National Center for Toxicological Research
Research Accomplishments and Plans

FY 2004 - 2005

Jefferson Laboratories of the FDA

Leaders in Health Science Research for the FDA

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Preface

The National Center for Toxicological Research (NCTR) plays a critical role in the U.S. Food and Drug Administration's (FDA) and Department of Health and Human Services' (DHHS) mission to promote and protect public health. The Center, a component of the Jefferson Laboratories of the FDA, is located in Jefferson, Arkansas, approximately 30 miles south of Little Rock.

The NCTR conducts FDA mission-critical, peer-reviewed critical path (translational) research that is targeted to develop a scientifically sound basis for regulatory decisions and reduce risks associated with FDA-regulated products. This research is aimed at evaluating the biological effects of potentially toxic chemicals or microorganisms, defining the complex mechanisms that govern their toxicity, understanding critical biological events in the expression of toxicity, and developing methods to improve assessment of human exposure, susceptibility and risk. Customized bioassessment of chemicals of vital interest to the FDA involves the coordination of expertise in the areas of biochemical and molecular markers of carcinogenicity, quantitative risk assessment, transgenics (mimicking responses in animal models by insertion or ablation of toxicologically relevant genes into a test animal or tissue culture), neurotoxicology, microbiology, chemistry, and genetic or reproductive/developmental toxicology.

Using its existing strengths in methods development, statistics, analytical chemistry and spectroscopy, NCTR is developing and standardizing new technologies, such as genomics, proteomics, metabonomics, and nanotechnology to identify and characterize early biomarkers of toxicity in our traditional toxicological models. In addition NCTR is using toxicoinformatics (data collection, interpretation, and storage of information about gene and protein expression) to manage and integrate data from these new technologies with traditional toxicological data to provide a basis for better predictive toxicology. Application of these new tools in animal surrogates will provide us with mechanistic biomarkers that will have more relevance for extrapolation of risk to humans; provide a better understanding of the present models used to assess risk in humans; and direct the development of more useful surrogate models that will increase our understanding of toxic responses in humans.

A significant contribution to our research accomplishments is the benefit gained by sharing knowledge through collaborations with scientific staff in all disciplines in other FDA Centers as well as in other government agencies, academia, and industry. One such example is the use of ArrayTrack, a software tool developed at the NCTR to store and analyze and interpret DNA microarray data. This tool is being used by the Center for Drug Evaluation and Research (CDER) in assessing phamacogenomic data voluntarily submitted by the regulated industry. This collaboration is one that identifies the FDA as a catalyst in the development of new standards that will facilitate drug development for the promotion and protection of the public health. ArrayTrack is also being considered as a useful regulatory tool for use by other agencies, as well as a research tool through a collaboration with the National Center for Toxicogenomics at the National Institute of Environmental Health Sciences.

In addition to methods and standards development, the NCTR conducts translational and applied research on compounds nominated by the FDA for evaluation by the National Institute of Environmental Health Sciences/National Toxicology Program (NIEHS/NTP). All of the studies

conducted at the NCTR are intimately associated with Secretary Leavitt's goals of transforming health care, advancing medical research, and securing the homeland. The NCTR views its public health role as a key element in the development and modification of toxicology safety standards through the application of innovative scientific research.

I am proud to present this report that summarizes NCTR research accomplishments for fiscal year 2004 and plans for fiscal year 2005.

Daniel A. Casciano, Ph.D.
Director, NCTR

NCTR Washington Operations

Science Advisory Board

Function

The NCTR Science Advisory Board (SAB) advises the Director in establishing, implementing, and evaluating the research programs that assist the Commissioner of the Food and Drug Administration (FDA) in fulfilling regulatory responsibilities. This external body of recognized scientific experts is a key component of the review and planning process and helps to ensure that the research programs at NCTR are scientifically sound and pertinent to the FDA.

FY 2004 Accomplishments

During FY04, one Site Visit was conducted by designated members of the NCTR SAB. There were no meetings of the full board scheduled.

On January 13-14, 2004, a two-day review was undertaken by the Site Visit Team (SVT) to evaluate the existing programs and future planned activities of the NCTR Division of Neurotoxicology. During the course of those two days, the Director and scientific staff of the Division of Neurotoxicology provided information on its scientific endeavors and accomplishments since its prior program review conducted in January 27-28, 1998.

The SVT determined that the division provides a valuable resource to the FDA, serving the latter's mission in developing and quantifying biomarkers and precursors associated with the neurotoxicity of drugs and foods. The SVT felt that it is incumbent upon the FDA to provide the necessary tools and support to the NCTR, to allow it to stay at the forefront of scientific matters that address drug and food safety issues.

The SVT registered its support of the research activities conducted by the Division of Neurotoxicology and indicated that it provides a unique interdisciplinary approach to regulatory issues confronted by the FDA, and that its matrix is unique as well. The SVT strongly recommended that this unique resource be preserved and supported in future years.

Science Advisory Board Membership Roster

NAME/TITLE	AFFILIATION	TERM ENDS	EXPERTISE
Dr. Daniel Acosta, Jr.* Dean, College of Pharmacy	University of Cincinnati	6/30/07	Pharmacology and Toxicology
Dr. Nancy Ann Gillett Sr. Vice-President Sierra Biomedical	Charles River Laboratories	6/30/07	Veterinary Medicine and Pathology
Dr. Jerry Kaplan Associate Dean for Research	University of Utah School of Medicine	6/30/04 [Term Expired]	Molecular Biology
Dr. John Groopman Toxicologist	Bloomberg School of Public Health Department of Environmental Health Sciences	6/30/06 [Resigned, effective 12/3/04]	Toxicology
Dr. Pat R. Levitt Director	Vanderbilt University, John F. Kennedy Center for Research and Human Development	6/30/06	Neurobiology
Dr. E. Albert Reese Vice-Chancellor and Dean	University of Arkansas College of Medicine	6/30/06	Physician
Dr. Alberto Luis Rivera-Rentas Dean	School of Environmental Affairs, Ana G. Mendez University System	6/30/06	Neurobiology Electrophysiology
Dr. Paul J. Catalano Associate Professor of Biostatistics	Harvard School of Public Health	6/30/06	Biostatistics
Dr. Kenneth R. Tindall Senior Vice-President	North Carolina Biotechnology Center	6/30/04 [Term Expired]	Biomedical Science, Genetics

*Chair

FDA Coordination Activities—Safety Testing

Function

The NCTR Office of Washington Operations serves as the Agency coordinator for activities of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and represents the Organization on Economic Cooperation and Development (OECD) matters related to the Test Guidelines Program of OECD.

The ICCVAM coordinates and advises on interagency issues on development, validation, and regulatory acceptance of new, improved, and alternative test methods and the national and international harmonization of such methods. Congress recently enacted the ICCVAM Authorization Act (Public Law 106-545, December 19, 2000) "to establish, wherever feasible, guidelines, recommendations, and regulations that promote the regulatory acceptance of new or revised scientifically valid toxicological tests that protect human and animal health and the environment while reducing, refining, or replacing animal tests and ensuring human safety and product effectiveness." As a result, ICCVAM, which was initially assembled as an *ad hoc* committee and had evolved to a standing committee, became a permanent committee.

ICCVAM's charge includes:

- Promote the scientific validation and regulatory acceptance of new, improved, and alternative test methods;
- Coordinate the review/evaluation of new/revised alternative test methods of interagency interest;
- Facilitate and provide guidance on test method development, the validation process, validation criteria, regulatory acceptance criteria, and submission requirements.
- Provide recommendations to federal agencies on the validation status of test methods and their regulatory suitability;
- Facilitate interagency regulatory acceptance and promote international harmonization and adoption of scientifically validated test methods; and,
- Facilitate awareness of and training for accepted test methods (end-users, regulators).

The Scientific Advisory Committee for Alternative Toxicological Methods (SACATM), established and chartered December 18, 2001, provides scientific and administrative advice to ICCVAM and its operational and scientific support center, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The SACATM held meetings in Bethesda, MD on March 10-11, 2004, and in Research Triangle Park, NC on October 20, 2004.

Information about ICCVAM and NICEATM can be found at http://iccvam.niehs.nih.gov. The SACATM charter, related *Federal Register* notices, and future meeting announcements can be found on the ICCVAM/NICEATM website at: http://iccvam.niehs.nih.gov/about/sacatm.htm.

FY 2004 Accomplishments

- Dr. Leonard Schechtman, NCTR, Chair of ICCVAM, and Dr. William Stokes, Director of NICEATM, continued their liaison activities with the European Commission's European Center for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC), located at the Joint Research Commission, Ispra, Italy.

- ICCVAM-NICEATM-ECVAM Collaborations ongoing:

  - Joint development and presentation of a justification for international guidance on the application of Good Laboratory Practice (GLP) to *in vitro* toxicity testing to the OECD GLP Working Group.

  - ECVAM-sponsored *in vitro* dermal irritation validation study. Representatives from the ICCVAM Dermal Corrosivity and Irritation Working Group (DCIWG) and NICEATM are serving as observers on the ECVAM management team for this study. NICEATM and DCIWG contributions to this effort involve input on the design of the validation study and identification of candidate reference chemicals for the project.

  - Joint ICCVAM-NICEATM-ECVAM Workshop on Validation Principles and Approaches for Toxicogenomics-based Methods. The workshop was held in December 2003 and was co-organized and co-chaired by Dr. Schechtman and Dr. Raffaella Corvi (ECVAM). The aims of the workshop were (a) to consider the validation and regulatory acceptance aspects of this technology as potential alternative predictive testing and screening methods that could reduce, refine, and replace animals, and (b) to establish the foundation that will facilitate future regulatory acceptance of scientifically valid Toxicogenomics-based test methods that could help in regulatory decision making. A manuscript describing the efforts of the workshop and the resulting conclusions and recommendations is being drafted.

  - Evaluation of ocular irritation assays for both *in vitro* and refinement alternatives. For this effort, ICCVAM-NICEATM is taking the lead in evaluating four current *in vitro* alternative test methods [the Bovine Corneal Opacity and Permeability (BCOP) assay, the Hen's Egg Test - Chorioallantoic Membrane (HET-CAM) assay, the Isolated Chicken Eye (ICE) Test Method, the Isolated Rabbit Eye (IRE) assay] for their ability to detect severe ocular irritants. ECVAM is taking the lead in evaluating current *in vitro* test methods for their ability to detect non-irritants and mild-to-moderate ocular irritants. ICCVAM-NICEATM and ECVAM are also developing a shared database of high quality *in vivo* rabbit ocular test method results. ICCVAM-NICEATM and ECVAM have also designated liaisons serving on the respective ocular focus groups of each organization (the ICCVAM Ocular Toxicity Working Group and the ECVAM Ocular Irritation Task Force).

- ICCVAM has put into practice the routine use of *Performance Standards* for validated and accepted ("pioneer") test methods that serve as a means by which to evaluate the reliability and accuracy of other ("me-too") test methods that are based on similar scientific principles

and measure or predict the same biological or toxic effect. The first implementation of such *Performance Standards* was the May 2004 NIH Publication No: 04-4510, *Recommended Performance Standards for In Vitro Test Methods for Skin Corrosion*.

- *The Revised Up-and-Down Procedure for Acute Toxicity (UDP).* The ICCVAM recommended this method as a valid replacement for the conventional LD50 (i.e., that dose producing lethality in 50% of the animals [median lethal dose]) test for hazard classification and concluded that its use could reduce the number of animals required for the conventional LD50 testing requirement by 60-70%. The Organization for Economic Cooperation and Development (OECD) has accepted the method as OECD test guideline 425, and it has been adopted by the United Nations Committee on Transport of Dangerous Goods.

- ICCVAM Biennial Report. The ICCVAM 2003 Biennial Report was published in February 2004. This report, which is available at http://iccvam.niehs.nih.gov, is required by Public Law 106-545 (the ICCVAM Authorization Act) and describes progress made in accordance with the Act.

- Six posters were presented by ICCVAM-NICEATM at the 2004 Annual Meeting of the Society of Toxicology (SOT); these (all available at: http://iccvam.niehs.nih.gov/) included:

  - ICCVAM Process for Nomination and Submission of New, Revised, and Alternative Test Methods (#1811)
  - The ICCVAM/NICEATM Process for Developing Test Method Performance Standards (#1812)
  - Phase I and II Results of a Validation Study to Evaluate *In vitro* Cytotoxicity Assays for Estimating Rodent and Human Acute Systemic Toxicity (#240)
  - Data Collection and Analysis Systems for an *In vitro* Cytotoxicity Validation Study (#241)
  - Estimation of False Negative Rates for the *in vivo* Rabbit Dermal Irritation Assay (#1298)
  - Estimate of False Negative Rates for the *in vivo* Rabbit Dermal Corrosion Assay (#1299)

- ICCVAM-NICEATM participated in a workshop at the 2004 Annual Meeting of the SOT: Workshop on Assurance of Animal Welfare in Research [AWR]: Coexistence of Toxicology Studies with Humane Endpoints. The program included the following:

  - Overview: A general definition of the issues, the goals of the workshop
  - Regulatory Issues: Toxicity Testing for Regulatory Purposes
  - Veterinary Medicine Issues: Development of relevant humane endpoints with the investigator and their utilization; the criteria determining when study interventions are necessary
  - IACUC Issues: Role of the IACUC in toxicology protocol review (husbandry, welfare endpoints)
  - Issues in Conduct of Toxicological Studies: Pressures to use more or fewer animals in both the academic and industrial settings

- European Perspective: How the UK and European Union address regulatory and scientific issues and humane endpoints in the care and use of laboratory animals

- ICCVAM Strategic Planning Meeting, National Institutes of Health, January 7-8, 2004. Participants assessed the current ICCVAM situation/endeavors and future prospects and examined various issues including (a) strengths of ICCVAM (b) ICCVAM challenges/areas for improvement, and (c) critical organizational issues ICCVAM will face over the next 3 years. From those discussions and mindful of the mandates of the ICCVAM Authorization Act, ICCVAM developed (1) an ICCVAM Mission, (2) an ICCVAM Vision, and (3) an ICCVAM Strategic Map to describe ICCVAM's Central Challenge, Strategic Priorities, and Strategic Objectives.

- OECD Expert Consultation Meeting (ECM) on Guidance Document (GD) 34 (*Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*), October 13-15, 2004. Invited experts from ICCVAM/NICEATM and other organizations participated in this ECM, which was directed at revising the draft GD34 and to ready it for consideration by the OECD National Coordinators at their spring 2005 meeting. This effort was undertaken to (a) broaden the document and have it express the current thinking on validation of test methods and their subsequent translation into OECD test guidelines, (b) make GD34 a more generic, less prescriptive, and less OECD-centric document, (c) transform GD34 into a document that embraced and complemented the validation principles and guidelines established by recognized validation bodies (e.g., ICCVAM, ECVAM), but not supplant them, (d) avoid potential incompatibilities between GD34 and the validation principles/guidelines of established international validation bodies, and (e) provide user-friendly guidance to all interested stakeholders.

Division of Biochemical Toxicology

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Research in the Division of Biochemical Toxicology ranges from animal bioassays to quantification of gene expression using molecular biology techniques. Shown is Dr. Jia-Long Fang.

Executive Summary

Introduction

The Division of Biochemical Toxicology conducts fundamental and applied research specifically designed to define the biological mechanisms of action underlying the toxicity of products either regulated by or of interest to the Food and Drug Administration (FDA). This research centers on assessing the toxicities and carcinogenic risk associated with specific chemicals and gene-nutrient interactions and the introduction of new techniques to assess toxicities and carcinogenic risk. The risk assessment research is firmly rooted in mechanistic studies focused on the understanding of toxicological endpoints, an approach that allows greater confidence in the subsequent carcinogenic risk assessments. Research within the Division capitalizes on scientific knowledge in the areas of biochemistry, organic chemistry, analytical chemistry, cellular and molecular biology, immunology, nutritional biochemistry, and pharmacology. It is supported by sound technical skills, the availability of state-of-the-art equipment, and internal and external collaborations and funding.

FY 2004 Accomplishments

A major emphasis within the Division is to conduct research on compounds nominated by the FDA for evaluation by the National Institute of Environmental Health Sciences, National Toxicology Program (NIEHS/NTP). This focus reflects the fact that the NCTR has superb animal facilities supported by a multi-disciplinary staff of scientists with strong mechanistic research experience; as such, the Center has the capability to conduct subchronic and chronic toxicological assessments in a rigorous manner to address the FDA's needs. While acknowledging the limitations of animal bioassays, these studies currently serve as the benchmark by which toxicological assessments are made by federal agencies, including the FDA. In addition to providing basic information on toxicological endpoints, such as cancer, these experiments form the basis for mechanistic studies to ascertain if the response detected in the experimental model is pertinent to humans.

During FY 2004, in response to an NTP nomination by the Center for Veterinary Medicine (CVM), division investigators completed an NTP final report on the carcinogenicity of malachite green, a therapeutic agent used in aquaculture. These studies indicated that leucomalachite green, a metabolite of malachite green, is a liver carcinogen in female mice. Division investigators also demonstrated that pyrrolizidine alkaloids, including riddelliine, monocrotaline,

retrorsine, heliotrine, lasiocarpine, and clivorine, are activated to genotoxins through a common dihydropyrrolizidine intermediate. These results indicate that DNA adducts derived from this intermediate can be used to assess exposure to carcinogenic pyrrolizidine alkaloids. Experiments continued on acrylamide, a carcinogen found in fried foods, in response to a request from the Center for Food Safety and Applied Nutrition (CFSAN). These investigations have emphasized dose-response relationships and the development of biomarkers for assessing exposure. During FY 2004, Division investigators demonstrated that acrylamide is metabolized to glycidamide, which is capable of forming covalent DNA adducts. These data support the concept that acrylamide is a genotoxic carcinogen. Further evidence in support of the genotoxicity of acrylamide through its metabolism to glycidamide was obtained by demonstrating that glycidamide is a more potent mutagen than acrylamide in various *in vivo* animal models.

An area of particular concern to the FDA, in particular CFSAN, is the potential toxicity of cosmetic ingredients due to their interaction with light. To address this concern, the NCTR, in collaboration with the NIEHS/NTP, constructed a phototoxicity facility that is located at NCTR. Studies at the NCTR Center for Phototoxicology have focused on the co-carcinogenic effects of simulated solar light and topically applied α- and ß-hydroxy acids, *Aloe vera*, and retinyl palmitate. More recently, experiments at the facility have been expanded to include chemicals found in tattoo inks, including those used in permanent make-up. During FY 2004, Division investigators discovered that Pigment Yellow 74, a dye found in many yellow tattoo inks, is metabolized by nitroreduction. This finding suggests that Pigment Yellow 74 could be converted *in vivo* to reactive intermediates similar to those found with aromatic amine carcinogens. Other Division investigators have been developing an experimental transgenic mouse model to study cutaneous melanoma. An important finding concerning this model has been the occurrence of spontaneous ocular melanoma.

Anti-retroviral drugs are being used to prevent the mother-to-child transmission of human immunodeficiency virus type 1, the virus responsible for acquired immunodeficiency syndrome. While effective in preventing viral transmission, the long-term consequences of perinatal exposure to these drugs are presently unknown. Division investigators have conducted a series of investigations to examine the genotoxic consequences of the anti-retroviral reverse transcriptase inhibitors zidovudine, lamivudine, stavudine, didanosine, and zalcitabine in neonatal mice. During FY 2004, these studies were expanded to assess the effects of transplacental and neonatal exposure of zidovudine and lamivudine in combination with the non-nucleoside reverse transcriptase inhibitor nevirapine and the protease inhibitor nelfinavir. In addition to investigating the carcinogenicity of these combinations, Division investigators have been measuring other endpoints (DNA incorporation, mutagenicity, and micronuclei induction) to determine the mechanisms for the adverse effects of these drugs.

Tamoxifen is an adjunct chemotherapeutic agent for the treatment of breast cancer and a chemoprotective agent for breast cancer prevention. Despite being beneficial in regard to breast cancer, tamoxifen is known to increase the risk of endometrial cancer in women. Division investigators have conducted experiments to elucidate the mechanisms for the induction of endometrial cancer, with emphasis on characterizing the DNA adducts formed from this drug. As part of this effort, mass spectral methods were developed with sufficient sensitivity to detect and quantify tamoxifen DNA adducts. During FY 2004, these methods were applied to

endometrial and breast samples obtained from women receiving the drug. The results suggest that the induction of endometrial cancer by tamoxifen does not involve the formation of tamoxifen-DNA adducts.

A strong emphasis within the Division has been in the area of nutritional folic acid deficiency and tumor progression. As part of this program, Division investigators evaluated the progressive changes in global DNA hypomethylation and promoter region hypermethylation in target and non-target tissues for carcinogenesis. Division investigators have also examined the metabolism of isoflavones found in red clover and demonstrated that metabolites of biochanin A and formononetin are potent inhibitors of cytochrome P450 1A1 and 1B1. This finding may provide a rational basis for the chemopreventive characteristics attributed to dietary isoflavones. In other work, Division investigators have developed analytical methods for the extraction and determination of six kavalactones and five ginkgo terpene trilactones found in dietary supplements and various functional foods.

FY 2005 Plans

In FY 2005, Division investigators will complete and defend the final NTP report on the carcinogenicity of a- and ß-hydroxy acids. NTP Toxicology Reports will be prepared on range finding studies for genistein, nonylphenol, ethinyl estradiol, and vinclozolin. An NTP final report will also be prepared on the genistein multigeneration chronic study. Mechanistic studies will continue on topically-applied *Aloe vera* and retinyl palmitate, and data will be compiled to assess time-to-tumor formation. Studies will continue to determine the effects of transplacental and neonatal exposure of zidovudine and lamivudine in combination with nevirapine and nelfinavir. In addition, chronic two-year bioassays will be initiated on *Aloe vera*, acrylamide, and glycidamide administered orally. Toxicokinetic data, including serum and tissue levels of acrylamide and glycidamide along with DNA adduct levels will be used to develop a PBPK model for acrylamide in food. This model will aid CFSAN in developing a valid risk assessment for acrylamide in food.

Investigators associated with the NCTR Center for Phototoxicology will continue to study the interaction of light with tattoo pigments. Specifically, photocarcinogenesis studies will continue on various tattoo inks using full spectrum simulated solar light. Investigation will be initiated to determine if tattoo inks can elicit an immune response, either through metabolism or photoactivation. Experiments will also be initiated to investigate potential toxic properties of nanoscale materials. Studies will also continue on the characterization of transgenic mouse models for photocarcinogenesis, with emphasis on the induction of cutaneous and ocular melanoma.

Results from the studies with endocrine-active compounds have indicated that soy-containing diets may protect against adrenal and renal toxicities. To elucidate the mechanism for this response, experiments will be initiated to investigate the protective effects of soy-containing diets against the renal toxicities of nonylphenol and di(2-ethylhexyl)phathalate. If soy is demonstrated to be protective, experiments will be conducted to determine the effect of soy on markers of cyst development, antioxidant systems, and cyclooxygenases.

There is increasing concern that certain components of kava may be hepatotoxic. Division investigators will attempt to identify these components by using bioassay-guided fractionations. Experiments with pyrrolizidine alkaloids will be expanded to determine if HPLC combined with mass spectrometry can be used to detect and quantify DNA adducts arising from these compounds. Finally, studies will continue to determine if global and locus-specific DNA hypomethylation could be a common mechanism in genotoxic and non-genotoxic hepatocarcinogenesis.

Ongoing Research Projects

PI: Ang, Catharina

Analytical Methodology Development for Assessing Bioactive Herbal Ingredients in Functional Foods (E0716101)

Objective(s): Develop qualitative and quantitative methods for determination of specific marker compounds, such as terpene trilactones (ginkgolides and biolobalide) and kava lactones in raw plant materials, dietary supplements, and functional food products containing ginkgo, kava kava, or their extracts. A minor objective of this proposed work is to include other minor compounds, which do not meet the selection criteria but may be of safety concerns. These compounds include ginkgolic acids, ginkotoxin, and urushiols in gingko products and unknown factors in kava.

2004 Accomplishments:

a. Identified acid-catalyzed degradation products of hyperforin, a key constituent of St. John's wort;
b. Developed analytical methods for 6 kavalactones and 5 ginkgo terpene trilactones in dietary supplements and various types of functional foods; and,
c. Published or submitted five papers.

2005 Plans:

a. Identify hepatotoxic kava constituents using bioassay-guided fractionation experiment with HepG2;
b. Determine the mutagenic activity of flavonoid glycoside from kava leaves using mouse lymphoma assay; and,
c. Develop analytical methods for functional foods containing Citrus aurantium (Bitter orange).

PI: Beland, Frederick

Perinatal Carcinogenicity of Drug Combinations used to Prevent Mother-to-Child Transmission of HIV (E0214111)

Objective(s): To determine the carcinogenicity, genotoxicity, and metabolism of antiretroviral drug combinations administered to mice transplacentally, perinatally, or neonatally.

2004 Accomplishments:

a. Initiated transplacental bioassay;
b. Conducted transplacental/neonatal range finding study;
c. Conducted *Tk*$^{+/-}$ mutagenesis assays;
d. Developed and validated an LC/MS method for analysis of AZT, 3TC, and two AZT metabolites in mouse serum/spleen and applied this method to a pharmacokinetic study of transplacental exposure; and,
e. Three papers submitted or published.
f. Continue investigations into mitochondrial proteome;
g. Complete transplacental pharmacokinetic study analysis of combination therapy and perform DNA incorporation studies; and,

h. Prepare pharmacokinetic study paper.

2005 Plans : a. Initiate transplacental neonatal bioassay; and
b. Continue *Tk*$^{+/-}$ mutagenesis experiments.

Genotoxicity and Carcinogenicity of Acrylamide and its Metabolite, Glycidamide, in Rodents – Range Finding/Subchronic/Two-Year Chronic Carcinogenicity Studies (E0215001)

Objective(s): To compare the carcinogenicity of acrylamide and its metabolite glycidamide in B6C3F1 mice and F344 rats treated chronically for two years.

2004 Accomplishments: a. Conducted range finding and subchronic study; and,
b. Conducted neonatal *Tk*$^{+/-}$ mutagenesis assay.

2005 Plans: a. Initiate chronic study; and,
b. Conduct additional neonatal *Tk*$^{+/-}$ mutagenesis assays.

DNA Adducts of Tamoxifen (E0701101)

Objective(s): The nonsteroidal antiestrogen tamoxifen, which is currently being used in clinical trials as a chemoprotective agent against breast cancer, has been associated with the induction of certain malignancies. In order to determine if tamoxifen is acting through a genotoxic mechanism, this project will characterize DNA adducts from suspected tamoxifen metabolites and develop methods for their detection and quantitation.

2004 Accomplishments: a. Conducted experiments on the tamoxifen analogue GW5638;
b. Conducted experiments on the tamoxifen analogue toremifene; and,
c. Two papers accepted.

2005 Plans: a. Continue experiments with toremifene; and,
b. Initiate experiments with *N*,*N*-didesmethytamoxifen.

PI: Boudreau, Mary

Effects of Aloe Vera Components on Cell Proliferation and DNA Adduct Formation in SKH-1 Mice Following Simulated Solar Light Exposure (E0214001)

Objective(s):

- Determine the dose response and acute kinetics of topical exposure to *Aloe vera* plant components on the structure of SKH-1 mouse skin in the absence of simulated solar light exposure;
- Determine the effects of topical exposure of *aloe vera* plant components on the amount of simulated solar light required to induce skin edema in the SKH-1 mouse;
- Determine the subchronic effects of repeated co-exposure to *Aloe vera* plant components and simulated solar light on skin cell edema, proliferation, and DNA damage in the SKH-1 mouse;
- Determine the tumor-promoting activities of *Aloe vera* plant components following simulated solar light tumor initiation; and,
- Determine the influence of *Aloe vera* components on simulated

solar light-induced tumor formations in mice.

