with homeostasis, development, reproduction, and/or behavior in a living organism or it's offspring. Certain classes of environmentally persistent chemicals such as polychlorinated biphenyls (PCBs), dioxins, furans, and some pesticides can adversely effect the endocrine systems of aquatic life and terrestrial wildlife. The University of Tennessee, Knoxville (UTN), developed a method for hazard scoring chemicals for the aquatic ecosystem. The Indiana Clean Manufacturing Technology and Safe Materials Institute at Purdue University (CMTI) later expanded the scoring system to include terms for worker hazard as well as terms for contamination of soil and air quality, and for stratospheric ozone depletion. We call the CMTI chemical hazard score the Purdue score. At West Virginia University, two improvements of the Purdue chemical hazard score are developed, a normalizing of the term for soil contamination, and addition of hazard score terms for ecosystem endocrine disruption. The results of incorporating endocrine disruption terms into the hazard scoring equations resulted in increased hazard rankings, often substantially increased, for 26 endocrine disrupting chemicals (EDCs) among 200 Superfund chemicals. Because data suggesting human endocrine disruption from such chemicals is still controversial, no endocrine disruptor term has been added to the human toxicity portions of the chemical hazard scoring system at this time. The third product of this work is assembly of a current consolidated list of (1) established or probable, mostly synthetic, industrial chemical and medication EDCs and (2) suspect (less certain) synthetic and natural (phytoestrogen) possible endocrine disrupting chemicals, with the goal of contributing to future development of quantitative structure activity relationship software for predicting whether an untested chemical is likely to be an endocrine disruptor. We conclude that enough endocrine disrupting chemicals are now identified to make an attempt at developing structure activity estimates of disrupting potential worthwhile. Further, we conclude that within a group of 200 chemicals of concern to the US EPA, the addition of endocrine disrupting terms to the Purdue score substantially increases its representativeness in reflecting ecological exposure hazard. We have developed this enhanced Purdue score risk management tool to be of assistance to industry.

Winkler S, Loosli F, Henrich T, Wakamatsu Y, Wittbrodt J. **The conditional medaka mutation eyeless uncouples patterning and morphogenesis of the eye.** Development 2000;127(9):1911-9.
Abstract: In early vertebrate eye development, the retinal anlage is specified in the anterior neuroectoderm. During neurulation, the optic vesicles evaginate from the lateral wall of the prosencephalon. Here we describe the temperature-sensitive mutation eyeless in the Japanese medakafish. Marker gene analysis indicates that, whereas, specification of two retinal primordia and proximodistal patterning takes place in the mutant embryo, optic vesicle evagination does not occur and subsequent differentiation of the retinal primordia is not observed. The mutation eyeless thus uncouples patterning and morphogenesis at early steps of retinal development. Temperature-shift experiments indicate a requirement for eyeless activity prior to optic vesicle evagination. Cell transplantation shows that eyeless acts cell autonomously.

Yamada T, Kunimatsu T, Sako H, Yabushita S, Sukata T, Okuno Y, Matsuo M. **Comparative evaluation of a 5-day Hershberger assay utilizing mature male rats and a pubertal male assay for detection of flutamide's antiandrogenic activity.** Toxicol Sci 2000;53(2):289-96.

Abstract: A 5-day Hershberger assay utilizing mature male rats and a pubertal male assay were evaluated for the ability to detect antiandrogenic compounds such as flutamide, an androgen receptor antagonist. Six days after the operation, implantation with two silicon capsules containing testosterone (T) (30 mg/capsule) in castrated rats provided the ventral prostate and seminal vesicle weights as well as serum T and luteinizing hormone (LH) levels equivalent to those of the controls (non-castrated, non-implanted rats). Castrated rats implanted with two T-capsules (6 rats/dose) were treated by gavage for 5 days with vehicle (0.5% carboxymethylcellulose) or flutamide (0.15, 0.6, 2.5, or 10 mg/kg/day). Flutamide produced significant decreases in weights of the seminal vesicles and the levator ani plus bulbocavernosus muscles (> or =0.6 mg/kg/day) and ventral prostate (> or =2.5 mg/kg/day), and an increase in serum LH levels (> or =2.5 mg/kg/day), but no changes in serum T levels. When age-matched intact male rats were treated with 10-mg/kg/day flutamide, a significant increase in serum T levels was observed concomitant with a tendency of increased LH. The organ weights were also decreased; however, the changes were less than those in the castrated, T-implanted rats. Immature intact male rats (10 rats/dose) were treated for 20 days with flutamide (0, 0.15, 0.6, 2.5, or 10 mg/kg/day). Flutamide produced significant decreases in weights of the seminal vesicles, ventral prostate, and levator ani plus bulbocavernosus muscles at 2.5 and 10 mg/kg/day. Serum LH levels, but not T levels, were increased at 10 mg/kg/day. Statistical significance of some of these changes was not observed in the 6 animals/dose examined. Our findings support that the Hershberger assay, in the current conditions, is the most sensitive among the assays examined and a useful short-term screening method for the detection of antiandrogenic compounds.

Yamauchi M, Kinoshita M, Sasanuma M, Tsuji S, Terada M, Morimyo M, Ishikawa Y. **Introduction of a foreign gene into medakafish using the particle gun method.** J Exp Zool 2000;287(4):285-93.
Abstract: We developed a procedure to introduce a foreign gene into fertilized eggs of medakafish (Oryzias latipes) using the particle gun method, which is one of the easiest and most reliable techniques for gene transfer. A plasmid construct with the green fluorescence protein (GFP) gene driven by the madakafish beta-actin gene promoter was successfully introduced into eggs, and the expression of GFP was observed in 20% of the primary transfectant (chimera) fish. In addition, germ line transmission of GFP was observed in 13% of the GFP-positive primary transfectant fish. The new application described here should enable us to investigate gene expression using the fish model on a routine basis without high technical sophistication. J. Exp. Zool. 287:285-293, 2000.

Yelon D, Ticho B, Halpern ME, Ruvinsky I, Ho RK, Silver LM, Stainier DY. **The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development.** Development 2000;127(12):2573-82.
Abstract: The precursors of several organs reside within the lateral plate mesoderm of vertebrate embryos. Here, we demonstrate that the zebrafish hands off locus is essential for the development of two structures derived from the lateral plate mesoderm - the heart and the pectoral fin. hands off mutant embryos have defects in myocardial development from an early stage: they produce a reduced number of myocardial precursors, and the myocardial tissue that does form is improperly patterned and fails to maintain tbx5 expression. A similar array of defects is observed in the differentiation of the pectoral fin mesenchyme: small fin buds form in a delayed fashion, anteroposterior patterning of the fin mesenchyme is absent and tbx5 expression is poorly maintained. Defects in these mesodermal structures

are preceded by the aberrant morphogenesis of both the cardiogenic and forelimb-forming regions of the lateral plate mesoderm. Molecular analysis of two hands off alleles indicates that the hands off locus encodes the bHLH transcription factor Hand2, which is expressed in the lateral plate mesoderm starting at the completion of gastrulation. Thus, these studies reveal early functions for Hand2 in several cellular processes and highlight a genetic parallel between heart and forelimb development.

zur Nieden NI, Ruf LJ, Kempka G, Hildebrand H, Ahr HJ. **Molecular markers in embryonic stem cells.** Toxicol In Vitro 2001;15(4-5):455-61.
Abstract: Embryonic stem cells are pluripotent cells derived from mouse blastocysts, which have the capacity to differentiate in vitro into a wide variety of cell types. Based on this potential the embryonic stem cell test (EST) has been developed, which represents an assay system for the classification of compounds for their teratogenic potential, based on the morphological evaluation of contracting myocard cells compared to the cytotoxic effects on undifferentiated stem cells and adult 3T3 fibroblasts. To expand the EST, the quantitative expression of the alpha- and beta-myosin heavy chain (MHC) genes under the influence of test compounds was studied employing real-time TaqMan PCR analysis. The molecular evaluation of the MHC genes allows a higher sensitivity for the classification of substances and the transfer of the EST to the molecular level allows to start experimental procedures at day 9 of culture. Thus, the modulated EST holds promise as a new easily quantifiable in vitro screening assay in teratology.

