SCIENCE ADVISORY BOARD (SAB) MEETING
JUNE 12, 2001
National Center for Toxicological Research

The meeting was reconvened and the concluding comments on the Endocrine Knowledge Base Program.

Dr. Rosenkrantz: Dr. Sheehan in your response to the EKB site visit team that the reason the information was not on the Internet was due to firewall issues. I understand that is a result of FDA regulations on the firewall issue. I recommend the SAB go on record as saying this problem needs to be fixed. Scientific work has to be communicated to the outside world and the only way to do that on a broad scale is through the Internet.

Dr. Sheehan: I agree, I don't know if there is a way to establish two systems, one with a strong firewall and one that allows greater access. It has been a frustration to us because we are not able to function as well nationally or internationally.

Dr. Casciano: The recommendation is very timely and recent leadership meetings with the new Commissioner have been directed toward transparency of the Agency and this is one more item to enhance that transparency. We appreciate your comments.

Dr. Rosenkrantz: There was some discussion about the microarray data analysis, Dr. Tindall would you like to comment.

Dr. Tindall: It occurs to me that the strong relationship with NIEHS should explore interactions with the new National Center for Toxicogenomics.

Dr. Kaplan: I don't understand why this group had to develop their own data analysis tool when there are so many available either proprietary or free. I recommend that you evaluate the available database software.

Dr. Rosenkrantz: I think the computational science group here has a great opportunity to review available software and make internal recommendations.

Dr. Tindall: All of the programs are likely to be more and more involved in various aspects of genomics, proteonomics, etc. so software, servers, and storage all need to be carefully evaluated. They are expensive propositions, making a choice is not easy and no software solution will be perfect but it is a very important consideration to have the infrastructure in place for getting the work done.

Dr. Rosenkrantz's motion to accept the response to the Endocrine Knowledge Base Site Visit report was approved by voice vote.

Division of Biochemical Toxicology Program Update, by Dr. Fred Beland, Director

The fundamental and applied research designed to define biological mechanisms of toxicity. The division is primarily interested in carcinogenicity; the focus is on assessment of carcinogenic risk and the introduction of new techniques to assess carcinogenic risk. The division has approximately 50 personnel assigned comprised of long-term permanent

researchers and investigators, several visiting scientists, and a varying number of postdoctoral students.

The distribution of funds drives much of the scientific research being conducted. Approximately 30% of the division's discretionary budget, used for supplies, travel, and equipment comes from the FDA. The other 60-70% comes primarily from the National Institute of Environmental Health Sciences (NIEHS) through an interagency agreement (IAG) with the National Toxicology Program (NTP). The majority of the research done within the division is funded externally however; the division is still meeting the agency needs but is obtaining funding from another source.

The Biochemical Toxicology Division focuses on four research areas, the NIEHS and FDA Interagency Agreement (IAG) research efforts in support of the NTP nominated chemical compounds, the neonatal mouse bioassay, dietary folate/methyl deficiency, and analytical methods development. Within the division the funding provided by the NTP IAG supports research efforts for fumonisin B1, chloral hydrate, urethane and ethanol, malachite green, endocrine disruptors, phototoxicity studies, dietary supplements, and anti-retroviral agents.

Fumonisin B1 was the first compound investigated under the NTP IAG. It was nominated by the Center for Food Safety and Applied Nutrition (CFSAN) and the study was conducted under the direction of Dr. Paul Howard. Fumonisin B1 is a known contaminant of corn and has been associated with esophageal cancer. It was nominated so a regulatory decision could be made regarding the safety levels. We learned that it is a good carcinogen, it causes kidney tumors in rats and liver tumors in mice, but it is not a genotoxic carcinogen. An important part of the program was the mechanistic studies used to determine why the compound caused tumors. Fumonisin appears to act through the inhibition of cell ceramide synthase and apoptosis, and the tumors appear to be resulting from regeneration in response to the apoptosis. It should be noted that the NTP has a discrete amount of funding for each nominated compound and if something very interesting is found, the NTP tends not to fund further investigation. At this point a request for additional study funds is made through internal funding. The mycotoxin also produces genotoxic compounds; there is an initiator and the fumonisin appears to act as a promoter. At this time, Dr. Howard is trying to isolate the ceramide synthase and to identify the genotoxic agents.

The second compound was Chloral Hydrate, which is used as a pediatric sedative. The EPA drinking water program showed that chloral hydrate was a mouse liver carcinogen. Here we have a situation where children are exposed to a liver carcinogen. It was nominated for study by the Center for Drug Evaluation and Research (CDER). We tried to develop a dose regime where we treated young animals and then discontinued the treatment to mirror the exposure to children. We gave the treatment by gavage. In our hands chloral hydrate was not a very good carcinogen. We think that the reason was because the EPA study involved continuous exposure and it acted more as a tumor promoter than as an initiator. The report is done.

Urethane is clearly a carcinogen and is in a number of food products. The Center for Food Safety and Applied Nutrition (CFSAN) needed a better dose response data to set regulatory limits. There was not a good animal study to indicate that ethanol was a carcinogen; it is clearly carcinogenic in humans but is associated with tobacco consumption. The question was how do urethane and ethanol interact? The study is complete and the final report is being written.

Malachite green is used without approval in the catfish farm industry as an antifungal agent. The Center for Veterinary Medicine (CVM) nominated the compound for further study to

determine if enforcement action is necessary. Malachite green is a triethylmethane dye reduced to leucomalachite green, which then becomes lymphophyllic and is found in fat tissue. Dr. Sandy Culp is conducting this study; the animals have been on the study for almost two years. Leucomalachite green is a very good liver carcinogen in female mice, and there is no question that this is the agent humans would be exposed to. The data has not been fully audited but the dose response is very clear. We are conducting some genotoxicity and DNA adduct identification studies. Working is also ongoing in collaboration with members of genetic toxicology as well as an *in vivo* mutagenicity study using the Big Blue Rat model. The protocol information will be available for the Center for Veterinary Medicine (CVM) within the next two years.

Dr. Barry Delclos is directing the endocrine disruptor studies. The issue is what is the dose response. There is a controversy over the type of response whether it is linear or an increase in toxicity at a very low dose. This is critical as we determine how to regulate a compound that does not have a linear response. The five compounds selected were genistein, methoxychlor, nonylphenol, ethanol estradiol, and the androgenic vinclozolin. All of the ranges finding studies for the five compounds are complete and based on the response the multigeneration studies have begun for genistein, nonylphenol, and ethanol estradiol. Mechanistic studies are underway looking at alteration of hormone levels and the induction of cytochrome P450. Collaborative studies with neurotoxicology on behavioral studies, and immunotoxicology at the Medical College of Virginia.

Dr. Paul Howard is doing phototoxicology studies with alpha and beta hydroxy acids. And Dr. Mary Boudreau is doing studies on aloe vera. Dr. Culp will be responsible for the retinyl palmate study.

Dietary supplements under study include genistein, riddelliine, and aloe vera. Riddelline is a compound that is found in some herbal teas. We are not doing the bioassay here, but we do know that it is a hepato carcinogen. The NTP asked if we could gather information to determine genotoxicity. It was found that ten DNA adducts were formed, two of which have been identified. Aloe vera is used in skin preparations and is also taken internally. The plant product includes several fractions, which include aloe vera gel, latex, a whole leaf preparation, etc. We do know that the fractions do contain anthroquinones, which could be genotoxic, so there are several preparations that require review.

In collaboration with Mimi Poirier at the MCI we started looking at the antiretroviral agent AZT. We developed an immunoassay and demonstrated the incorporation of AZT into the DNA. Our concern is that women who are pregnant are given the agents to prevent development of HIV in the unborn child. We are not suggesting the antiretroviral agent should not be used, t is beneficial for the pregnant woman to be treated. Our question is, is there a safer regime to follow? It has been demonstrated in a transplacental model that AZT is carcinogenic. NTP has an antiretroviral program to look at AZT (zidovudine), 3TC (lamivudine), which are in the family of reverse transcriptase inhibitors called nucleoside analogs, and nevirapine and nelfinavir which are also reverse transcriptase inhibitor in a class called non-nucleoside analogs. Protocols have been prepared using AZT, 3TC +/- nevirapine and AZT, 3TC +/- nelfinavir, the animals will be treated transplacentally only, neonatally only, and both transplacentally and neonatally. We will do studies to determine carcinogenicity, genotoxicity, metabolism, and incorporation into DNA.

We know that the neonatal mouse bioassay responds to very genotoxic compounds, but there are other compounds that the assay is sensitive to. At this time the following chemicals are

undergoing tests using this assay: benzodiazepines, antihistamines, lipid peroxidation products, estrogens/antiestrogens, proton pump inhibitors, mycotoxins, known human carcinogens, and antiretroviral agents.