2004 Accomplishments:
a. Completed Inlife phase of chronic dermal studies on *Aloe vera*; and,
b. Preliminary results based on animal removal curves and time-to-first reported tumor curves suggest that *Aloe vera* whole leaf extract and *Aloe vera* gel may enhance the photo-carcinogenicity of simulated solar light.

2005 Plans:
a. Review image files of mice and attempt to correlate recorded time-to-first tumor data on the Multigen Support System with that observed on the image files;
b. Prepare a review article on the photobiology and toxicological potential of *Aloe vera* plant products;
c. Examine mouse skins from Aloe- and UV-exposed animals for DNA photoproducts and p53 in mutant conformation as a marker for apoptosis; and,
d. Examine *in vitro* expression of ODC and COX following exposure to *Aloe vera* and UV light.

Bioassays in the F-344 Rat and the B6C3F1 Mouse Administered Aloe Vera Plant Constituents in the Drinking Water (E0214201)

Objective(s): The use of *aloe vera* is not limited to over-the-counter dermal therapeutics and cosmetics. *Aloe vera* is also taken internally, and *Aloe vera* for internal consumption is also widely used as a prophylaxis and treatment for a variety of unrelated systemic conditions. In view of the complexities inherent in aloe pharmacology and the inconsistencies reported in literature, the objective of these studies is to conduct bioassays in rats and mice using standardized preparations of *aloe vera* to explore the limits of safety for the *aloe vera* leaf constituents present in commercial products.

2004 Accomplishments:
a. Completed subchronic (13-week) dosed-water studies on *Aloe vera* whole leaf extract; and,
b. Dose-related hyperplastic changes were observed in rats and, to a lesser degree, in mice in the large intestine from the cecum to the rectum. The effects were most pronounced in the number of proliferating goblet cells within the mucosal layer.

2005 Plans:
a. Initiate 2-year bioassay; and,
b. Examine colon tissues for early markers of colon cancer, in particular for aberrant crypt foci.

PI: Chou, Ming

A Study of Genotoxic Mechanisms of Carcinogenic Pyrrolizidine Alkaloids and Pyrrolizidine Alkaloid N-Oxides (E0710401)

Objective(s):
- Characterize the structures of the eight DHP-derived DNA adducts;

- Study metabolism of retronecine-based pyrrolizidine alkaloids, heliotridine-based pyrrolizidine alkaloids, otonecine-based pyrrolizidine alkaloids, and pyrrolizidine alkaloid N-oxides by liver microsomes of F344 rats, B6C3F, mice, and humans of both sexes, and compare metabolism profiles;
- Study the DNA adduct formation *in vitro* (from liver microsomal metabolism of the pyrrolizidine alkaloids described above in the presence of calf thymus DNA) and *in vivo*, and determine whether or not the same set of DHP-derived DNA adducts is formed in all cases;
- Determine whether or not the levels of DHP-derived DNA adducts from different types of necine-based pyrrolizidine alkaloids formed in target tissues (liver) are significantly higher than those in non-target tissues;
- Determine whether or not pyrrolizidine alkaloid N-oxides can be metabolized by rat and mouse liver microsomes to the parent pyrrolizidine alkaloids and whether or not DHP-derived DNA adducts are formed in significant amounts both *in vivo* and *in vitro*;
- Determine whether or not some dietary supplements sold in the United States contain genotoxic pyrrolizidine alkaloids;
- Determine the effect of liver carboxyesterases on DHP-derived DNA adduct formation from rat and human liver microsomal metabolism in the presence of calf thymus DNA;
- Determine the effect of liver carboxyesterase inhibitors on DHP-derived DNA adduct formation from rat and human liver microsomal metabolism in the presence of calf thymus DNA; and,
- Determine the effect of Chinese herbs, such as licorice, and their active components, such as glycyrrhizin and glycyrrhetinic acid, on inhibition of DHP-derived DNA adduct formation *in vivo* and *in vitro*.

2004 Accomplishments:

a. Continued to examine if DHP-derived DNA adducts can serve as biomarkers of tumorigenic pyrrolizidine alkaloids;
b. Examined cell-specific activation and mutagenicity of riddelliine;
c. Examined the microsomal reduction of pyrrolizidine alkaloid N-oxides; and,
d. Submitted seven papers.

2005 Plans:

a. Compare ^{32}P-postlabeling/HPLC with LC-ES/MS/MS for the analysis of pyrrolizidine alkaloid DNA adducts;
b. Quantify genotoxic pyrrolizidine alkaloids in herbal plants and dietary supplements;
c. Determine whether tumorigenic pyrrolizidine alkaloids induce oxidative DNA damage; and,
d. Study the effects of hepatic carboxyesterase on metabolic activation of pyrrolizidine alkaloids.

PI: Delclos, Kenneth

Genistein: Evaluation of Reproductive Effects Over Multiple Generations and the Chronic Effects of Exposure during Various Life Stages (E0213201)

Objective(s):
- Determine the effects of genistein, a naturally occurring isoflavone, on reproduction and on the development of reproductive and selected other hormone-sensitive organs when administered to CD rats over multiple generations;
- Determine if subtle effects observed in the dose range-finding study are magnified through multiple generations;
- Evaluate the reversibility of any observed effects; and,
- Evaluate the chronic toxicity of genistein, particularly potential induction of cancer of the reproductive organs, following exposures that will include various life stages (in utero through early adulthood, in utero and continuous for 2 years after birth, in utero and lactational only, and postweaning only).

2004 Accomplishments:
a. Pathology work group review, data analyses, and statistical analyses were completed;
b. Genistein reduced mammary gland fibroadenomas but increased mammary gland adenomas/adenocarcinomas; and,
c. Evaluated lactational transfer of genistein in SD rats.

2005 Plans:
a. Complete and defend NTP report; and,
b. Prepare manuscripts.

para-Nonylphenol: Evaluation of Reproductive Effects over Multiple Generations (E0213501); **on Pathways of Sex Steroid Synthesis, Metabolism, and Response in Male CD (Sprague-Dawley) Rats** (E0213511)

Objective(s):
a. Determine the effects of p-nonylphenol, an intermediate in the production of surfactants and other industrial products, on reproduction and on the development of reproductive and selected other hormone-sensitive organs when administered to CD rats over multiple generations;
b. Determine if subtle effects observed in the dose range-finding study are magnified through multiple generations; and,

2004 Accomplishments:
a. Evaluate the reversibility of any observed effects.
b. Pathology analyses completed and statistical analyses began.

2005 Plans: Complete statistical analysis and prepare technical report.

Ethinyl Estradiol: Evaluation of Reproductive Effects over Multiple Generations, and the Chronic Effects of Exposure during Various Life Stages (E0213801)

Objective(s):
- Evaluate the effects of ethinyl estradiol, a potent synthetic estrogen widely used in prescription drugs, on reproduction and on the development of reproductive and selected other hormone-sensitive organs when administered to CD rats in the diet over multiple generations;
- Determine if subtle effects observed in the dose range-finding study are magnified through multiple generations;
- Evaluate the reversibility of any observed effects; and,
- Evaluate the chronic toxicity of ethinyl estradiol, particularly the potential induction of cancer of the reproductive organs, following exposures that will include various life stages.

2004 Accomplishments: Draft pathology report completed.

2005 Plans: Conduct pathology working group review, initiate statistical analysis, initiate report preparation.

The Effects of Dietary Genistein on the Growth of Chemically Induced Mammary Tumors in Ovariectomized and Intact Rats (E0702701)

Objective(s): This study will determine whether or not, in the absence of endogenous ovarian estrogens, dietary genistein can promote or suppress the growth of neoplastic mammary tissue at various stages of the carcinogenic process.

2004 Accomplishments:
a. All experimental work completed; and,
b. Two manuscripts published/submitted.

2005 Plans: Prepare final report and manuscripts.

Effects of Endocrine Active Agents on Bone (E0710601)

Objective(s):
- We hypothesize that the administration of the endocrine active agents genistein and ethinyl estradiol (EE2) will alter bone growth and remodeling, and that the direction and extent of the effect will depend on the window of exposure to the compounds. Utilize the experience of Bionetics staff and tissues available from the on-going endocrine disruptor studies to address an important health concern.

2004 Accomplishments:
a. All data collection was completed; and,
b. One manuscript on the genistein study submitted. Minimal treatment effects were observed.

2005 Plans: Complete final report and manuscript.

Dietary Modulation of the Renal Toxicity of p-nonylphenol (NP) and di(2-ethylhexyl)phthalate (DEHP) (E0714201)

Objective(s):

- Demonstrate that the cystic kidney disease previously shown to be induced by nonylphenol in developing NCTR CD rats fed a soy-free diet is decreased in incidence and/or severity in rats fed soy-containing diets;
- Evaluate the renal toxicity of dietary DEHP in developing rats maintained on a soy-free diet;
- Evaluate potential early markers of renal cystogenesis in nonylphenol- and DEHP-treated rats and their modulation by soy-containing diets;
- Evaluate the roles of modulation of antioxidant defenses and cyclooxygenase activities in the protective effect of soy against nonylphenol- and, if demonstrated, DEHP-induced renal toxicity; and,
- As secondary objectives in the above studies, the effect of diet on hepatic, testicular, and lung toxicity of DEHP will be assessed.

2004 Accomplishments:

a. Animals were dosed with nonylphenol under conditions that have been shown to induce renal toxicity, and tissues were taken at various stages of development to evaluate early indicators of toxicity and the modulation of these indicators by the soy diets; and,

b. An evaluation of kidney, lung, and testicular toxicity of DEHP was started.

2005 Plans: Continue experiments as outlined in protocol.

PI: Doerge, Daniel

Genotoxicity, Mutagenicity and Exposure Biomarkers of Acrylamide and Its Metabolite, Glycidamide, in Rodents (E0214601)

Objective(s):

- Synthesize chemically and characterize spectroscopically the major glycidamide-DNA adducts;
- Develop and validate LC-ES/MS assays to quantify the major glycidamide-DNA adducts;
- Determine glycidamide-DNA adduct levels in rodent tissues following short-term exposures of rodents to acrylamide and to glycidamide;
- Determine toxicokinetics and compare bioavailability of acrylamide and glycidamide following exposure by intravenous, oral gavage, and dietary administration;
- Correlate the levels and kinetics of glycidamide-DNA adduct in target tissues and circulating lymphocytes with acrylamide- and glycidamide-hemoglobin adducts in rodent exposure studies for future use in monitoring human exposure through occupation, smoking, and the diet; and,

- Determine *in vivo* mutagenesis of acrylamide and glycidamide using transgenic mice (Big Blue).

2004 Accomplishments:

a. A paper was published describing the identification of new DNA adducts from glycidamide and the development/validation of a quantitative method for two of them;
b. A paper was published describing a preliminary toxicokinetic analysis of acrylamide and glycidamide in mouse serum coupled with analysis of liver DNA adducts;
c. A method for quantification of acrylamide in rodent diets was developed/validated, and the serum toxicokinetics and accumulation of liver DNA adducts from low levels of acrylamide in the autoclaved diet basal was described. This work also identified a replacement diet that is irradiated and contains much lower acrylamide content; and
d. A toxicokinetic analysis of acrylamide and glycidamide in B6C3F1 mice was accepted for publication in TAP. This publication provided important new insight into the metabolism of acrylamide and the formation of DNA adducts. This work was the first to determine bioavailability of acrylamide from water and food, critical information for the FDA Action Plan for acrylamide. It will be an important data set for a PBPK model for acrylamide, a critical tool needed for the JECFA review/risk assessment of acrylamide to be held next year;
e. A publication describing the tissue distribution of DNA adducts from acrylamide and glycidamide treatment from single and repeat dosing was submitted for publication; and
f. A publication describing the toxicokinetic analysis of acrylamide and glycidamide in F344 rats is in progress and provides additional new information for understanding the bioavailability, metabolism, disposition, and formation of DNA adducts needed for the PBPK model.

2005 Plans:

a. A publication describing the mutagenicity of acrylamide and glycidamide *in vivo* (*hprt* and *cII* genes) is being prepared;
b. Consolidate all data sets, including serum and tissue toxicokinetics with DNA and adducts of acrylamide and glycidamide, into a human PBPK model for acrylamide in food. This work is unique in its scope and is leading FDA/CFSAN and world efforts to determine a valid risk assessment for acrylamide in food; and,
c. Participate in the JECFA review of acrylamide and write the JECFA document with CFSAN. A CRADA funding of this project by JIFSAN is currently being negotiated.

Development of Methods for Analysis and Confirmation of B-Agonists (E0694501)

Objective(s):

- Develop determinative and confirmatory procedures using Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass

Spectrometry (LC-APCI/MS) for multiresidue screening ß-agonists in livestock tissues;

- Develop synthetic procedures to produce authentic ß-agonist standards for use in regulatory screening. These methods will provide the flexibility to adapt to the potential for "designer drug" modifications by clandestine laboratories; and,
- Explore the use of packed-column supercritical fluid chromatography (SFC) coupled to APCI/MS as a more efficient technique for chromatographic separation in the screening of large numbers of ß-agonists in livestock tissues.

2004 Accomplishments: A manuscript describing a multi-residue confirmatory procedure is in review. The LC/MS/MS method has been transferred to the USDA Food Safety and Inspection Service Western Regional Laboratory.

Effect of Soy-Containing Diets on Ammonium Perchlorate-Induced Thyroid Toxicity in Sprague-Dawley Rats (E0716301)

Objective(s): Determine the effect of dietary soy and genistein, the principal soy isoflavone, on the dose-response characteristics for perchlorate-induced thyroid toxicity in male Sprague-Dawley rats.

2004 Accomplishments: All exposures and sacrifices have been completed. Pathology results are pending. Thyroid and pituitary tissues have been collected for genomic and/or proteomic analysis. This study is unique in addressing diet/environmental toxicant interactions for an emerging FDA need for information regarding perchlorate exposure assessment and thyroid toxicity risk assessment.

Human Studies of Isoflavone Safety and Efficacy (S00607)

Objective(s): Bioanalytical analysis of soy isoflavones (and metabolites) in support of clinical trials at the University of Miami and Wayne State University.

2004 Accomplishments:

a. Novel LC/MS methods have been used to measure isoflavones from blood spots, saliva, and hair for use in clinical trials of soy infant formula in collaboration with NIEHS, University of Pennsylvania, Brigham University and Women's Hospital. Study with infants now started and in the first phase;
b. A NovaSoy-bone study to be conducted at University of Miami was funded by NIH as of July 2004. A manuscript describing single and multiple dosing pharmacokinetics based on pilot study of Novasoy at the University of Miami has been submitted; and,
c. Completed analysis of six hundred serum samples from a breast cancer case-control study conducted at the University of Southern California to investigate possible interactions of tamoxifen with soy isoflavone consumption and changes in breast cancer incidence/survival.

Phytoestrogens and Aging: Dose, Timing, and Tissue (E0721001)

Objective(s): Evaluate the potential benefits or detrimental effects of dietary phytoestrogens on breast cancer progression, adipose tissue, and the brain, using well-established laboratory animal models.

2004 Accomplishments:

a. A P01 project with investigators at the University of Illinois, entitled, "Dietary estrogens and aging: Age of exposure, dosage and target tissues as determinants of biological activity@ was funded for five years;

b. A paper describing work on transcriptional activation of equol enantiomers through estrogen receptors a and ß was published. This paper is unique in describing real vs. putative biological activities of equol; and,

c. Another paper was published describing the effect of soy processing on tumor growth stimulation by genistein of human mammary carcinoma cells in the athymic mouse model. A related paper describing the effect on metabolism and disposition of soy isoflavones from soy processing is ready for submission. These papers are unique in describing the effect of dietary matrix on biological effects of soy isoflavones, in both whole soy foods and in purified dietary supplements.

2005 Plans:

a. Serum and tissue samples will be analyzed from rodents to complete work on the genistein PBPK model (developed in collaboration with John Young) and to extend the model to include equol; and,

b. Major goals include defining the role that soy matrix plays in modifying biological effects (mammary tumor growth stimulation, adipose tissue proliferation, cognitive function, genomic and proteomic analysis) from several commercial products, including whole soy, soy protein isolates, and purified dietary supplements. This work is unique in its scope and will focus on critical issues of isoflavone estrogenicity, metabolism/disposition, target tissues, and life-stage susceptibility. Such investigations are critical to FDA safety assessments of soy foods and dietary supplements.

PI: Fu, Peter

Effect of Topically Applied Skin Creams Containing Retinyl Palmitate on the Photocarcinogenicity of Simulated Solar Light in SKH-1 Mice (E0214301)

Objective(s): Study the effects of topically applied skin cream containing retinyl palmitate on the photocarcinogenicity of simulated solar light in SKH-1 mice.

2004 Accomplishments:

a. The one year chronic tumorigenicity bioassay with simulate solar light was completed and the animals are in pathology;

b. Mechanistic studies of the photoirradiation of retinyl palmitate (RP), anhydroretinol (AR), and 5,6-epoxy-RP by UVA in the

presence of a lipid, methyl linoleate indicated the formation of lipid peroxide in a light dose-response relationship; and,

c. A manuscript entitled "Photodecomposition of retinyl palmitate in ethanol and methanol by UVA light" was submitted.

2005 Plans:

a. Complete clinical evaluation of the animals of one year chronic tumorigenicity bioassay for determination of tumor formation based on the methodology established by the Argus Laboratory;
b. Based on the clinical evaluation described above, determination of the (i) tumor type; (ii) tumor location; (iii) tumor size; and (iv) date of first appearance of tumor formation of each tumor type (particularly the flat and stalk tumors);
c. Relate time-to-tumor and type of tumor to particular exposure;
d. Study of skin absorption and metabolism of RP *in vitro* and *in vivo* in the presence and absence of light irradiation; and,
e. If RP does enhance photocarcinogenicity, study the mechanisms of enhancement of photocarcinogenicity.

PI: Howard, Paul

Effect of Topically Applied Skin Creams Containing Glycolic and Salicylic Acid on the Photocarcinogenicity of Simulated Solar Light in SKH-1 Mice (E0213701)

Objective(s): Determine if the application of creams containing a- and ß-hydroxy acids to the skin of male and female SKH-1 hairless mice alters the tumor incidence induced by simulated solar light in the mouse skin

2004 Accomplishments: The final report is being written and the data are being audited by NCTR QA.

2005 Plans: The NCTR final report will be completed, and the NTP Technical Report will be completed and defended.

Methodology for Safety Testing of pigments used for Tattooing, Including Permanent Make-up (E0710501)

Objective(s):

- Determine the chemicals in tattoo pigments and their metabolism *in vitro*;
- Develop methodology for tattooing SKH-1 hairless mice in a quantitative and reproducible manner;
- Determine the extent of inflammation induced by the implanted pigment and determine the time of recovery following tattooing;
- Determine the acute toxicity of several tattoo inks and permanent make-up inks in SKH-1 hairless mice in the presence and absence of simulated solar light; and,
- Determine if tattoo pigments are photococarcinogenic in the SKH-1 hairless mouse using simulated solar light.

2004 Accomplishments:

a. Determined that pigment yellow 74 (PY74) is the primary pigment in many yellow tattoo inks, and that it is metabolized by ring hydroxylation and nitroreduction. One manuscript has been

submitted to CFSAN for clearance for publication;
b. The nitroso-derivative of PY74 (NO-PY74) was synthesized and its reactivity investigated. It formed a DNA adduct when reduced with ascorbate, and the DNA adduct was detected in DNA isolated from mouse lymphocytes treated with NO-PY74;
c. Investigated the photodecomposition of PY74 and PO13. One manuscript on the PY74 studies was accepted for publication (*Photochemistry & Photobiology*), and one on PO13 photodecomposition is being written;
d. Determined the immunological impact of the tattooing process. A manuscript on the method has been reviewed at NCTR and CFSAN and will be submitted for publication;
e. Conducted subchronic study on 16 tattoo pigments; and,
f. Initiated photocarcinogenesis study with 6 tattoo pigments.

2005 Plans:
a. An addendum is being submitted to support additional studies on the photoactivation of tattoo inks;
b. Determine the immunogenic potential of specific tattoo inks;
c. Continue photocarcinogenesis study; and,
d. Continue photodecomposition studies.

Nanoscale Material Toxicology Methods Development (P00639)

Objective(s):
- Assess methodology for the detection of nanoscale particles; and,
- Provide preliminary data to support the direction of a research protocol.

2004 Accomplishments: Conduct preliminary experiments.

PI: Pogribna, Marta

Folic Acid Metabolism in Children with Down Syndrome (E0708501)

Objective(s): Determine whether supplementation with the nutrients folic acid and betaine will increase plasma levels of methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine SAH, which have shown to be low in children with Down Syndrome

2004 Accomplishments:
a. Twenty-six participants have been enrolled to date. Nineteen have completed the study, two are still following supplementation protocol, consent process was initiated with one participant, and four participants were lost to follow up; and,
b. Concentration of total thiols, adenosine, S-adenosylhomocysteine (SAH), S-adenosylmethionine in plasma of 18 participants before and after supplementation was determined. There was a statistically significant increase in the plasma methionine level and plasma glutathione. Levels of SAH, SAM, and total homocysteine also showed improvement.

2005 Plans:
a. At least 30 children with Down syndrome are necessary to complete the supplementation protocol to have the minimal number of participants for data analysis. Recruitment of qualifying participants is slow but steady; and,
b. Assess DNA damage before and after nutritional supplementation.

PI: Pogribny, Igor

Mechanisms and Consequences of DNA Damage and Methylation Dysregulation during Rat Hepatocarcinogenesis (E0712801)

Objective(s):
- Confirm that the presence of uracil and abasic sites in preneoplastic DNA from folate/methyl deficient rats creates nonproductive high affinity binding sites for the DNA methyltransferase that compromise normal DNA methylation at the replication fork resulting in genome-wide hypomethylation;
- Determine a) whether the double-stranded loss of cytosine methylation is maintained in folate/methyl deficient rats after nutritional repletion of methyl donors or whether the original methylation pattern and chromatin structure can be reestablished; and b) whether the increase in dnmt1 expression is stimulated by global loss of methyl groups and whether dmnt1 expression is decreased by methyl repletion;
- Determine the temporal relationship between the appearance of DNA lesions and site-specific methylation, within the CpG island of the p16 promoter region, in p16 gene expression with alterations in local chromatin structure and DNA methyltransferase mRNA levels and activity; and,
- Use microarray slides printed with the rat cDNA library in collaboration with investigators in the Functional Genomic Center as a tool to screen for methylation-related down-regulation of candidate genes in hepatic preneoplastic foci, preneoplastic nodules, and tumor tissue from folate/methyl deficient rats.

2004 Accomplishments:
a. Isolated DNA and RNA from liver tissue of all rats;
b. Measured the status of global and gene-specific DNA methylation (p16, ER) in liver tissue at each time point as described in the protocol;
c. Measured the SAM, SAH contents in liver tissue at each time point;
d. Started preliminary studies of alterations in chromatin structure during hepatocarcinogenesis using chromatin immunoprecipitation assay; and,
e. One manuscript was published and two are in preparation.

2005 Plans:
a. In collaboration with the Functional Genomic Center determine the genes that are stably up-regulated and down-regulated during hepatocarcinogenesis induced by folate/methyl deficiency;
b. Evaluate the methylation profile in up-regulated and

down-regulated genes, and correlate it with changes in gene expression pattern during hepatocarcinogenesis;

c. Using methylation sensitive arbitrary-primed PCR, determine the regions of genomic DNA in which alterations in DNA methylation occurs early during carcinogenesis, and measure the expression of corresponding genes; and,

d. Using ChIP assay, determine the alterations in chromatin structure during early stages of hepatocarcinogenesis.

Global and Locus-specific DNA Hypomethylation: A Common Mechanism Involved in Genotoxic and Non-genotoxic Rat Hepatocarcinogenesis (E0718101)

Objective(s):

- Determine if the temporal alteration in genomic methylation profile in preneoplastic liver tissue, observed in the folate/methyl deficient model of rat endogenous hepatocarcinogenesis, also occurs in other carcinogenesis model;
- Identify genes that are steadily up-regulated or down-regulated in target tissue during the promotion stage of carcinogenesis; and,
- Evaluate whether or not the global and locus-specific DNA hypomethylation, along with aberrant expression of related genes and changes in chromatin conformation, is specific only to target tissues and may be used for early detection of chemicals with carcinogenic potential.

2005 Plans:

a. Start experiments to develop short-term test for carcinogenicity and toxicity based on assessment of changes in gene expression and DNA methylation patterns. Perform 3 experiments with tamoxifen, PhIP, and WY-14643;

b. Isolate DNA from target and non-target tissues of animals exposed to different type of carcinogens, and evaluate the genomic and regional methylation profile during early carcinogenic process by using cytosine extension assay and methylation-sensitive arbitrary PCR; and,

c. Isolate RNA and determine the expression pattern of the genes in tissue that undergoes carcinogenesis by employing microchip array technology.

PI: Tolleson, William

Photoinduction of Cutaneous Malignant Melanoma in TP-ras/ink4A (+/-) Transgenic Mice (E0708901)

Objective(s):

- Characterize photochemical DNA damage in the skin of TP-ras/ink-4a mice exposed to UVA+UVB radiation;
- Determine whether cutaneous malignant melanoma can be induced in neonatal TP-ras (+) ink4a (+/-) transgenic mice using UVA+UVB radiation;
- Identify photochemically induced mutations within the ink4a/p16/CDKN2A and p53 loci in tumor tissues; and,

- Determine whether UVA+UVB exposure at an early age creates a greater risk for developing cutaneous melanoma in TP-ras (+)ink4a(+/-) mice compared with chronic UVA+UVB exposure of older animals.

2004 Accomplishments:

a. Methods were developed for isolation of total RNA from follicular cells collected using laser microdissection of frozen skin section;
b. Continued photocarcinogenesis study; and,
c. Spontaneous ocular melanoma arising in TP-ras (+) ink4a/Arf (-/-) animals (in progress) - TP-ras (+) ink4a/Arf null males were found to develop ocular melanoma in addition to cutaneous melanoma. A paper describing these results has been submitted.

2005 Plans:

a. Complete photocarcinogenesis study;
b. Expression of genes associated with oxidative stress (OGG-1, COX-2), photoimmunosuppression (IL-10), UV responsive transcription factors (ETR-101, EGR-1/ETR-103, c-fos, c-jun), and the melanocytic phenotype (mitf, TRP-1, TRP-2, silver) will be assessed in follicular melanocytes isolated from neonatal mouse skin frozen sections using laser microdissection and real time RT-PCR analysis; and,
c. Representative UV signature mutations of the p53 and ink4a/Arf loci will be measured.

Completed Research Projects

PI: Culp, Sandra

Two-Year Bioassay in Mice Administered Malachite Green or Leucomalachite Green in the Diet (E0212701)

Objective(s): Determine the risk associated with exposure to malachite green or leucomalachite green.

Two-year Bioassay in Rats Administered Malachite Green or Leucomalachite Green in the Diet (E0212801)

Objective(s): Determine the risk associated with exposure to malachite green or leucomalachite green.