Akella SS, Beck MJ, Philbert MA, Harris C. **Comparison of in vitro and in utero ethanol exposure on indices of oxidative stress.** In Vitr Mol Toxicol 2000;13(4):281-96.
Abstract: Prenatal ethanol exposure produces neural tube defects and growth retardation in experimental animals. Because ethanol's teratogenic effects may involve oxidative stress and effects may differ in vitro and in utero, glutathione, cysteine and ATP were evaluated in gestational day 10 rat conceptuses exposed to ethanol. Cultured embryos exposed to ethanol (1.5 or 3.0 mg/mL) maintained a concentration-dependent decrease in glutathione of 21 or 35%, respectively, at 6 h; visceral yolk sac (VYS) glutathione (GSH) decreased by 22 or 18%, respectively, at 3 h. Maternal ethanol exposure (4.5 g/kg) decreased glutathione by 30% in embryos and VYSs at 3 h, but values rebounded. Cultured embryonic cysteine decreased after 30 min by 42% with both doses and after 6 h by 32 or 38% with 1.5 or 3.0 mg/mL, respectively. Ethanol (1.5 mg/mL) increased VYS cysteine by 35% after 30 min. In utero ethanol exposure decreased embryonic cysteine by 58% at 3 h. Ethanol (1.5 mg/mL) decreased adenosine triphosphate (ATP) by 30-60% in embryos and VYSs at 30 min. After 6 h, embryonic ATP decreased by 41 and 30% with 1.5 and 3.0 mg/mL, respectively, while VYS ATP decreased by 38% with 1.5 mg/mL. In utero ethanol exposure decreased ATP by 31% at 3 h in VYSs. While decreases in GSH and cysteine were evident earlier in utero than in vitro, values returned to control suggesting embryos exposed in utero respond rapidly to chemical-induced oxidative stress due to maternal protective mechanisms. Differences between in vitro and in utero responses to ethanol have important implications for interpretation of in vitro developmental studies.

Manabe N, Sugimoto M, Morita M, Tanaka T, Yamaguchi M, Miyamoto H. **[A novel in vivo 31P-nuclear magnetic resonance technique for assessment of teratogenicity induced by environmental endocrine disrupters in mice].** Nippon Rinsho 2000;58(12):2482-5 [Jpn].

Abstract: Novel in vivo 31P-nuclear magnetic resonance spectoroscopy(NMR) and NMR-imaging techniques for accurate and noninvasive assessment of teratogenicity induced by environmental endocrine disrupters(EDs) were developed. Mice with pregnancy were administered ED at extremely low dose, and then in vivo 31P-NMR spectra of embryos were acquired noninvasively and quantitatively to evaluate the energy metabolism. A significant decrease in embryo ATP level was seen, but no significant changes were detected by conventional histological and biochemical methods. In conclusion, in vivo NMR techniques are highly sensitive(at least 100-fold more sensitive than conventional methods) and are useful for toxicological assessment of environmental pollutants.

Schmid TE, Lowe X, Marchetti F, Bishop J, Haseman J, Wyrobek AJ. **Evaluation of inter-scorer and inter-laboratory reliability of the mouse epididymal sperm aneuploidy (m-ESA) assay.** Mutagenesis 2001;16(3):189-95.
Abstract: The mouse epididymal sperm aneuploidy (mESA) assay using 3-chromosome fluorescence in situ hybridization (FISH) was recently developed for assessing the aneugenic potential of chemicals on male germ cells. This study was designed to identify the major technical factors that affect inter-scorer and inter-laboratory variability of the mESA assay. Two laboratories participated in this study (GSF and Lawrence Livermore National Laboratory, LLNL). Mice (102/ElxC3H/El) F(1) were exposed in one laboratory (GSF) to vinblastine (VBL; single intraperitoneal injection of 0, 0.5, 1.0 or 2.0 mg/kg), one of the 10 priority compounds of the Commission of the European Communities (CEC) Aneuploidy Program. Twenty-two days later the mESA assay was applied to analyze sperm aneuploidy. In the initial evaluation, small but statistically significant differences were found between the two laboratories in baseline frequencies and there was also disagreement in the determination of a VBL aneuploid effect. Therefore, experiments were conducted to identify the sources of the inter-laboratory differences and technical factors that affected assay reliability and the VBL study was repeated. A harmonization experiment was conducted by bringing the microscope scorers from both laboratories to the same site (LLNL) for a cross-training exercise. Following this exercise, a second group of VBL-treated and control mice were evaluated, and we concluded that VBL is not a sperm aneugen. Our research has identified scoring criteria as the major source of inter-laboratory variation and emphasizes the importance of strict technical controls for the mESA assay, including controlling slide preparations for treatment-induced reductions in sperm count, coding of slides and selection of statistical tests. These considerations are particularly important for the interpretation of small effects (< or =2-fold) on sperm aneuploidy. Our findings suggest that 2-fold differences in frequencies can result from differences among scorers, samples and treatment groups, and are readily within the normal variation for the mESA assay. Such small differences should be viewed with caution until independently confirmed.

Spearow JL, Barkley M. **Reassessment of models used to test xenobiotics for oestrogenic potency is overdue.** Hum Reprod 2001;16(5):1027-9.
Abstract: Product safety bioassays need to include data from animals with susceptible genotypes or the potential for environmental compounds to disrupt reproductive development in hormonally sensitive populations may be greatly underestimated. The continued use of resistant animal models is likely to result in allowable releases of toxic levels of oestrogenic agents that could differentially disrupt reproductive development and function of sensitive genotypes, leading to reproductive failure and loss or extinction of susceptible individuals, populations and species. Rather than ignoring the role of genetic

differences in susceptibility to oestrogenic agent-induced carcinogenicity and endocrine disruption, government agencies should support efforts to identify the genetic mechanisms involved in these responses, and to screen for and develop strains of mice and rats which are sensitive to the induction of genotoxicity/carcinogenicity as well as the inhibition of reproductive development and function by oestrogenic agents. Such sensitive strains would be even more optimal for testing chemicals for endocrine disruptor activity.

MISCELLANEOUS

Ait-Aissa S, Porcher J, Arrigo A, Lambre C. **Activation of the hsp70 promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity.** Toxicology 2000;145(2-3):147-57.
Abstract: Stress proteins (heat shock proteins, HSPs) have been proposed as general markers of cellular aggression and their use for environmental monitoring is often suggested. The aim of this work was to study the potency of various environmentally relevant organic and inorganic chemicals to induce the expression of the HSP70 marker. For this purpose, we used an established HeLa cell line containing the chloramphenicol acetyl transferase (CAT) gene under the control of the hsp70 promoter. The screening of three metallic and 15 organic chemicals revealed differences in their capacities to induce the hsp70 promoter. The three metals tested (cadmium, zinc and mercury) were able to induce a stress response. Some organochlorine compounds (chlorophenol derivatives, tetrachlorohydroquinone, 3, 4-dichloroaniline, ethyl parathion and 1-chloro-2,4-dinitrobenzene) induced a response, whereas other common halogenated pesticides or aromatic hydrocarbons (e.g. benzo(a)pyrene, 2, 4-dichlorophenoxyacetic acid, endosulfan, diuron, 4-nonylphenol) did not. The potency to induce hsp70 was significantly correlated to the octanol-water partition coefficient (log K(ow)) of the inducing chemicals, except for 1-chloro-2,4-dinitrobenzene and ethyl parathion. Cytotoxicity assays run in parallel to the induction measurements revealed that the three metals were effective at non cytotoxic doses whereas all organic compounds, except tetrachlorohydroquinone and 1-chloro-2,4-dinitrobenzene, induced the promoter at cytotoxic doses. These results suggest that hsp70 is induced by different mechanisms of toxicity. We propose that this model can be used in mechanistic studies for the detection of toxic effects of certain pollutants.