Dr. Jill James is the director for investigative studies on dietary folate and methyl deficiency. The studies look at nucleotide pool imbalance and methylation dyregulation during hepatocarcinogenesis, folate-dependent homocysteine metabolism, and methylene tetrahydrofolated reductase polymorphisms and Down syndrome. These studies have allowed additional funding to come into the Center through the Center for Disease Control (CDC) and the Arkansas Children's Hospital.

The forth area we focus on is analytical methods development. There are two major laboratories working on this the first is immunochemical methods primarily by Dr. Dean Roberts. He has developed antibodies against fumonisins which has allowed us a system of purification of ceramide synthase he has developed antibodies against specific DNA adducts and more recently catechol estrogens as part of funding from the Office of Women's Health within the FDA. The last area is the mass spectrometry group headed by Dr. Dan Doerge; he has done work with genistein and daidzein as part of the endocrine disruptors in collaboration with Dr. Barry Delclos. We have developed mass spectrometry methods for oxidative DNA damage from the DNA adducts which come from tamoxifen and we are expanding these studies to include DNA adducts that come from components found in hormone replacement therapy.

Dr. Acosta: You indicate the portion of funding for your research from the NTP, if there would be a decrease in funding externally how would the division be effected?

Dr. Beland: Personally I would go out and solicit funds from another source.

Dr. Acosta: Are your investigators looking for sources of external research funds?

Dr. Beland: It has always been encouraged. Approximately seven years ago a site visit team suggested we concentrate more on FDA funding, a year or two later the focus shifted once again to external funding. Philosophically we have to take care of ourselves.

Dr. Casciano: With the NIH budget on its way to doubling and the majority of that going towards extramural funding, we feel fairly stable. There are very few places in the world that can do what this group is capable of doing and our uniqueness allows us to be participants as well. The excellent mechanistic work that is done which the NTP is not interested in is applicable to future FDA questions so I think the good science will be continued to be supported.

Dr. Beland: We have Office of Women's Health money as seed money for research that has in turn brought in funding from the CDC. We do not mind trying to obtain external funding.

Division of Molecular Epidemiology Program Update by Dr. Lionel Poirier on behalf of Dr. Fred Kadlubar, Director

The major research areas of the Division of Molecular Epidemiology are the identification of genetic polymorphisms that influence carcinogen metabolism, DNA repair, and individual cancer susceptibility, a second area is chemoprevention. The division consists of approximately 25 people here at NCTR with a number of collaborators at the University of Arkansas for Medical Sciences working with Dr. Nicholas Lang at UAMS-VA.

The focus today will be on the work of the seven senior staff members. The Director, Dr. Fred Kadlubar's interests are on genetic polymorphisms, DNA adduct detection in humans, and molecular epidemiology studies. He is also developing a DNA microchip for large-scale population based studies. He has asked that I present one of his studies, which tries to correlate cytochrome P450s with the onset of puberty in young girls. There are three sets of girls who were examined, African-American, Hispanic, and Caucasian 9½ years +/- three years of age. Several biological parameters were examined and the onset of their puberty was determined from the Tanner breast scores with a T2B designation as the gauge of initial breast growth. Blood samples were taken from the girls and they were genotyped with CYP-17, CYP1A2, CYP1B1, and CYP3AP4.

The result with the cytochrome P450-17 with two different alleles, the A1 and the A2. The homozygous with the A1 had a 50-50-proportion split with the girls who were entering puberty at that age and those who had not. The CYP17 A1 homozygous, the CPY17 A1-A2 heterozygous, or the CYP17 A2-A2 homozygous there was a 50-50 split with the girls who had entered puberty and those who had not. With the CYP3A4 however with the homozygous 1A-1A and the heterozygous 1A-1B there was again a 50-50 split in the girls who had entered puberty compared to those who had not. But in those who were 1B-1B homozygous there was a big difference in the proportion who had entered puberty with a higher proportion of girls who had entered puberty who were homozygous for the 1B-1B.

Another person who is key in the group at this time is Dr. Brian Coles the work being presented for Dr. Coles was done in collaboration with a number of investigators to include Dr. Kadlubar, Dr. Christine Ambersone, and Dr. Carol Sweeny. Dr. Coles is an expert in the enzyme glutathione transferase. There are three major studies he has been working on, a case control study on colorectal cancer with respect to the H-glutathione transferase A-1 polymorphism, a retrospective case study on survival of breast cancer after chemotherapy with respect to GS phenotype, and the recurrence of colorectal polyps with GST genetic polymorphism, MTHFR polymorphism, and glutathione peroxidase polymorphism.

Dr. Coles has worked on a study examining the amount of GSTA-1 protein in the livers of humans compared to the amount of GSTA-2 protein in the same livers. There was no evident correlation between the two, if however if the GSTA-1 is subdivided into its three allelic forms, the A1 homozygous A, the A1 heterozygous AB, and the A1 homozygous B, you find a significant linear correlation which is different in each case between the GSTA1 levels and GSTA2 levels. This has some practical significance in the study of Dr. Carol Sweeny comparing the overall survival of women with each of the previous phenotypes with their phenotypes of GSTA1. The women who have the weaker from of glutathione transferase, the BB, survived longer indicating the detoxification of the chemotherapeutic agent increased the efficiency of the chemical treatment with the alkaloiding agent. Dr. Coles will be looking in the future at breast cancer response to chemotherapy, and increase the study populations that will be examined, looking at pharmacokinetics variations and altered enzyme kinetics. He will also examine additional polymorphism and GSTs in the protein and the gene, and of tissue specific GST expression as potential factors in individual variation in the disease and chemotherapeutic response. In addition, this group has a large-scale study done in collaboration with the University of Arizona Cancer Center examining GST.

A new addition to the Division is Dr. Jung Jen Chen, who is just getting underway in the four areas of research that he will undertake here. The validation of DNA sniff microassay chip for examination of large scale population based studies, investigation of genetic and epigenetic

alterations of specific cells using laser capture micro dissection, examinations of mutations in mitoconjugal DNA to look at the perspective role of oxidative damage in carcenogenisis, and the determination of hyper methylation of GST1 promoter as an early marker of prostate cancer in men. This is actually has been started by other groups in Australia and there is a good correlation between hypermethylation of this gene with the detection of early forms of prostate cancer. I am hoping to persuade Dr. Chen to include additional genes in his studies of hypermethylation.

Another investigator within the Division is Dr. George Hammonds who works half time here at the Center and half as a teacher at Philander-Smith College. Dr. Hammonds collaborators with other investigators within the Division principally with Dr. Beverly Lyn-Cook. He has two recent projects of particular interest. One was examination of hepatic DNA methyl transferase activity in smokers and the other was determination of individual methylation profiles, gene expression, and the enzyme activity of CYP1A2 in human liver. Dr. Hammonds has one paper in publication and another in press. The first is on the elevation of DNA methyl transferase in the livers of smokers than in the livers of nonsmokers. Dr. Peter Jones, who gave a seminar here approximately two months ago, particularly praised this work. Dr. Hammonds has also shown hypermethylation in the promoter region of the CYP1A2 gene and that it was associated with decreased expression. Generally the methylation of the promoter regions of genes is commonly associated with decreased expression of these genes. Dr. Hammond's recent results reflect an elevated level of methylation with a lesser-methylated state.

Dr. Beverly Lyn-Cook is a senior investigator in the Division who studies *in vitro* toxicity of pancreatic cells in culture. Who two major areas of research are biomarkers of pancreatic cancer and she is working to establish biomarkers of cancer in high risk groups such as smokers versus nonsmokers and to develop *in vitro* predictive bioassays for chemopreventive agents. She also looks at the toxicity of agents such as nicotine, soy, and tea components on pancreatic cells in culture. She is determining the mechanistic actions of such agents. A recent result from the genistein on the expression of K-RAS on pancreatic cells in culture and genistein induces the expression of K-RAS of the pancreatic cells in culture. In the future, Dr. Lyn-Cook will undertake mechanistic studies on the biological and pharmacological actions of chemopreventative agents, she will conduct sight specific methylation studies over the promoter region of the IGF gene and lymphocytes from a case control study of colon adenomas and will also look at the global hypomethylation studies on H and K-RAS methylation patterns in human lymphocytes, again from a case control study.