PI: Delclos, Kenneth

Range-Finding Study for the Evaluation of the Toxicity of Nonylphenol Administered in the Feed to CD (Sprague-Dawley) Rats (E0212501)

Objective(s): Determine the doses of nonylphenol for use in a multigeneration bioassay for assessing the effects of this compound on the development of the reproductive tract, reproduction, and neurological and immunological function.

Range-Finding Study for the Evaluation of the Toxicity of Vinclozolin Administered in the Feed to CD (Sprague-Dawley) Rats (E0212601)

Objective(s): Determine the doses of vinclozolin for use in a multigeneration bioassay for assessing the effects of this compound on the development of the reproductive tract, reproduction, and neurological and immunological function.

Range-Finding Study for the Evaluation of the Effects of Ethinyl Estradiol Administered in the Feed to CD (Sprague-Dawley) Rats During Development (E0212901)

Objective(s): Determine the doses of ethinyl estradiol (EE2) for use in a multigeneration bioassay for assessing the effects of this compound on the development of the reproductive tract, reproduction, and neurological and immunological function.

A Comparison of Weight Gain and Fertility in CD Rats Fed a Standard Diet (NIH-31) or a Soy- and Alfalfa-free, Casein-containing Diet (NIH-31C) (E0213001)

Objective(s): Evaluate effects of NIH-31C on fertility by comparing pregnancy rates, litter size, and weight in CD rats treated according to the treatment regimen to be used in the F0 generation of the multigeneration.

Optimization of Procedures for 1) laser capture microdissection of rat kidney for gene and protein expression studies and 2) measurement of renal cyclooxygenases, antioxidant enzymes, and isoprostanes (P00619)

Objective(s):

- Determine optimal parameters for laser capture microdissection to collect distinct renal cell populations for analysis of mRNA and proteins;
- Optimize conditions for the measurement of cox-1, cox-2, glutathione peroxidase, superoxide dismutase and quinone reductase; and,
- Evaluate the feasibility of utilizing commercial ELISA kits for the determinations of prostaglandin and isoprostane levels in renal cortex and medulla.

Toxic Hazards from Antithyroid Chemicals (E0692001)

Objective(s):

- Determine inhibition mechanisms for environmental goitrogens using purified thyroid peroxidase and lactoperoxidase;
- Determine the mechanism for covalent binding suicide substrates to purified peroxidases using electrospray-mass spectrometry to analyze intact adducted proteins and/or proteolytic fragments;
- Determine mechanism of goitrogen uptake into isolated thyroid cells in primary culture and subsequent inhibition of iodination/coupling reactions involved in thyroid hormone synthesis; and,
- Determine the structure-activity relationship for uptake of goitrogens into the thyroid and inhibition of thyroid hormone synthesis rats.

Measurement of Oxidative DNA Damage in Normal and Hepatitis C-Infected Human Liver (E0706401)

Objective(s):

- Develop simple synthetic methods to produce stable labeled analogs of 8-oxo-dG, etheno-dA, etheno-dC, and M1-dG;
- Develop an automated on-line sample preparation method to maximize detection sensitivity for 8-oxo-dG, etheno-dA, etheno-dC, and M1-dG, in a single sample analysis, using liquid chromatography and tandem mass spectrometry;
- Apply methodology to the analysis of hepatic DNA from humans and animals; and,
- Determine feasibility for application to clinical trials of therapeutic agents and toxicity/carcinogenicity testing in experimental animals.

PI: Howard, Paul

Assessment of the Phototoxicity of Lemon and Lime Oil Furocoumarins, including Oxypeucedanin (E0215401)

Objective(s):

- Determine the types of DNA damage caused by photo irradiation of several furocoumarins *in vitro* in the presence of DNA;
- Determine the photomutagenicity of furocoumarins in the *Salmonella* typhimurium forward mutation assay in the presence and absence of light;
- Determine the effect of route of administration on the pharmacodynamics of oxypeucedanin *in vivo*; and,
- Determine the DAN damage and gene expression changes that occur in mice administered furocoumarins and irradiated with light for 13 weeks.

Development of a Research Plan and Research Protocol for Furocoumarins in Lemon and Lime Oil (P00624)

Objective(s):

- Conduct a literature search and summarize the occurrence, use, pharmacokinetics, toxicity, mutagenicity, and carcinogenicity of lemon and lime oil furocoumarins; and
- Develop a draft Research Protocol for submission to the NCTR Protocol Review and IAG Toxicology Study Selection and Review Committee.

PI: Tolleson, William

The Role of Human Metabolism in Endocrine Disruption (E0702301)

Objective(s): Humans may be exposed to compounds in the diet or in the environment that disrupt endogenous endocrine responses in various tissues. We propose to utilize cell biological approaches to determine the role of human cytochromes P-450, UDP-glucuronosyltransferases, and sulfotransferases in the antiestrogens. The relative abilities of the various human enzyme systems, expressed by individual cell lines to alter the extent of green fluorescent protein synthesis, will indicate those human enzyme activities that activate or deactivate endocrine disrupting agents.

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Division of Biometry and Risk Assessment

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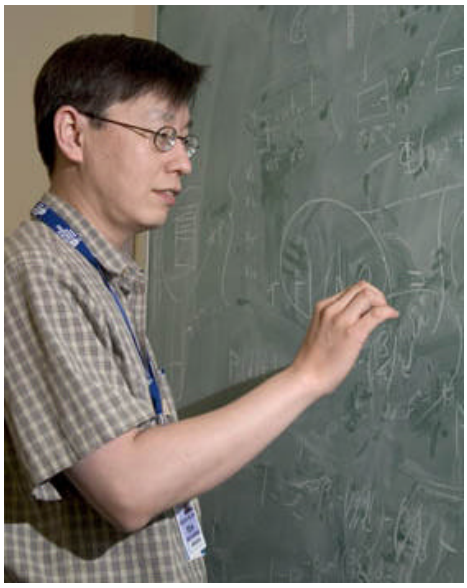

Development of Novel Methods for Detection and Prediction of Cancer: CERP (Classification by Ensemble of Random Partitions)

Executive Summary

Introduction

Risk assessment is a process for determining the extent of health hazard as a function of the conditions of exposure to toxic substances. The Division of Biometry and Risk Assessment conducts research to develop new and improved methods for assessing human health risks associated with exposure to chemicals and biological organisms. The Division currently is comprised of four mathematical statisticians, two research biologists, two information technology specialists, and one program support specialist. Recruitment is underway for two postdoctoral fellows. Division scientists conduct both individual research within the Division and collaborative research with scientists from other NCTR Divisions, other FDA centers, other government agencies, and academic institutions.

In June 2002 the Center for Toxicoinformatics was established within the Division. The mission of the Center is to conduct research in bioinformatics and chemoinformatics and to develop and coordinate informatics capabilities in support of NCTR's toxicological research in genomics, proteomics, and metabolomics. In August 2004, this Center was combined with similar Centers of Excellence to form NCTR's new Division of Systems Toxicology. Consequently, two computational chemists were reassigned from Biometry to the new Division. However, because these two scientists were in the Division during most of FY 2004, their accomplishments are included herein.

The main functions of the Division of Biometry and Risk Assessment include:

- Developing statistical testing methods and predictive systems for identifying potential health hazards associated with toxic substances;
- Developing biometrical methods for estimating risks associated with toxic substances to enable setting exposure levels that correctly reflect underlying uncertainties;
- Developing mathematical models for better representation of internal exposure levels and of biological mechanisms in order to reduce uncertainty in estimates of risk;
- Providing analytical expertise to NCTR scientists on the design, conduct, and analysis of research studies to evaluate the toxicity of regulated products;
- Assisting other FDA Centers in conducting risk assessments for the regulation of specific products and in investigating generic risk assessment issues;

- Participating in interagency risk assessment activities to maintain knowledge of the state of the art, and to promote the improvement and unification of risk assessment practices across agencies; and,
- Coordinating research and support in toxicoinformatics at NCTR relative to data arising from new technologies in genomics, proteomics and metabolomics. (In August 2004, this function was moved to the Division of Systems Toxicology.)

FY 2004 Accomplishments

During FY 2004, scientists in the Division engaged in research addressing a variety of problems in biometry, risk assessment, and toxicoinformatics relevant to science-based regulation. Research projects included:

- Developing a new criteria for assessing model uncertainty in microbial risk assessment and new techniques for deriving risks and doses that correctly reflect that uncertainty;
- Developing sensitive-subpopulation models for the spread of infection and disease caused by foodborne pathogens, with special emphasis on the kinetics of infection by *Cryptosporidium parvum;*
- Using statistical class-prediction tools to classify chemicals with respect to liver carcinogenicity in rodents, based on chemical descriptors and physical properties;
- Accounting for key sources of variation and ensuring adequate sample size in the design and analysis of functional genomics experiments aimed at detecting differential gene expression using microarrays;
- Developing novel computer-based classification systems to be used in the regulatory review process for chemicals that lack specific toxicity data;
- Developing robust, survival-adjusted statistical estimators and tests for improved assessment of tumorigenicity in long-term rodent bioassays;
- Developing novel normalization methods and statistical multiple-testing strategies for analyzing cDNA array data on gene expression;
- Developing new techniques for quantitative risk assessment, with emphasis on FDA-regulated products; and,
- Developing an integrated system of databases, libraries, and analytical tools for toxicoinformatics.

FY 2005 Plans

For FY 2005, scientists in the division will conduct research on the spread and assessment of microbiological pathogens; research to develop improved methods of data mining and class prediction, including risk/benefit assessment; research related to the design, analysis, and interpretation of genomics studies; research on physiologically based pharmacokinetic (PBPK) models; and research on improved analytical methods for both long-term and short-term tumorigenicity studies.

Planned research activities, identified by project number will include:

- Developing novel statistical procedures for assessing dose-response-model uncertainty in microbial risk/safety assessment and for appropriately reflecting such uncertainty in model-derived exposure levels (E0704501);
- Conducting animal experiments on host susceptibility and strain virulence to predict the spread of microbial pathogens through a population, for use in the development of counter-terrorism measures for agents like *Cryptosporidium parvum* (E0708201);
- Developing computer-based systems to predict the risk of organ-specific toxicity using multiple inputs based on chemical structures and properties (E0708301);
- Developing statistical adjustments to account for the simultaneous testing of multiple genes for differential expression among comparison groups (E0711201);
- Developing a Windows-based program to implement a multi-component (parent chemical and up to three metabolites) PBPK model that accommodates postnatal growth in laboratory animals and humans (E0713001);
- Developing network architecture to identify precursor genes, co-expressed genes and target genes for constructing genetic profiles of risk (E0715901);
- Developing robust, survival-adjusted statistical tests (E0717101) and novel estimators of tumor progression time (E0717201) for data from long-term tumorigenicity bioassays for hazard identification;
- Developing robust statistical designs for functional genomics studies aimed at hazard identification (E0718401);
- Developing novel risk/benefit classification models for safety and efficacy in regulatory decision making (E0722001); and,
- Developing and comparing ensembles of classifiers to enhance class prediction for a variety of applications, including risk categorization based on biomarkers of disease and carcinogenicity classification based on chemical descriptors (E0722101).

Research will also continue on all other active projects. In addition, Division scientists will continue to engage in extensive collaborative research outside the Division, providing statistical and pharmacokinetic expertise to projects initiated by scientists in other NCTR divisions and other FDA Centers. Provision of oversight to on-site contract activities associated with statistical analyses and experimental support will continue.

Public Health Significance

Human health risk estimates influence the regulation of exposure to toxic substances, thereby affecting both the health of the U.S. population and the health of the U.S. economy. The nature of the research carried out in the Division of Biometry and Risk Assessment is diverse, with projects characterized by development of mathematical and statistical theory and methods for risk assessment; biological experimentation and pharmacokinetic modeling with specific agents; and development of computational systems for predicting toxicity through knowledge discovery in databases. The ultimate goal of the research carried out in the Division is to improve the regulation of natural or synthetic toxic substances occurring in foods, drugs, cosmetics, biologics, medical devices, and animal drugs. Continued significance to the FDA is fostered through interactions with individuals and committees at other FDA Centers involved in

evaluations of risk for the regulation of specific products. Participation by Division scientists on interagency risk-assessment committees ensures relevance of the Division's research not only to FDA's regulatory needs, but also to broad public health issues.

Ongoing Research Projects

PI: James J. Chen, Ph.D.

Design and Analysis of Gene Array Data *(*E0711201)

Objective(s): Develop statistical and computational procedures for the design, analysis, and interpretation of gene expression data from microarray experiments.

Accomplishments:

a. Chen, J.J., Delongchamp, R.D., Tsai, C.A., Hsueh, H.M., Sistare, F., Thompson, K.L., Desai, V.G. and Fuscoe, J.C., Analysis of variance components in gene expression data, *Bioinformatics*, 20:1436-1446, 2004.

b. Tsai, C.A., Hsueh, H.M. and Chen, J.J., A generalized additive model for microarray gene expression data analysis, *Journal of Biopharmaceutical Statistics*, 14:553-573, 2004.

c. Wang, S.J. and Chen, J.J., Sample size for identifying differentially expressed genes in microarray experiments, *Journal of Computational Biology*, in press, 2004.

d. Chen, J.J., Microarrays in pharmacogenomics (Review article), *Journal of Biopharmaceutical Statistics*, 14:535-537, 2004.

e. Tsai, C.A. and Chen, J.J., Significance analysis of ROC indices for comparing diagnostic markers: applications to gene microarray data, *Journal of Biopharmaceutical Statistics*, accepted, 2005.

f. Tsai, C.A., Chen, C.H., Lee, T.C., Ho, I.C., Yang, U.C. and Chen, J.J., Gene selection for sample classifications in microarray experiments, *DNA and Cell Biology, a*ccepted, 2005.

PI: Delongchamp, Robert

Statistical Design and Analysis of Functional Genomic Studies that Estimate Changes in Gene Expression Using cDNA Arrays (E0718401)

Objective(s): Develop efficient designs and analytical procedures for simultaneously interrogating multitudes of genes in functional genomic experiments.

Accomplishments:

a. Delongchamp, R.R., Bowyer, J.F., Chen, J.J. and Kodell, R.L., Multiple-testing strategy for analyzing cDNA array data on gene expression, *Biometrics*, 60:774-782, 2004.

b. Delongchamp, R.R. and Kodell, R.L., Comments on validation of normalization and background correction for microarray data (Invited commentary), *ICSA Bulletin*, 44-47, July 2004.

c. Parrish, R.S. and Delongchamp, R.R., Normalization. In: *DNA Microarrays and Statistical Genomic Techniques: Design, Analysis, and Interpretation of Experiments* (D.B. Allison, G.P. Page, T.M. Beasley and J.W. Edwards, eds.), New York: Marcel Dekker. In press, 2004.

d. Delongchamp, R.R., Velasco, C., Razzaghi, M., Harris, A. and Casciano D., Median-of-subsets normalization of intensities for cDNA array data, *DNA and Cell Biology*, accepted, 2005.

PI: Kodell, Ralph

Dose-Response Modeling for Microbial Risk Assessment (E0704501)

Objective(s):
- Develop improved models for estimating probabilities of infection and disease.
- Develop methods for incorporating model uncertainty into microbial risk assessment.

Accomplishments: Moon, H., Chen, J.J., Gaylor D.W., and Kodell, R.L., A comparison of microbial dose-response models fitted to human data. *Regulatory Toxicology and Pharmacology.* 40:177-184, 2004.

Modification and/or Application of Quantitative Risk Assessment Techniques for FDA-regulated Products (S00174)

Objective(s): Improve quantitative methods for assessing risk and to apply to FDA-regulated products as needed.

Accomplishments:

a. Kodell, R.L. and Turturro, A., Risk-assessment implications of mechanistic model's prediction of low-dose nonlinearity of liver tumor risk for mice fed fumonisin B1, *Nonlinearity in Biology, Toxicology and Medicine,* 2:35-43, 2004.

b. Piegorsch, W.W., West, R.W., Pan, W. and Kodell, R.L., Low-dose risk estimation via simultaneous statistical inferences. *Applied Statistics* 54, in press, 2005.

c. Piegorsch, W.W., West, R.W., Pan, W. and Kodell, R.L., Simultaneous confidence bounds for low-dose risk assessment with non-quantal data. *Journal of Biopharmaceutical Statistics,* accepted, 2005.

d. Nitcheva, D.K., Piegorsch, W.W., West, R.W. and Kodell, R.L., Multiplicity-adjusted inferences in risk assessment: benchmark analysis with quantal response data. *Biometrics,* accepted, 2005.

e. West, R.W. and Kodell, R.L., Changepoint alternatives to the NOAEL. *Journal of Agricultural, Biological and Environmental Statistics,* accepted, 2005.

f. Razzaghi, M. and Kodell, R.L., Quantitative risk assessment for developmental neurotoxic effects. *Risk Analysis,* accepted, 2005.

g. Joo, J., Ahn, H., Delongchamp, R.R., Nowell, S.A. and Lang, N.P., A mixture-of-genotypes model for the distribution of thermostable phenol sulfotransferase activity, *Biometrical Journal,* accepted, 2005.

h. Chen, J.J., Chen, Y.J. and Kodell, R.L., Designs and models for mixtures: assessing cumulative risk. In: *Quantitative Methods for Cancer and Human Health Risk Assessment* (L Edler and C Kitsos, eds.). Wiley: New York, 2005.

PI: Moon, Hojin

Development of Improved Survival-adjusted Tests for Animal Carcinogenicity Data (E0717101)

Objective(s): Modify and extend the Peto and Poly-k tests in order to improve their robustness to various underlying tumor-onset distributions and to various competing-risks survival distributions.

Accomplishments:

a. Moon, H., Ahn, H. and Kodell, R.L., A weight-adjusted Peto's test when cause of death is not assigned. *Environmental and Ecological Statistics*, in press, 2004.

b. Moon, H., Ahn, H. and Kodell, R.L., A bootstrap-based age-adjusted poly-k test. *Statistics in Medicine*, in press, 2004.

PI: Tong, Weida

Development of a Novel Class Prediction Method, Decision Forest, for Analysis of Genomic and Proteomic Data (0716901)

Objective(s): Develop a strategy for combining the predictions of multiple decision trees to improve class prediction.

Accomplishments:

a. Tong, W., Xie, Q., Hong, H., Shi, L., Fang, H. and Perkins, R.G. Assessment of prediction confidence and domain extrapolation of two structure-activity relationship models for predicting estrogen binding activity. *Environmental Health Perspectives* 112(12): 1249-1254, 2004.

b. Hong, H., Tong, W., Perkins, R.G., Fang, H., Xie, Q. and Shi, L., Multi-class decision forest – a novel pattern recognition method for multi-class classification in microarray data analysis. *DNA and Cell Biology*, accepted, 2005.

c. Tan, Y., Shi, L., Wang, C., Hwang, G. and Tong, W., Mulit-class tumor classification by discriminant partial least squares using microarray gene expression data: assessing the robustness of gene selection and the quality of classification models. *Computational Biology and Chemistry*, accepted, 2005.

General Toxicoinformatics Support (S00617)

Objective(s): Provide toxicoinformatics support for NCTR research and FDA review involving genomics, proteomics, and metabolomics data.

Accomplishments:

a. Tong, W., Fang, H., Hong, H., Xie, Q., Perkins, R.G., Anson, J.F. and Sheehan, D.M., Regulatory applications of SAR/QSAR for priority setting of endocrine disruptors – a perspective. *Pure and Applied Chemistry* 75(11-12):2375-2388, 2003.
b. Tong, W., Cao, X., Harris, S.C., Sun, H., Fang, H., Fuscoe, J., Hong, H., Xie, Q., Perkins, R.G., Shi, L. and Casciano, D.A., ArrayTrack – supporting toxicogenomic research at the FDA's National Center for Toxicological Research (NCTR). *Environmental Health Perspectives – Toxicogenomics* 111(15):1819-1826, 2003.
c. Shi, L., Tong, W. and Lu, X., Biocheminformatics: integrating bioinformatics and chemoinformatics for drug discovery and development. *European Biopharmaceutical Review*: 59-62, 2003.
d. Tong, W., Harris, S.C., Cao, X., Fang, H., Shi, L., Sun, H., Fuscoe, J., Harris, A.J., Hong, H., Xie, Q., Perkins, R.G. and Casciano, D.A., Development of a public Toxicogenomics software for microarray data management and analysis. *Mutation Research*, accepted, 2004.

PI: Turturro, Angelo

Development of a Model for the Transmission Kinetics of Infection by Cryptosporidium parvum with Acquisition of Data on Key Parameters (E0708201)

Objective(s): Compare infectivity susceptibility among various subpopulations of animals characterized by age, pregnancy, immunocompetence, stress, and diet.

Accomplishments:

a. NCTR's quarantine facility has been modified to facilitate conduct of the study;
b. Special racks and changing stations for *Cryptosporidium parvum* containment have been purchased;
c. All animal care and safety issues have been addressed;
d. The PI has obtained training in handling the protozoan; and,
e. All supplies and laboratory equipment have been procured.

PI: Young, John

Computational Predictive System for Rodent Organ-Specific Carcinogenicity (E0708301)

Objective(s): Develop an expert system to predict rodent carcinogenicity using modern SAR technology and statistical approaches.

Accomplishments:

a. Young, J.F., Tong, W., Fang, H., Xie, Q., Pearce, B.A., Hashemi, R., Beger, R.D., Cheeseman, M.A., Chen, J.J. and Kodell, R.L., Building an organ-specific carcinogenic database for SAR analyses. *Journal of Toxicology and Environmental Health* Part A 67:1363-1389, 2004.
b. Beger, R.D., Young, J.F. and Fang, H., Discriminant function analyses of liver-specific carcinogens. *Journal of Chemical Information and Computer Sciences* 44(3):1107-1110, 2004.

Completed Research Projects

PI: Chen, James

Cumulative Risk Assessment for Chemical Mixtures (E0708701)

Objective(s): Develop and apply the relative potency factors approach for estimating the risk from combined exposures to a set of chemicals having a common mode of action.

PI: Delongchamp, Robert

Mortality Among Atomic Bomb Survivors who were Exposed In Utero (E0702901)

Objective(s):
1. Estimate the dose-response relationship between noncancer mortality and radiation exposure;
2. Assess the effect of gestational age at exposure on mortality; and,
3. Appraise the role of severe mental retardation in mortality.

An Investigation of the Effects of Adjusting Intensities from cDNA Arrays on the Assessment of Differential Gene Expressions (E0709601)

Objective(s):
- Evaluate the advantages/disadvantages of using either the mean or median for normalizing array data in the presence of nuisances;
- Determine an optimal size of subsets for normalizing data in the presence of nuisances that merit their use; and,
- Assess the bias induced by nuisances and the extent to which normalization procedures are able to remove them.

Publications

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Division of Genetic and Reproductive Toxicology

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Dr. Carrie Valentine develops a new assay for quantitative risk assessment.

Executive Summary

Introduction

The Division of Genetic and Reproductive Toxicology (DGRT) conducts basic and applied research to address specific high priority issues regarding genetic and reproductive/developmental toxicology. Division research is directed toward developing and validating new methods that can be used for the identification of potentially hazardous food additives, human and animal drugs, biological therapies, and medical devices. In addition, in collaboration with other NCTR scientists, DGRT utilizes the methodologies that it develops to conduct research to understand the potential toxicity of specific high priority drugs, dietary supplements, and/or other agents. For example, AIDS therapeutic drugs (including zidovudine, lamivudine, nelfinavir and nevirapine), acrylamide, and bitter orange are undergoing extensive evaluations in cross-Division collaborative research efforts.

Currently there are four basic focus areas in the Division research program. Genetic Toxicology research addresses the development of methods to assess the potential for chemicals to negatively impact human genetic material or the function of the genetic material. Reproductive/Developmental Toxicology focuses on methods to understand normal human development and how chemicals might alter normal development. In addition to these disciplinary research areas, the Division conducts research to understand the impact of diet. This research primarily focuses on understanding the physiological and genetic consequences of dietary modulation and on the potential hazards of dietary supplements. While the Center for Functional Genomics recently has been moved from the Division into the new Division of Systems Toxicology, DGRT continues to conduct research utilizing genomic technologies to address a variety of scientific questions.

FY 2004 Accomplishments

A series of ACB-PCR genotypic selection methods were developed that can directly measure specific mutations in genes (oncogenes and tumor suppressor genes) involved in tumor induction. These assays were used to measure mutations in tumors resulting from solar light exposure and in mice treated with 4-aminobiphenyl. ACB-PCR can detect mutations when they occur in as few as 1 cell in 100,000 cells. These studies have yielded significant new information. For instance, all the skin tumors resulting from solar exposure had relatively high frequencies of *p53* mutations, indicating that while this mutation was not the initiating event for the tumor, it was important for tumor formation.

Division scientists completed and published results from studies evaluating the genotoxicity of several specific chemicals (zidovudine, didanosine, malachite green, leucomalachite green, azathioprine, comfrey, and riddelliine) of regulatory interest to the FDA. Many of these compounds were nominated by the FDA for evaluation and the research on them supported through an IAG with the NTP/NIEHS. Studies evaluating the *in vivo* mutagenicity of acrylamide, which has recently been detected in baked goods, also are ongoing, and preliminary results on the *in vivo* genotoxicity of acrylamide and its major metabolite glycidamide were recently published.

Studies were completed and published that described the phenotype of the thymidine kinase mouse model, the effects of maternal folate-deficiency, alterations in gene expression profiles following *in vitro* exposure to ionizing radiation, the effects of dietary restriction on neoplastic pathology in male Sprague-Dawley rats, and the age-dependent sensitivity of mice to the mutagenicity of *N*-ethyl-*N*-nitrosourea.

The Center for Functional Genomics (now located in the Division of Systems Toxicology) completed a series of experiments establishing parameters required to generate high quality microarray data, methods for handling and interpreting microarray gene expression data, and a baseline study evaluating the effect of time-of-day on gene expression.

FY 2005 Plans

DGRT scientists will continue with the development of a new two-transgene *in vivo* assay using a fluorescent green protein as a reporter of mutation and another gene that controls its production as the mutational target sequence. When a chemical causes a mutation in the controlling gene, the cell produces a fluorescent protein that becomes visible under UV light and can be quantified using flow cytometric instrumentation. Also, DGRT scientists will apply their new technology measuring specific rare mutations in cancer-causing genes to studies involving colon cancer in humans, a skin cancer model in mice, and a colon cancer model in rats. The rat studies will involve examining mutations in cells with specific morphologies related to cancer induction; the cells will be isolated using laser capture microdisection.

Another new approach for directly analyzing mutations will be developed. This assay uses fluorescent probes to detect mutation in the endogenous, X-linked *PIG-A* gene. In theory, detection of mutations in this gene does not require cell culture (as do many other *in vivo* mutation detection methods) and lends itself to both *in situ* and high through-put analyses in humans and animal models. All of these properties make *PIG-A* an attractive reporter gene for *in vivo* mutation studies. Experiments are being conducted to determine the level of sensitivity of the analysis using cultures of cells that are wild-type and mutant for *PIG-A*.

The AIDS drug project will be expanded with two new studies. The drug treatments in these studies will model the use of these agents to prevent the transmission of the HIV virus from infected pregnant women to their children. Human clinical data suggest that a major target for the toxicity of AIDS therapeutic agents is the mitochondria, and studies will be conducted to evaluate the long-term effects of perinatal treatments to mice on mitochondrial DNA copy number and mutation. In an additional study, an NTP-sponsored experiment will evaluate the

ability of *p53* haplodeficient ($p53^{+/-}$) mice to detect the tumorigenicity of AIDS drugs in a relatively short-term bioassay.