Altvater T, Hendrich C, Noth U, Rader CP, Stach R, Schutze N, Eulert J, Thull R. **[Cytotoxicity study of high gold content Degutan surfaces of various degrees of roughness with fibroblasts (BALB 3T3) and osteoblasts (hFOB 1.19)].** Biomed Tech (Berl) 2000;45(9):238-42 [Ger].
Abstract: The cytotoxicity of Degutan surfaces with different degrees of roughness, and the effect of surface structures on osteoblast proliferation and differentiation, was investigated with standardised cell culture systems. Fibroblast cell lines (BALB/3T3) and osteoblast cell lines (hFOB 1.19) were used. The number and variability of the cells were determined for assessment of proliferation and alkaline phosphatase activity, collagen I and osteocalcin production were used as parameters for differentiation. In the early phase, the largest numbers of cells and greatest proliferation were measured on polished Degutan surfaces. In the late phase, however, larger numbers of cells and a greater degree of proliferation were to be seen on sandblasted and sandblasted/heat-treated Degutan surfaces. No differences were found for collagen I, osteocalcin production or alkaline phosphatase activity. Neither

the osteoblasts nor the fibroblasts revealed a toxic effect of Degutan. The results for osteoblast differentiation correlate with recent studies on identical structured titanium surfaces. In view of the immeasurable amount of ion release, Degutan may be considered an ideal model for an inert material surface.

Billington R, Carmichael N. **Setting of acute reference doses for pesticides based on existing regulatory requirements and regulatory test guidelines.** Food Addit Contam 2000;17(7):621-6.
Abstract: Reference doses (RfD) for the definition of tolerable food residues have traditionally been based on the lowest no observed adverse effect level (NOAEL), which usually comes from chronic toxicity studies. While this is generally agreed to be a safe approach to evaluate the overall significance of expected consumption it is clear that it is not appropriate for evaluation of the toxicological significance of residues in a single meal. Standard acute toxicity tests are not designed to generate an NOAEL, from which an RfD can be derived. They are more appropriate to evaluating risk following accidental high exposure to the product itself rather than to food residues. A typical toxicological database for a pesticide active substance contains studies which may be appropriate, on a case-by-case basis, to evaluate shorter term endpoints of interest for specific molecules, such as developmental or acute neurotoxicity studies. However, their specificity limits their scope of application. General toxicological endpoints are well covered by short-term, 28- or 90-day, guideline studies. However, neither of these studies is ideal for setting of an acute RfD (ARfD) as the treatment period is significantly longer than the duration of consumer exposure. This could be balanced by applying a reduced safety factor to the NOAEL to set the ARfD. Alternatively, a test guideline could be designed to generate a relevant acute NOAEL but the time necessary for development, validation and acceptance of such a guideline means that an interim approach is, in any case, necessary.

Bois FY. **Applications of population approaches in toxicology.** Toxicol Lett 2001;120(1-3):385-94.
Abstract: Many experimental or observational studies in toxicology are best analysed in a population framework. Recent examples include investigations of the extent and origin of intra-individual variability in toxicity studies, incorporation of genotypic information to address intra-individual variability, optimal design of experiments, and extension of toxicokinetic modelling to the analysis of biomarker studies. Bayesian statistics provide powerful numerical methods for fitting population models, particularly when complex mechanistic models are involved. Challenges and limitations to the use of population models, in terms of basic structure, computational burden, ease of implementation and data accessibility, are identified and discussed.

Brown MD, Schatzlein A, Brownlie A, Jack V, Wang W, Tetley L, Gray AI, Uchegbu IF. **Preliminary characterization of novel amino acid based polymeric vesicles as gene and drug delivery agents**. Bioconjug Chem 2000;11(6):880-91.
Abstract: The amino acid homopolymers, poly-L-lysine and poly-L-ornithine, have been modified by the covalent attachment of palmitoyl and methoxypoly(ethylene glycol) (mPEG) residues to produce a new class of amphiphilic polymers-PLP and POP, respectively. These amphiphilic amino acid based polymers have been found to assemble into polymeric vesicles in the presence of cholesterol. Representatives of this new class of polymeric vesicles have been evaluated in vitro as nonviral gene delivery systems with a view to finding delivery systems that combine effective gene expression with

low toxicity in vivo. In addition, the drug-carrying capacity of these polymeric vesicles was evaluated with the model drug doxorubicin. Chemical characterization of the modified polymers was carried out using (1)H NMR spectroscopy and the trinitrobenzene sulfonic acid (TNBS) assay for amino groups. The amphiphilic polymers were found to have an unreacted amino acid, palmitoyl, mPEG ratio of 11:5:1, and polymeric vesicle formation was confirmed by freeze-fracture electron microscopy and drug encapsulation studies. The resulting polymeric vesicles, by virtue of the mPEG groups, bear a near neutral zeta-potential. In vitro biological testing revealed that POP and PLP vesicle-DNA complexes are about one to 2 orders of magnitude less cytotoxic than the parent polymer-DNA complexes although more haemolytic than the parent polymer-DNA complexes. The polymeric vesicles condense DNA at a polymer:DNA weight ratio of 5:1 or greater and the polymeric vesicle-DNA complexes improved gene transfer to human tumor cell lines in comparison to the parent homopolymers despite the absence of receptor specific ligands and lysosomotropic agents such as chloroquine.

Burse VW, Najam AR, Williams CC, Korver MP, Smith BF Jr, Sam PM, Young SL, Needham LL. **Utilization of umbilical cords to assess in utero exposure to persistent pesticides and polychlorinated biphenyls.** J Expo Anal Environ Epidemiol 2000;10(6 Pt 2):776-88.
Abstract: In support of a study to relate developmental and cognitive effects with prenatal exposure to selected environmental toxicants, we developed and applied an analytical method to determine the concentration of two persistent pesticides, hexachlorobenzene (HCB) and p,p'-dichlorodiphenyldichloroethylene (DDE), and 32 specific polychlorinated biphenyl (PCB) congeners in 316 umbilical cords taken in 1986-1987 from women of the Faroe Islands. The analytical method consisted of homogenization of the cords, partitioning, microsilica gel column chromatography for clean-up, and dual-column capillary gas chromatography (DB-5 and DB-1701) with electron capture detection. Several quality control parameters were followed to monitor the performance of the method. Important criteria used before reporting unknown data were the recovery of in vitro-spiked analytes from a bovine umbilical cord (BUC) and the percentage lipid obtained for a Certified Reference Material (CRM)-350 of mackerel oil (MO). Recoveries of analytes that had been spiked at two concentration ranges (0.26-0.95 ng/g whole weight; 0.35-2.42 ng/g whole weight) into bovine cords ranged from 38.5% to 158% and from 50.4% to 145%, respectively, with a median recovery of 77.7%. Measurement of the percentage lipid for CRM-350 ranged from 73.8% to 107% with a median lipid value of 96.0%. The most prevalent analytes detected (%) in unknown umbilical cords were HCB (100), DDE (100), Ballschmiter/Zell PCBs 153 (100), 138 (98), 180 (98), 170 (93), 118 (88), 187 (86), and 146 (83), with corresponding median concentrations (ng/g whole weight) of 0.17, 1.19, 0.38, 0.30, 0.17, 0.11, 0.12, 0.09, and 0.07, respectively. Total PCB--sum of all measurable PCB congeners--had a median concentration of 1.37 ng/g whole weight. The analytes, which were very low in lipid content were also quantified on a lipid-adjusted basis, which provided an analytical challenge in these umbilical cord samples. The gravimetrically measured lipids in the human specimens ranged from 0.01% to 1.43% (median of 0.18%). In the pooled BUCs, our lipid measurements varied from 0.05% to 0.33% with a median value of 0.13%. The utility of using the umbilical cord as a matrix to assess in utero exposure to persistent environmental pollutants, compared with the use of umbilical cord blood or mother's blood, is worthy of debate.