The last two studies are being done in conjunction with an IAG with the National Cancer Institute. I examine DNA methylation and cancer risk in humans and experimental animals as well as abnormal methyl metabolism in non-neoplastic diseases. One of the recent studies was examining the effects of dietary homocysteine on disease. Homocysteine has been postulated to be a risk factor in the development of heart disease. This study looked at different levels of homocysteine in the diet; this study showed that the level of homocysteine in the blood is proportional to the level of homocysteine in the diet. The effect of increasing levels of homocysteine in the blood on the formation of pre arthrosporadic lesions in the blood vessels. This is a two-stage study that was done in collaboration with a group at the University of Arkansas. The first stage is actually the wounding of the carotid artery and the second is feeding the high homocysteine diet and studying the development of the arthrosporadic plaques. The continued feeding of the homocysteine enhanced the plaque formation and the rate of formation was proportional to the amount of homocysteine in the diet and in the blood. The most recent findings made in this study are that homocysteine raises the plasma level of homocysteine in the blood and accelerates the formation of arthrosporadic plaques. In

diabetics, a high plasma level of homocysteine is accompanied of elevated blood levels of S-adenosylmethionine, S-adenosylhomocysteine (SAM) this is a reflection of the hypermethylating environment that is produced by accumulating levels of homocysteine. Finally in both rats and humans the availability of SAM, the body's chief physiological methyl agent, appears to be inversely proportional to caloric intake. The greater the caloric intake the less SAM seems to be available. In rats this is a collaborative effort with Dr. Ming Chou, and it is fairly dramatic effect. In humans the effect is not so striking it is fairly subtle and was made in a collaborative study with Dr. Robert Delongchamp.

Future projects of this research are a continued collaboration with NCI in methylation in case-controlled colon adenoma study, to extend the collaboration on DNA gene methylation in rats undergoing hepatocarcinogenesis by methyl depravation, to complete collaborative clinical studies on all abnormal methyl metabolism associated with non-neoplastic disease such as diabetics and arteriosclerosis, and to organize a TRANS/HHS workshop on diet, DNA methylation processes, and health. Enough interest has been elicited by the National Cancer Institute that a group there is willing to sponsor an all National Institute of Health plus FDA workshop on the different diseases and the overlapping mechanisms that seem to be impacted upon or involved with methyl deficiency.

Dr. Richard Kennedy: The study design for the homocysteine diet, was this in the folate deficient rat?

Dr. Poirier: Yes. The diet is similar to the one used to establish cancer in rats although this modified diet is folic acid deficient. In previous studies I kept the folate constant. The effect of homocysteine per se, is only now being investigated in the chow diet.

Dr. Kennedy: I can tell you that we see a direct cardiac effects of hyper homocysteine anemia are different when studied alone versus when studied in the folate deficient rat. We are seeing a wasting phenomenon in the folate deficient rat after about three months. They are losing about 30% of the body mass and we are getting a big increase in collagenase activity. I think the model will require a lot of study.

Dr. Poirier: Is this the study with Lisa Brown?

Dr. Kennedy: It started there and came to cardiology where they are looking specifically at cardiac aspect. We piloted using the folate deficient diet versus the diet, which was just too much homocysteine. We see dramatically different results with the two diets. We were concerned about making the animals folate deficient for a period of over three months. We went to a diet where folate is maintained, and a normal homocysteine diet and we see different effects on cardiac muscles. The vasculature study has just started.

Dr. Poirier: Thank you.

Dr. Kaplan: How do you do mass spectrometry here?

Dr. Casciano: We have a core facility that you will here about from Dr. Turesky.

Division of Genetic and Reproductive Toxicology Program Update by Dr. Martha Moore

The Division Staff, has 31.8 FTE (full time equivalent) Government positions. These staff members are composed of 14 principal research scientists, 3 staff fellows, 12.8 support

scientists, and 2 administrative support personnel. There are six positions for Oak Ridge Institute for Science Education (ORISE) Post Docs and we have three personnel working now. We also have one ORISE Pre Doc who is already an MD, and is working on his Ph.D. here with Dr. Bob Heflich.

The division has four research focus areas, we have two disciplinary areas genetic toxicology and reproductive developmental toxicology. We also have some very substantial programs which cross-cut the diet and nutrition program, e.g. caloric restriction and dietary restriction program which at one time was very large here at NCTR. That program has focused down substantially but still exists and is part of this division. We are expanding that area to move more into general nutrition, dietary supplements, etc. so we are broadening the research area. It has aspects in both in genetic and developmental and reproductive toxicology. We are also moving toward use of the new tools of genomics and proteomics. Our biggest challenge is how to use these tools to answer questions.

Senior Investigators:

Genetic Toxicology from a disciplinary standpoint is the largest part of the division. Within Genetic Toxicology the group is specialized and is a center for excellence in the area of in vivo mutagenesis. The in vivo team is led by Dr. Bob Heflich, who has been directly involved in most of the genetic toxicology in vivo studies done here at NCTR and has served as a mentor for almost each one of the people within the group. The group includes, Dr. Ben Aidoo who was responsible for the development of the rat *hprt* gene mutation assay. Dr. Tao Chen, received his Ph.D. while here and is currently a staff fellow, he did much of the work helping Dr. Aidoo grow lymphocytes to make the assay work. Dr. Chen has also done some work with the in vitro mouse lymphoma assay trying to understand mutation in that setting. Dr. Magomed Khaidakov is a post doc who also works with Dr. Aidoo, and has an interest in mitochondrial mutations. Dr. Vasily Dobrovolsky is a staff fellow, who has been very successful in his work in developing the TK heterozygous mouse model. Dr. Jim Fuscoe, the newest member of the staff, has done quantitative mutagenesis, molecular mutagenesis, and most recently has used molecular techniques to quantitate genomic rearrangements; he came to NCTR to help develop the genomic proteomics program within the division. Dr. Manju Manjanatha has done a tremendous amount of work understanding the Big Blue Mouse and Big Blue Rat models. Dr. Page McKinzie, a post doc, and Dr. Barbara Parsons, a staff fellow work, are working together as a team developing new techniques for genotypic selection. Dr. Suzanne Morris the in vitro team leader, has a substantial program working with the human lymphoblastoid cell lines and trying to understand mutation at TK and also understanding how the P53 phenotype impacts mutation. Dr. Morris is currently doing a study on genistein in the P53 mouse. Dr. Carrie Valentine is developing the FIX in vivo gene mutation assay. Dr. Bruce Hass is continuing his work with keratinocyte to develop an *hprt* assay.

Reproductive/Developmental Toxicology is a smaller focus group with four members. Dr. Dan Sheehan and Bill Branham work on the endocrine knowledge models. Ming Lu works on cell cycle kinetics and apoptosis. Dr. Deb Hansen, who with Dr. Sheehan's retirement becomes the lead for this group, is working to understand how folate impacts the development of the neural tube and how the normal neural tube closure process occurs.

The Diet and Nutrition group is one of our crosscutting teams. Dr. Ritchie Feuers is the team lead and he and several individuals, Dr. Peter Duffy, Dr. Varsha Desai a post doc, and Ming Lu have been involved in the caloric restriction and dietary control studies. Dr. Ben Aidoo is doing a study with genistein that is funded by the Office of Women's Health. Dr. Deb Hansen is

working with folate as a dietary issue. Dr. Bob Heflich and Sachin Bendre, an MD working on his Ph.D. at UAMS, are working on development of a study to understand the impact of sever malnutrition on the induction of somatic cell mutations. Dr. Suzanne Morris is also doing a study with genistein and P53 mice.

We are starting to move into the Genomics and Proteomics area and we have done a fair amount of work. Dr. Jim Fuscoe will take the lead role in solidifying this program and deciding where we should go forward. A number of senior investigators will be involved in this effort and I am confident it will be successful. Dr. Ritchie Feuers and Dr. Varsha Desai have a great deal of expertise in doing 2-D gels. Dr. Angela Harris, just completed her Ph.D. requirements, and has been working with both micro and filter arrays and has done much work to further our understanding how filter arrays work and the problems in using filter array technology. She has also done work to further our understanding of liver toxicity. Dr. Bob Delongchamp, in his work, has tried to computerize the data generated and to normalize the data. Dr. Suzanne Morris is working in an international collaboration to try to understand the microarray technology.

The framework for the Division of Genetic and Reproductive Toxicology (DGRT) is basic applied research to improve regulatory decision making (risk assessment). In the process we do generate chemical specific information. We do this research in support of the various centers and the Office of Women's Health. We are not currently in collaboration with each of the centers but we do have open dialogs to understand their needs and build our program. The framework of the specific research is hazard characterization and dose response assessment. Much of our research is done in rodents and cell cultures but ultimately we want to know what happens to people.

In hazard characterization there are several areas. The first is to develop new methods (the TK+/- Mouse Model, PhiX174 gene mutation, keratinocyte *hprt*, MutEx/ACB-PCR genotypic selection, endocrine knowledge base, fluorescent markers) and then to characterize and understand these new methods. Next we interpret the data (*in vivo laci* and *hprt*, *in vivo* mouse TK assay, human lymphoblastoid TK assay, filter arrays, mouse lymphoma TK assay) and use this information for regulatory decision making. The modes of action for toxicants plays in both hazard characterization and selection of dose response models (DNA sequence analysis, chromosomal mutations, genomic rearrangements, gene expression and protein production, folate and neural tube development, impact of dietary restriction on somatic mutation and physiological parameters). From here we are faced with the rodent/human extrapolation, response of hepatocytes to toxicants, response to dietary restriction and nutritional changes. Lastly, we develop the necessary guidelines so that we can provide information to the centers on how assays are done and which should be required (mouse lymphoma TK assay, *in vivo* gene mutation assays).