In collaboration with the Division of Biometry and Risk Assessment and private-sector researchers, DGRT scientists will develop approaches to use dose-response *in vivo* mutation data to inform risk assessment. Models are being applied to the data in order to ascertain the mode-of-action of rodent carcinogens. This information is of critical significance to regulatory agencies doing quantitative risk assessments, including the FDA.

Work will continue on several ongoing projects including: (1) An Office of Women's Health project that is investigating whether genistein can decrease the induction of carcinogen-caused mutations; (2) A collaborative project with the University of Arkansas for Medical Sciences investigating the influence of biotin on the developing embryo; (3) The further development and characterization of the FX174 transgenic mouse, particularly as it applies to detecting mutation in mouse skin following exposure to solar light; (4) NTP projects investigating the genetic toxicity of AIDS therapeutic drugs and the developmental toxicity of bitter orange; (5) Evaluation of flow cytometry for the high through-put analysis of micronucleus frequency in mice, rats, monkeys, dogs, and humans; and (6) A project to investigate whether the developing embryo and/or the neonate is particularly sensitive to the induction of mutation following exposure to known carcinogens.

Public Health Significance

Genetic toxicology is concerned with the ability of chemicals to alter genetic material. The FDA requires that petitioners provide data evaluating the potential genetic toxicity of their products as a part of the product approval process. Because genetic damage is believed to be important in tumor development, this information is used as a part of the evaluation of suspected carcinogens. Regulatory decisions are based not only on the identification of potentially genotoxic substances, but also on an understanding of their mode of action. The research within the Division centers on the development and validation of new methods to assess genetic risk. Bacterial and tissue culture approaches are commonly used to detect potential genotoxicity and to generate hypotheses concerning the basic mechanisms of genotoxicity. While the Division utilizes *in vitro* approaches, it specializes in the development and validation of *in vivo* mammalian systems and the incorporation of these methods into risk assessment strategies. An increased understanding of mutational mechanisms, combined with test systems with an increased ability to detect genetic damage, will provide the FDA with better information for decision making. As new assays are validated, Division scientists will continue to work with international scientists to assure the harmonization of protocols and the development of guidelines to assess genetic hazards.

Reproductive/Developmental Toxicology is important to the Agency because one of the difficult challenges facing the FDA is the identification and regulation of chemicals, food additives, and biological therapies that may produce birth defects. Such defects affect 7% of humans at birth, another 7% have low birth weights, and at least 25% of pregnancies end in spontaneous abortion. The Division specializes in research to understand how toxicants may induce birth defects such as neural tube defects. Current research addresses the role that the vitamin folic acid plays in the normal closure of the neural tube. This research supports current thinking that diet may play a

role in the development of normal offspring, and that interaction between diet and toxicants may be important in producing certain birth defects.

Genomic technologies are beginning to provide new tools for making better public health decisions. International research efforts are providing the scientific and medical community with an increased understanding of the genetic material and how it functions in both humans and rodents. Utilizing this information, new molecular technologies are being rapidly developed and can be used to evaluate structural and functional changes to the genetic material of both rodents and humans. The Division is using new technologies in combination with more traditional approaches to address various research questions. While current technologies in the field of genetic and reproductive/developmental toxicology generally evaluate single endpoints, these new genomic technologies are providing the opportunity to detect alterations in a number of different endpoints. In the future, these new approaches to evaluating toxicity will allow for the integration of information across the various types of adverse health outcomes. For instance, when these technologies are fully developed, it will be possible to concurrently evaluate chemicals for their ability to cause cancer, to impact the nervous system, to cause birth defects, and to modify the immune function.

Ongoing Research Projects

PI: Aidoo, Anane

Evaluation of the Effects of Daidzein and Genistein (Hormone Replacement Agents) on the Genotoxic and Carcinogenic Activity of the Model Mammary Carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) in Ovarioectomized Transgenic Big Blue Rats (E0707001)

Objective(s): Determine whether daidzein, genistein, or estradiol supplementation to ovariectomized rats, singly or in combination, alters the following in mammary tissues,

- DNA adducts produced by DMBA,
- Frequency and types of mutations produced by DMBA, and
- Tumor formation by DMBA and types of *p53* and *H-ras* mutations in the tumors.

ADDENDUM: An Efficient Regulatory Method for Evaluating Chromosomal Damage: Analysis of Micronucleus in Different Rat Strains by Flow Cytometry (E0714011)

Objective(s): Addendum requesting 50 additional animals, including both normal (intact spleen) and splenectomized Sprague Dawley rats.

PI: Chen, Tao

Comparison of Mutation Induction and Types of Mutations in the *cII* Gene of Big Blue Mice Treated with Carcinogens as Neonates and Adults (E0709001)

Objective(s):

- Determine the mutant frequencies in the *cII* gene of lambda/*lacI* transgenic mice treated with ethylnitrosourea, a direct-acting carcinogen, and the modifying role of age, sex, and target organ on mutant induction;
- Compare mutant frequencies in the *cII* gene of livers from transgenic mice exposed as neonates and adults to different doses of aflatoxin B1, a human hepatocarcinogen that requires metabolic activation;
- Determine the effect of exposure of neonatal and adult Big Blue mice to 17 ß-estradiol, a human hormone carcinogen, on subsequent spontaneous and carcinogen-induced mutations in the *cII* gene of the target organs; and
- Determine the types of *cII* mutations in the mutants from the studies listed above.

DNA Adduct Formation, Mutations and Patterns of Gene Expression in Big Blue Rats Treated with the Botanical Carcinogens Riddelliine, Aristolochic Acid (AA), and Comfrey (E0710001)

Objective(s):

- Treat Big Blue rats subchronically with riddelliine, AA, and comfrey using procedures appropriate for tumor induction;
- Analyze DNA adduct formation in the target tissues for carcinogenesis and in spleen lymphocytes;
- Determine the *cII* mutant frequencies and the types of *cII* mutations in

the target tissues of treated rats;
- Determine global gene expression patterns in the target and surrogate tissues of treated rats; and
- Correlate gene expression patterns with DNA adduct formation and mutation induction in treated rats.

Further Evaluation of the Types of Genetic Events Detected by the Mouse Lymphoma Assay (MLA) and the Role of the Assay in Mechanistically Based Risk Assessment (E0711701)

Objective(s):
- Determine if the L5178Y/*Tk*$^{+/-}$ MLA detects both aneuploidy and mitotic recombination;
- Determine if MLA cells have active recombinase functions, which lead to a large proportion of mutants that result from recombinase-mediated rearrangements; and
- Determine what is/are the fundamental genetic mechanism(s) causing the small and large colony *Tk* mutant phenotypes.

PI: Dobrovolsky, Vasily

Transgenic Mouse Model for Detecting *In Vivo* Mutation Using a Green Fluorescent Protein Reporter (E0713801)

Objective(s):
- Produce two lines of transgenic mice expressing the tetracycline-repressor protein;
- Investigate the efficiency of *in vivo* repression of green fluorescent protein (GFP) in various tissues of different lines of the double-transgenic mice; and,
- Determine the frequency of spontaneous and ?-ray-induced *TetR* mutation in lymphocytes of double-transgenic mice using flow cytometry.

PI: Duffy, Peter

Effect of Different Levels of Caloric Restriction (CR) on Physiological, Metabolic, Biochemical, Immunological, Molecular, and Body Composition Variables in Rats (E0692401)

Objective(s):

- Determine how various levels and durations of CR affect physiological function, enzymes related to intermediary and drug metabolism, hormonal regulation, and blood chemistry;
- Determine the relationships among body fat (BF), fat free mass (FFM), total body water (TBW), and total body electrical conductivity (TOBEC) as a function of strain, age, mass, and nutritional status in rats;
- Validate and automate the use of a new noninvasive electromagnetic scanning device to measure BF, FFM, and TBW, and compare the results to a conventional chemical fat extraction technique;
- Determine if CR alters the relative quantity and disposition of various types of lipids such as cholesterol, phospholipids, and free fatty acids, in various tissues, as well as in urine, feces, and blood serum;
- Develop control data related to CR that can be used by CFSAN to evaluate the toxicity and efficacy of low calorie foods, food additives, and food substitutes;
- Determine temporal and environmental factors that modulate the effects of CR;
- Develop experimental methods for utilizing a low level of CR (less then 30% calorie reduction) to increase the survival rate and to decrease variability in the chronic bioassay; and provide the concomitant control data for comparison; and
- Develop control data for a reference purified diet that has been formulated to conform to long-term nutrient requirements of rodent animal models typically utilized in toxicology and nutrition studies.

PI: Hansen, Deborah

Developmental Toxicity of Bitter Orange in Rats (E0214701)

Objective(s): Determine the potential developmental toxicity of synthetic synephrine and *Citrus aurantium* extract (bitter orange) in rats.

Physiological Effects of Bitter Orange in Rats (E0214901)

Objective(s): Determine the potential physiological effects of synthetic synephrine as well as an extract from the botanical *Citrus aurantium* alone and in combination with caffeine in rats.

Mechanism(s) of Folate-Responsive Dysgenesis (E0707401)

Objective(s):

- Determine if there is concordance between the expression of the folate receptor (FBPI) and the most proliferative cohorts of neural tube and neural crest cells during defined 12-hour windows on each day of gestation from gestation day (GD) 5 to GD 15, and determine if the loss of these cohorts of cells during these windows of antifolate exposure gives rise to recognizable neural tube defects and neurocristopathies in the fetus at term;
- Characterize the basal expression of FBPI isoforms and the extent and mechanism of FBPI regulation in the placenta and various fetal tissues on GD 17 among cohorts of dams fed a folate-deficient or folate-replete diet;
- Determine if sustained quenching of placental cytotrophoblast FBPI by antisense FBPI cDNA overexpression from GD 8 to GD 16 during maternal folate deficiency has an adverse impact on cytotrophoblastic proliferation leading to small placentas and global growth retardation of fetuses; and,
- Demonstrate that neural tube closure and neural crest cell function in the whole mouse embryo at GD 8.5 can be perturbed by down-regulating FBPI expression in neural tube cells through the introduction of antisense oligonucleotides to the 43-kDa trans-factor, which is required for FBPI transcription.

Examination of Embryonic Gene Expression during Neural Tube Closure (E0710901)

Objective(s):

- Construct a SAGE library of expressed genes from control untreated GD 8.0 and GD 8.25 CD-1 mouse embryos;
- Construct a SAGE library of expressed genes from GD 8.25 CD-1 mouse embryos treated with a teratogenic dose of valproic acid on GD 8.0;
- Compare the libraries to determine which genes are up- or down-regulated by valproic acid treatment;
- Use Northern blot techniques to determine if the mRNA transcripts for these genes are indeed increased or decreased in expression compared to control embryos;
- Use Northern blot techniques to determine a time-course of altered gene expression for genes of interest;
- Examine expression of some of these genes after treatment with teratogenic or non-teratogenic doses of valproic acid, valproate analogs, or another developmental toxicants; and,
- Use in situ hybridization, laser capture microdissection, and Northern blot techniques to determine if altered gene expression is specific for subsets of embryonic cells.

Mechanism of Biotin Deficiency-induced Malformations (E0713301)

Objective(s):
- Determine if palatal tissue from biotin-deficient embryos is able to fuse *in vitro* in either biotin-sufficient or -deficient medium;
- Determine if arachidonic acid increases palatal fusion and improves limb development and increases the length of the long bones *in vitro* from biotin-deficient mouse embryos;
- Determine if prostaglandin E2 increases palatal fusion and improves limb development and increases the length of the long bones *in vitro* from biotin-deficient mouse embryos;
- Determine if malonyl CoA increases palatal fusion and improves limb development and increases the length of the long bones *in vitro* from biotin-deficient mouse embryos;
- Determine fetal arachidonic acid content and synthesis *in vivo*; and,
- Determine if arachidonic acid is able to prevent biotin deficiency-induced orofacial clefting and limb hypoplasia *in vivo.*

PI: Hass, Bruce

Identification of Target Sites for UVB Irradiation in Gene *A* of F X174 contained as a Transgene in Mouse Embryonic PX-2 Cells (E0710101)

Objective(s):
- Determine the dose-survival response of PX-2 cells to UVB/UVA light in order to determine UV doses that optimize mutation induction and cell survival;
- Determine the induced mutant frequency in gene *A* of FX174 by a forward mutation assay using cultures of PX-2 cells exposed to UVB; and,
- Sequence the UVB/UVA-induced mutants from treated and untreated cultures to identify specific target sequences.

UV-Induced Mutations in Mouse Epidermis using Gene *A* of F X174: Proof of Principle (E0718701)

Objective(s): Establish that a UVB-induced dose response in mutant frequency of mouse epidermis can be detected by the gene *A* forward assay for FX174 analyzed by single bursts.

PI: Heflich, Robert

Effect of Azathioprine on Somatic Cell and Germline *Hprt* Mutant Frequencies in the Mouse (E0709901)

Objective(s): Test the hypothesis that *in vivo* selection by azathioprine affects both somatic cell and germline *Hprt* mutant frequencies using the mouse.

PI: McKinzie, Page

ACB-PCR Measurement of Azoxymethane-induced Rat *K-ras* codon 12 GGT? GAT and GGT? GTT Mutations in Colonic Aberrant Crypt Foci Isolated using Laser-capture Microdissection (E0714901)

Objective(s):
- Use newly established PCR-based methods to quantify the rat *K-ras* codon 12 GGT ? GAT and GGT ? GTT mutant fractions in rat colonic mucosa, aberrant crypt foci, and tumors at specified times after colon tumor initiation by azoxymethane treatment; and,
- Use this data in conjunction with *K-RAS* mutant fraction data generated from studies of human colon to determine how rodent data can be extrapolated to human disease.

PI: Morris, Suzanne

Effect of *p53* Genotype on Gene Expression Profiles in Mice Exposed to the Model Mutagen, *N*-ethyl-*N'*-nitrosourea (ENU) (E0712901)

Objective(s):
- Determine the effect of mutation in the *p53* tumor suppressor gene on gene expression profiles in young and aged mice; and,
- Determine the effect of mutation in the *p53* tumor suppressor gene on gene expression profiles in young and aged mice exposed to the model mutagen, *N*-ethyl-*N*-nitrosourea.

PI: Parsons, Barbara

Analysis of *p53* Codon 270 CGT? TGT Mutation in Simulated Solar Light (SSL)-induced Skin Tumors and Exposed Mouse Skin (E0715201)

Objective(s):
- Develop an ACB-PCR detection method for the mouse *p53* codon 270 CGT? TGT mutation;
- Measure the frequency of this mutation in mouse skin tumors;
- Measure the frequency of this mutation in skin tissue from tumor-bearing animals; and,
- Measure the frequency of this mutation in skin exposed to decreasing levels of SSL.

Measurement of Cancer-Associated Gene Mutation in Colon Tumor and non-Tumor Tissue (E0716001)

Objective(s):
- Determine *K-RAS* codon 12 GGT**?** GAT and GGT**?** GTT mutant frequencies in human colonic aberrent crypt foli (ACF), adenomas, and carcinomas; first by DNA sequencing and, if mutation is not detected, then by ACB-PCR;
- Determine *K-RAS* codon 12 GGT**?** GAT and GGT**?** GTT mutant frequencies in tumor margin samples and tumor-distant, normal-appearing colonic epithelium from colon cancer patients; and,
- Determine *K-RAS* codon 12 GGT**?** GAT and GGT**?** GTT mutant frequencies in autopsy samples of colonic epithelium from colon-disease-free individuals.

PI: Valentine, Carrie

Evaluation of the Potential of the Gene *A* Forward Mutational Assay of F X174 for Improving the Sensitivity of Transgenic Mutation Assays (E0711501)

Objective(s):
- Determine the appropriate experimental conditions to identify single bursts of mutations fixed *in vivo*;
- Develop a microplate scoring method that will identify *in vivo* bursts within numerous aliquots;
- Determine the spontaneous mutant frequency and ENU-induced mutant frequency by single burst analysis for mouse splenic lymphocytes; and,
- Continue development of a frameshift assay for FX174 in gene *J* with our collaborator, Dr. Bentley Fane, of the University of Arizona.

Creation of a Web-based Database for Mutations Associated with Exon-skipping (E0720101)

Objective(s): Create and update a public web site database with reported exonic mutations associated with exon loss. The database will be posted on a public web site that is searchable by gene characteristics and will be monitored for use.

Completed Research Projects

PI: Bishop, Michelle

Fluorescence-based Detection of Oxidative DNA Damage in Cells Treated *In Vitro* using Flow Cytometry and Fluorescence Microscopy (P00441)

Objective(s):
- Develop a sensitive and reliable method for the detection of 8-hydroxydeoxyguanosine in cells by flow cytometry and fluorescence microscopy;
- Optimize conditions for the assay; and
- Apply the methods developed to evaluate a free-radical mechanism for drug-or chemical-induced DNA damage in cells.

PI: Branham, William

Development of a Statistically Robust 3D-QSAR Model to Predict *In Vitro* Rat Uterine Estrogen Receptor Binding Activity (E0290001)

Objective(s): Develop and validate a statistically robust model for prediction of isolated rat uterine estrogen receptor relative binding affinity (RBA) that can be used as part of a prioritization scheme to identify chemicals for further *in vitro*/*in vivo* screening tests.

Development of a Statistically Robust Rat Androgen Receptor (AR) 3D-QSAR Model for Predicting Relative Binding Affinity (RBA) of Untested Chemicals (E0290101)

Objective(s):
- Develop and validate a statistically robust 3D-QSAR model to predict *in vitro* rat AR RBA; and
- Provide an alternative and/or supplemental method to prioritize chemicals for entry into Tier 1 screening under the EPA's Screening and Testing Program for endrocrine disruptors.

PI: Dobrovolsky, Vasily

Validation of the Mouse Targeted *Tk*$^{+/-}$ *In Vivo* System for Use in Mutagenicity Studies (E0701801)

Objective(s):
- Expand a colony of transgenic *Tk*$^{+/-}$ mice using breeding of *Tk*$^{+/-}$ founders and C57Bl/6 mice and transfer the *Tk*$^{+/-}$ genotype to a C57BL/6 background;
- Determine spontaneous mutant frequencies for the *Tk* and *Hprt* genes in splenic T-lymphocytes from mice of different ages;
- Induce mutations in *Tk*$^{+/-}$ transgenic mice using treatment with the point mutagen ENU and the clastogens BLM and ?-radiation, and measure the kinetics of mutant induction in the *Tk* and *Hprt* genes;
- Breed transgenic *Tk*$^{+/-}$ parents in an attempt to derive *Tk*$^{-/-}$ knockout mice, and study the biological significance of the *Tk* gene in mice; and
- Determine how the *Tk*$^{-/-}$ genotype may effect micronucleus frequencies and mutant frequencies in the *Hprt* gene.

Addenda:
- Establish routine microbiological surveillance of the colony as indicated in the addendum. This surveillance will be conducted on sentinel animals removed from the colony on an approximately monthly basis, and will consist of tests for the microorganisms listed in the addendum;
- Extend the range of mutagens tested in $Tk^{+/-}$ mice through a collaborative arrangement with Dr. Vernon E. Walker of the Wadsworth Center, New York Department of Health, Albany, NY, and Dr. Rogene Henderson of the Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM.
- Breed $Tk^{+/-}$ mice with $Pms2^{+/-}$ mice in order to derive $Tk^{+/-}$ mice that can be used for evaluating LOH mutation and that are also deficient in the Pms2 gene product.

Evaluation of the *Tk*$^{-/-}$ Knockout Mouse as a Model of Systemic Lupus Erythematosus (SLE) (E0706901)

Objective(s): Investigate whether the *Tk*$^{-/-}$ genotype in mice is lupus prone. Particular emphasis will be given to documentation of the putative immune-complex mechanism of the renal disease and in-depth evaluation of the immune system in *Tk* KO mice, seeking comparison with published characteristics of SLE in mice and humans.

PI: Heflich, Robert

ADDENDUM: Micronucleus and Gene Mutation Analysis in Female Big Blue B6C3F1 Mice Administered Malachite Green and Leucomalachite Green in the Diet (Addendum to E0212821) (E0212841)

Objective(s): Assess the genotoxicity of malachite green and leucomalachite green in relation to DNA adduct formation in female Big Blue mice.

PI: Manjanatha, Mugimane

ADDENDUM: Micronucleus and Gene Mutation Analysis in F344 Big Blue Rats Administered Leucomalachite Green in the Diet for 4, 16, and 32 weeks (E0212821)

Objective(s): Assess the mutagenicity of leucomalachite green in relation to DNA adduct formation in tissues of Big Blue rats.

Addendum: Expand the analyses of the remaining rats from the 32-week dose groups of E212821 to include additional indicators of hepatic toxicity.

PI: McKinzie, Page

Development of Techniques for DNA Isolation from Formalin-fixed Archived Tissue using Laser Capture Microdissection (LCM) Suitable for Subsequent Allele-specific Competitive Blocker PCR (ACB-PCR) (P00613)

Objective(s): Training of the investigator in the use of LCM techniques and determining a working protocol for using LCM to isolate cells from archived tissue for performing ACB-PCR studies.

PI: Mittelstaedt, Roberta

ADDENDUM: Measurement of *H-ras* Codon 61 CAA? AAA Mutation in Mouse Liver DNAs using the MutEx/ACB-PCR Genotypic Selection (E0704121)

Objective(s): Quantify and identify *lacI* mutations in liver DNA of mice treated as neonates with 4-aminobiphenyl in order to establish mutation induction and specificity as an early event in hepatic tumorigensis.

PI: Morris, Suzanne

***p53* Transgenic Mouse Evaluations of Genistein: 14-day Studies** (E0213601)

Objective(s):

- Determine the toxicity of genistein in the C57BL/6J strain and select doses for 36-week tumorigenicity studies;
- Identify the potential carcinogenicity of genistein in the *p53* transgenic mouse model;
- Determine if the the carcinogenicity of genistein relates to changes in the rates of cell death and cell proliferation; and
- Determine if exposure to genistein results in an increase in the mutant frequency in the *Hprt* reporter gene in the splenic lymphocytes of *p53* mice.

PI: Parsons, Barbara

Measurement of *H-ras* Codon 61 CAA? AAA Mutation in Mouse Liver DNAs using MutEx/ACB-PCR Genotypic Selection (E0704101)

Objective(s):

- Quantify somatic mutations in liver DNA of mice treated with 4-aminobiphenyl in order to establish and evaluate MutEx/ACB-PCR genotypic selection as an approach for human risk assessment; and
- Determine whether or not the MutEx/ACB-PCR genotypic selection is sensitive enough to measure the spontaneous frequencies of *H-ras* codon 61 CAA**?** AAA mutation in three different mouse models: B6C3F1, C57BL/6, and the Pms2 mismatch- repair-deficient transgenic mouse.

Addendum: Due to failure of a freezer, liver tissues being collected under the master protocol were thawed. The livers of the one-month post-treatment time point of the newborn mouse assay were destroyed. Additional animals and resources provided by this addendum will be used to repeat the one-month timepoint of the B6C3F1 newborn mouse assay.

PI: Shaddock, Joseph

ADDENDUM: Lymphocyte *Hprt* Mutant Frequencies and Types of Mutations in Pms2 Mice Treated as Neonates with Solvent or with 4-aminobiphenyl (E0704131)

Objective(s): Quantify and identify the *Hprt* mutations in spleen lymphocytes of $Pms2^{+/+}$, $Pms2^{+/-}$, and $Pms2^{-/-}$ mice treated as neonates with either dimethlysulfoxide (solvent control) or with 4-aminobiphenyl.

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Dr. Michael Adjei streaking bacterial isolates .

Executive Summary

Introduction

The Division of Microbiology at the National Center for Toxicological Research (NCTR) serves a multipurpose function with specialized expertise to perform fundamental and applied research in microbiology in areas of the Food and Drug Administration's (FDA's) responsibility in toxicology. The Division of Microbiology also responds to microbial surveillance and diagnostic needs for research projects within the NCTR and FDA. Projects are selected based on FDA priorities and programmatic expertise. The research program is divided into five focal areas: 1) Foodborne pathogens, food safety and methods development; 2) Antimicrobial resistance; 3) Gastrointestinal microbiology and host interactions; 4) Environmental biotechnology; and 5) Microbiological surveillance and diagnostic support of research.

Ongoing Research Projects

The Division of Microbiology research scientists continue to provide valuable information to FDA for evaluating key regulatory issues in food safety and environmental biotechnology, with special emphasis on antimicrobial resistance in the food animal production environment.

Foodborne Pathogens, Food Safety, and Methods Development

In recent years, there has been increasing concern by the public concerning the safety and quality of food. Scientists in the Division of Microbiology have collaborated with the Center for Veterinary Medicine (CVM), Center for Food Safety and Applied Nutrition (CFSAN), and Office of Regulatory Affairs (ORA) on a variety of projects to identify and characterize foodborne hazards more rapidly and accurately.

Salmonellosis, a disease caused by the foodborne pathogen *Salmonella*, accounts for nearly one-third of the deaths from foodborne illnesses in the U.S. Although a CDC report found that *Salmonella* infections have declined by 17% in the U.S. since 1996, this pathogen remains a serious threat to the food industry. There is limited information available on the prevalence and genetic characterization of *Salmonella* isolates from turkey farms. In collaboration with West Virginia University (WVU) scientists, who provided *Salmonella* isolates, we evaluated the prevalence and sources (and/or vectors) of *Salmonella* colonization on a turkey farm in WV; measured the intrinsic antibiotic resistance in *Salmonella* isolates using the disk diffusion assay

and minimum inhibitory concentration (MIC) broth dilution methods; and determined the genetic diversity of *Salmonella* isolates using molecular genetic techniques to delineate possible transmission pathways and bacterial source tracking to show how the pathogen moves within the production facility. Based on the data from these studies, the agency can make recommendations on the use of antibiotics by the turkey industry and develop HACCP guidelines for controlling *Salmonella* in the poultry industry.

In another study, forty-nine *E. coli* isolates from food samples were characterized for virulence genes by multiplex polymerase chain reaction (PCR) targeting *stx1, stx2, stx2e, eaeA, CNF1, CNF2, Einv, LTI, STI,* and *STII* toxin genes. Only ten strains showed the Shiga toxin *stx1* and *stx2* genes, and none showed the other genes. The Shiga toxin positive *E. coli* strains (STEC) all were isolated from soft and cottage cheeses. These *E. coli* isolates were serotyped and belonged to O18, O8, O57w, O79, O44, and O128 groups. Pulsed field gel electrophoresis analysis generated a unique profile for each strain. Analysis of the biotypes of STEC isolates showed variations, and four strains revealed characteristics compatible with *E. coli* O157:H7. These results suggest that strains of *E. coli,* isolated from ready-to-eat food, represent an important reservoir of virulence genes.

Vibrio parahaemolyticus is a marine bacterium that causes enteritis in humans through consumption of seafood. Outbreaks of *V. parahaemolyticus* gastroenterititis in the United States emphasized the need to develop molecular methods for identification and differentiation of these pathogenic organisms. Scientists in the Division of Microbiology have cloned and sequenced a phage-related/chemotaxis-related gene from *V. parahaemolyticus* O3:K6 strains. The pandemic *V. parahaemolyticus* O3:K6 strains have this unique gene. Most of the *V. parahaemolyticus* O3:K6 strains isolated from patients and environment produce thermostable direct hemolysin (TDH) but did not produce another TDH-related hemolysin (TRH). To identify specifically these virulent O3:K6 strains, a multiplex PCR method was developed by targeting both genes simultaneously.