Butowt R, Jeffrey PL, von Bartheld CS. **Purification of chick retinal ganglion cells for molecular**

analysis: combining retrograde labeling and immunopanning yields 100% purity. J Neurosci Methods 2000;95(1):29-38.
Abstract: Retinal ganglion cells (RGCs) from embryonic and posthatch chickens were 100% purified by a novel combination of three steps: (1) Retrograde labeling by injection of the fluorescent carbocyanine tracer DiI into the optic nerve, (2) immunopanning of dissociated retinal cells with Thy1 antibodies, and (3) micro-aspiration of labeled RGCs into glass capillaries. The retina was dissected and dissociated with trypsin 12-15 h after the injection of DiI. DiI-labeled cells were identified on immunopanned dishes by fluorescence and collected for molecular analysis within 3 h after dissociation. This technique allowed the collection of up to 500 RGCs per capillary tube and 1500 labeled RGCs per retina. Extraction of RNA and molecular analysis by RT-PCR from 600 RGCs shows that expression of rare genes, such as those of neurotrophic factors, can be detected. This is the first description of a rapid and reliable technique for a 100% purification of RGCs with sufficient yield for molecular analysis of rare gene expression. The protocol can be modified for the purification of other cell types. The advantages and limitations of the three-step purification method are compared with previous RGC purification protocols.

Fukuda K. **Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering.** Artif Organs 2001;25(3):187-93.
Abstract: We have isolated a cardiomyogenic (CMG) cell line from murine bone marrow stroma. Stromal cells were immortalized, treated with 5-azacytidine, and spontaneous beating cells were repeatedly screened for. The cells showed a fibroblast-like morphology. However, this morphology changed after 5-azacytidine treatment in about 30% of the cells, which connected with adjoining cells after 1 week, formed myotube-like structures and began spontaneous beating after 2 weeks, and beat synchronously after 3 weeks. These cells expressed atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Electron microscopy revealed a cardiomyocyte-like ultrastructure including typical sarcomeres and atrial granules. They had sinus node-like or ventricular cell-like action potentials. Analysis of the isoform of contractile protein genes, such as myosin and alpha-actin, indicated that their phenotype was similar to fetal ventricular cardiomyocytes. These cells expressed Nkx2.5, GATA4, TEF-1, and MEF2-C mRNA before 5-azacytidine treatment, and expressed MEF2-A and MEF2-D after treatment. This new cell line provides a powerful model for the study of cardiomyocyte transplantation.

Furler M, Demuth D, Althaus FR, Nageli H. **[Computer-supported poisonous plant information system for veterinary medicine].** Schweiz Arch Tierheilkd 2000;142(6):323-31 [Ger].
Abstract: Animals poisoned by plants are the subject of an increasing number of inquiries made to poison control centres. The most frequent questions are concerned with the identification of potentially toxic species and the choice of adequate therapeutic strategies. To meet this growing demand for information, we generated a database on poisonous plants to be used by veterinary practitioners. Relevant data were selected from the scientific literature and organised according to the requirements of a structured database. As a result, we now introduce a user-friendly decision support system that is equipped with several search functions for fast and efficient retrieval of data. The information provided for each plant includes the degree of toxicity, major toxic constituents, their mechanism of action, pathological findings, clinical symptoms with brief case reports, therapeutic guidelines and references. In addition, each species is accompanied by a botanical description with photographic illustrations. This

information tool on poisonous plants is available via the internet, or compact disc, and can be accessed on Macintosh, Windows or UNIX using a browser that supports HTML 3.

Gray SA, Kusel JK, Shaffer KM, Shubin YS, Stenger DA, Pancrazio JJ. **Design and demonstration of an automated cell-based biosensor.** Biosens Bioelectron 2001;16(7-8):535-42.
Abstract: Cell-based biosensors have the capacity to respond to a wide range of analytes in a physiologically relevant manner and appear well-suited for toxicity monitoring of both known and unknown analytes. One means of acquiring cellular functional information for biosensor applications involves extracellular recording from excitable cells, which can generate noninvasive and long-term measurements. Previous work from our laboratory described a prototype portable system capable of high signal-to-noise extracellular recordings, in spite of deficiencies in thermal control, fluidics handling, and absence of data acquisition (DAQ) capability. The present work describes a cell-based biosensor system that incorporates low noise amplifier and filter boards, a two-stage thermal control system with integrated fluidics and a flexible graphical user interface for DAQ and control implemented on a personal computer. Wherever possible, commercial off-the-shelf components have been utilized for system design and fabrication. The system exhibits input-referred noise levels of 5-10 &mgr;V(RMS), such that extracellular potentials exceeding 50-60 &mgr;V can be readily resolved. In addition, the biosensor system is capable of automated temperature and fluidics control. Flow rates can range from 0-2.5 ml/min, while the cell recording chamber temperature is maintained within a range of 36-37 degrees C. To demonstrate the capability of this system to resolve small extracellular potentials, recordings from embryonic chick cardiac myocytes have been performed.

Gunatilleka AD, Poole CF. **Models for estimating the non-specific toxicity of organic compounds in short-term bioassays.** Analyst 2000;125(1):127-32.
Abstract: The solvation parameter model is used to construct equations for the estimation of the non-specific toxicity of neutral organic compounds to five organisms used for short-term toxicity testing. For the bacteria Vibrio fischeri (Microtox test) and Pseudomonas putida, the protozoan Tetrahymena pyriformis (Tetratox test), the green alga Scendesmus quadricauda and the brine shrimp Artemia salina, the main factors resulting in increased non-specific toxicity are size (dominantly) and lone-pair electron interactions, with hydrogen-bond basicity the most important solute property reducing toxicity. Species differences in relative non-specific toxicity are largely related to differences in cohesion and hydrogen-bond acidity of the biomembranes. The models for non-specific toxicity are proposed as an alternative to the octanol-water distribution constant for the determination of baseline toxicity. Failure of the octanol-water distribution constant to model non-specific toxicity is quantitatively explained by its inability to adequately characterize the sorption properties of the biomembranes for compounds with varied properties.

Hol WG. **Structural genomics for science and society.** Nat Struct Biol 2000;7 Suppl:964-6.
Abstract: The field of robotics is affecting structural biology, enabling the era of structural genomics. The potential impact on protein fold prediction, biology, protein engineering and medicine is immense. Unraveling mysteries in the protein structure universe will require a dedicated effort for decades to come with computational toxicology as possibly a century long challenge.

Holmes CJ. **Pre-clinical biocompatibility testing of peritoneal dialysis solutions**. Perit Dial Int 2000;20 Suppl 5:S5-9.
Abstract: Pre-clinical biocompatibility testing of peritoneal dialysis (PD) solutions has become an integral part of new solution development. The construction of a pre-clinical screening program for solution biocompatibility should take a hierarchical approach, starting with in vitro cell viability and function assays. The selection of cell types and assay systems for the in vitro studies should be broad enough to permit a balanced interpretation. Whenever possible, animal models are recommended for the next hierarchical level of testing, followed by human ex vivo study designs. Designs of the latter sort provide evidence that a new solution formulation is exerting an altered biological response in vivo; the response is not purely an in vitro artifact or restricted to a given animal species. This article discusses the various approaches available for biocompatibility testing during the pre-clinical phase of solution development, with an emphasis on the advantages and drawbacks of each method.