Dose response assessment is composed of several issues, relevant doses (dose selection, biomarkers, genomics/proteomics) the susceptibility/variability (fetus/newborn/young child, repair deficiency: PMS2-mismatch mice, diet: antioxidants, dietary restriction, phytoestrogens), the rodent/human extrapolation (liver toxicity, biomarkers, diet and nutrition, genomics/ proteomics) the cancer/noncancer risk assessment, and finally the development of quantitative models (mechanistic commonality, genomics/proteomics).

We are currently involved in studies for chemical specific information on genistein, coumestrol, leucomalachite green, AZT (and other combination drugs for the treatment of AIDS), and UV light/phototoxicity studies.

In closing, the cross agency collaboration is very important is the only way we will get done what needs to be accomplished. We are currently have active cross-divisional collaboration activities with Biochemical Toxicology, Biometry and Risk Assessment, Neurotoxicology, and the Endocrine Knowledge Base, and cross agency collaboration with the Office of Women's Health, CDER, CFSAN, NIEHS, NTP, and we are talking with the Center for Biologics.

Dr. Acosta: Do you have an internal central organizing group for your proteomics genomics efforts?

Dr. Moore: We are starting that.

Dr. Casciano: There has been an active commitment to support of analytical infrastructure to participate in collaboration with biologists in bioinformatics. Dr. Jack Lay in Chemistry, Dr. Angela Harris in Genetic and Reproductive Toxicology, Dr. Bob Delongchamp in Statistics, and Dr. Weida Tong with ROW Sciences contractor have been meeting for the last year to develop methodology and mechanisms for enhancing team efforts in these highly complex areas. As well as Dr. Moore's explanation of our work with NIEHS and Biologics.

Dr. Moore: There has been a good deal of discussion and dialogue and we should develop a plan that will be very useful to the agency.

Dr. Marilyn Lightfoot: Is there an Agency wide genomics/proteomics working group and are you working and participating with them?

Dr. Casciano: We are participating with the Office of Science to coordinate the various efforts of the difference Centers and to generate common goals with minimal repetition.

Division of Chemistry Update, Dr. Robert J. Turesky

The organization of the Chemistry division has three different units, a strong commitment to the NTP Coordination support led by Dr. Paul Sittion, Mass Spectrometry Branch led by Dr. Jack Lay which conducts both fundamental research and support chemical mass spectrometry service work, and the Analytical/Biomarker Branch led by Dr. Dwight Miller. There are additional research efforts ranging from analytical chemistry, toxicology, NMR spectrometry, spectroscopy, computational chemistry, and biomarker work.

The mission statement for the Division of Chemistry is to: Utilize chemical research techniques, including analytical chemistry, mass spectrometry and NMR spectrometry, spectroscopic and computational methods to implement into intra Divisional, intra Center, and FDA relevant research initiatives in toxicology, risk assessment, and regulatory compliance.

This division has historically been involved with essential support service work for the NTP programs as well as for the other divisions providing the knowledge and analytical chemistry and spectrometry. Over the past year, Dr. Casciano has asked and encouraged a number of scientists within our division to initiate their own protocols in fundamental research.

Some of the Key Research Projects of the Division of Chemistry include:

Dr. Rick Beger, protocol E0706801, Spectrometric Data Activity Relationship (SDAR) Models for Compounds Binding to Receptors of Toxic Responses: Predictive Toxicology

Dr. Fred Evans, protocol E0707801, NMR spectroscopy of drug purity and public health implications

Dr. Jack Lay, protocol E0700501, Rapid identification of intact whole bacteria based upon spectral patterns using MALDI-TOF MS (matrix assisted laser d-ionization – time of flight mass spectrometry)

Dr. Dwight Miller, protocol E0687401, Fresh Tag Sensor™ technology for product safety, quality, and rapid screening of explosives

Dr. John Wilkes, protocol E0693101, Rapid screening, and identification of complex mixtures by pyrolysis-mass spectrometry with pattern recognition methods

Dr. Catherine Ang, E0705601, Chemical characterization of critical chemicals, components, constituents, and biologics for selected medicinal botanical products

Dr. Julian Leakey and Dr. Catherine Ang, project X00031, (through the Office of Women's Health) Impact of dietary supplements on woman's health issues

Dr. Daniel Buzatu, protocol E07077.01, Comparison of principal components analysis (PCA) and artificial neural networks (ANN) for the prediction of qualitative and quantitative biological end points from spectrometric data

Dr. Robert Turesky, Risk assessments of dietary contaminants

The Division of Chemistry has a very strong support relationship for the NTP research programs. We conduct a great deal of analytical chemistry measuring dose certification, stability, and homogeneity, which is a challenging task involving 30% of our division. We survey diet, bedding, and drinking water on a wide range of NTP initiatives. In addition the division has active collaborations with the FDA Center for Veterinary Medicine (CVM), with the various drug residues including Amoxicillin, erythromycin, Lincomycin, and sulfa drugs which require development of determinative methods that achieve CVM method trial ruggedness and testing requirements for reliability. Our division works very hard to establish and validate these methods and when the priorities change with CVM and some of the drug residues which we were establishing have dropped down in priority some of the methods aren't being validated by other laboratories within the FDA which can be somewhat frustrating.

I am a very strong proponent of mass spectrometry use in toxicology and risk assessment. In the past few years our Division has used MS with regard to the food safety initiative for characterization and identification of various bacterial species and problems associated with microbial contamination. There are no rapid screening tools to identify bacteria, but mass spectrometry has been a great boon to these types of investigations. According to the Center for Disease Control (CDC) in 1999, as a direct result of microbial contamination of food there were: 76,000,000 food-borne illnesses in the United States, 325,000 reported hospitalizations and 5,000 deaths, with 64% of the deaths due to unknown organisms. Dr. Jack Lay was able to use MADLI-TOF and extracts of *V. parahaemolyticus* bacteria to identify regional outbreaks of this seafood pathogen. He was able to identify strains of seafood pathogens from the Pacific Northwest (Washington *Vibrio* Parahaemolyticus 10290 and *Vibrio Parahaemolyticus* 10293, which were reproducible but significantly different from strains associated with the Gulf coast (*Vibrio Parahaemolyticus* 2030). It is possible using mass spectrometry to differentiate the different strains of food borne pathogens. Another application was in an acid resistant bacteria

(*S. flexneri* and e. coli) in this instance Dr. Lay took whole bacteria and did MALDI-TOF MS to find common proteins within these two different strains which might be an explanation or a protein that confer acid resistance. He was not able to do classical MS peptide sequencing due to lack of instrumentation, he isolated different fractions and then ran MALDI-TOF until he got the appropriate fractions that had these protein bands, and then ran classical Edmond degradation. The sequence actually showed these were two different acid resistant proteins. This work today would be much more straightforward because with the QTAR, which we will have in the near future, we will be able to do peptide sequencing by MS analysis. This is a standard procedure used by FDA and other institutes in the United States and worldwide in characterization of bacteria.

We have had a number of different milestones in this area:

1. MALDI can differentiate bacteria by genus, species, and strain:
   J. O. Lay, Jr., "MALDI TOF Mass Spectrometry and Bacterial Taxonomy" Trends in Analytical Chemistry, 19, 507 (2000).

2. Specific Biomarkers for virulence can be detected by MALDI:
   R. D. Holland, C. R. Duffy, F. Rafii, J. B. Sutherland, T. M. Heinze, C. L. Holder, K. J. Voorhees and J. O. Lay, Jr., "Identification of Bacterial Proteins Observed in MALDI TOF Mass Spectra from Whole Cells", Anal. Chem. 71:3226-3230 (1999).

3. Biomarker proteins can sometimes be detected in contaminated media without pre-MS culture steps:
   R. D. Holland, F. Rafii, T. M. Heinze, J. B. Sutherland, K. J. Voorhees, and J. O. Lay, Jr. "MALDI TOF/MS detection of bacterial biomarker proteins isolated from contaminated water, lettuce and cotton cloth" Rapid Communications in Mass Spectrometry, 14:911 (2000).

We will continue to use these methods in areas such as proteomics, which requires the same types of skills. We may also use them in applications of bioterrorism. The fact that we can actually detect proteins with intact cell bacteria may make it possible to actually do this with malignant type cells and get biomarkers rapidly or ultimately even *in vivo*. In the future we also hope to:

- Determine correlation of toxicity and strain types with MALDI spectra
- Develop more powerful MS methods (MALDI/FTMS)
- Make more accurate assignment of biomarker (protein) identity

Benefits to FDA include:
- Differentiation of strains from more difficult Vibrio species
- Detection of biomarkers associated with antibiotic resistance
- An application to FDA programs in bioterrorism, proteomics, and even characterization of other cell types, possibly malignant cells, by MS.