In collaboration with the Division of Chemistry, researchers in the Division of Microbiology have been involved in creating a mass spectrometric database for the detection of pathogens and potential pathogens from food and designing strategies to differentiate harmless materials from pathogens by the use of mass spectrometry.

Antimicrobial Resistance

Reports of antimicrobial-resistant bacteria from farms, animal carcasses, and aquaculture facilities are raising concerns that antimicrobial use in food-producing animals may play a role in selecting for antibiotic resistance. The research and regulatory issues on antimicrobials used in food-producing animals are of great importance to the FDA. A number of collaborative research projects with other FDA Centers are being conducted in the Division of Microbiology.

In FY 2004, researchers in the Division of Microbiology collected poultry litter, feed, and water samples from farms to isolate *Salmonella, Campylobacter*, and *Escherichia coli* to determine if they are fluoroquinolone-resistant. Molecular methods were developed to screen for fluoroquinolone resistance genes in isolates from chicken and turkey farms. Two hundred samples of poultry litter, feed, and water were collected to isolate bacteria to determine if they

were fluoroquinolone-resistant. Fifty *E. coli* and forty-five *Campylobacter* spp. resistant to fluoroquinolones were characterized by ribotyping, restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), and polymerase chain reaction (PCR). The *gyrA* and *parC* genes were amplified and sequenced from *E. coli* and *Campylobacter* spp. resistant to fluoroquinolones to determine the point mutations. Sequencing of the quinolone resistance-determining region of the *gyrA* gene suggested that a higher level of resistance to ciprofloxacin was associated with double mutations, but the mutants with a low level of resistance had only a single mutation. Fluoroquinolone resistance was present among different serotypes and ribotypes, and drug resistance profiles suggest that the incidence of resistance does not indicate clonal populations in *E. coli* and *Campylobacter* spp. from poultry.

Researchers in the Division of Microbiology have isolated 48 strains of tetracycline-resistant *Aeromonas* spp. from catfish intestine. Morphological and biochemical analysis indicated that 37 strains were tentatively identified as *A. veronii,* and 11 strains were *A. hydrophila*. Currently scientists in the Division of Microbiology are adapting a molecular method for identification of the isolates.

Recently, we isolated three variants of *Enterococcus faecium* that showed differences in their vancomycin sensitivity, PFGE, and *Eco*R1 RFLP profiles. Sequence analysis indicated the presence of novel mutations in the *vanR*, *vans,* and *vanH* genes and the presence of an insertion element, IS*1251,* in a non-coding region between the *vanS* and *vanH* genes. Since vancomycin exposure of the parent generated these variants, vancomycin is believed to have caused these mutations that are responsible for higher-level resistance to vancomycin. Results of the study suggest that the clinical use of vancomycin could have serious consequences for later patients undergoing vancomycin therapy. The use of vancomycin may generate hyper-resistant strains that are difficult to manage.

An oligo-based microarray method was developed for the detection of 131 antibiotic resistance markers representing 20 different antibiotics. The initial data were generated by antisense-oligo hybridization. In addition, a new and efficient RNA isolation method was developed to prepare RNA from Gram-positive and Gram-negative bacteria. The RNA developed by this new method provides qualitative data similar to the RNA obtained by standard commercial methods.

Researchers in the Division of Microbiology have compared fluoroquinolones with different structures for their potency against bacteria and their ability to prevent the selection of resistant mutants. They have evaluated the structural variation in fluoroquinolones, which results in their effectiveness and the prevention of mutant development in bacteria. They have found that different resistant mutants develop in bacteria exposed to different fluoroquinolones. They also have investigated various mechanisms that bacteria employ to evade bactericidal activities of antimicrobial agents. In collaboration with researchers in universities, they have evaluated natural compounds that enhance antimicrobial activities of currently available antimicrobial agents.

Competitive exclusion products consist of mixtures of bacteria originally isolated from chicken intestinal tracts. The products are sprayed on newly hatched chicks to promote colonization by a harmless bacterial flora that prevents human pathogens from colonizing the birds later in life. In

2004, we evaluated the antimicrobial drug resistance patterns of multiple-antibiotic-resistant bacteria isolated from a competitive exclusion product. They were isolates of *Lactococcus lactis* and *Enterococcus faecalis.* We wanted to determine whether these bacteria that are resistant to vancomycin, erythromycin, and tetracycline can transfer their drug-resistance to recipient strains of *Enterococcus* spp. isolated from the competitive exclusion product and a laboratory strain of *Staphylococcus aureus.* The results of these studies will indicate the probability of resistance transfer between bacteria in the competitive exclusion product.

It has become increasingly important that alternatives to antibiotic therapy be developed for the prevention and treatment of diseases caused by *Staphylococcus aureus*. A promising therapeutic approach to treat staphylococcal infections is to target important steps in the pathogenesis of disease, thereby reducing the severity of infection and retarding disease progression. This would augment antimicrobial therapies and allow the patient's own defenses to control and eliminate the infection. Passive immunization is an attractive approach to rapidly neutralize toxins, enzymes, and cell wall-associated proteins, which are important in life-threatening disease. In order to develop an alternative approach, like passive immunotherapy, for the prevention and treatment of staphylococcal disease, we believe a comprehensive analysis of all extracellular proteins produced by a number of representative *S. aureus* strains is required. Most extracellular proteins produced by *S. aureus* are virulence factors and involved with some aspect of disease.

Research using one-dimensional polyacrylamide gel electrophoresis and liquid chromatography (LC)-mass spectrometry (MS) in tandem has generated a profile of proteins found in the spent medium of *S. aureus* UAMS-1 and its regulatory mutants (*sar*, *agr*, and *sar agr*). Some 624 proteins have been identified thus far, when searched against the proteomic database of *S. aureus* MW2, a clinical isolate for which a proteomic database has been established. In addition, because regulation of virulence genes by *sar* and *agr* is not always an all-or-none process, we have been able to utilize both peptide and protein scores generated from the LC/MS/MS data to estimate the amounts of certain proteins whose expression is controlled by these regulators. Work is now underway to develop a more quantitative assessment of proteins utilizing data generated from the LC/MS/MS analysis. It is anticipated that such a profile of *agr* and *sar* regulated proteins will provide the premise for further investigation of these proteins as potential targets for anti-staphylococcal vaccine and drug development.

Gastrointestinal Microbiology and Host Interactions

The human gastrointestinal tract is populated with a complex and diverse population of anaerobic bacteria. These bacteria play an important role in human health, acting as a barrier to colonization of the intestinal tract by pathogenic bacteria, as well as contributing to the digestion of dietary components and metabolism of drugs, xenobiotics, and nutrients. Shifts in the composition of the population of intestinal microbiota may contribute to increased susceptibility to infection, or altered metabolic potential. We are developing methods to monitor changes in the bacterial population within the human gut, particularly when exposed to residual levels of antimicrobial compounds. An oligonucleotide array that targets 40 of the most common intestinal bacteria has been developed in the Division of Microbiology. We have improved the sensitivity of this method when used in a membrane-array format. We have, however, detected some cross-reactivity between related bacterial species in the array, which will need to be

addressed for this method to be more useful. We are also using genus-specific probes to monitor larger population shifts and testing real-time PCR approaches for more accurate quantitation of changes in individual bacterial species.

Assessing the safety of drugs and other compounds involves understanding their effects on the gastrointestinal tract microbiota, manifested in FDA Guidance for Industry #52. *Lactobacillus* species are currently being studied in this Division because they serve as important indicators of gastrointestinal and vaginal tract health and are heavily used by consumers intentionally, as probiotic supplements, or unintentionally in microbially fortified foods. Although they are afforded GRAS status by the FDA, little is known about either their antimicrobial resistance profiles or whether these profiles are subject to change. We determined that these organisms are generally resistant to high levels of aminoglycosides. However, under conditions typical of the gastrointestinal tract where they encounter bile acids, they become sensitive to aminoglycosides, indicating that treatment with such compounds may have profound effects on *Lactobacillus* populations. Other studies assessing the effect of endogenous steroid hormones, similar in basic structure to bile acids, are currently being conducted as are studies assessing the resistance to microbicides and spermicides in conjunction with vaginal health. We are also currently working on molecular methods to genetically manipulate *Lactobacillus* to further all of these studies. This work underscores the differences that may exist between resistance profiles observed in the laboratory versus the actual scenario in the environment.

Scientists in the Division of Microbiology developed a biological assay for measurement of the concentrations of antimicrobial drug residues in food that cause a failure of the intestinal microflora to prevent infection by *Salmonella*. The assay provides a means to directly observe detrimental effects of antibiotics on the intestinal barrier against food poisoning. The highlights of this work, over the past year, have been the preparation of a model human intestinal microflora that provides protection against *Salmonella* invasion of the human intestinal cell line, Caco-2 and the use of the protection assay to measure concentrations of 22 antimicrobial drugs that break down the protective intestinal barrier. These results will help us calculate more accurate levels of Acceptable Daily Intake of residues of these drugs in foods.

A specialized animal facility was built to maintain mice without any bacteria in their intestinal tracts. We received breeding pairs of germfree normal BALB/c and Tge26 mice, which have deficient immune systems, from North Carolina State University. The mice were bred in germfree isolators and colonized with a model human intestinal flora. Some of these mice were colonized with a commercial mixture of probiotic bacteria (considered to be helpful for protection against food poisoning bacteria) and others were colonized with *Salmonella* or *Campylobacter*, bacteria that cause food poisoning in humans. The effects of these bacteria on the immune systems of the mice and on the populations of the bacteria in their intestinal tracts are being measured. These studies will give us a better understanding of how changing bacterial populations in the intestinal tract though the use of probiotics affect the mechanisms that protect us from food poisoning.

Researchers in the Division of Microbiology have investigated the effect of intestinal microflora on the activities of plant hormones, such as phytoestrogens. They have found that bacteria

residing in the guts of some individuals can change these compounds to useful metabolites, while in others these compounds are changed to compounds that do not benefit the consumers.

The pigments used in tattoos and topically applied colorants are subject to FDA regulation. The metabolism of the colorants by the skin and intestinal microflora, and the potential toxicity of the reaction products to the human body are currently being studied in the Division. This investigation will provide valuable information on the toxicity of colorants metabolized by bacteria and their enzymes. We have identified, cloned, and over-expressed azoreductases from a skin bacterium, *Staphylococcus aureus*, and an intestinal bacterium, *Enterococcus faecalis*. The properties of the azoreductases indicate that the enzymes have a broad spectrum of substrate specificity and are capable of degrading a wide variety of azo dyes. To understand the mechanism of dye degradation, it is essential to know the structures of the azoreductases. In cooperation with investigators at the University of Georgia, a crystal of 2.5Å in size has been obtained from the *E. faecalis* azoreductase. A microarray method to monitor intestinal bacterial species predominant in azo dye reduction has been developed.

The anaerobic fungi represent an important part of the gut microflora of herbivorous mammals. They produce a wide range of enzymes capable of hydrolyzing many compounds and have potential to contribute substantially to drug degradation in the alimentary tract of the host animal. Anaerobic fungi are resistant to penicillin, streptomycin, and chloramphenicol, and these antibiotics may be metabolized and degraded. However, the mechanism of the deactivation and degradation are not known. In cooperation with investigators at USDA/ARS, we are studying important hydrolytic enzymes from rumen anaerobic fungi.

Environmental Biotechnology

The environmental fates of veterinary drugs and factors that influence the biodegradation of antibiotics used in farm animals and aquaculture have been investigated. Scientists in the Division of Microbiology are determining the pathways used by bacteria and fungi for the biodegradation of fluoroquinolone drugs in the environment. They have shown that two mold fungi, *Cunninghamella elegans* and *Penicillium chrysogenum*, transform flumequine, a fluoroquinolone used in aquaculture in some countries outside the USA. Liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy have been used to show that fungi oxidize the flumequine molecule to metabolites containing hydroxyl and keto groups. In addition, they have shown that several bacteria in the genus *Mycobacterium* transform norfloxacin, which is approved in the USA not only for human clinical use but is used for animals elsewhere. The bacteria add hydroxyl, acetyl, and nitroso groups to the norfloxacin molecule, and they also break one of the rings. Parallel studies with 1-phenylpiperazine, a model compound, show that mycobacteria also metabolize it in similar ways.

Benzoquinolines are environmental pollutants produced during the burning of fossil fuels. Scientists in the Division of Microbiology have dosed cultures of the soil fungus *Umbelopsis ramanniana* with three different benzoquinolines and shown that the fungus transforms them to eight different oxidized metabolites. At least one of the metabolites produced from each benzoquinoline was mutagenic, so the process could not be considered a detoxification step.

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of organic compounds whose environmental fate is of concern because some PAHs have mutagenic, ecotoxic, and carcinogenic potential. Scientists in the Division of Microbiology have elucidated the biodegradative pathways of benzo[*a*]pyrene, benz[*a*]anthracene, and 7,12-dimethylbenz[*a*]-anthracene and the enzymes involved in PAH metabolism. *Mycobacterium vanbaalenii* PYR-1 is capable of degrading a number of polycyclic aromatic hydrocarbons (PAHs) to ring cleavage metabolites via multiple pathways. Proteomic and genomic techniques have been developed to characterize protein expression and the genes involved in the bacterial metabolism of PAHs. Molecular cloning, sequencing, and functional characterization of dioxygenases, cytochromes P450 and putative enzymes involved in the degradation of phenanthrene and phthalate were determined. This research increases our understanding of the environmental fate of PAHs for developing practical PAH bioremediation strategies in the future.

Microbiological Surveillance and Diagnostic Support of Research

The primary mission of the Surveillance/Diagnostic Program in the Division of Microbiology is the assurance that NCTR research data is not compromised by the use of infected or unhealthy experimental animals. Additionally, Surveillance/Diagnostic personnel provide Division of Microbiology and other NCTR researchers assistance with microbe identification, media preparation, and stock culture maintenance. For FY 2005, the development of molecular biology techniques for the detection of pathogenic microorganisms will be given highest priority.

FY 2005 Plans

Research will continue on a number of ongoing projects, including:

Foodborne Pathogens, Food Safety, and Methods Development

In an effort to promote FDA's mission by better ensuring that the nation's food supply is safe and sanitary, we will be addressing the issue of microbial pathogen contamination in seafood and the development of improved molecular diagnostic methods for detecting their presence in food samples.

- Characterize *Salmonella* and *Vibrio* spp. isolated from seafood samples by molecular techniques. After characterization, a rapid microarray method will be developed to detect these pathogens in ocean derived products. The results of this study will be used as a template for development of a diagnostic gene chip capable of simultaneous detection of multiple foodborne pathogens.
- Continue to collaborate with other Divisions to use flow cytometry to facilitate isolation of single bacteria from contaminated samples for rapid bacterial identification and for pyrolysis mass spectrometry.

Antimicrobial Resistance

- Develop collaboration with CFSAN involving whole-genome *E. coli* microarrays to study the effect of steroid hormones and multiple drug efflux pump expression on gene expression in this model organism.
- Develop a CRADA with a pharmaceutical company to determine the fate of cephalosporin antibiotics when exposed to the intestinal microbiota of the treated animal. Research will center on the third-generation cephalosporin, ceftiofur, which is approved solely for veterinary use. Preliminary evidence suggests that this drug is rapidly degraded by an as yet undetermined process in the bovine intestine. Our approach will involve determining the rate of inactivation/degradation of this antibiotic, the isolation of bacteria showing resistance to ceftiofur, and characterization of the resistance mechanisms of these isolates.
- Continue to develop a proteomic approach for identifying *Staphylococcus aureus* extracellular proteins to include a quantitative analysis utilizing ^{12}C6- and ^{13}C6-arginine incorporation into protein.
- Continue to improve the microarray screening method for antibiotic resistance markers.
- Continue to address the genetic diversity among *Salmonella* isolates, using multilocus sequence typing methods, and develop microarray probes to detect the virulence and antibiotic resistance genes in *Salmonella.*
- Continue to evaluate the contributions of chemical structures of fluoroquinolones on the mechanisms of resistance development in bacteria and the effects of mutations in target enzymes on the resistance and growth of bacteria exposed to antimicrobial agents.

Gastrointestinal Microbiology and Host Interactions

- Continue to evaluate the metabolism of phytoestrogens and other compounds of interest by colonic microflora of humans and experimental animals and the role of bacterial enzymes in the metabolism of these compounds.
- Continue to study genes encoding azoreductases from skin and intestinal bacterial species. Site-directed mutagenesis techniques will be employed to study the enzyme activity center, an FMN binding motif of the recombinant azoreductases from skin bacteria. DNA probes and antibodies for *E. faecalis* and *S. aureus* azoreductases will be used for screening similar genes in skin and intestinal microflora to determine the distribution of the azoreductase genes across various predominant bacteria and the enzyme expression levels in these microorganisms.
- Evaluate DNA microarray approaches for studying effects of antibiotics and food supplements on intestinal microflora.
- Evaluate the mechanisms of inactivation and the degradation of cephalosporins by anaerobic fungi from the bovine rumen and the identification of the degradation products.

Environmental Biotechnology

- Determine the metabolites produced during the microbial degradation of fluoroquinolone antimicrobial residues by bacteria and fungi found in the environment.
- Continue to develop proteomic and genomic approaches for characterizing polycyclic aromatic hydrocarbon degradation pathways.

Public Health Significance

The microbiological safety of food has become an important concern of consumers, industry, and regulatory agencies. The FDA gives a high priority to protecting the public from microbial contamination of the food supply. We will continue to develop rapid and sensitive methods to detect foodborne pathogens and determine the mechanisms of pathogenesis. This information will be of value for the FDA food safety policies.

The FDA, various national and international committees, and the general public are concerned about the increased multiple antimicrobial resistance that has recently been found among pathogenic microorganisms. This may be due in part to the veterinary use of antimicrobials, which will potentially bring about a general increase in the numbers of antimicrobial-resistant bacteria in food animals and the environment and increased amounts of antimicrobials and their biotransformation products in meat, milk or egg products that could affect consumers via the intestinal microflora. The issue of microbial drug resistance has significance both to health and regulatory agencies. The FDA has expressed an interest in research that would determine whether antimicrobial resistance occurs in bacteria isolated from animal feeds containing antibiotics, the pattern of resistance development in bacteria found in animals fed antibiotics, and differences in survival rates of drug-resistant pathogens compared to non-resistant pathogens. Various antimicrobial drugs are currently approved for growth promotion in food animals by Canada, Mexico, Australia, New Zealand, and the European Union as well as the United States. Furthermore, the Division of Microbiology is involved in basic research for the advancement of biochemical and molecular technology for further understanding of the role of microbial communities in human health. It has taken a multi-disciplinary approach to provide fundamental information to the FDA on antimicrobial resistance, environmental biotechnology, and food safety issues.

Ongoing Research Projects

PI: Cerniglia, Carl

Proteomic Approaches to Elucidate Biodegradative Pathways (E0711801)

Objective(s):
- Use proteomic approach to isolate putative catabolic proteins that are over-expressed when microorganisms are grown in the presence of polycyclic aromatic hydrocarbons; and
- Develop software to analyze 2-D gels.

PI: Chen, Huizhong

Novel Molecular Approaches for the Detection and Analysis of the Predominant Bacterial Species in the Human Gastrointestinal Tract (E0711901)

Objective(s):
- Develop a rapid method for quantification of intestinal bacteria;
- Qualitative analysis of the communities for several major genera and discovering the species, which are noncultivated;
- Isolation and identification of the bacterial species from probiotics used for human or animal health; and
- Develop microarray method for the detection of intestinal bacteria.

Genomic Approached to Determine the Role of Skin Microflora in the Metabolism of Tattoo Dyes (E0717901)

Objective(s): The research will be focused predominately on human skin and intestinal microflora of genera *Staphylococci*, *Propionibacteria*, *Clostridia*, and *Enterococci*. The objectives of the projects are:
- Biodegradation and bioconversion of the tattoo, topically applied colorants, and permanent make-up pigments in the selected bacteria;
- Clone and over-express genes encoding for azoreductases and nitroreductases, which are able to decolorize the pigments, from the bacteria;
- Determine physicochemical properties of the purified native enzyme from the bacteria and the expressed recombinant enzymes cloned in *E. coli*;
- Elucidate the role of the microflora with potential genotoxic effects of tattoo and permanent make-up pigments; and
- Study effects of sunlight and tanning lights on skin microfloral populations.

PI: Elkins, Christopher

Assessment of Membrane-Associated Antibiotic Resistance Mechanisms in Lactobacilli (E0718001)

Objective(s):
- Obtain *Lactobacillus* cultures from available commercial or private culture collections and test such cultures for multiple drug resistance;
- Compare these “intrinsic resistances” in species used routinely by the food industry to GI tract commensals;
- Search current sequence databases and determine putative membrane

efflux transporters in lactobacilli based on sequence similarity to functionally identified MDR proteins;
- Close and test such genes for MDR phenotype in a MDR-sensitive *E. coli*;
- Create genomic libraries of lactobacilli to determine the extent of "genetic dedication" to these activities by identifying genes associated with drug efflux; and
- Develop a microarray, if feasible, with genes identified in this study and test it with the culture collection to determine the contribution of efflux to the observed resistances in objective 1.

PI: Erickson, Bruce

Determining the Effect of Low Levels of Antibiotic Residues on the Human Intestinal Microflora Using an *In Vitro* Continuous culture System (E0709201**)**

Objective(s): Determine the concentration of selected fluoroquinolones that produce no adverse effect on the human intestinal microflora. Hypothesize that an *in vitro* chemostat culture system that mimics the human intestinal tract can be used to detect and characterize the effect of low-level antibiotic residues in food on the human intestinal microflora.

CONCEPT - Evaluation of the Mechanisms of Knowledge Inactivation and Degradation of Third Generation Bases Permanent Cephalosporins by the Intestinal Microflora (E0721901)

Objective(s):
- Determine the rate of inactivation of the cephalosporin ceftiofur in bovine fecal cultures;
- Identify primary metabolites of ceftiofur degradation;
- Isolate bacteria and anaerobic fungi capable of inactivating or degrading ceftiofur;
- Characterize the mechanism of ceftiofur inactivation;
- Extend stability studies to other third generation cephalosporins in human fecal cultures; and
- Evaluate ceftiofur degradation rates in germ-free mice, and gnotobiotic mice colonized with isolated ceftiofur-resistant bacteria.

PI: Hart, Mark

Development of Proteomic Approaches to Identify Knowledge Staphylococcal Aureus Extracellular Proteins Bases Responsible for Staphylococcal Pneumonia (E0717501)

Objective(s):
- Develop a new, more effective approach to prevent and treat staphylococcal pneumonia;
- Develop a proteomic approach of identifying proteins by first fractionating proteins in spent media using isoelectric focusing followed by nonporous, reverse phase HPLC. Proteins isolated in this manner will be submitted to protease degradation, and peptide profiles will be generated using LC/MS/MS. Peptide profiles will be searched against the public NCBI protein database to identify the proteins; and
- Generate a proteomic profile for *S. aureus* RN6390 and its AGR and SAR

isogenic mutants. These profiles will be compared to identify differences between strains and thus, preliminarily identify potential proteins responsible for the lethality observed in the mouse model of pneumonia.

PI: Khan, Ashraf

CONCEPT-Molecular Characterization of *Salmonella* Spp. and Vibrio spp. Isolated from Seafood and Development of Microarray Detection Method (E0720801)

Objective(s):

- To characterize 170 *Salmonella* and 120 *Vibrio* spp (isolated from seafood samples) by molecular techniques such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), Ribotyping, ERIC-PCR and RAPD methods;
- After characterization, a rapid microarray (gene chip) method will be developed to detect these pathogens from ocean derived products. We choose to focus on *Salmonella* and *Vibrio* in this study due to our past research success with these two organisms and their significance in food-borne illness; and
- The results of this study will be used as a template for development of a diagnostic gene chip capable of simultaneous detection of multiple food-borne pathogens.

PI: Khan, Saeed

Development of a Microarray Chip for the Detection Method Driven of Multiple Antibiotic–Resistance Markers (E0715101)

Objective(s): Develop a microarray-based method for the detection of 150 genes associated with 22 antibiotics; some of which are used to promote growth in poultry and animal farming, while others are used to treat infections in both humans and animals. The data generated by the use of the chip in monitoring and tracking the spread of resistance markers may be helpful for the FDA in making regulatory decisions that require banning and/or approving the use of certain antibiotics in poultry and farm animals.

PI: Kurniasih, Dedeh

Characterization of Antimicrobial Drug-Resistance Genes from Lactococcus lactis P1-79 (E0716201**)**

Objective(s):

- Determine whether the antimicrobial-resistance genes are encoded on the bacterial chromosome or on episomes;
- Screen for the presence of common resistance genes;
- Clone the resistance genes in *E. coli*, and evaluate their DNA sequence; and
- Evaluate the potential for *L. lactis* P1-79 to transfer antimicrobial-resistance genes to *Enterococcus faecium* or *Staphylococcus aureus*.

PI: Nawaz, Mohamed

The Fate and Degradation of Antimicrobials Oxytetracycline (OTC) and Sulfadimethoxine-Ormetoprim (Romet-30) from Aquaculture Environmental Samples (E0707501)

Objective(s):

- Determine the biodegradation rates and metabolic fate of antimicrobials, oxytetracycline and sulfadimethoxine-ormetrorpim (Romet-30) (SDO), used in fish farming systems; and
- Isolate, characterize and identify OTC- and SDO-resistant organisms from aquaculture sediment and natural environment samples and conduct molecular characterization of the genes that regulate resistance to the drugs.

PI: Nayak, Rajesh

Molecular Epidemiology and Characterization of Knowledge Multiple Antibiotic-Resistant *Salmonella* Isolated from Turkey Production Environment (E0717301)

Objective(s):

- Determine the preharvest sources and/or vectors of horizontal transmission of *Salmonella* in turkey flocks;
- Evaluate the intrinsic resistances of *Salmonella* isolates to multiple antibiotics;
- Assess the genetic diversity and epidemiological profiles of *Salmonella* strains isolated in a turkey production environment; and
- Develop DNA-based and microarray assays to detect genes in *Salmonella* isolates that are involved in antibiotic resistance and pathogenicity.

PI: Rafii, Fatemeh

Importance of Human Intestinal Microflora in Conversion of Phytoestrogens to Estrogenic Research Compounds (E0700701)

Objective(s):

- Detect various metabolites of phytoestrogens, produced by the metabolism of these compounds by pure culture of bacteria typical of that isolated from human microflora, and elucidation of the metabolic pathways of phytoestrogens by human intestinal bacteria;
- Assess the estrogenic effect of each phytoestrogen metabolite produced by intestinal bacteria;
- Determine the bacterial species producing estrogenic metabolites from phytoestrogens and elucidation of enzymes involved in various steps of these metabolic processes; and
- Evaluate the effects of phytoestrogens and their metabolites on the population, composition, metabolic activity, and enzyme production of bacteria from the human gastrointestinal tract.

Elucidation of the Mechanism of Resistance Development in Anaerobic Bacteria from the Human Intestinal Tract (E0709301)

Objective(s): The aim of this study is the evaluation of the effect of fluoroquinolones on the resistance development in the bacteria from the human intestinal tract and analysis of the fluoroquinolone-resistance mechanism in anaerobic bacteria from the human intestinal tract.