Hug TS, Prenosil JE, Morbidelli M. **Optical waveguide lightmode spectroscopy as a new method to study adhesion of anchorage-dependent cells as an indicator of metabolic state.** Biosens Bioelectron 2001;16(9-12):865-74.
Abstract: Optical Waveguide Lightmode Spectroscopy (OWLS) is based on measurements of the effective refractive index of a thin layer above the waveguide. Its potential as a whole-cell biosensor was demonstrated recently monitoring adhesion and spreading of Baby Hamster Kidney (BHK) cells on-line. In this work the OWLS is shown to be a promising tool to study the adhesion, morphology and metabolic state of fibroblasts in real time. A new design of the measuring chamber allowed simultaneous observation by phase-contrast microscopy and made the adsorbed cell density controlable and reproducible. The OWLS signal correlated quantitatively with the contact-area between the fibroblasts and the waveguide. The OWLS signals for adhesion and spreading of three different fibroblast cell lines were in good agreement with their morphology identified by phase-contrast microscopy. The cell adhesion and cell shape changes were examined in three scenarios: (a) serum-induced spreading of the surface attached fibroblasts was followed until it was completed, and the OWLS signal remained constant for over 12 h; (b) the fully spread cells were exposed to the microtubuli-disrupting colchicine and a decrease of the OWLS signal was monitored; (c) in a similar experiment with benzalkonium chloride, a strong skin irritant, a concentration-dependent response of the signal was found. The results show the strength of the OWLS method for monitoring the adhesion behavior of anchorage-dependent cells such as fibroblasts. It has a great potential as a whole-cell biosensor for high throughput screening in toxicology.

Jennings VL, Rayner-Brandes MH, Bird DJ. **Assessing chemical toxicity with the bioluminescent photobacterium (Vibrio fischeri): a comparison of three commercial systems.** Water Res 2001;35 (14):3448-56.
Abstract: The inhibition of light emitted by the bioluminescent bacterium, Vibrio fischeri, is the basis for several toxicity bioassays. The inhibitory effects of 81 chemicals, after 5 min contact time, were studied at eight concentrations using reagents from three commercial assay systems (ToxAlert 10, Microtox and LUMIStox). Solubility in water was the limiting factor in determining the selection of chemicals for study. The effective nominal concentrations (EC) resulting in 20, 50 and 80% inhibition were determined using Ln dose/Ln gamma plots and the results obtained for each system were compared by

linear regression. The chemical concentrations producing 10-90% inhibition extended over 9 orders of magnitude and ranged from a minimum of 0.001 ppm to a maximum of 1,000,000 ppm. The toxicity of many chemicals was apparently related to their pH in solution and at high chemical concentrations, to osmotic imbalance. The fact that the same operator tested the same solutions simultaneously on three different systems reduced sources of error and variability and improved the consistency and reliability of the results. Only five compounds gave EC 50s that varied more than three-fold between assays. These data provide comparisons of toxicity that have not been previously available and demonstrate that, when used under standardised conditions, these bioluminescence-based toxicity assays produce very similar results.

Laval-Gilly P, Falla J, Klestadt D, Henryon M. **A new approach to evaluate toxicity of gases on mobile cells in culture.** J Pharmacol Toxicol Methods 2000;44(3):483-8.
Abstract: A novel technique is described that measures the degree of toxicity of short-term exposure to gaseous pollutants or other chemical compounds on cultured cells, in 30 min. This technique, based on the study of the mobility properties of activated macrophages, consists of an image analysis procedure incorporating a specific exposure chamber (EC). The EC, which is developed from commercial culture flasks (50 ml, 25 cm(2) of culture surface), was first used to maintain cells in culture conditions, overnight, prior to the assay. In order to measure toxicity, it was then connected to the gaseous pollutant or chemical source. After exposing the culture medium and cells to the gas stream for 10 min, fMLP, a chemotactic factor, was added and the mobility of the macrophages measured by superimposing sequential analogue images captured by a CCD camera that were digitised and analysed using a software developed for this purpose. For example, the effect of ozone on macrophage-like cell (THP-1) was investigated. After exposure to 0.1 and 0.5 ppm, cells lost, respectively 79% and 90% of their mobility, compared to the control sample.

Laval L, Leveque P, Jecko B. **A new in vitro exposure device for the mobile frequency of 900 MHz.** Bioelectromagnetics 2000;21(4):255-63.
Abstract: A wire patch cell has been designed for exposing cell cultures during in vitro experiments studying possible effects of mobile radio telephone. It is based on the wire patch antenna which works at 900 MHz with a highly homogeneous field inside the antenna cavity. The designed cell structure is symmetric and provides a rather homogeneous field distribution in a large area around its centre. Moreover, the exposure cell can irradiate equally up to eight 35 mm Petri dishes at the same time, which enhances the statistical biological studies. To improve the specific absorption rate (SAR) homogeneity inside each sample, each dish is placed into another 50 mm dish. This way, SAR inhomogeneity is always proper for biological studies (below 30%). The main advantage of this new device is that it can provide SAR levels 20 times higher than those induced by classical Crawford transverse electromagnetic (TEM) cell. Moreover, this small open device is easy to construct and fits into an incubator. However, to be used for in vitro, the wire patch cell is a radiating element with the same radiating pattern as a dipole, and thus some absorbing materials are necessary around the system when used for in vitro experiments. Secondly, because of its narrow bandwidth, it is difficult to maintain its working frequency. To overcome this problem, a matching device is integrated into the test cell. In this paper, we present a detailed explanation of the cell behavior and dosimetric assessments for eight 35 mm Petri dishes exposed. Simulations using the Finite Difference Time Domain technique and experimental

investigations have been carried out to design the cell at 900 MHz. The numerical dosimetry was validated by dosimetric measurements. These investigations estimated the dosimetric precision at 11%.

Lockett T, Reilly W, Manthey M, Wells X, Cameron F, Moghaddam M, Johnston J, Smith K, Francis C, Yang Q, et al. **Tris lipidation: a chemically flexible technology for modifying the delivery of drugs and genes**. Clin Exp Pharmacol Physiol 2000;27(7):563-7.
Abstract: 1. One of the major challenges in the development of pharmaceuticals is their formulation with other materials to give them the desired bioavailability profile when administered into the body. 2. We have developed a flexible platform technology (Tris lipidation) to simply and effectively alter the lipophilicity of drugs. As implied by the name, the technology uses the common buffer Tris as a linker between the drugs of interest and a domain of variable hydrophobicity. 3. We demonstrate, using a mouse melanoma model, that Tris-lipidated conjugates of the widely used cytotoxic and anti-inflammatory drug methotrexate (MTX) display enhanced potency in the local treatment of tumours and reduced systemic toxicity when compared with the unconjugated drug. 4. With genes now being predicted to be the pharmaceuticals of the future, we show that Tris-lipidated cationic peptides can efficiently deliver DNA into (transfect) cells in culture. Furthermore, by comparing the abilities of variants of these Tris-based cationic lipids to transfect cultured cells, we demonstrate that modifications made to variable regions of Tris-lipidated compounds can dramatically alter their delivery profiles.

Lohse PA, Wright MC. **In vitro protein display in drug discovery.** Curr Opin Drug Discov Devel 2001;4(2):198-204.
Abstract: Nucleic acid-encoded libraries have been used at different stages of the drug discovery process for the identification of polypeptide ligands and for target identification. Traditionally, phage display screening systems have been used to explore large libraries of peptides and proteins. Lately, novel protein selection technologies have been developed that work entirely in vitro and use the polymerase chain reaction (PCR) rather than cells to amplify genetic material. The simplicity of the linkage between the protein and its encoding nucleic acid leads to several advantages, including the use of larger libraries without the biases of cell-based amplification, greater control over binding conditions and the ease with which PCR-based mutagenesis and recombination can be incorporated. This review focuses on the latest improvements in this new generation of in vitro protein display techniques and discusses their applications to the drug discovery process.