As a complementary tool to MALDI in bacterial speciation and characterization, Dr. John Wilkes and the Mass Spectrometry Branch has utilized Metastable Atom Bombardment Time of Flight Mass Spectrometry (MAB-TOF MS) an alternative approach to characterize bacterial identification. This technique has actually been around quite some time and much of the work has been done by pyrolysis prior to MS analysis. Pyrolysis breaks down macromolecules into smaller constituents. Initial work is done by electron impact, which further obliterated these markers, and they were not able to get any useful pattern or profile for characteristics. The

technique was just to aggressive. What Dr. Wiles has done is look at MAB-TOF MS, which is a much softer technique and ionization method. He has had some complications because culture and bacteria in different labs in ostensibly identical conditions can give different profiles because the slightest changes in pH, salt concentration, or temperature can elevate or down regulate various proteins. Dr. Wilkes has developed an algorithm to normalize this.

Our current goals and objectives using MAB-TOF MS include:
- Rapid chemotaxonomic strain-specific bacterial identification
- Development of bacterial databases and search strategies
- Applications to food/seafood borne bacteria, especially Vibrio species (CFSAN & ORA)
- Development of patents for new methods
- Identification of bacteria without a prior cell-culture step.

Key Findings to date:
- Demonstrated that a multiplicity of laboratory variables distort mass spectral fingerprints.
- Patented a simple algorithm to correct for such method-related spectral changes.
- The correction is more practical than using identical conditions.
- US Pat. App. No. 60/239,549 filed 10/10/2000

Future Experimental Directions
- β-test Py-MAB-TOF- MS (from Dephy, Montreal) at NCTR.
- Assemble and validate a 200-sample spectral database using bacteria from CFSAN and ORA reference collections.

Future Computational Directions
- License the patent on using a spectral correction method to mitigate laboratory-based variations.
- Develop similar algorithms to transform spectra from environmental samples to their equivalent laboratory (database) spectra.

FRESH TAG™ Technology

Industry has always wanted a rapid screening tool to ensure product quality and freshness. With Dr. Dwight Miller's strong background in synthetic organic chemistry, he has developed some very selective tools and techniques for measuring freshness, which he named Fresh Tag™. In the fish industry, during storage trimethylamine N oxide will be converted via bacteria to trimethylamine dimethylamine. Dr. Miller has used the chemistry and biochemistry of fish metabolism to generate a tag agent that can show when fish have gone bad through a measurement of the elevation of these volatile bases. He has both a commercial version that could be used in a stock room and a consumer version that could be placed into the product package. Not only does the tag ensure product safety or freshness, it can go beyond protecting the consumer and can be used as a marketing tool. For example, the company can show their product is really outstanding, they can show that they take all the necessary precautions to ensure freshness while some of their competitors may not.

In addition to the Fresh Tag™ product, Dr. Miller is working on another method the Indole Test shrimp tryptophane will degrade to form indole. Dr. Miller uses both an analytical quantitative method by GC-MS and a colorimetric method by derivatization with benzyl. The derivatives can be correlated to a quantitated GC-MS method so that they have developed a rapid indole test method for shrimp. The method steps are to: grind 20 grams of shrimp in 50 mL toluene and 5

mL 5% TCA for 1 minute, centrifuge this puree for 30 minutes at 3500 rpm and decant off toluene layer, filter extract through a 0.45 mm syringe filter into a beaker containing anhydrous Na_2SO_4, then use the GC-MS or colorimetric method to determine freshness. I have also asked him to consider rapid screening tests for aldehydes and sulfides.

In addition he has expanded this to an IAG with the FAA where he is developing a method to be used in the federal aviation industry; N-oxides will oxidize a benzidiene derivative to give a highly colored ground color, which can be set with a tag agent, that can then be used in the federal aviation industry. The things he is trying to optimize now are things such as sensitivity, specificity, and the speed of reaction.

Dr. Catherine Ang in collaboration with CFSAN has been establishing methods to isolate various bioactive components in medicinal or herbal medications. There has been a great deal of discussion and controversy because some of these chemicals have pharmacally active components such as hyperforin which is a known inducive of P435A4 which competes with other P450s phase two enzymes which may alter pharmacological activities of a number of different drugs and medications and this is a genuine health problem.

More recently I have asked that she utilize some of her expertise in the chemistry of these compounds to work with Dr. Julian Leakey who is setting up a program on the potential toxicity of some of these different constituents. Dr. Leakey is looking at various human cell based assays and accessing the activity of some of these herbal medications and their influence on key enzymes, up regulation, down regulation, and metabolic activities.

Because of Dr. Ang's skills she has been able to isolate hyperforin which is found in St. John's Wort and is one of the key etiological agents under questions. We are able now to conduct some *in vitro* bioassays to better understand the biochemistry of these molecules. This work will be extended to human hepatocyte work looking at metabolism of various ingredients within St. John's Wort.

Research Progress:

- Extraction and LC methods developed for 4 SJW components in tea powder, fortified drinks, puffs and snack bars
- Methods Developed for 5 phenolic compounds in echinacea capsules and tablets
- Potential Toxicity of Herbal Constituents
  Investigators: J. Leakey, C. Ang, R. Cecotti, and Y. Cui.

Objectives:

1. To develop human cell-based assays to determine whether a test substance affects key enzymes involved in the metabolism of pharmaceuticals.
2. To use these assay systems to investigate potential drug-herb interactions between prescribed pharmaceuticals and dietary supplements.

Preliminary Findings:

- Developed methods for isolating hyperforin, the major active ingredient of St. John's Wort.
- Developed or procured battery of cell lines expressing major isoforms of human drug metabolizing enzymes: used in inhibition assays.
- Established that constituents of Echinacea inhibit enzymes conjugating estrogens

Future Work:

- Develop human hepatocyte-based assay systems for measuring drug metabolizing enzyme induction.
- Isolate and identify the inhibitory constituents of Echinacea and St. John's Wort.
- Investigate the metabolism of active ingredients of St. John's Wort by human enzymes.
- Apply inhibition and induction assays to other herbal products.
- Establishment of microarray and proteomic technology for elucidation of mechanisms

Dr. Rick Beger in collaboration with Dr. Jack Lay, Dr. Dwight Miller, and Dr. John Wilkes has been doing some work on the relationship between structure-activity relationships (SAR) and spectrometric data-activity relationships (SDAR) modeling (Protocol E0706801). Using C13 NMR in particular, which gives a great deal of information on electron density molecules, configuration, and confirmations. To use C13 NMR to aid in predicting function with various receptors. There are some unique things that C13 NMR can provide that other spectrometric methods may not. An example of some of the success of SDAR and QSDAR models include:

- SDAR model of 108 compounds binding to the estrogen receptor using NMR and MS data. Through principle component analysis he has done further separation into different components as strong binders, weak binders, and moderate binders at binding of various
- QSDAR model of 26 poly- chlorinated dibenzofurans binding to the aryl receptor using predicted NMR data.

SDAR Publications and Patents:
- 13C NMR and EI Mass Spectrometric Data to Produce a Predictive Model of Estrogen Receptor Binding Toxicology and Applied Pharmacology. 169: 17-25, 2000.
- Producing 13C NMR, Infrared Absorption and EI Mass Spectrometric Data Monodechlorination Models of Chlorobenzenes, Chlorophenols, and Chloroanilines J. Chem. Inf. Comput. Sci. 40:1449-1455, 2000.
- Developing 13C NMR Quantitative Spectrometric Data-activity Relationship (QSDAR) Models to the Corticosteroid Binding Globulin. J. Comput.-Aided Molec. Design.
- Models of Polychlorinated Dibenzodioxins, Dibenzofurans, and Biphenyls Binding Affinity to the Aryl Hydrocarbon Receptor Developed Using 13C NMR Data. J. Chem. Inf. Comput. Sci.
- Patent Pending for "Methods for Predicting the Biological, Chemical, and Physical Properties of Molecules from Their Spectral Properties."

Future Directions of SDAR:
- Protocol E0706801: "Continuing to develop SDAR models for the Ames test, neuraltoxicity (Neurotox), and other toxic endpoints"
- Protocol E0706811: "Developing new strategies for spectrometric models of toxicity" (ROW)
- Protocol E0708301: "Computational predictive system for rodent organ-specific carcinogenicity" (in collaboration with Biometry, CDER, ROW)
- Producing hybrid spectrometric models that incorporate three-dimensional structural information directly into the SDAR model.

Much of his work is retrospective, he is confirming toxicological data. I would like to see if we could use these methods to predict things. I hope that in the near future we will be able to establish some of these techniques in combination with e.g. DNA adducts, metabolites, or other endpoints of compounds we are not sure what the risk is to humans to see if we can help streamline types of studies we have done and focus on this area as well.