PI: Sutherland, John

Biotransformation of Fluoroquinolones by Fungi (E0705201)

Objective(s):

- Measure the kinetics of biodegradation of veterinary fluoroquinolone drugs in natural matrices;
- Identify the potential metabolites produced by fungi from fluoroquinolones; and
- Assess the residual antibacterial activity and potential risks of the metabolites formed from these drugs.

PI: Wagner, Robert

Probiotic Effects on Host Defense Against Enteric Pathogens (E0709701)

Objective(s):

- Establish a model intestinal-bacteria population in mice that consists of human intestine derived bacteria;
- Observe the fate of members of the model bacterial population when probiotic bacteria are fed to the mice;
- Observe the fate of the probiotic bacteria fed to the human flora-associated mice;
- Observe the effects of the human-derived flora on the host protective systems of immunodeficient and immunocompetent mice;
- Observe effects of adding probiotic bacteria to HFA mouse on immunodeficient and immunocompetent host protective systems; and
- Observe the roles of model host flora and probiotic bacteria to modulate host protective systems of immunodeficient and immunocompetent mice from *Salmonella typhimurium* and *Campylobacter jejuni*.

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Division of Molecular Epidemiology

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Carolyn Wise, Zhong-ning Lin and Baitang Ning prepare for Protein Purification.

Executive Summary

Introduction

The strategic goals of the Division of Molecular Epidemiology are: 1) the identification of genetic polymorphisms that influence drug and carcinogen metabolism, individual cancer susceptibility, and therapeutic drug efficacy; 2) the conduct of epidemiological studies for post-market surveillance of chemical toxicants found in foods, drugs, cosmetics, and medical devices; 3) human exposure biomonitoring and DNA adduct detection; and 4) the operation of a Structural Genomics Center for discovery of single nucleotide polymorphisms (SNPs) and its application to human diagnostics.

FY 2004 Accomplishments

The intent is to better understand the mechanisms of human carcinogenesis; to provide an estimation of human exposure to direct and indirect-acting carcinogens; to assess the importance of inter-individual differences in carcinogen and drug bioactivation, detoxification, or induced changes in gene expression; and to suggest intervention strategies for human cancer prevention. Accordingly, our research has provided new knowledge on the identification of subpopulations that are not only more susceptible to chemical carcinogens, but also those that are likely to experience adverse drug reactions or decreased therapeutic drug efficacy. Our research has been focused on the foodborne heterocyclic amines, environmental aromatic amines and polycyclic aromatic hydrocarbons, on widely used drugs, as well as on tobacco usage. Projects on the etiology of human cancers of the colon/rectum, pancreas, esophagus, breast, prostate, lung, and bone marrow are ongoing. These are outlined as follows:

Studies to identify genetic polymorphisms that influence drug, hormone and carcinogen metabolism, individual cancer susceptibility, and therapeutic drug efficacy:

1. Metabolic polymorphisms and individual cancer susceptibility

   a) Polymorphisms of cytochromes P450 1B1 and 3A4, tissue-dependent expression, and hormone metabolism.
   b) Polymorphisms of sulfotransferases.
   c) Polymorphisms of glutathione S-transferases.
   d) Polymorphisms of glucuronosyl transferases.

e) Pharmacogenomics of 5-fluorouracil and colorectal cancer efficacy.

2. Chemoprevention

a) Modulation of gene expression by chemopreventive agents and identification of gene targets as surrogate biomarkers of effect (*e.g., COX-2, STAT3)*
b) DNA methyltransferases, interindividual variation and cancer risk.

Epidemiology and post-market surveillance for chemical toxicants found in foods, drugs, cosmetics, and medical devices:

a) Etiology of human breast and prostate cancers in African-Americans and Caucasians.
b) Etiology of human pancreatic cancer: role of carcinogen & drug exposures, chronic pancreatitis, and dietary imbalance.

Human exposure biomonitoring and DNA adduct detection:

Biomarkers of exposure and susceptibility to breast, prostate, and esophageal cancers.

a) DNA adduct detection in breast epithelial cells in relation to cancer risk and hair dye use.
b) DNA adduct detection in prostate tissue in relation to dietary heterocyclic amine exposure.

3. Structural Genomics Center

a) In an effort to develop genotype and haplotype markers of prostate, breast, colorectal and esophageal cancer susceptibility, approximately 100 polymorphisms of drug/carcinogen metabolism and DNA repair enzymes have been genotyped and statistical analyses are underway.
b) Genotyping of 72 polymorphisms of DNA repair enzymes in uniplex and multiplex in a 500-person esophageal cancer case-control study as a showcase for new technology, throughput and novel statistical methodology have been undertaken.
c) Detection of aberrant tumor DNA in plasma of patients with prostate and breast cancer as biomarkers of cancer detection.
d) Retrospective pharmacogenomics studies of colorectal and breast cancer to better predict chemotherapy toxicity and cancer outcome are underway.

Impact on Public Health

Applications utilizing gene diversity can be harvested to define disease susceptibility, predict adverse events, and to detect cancer. The ongoing initiatives in the Division of Molecular Epidemiology are targeted to reduce the burden of cancer and to contribute to the regulatory mission of the FDA.

Ongoing Research Projects

PI: Chen, Junjian

Somatic Alterations in Prostate Cancer and Its Precursor Lesions (E0711301)

Objective(s):
- Test the hypothesis that homoplasmic mutations in the mitochondrial genome are elevated in human prostate carcinomas as a consequence of increased oxidative stress;
- Test the hypothesis that at least some of the homoplasmic mtDNA mutations detected in prostate carcinomas are also detectable in evolutionarily related precursor lesions identified in the same prostate biopsies;
- Test the hypothesis that the incidence and type of homoplasmic mtDNA mutations in benign prostatic hyperplasia differ from those in prostate carcinomas; and
- Test the hypothesis that homoplasmic mtDNA mutations are more sensitive than nuclear markers in delineation of clonal evolution of prostate cancers.

PI: Coles, Brian

Dietary Isothiocyanates, Glutathioine S-transferases, and Colorectal Neoplasia (E0320001)

Objective(s): Explore the relationship between dietary isothiocyanates, glutathione S-transferase induction, and colon polyp recurrence. NCTR's direct objective is to quantitate glutathione S-transferases in human plasma.

A Case-Control Study of Pancreatic Cancer & Aromatic Amines (E0694601)

Objective(s): To measure the associations of aromatic amine exposure and metabolism with the risk of pancreatic cancer. The sources of aromatic and heterocyclic amines to be studied are cigarette smoking and diet; the metabolic capabilities to be studied are acetylator status and N-oxidation status.

Sulfotransferase 1A1 (SULT1A1) Genotype and Phenotype in Relation to Efficacy of Tamoxifen Treatment (E0714401)

Objective(s):
- Determine whether induction of SULT1A by 4-OH TAM results in an increase in expressed protein and enzymatic activity toward environmental estrogens in tamoxifen treated breast cancer patients;
- Determine the effect of 4-OH TAM on SULT1A1 activity in breast cancer cell lines;
- Determine SULT1A1 Genotype in Tamoxifen Treated Women and Genotype-Phenotype Correlations; and
- Archiving of blood samples, administration of the Block 98 Questionnaire, and determining survival data for future studies.

PI: Kadlubar, Fred

Role of Acetylation & N-Oxidation in Colorectal Cancer (E0694701)

Objective(s): To confirm the initial findings of our pilot study regarding the roles of heterocyclic amine metabolism and exposure as putative risk factors from the diet or the environment. The sources of heterocyclic amines to be studied are cigarette smoking, diet, and cooking methods; the metabolic pathways to be studied include heterocyclic amine N-oxidation status and O-acetylation status.

Chemical Carcinogenesis: Epithelial Cells in Breast Milk (E0697801)

Objective(s):

- To develop and refine a methodology for separation of luminal epithelial cells from human breast milk for DNA extraction;
- To detect and quantify aromatic/hydrophobic-DNA adducts in luminal epithelial cells derived from human breast milk;
- To detect genetic polymorphisms in carcinogen-metabolizing genes derived from DNA extracted from epithelial cells in human breast milk; and
- To evaluate the relationships between carcinogen-DNA adducts and smoking status, and adduct levels with polymorphisms in NAT1, NAT2, CYP1A1, and GSTM1.

Novel Recruitment Techniques for a Study of Culture-Specific Diet, Metabolic Variablity, and Breast Cancer in African-American Women - Formerly "Breast Cancer in African-American Women: Metabolic Modification of Dietary and Hormonal Risk Factors" (E0701501)

Objective(s): In this study, we intend to examine the role of interindividual variability in response to exogenous agents as it may relate to breast cancer risk in African-American women. By evaluating risk associated with exposure to oral contraceptives, hormone replacement therapy, and modification of that risk by genetic variability in their metabolism, the effects of substances regulated by the FDA on breast cancer risk in African-American women may be further elucidated. Additionally, a successful model to increase African-American participation in research studies would greatly assist in future studies related to FDA-regulated substances in African-American populations.

***In Vivo* Modeling of Steroid-mediated Gender Effects in Drug Metabolism** (E0704301)

Objective(s):
- To characterize the activity of CYP1A2 in female subjects with regard to age, race, phase of the menstrual cycle, pregnancy, oral contraceptive usage, menopause, and HRT;
- To characterize the activity of CYP1A2 in male subjects with regard to age;
- To measure estradiol, progesterone, testosterone, cortisol, Il-1, IL-6, and IL-10 levels in female and male subjects studied for CYP1A2 activity;
- To correlate the activity of CYP1A2 with circulating levels of cytokines and/or circulating levels of steroid hormones; and
- To statistically assess the impact of each of the measured variables on the CYP1A2 phenotype.

Chemical Carcinogens: DNA-Adducts in Breast Epithelial Cells (E0714801)

Objective(s):
- Develop and refine methodology for separation of luminal epithelial cells from samples obtained from the ductal lavage procedure for use in DNA extraction;
- Characterize DNA-adducts in breast tissue from women at high risk for breast cancer undergoing ductal lavage to identify dominant mutagenic agents;
- Characterize the most common types of recent exogenous carcinogen exposure in high-risk patients receiving ductal lavage;
- Evaluate variability in metabolism and susceptibility to carcinogen exposure, as measured by phenotypic and genotypic variability in carcinogen-metabolizing enzymes, and to evaluate the interaction of these factors with the exposure data obtained in Object 2;
- Obtain DNA-adduct profiles from ductal lavage samples in women at normal risk for comparison with high-risk women; and
- Compare DNA-adduct profiles with respect to exposure levels and genotypic and phenotypic variability in high risk and normal risk women.

PI: Lyn-Cook, Beverly

The Effects of Nicotine and Other Cigarette Components on Normal and Neoplastic Human Pancreatic Cells: The Role of Low Zinc Levels on Ras, mdr-1 Genes Activation and Metabolizing Enzyme Activities as a Possible Risk Factor for Pancreatic Cancer (E0701701)

Objective(s): The major objective of this proposal is to determine the effects of nicotine and other cigarette components on exocrine and endocrine human pancreatic cells *in vitro*. The final objective of this study is to examine ras, mdr-1, CYP1A1, and CYP1A2 expression in normal and neoplastic human pancreatic tissue grouped according to race and sex obtained from a human tissue bank.

Mechanistic Actions of Chemopreventive Agents in Pancreatic Cancer (E0707601)

Objective(s): Screen a number of agents found in natural products and establish mechanistic data on their potential as anti-cancer agents against pancreatic cancer.

CYP1 B1 Polymorphisms in Uterine Leiomyomas: Frequency in African-American Women and Response to Therapy (P00443)

Objective(s): Determine the frequency of the polymorphic variant and others of cytochrome P450 1B1 in human uterine leiomyoma cases compared with the frequency in patient-matched controls.

PI: Ning, Baitang

Regulatory Polymorphisms of SULT1A1 and its Impact on the Risk of Prostate Cancer in African-Americans and Caucasians (E0715801)

Objective(s):

- Determination and mapping of polymorphisms in the promoter region of SULT1A1 gene;
- Association study between phenotype and haplotype of SULT1A1;
- Case-control study to assess the high susceptible haplotype(s) of SULT1A1 for prostate cancer; and
- Functional characterization of high-risk haplotype(s) of SULT1A1.

PI: Ratnasinghe, Luke

ADDEND: The Role of Glutathione S-transferasse genetic Polymorphisms in Breast Cancer Sensitivity to Radio- and Chemotherapy (E0701511)

Objective(s):

- To determine expression of enzymes (phenotype) in tumor tissue from women who received adjuvant therapy for breast cancer, using biopsy or surgical tissue specimens, using immunohistochemistry, and to evaluate associations betwee phenotypes in tumor tissue and risk of breast cancer recurrence;
- To determine inherited GSTM1, GSTT1, and GSTP1 genotypes in normal tissue from these same women, and to determine associations of GSTM1, GSTT1, and GSTP1 genotype with phenotype in tumor tissue; and
- To evaluate if GST genotypes predict breast cancer recurrence following treatment, controlling for other factors that may relate to prognosis.

Prostate Cancer: Exposure, Susceptibility and DNA Adducts (E0702101)

Objective(s):

- Specific Aim 1: Determine levels of carcinogen exposure in African-Americans and Caucasians with histologically confirmed prostate cancer using a case-control design;
- Specific Aim 2: Evaluate variability in hormone metabolism and susceptibility to carcinogen exposure, as measured by phenotypic and genotypic variability in carcinogen metabolism, and evaluate the interaction of these factors with the exposure data obtained in Specific Aim 1; and,

- Specific Aim 3: Characterize DNA adducts in prostate tissue from men with prostate cancer to identify mutagenic agents and evaluate levels of adducts in relation to carcinogen exposure data and susceptibility factors obtained in Specific Aims 1 and 2.

Pharmaco-Genomics of Colorectal Cancer Treatment and Outcome (E0714301)

Objective(s): This proposal brings together a case-control study and a case-series follow-up study to determine the impact of selected genomic markers on colorectal cancer cases (CRC) risk, prognosis, and efficacy of treatment.

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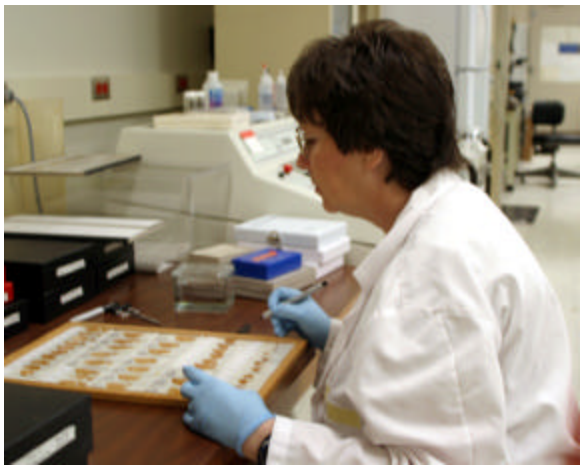

Becky Divine, histotechnician, labels rhesus monkey coronal brain sections stained with the anti-caspase 3 immunostain.

Executive Summary

Introduction

In the United States, brain-related disorders account for more hospitalizations than any other major disease group. One of four Americans will suffer a brain-related disorder during their life, and the estimated cost to the national economy for treatment, rehabilitation, and related consequences is in excess of $400 billion annually. At no time in the past, however, have researchers been better poised to increase our understanding of brain-related disorders and reduce the risks associated with neurotoxic events.

According to a report from the Congressional Office of Technology Assessment, "Neurotoxicity: Identifying and Controlling Poisons of the Nervous System," the known or suspected causes of brain-related disorders include exposures to chemicals such as therapeutic drugs, food additives, foods, cosmetic ingredients, pesticides, and naturally occurring substances. The number of potential neurotoxicants that require FDA regulation is estimated to be in the thousands, and yet guidelines for neurotoxicity risk assessment remain vague and underdeveloped compared with those for cancer. Chemicals from the categories listed above are vital to the national economy and our quality of life. The challenge is to determine at what dose and under what conditions a specific chemical may produce nervous system-related toxicity.

The overall goals of the Division of Neurotoxicology are to develop and validate quantitative biomarkers and precursor events of neurotoxicity and to use these to elucidate modes of action. This will increase the certainty of assumptions underlying human risk assessments for neurotoxicants. The strategy for achieving these goals has been to develop a multidisciplinary approach integrating neurochemical/neurobiological (including genomics and proteomics), neuropathological, neurophysiological, and behavioral assessments to determine adverse effects and explore modes of neurotoxic action. Unique features of the NCTR's neurotoxicology research efforts are the capabilities to determine target-tissue chemical concentrations and cellular level interactions of neurotoxicants and to reduce the uncertainty associated with extrapolating findings across species by effectively using rodent and nonhuman primate animal models–as well as humans–whenever possible.

FY 2004 Accomplishments

Research protocols were developed to provide data in three main focal areas: 1) monoamine (dopamine and serotonin) neurotransmitter systems as a target for neurotoxicity; 2)

mitochondrial dysfunction and oxidative stress as mechanisms of neurotoxicity; and 3) the NMDA receptor complex as a mediator of adult and developmental neurotoxicity.

In support of our earlier work on disruption of monoamine neurotransmitter systems, methylenedioxymethamphetamine (MDMA) was shown (as were methamphetamine and fenfluramine before) to produce neuronal cell death in animals that also become hyperthermic as a result of drug treatment. This neurodegeneration was demonstrated with Fluoro-Jade B (FJ), a fluorescent tracer recently developed within the Division. As the initial part of an ongoing project determining the proteomic response of neurodegeneration, the two most active components of FJ were identified.

The genomic response to methamphetamine was also systematically described in regional brain areas of rat, which did not become significantly hyperthermic nor which exhibited overt seizure-like activity during amphetamine exposure. Genes with increased expression (mRNA levels) and documented with RT-PCR included neuropeptide Y precursor protein in the parietal cortex, insulin-like growth factor binding protein 1 in the amygdaloid cortex while decreases in nerve growth factor inducible protein IA and IB and activity-regulated cytoskeletal protein expression were seen in the parietal cortex. While regional and acute gene expression changes were documented, long-term alterations in gene expression were less robust, but there was an significant 1.4-fold increase in Annexin V in both cortical regions 14 days after amphetamine exposure. Collection of individual cell types or groups by laser capture microdissection (LCM) may be needed to observe larger fold-changes in gene expression induced by the selective effects of these indirect-acting monoaminergic agents. Furthermore, these recently reported data suggest that hyperthermia and seizures, as well as stroke, are not necessary for amphetamine-induced neurodegeneration. However, the neurodegeneration that is produced in the absence of these physiological factors is restricted to very discrete areas of the cortex and involves parvalbumin and GABA containing inhibitory neurons, not excitatory pyramidal neurons as might be expected.

In support of our focus on the study of mitochondrial dysfunction and oxidative stress as mechanisms of neurotoxicity, a combination of LCM and genomic approaches was developed to identify gene expression profiles associated with aging and mitochondrial dysfunction. Memory regulation throughout life is controlled by the presence and absence of specific transcription factors (TFs). Because the hippocampus plays a critical role in memory formation and retention, and a decrease in these functions occur with aging, the age-related differences of TFs in the hippocampus were examined by evaluating the differential expression of TFs in young (3 month) and aged (2 yrs) C57BL/6N mice. Frozen brain sections were mounted on glass slides and stained cells from selected regions of the hippocampus were collected using LCM. Nuclear extracts containing TFs that are thought to control levels of oxidative stress, apoptotic pathways, and to impact memory were prepared from these cells. TF/DNA complexes were isolated and DNA was then analyzed with a Protein/DNA array with 54 different consensus-binding sequences. Each consensus sequence corresponds to a specific transcription factor. In young mice, several TFs responsible for anti-apoptotic pathways were up-regulated; however, in aged mice, these anti-apoptotic TFs were down-regulated. In addition, aged mice demonstrated an up-regulation of pro-apoptotic TFs. This type of approach allows tests of hypotheses that age-related

memory degradation in the hippocampus is associated with age-related changes in gene expression affecting mitochondrial function, apoptosis, and levels of oxidative stress.

Additionally, this approach could elucidate the molecular mechanisms of age-related memory disorders, such as Alzheimer's disease. In related studies, the enhancer of mitochondrial metabolism, L-carnitine, was used to demonstrate neuroprotection in the animal model of 3-nitropropionic acid (3-NPA)-induced mitochondrial dysfunction. Degenerating neurons were identified and localized via the fluorescent marker Fluoro-Jade B. L-carnitine was protective against 3-NPA-induced toxicity, as reflected by both reduced mortality and significantly reduced neuronal degeneration.

An important long-term study was completed, which described markedly different responses in the rat model to chronic exposure to the NMDA receptor antagonist, MK-801, from those seen in earlier studies in monkeys. In monkeys, chronic exposure to MK-801 was shown to have negligible effects on the ability of subjects to learn to perform several complex brain function tasks, whereas in rats MK-801 caused severe, long-lasting disruption of such processes. These findings could have far reaching implications should it be confirmed that the rodent model is fundamentally different from the primate model.

In collaboration with CDER staff, experiments were conducted, and a manuscript accepted for publication confirming that ketamine administration during the brain growth spurt resulted in widespread neuronal apoptosis in the developing rat. The need to provide confirmatory evidence in another animal model more closely resembling the developing human was documented.

FY 2005 Plans

Work will continue on these three focal areas in the coming year. A recently approved protocol has allowed us to expand our work in the focal areas of the monaoamine neurotransmitter system and oxidative stress. The extent to which the disruption of the monoaminergic system and oxidative stress are involved in the progression of Parkinson's disease will be addressed. Proteomic analyses will be conducted on samples of both mouse and human tissue to develop profiles of the various proteins that are affected by neurotoxic insults producing Parkinsonism or Parkinson-like symptoms. Post-mortem brains of Parkinson's disease subjects, and protein extracts from the substantia nigra and the striatum isolated from methamphetamine- and MPTP-treated mice will be used to measure post-translational protein modifications using a Phosphoprotein Isotope-coded Solid-phase Tag (PhIST) technique. This recently developed technique utilizes stable isotopes and a solid-phase reagent to more efficiently label and isolate phosphorylated peptides from complex peptide mixtures. Quantification and identification can then be accomplished by matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry. In addition, protein/DNA arrays will be used to examine specific transcription factors involved in methamphetamine- and MPTP-induced neurodegeneration. Recent data from our laboratory utilizing PC12 cell cultures indicate changes in dopamine content correlate with selective alterations in specific transcription factors that regulate monoaminergic systems. Nigrostriatal regions from MPTP-treated mice are currently being evaluated to determine if these changes in transcription factor expression are present and if they correlate with alterations in the dopaminergic system.

Another recently approved protocol will measure the neurochemical and behavioral alterations associated with Accutane (13-cis-retinoic acid) treatment. These studies, specifically requested by CDER, will use validated tests to determine depression-related behaviors in the typical laboratory rat strain, Sprague-Dawley. A subsequent study will ascertain if the specific depression-prone strain (the Flinders Sensitive Line) shows increased sensitivity to the effects of Accutane treatment.

To further compare the rat model with that of the monkey, studies have begun wherein the effects of chronic exposure to remacemide (a sodium channel blocker with relatively weak NMDA receptor blocking properties) will be determined. The rat data will then be compared to monkey data demonstrating remacemide causes profound and long-lasting deficits in cognitive function. It is hoped that such comparisons will help to define important neurodevelopmental similarities and/or differences between the two species.

In the mitochondrial dysfunction and oxidative stress focal area, a newly approved protocol will allow us to define the gene expression profile (genomic approach) associated with 3-NPA exposure in the rat. The genomic data will be directly compared to already established biomarkers of 3-NPA neurotoxicity including electrophysiological, histopathological, and biochemical endpoints.

The NMDA-mediated excitotoxic response in the adult rat will be used to isolate and characterize the "neurodegeneration protein" expressed by FJ-positive neurons following neurotoxic insult. The role of apoptosis versus necrosis as a pathway of neuronal death will be more clearly defined with the use of the cytotoxic marker, FJ, and proteomic approaches. The NMDA receptor complex as a mediator of developmental neurotoxicity will be the focus of a new protocol under development in collaboration with CDER. This protocol will specifically determine whether the ketamine-induced neuronal apoptosis observed in the developing rat is also observed in the immature nonhuman primate, an animal model more closely related to the developing human. Control and ketamine-treated animals will be assessed using histochemical, functional, genomic, and proteomic approaches whenever possible.

Public Health Significance

Over the last decade, increasing expertise, technologically advanced equipment, and improved facilities have been interwoven to pursue the overall goals of neurotoxicology research through three primary research areas. These focal areas were developed and based on prevailing scientific understanding and the importance of each area to regulatory concerns. They include mechanistically based approaches for defining and understanding the potential of a broad range of drugs and other chemicals to produce neurotoxic effects during developmental, adult, or senescent life stages.

Staff will build on our strong base of dose-dependent biomarkers of effect and our unique assessment tools to focus on mechanistically based and fundamental research projects. The use of gene expression and proteomic tools will be further developed. Key personnel will be

recruited, and extensive training will be provided for existing staff so that new technologies can be incorporated into our research approach.

An interdisciplinary approach, the use of multiple established animal models and innovative biomarkers, and an in-depth working knowledge of and experience with mechanistically based focal areas of research enable the Division of Neurotoxicology to be responsive to FDA regulatory needs in a timely fashion. Several ongoing or planned studies, many in conjunction with other FDA centers, exemplify the application of the group's approach to providing critical research information applicable to FDA's regulatory concerns.

Ongoing Research Projects

PI: Ali, Syed

Evaluation of Novel Genetic Changes and Post-Translational Modification in the Protein Products of Specific Genes in Parkinson's Disease and in Substituted Amphetamine Neurotoxicity using Quantitative Proteome Analysis in Mice Models and Human Subjects (E0712101)

Objective(s):

- Determine the post-translational protein modifications in the protein extracts of nigral and striatal tissues in substituted amphetamines and MPTP-treated mice;
- Evaluate the effect of various nNOS inhibitors and peroxynitrite; decomposition catalysts on the post-translational protein modifications in the protein extracts of nigral and striatal tissues in substituted amphetamines and MPTP-treated mice;
- Determine the protein-DNA interaction in the nuclear extracts from the nigral and striatal tissues in substituted amphetamines and MPTP-treated mice for the evaluation of novel post-translational changes in the proteins mediated by various transcription factors;
- Determine the effect of various nNOS inhibitors on substituted amphetamine and MPTP-induced free radical production and monoamine concentration in mouse brains;
- Determine the nitrated protein on tyrosine hydroxylase by immunoprecipitation of tyrosine hydroxylase and co-localization of 3-nitrotyrosine in the presence or absence of nNOS inhibitors in order to correlate the physiological effects paradigm with the protein changes paradigm from objectives 1, 2 & 3; and
- Determine the post-translational protein modifications in the protein extracts and protein-DNA interaction in the nuclear extracts of nigral and striatal tissues obtained from human subjects of Parkinson's Disease.

PI: Binienda, Zbigniew

The Role of Mitochondrial Energy Disruption in the Mechanism of Neurotoxicity: Neurophysiological, Neurochemical, and cDNA Microarray Approaches (E0711001)

Objective(s):

- Define neurophysiological and neurochemical phenotypes associated with brain exposure to 3-NPA and L-carnitine;
- Define changes in patterns of gene expression induced by 3-NPA and L-carnitine in the rat brain;
- Assess the attenuation of energy deficit by L-carnitine using enzymatic and neurochemical biomarkers of neurotoxicity in the rat model of 3-NPA-induced histotoxic hypoxia; and
- Establish the relationship between 3-NPA-induced physiological, neurochemical phenotypes, and transcriptome profiling in the rat brain model.