Pauwels A, Cenijn PH, Schepens PJ, Brouwer A. **Comparison of chemical-activated luciferase gene expression bioassay and gas chromatography for PCB determination in human serum and follicular fluid**. Environ Health Perspect 2000;108(6):553-7.
Abstract: We assessed exposure to dioxin-like compounds using chemical and bioassay analysis in different matrices in a female population. A total of 106 serum and 9 follicular fluid samples were collected from infertile women attending Centers for Reproductive Medicine in Belgium from 1996 to 1998. Major polychlorinated biphenyl (PCB) congeners were quantified by chemical analysis using gas chromatography with electron-capture detection, and the chemical-activated luciferase gene expression (CALUX) bioassay was used to determine the total dioxin-like toxic equivalence (TEQ) of mixtures of polyhalogenated aromatic hydrocarbons present in body fluids, such as serum and follicular fluid. To the best of our knowledge, this is the first investigation to determine TEQ values by the CALUX bioassay in

follicular fluid. The TEQ levels in both matrices are well correlated (r = 0.83, p = 0.02). As the chemical and bioassay analysis executed in this study do not cover the same span of polyhalogenated aromatic hydrocarbons, we did not expect totally correlated results. Moreover, the sample workup and quantification of the analytes differed completely. Nonetheless, the TEQ values in human extracts correlated well with the sum of four major PCB congeners chemically determined in both serum and follicular fluid. These results indicate that the CALUX bioassay may serve as a simple, relatively inexpensive prescreening tool for exposure assessment in epidemiologic surveys.

Sakai Y, Shoji R, Kim BS, Sakoda A, Suzuki M. **Cultured human-cell-based bioassay for environmental risk management**. Environ Monit Assess 2001;70(1-2):57-70.
Abstract: Among bioassays for evaluating various impacts of chemicals on humans and ecosystems, those based on cultured mammalian-cells can best predict acute lethal toxicity to humans. We expect them to be employed in the future in environmental risk management alongside mutagenicity tests and endocrine-disrupting activity tests. We recently developed a disposable bioassay device that immobilizes human hepatocarcinoma cells in a small micropipette tip. This enables very quick (within 2 h) evaluation of acute lethal toxicity to humans. For bioassay-based environmental management, 2 promising approaches have been demonstrated by the US-EPA: toxicity identification evaluation (TIE) and toxicity reduction evaluation (TRE). The Japanese Ministry of Environment has been supporting a multi-center validation project, aimed at assembling a bioassay database. To make full use of these resources, we present a numerical model that describes contribution of individual chemical to observed toxicity. This will allow the selection of the most effective countermeasure to reduce the toxicity. Bioassay-based environmental risk management works retrospectively, whereas impact assessment using substance flow models and toxicity databases works prospective. We expect that these 2 approaches will exchange information, act complementarily, and work effectively in keeping our environment healthy in the 21 st century.

Sharif KA, Goldman ID. **Rapid determination of membrane transport parameters in adherent cells.** Biotechniques 2000;28(5):926-8, 930, 932.
Abstract: Reported here is a new method that permits rapid (approximately 5 s) determinations of membrane transport phenomena in cells grown in monolayers at the base of 17-mm glass scintillation vials. The method is convenient, cost effective and requires no special apparatus. Initial uptake rates, steady-state and free substrate levels are demonstrated in ZR-75-1 breast cancer and Chinese hamster ovary cell lines using methotrexate, a model agent transported by the reduced folate carrier. The technique should be applicable to the study of the transport properties in a broad range of substrates and cells in monolayer culture.

Simons CT, Dessirier JM, Jinks SL, Carstens E. **An animal model to assess aversion to intra-oral capsaicin: increased threshold in mice lacking substance p.** Chem Senses 2001;26(5):491-7.
Abstract: Despite the widespread consumption of products containing chemicals that irritate the oral mucosa, little is known about the underlying neural mechanisms nor is there a corresponding animal model of oral irritation. We have developed a rodent model to assess aversion to capsaicin in drinking water, using a paired preference paradigm. This method was used to test the hypothesis that the neuromodulator substance P (SP) plays a role in the detection of intra-oral capsaicin. 'Knockout' (KO)

mice completely lacking SP and neurokinin A due to a disruption of the preprotachykinin A gene and a matched population of wild-type (WT) mice had free access to two drinking bottles, one containing water and the other capsaicin at various concentrations. Both KO and WT mice showed a concentration-dependent aversion to capsaicin. KO mice consumed significantly more capsaicin than WT at a single near threshold (1.65 microM) concentration, indicating that SP plays a limited role in the detection and rejection of oral irritants.

Smirnova OA. **Mathematical modeling of mortality dynamics of mammalian populations exposed to radiation.** Math Biosci 2000;167(1):19-30.
Abstract: A mathematical model is developed which describes the dynamics of radiation-induced mortality of a non-homogeneous (in radiosensitivity) mammalian population. It relates statistical biometric functions with statistical and dynamic characteristics of a critical system in organism of specimens composing this population. The model involves two types of distributions, the normal and the log-normal, of population specimens with respect to the radiosensitivity of the critical system cells. This approach suggests a new pathway in developing the methods of radiation risk assessment.

Strathmann M, Griebe T, Flemming HC. **Artificial biofilm model--a useful tool for biofilm research**. Appl Microbiol Biotechnol 2000;54(2):231-7.
Abstract: For biofilm studies, artificial models can be very helpful in studying processes in hydrogels of defined composition and structure. Two different types of artificial biofilm models were developed. Homogeneous agarose beads (50-500 microm diameter) and porous beads (260 microm mean diameter) containing pores with diameters from 10 to 80 microm (28 microm on average) allowed the embedding of cells, particles and typical biofilm matrix components such as proteins and polysaccharides. The characterisation of the matrix structures and of the distribution of microorganisms was performed by confocal laser scanning microscopy. The physiological condition of the embedded bacteria was examined by redox activity (CTC-assay) and membrane integrity (Molecular Probes LIVE/DEAD-Kit). Approximately 35% of the immobilised cells (Pseudomonas aeruginosa SG81) were damaged due to the elevated temperature required for the embedding process. It was shown that the surviving cells were able to multiply when provided with nutrients. In the case of homogeneous agarose beads, cell growth only occurred near the bead surface, while substrate limitation prevented growth of more deeply embedded cells. In the porous hydrogel, cell division was observed across the entire matrix due to better mass transport. It could be shown that embedding in the artificial gel matrix provided protection of immobilized cells against toxic substances such as sodium hypochlorite (0.5 mg/l, 30 min) in comparison to suspended cells, as observed in other immobilized systems. Thus, the model is suited to simulate important biofilm matrix properties.

Sudo T, Ito H, Ozeki Y, Kimura Y. **Estimation of anti-platelet drugs on human platelet aggregation with a novel whole blood aggregometer by a screen filtration pressure method**. Br J Pharmacol 2001;133(8):1396-404.
Abstract: 1. The effects of anti-platelet drugs on human whole blood aggregation were evaluated using a novel whole blood aggregometer by a screen filtration pressure (SFP) method. 2. The SFP whole blood aggregometer was found to successfully detect whole blood aggregation induced by ADP, collagen and TRAP by measuring the SFP of blood samples. The platelet aggregation threshold index (PATI), the

concentration of agonist required with an inducing pressure rate of 50%, varied time-dependently after collection of blood. High values for ADP and collagen were noted immediately after blood collection, suggesting low aggregation activity of platelets, and gradually increase thereafter. 3. Cilostazol (phosphodiesterase 3 inhibitor), dipyridamole, aspirin and tirofiban all inhibited whole blood aggregation in vitro. Inhibitory effects of cilostazol and dipyridamole, but not tirofiban, were markedly enhanced 6 or 7 fold by long pre-incubation (60 min), compared with short pre-incubation (2 min). Such enhancement was only observed with ADP- and not collagen-induced whole blood aggregation. A similar phenomenon was also observed for aggregation with platelet rich plasma (PRP). Cilostazol inhibition of ADP-induced platelet aggregation was more potent with PRP than whole blood (PATI(200) =3.80+/-0.95 microM for whole blood; 2.04+/-0.61 microM for PRP). Inhibitory effects of dipyridamole were attenuated in PRP without erythrocytes. 4. These results demonstrate that the SFP aggregometer can sensitively detect anti-platelet aggregatory effects of various kinds of drugs. So that it is a useful tool for evaluation of anti-platelet drugs.