Dr. Dan Buzatu, in collaboration with Dr. Jack Lay, are taking complex chemical spectral data from C13 NMR and setting up artificial neural networks to interpret the data and look at the different predictive biological endpoints. One experiment using QSDAR-ANN model reflected excellent results for 28 poly-chlorinated biphenyl, dioxin, and furan toxic equivalence factors (TEFs) using predicted C13 NMR spectra.

Publications : Predicting Toxic Equivalent Factors from NMR Spectra for Dioxins Furans and PCBs Using Principle Components Analysis and Artificial Neural Networks, Environmental Health Perspectives, manuscript in preparation (2001).

Future Directions:
- Currently developing a quantum mechanical parameter based neural network model for the prediction of TEFs for the dioxins and dioxin-like compounds.
- Development of an internet parallel distributed neural network to allow for the handling of large data sets as well as increasing the efficiency of the neural network.

Dr. Fred Evans has been establishing methods to determine whether NMR can be used as a rapid screening tool for measuring adulteration, contamination of drugs, using genistein as a model system. The associated protocol is “A New Approach to the NMR Spectroscopy of Drug Purity and the Public Health Implications” (Protocol E070781). The objectives of the protocol:

1. Determine properties and develop procedures for use of NMR spectrometer at the NCTR under high dynamic range conditions
2. Develop concepts and methodology for application of spectroscopy to investigation of very-low-level impurities in drugs using results on genistein as a model

In vivo NMR for Detection of Biomarkers and the Intermediates of Metabolic Pathways

Cost to Upgrade NMR ~ $350,000 or New NMR ~ $550,000
Cost of labeled compounds ~ $15,000/year

Mass Spectrometry Applications in FDA Research Initiatives:
- Allergenicity
- Bacteria Taxonomy/Speciation
- Bioterrorism
- Drug Purity (Chemicals and Recombinant Proteins)
- Ion Mobility - MS (Protein confirmation determination)
- Microbial biotransformation of drugs and antibiotics
- Proteomics
- Quality Assurance and Compliance Programs
- Rapid through-put analysis
- Redox Status (Vitamins, Lipids, Proteins, DNA)
- Risk Assessment (Biomarkers, DNA- and Protein Adducts, DNA Damage, Metabolites)

In order for us to have a successful infrastructure we for proteomics have recently received funding to obtain the following MS instrumentation (available or planned):

Instrument	Application
Triple Quadrupole LC/MS ESI	MW determination for isolated proteins confirmation of MW for peptides/small proteins
Quadrupole TOF MS	MALDI and LC/ESI for sequencing especially for tagged proteins in measurement or relative levels of expression
SELDI	MALDI of affinity surfaces rapid screening of dirty samples for end-point specific proteins {Offsite}
MALDI TOF MS {at UAF} MALDI FTMF {at UAF}	MW determination for proteins and digests more accurate mass assignments and analysis of whole cells

In addition to the appropriate equipment and tools for proteomics, we need to be able to recruit and hire people with the skills and techniques to make this run. This will be our challenge.

There is interest in BSE detection, there is a possibility through ion mobility mass spectrometry which separates proteins by confirmation to actually separate prions then detected by mass spectrometry. Dr. Alex Strasbourg will establish this methodology using model compounds and this may potentially have the possibility to do BSE detection.

NMR Spectroscopy Applications in FDA Research Initiatives:
- Computational Chemistry
- Metabolomics
- Proteomics - Drug Interaction
- Drug Purity

Dr. Rick Beger is exploring in collaboration with Dr. Jill James looking at the metabolism modulation of tetrahydrofolate in Down Syndrome. Some of which can only be done with NMR.

Areas Supported in FY-2001
- Ethinyl Estradiol on Bone Growth in Rats
- Erythromycin from Farmed Animals
- Malachite Green/Leuco Malachite Green in Mice
- Retinyl Palmitate: Isolation & Detection
- DNA Adducts of Tamoxifen
- Dietary Supplements & Herbals: Identification of Bioactive Ingredients
- Endocrine Disrupters: Genistein & Daidzein
- Phytoestrogen Conversion to Estrogenic Compounds: Genistein & Daidzein
- Fluoroquinolone Biotransformation by Fungi
- Microbial Degradation of Drugs & Feed Additives in Aquaculture
- Antihistamine Drugs in Neonatal Mouse Cells

Dr. Catherine Donnelly: There is a lot of overlap in some of the initiatives in the micro division, are there attempts to do formal collaboration and coordinate efforts?

Dr. Turesky: There is overlap but they are collaborative in nature. Historically our division has had strong collaboration with the Microbiology Division, particularly addressing things such as

the microbial antibiotic resistance that requires analytical chemistry and mass spectrometry. However, some of the other work that has been done by characterization of various proteins was initiated in our division.

Dr. Jack Lay: This is actually larger than just an NCTR project, the Vibrio species we have looked at have come from Dolphin Island. We have also transported the technology to the Center for Food Safety and Nutrition (CFSAN) and they have a capability for doing this and they are now sequencing the proteins we found that allow us to differentiate the Vibrios. There are several microbiologist from NCTR and other FDA facilities involved in this as well as chemist.

Dr. Casciano: There is a natural fit, these two groups have been collaborating together for years because of their scientific interests. Microbiology and chemistry has a support and research function and they leverage with each other to answer some of the questions.

Dr. Donnelly: There is also further work that can be done on identification of some of the poultry strains, you could apply you mass spectrometry identification to derive more information. The common interests are very exciting.

Dr. Casciano: Dr. Cerniglia's group is developing 2-D gel electrophoresis techniques to look at proteins as a collaborative effort, he will do the PCR to do the comparison.

Dr. Lay: The comment about PCR is very interesting because when we looked at the Texas Gulf Coast and the Pacific Northwest outbreaks of *Vibrio Parahaemolyticus* mass spectrometry only differentiated those into two groups. The PCR method differentiated the single outbreak in the Pacific Northwest into as many as nine different outbreaks even though it was a single outbreak. The methods are complimentary because they detect different kinds of markers that are orthogonal with respect to classifying the bacteria.

Dr. Rosenkrantz: There were some questions on the commercialization of FreshTag™. Are there other mechanisms for commercializing the FreshTag™ that could be used?

Dr. Dwight Miller: FreshTag™ is under license to Cox Technologies in Charlotte, North Carolina. We have worked with them from the initial prototype, which was put on a piece of paper, and it actually increases the surface area of FreshTag™ and increases the speed of reaction. As you saw in the slide, the one with the plastic laminated disk that is made porous with the actual matrix that we use with FreshTag™. In terms of marketability the slide underneath it is being looked at by people like Tenneco that do packaging to build FreshTag™ into a packaging material so that you would have a zip lock bag that if you put the fish product or another food that produces ammonia in the bag it will give a clear signal as soon as it decomposes. The aldehyde has been brought up by the seafood industry itself in that you get lipid peroxidation in cold storage in addition to DMA as a cold storage problem. Formaldehyde and DMA are formed by autolytic oxidation in the seafood at –20 degrees. The process is a autolytic and possibly enzymatically generated hydroperoxide which in turn forms the aldehydes from the lipids and the detection you saw of the little green dots in under development for inclusion directly into a package for detection of that. As it turns out the major compounds of decomposition, ammonia, acids in the case of carbohydrates, sulfurs (hydrogen sulfide that would occur from salmonella or degradation of sulfur containing compounds in the proteins), and aldehydes can all be detected. This is one of the things Tenneco has expressed interest in, that we detect all of these things in one step.

Dr. Acosta requested comments or questions from the public, hearing none the meeting continued.

Division of Biometry and Risk Assessment Update, by Dr. Ralph Kodell

There are several mathematical statistician's in the division as well as support staff.

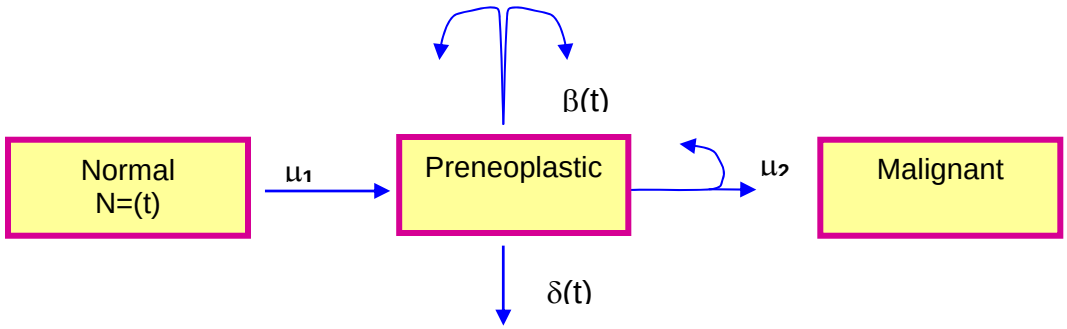
James Chen, Ph.D.	Mathematical Statistician
Robert Delongchamp, Ph.D.	Mathematical Statistician
Yi-Ju Chen	Post Doc, Mathematical Statistician
Daniel Molefe, Ph.D.	Post Doc, Mathematical Statistician
Cruz Velasco, Ph.D.	Post Doc, Mathematical Statistician
Angelo Turturro, Ph.D.	Research Biologist
John Young, Ph.D.	Research Biologist
John Appleget	Computer Specialist
Bruce Pearce	Computer Specialist
Susan Taylor	Program Support Specialist
Qi Zheng, Ph.D.	Staff Fellow

Research Highlights:

Fumonisin B1 Risk Modeling, Qi Zheng et al.