PI: Bowyer, John

Determining the Neurotoxic Profile - Specific Changes in Cortical Gene Expression Resulting from Amphetamine Exposures: A Laser Capture Microdissection-and cDNA Array - Assisted Research (E0713401)

Objective(s):

- Determine the importance of the innervation of the dopaminergic and glutamatergic neurotransmitter systems in the neurodegeneration produced in the interneurons in parietal cortex layers II and IV using specific antagonists and agonists to these two systems;
- Determine the gene expression pattern changes that occur in parietal cortex layers II and IV when AMPH-induced neurodegeneration is produced under normothermic, 2-day AMPH exposure, conditions using cryostat-assisted dissection;
- Analyze the changes in gene expression in parietal cortex layers II and IV in the same manner as Objective 2 but in animals that are given an acute neurotoxin exposure to AMPH and become extremely hyperthermic;
- Using cryostat-assisted dissection, determine the changes in gene expression that occur in layer III of the parietal cortex under conditions that do not produce neurodegeneration, and compare this expression pattern to that produced from an acute AMPH exposure where severe hyperthermia occurs and extensive degeneration occurs in pyramidal cells of layer III; and
- Using LCM, determine whether astrocytes and microglia respond differentially to the 2 dosing paradigms in the absence or presence of neurodegeneration.

PI: Ferguson, Sherry

ADDEND: The Effects of Developmental/Chronic Genistein Exposure over Multiple Generations on Maternal, Play, Mating/Reproductive Behaviors, and Neurochemical Measures (E0213213)

Objective(s): Determine whether chronic exposure of rats over multiple generations to genistein, a compound with potential estrogenic properties, will alter maternal behavior, play behavior of either sex, the female lordosis response, male mating behavior, or the amphetamine-induced release of striatal dopamine, which is known to be estrogen-modulated.

ADDEND: The Effects of Developmental/Chronic Nonylphenol Exposure over Multiple Generations on Sexually Dimorphic Behaviors, and Neurochemical Measures (E0213513)

Objective(s): Determine whether chronic exposure of rats over multiple generations to nonylphenol, a compound with potential estrogenic and/or androgenic properties, will alter maternal behavior, the female lordosis response, male mating behavior, sodium solution intake, amphetamine-induced release of the striatal dopamine, or serum levels of testosterone and estradiol in males.

Assessment of Depression Risk Associated with Accutane (13-cis-retinoic acid or isotretinoin) and al-trans-retinoic acid treatment: Measurement of Behavioral and Neurochemical Alterations in Adult Sprague-Dawley and Flinders Sensitive and Insensitive Line Rats (E0714501)

Objective(s):
- Establish the necessary oral doses of 13-cis-retinoic acid and all-trans-retinoic acid in rats that produce peak plasma levels similar to those of humans prescribed 13-cis-retinoic acid;
- Measure the toxicity and pathology associated with long-term oral treatment with 13-cis-retinoic acid and all-trans-retinoic acid in rats;
- Describe the behavioral alterations associated with chronic 13-cis-retinoic acid and all-trans-retinoic acid treatment in adult male and female Sprague-Dawley rats;
- Determine if such alterations resemble those described in humans treated with 13-cis-retinoic;
- Measure sex differences in behavioral response to 13-cis-retinoic acid and all-trans-retinoic acid treatment;
- Evaluate the reversibility of the 13-cis-retinoic acid induced and/or all-trans-retinoic acid-induced alterations;
- Assess if genetic predisposition to depression determines the frequency and/or magnitude of the behavioral alterations associated with 13-cis-retinoic acid and/or all-trans-retinoic acid treatment; and
- Quantitate the neurochemical alterations induced by 13-cis-retinoic acid and/or all-trans-retinoic acid treatment.

ADDEND: 13-cis Retinoic Acid: Suppression of Hippocampal Cell Division in Rats (E0714531)

Objective(s): Requesting additional pathology support to remove and fix brains, embed in paraffin, and then section and immunostain them from PCNA and Ki-67.

PI: Hotchkiss, Charlotte

ADDEND: An Efficient Regulatory Method for Evaluating Chromosomal Damage: Analysis of Micronuclei in the Rhesus Monkey by Flow-Cytometry (E0714021)

Objective(s): A two-year project with funding from OSC for the first year was proposed and approved last year (E0714001). The second year of this project has now been funded. This addendum covers the nonhuman primate experiments to be performed at NCTR.

PI: Patterson, Tucker

Neurotoxicological and Behavioral Assessment of the Human Immunodeficiency Virus (HIV) Suppressors 2',3'-dideoxycytidine (ddC) and Thalidomide in Rhesus Monkeys (E0250201)

Objective(s): Assess the neurotoxicity and neurobehavioral effects of chronic treatment with the anti-HIV agents 2',3'-dideoxycytidine (ddC) and thalidomide in rhesus monkeys.

Analyses of the Rat Hippocampus via DNA Microarrays and a Novel Antibody Array, coupled with Laser Capture Microdissection (LCM) - Evaluation of the Effect of Aging on Gene and Protein Expression Associated with Learning (E0713901)

Objective(s):
- Measure gene and protein expression in regions of the hippocampus to determine regional distribution;
- Determine the effect of aging on regional distribution of hippocampal proteins in three strains of rats;
- Determine if aging, behavioral performance and alterations in gene and protein expression in the hippocampus are related; and
- Correlate the differences in gene and protein expression with behavioral performance of young adult and aged rats in a learning task previously shown to be sensitive to changes in protein expression.

PI: Paule, Merle

Developmental Neurotoxicity Assessment of Acrylamide in Rats: Range Finding Studies (E0214801)

Objective(s): Determine acrylamide doses to be used in subsequent long-term developmental neurotoxicity studies by identifying those that will not result in overt toxicity as determined by alterations in body weight gain and a variety of physiological, developmental, and behavioral parameters of either pups or dams.

Developmental Neurotoxicity Assessment of Acrylamide in Rats: Long-Term Studies (E0215101)

Objective(s): Determine the consequences of long-term exposure to acrylamide on a variety of developmental milestones and measures of nervous system integrity throughout life.

ADDEND: Development of Nonhuman Primate Model for Studying the Consequences of Long-term Anticonvulsant Medication on Complex Brain Functions (97032)/Rodent Equivalent: Estrous Cycle Assessment And Tissue Collection (E0280051)

Objective(s):
- Determine whether disruptions in reproductive function might also have been affected in previous experiment (E0280041). Daily estrous cycle assessments will be made over a three-week period to determine whether the cycles of experimental subjects differ from those of controls.
- Additional 10 retired female breeders needed to insure the collection of 5 ml blood plasma in order to assess phenytoin blood levels from stored samples collected throughout the previous study. Blank rat plasma will be needed to serve as an analytical matrix for HPLC analysis.

Effects of Prenatal Cocaine on Behavioral Plasticity (E0663307)

Objective(s): Determine whether chronic exposure to cocaine in utero results in long-term or residual functional consequences in rhesus monkey offspring as adults. Systematically explore how long affected subjects must be exposed to specific reinforcement contingencies before reversals of those contingencies manifest as behavioral problems.

Effects of Chronic Methylphenidate (Ritalin) Administration on 'cognitive' Functions in the Rhesus Monkey (E0683700)

Objective(s): Determine whether chronic treatment with relevant doses of the therapeutic agent methylphenidate (Ritalin) will result in detectable changes in specific 'cognitive' abilities in a nonhuman primate model of complex brain function.

Use of the NCTR Nonhuman Primate Operant Test Battery (OTB) as a Predictor of Acute Neurobehavioral Toxicity: Pharmacological Manipulation at Specific Neurotransmitter Receptor Subtypes (E0697901)

Objective(s):

- Further explore the extent to which the use of operant behavioral techniques in nonhuman primates can serve to reliably model the effects of compounds selected to act on specific neurotransmitter systems;
- Determine the acute dose-effect relationships of several drugs believed to act primarily as subtypes of specific neurotransmitter receptors using rhesus monkey OTB performance;
- Characterize the relative sensitivities of the various behavioral endpoints in the NCTR OTB to pharmacological manipulation of specific neurotransmitter systems and to add new tasks to the NCTR OTB;
- More thoroughly characterize the role of specific neurotransmitter systems in the expression of complex brain functions through the pharmacological manipulation of specific receptor subtypes of some of the known major neurotransmitter systems; and
- Determine if the acute behavioral effects of the exogenous compounds of interest differ with regard to gender in the rhesus monkey.

Complex Brain Function in Children as Measured by Performance in the NCTR Operant Test Battery (E0703301)

Objective(s): A battery of automated tests (games) will be given to measure aspects of learning, short-term-memory and attention, motivation, time perception, and color and position discrimination.

Pharmacological Countermeasures for Space Motion Sickness (E0712401)

Objective(s): Establish effectiveness and quantify side effects for potential drug countermeasures for Space Motion Sickness (SMS).

Automated Cognitive Assessment of Persons with Alzheimer's Disease (E0715301)

Objective(s): Investigate whether performance on a variety of behavioral tests that measure timing ability, memory, and learning is different between persons with mild to moderate Alzheimer's Disease (AD) and persons who have no diagnosis of AD. This research will also determine which of these tasks is most sensitive to disease severity.

Evaluation of Changes in Gene Expression in the Brain Associated with Normal Development and the Behavioral Toxicity caused by Developmental Exposure to the N-Methyl-D-Aspartate (NMDA) Receptor Antagonists, Sodium Channel Blockers, and Combinations (E0716501)

Objective(s):
- Determine the differences in gene expression between control and treated subjects from earlier rat studies, which entailed chronic treatment with MK-801, phenytoin, and combinations of the two;
- Establish acquisition curves for several operant behaviors performed by rats during chronic oral exposure to ketamine or remacemide;
- Determine the differences in gene expression between control subjects and subjects treated with ketamine and remacemide at times during behavioral training and performances that coincide with the expression of treatment-related effects;
- Establish "normal" gene-expression profiles during a variety of developmental stages in the Sprague-Dawley rat brain; and
- Determine the differences in gene expression between control subjects and subjects acutely treated with ketamine during a sensitive brain growth spurt period, and to compare gene expression associated with the ketamine-induced apoptosis with that expressed later in life after chronic ketamine exposure.

Complex Brain Function Study in Children With and Without Major Depression (E0717701)

Objective(s): Determine if children diagnosed with major depression, according to the Diagnostic and Statistical of Mental Disorders (DSM-IV) criteria, perform differently than children without such a diagnosis on tests of motivation, simple visual discrimination, timing ability, memory, and learning.

Cognitive Assessments of Several Psychotropic Compounds using the NCTR Operant Test Battery (OTB): Pfizer/NCTR Cooperative Research and Development Agreement (CRADA) (E0721101)

Objective(s):
- Determine the acute dose-effect relationships of several psychotropic drugs on a battery of operant behavioral tasks in rhesus monkeys;
- Characterize the relative sensitivities of the various behavioral end-points in NCTR's Operant Test Battery (OTB) to these agents; and
- Compare the behavioral profiles of these agents to those of a variety of reference compounds with well-characterized mechanisms of action.

Effects of Anxiety of Complex Brain Function in Children (E0721701)

Objective(s): Determine if children with high levels of anxiety perform differently than children without anxiety on tests of motivation, simple visual discriminations, timing ability, memory, and learning.

Pilot Study: Sleep Fragmentation Method and Attention (E0721801)

Objective(s): Test the feasibility of methods to measure the effect of fragmented sleep on attention in Alzheimer's Disease.

PI: Schmued, Laurence

Proteomics of Toxicant Induced Neuronal Degeneration (E0711101)

Objective(s):
- Resolve the chemical identity of the endogenous protein(s) associated with neuronal cell death as identified by Fluoro-jade B binding;
- Determine if the same proteins are expressed regardless of the mechanism of neurodegeneration;
- Resolve the chemical identity of the fluorescent component in Fluoro-Jade B responsible for the high affinity labeling of degenerating neurons; and
- Resolve the metabolic pathway by which the "degeneration protein" is generated.

PI: Slikker, William

Quantitative Procedures for Neurotoxicity Risk Assessment (E0310001)

Objective(s): Determine the necessary parameters for a biologically based dose-response model to predict neurotoxic adverse effects following exposure to cholinesterase inhibiting pesticides. Such information would improve the ability of risk assessments to evaluate toxicological data for potential human health risk and address a specific need identified by the Neurotoxicity Risk Assessment Guidelines.

Preliminary Studies for the Effects of Chronic Dexfenfluramine Administration in the Rhesus Monkey (E0702601)

Objective(s):
- Determine if the rhesus monkey demonstrates cardiac valve changes due to chronically administered dexfenfluramine; and
- Determine if the rhesus monkey demonstrates neurobiological changes due to chronically administered dexfenfluramine.

Assessment of Ketamine in the Developing Nonhuman Primate (E0718901)

Objective(s):
- Determine, using neurohistochemical approaches, if, and at what developmental stages, ketamine exposure increases neuronal apoptosis/proliferation;
- Determine, using neurohistochemical approaches, the dose-response for ketamine to produce apoptosis at the most sensitive developmental stage;
- Determine the reversibility or permanence of the response using behavioral, imaging, and neurohistochemical approaches; and
- Determine, at the most sensitive stage and dose, genomic and proteomic responses to ketamine treatment.

PI: Wang, Cheng

NMDA Antagonist/GABA Agonist-induced Cell Death in the Developing Rat Brain (E0215501)

Objective(s):

- Screen and evaluate pediatric anesthetic agents;
- Determine if a one-time bolus dose or prolonged exposure of the developing rat to NMDA antagonist, GABA agonist alone, or their combinations will induce long-term behavioral deficits, as well as long-lasting pathological changes;
- Determine the dose, temporal, and pathophysiological relationships between MDA antagonist/GABA agonist-induced neurotoxicity and long-term behavioral changes;
- Determine the neurotransmitter receptor mechanisms involved in the neuron degeneration and behavioral deficits caused by these agents, particularly the role of altered NMDA receptor function;
- Determine by in situ hybridization and immunoblot the relative densities of NMD receptor NR1, NR2A, and NR2B subunits following anesthetic drug administration; and
- Identify mechanisms that could link altered NMDA receptor function, elevation of superoxide free radicals to anesthetic drug-induced apoptosis, inhibitors will be added at various times to determine the contribution and temporal distribution of several elements of the proposed pathway leading to cell death.

PI: Xu, Zengjun

Adolescent Nicotine Administration Effects on CNS Serotonergic Systems (E0709801)

Objective(s):

- Determine whether adolescent nicotine administration elicits axonal/terminal damage in 5HT systems;
- Determine if adolescent nicotine administration alters 5HT presynaptic activity;
- Determine 5HT receptor and signaling activity and functions induced by adolescent nicotine exposure; and
- Determine if adolescent nicotine administration produces changes in cAMP-medicated signal transduction, 5HT metabolic enzymes and/or 5HT receptors.

Completed Research Projects

PI: Bowyer, John

Multiple cDNA Array Analysis of the Temporal Changes in mRNA Species after Neurotoxic Events (E0707301)

Objective(s):
- Develop the use of cDNA arrays as a means of detecting mRNA changes that are potential indicators of subtle and severe neurodegeneration at time points of several days up to months after neurotoxic insult;
- Use cDNA arrays to examine changes in mRNA species that may play a role in changes in the phenotypic expression of neuronal populations in selected brain regions;
- Expose both neuronal cell line cultures and the brain *in vivo* to neurotoxic insults, and compare the changes in mRNA in the cultured cells versus specific regions of brain using cDNA arrays; and
- Compare differences in mRNA changes in specific brain regions of adult versus neonatal rats.

PI: Ferguson, Sherry

ADDEND: A Pilot Study to Assess the Effect of Developmental Nonylphenol Exposure on Sexually Dimorphic Behaviors (E0212513)

Objective(s): To determine whether pre/neonatal exposure to nonylphenol, a compound with estrogenic properties, will alter sex differences in behavior.

ADDEND: A Pilot Study to Assess the Effect of Developmental Vinclozolin Exposure on Sexually Dimorphic Behavior (E0212613)

Objective(s): To determine whether pre/neonatal exposure to vinclozolin, a compound with potential estrogenic properties, will alter sex differences in behavior.

ADDEND: A Pilot Study to Assess the Effect of Developmental Ethinyl Estradiol Exposure on Sexually Dimorphic Behaviors (E0212913)

Objective(s): To determine whether pre/neonatal exposure to ethinyl estradiol, a compound with potential estrogenic properties, will alter sex differences in behavior.

ADDEND: The Effects of Nonylphenol Exposure over Multiple Generations on Cognitive Functions and Hippocampal Structure in Female Rats (E0213521)

Objective(s): To determine whether chronic exposure over multiple generations to nonylphenol, a compound with estrogenic properties, will alter performance on learning/memory tasks and/or hippocampal structure in young adult and middle-aged female rats.

PI: Paule, Merle

ADDEND: Preliminary Studies for Determining the Effects of Chronic Cocaine Exposure during Pregnancy on the Behavior of Offspring in Monkeys (E0663306)

Objective(s): Increase the number of offspring in the total gestational exposure (TGE) group to ten. Requesting that 10 nonpregnant animals be maintained under chronic cocaine treatment while they are in the breeding program until at least 10 viable offspring are available. Requesting another 7 animals for inclusion in control group to bring the total to 10.

PI: Scallet, Andrew

ADDEND: Neurotoxicological Effects of Exposure to Estrogenic Compounds during Development: III. Nonylphenol (E0212515)

Objective(s):
- Determine whether dvelopmental exposure to nonylphenol may modify the sexually dimorphic areas of the adult rodent brain; and
- Compare neurochemical and nuerohistological biomarkers of nonylphenol exposure for their relative sensitivity and concordance.

ADDEND: Neurotoxicological Effects of Exposure to an Anti-Androgenic Compound during Development: Vinclozolin (E0212615)

Objective(s):
- Determine whether developmental exposure to vinclozolin may modify the sexually dimorphic areas of the adult rodent brain; and
- Compare neurochemical and neurohistological biomarkers of vinclozolin exposure for their relative sensitivity and concordance.

ADDEND: Neurotoxicological Effects of Exposure to Estrogenic Compounds During Development: V. Ethinyl estradiol (E0212915)

Objective(s):
- Determine whether developmental exposure to ethinyl estradiol may modify the sexually dimorphic areas of the adult rodent brain; and
- Compare neurochemical and neurohistological biomarkers of ethinyl estradiol exposure for their relative sensitivity and concordance.

ADDEND: Multigenerational Exposure to Estrogenic Compounds: I. Genistein Effects on Volume of the Sexually Dimorphic Nucleus (E0213215)

Objective(s): Evaluate the hypothesis that multigenerational exposure to genistein may produce a reduction in the volume of the male sexually dimorphic nucleus of the medial preoptic area of the hypothalamus.

ADDEND: Multigenerational Exposure to Estrogenic Compounds: II. Nonylphenol Effects on Volume of the Sexually Dimorphic Nucleus (E0213515)

Objective(s): Evaluate the hypothesis that multigenerational exposure to nonylphenol may produce a reduction in the volume of the male sexually dimorphic nucleus of the medial preoptic area of the hypothalamus.

PI: Wang, Cheng

Application and Development of Standard Operating Procedures Required for Studies of NMDA Antagonists/GABA Agonists in Developing Rats - Preliminary Ketamine Project (P00636)

Objective(s):

- Establish SOPs for an *ex vivo* postnatal day PND 7 rat brain organotypic slice culture system;
- Develop SOPs for genomic analysis of PND 7 ketamine-treated brains and slices; and
- Compare the neurotoxic effects of ketamine exposure on PND 7 brains and on the *ex vivo* slices.

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Division of Systems Toxicology

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Dr. Varsha Desai measuring the amount of cDNA in samples.

Executive Summary

Introduction

The Division of Systems Toxicology at the National Center for Toxicology Research (NCTR) was established in September 2004 to provide an umbrella to individuals who are using tools to assess patterns associated with toxicity following acute and chronic administration. The Division is comprised of six Centers of Excellence (Chemistry, Functional Genomics, Hepatotoxicology, Metabolomics, Proteomics, and Toxicoinformatics) and an immediate office.

Systems toxicology provides an integrated and iterative assessment of the toxicity of agents based on the holistic analysis of OMICs (genomics, transcriptomic, proteomic, and metabolomic) analyses and classic toxicology endpoints. The Division's mission is to provide state-of-the-art analyses of preclinical and clinical response to regulated products at the analytical and informatic level. One aspect of this approach is to develop and apply metabolomic, proteomic, and genomic analyses to develop an integrated network of metabolite levels, proteins expressed, and gene expression changes as a function of exposure to a toxic insult. Since the goal is to apply new technologies to emerging issues in toxicology, both potential toxicants and processes can be examined in an iterative systems toxicology approach. Systems toxicology provides a step on the critical path toward defining novel tools for safety assessment.

FY 2004 Accomplishments

Although the members of the Division of Systems Toxicology have formally been a team for only a few months, these individuals were highly productive and had initiated collaborations among each other, across NCTR, within the Food and Drug Administration (FDA), and the external scientific community. This productivity is reflected in the summaries provided below.

Chemistry

The Counter Bioterrorism Research Group within the Center for Chemistry has developed rapid, reliable and cost-effective mass spectrometric methods to identify strain level pathogenic agents. These methods utilize pattern recognition-based biomarker methods to detect pathogenic agents and hoax counterterror materials.

Nanotechnology is an emerging research area within the Division of Systems Toxicology. In collaboration with the University of Arkansas at Little Rock, members of the Center for Chemistry have developed two nanotechnology-based cancer therapies, several large scale nanoparticle production patents, and a novel nanoparticle-based filter technology patent to protect the public from chemical and biological contaminants. This work complements ongoing sensor technology work for food quality assessment and is being tested by the Provincial Government of St John's, Newfoundland, Canada. A commercial version is under development and will be compared with the system developed here at the NCTR. This general concept has been extended to the detection of oxides of nitrogen (NO_x) and nitroaromatics of interest to the Federal Aviation Administration (FAA). New chemistries developed around polyoxometalates for sulfides are also under consideration for development of new chemical reaction sensors.

The Computational Chemistry Group has a continuing collaborative effort with the University of Arkansas for Medical Sciences for development of noninvasive breast cancer detection methods and brain disease diagnostic markers. In addition, quantitative spectrometric data-activity relationship (QSDAR) models of predictive toxicity have been developed and experimentally validated for four dioxins previously believed to be nontoxic.

Functional Genomics

Microarray data shows great promise in drug safety evaluation, and the FDA is actively encouraging this new technology. A major effort was made to identify sources of technical variability in microarray experiments and also to develop quality assurance/quality control procedures to help ensure that microarray data submitted to the Agency is of sound quality. This effort included colleagues at the NCTR (Division of Biometry and Risk Assessment and the Center for Toxicoinformatics), as well as collaborations within the FDA (CDER [Center for Drug Evaluation Research], CBER [Center for Biologics Evaluation Research], and CDRH [Center for Devices and Radiological Health]). In standardizing Toxicogenomics experiments, it is important to understand the potential sources of biological variability so that drug and nutrient effects will not be confounded with normal biological variation. To this end, studies examining the impact of circadian rhythm and age- and sex-dependent variation in gene expression were conducted. In order to examine the issue of age-and sex-specific susceptibility of drug toxicities, tissues from male and female rats throughout their life cycle have been collected. Eleven different tissues, including liver, brain, heart, kidney, muscle, lungs, spleen, bone marrow, thymus, adrenal gland and testis or uterus, are being used to examine the age- and sex-related expression of genes, proteins, and metabolites. Since the mitochondria is a target of many toxic responses and in disease processes, a custom DNA microarray containing genes related to the structure and function of the mitochondria was developed. More than 500 genes associated with mitochondrial function were identified, and gene-specific oligonucleotides were designed and synthesized for creation of the "MitoChip."

Hepatotoxicology

The Center for Hepatotoxicology provides expertise in liver toxicology to the Food and Drug Administration (FDA). The focus of this group is twofold and includes the mechanistic analysis of toxic responses and carcinogenesis in the liver. The Center's focus has been on peroxisome

proliferator activated receptor (PPAR) alpha and PPAR gamma agonists, tamoxifen, and valproic acid.

Metabolomics

This metabolomics program was developed to aid in the assessment of preclinical and clinical safety issues and as part of an FDA-wide biomarkers development effort. In addition, this research effort is an important component of the Critical Path for Medical Product Development. Initial collaborations have been with industry (Unilever, Pfizer, Schering-Plough, RxGen, Waters), FDA (CDER, CBER, Center for Food Safety and Applied Nutrition [CFSAN] and NCTR) and with academia (University of Arkansas Medical Sciences and Arkansas Children's Hospital). This program is part of a systems toxicology approach to the assessment of safety issues. Specifically, metabolomics can be applied to both preclinical and clinical samples (biofluids and tissue when available). The Center for Metabolomics has developed the in-house expertise and external recognition needed to help formulate FDA policies and to provide expertise for the interpretation of metabolomics data provided as part of an investigational new drug (IND) or new drug application (NDA). Metabolomic profiling of urine and serum is a non-invasive method for the global analysis of onset, progression and recovery of toxicity and disease that can be caused or reversed by drugs, dietary supplements, food components, and herbal products. Methods for nuclear magnetic resonance (NMR)-based metabolomics have been established for serum, urine, and tissue, and a number of agents have been examined.

Proteomics

The Center for Proteomics has established methods to help elucidate the biological mechanisms associated with toxicity using mass spectrometry (MS)-based proteomics. The development of mechanisms to integrate proteomic data with that of other OMICs is underway. This work complements the development of software for large protein identification studies and is being extended to assist in quantitative comparisons. This work is necessary to allow a higher sample number to be examined to permit both dose and time studies in support of toxicology studies. The Center worked in collaboration with members, within the Division of Microbiology, in order to identify enzymes that detoxify polyaromatic hydrocarbons, an important environmental contaminant in soil in *Mycobacterium vanbaalenii* PYR-1. In addition, proteomics efforts have led to the identification of extracellular proteins produced by *Staphylococcus aureus* that may be useful targets for immunotherapeutic inactivation. The Center is also working with the Division of Neurotoxicology and the Center for Hepatotoxicology. Two current projects include development of the rat liver mitochondrial proteome and the mouse liver proteome.

Toxicoinformatics

The Center for Toxicoinformatics conducts research in bioinformatics and chemoinformatics. The goals of the toxicoinformatics group are to develop methods for the analysis and integration of OMICs datasets. As part of this initiative, the toxicoinformatics group acts to develop and coordinate informatics capabilities within NCTR, across FDA centers, and in the larger toxicology community. ArrayTrack software has been developed and implemented for integration of singular nucleotide polymorphism (SNP), genomic, and proteomic data. Initial

integration of metabolomic with the microarray and proteomic data has been started. Specifically, surface-enhanced laser desorption and ionization (SELDI)-based proteomic analysis, microarray array quality control, and test-mining approaches have been developed. ArrayTrack is being used to support research and regulatory submissions at NCTR, CDER, CVM (Center for Veterinary Medicine), and CFSAN.