Teuschler LK, Gennings C, Stiteler WM, Hertzberg RC, Colman JT, Thiyagarajah A, Lipscomb JC, Hartley WR, Simmons JE. **A multiple-purpose design approach to the evaluation of risks from mixtures of disinfection by-products.** Drug Chem Toxicol 2000;23(1):307-21.
Abstract: Drinking water disinfection has effectively eliminated much of the morbidity and mortality associated with waterborne infectious diseases in the United States. Various disinfection processes, however, produce certain types and amounts of disinfection by-products (DBPs), including trihalomethanes (THM), haloacetic acids, haloacetonitriles, and bromate, among others. Human health risks from the ubiquitous exposure to complex mixtures of DBPs are of concern because existing epidemiologic and toxicologic studies suggest the existence of systemic or carcinogenic effects. Researchers from several organizations have developed a multiple-purpose design approach to this problem that combines efficient laboratory experimental designs with statistical models to provide data on critical research issues (e.g., estimation of human health risk from low-level DBP exposures, evaluation of additivity assumptions as useful for risk characterization, estimation of health risks from different drinking water treatment options). A series of THM experiments have been designed to study embryonic development, mortality and cancer in Japanese medaka (Oryzias latipes) and liver and kidney endpoints in female CD-1 mice. The studies are to provide dose-response data for specific mixtures of the 4 THMs, for the single chemicals, and for binary combinations. The dose-levels and mixing ratios for these experiments were selected to be useful for development and refinement of three different statistical methods: testing for departures from dose-additivity; development of an interactions-based hazard index; and use of proportional-response addition as a risk characterization method. Preliminary results suggest that dose-additivity is a reasonable risk assessment assumption for DBPs. The future of mixtures research will depend on such collaborative efforts that maximize the use of resources and focus on issues of high relevance to the risk assessment of human health.

Toussaint O, Dumont P, Dierick JF, Pascal T, Frippiat C, Chainiaux F, Magalhaes JP, Eliaers F, Remacle J. **Stress-induced premature senescence as alternative toxicological method for testing the long-term effects of molecules under development in the industry.** Biogerontology 2000;1(2):179-83.
Abstract: No alternative in vitro method exists for detecting the potential long-term genotoxic effects of molecules at subcytotoxic concentrations, in terms of days and weeks after exposure(s) to the molecule

tested. A theoretical model of cellular senescence led to the concept that subcytotoxic stresses under any molecules at subcytotoxic doses, such as molecules under development in the pharmaceutical, cosmetics and food industry, might lead human fibroblasts into a state closely related to in vitro senescence. This concept was then experimentally confirmed in vitro: many biomarkers of replicative senescence of human fibroblats were found 72 h after their exposure to various kinds of stressors used at non-cytotoxic concentrations. This phenomenon has been termed stress-induced premature senescence (SIPS). Moreover, proteomics studies have revealed that, besides their effects on the appearance of the biomarkers of senescence, sublethal stresses under a variety of stressors also lead to long-term specific changes in the expression level of proteins which are stress-specific. These changes have been coined the molecular scars of stress. The proteins corresponding to these molecular scars may be identified using the latest developments in mass spectrometry. This model of stress-induced premature senescence may be applied to the toxicological sciences when testing for the potential irreversible long-term effects of molecules on the cell fate.

van Acker FA, van Acker SA, Haenen GR, Bast A, van der Vijgh WJ. **In vitro screening of antitumour agents for cardiotoxicity by means of isolated mouse left atria**. Anticancer Res 2000;20 (6B):4483-7.
Abstract: Cardiotoxicity, a side-effect that can occur after treatment with an anticancer drug, has severe clinical implications. Therefore, a model is desired to screen new anticancer drugs or drug combinations for possible cardiotoxic side-effects. In the present study we tested the applicability of the electrically stimulated isolated mouse left atrium model using a wide range of anticancer drugs with known cardiotoxicity. It appeared that the cardiotoxicity observed in our model, i.e. the negative or positive inotropic effects of the drugs on the isolated atrium, corresponded with the observed cardiotoxicity in animals and/or humans. It is therefore concluded that our model can be used to wam for possible cardiotoxic side-effects of anticancer drugs in vivo.

van Vliet-Vroegindeweij C, Wheeler F, Stecher-Rasmussen F, Moss R, Huiskamp R. **Microdosimetry model for boron neutron capture therapy: I. Determination of microscopic quantities of heavy particles on a cellular scale.** Radiat Res 2001;155(3):490-7.
Abstract: Due to the limitations of existing microdosimetry models, a new model called MICOR has been developed to analyze the spatial distribution of microscopic energy deposition for boron neutron capture therapy (BNCT). As in most existing models, the reactions independent of the incident neutron energy such as the boron and the nitrogen capture reactions can be considered. While other models do not include reactions that are dependent on the neutron energy such as the proton recoil reaction, the present model is designed so that the energy deposition resulting from these reactions is included. The model MICOR has been extended to enable the determination of the biological effects of BNCT, which cannot be done with the existing models. The present paper describes the determination of several microscopic quantities such as the number of hits, the energy deposition in the cell nucleus, and the distribution of lineal and specific energy deposition. The companion paper (Radiat. Res. 155, 000-000 2001) deals with the conversion of these microscopic quantities into biological effects. The model is used to analyze the results of a radiobiological experiment performed at the HB11 facility in the HFR in Petten. This analysis shows the value of the model in determining the dose depositions on a cellular scale and the importance of the extension to the energy deposition of the proton recoil.

Vo-Dinh T, Alarie JP, Cullum BM, Griffin GD. **Antibody-based nanoprobe for measurement of a fluorescent analyte in a single cell.** Nat Biotechnol 2000;18(7):764-7.
Abstract: We report here the application of an antibody-based nanoprobe for in situ measurements of a single cell. The nanoprobe employs antibody-based receptors targeted to a fluorescent analyte, benzopyrene tetrol (BPT), a metabolite of the carcinogen benzo[a]pyrene (BaP) and of the BaP-DNA adduct. Detection of BPT is of great biomedical interest, since this species can serve as a biomarker for monitoring DNA damage due to BaP exposure and for possible precancer diagnosis. The measurements were performed on the rat liver epithelial clone 9 cell line, which was used as the model cell system. Before making measurements, the cells were treated with BPT. Nanoprobes were inserted into individual cells, incubated 5 min to allow antigen-antibody binding, and then removed for fluorescence detection. We determined a concentration of 9.6+/-0.2x10(-11) M for BPT in the individual cells investigated. The results demonstrate the possibility of in situ measurements inside a single cell using an antibody-based nanoprobe.