There was an NTP IAG Study in rats and mice (Dr. Paul Howard, PI). The only two dose related endpoints deemed statistically and biologically significant were liver tumors in female mice and kidney tumors in male rats. At that time Dr. Bern Schwetz, the Director of NCTR, directed and encouraged us to engage in this risk assessment for the mechanistic data and traditional bioassay data to try to use that in a biologically based model to do a comprehensive risk assessment if possible. The clients being hopefully, Center for Food Safety and Applied Nutrition (CFSAN) and the Center for Veterinary Medicine (CVM). This approach aslo fit well with two recommendations of the Science Advisory Board (SAB) Site Visit Team (SVT) on their last evaluation of our program. They said that we ought to determine projects that related to the Food Safety Initiative and to try to find projects for intra-division collaboration. The basic data we tried to model were two types of female mouse liver tumors, hepatocellular adenoma or carcinoma. We used adjusted tumor rates at 104 weeks of 11.7% background incidence, 6.5% at 5 ppm, 2.1% at 15 ppm, and 42.7% at 50 ppm, and 88.3% at 80 ppm. We wanted to look at the down turn to see if it would be mimicked in any type of prediction that we made.

We used a mathematical model of Mogafkar, Vincent, Knudson (MVK) two-stage, cell-proliferation model to predict probability of tumor at 104 weeks.



This model assumes there is a pool of normal cells, in this case normal liver cells annotated by the number of those cells. These cells can undergo a first mutation at rate muse of one to become pre-neoplastic. The pre-neoplastic cells can give birth to two new daughter cells that are pre-neoplastic or they can die. The birth rate being $\beta(t)$ we make this time dependent with the death rate being $\delta(t)$ which is also time dependent. The pre-neoplastic cells can undergo a second mutation to produce one daughter cell that is pre-neoplastic and one daughter cell that is malignant cell. This is where the model says cancer has occurred.

We wanted to take the mechanistic data and identify and estimate these various parameters to do the probability of cancer prediction at 104 weeks. The hypothesis was that Fumonisin B1 affects the incidence of liver tumor formation in mice by increasing the death rate of liver cells which leads to compensatory proliferation. To implement the model:

- Use allometric relationship between liver weight and body weight, LW(t)=a[BW(t)]b, to estimate the liver weight
- Estimate the number of cells in the liver by N(t) = LW(t) / CW
- Estimate the net growth rate of the liver using d[log LW(t)] / dt
- Use PCNA data to estimate the cell birth rate, $\beta(t)$
- Estimate the cell death rate by $\delta(t) = \beta(t)$ - d[log LW(t)] / dt
- Relate differential effect of FB1 on $\delta(t)$, and consequently, $\beta(t)$ by level of sphinganine in liver
- Infer mutation rates, $\mu 1$ and $\mu 2$, (constant w.r.t. FB1 and time) from tumor data

Female Mouse Liver Tumors, tumor incidence at 104 weeks, hepatocellular adenoma or carcinoma

Observed:	.117	.065	.021	.427	.883
Predicted:	.091	.084	.105	.284	.992

Male and Female Mouse Liver Tumors

Male	Observed:	.268	.211	.190	.213	.213
	Predicted:	.199	.201	.198	.233	.237
Female	Observed:	.117	.065	.021	.427	.883
	Predicted:	.091	.084	.105	.284	.992

Fumonisin B1 Summary: We concluded that the data and model are consistent with hypothesis. We presented this information in papers and a poster session at the FDA Workshop on Fumonisins Risk Assessment held in February, 2000. We were invited to present the information by CFSAN organizers. We published articles in Food Additives and Contaminants, 2001 and CFSAN/CVM were receptive of our information. Subsequently Dr. Mike Bolger who is in charge of CFSAN's risk assessment for fumonisins and also chairs a subcommittee of the FAO/WHO JECFA (Feb 01) used our work extensively in a draft report on fumonisins. There is still a question of the kidney tumor risk in male rats which remains to be addressed.

Key Research Projects:

Dr. Angelo Turturro is the Principle Investigator the *Cryptosporidium parvum* study (Protocol E07082.01). This study is partially funded by an IAG with the Environmental Protection Agency National Center for Environmental Assessment (EPA-NCEA), in Cincinnati (B. Bodin). We

received input on the protocol from CFSAN (R. Buchanan, G. Jackson, M. Miliotis) and Dr. Carl Cerniglia. This is a first for NCTR where we have an animal study with an infectious agent.

Cryptosporidium parvum is a protozoan and is a common contaminant of drinking water, hence EPA's interest. It is also a contaminant of the food supply through improper hygiene. The objective of the study to develop a model for transmission dynamics of *Cryptosporidium parvum* (Cp) in human outbreaks. Subobjectives were to standardize the dose of Cp strains in the neonatal mouse (three isolates of varying potency) and to establish an appropriate animal model. Through Dr. Turturro's experience in the PCR study he selected the Brown Norway rat. The C57Bl/6 mouse (Dex) unless it is chemically suppressed does not show the same level of zero positive individuals as the normal human population. Other objectives to investigate subpopulations with varying degrees of immunocompetence. Using three age groups - young, adult, elderly, pregnant, immunosuppressed similar to AIDS, and physiologically stressed through diet or exercise. The protocol has been through the review process, has been revised, has been re-submitted, and is awaiting Director signature.

Another part of the IAG with EPA-NCEA, Cincinnati (G. Rice, L. Teuschler) is Cumulative Risk Assessment for Chemical Mixtures, led by PI, Dr. James Chen, Yi-Ju Chen et al. (Protocol E07087.01). The objective is to develop and apply a Relative Potency Factor (RPF) methodology for estimating the cumulative risk from exposure to a mixture of chemicals having a common mode of action (e.g., organophosphates which induce cholinesterase inhibition) and as part of the EPA mandate Food Quality Protection Act (FQPA) of 1996. The specific aims are to use an expanded definition of dose addition to develop a risk estimation method that does not depend strictly on parallelism of log-dose-response curves. Generally speaking when people think in terms of dose addition and do dose response modeling we are talking about on a log scale looking for parallel curves. We think that does not have to strictly apply to have dose addition in a broader sense. In addition we want to develop a classification algorithm for clustering chemicals into several constant relative potency subsets. You could have subsets of chemicals in this mixture that do have a common constant relative potency so you can segregate these out and apply the more general method.

This method uses actual dose-response functions of mixture components, not just ED10s, say (like TEF, HI, etc.). If the RPF is constant across all chemicals, then it is invariant to the choice of index chemical. It can be used even when the RPF differs for different subsets of chemicals in the mixture. The protocol is in review at this time.

Dr. John Young is heading the Computational Toxicology effort (Protocol E07083.01) with collaboration with ROW Sciences, the NCTR Chemistry Division, and CFSAN. The objective is to develop an expert computational system for prediction of organ-specific rodent carcinogenicity by applying structure activity relationships (SAR) in conjunction with data on short-term toxicity tests (STT), and nuclear magnetic resonance (13C-NMR) spectroscopy. The motivation is the FDA's need to bring safe products to market more quickly and to screen out unsafe products reliably. CFSAN (M. Cheeseman) indicated it would be very useful to streamline toxicity testing, e.g., require sponsor to conduct target-specific toxicity based on system's prediction.

We are looking at the 1,298 chemicals in the Carcinogenic Potency Database (CPDB). We are considering three groups, Group 1: carcinogenicity in liver, Group 2: carcinogenicity, but not in liver, and Group 3: no carcinogenicity in any organ. We will add data on SAR, STT and NMR mass spectrometry. There are 392 NTP chemicals in CPDB, 342 are positive in liver for ≥ 1 species-sex combo. For a good mix of positive/negative, we might need to do species-specific

prediction sex-specific prediction. For the training set we will use 392 NTP chemicals in the CPDB and for the testing set we will use 288 literature chemicals in the CPDB. There are 282 pharmaceuticals in the CDER database with only 33 of those positive in liver for ≥ 1 species-sex combo and could serve as a good test case for our strategy. The protocol was recently approved and implemented.