FY 2005 Plans

In FY 2005, the Division of Systems Toxicology will investigate the toxicity of selected liver, renal, and cardiovascular toxins using an integrated OMICs platform coupled with informatics and modeling analysis. This systems biology approach takes an integrative and iterative approach to test questions following perturbation of biological systems. In order to accomplish its mission, the Systems Toxicology group will:

- Develop an integrated, state-of-the-art OMICs platform (consisting of microarray, NMR- and MS-based metabolomic and proteomic signatures) that can perform analyses of compounds-of-interest to FDA and to provide the technical expertise to the Agency in genomic, proteomic, and metabolomic interpretation and guidance;
- Perform integrated OMICs research in conjunction with classic toxicology studies to provide a systems toxicology approach in support of the FDA Critical Path;
- Develop a systems toxicology approach to the integrative analysis of OMICs data with conventional toxicology assessments;
- Utilize a series of liver, renal, and cardiovascular toxins to demonstrate the utility of integrated OMICs analyses;
- Develop computational models of toxicity and biomarker pattern identification;
- Continue to develop nanotechnology and sensor technology efforts;
- Determine differences in gene, protein, and metabolite expression patterns in the rat heart as a function of age and sex;
- Perform hepatic gene expression analyses of AIDS therapeutics, herbal compounds, tattoo inks, and PPAR alpha and PPAR gamma agonists;
- Develop a MitoChip to assess the mitochondrial toxicity of model compounds in different tissues;
- Develop a rat mitochondrial proteome map for use in conjunction with MitoChip analyses;
- Establish NMR- and MS-based metabolomic analysis of liver, renal, and cardiovascular toxins;
- Develop biomarkers of liver injury and disease using NMR- and MS-based metabonomic, SELDI-based proteomic, and microarray analysis; and
- Expand the liver toxicity knowledge base using text mining and other informatics tools.

Public Health Implications

The driving factors in the use of integrated OMICs analyses is to be able to better predict risk in humans based on an increased understanding of mechanisms of toxicity and of the cross-species similarities and differences that inform our understanding of the impact of exposure and susceptibility on risk. As toxicology moves from developing an understanding of acute toxicity to chronic disease, our models and applications must change. Systems toxicology provides a

framework in which to develop the methodology required for this change. The root of toxicology is a system-based approach, which looks at impacts from exposure in whole organisms, target tissues, and isolated cells or organelles, but which integrates this information to provide risk analyses for individuals and for populations. The inclusion of OMICs data into the data gathering phase of toxicology permits a more holistic analysis of the perturbation of biological systems, by selected exposures to permit a better understanding of the mechanism of toxicity of a given agent and the conditions under which exposure to that agent is toxic in a model system and in the human.

Ongoing Research Projects

PI: Beger, Richard

Methods for Predicting Toxicological Properties of Molecules from Their NMR Chemical Shifts Through-bond and Through-space Distance Connectivity Patterns (E0712601)

Objective(s): Produce models that use NMR data and infuse three-dimensional atom-to-atom through-bond connectivity and atom-to-atom through-space intramolecular distance information into a three-dimensional pattern that can be used by pattern recognition software to build a model of a biological or toxicological endpoint. The results of the 3D-QSDAR models will be compared to the results of QSDAR and QSAR models from protocols E0706801, E707701, and E0708301.

Case-Control Study of NMR Metabonomic Signatures for Prostate, Breast, and Colorectal Cancer (E0717601)

Objective(s): Determine if there are unique NMR spectral signatures in urine and/or serum obtained from prostate, breast, and colorectal cancer patients compared to controls.

Preclinical Metabonomic Biomarkers of Toxicity and Disease (E0720401)

Objective(s): Examine the utility of metabonomics as an approach to produce predictive models of cardiovascular, renal, neural, and hepatic toxicity. The models will be built using a variety of pattern recognition technologies to determine how temporal endogenous metabolic changes found in NMR and/or MS spectra of urine, serum, and tissue related to toxicity and disease state.

General Support for Center for Metabolomics (S00624)

Objective(s): Provide the NCTR Metabolomics Support

PI: Buzatu, Dan

The Development of Dynamic Mass Spectral/Pattern Recognition Based Methods for the Rapid Identification of Bioterror Agents (E0714601)

Objective(s): Develop the necessary computational capability to enable the rapid identification of pathogen/non-pathogen microorganisms, non-biological hoax materials, and mixtures of all mentioned collected real world situations. An analysis will be done of the salient spectral features necessary for identifying these substances, and the effect of both instrumental and pattern definition techniques on the ability to use these features for rapid identification.

Analysis of Proton MRS Data Using a Distributed Artificial Neural Network (E0719501)

Objective(s): Evaluate whether a self-optimizing, parallel distributed neural network can use the data from *in vivo* proton magnetic resonance spectroscopy (MRS) exams to provide additional information about a brain lesion. If so, this project will lead to improved brain tumor diagnoses from proton MR spectra.

PI: Desai, Varsha

Development of "Mitochip" a Glass-based Oligonucleotide Microarray Containing Mitochondrial and Nuclear Genes Associated with Mitochondrial Function (E0718601)

Objective(s):
- To develop a "MitoChip" containing genes associated with mitochondrial function such as oxidative phosphorylation, B-oxidation of free fatty acids, tricarboxylic acid cycle, apoptosis, as well as genes involved in the replication, transcription, translation of mitochondrial DNA, DNA repair, and regulation of DNA copy number;
- Validate the developed "MitoChip" by evaluating gene expression profiles of AZT, an anti-HIV drug, and 3-NPA, a neurotoxin that are known to alter mitochondrial function; and
- Verify the relative expression levels of differentially expressed genes by real-time quantitative PCR.

PI: Dragan, Yvonne

SV40 T Antigen Transgenic Rats: Breeding Colony Development (E0717801)

Objective(s): Develop a colony of albumin-SV40 transgenic rats at NCTR.

Biomarkers of Liver Disease and Toxicity (E0718801)

Objective(s): Develop biomarker profiles for normal individuals and those with liver diseases or toxicity.

Training in Hepatocyte Perfusion and Hepatic Cell Isolation (P00610)

Objective(s): Train member(s) of the Hepatotoxicology Lab in primary liver cell isolation and culture. The long-term goals will be to obtain signature gene and protein expression patterns of each cell type for comparison to toxin-induced changes. Training must be provided to give confidence in the integrity of liver cells following perfusion, separation, and culture of the liver cells.

PI: Edmondson, Rick

General Support for Center for Proteomics (S00626)

Objective(s): Provide NCTR Proteomic Support

PI: Feuers, Ritchie

Memphis Study: Evaluation of Calorically Restricted Human Surgical Samples Received from Department of Surgery, University of Tennessee, Memphis (E0699801)

Objective(s): Determine whether rodents and humans behave biologically in the same manner when calorically deprived but nutritionally supplemented.

Methods for Support of a Functional Proteomics Facility at NCTR (E0713501)

Objective(s):
- Establish and standardize for routine use procedures for whole cell and subcellular organellar isolation for a variety of tissues;
- Develop and standardize specific and sensitive markers of cell type and organellar purity and yield;
- Identify, adapt, develop, and standardize appropriate 2-D protein separation techniques; and
- Integrate results of specific aims 1-3 to provide "front-end" components of a functional proteomics facility.

PI: Fuscoe, James

Assessment of the Global Gene Expression Changes during the Life Cycle of Rats (E0712201)

Objective(s):
- Use the NCTR rat microarray chip to quantitate the relative expression of approximately 4000 genes in the liver of rats at the following ages: 2 wks, 5 wks, 6 wks, 8 wks, 15 wks, 21 wks, 52 wks, 78 wks, and 104 wks. These data will serve as a baseline measurement of gene expression that will be available for future studies on drug metabolism, toxicity, and susceptibility.
- Verify the relative expression levels by quantitative PCR or Northern analysis

Evaluation of Performance Standards and Statistical Software for Regulatory Toxicogenomic Studies (E0716801)

Objective(s): Supply the experimental and statistical analyses necessary to help develop a consensus within FDA as to what performance standards would be beneficial for assessing the quality of microarray data submitted to the FDA on sponsor-selected platforms. The experimental results and conclusions from this inter-center project will be shared with other consortial microarray standardization efforts and made publicly available through publication.

Prioritizing Sources of Variability in Genomic Profiling Data for Standards and Guidance Development (E0720601)

Objective(s): Prioritize sources of variability in microarray data in order to determine how to focus additional experimental queries, guidance development, and experimental standards. The outcome should be an enhanced capability to address standards development and accept new technologies as they arise.

General Support for Center for Functional Genomics (S00616)

Objective(s): The Center for Functional Genomics is a centralized facility to handle all aspects of microarray printing and processing. Its objectives are 1) to provide NCTR investigators with access to high quality microarray technology, for the investigation of biological mechanisms of action underlying the toxicity of products regulated by the FDA, and related fundamental and applied research; 2) to create a validated toxicogenomics database that will be a resource for the scientific and regulatory community; 3) to be a focal point and scientific resource for issues in toxicogenomics, and 4) to utilize advances in genomics to address issues critical to the FDA mission. In addition, the CFG will provide continual development of new and better approaches to microarray technologies, including larger gene collections, custom microarrays, validated gene expression databases, experimental design, and tools for handling and analyzing microarray data.

PI: Miller, Dwight

Application of Solid Phase Detection Systems to Explosives in Airplane Cargo (E0708101)

Objective(s):
- Detection of ammonia - formulation, measurement of Am concentrations around container of ammonium nitrate, reformulation of Fresh Tag chemistry for label type detection, and development of PE or PVC film Shrink Rap detector;
- Detection of acids, and
- Detection of oxidizers such as peroxides and NO or No2.

Innovative, Static, and Dynamic Chemical Sensors - Litmus CRADA (E0719901)

Objective(s):
- To continue development of simple, inexpensive, field-compatible methods to monitor biochemical indicators of food quality;
- To support development of manufacturing techniques that maintain food quality indicator (FQI) performance; and
- Develop analytical laboratory procedures to confirm the colorimetric result and methods.

General Support for the Chemistry Staff of the Division of Systems Toxicology (S00627)

Objective(s): Provide chemistry support to NCTR.

PI: Tong, Weida

Development of a Novel Class Prediction Method, Decision Forest, for Analysis of Genomic and Proteomic Data (E0716901)

Objective(s):
- Develop the two-class Decision Forest method. The method will be developed on several publicly available gene expression and SELDI-TOF data sets, and the results will be compared with others that derive from traditional classification techniques;
- The multi-class Decision Forest method will be also developed in this protocol. The method will be demonstrated on a gene expression data set to classify the pediatric acute lymphoblastic leukemia (ALL) subtypes.

General Support for Center for Toxicoinformatics (S00617)

Objective(s): Provide toxicoinformatics support for the center-wide research.

ODASI (Omics Data Analysis Solutions Initiative) Committee (S00632)

Objective(s): The committee will review concept papers to identify the needs for bioinformatics support, interact with PIs to provide suggestions about scope and procedure of data manaement and analysis required for the protocol, and recommend appropriate experts to assist the project for experiment design, data analysis and pattern recognition.

PI: Wilkes, Jon

Combining MAB/MS with Pattern Recognition to Sub-type Bacteria (E0707901)

Objective(s): This work is intended to demonstrate the validity of the combination of pyrolysis/metastable atom bombardment (MAB)/mass spectrometry (PyMAB/MS) with computerized pattern recognition (PattRec) for bacterial sub-typing. The work should produce a scientifically and technologically validated basis for commercial licensing of an NCTR-patented process: a method for assembling coherent spectral data bases for use in rapid chemotaxonomy at the strain and sub-strain level.

Evaluation of Pyrolysis MAB/Tof MS and MALDI/Tof MS for Rapid Characterization of Presumptive Bio-terror Agent Samples (E0714701)

Objective(s): The suitability of mass spectral data obtained from both pyrolysis metastable atom bombardment MS and matrix-assisted laser desorption/ionization time-of-flight MS techniques will be evaluated for the purpose of rapidly characterizing presumptive bio-terror agent samples. This includes analysis of the salient spectral features necessary for identifying microorganisms from contaminated samples and differentiating tainted samples from hoax sample materials collected from the environment, as well as evaluating the effects of both instrumental and pattern definition techniques on the ability to use these features for rapid identification.

Publications

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Huber, W.W., Teitel, C.H., Coles, B.F., King, R., Wiese, F.W., Kaderlik, K., Casciano, D.A., Shaddock, J.G., Mulder, G.J., Kadlubar, F.F. and Ilett, K., Potential Chemoprotection by the Coffee Components Kahweol and Cafestol Palmitates Via Regulation of Hepatic N-Acetyltransferase and Glutathione S-Transferases, *Env and Mol Mutagenesis* (Special Issue). Accepted: 5/19/2004 (E0694701).

Tong, W., Harris, S.C., Cao, X., Fang, H., Shi, L., Sun, H., Fuscoe, J., Harris, A.J., Hong, H., Xie, Q., Perkins, R.G. and Casciano, D.A., Development of a Public Toxicogenomics Software for Microarray Data Management and Analysis, *Mutation Research*. Accepted: 12/9/2003 (S00617).

Division of Veterinary Services

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Ventilated rack systems provide state-of-the-art biocontainment housing for NCTR research animals.

Executive Summary

Introduction

The Division of Veterinary Services (DVS) provides professional and technical support to the various NCTR research divisions and Centers of Excellence in their efforts to conduct peer-reviewed scientific research that supports and anticipates the FDA's current and future regulatory needs. The Division provides administration for the Center's Animal Care and Use Program, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Included within the division are the contracted services for animal care, diet preparation, and pathology, all of which are staffed by on-site contract employees.

FY 2004 Accomplishments

Immediate Office

The Division provided oversight and management of all laboratory animal facilities at NCTR. Divisional personnel were responsible for breeding, rearing, and/or acquiring and quarantining all experimental animals used on-site. Personnel submitted annual reports assuring compliance with federal regulations and NIH guidelines relative to our Animal Care and Use Program and participated in semi-annual program reviews, facility inspections, and experimental protocol reviews as part of the NCTR Institutional Animal Care and Use Committee proceedings. The NCTR Animal Care and Use Program was site-visited by AAALAC during August 2004, and was successful in retaining its fully accredited status. Divisional personnel served as government project officers for the pathology services, animal care and diet preparation, and rodent bedding contracts for the Center. Divisional personnel also planned and implemented the NCTR Laboratory Animal Care Technician Recognition Week and the Annual Arkansas Branch AALAS Meeting. As a member of the FDA Research Animal Committee, the director performs peer reviews of the Animal Care and Use Program Description Documents of each Center and provides "mock" AAALAC site visits to those facilities.

Animal Care/Diet Preparation Services

During 2004, the average number of experiments supported per month by contract animal care personnel was 33. These experiments entailed as a minimum the daily animal care support of an

average of 8,000 rodents, many individually housed, and 100 rhesus monkeys. Technical manipulations for these studies included one or more of the following procedures: tattooing, tumor palpations, injections (SQ, IM and/or IV), oral gavage (including procedures for neonatal mice), behavioral assessments on rats and rhesus monkeys, application of topical creams, biological sample collections (including cardiac puncture on neonatal mice), rodent date-mating, quarantine of rodents and rhesus monkeys, physical exams and pregnancy exams on rhesus monkeys, micro chip implantation, and humane euthanasia. Ninety-five percent of the contract animal care staff is certified at one of three levels of certification by the American Association of Laboratory Animal Science (AALAS), and eight of the animal care supervisors are Certified Managers of Animal Resources (CMAR). Contract diet preparation personnel provided consultation, nutritional support, and diet preparation services for all studies requiring dosed feed, dosed water, or dosed topical skin creams. Additionally, personnel processed standard rodent chow and water for all studies not requiring dosed feed or water. Quality assurance personnel performed quality control audits of contractor-performed procedures and updated all animal care and diet preparation standard operating procedures (SOPs). Contract personnel currently adhere to 262 Animal Care SOPs and 74 Diet Preparation SOPs in support of NCTR research activities.

Pathology and Pathology-related Services

During 2004, two trainees completed the laboratory technician apprenticeship training program and became eligible to take the histotechnician registry exam. Pathology contract personnel are currently testing the newly developed "paperless" system for collecting and reporting of pathology data and tracking of specimens through the pathology process. The new system is designed to link gross pathology data with microscopic data and all in-house processing tasks, including the archiving of specimens. The new system is expected to streamline our entire pathology process. Personnel have developed new RNA isolation protocols and short immunohistochemistry protocols for use with the new laser capture microdissection system to support current and future research efforts at the Center. A Virtual Microscopy/Pathology System (ScanScope) has been procured and installed in the pathology section. Plans are to use the scope to input, store, and retrieve the vast number of images collected for the phototox studies. In addition, this system may replace the traditional Pathology Working Group (PWG) process as it allows the sharing of slides/images via the internet with multiple off-Center sites thus eliminating the need for the PWG members to meet in a single location for viewing the specimens of interest. New assays developed for labeling of cells and structures of interest in frozen liver sections include: histone1, beta-catenin, beta-actin, anti-ED2-like antigen, CK-7, and GFAP IHC. The NTP quality assessment and peer review of pathology date for Genistein was accomplished. Personnel gave Quality Control and Quality Assurance presentations at the FDA's BioResearch Monitoring Course for FDA auditors. Pathology personnel completed annual training in animal care and use required by the NCTR IACUC. During 2004, pathology contract employees authored or co-authored multiple publications or presentations.

FY 2005 Plans

- Plan to continue to support the research mission of NCTR including future BSL/ABSL work once the biocontainment facility has been completed.

- Plan to procure a new Animal Care and Diet Preparation Contract for the Center.
- Plan to continue supplying methods development and support, both technical and professional, needed to accomplish the NIEHS IAG work at NCTR.
- Plan to add methodologies in pathology to support research proposed by personnel of the new Systems Toxicology Division at the Center.
- Plan to continue a quality laboratory animal care program that is consistent with state and federal laws, regulations, and guidelines.
- Plan to continue to assist FDA in maintaining AAALAC accreditation of its various laboratory animal care and use programs.

Public Health Significance

FDA's mission is to protect and promote the nation's public health. Animal related studies such as those being conducted by the NCTR research community greatly enhance the Agency's ability to meet this public health mission. The Division of Veterinary Services (DVS) has the facilities, equipment, and personnel to actively support this vital interdisciplinary research.

The "gold standard" for laboratory animal care and use programs is accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Such accreditation is widely accepted by the scientific community and indicates that the accredited organization conforms to all government policies and regulations, and that it indorses the highest quality care for the animals involved in their animal use activities. DVS personnel oversee the NCTR Laboratory Animal Care and Use Program, which has been accredited by AAALAC International since 1977. The DVS director, working through the FDA Research Animal Council (FRAC), has assisted, and will continue to assist other FDA Centers in obtaining and maintaining accreditation of their animal care and use programs.

NCTR Collaborative Efforts

Solving today's public health issues requires innovative, multidisciplinary, integrative approaches. The National Center for Toxicological Research (NCTR), with its internationally recognized research staff, unique facilities, and scientific capabilities, provides the opportunity to conduct collaborative research that addresses a wide variety of specific public health questions. Throughout its history, NCTR has actively sought and participated in cooperative partnerships with other scientific and regulatory organizations. These opportunities to leverage resources, both public and private, have led to substantial research advances that have resulted in significant improvements in long-term public health.

As a government agency, NCTR can enter into financial partnerships with agencies via two mechanisms–Interagency Agreements (IAGs) (partnerships with other government agencies) or Collaborative Research and Development Agreements (CRADAs) (partnerships with nongovernmental organizations, nonprofit organizations and private companies).

Interagency Agreements (IAGs)

In 1992 the Food and Drug Administration (FDA) entered into an Interagency Agreement (IAG) with the National Institute for Environmental Health Sciences (NIEHS). The design for this agreement concentrated on FDA's priority National Toxicology Program (NTP) nominations of chemicals/agents and utilized the unique resources and facilities at the National Center for Toxicological Research (NCTR). The research conducted under the IAG provided FDA the ability to better assess the safety of a number of FDA-regulated products.

The 1992 agreement provided support for five FDA priority chemical/agent NTP nominations. The agreement has expanded to include collaborative research on five commonly accepted endocrine disrupter compounds, which include three multigeneration studies and two chronic cancer studies. Currently the IAG includes the evaluation of AIDS therapeutic drugs, dietary supplements, mycotoxins, such as fumonisin, and acrylamide, a chemical produced when some food products, such as potatoes, are cooked at high temperatures. In 1998, NCTR opened a FDA/NIEHS Phototoxicity Research and Testing Laboratory. The facility is state-of-the-art, testing compounds applied to the skin in simulated solar light.

All research under the NIEHS/NTP IAG is designed with input from FDA regulatory scientists, NCTR and NIEHS scientists, experts from universities, and often experts from the regulated industry. The IAG utilizes resources from public funds and exceptional scientific expertise to provide the best possible assessment of product safety resulting in accomplishment of the missions of the FDA and NIH.

In addition to the IAG with NIEHS/NTP, NCTR has received support from other governmental agencies. For example, the Environmental Protection Agency (EPA) has supported NCTR in conducting a broad area of research on neurotoxicity risk assessment and risk assessment associated with waterborne and foodborne pathogens. Currently, research is being conducted for an infectivity model for *Cryptosporidium*, which can potentially contaminate drinking water and the food supply.

The Federal Aviation Administration (FAA) has entered into an IAG with scientists at the Center to develop rapid sensor detection methods to screen for explosives in counterterrorism. Additionally, the National Cancer Institute (NCI) is supporting a study at the NCTR evaluating the role of dietary constituents (e.g., methionine, choline, folate, alcohol) in the early phases of carcinogenesis (liver and possibly mammary) and effects on global and site-specific DNA hypomethylation.

Collaborative Research and Development Agreements (CRADAs)

NCTR actively pursues and maintains partnerships with nongovernmental organizations, nonprofit organizations and private companies through Collaborative Research and Development Agreement or CRADAs.

A CRADA has been developed between NCTR and the University of Arkansas at Little Rock (UALR). Under this partnership arrangement, scientists from NCTR's Division of Neurotoxicology and the UALR explore their initial observations that animals exposed to cocaine during gestation fail to adapt to important changes in their environment. The studies examine additional aspects of behavioral adaptability by changing "the rules of the game" for a variety of behavioral tasks.

The SAS system, a primary statistics tool used by FDA, and ArrayTrack, DNA microarray data management, mining, analysis, and interpretation software developed by the NCTR/FDA, are readily available both for FDA scientists and reviewers. A CRADA with the SAS Institute will combine ArrayTrack with SAS Scientific Discovery Systems (SDS) to provide the FDA scientist and reviewers key capabilities for analyzing data from toxicogenomic/pharmacogenomic studies for scientific research and regulation.

The effects of a variety of psychotropic agents on important cognitive processes, such as short-term memory, learning, visual and position discrimination, time perception and motivation, on the nonhuman primate will be supported by a CRADA with Pfizer. These data will help further characterize the influence of specific neurotransmitter manipulations on cognitive function and identify specific cognitive domains most likely to be affected by other drugs with similar mechanisms of action.

AstraZeneca is currently supporting a study to determine whether ketamine, an NMDA receptor antagonist frequently used as an anesthetic in children, and remacemide, an antiepileptic agent with both NMDA receptor antagonist and sodium channel blocking properties, cause adverse effects similar to those noted in previous studies. Researchers have confirmed that administration of ketamine during the brain growth spurt results in widespread neuronal apoptosis in the rat. Further evidence in a nonhuman primate model is in progress.

The NCTR Division of Chemistry developed small disks called Food Quality Indicators (FQIs) as rapid chemical sensors to assess food for freshness. These FQIs were evaluated by the Canadian Centre for Fisheries Innovation, St. John's, Newfoundland, Canada. This independent evaluation confirmed that the FQI is rapid, sensitive, rugged, and simple enough that multiple

analysts can obtain results of equal and high quality. A CRADA has been developed with Litmus to develop a commercial outlet for this FQI and also to support the further development of FQI technologies.

NCTR's Division of Neurotoxicology is collaborating with Sigma Tau Research, Inc. to characterize the early genomic biomarkers of mitochondrial dysfunction, a frequently observed effect of neurotoxicants. This research may provide information on a standardized microarray system that will allow for the screening of agents with the potential to predict brain injury.
A CRADA between the NCTR Metabonomics research team and RxGen will develop a method for predicting human hepatotoxicity through the identification of characteristic high-resolution proton nuclear magnetic resonance (NMR) spectroscopic profiles in biofluids and liver tissue.

University Interactions

NCTR scientists actively pursue collaborations with individuals and departments of universities. Currently, many NCTR scientists hold adjunct academic positions, and research collaborations exist with more than 20 universities. Thus, the NCTR staff is collaborating with various university staffs to solve problems of mutual interest to FDA and the respective university. Academic collaborations include mutual use of specialized equipment, sharing of research samples to maximize the gain of information from a project, and the exchange of staff between the institutions for lectures, seminars, and conduct of research.

Of particular importance are the close collaborations between NCTR and the University of Arkansas for Medical Sciences (UAMS) in Little Rock, AR. In addition to the adjunct positions held by NCTR scientists at the UAMS, NCTR participates in the UAMS Interdisciplinary Toxicology Program through which graduate students receive a Ph.D. in toxicology. Many of the graduate students perform research for their dissertations in an NCTR laboratory under NCTR staff supervision.

Other collaborations with UAMS scientists include projects that: 1) investigate the biological effects of ephedrine, ethanol, baby diets, cardiovascular disease, and aging on health using the metabonomics approach; 2) investigate the influence of biotin on the developing embryo; 3) identify the brain tumor diagnostic method to achieve tissue characterization; 4) assess DNA from breast epithelial cells for the presence of carcinogen adducts, 5) develop methodology and determine the critical biotransformation pathways involved in adduct formation and assess possible differential sensitivity in normal-risk women as opposed to women at high risk for breast cancer; 6) conduct the first LCM-based study on genetic changes of the mitochondrial genome in prostate cancer and precursor lesions; and 7) develop a microarray-based method for the detection of 150 genes associated with 22 antibiotics, some of which are used to promote growth in poultry and animal farming while others are used to treat infections in both humans and animals.

NCTR staff in the Division of Neurotoxicology has access to a Complex Brain Function Laboratory at the Arkansas Children's Hospital (ACH). Results of behavioral studies obtained in animals using the Operant Test Battery at NCTR are verified in humans at ACH.

NCTR staff collaborates on a number of grants with area universities. These include a NASA-funded UAMS (Department of Otolarynology) project designed to provide information on the efficacy of several drugs used as anti-space motion sickness therapies and their effects on cognitive function as assessed using the NCTR Operant Test Battery. Grant funding via UAMS/UALR and NIH has provided support for studies to examine the ability of the NCTR Operant Test Battery to detect and monitor cognitive dysfunction in Alzheimer's patients.

Recent projects that include collaborations with area universities and hospitals include those with Central Arkansas Veterans Healthcare System (CAVHS), ACH, and the University of Arkansas. A project with UAMS and CAVHS will identify the measurement of cancer-associated gene mutation in colon tumor and non-tumor tissue. Collaborations with UAMS/UALR/ACH have begun on a project to determine if children diagnosed with major depression perform differently than children without such a diagnosis on tests of motivation, simple visual discrimination, timing ability, memory, and learning. In addition, the University of Arkansas and NCTR scientists will address the extent to which the disruption of the monoaminergic system and oxidative stress are involved in the progression of Parkinsonism or Parkinson-like symptoms.

NCTR scientists collaborating with universities in the U.S. and abroad have resulted in, at no cost to FDA, a number of visiting scientists who come to NCTR to pursue research in areas developed by NCTR scientists. Thus far, NCTR has hosted more than 36 visiting scientists from the U.S. and 15 foreign countries. These visiting scientists not only contribute valuable scientific expertise to NCTR research programs, but many return to their respective institutions to continue research on problems of interest to FDA and NCTR.