Voros J, Graf R, Kenausis GL, Bruinink A, Mayer J, Textor M, Wintermantel E, Spencer ND. **Feasibility study of an online toxicological sensor based on the optical waveguide technique.** Biosens Bioelectron 2000;15(9-10):423-9.
Abstract: Morphological properties of the cells often change as an early response to the presence of a pharmacologically acting toxic substance [Etcheverry, S.B., Crans, D.C., Keramidas, A.D., Cortizo, A. M., Arch. Biochem. Biophys. 338 (1997) 7-14]. Recently it has been shown that living animal cell adhesion and spreading can be monitored online and quantitatively via the interaction of the cells with the evanescent electromagnetic field present at the surface of an optical waveguide [Ramsden, J.J., Li, S. Y., Heinzle, E., Prinosil, J.E. Cytometry 19 (1995) 97-102]. In the present study, optical waveguide lightmode spectroscopy (OWLS) and confocal laser scanning microscopy (CLSM), which provides information about the shape of the cells at the surface, were compared under identical experimental conditions. This allowed for the correlation between the cell-shape information from CLSM and the cell-surface interaction measurements from OWLS. The proposed design of the microsystem sensor involves the establishment of a cell layer on the surface of the waveguide and the subsequent online measurement of the morphological response of the cells to various toxic substances. In the present study, the setup was evaluated using cells from an osteoblastic MC 3T3-E1 cell line, and sodium hypochlorite was used as model toxic substance. Comparing the OWLS signal to the morphological response measured by CLSM reveals that OWLS is effective in monitoring not only cell attachment and spreading but also the cellular response to toxic compounds (i.e. by means of change in cell morphology). For the model toxin, the OWLS measurements indicate that, at concentrations above 0.01%, the cells exhibit a clearly discernable morphological effect (i.e. a decrease in average cell contact area). Thus, the potential of an on-line sensor based on OWLS to applications in toxicology, pharmacy and biocompatibility was demonstrated.

Wu JT, Zeng H, Deng Y, Unger SE. **High-speed liquid chromatography/tandem mass spectrometry using a monolithic column for high-throughput bioanalysis.** Rapid Commun Mass Spectrom 2001;15 (13):1113-9.
Abstract: With the ever-increasing workload from a variety of in vitro and in vivo screening procedures,

new analytical methodologies to perform bioanalysis in an accurate and high-throughput manner are in great demand. In this work, monolithic columns were used instead of conventional particulate HPLC columns to perform chromatographic separations. Because the pressure drop on a monolithic column was considerably lower than that on a particulate column, a high flow rate (6 mL/min) was used for a 4.6 x 50 mm monolithic column with a total backpressure of about 61 bar measured using acetonitrile/water (50:50). The capability of using a regular column length at high flow rates, combined with the extremely small dependency of separation efficiency on linear flow velocity, allowed for the generation of sufficient chromatographic resolving power in a significantly reduced runtime. As demonstrated in this work, a plasma extract of a mixture of tempazepam, tamoxifen, fenfluramine, and alprozolam were baseline separated within a total analysis time of one minute. An average peak width at half maximum of approximately one second was noted using a generic broad gradient. It was also found that the separation efficiency and signal/noise (S/N) ratios for this separation remained almost constant at flow rates of 1, 3, and 6 mL/min, respectively. The ruggedness of the separation was evaluated by injecting 600 plasma extracts containing the replicates of a standard curve of the above mixture during an overnight run. The chromatographic retention time, separation quality, peak response and sensitivity were highly reproducible throughout the run. This high-speed liquid chromatography/tandem mass spectrometry (LC/MS/MS) system has been used routinely in the authors' laboratory to support drug discovery programs. Copyright 2001 John Wiley & Sons, Ltd.

Xiang J, Srivamadan M, Rajala R, Jia Z. **Study of B72.3 combining sites by molecular modeling and site-directed mutagenesis.** Protein Eng 2000;13(5):339-44.

Abstract: A B72.3 Fab/sTn(2) complex was modeled from the known structure of B72.3 Fab and the dimeric Tn-serine cluster (sTn(2)). In the complex model, the side chains of 15 heavy- and light-chain complementarity-determining region (CDR) residues and the main chains of two light-chain CDR residues contact the sTn(2) epitope. Among 15 CDR residues which contact sTn(2) in the model, two heavy-chain residues (Ser95 and Tyr97) and light-chain CDR residue (Tyr96) have been confirmed in a previous study. To test the accuracy of the computational model, further site-directed mutagenesis was performed by alanine scanning on the remaining 12 residues that are predicted in the model to have side-chain interactions with sTn(2). Of these 12 mutants, eight that are all from the heavy-chain (His32Ala, Ala33Leu, Tyr50Ala, Ser52Ala, Asn52Ala, Asp56Ala, Lys58Ala and Tyr96Ala) had significantly reduced sTn(2) affinities, and four consisting of three light-chain mutations (Asn32Ala, Trp92Ala and Thr94Ala) and one heavy-chain mutation (His35Ala) retained wild-type sTn(2) affinity. On the whole, this evidence suggests that the complex model, although not perfect, is correct in many of its features. In a more general vein, these results lend credibility to the computational modeling approach for the study of the molecular basis of antigen-antibody complexes.

Yeap BY, Davidian M. **Robust two-stage estimation in hierarchical nonlinear models.** Biometrics 2001;57(1):266-72.

Abstract: Hierarchical models encompass two sources of variation, namely within and among individuals in the population; thus, it is important to identify outliers that may arise at each sampling level. A two-stage approach to analyzing nonlinear repeated measurements naturally allows parametric modeling of the respective variance structure for the intraindividual random errors and interindividual random effects. We propose a robust two-stage procedure based on Huber's (1981, Robust Statistics)

theory of M-estimation to accommodate separately aberrant responses within an experimental unit and subjects deviating from the study population when the usual assumptions of normality are violated. A toxicology study of chronic ozone exposure in rats illustrates the impact of outliers on the population inference and hence the advantage of adopting the robust methodology. The robust weights generated by the two-stage M-estimation process also serve as diagnostics for gauging the relative influence of outliers at each level of the hierarchical model. A practical appeal of our proposal is the computational simplicity since the estimation algorithm may be implemented using standard statistical software with a nonlinear least squares routine and iterative capability.

Dempster AM. **Nonclinical safety evaluation of biotechnologically derived pharmaceuticals.** Biotechnol Annu Rev 2000;5:221-58.
Abstract: The primary objectives of nonclinical safety evaluation for pharmaceutical products are to identify potential target organ toxicity, provide a safe starting dose for clinical trials, and establish dose-response relationships. These objectives do not differ in concept for either small molecular weight compounds or biotechnologically derived pharmaceuticals; they are important for both. The complex structural and biological characteristics of biotechnologically derived pharmaceuticals, however, dictate that different approaches to their safety evaluation are needed. Although their novel mode of production initially raised concerns about their safety, improvements in analytical and manufacturing procedures have largely minimized the perceived risks. It is primarily their exaggerated pharmacodynamic properties that produce the toxicity observed in nonclinical studies. Even though most of these products will require a case-by-case, scientifically based approach, knowledge gained from both nonclinical and clinical evaluation of these novel products have highlighted some general principles with regards to their safety evaluation. These principles include the importance of evaluating species in which the biotechnologically derived pharmaceutical is biologically active, the potential impact of immunogenicity on the interpretation of multiple dose toxicity study results, and the need for both highly sensitive and specific analytical methods to measure their pharmacodynamic properties. An understanding of these principles forms the basis for the development of a scientifically sound nonclinical safety evaluation program.

Hojelse F. **Preclinical safety assessment: in vitro -- in vivo testing.** Pharmacol Toxicol 2000;86 Suppl 1:6-7.
Abstract: In vitro--and in vivo preclinical safety tests on drug candidates needed before first dose in man and before marketing authorisation are as follows: The acute and repeated dose toxicity studies, the reproductive toxicity studies, the genotoxicity studies, the carcinogenicity studies and finally the safety pharmacology studies. The Safety Assessment of the results with respect to predictability for humans is discussed, as well as new tests under validation. Suggestions for changes in the future of Non-Clinical Safety tests are mentioned.