The next project is Photocarcinogenicity Theory and Methods, I am the PI on this with help from Dr. Daniel Molefe (Protocol E07061.01). We are working with Dr. Paul Howard of the NCTR Phototoxicity program who has set up a CRADA with ARGUS Laboratories (S00213) to obtain data for us to use as we develop our methodology and has obtained funding for a Post Doc through the NTP (Protocol E02037.01). This will meet the needs of CFSAN (cosmetics), and CDER (dermal applied drugs) (K. Lin) and will not be restricted solely to photocarcinogenicity but can be applied to any studies where there are multiple tumors that are externally observed (i.e., skin, mammary tumors, etc.) One of the paradigms for drugs is that short term tests can replace one aspect of the long term study.

We will use the statistical approaches of the standard testing method, the log-rank test for differences in distributions of time to first observed tumor. We will also use a new testing method to test for difference in number of induced tumors and/or to test for difference in distributions of time to observation of tumors. You can then contribute this to the carcinogen inducing more tumors or simply changing the latency period. Thus far we have developed a model for repeated-exposure case and a computational optimization procedure. We have analyzed data on the first of eight ARGUS studies and we intend to compare this data using both the log-rank and Dunson's (NIH) methods. The protocol is ongoing.

Analysis of cDNA Microarray Data is headed by Dr. Bob Delongchamp, and Dr. Cruz Velasco (Protocol E07096.01). cDNA microarrays are popular new biotech tool you get vast amounts of data on gene expression quickly. There are various statistical, experimental design, and analysis and interpretation issues that remain to be worked out. We know you need to replicate arrays and genes within an array if possible. Data analysis issues are: adjustment for nuisance sources of variation, appropriate methods for assessing differences, adjustment for multiple comparisons, and identification of genetic profiles. The field is at this juncture, microarray data first needs to be normalized, there are several sources of variation that must be accounted for. Then you begin statistical analysis. The objectives are data analysis, development of appropriate methods for assessing differences in individual genes, clusters of genes (genetic profiles), and adjustments for multiple comparisons of different error rates (ER), e.g., Per Comparison Error Rate (PCER), Family Wise Error Rates (FWER), and False Discover Rate (FDR). This protocol is currently in development.

We are trying to stay abreast of the newest technology through staff enrichment. We are trying to take short courses and attend various conferences (UCLA Functional Genomics (Chen), IBS/ENAR Conference (Chen, Delongchamp, Kodell), Gordon Conference on Bioinformatics (Zheng), Genetic and Evolutionary Computation Conference (Pearce)) and to visit various laboratories (Academia Sinica, Taiwan (Chen, 2 weeks), Visualization, classification (C-H Chen), Jackson Lab. (Delongchamp, 1 month), and to visit other FDA Centers, and to host various visiting scientists.

Dr. Nancy Gillett: I was interested in on your comments on the risk modeling of fumonisin. I was wondering if you planned to do that with any of the other NTP products and also are people in your group who, if you are planning to do more, involved in some of the protocol design so that you get the appropriate data at the earlier sacrifice time?

Dr. Beland: We do want to be up-front on the design of the NTP protocols. We have been involved in design. As far as risk assessment and modeling we have not done any of that and do not have any immediate plans at this time.

Dr. Casciano: Protocol development is high scrutinized and there are statisticians involved in that review. I have asked Dr. Kodell's group to think about utilizing the expertise they have developed in chemical dose response, database mining, statistical analysis, and statistical development of new analytical tools to applications in the new fields because we consider this to be future toxicology as well.

Dr. Gillett: I was just wanting to make the point that the purpose of these very large, expensive, bioassays is to get risk assessment to humans and therefore the application of risk modeling is important.

Dr. Beland: We will involve Dr. Kodell's group. At this time we have only completed two compounds, fumonisin and chloral hydrate. Chloral hydrate did not cause cancer. The big issue that will come up is urethane and alcohol and how we treat the interaction of these two compounds.

Dr. Gillett: It is important to get involved in the front end of the design.

OPEN DISCUSSION:

Dr. Gillett: I am still unclear is there a provisional committee for genomics and proteomics at NCTR?

Dr. Casciano: The initial interaction has been to gather interested individuals who have interested in using microarray technology in building databases and evaluating the databases. We have committees on computational science. Identified interested individuals creating a new group of individuals with both a cellular and molecular interest. The dialog is also ongoing with the various centers regarding our various techniques and how best to use them to supply the data needed by the FDA for regulatory decision making.

Dr. Lightfoot: Question for Dr. Beland regarding antiretroviral agents and a study in pregnant women. One of the few the success stories for the search for antiretroviral agents is that of pregnant women treated with AZT. What was the motivation for the research? Are you looking to see if AZT is more mutagenic than therapeutic in the babies of women with AIDS?

Dr. Beland: Women and their babies are being treated with multi drug therapy now and the question is, is this safer than AZT by itself or is it going to cause complications in the children who are not HIV positive later on. We are looking at safety issues, we are not saying discontinue the treatment, but rather are there certain treatments that may be safer.

Dr. Lightfoot: What motivated the study?

Dr. Beland: The motivation for the study was the demonstration that AZT is carcinogenic when administered transplacentally and neonatally to mice. There have also been some mitochondrial toxicities and death reported in children who have been treated with AZT and 3TC in Europe.

Dr. Pat Hansen: I enjoyed the Biometry group presentation; CFSAN is very interested in pursuing more of these structure activity approaches and approaches to constructing tier testing schemes that we can give as both guidance to petitioners as well as using for in internal review guidance for our own staff. The utility of the work being done in Biometry is excellent.

Dr. Casciano: The idea is to help us develop the regulatory questions.

Dr. Linda Youngman: Being new to the government and as my first time to be at NCTR it has been a real learning experience, I didn't know the scope of the work being done here. I see a hugh amount of scope for future collaboration with the Office of Research in Laurel, MD. I've already spoken to some of the Division Director's after their presentations so I hope that collaborative projects can be considered.

Dr. Catherine Donnelly: I think the last two days have been incredibly valuable. There is such great work being done here at NCTR. How you get that work out to the other Center's and to the public so that other division's and constituents recognize it is vital. Dr. Casciano, you raised the question "Is it time for NCTR to have a review as an agency?". When I listened to some of the presentations I got the impression that many of the initiatives and projects are PI directed, but I'm not sure that they fit within the context of the overall goals of NCTR. I think you need to have a very simplistic annual plan and to establish strategic goals so that it is really clear for reviewers when they come in to look at the way the program is being managed. There are typically five central goals for a program and each project has to fit within one of the goals. It would seem to me that it would be very easy for NCTR to sit down and draft an annual plan and strategic goals and to have each project fit within this strategic framework. You would then get the sense that the projects are not simply PI driven because of PI interest or because of extramural funding. It would also be easy to see that the project fits not only within the strategic goals of NCTR but perhaps the goals of CFSAN, CVM, or one of the other centers. That way I think you would build a constituency base across the center's. The frustration I have is that there is some fundamentally excellent work going on but perhaps you folks are the only ones that know about it.

Dr. Casciano: We have been trying to do that over the last nine years. The work that is going on relative to the NTP is a direct response to FDA needs. I said FDA needs because there are five product centers each of which would like us to have a strategic goal directed specifically towards them. Each one of the product centers has different mandates and methodologies for doing something as simple in concept as risk assessment. We establish our strategic goals from the point of view of, prior to development of a protocol there is a concept statement or paper prepared. That concept statement can be one page or five pages long, whatever it takes to communicate to me what the scientists is interested in doing. The scientist are very creative and in the last eight to ten years they have greater communications with the product centers and have a greater sense of what their needs are. If I understand and agree with the concept, I send it to the five product centers for comments. The rational is to get input from the regulatory centers so they can have interaction with the scientist on the front end. If there are three or four objectives that I think will meet the needs or requirements of the centers, then the PI develops the protocol. We do write strategic goals that are broad enough to cover the mandates of all of the centers. They are directed towards methods development which is more long term.

Dr. Donnelly: I think that might be a nice framework for publicity.

Dr. Casciano: We are not very good at publicity, we are not very good at conveying our strength.

Dr. Rosenkrantz: Do you have a plan for the site visit teams for the coming year?

Dr. Casciano: We are planning a site visit for the early fall or winter for the Chemistry Divisions. We generally review every two or three years.

Dr. Acosta: It might be good to have a schedule of the past site visits and reviews.

Dr. Casciano: Thank you for your extended interaction with the NCTR staff. I hope it has been beneficial. We do listen to your recommendations and do try to respond.

Dr. Acosta: We appreciate the fact that all of the Division heads were present. It is evident you are very proud of all of the hard work of your staff, that you have very high standards and operate in a very professional manner.

The meeting was closed for Executive Session.