Site Visit Team Report

Review of the Division of Biochemical Toxicology

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Executive Summary

The Site Visit Team members were uniformly positive concerning the achievements of the Division of Biochemical Toxicology. This was the first review to take place in the last ten years due to funding issues. In that time the Division has accomplished a lot with limited resources. These accomplishments include completion of a large number of NTP technical reports, major discoveries concerning the mechanism of toxicity of compounds nominated by stakeholders in Federal Agencies, and the publication of 60-80 research articles per year in some of the leading journals. Dr. Frederick Beland, Director of the Division must take credit for these achievements.

The Site Visit Team members also identified areas that require attention if the NCTR is to maintain a preeminent role in the NTP and more broadly in toxicological research. These are listed below:

1. The Leadership of NCTR and the Division should provide additional guidance for the strategic direction of research programs. While it is evident that there has been an increased focus on the research needs of the FDA, it is not clear that the issues of highest importance to the agency are receiving the highest priority for efforts at NCTR. While additional input from leadership on strategic direction is encouraged, it is important that the scientific staff retains the responsibility for determining the approaches to address these issues.

2. The presentation of scientific programs and results should be more highly focused on the projects rather than on investigator to assure that the value of the program is fully understood. The current presentation format resulted in some projects resurfacing several different times leaving the listener/reader to synthesize the pieces of the program. The presentation of the various programs should more clearly articulate the issue as well as the hypothesis or objective that is being addressed in the research effort. In the current presentation of material both written and orally, these points were well characterized in some cases but very unclear or more frequently very vague. It was noted that presentations were most frequently made by male staff which raised the question whether presentation visibility was appropriately shared by male and female staff.

3. There should be greater focus and inclusion of pharmacokinetics (PK) and toxicokinetics (TK) in the research efforts in the Division. Currently some programs appear to have extensive efforts in these areas while other programs have minimal to no PK and TK which is required to interpret the data and to determine human relevance of animal study results.

4. Additional professional expertise would greatly assist the productivity of a number of programs.

- Immunologist: With the planned direction of several programs, it would be very advantageous to have on site expertise in immunology rather than relying on expertise located at NTP in North Carolina.
- Experimental Pathologist: While the diagnostic needs to support the current research program are adequately met through a contract for this expertise, an on site experimental pathologist would greatly assist in experimental design and study interpretation particularly related to very specialized studies where routine diagnostic pathology expertise in not sufficient.

- Synthetic chemist: The extensive analytical capabilities and expertise of the Division result in the need for synthesis of numerous chemical standards that may not be readily available through commercial sources or through private contacts. Currently the need for chemical synthesis is being at least partially met by very excellent staff but these staff has broad project responsibilities.
- Electron microscopy: The entry into nanotechnology work requires precise characterization of material and localization of material that in some cases is best accomplished by electron microscopy. This expertise is essential and must be provided at NCTR or through a rapid and interactive external collaboration.

5. The Division has adopted "a promotion from within policy" to fill vacant leadership positions. While the appointments made are adequate, the failure to conduct national searches for the best talent will mean that the Division cannot reach its full potential.

6. The analytical instrumentation which is key to the successful mission of the Division is aging. The mass spectrometers except for a single newly purchased instrument are approaching obsolescence. Thus investigators are unable to reap the benefit of the high-sensitivity and fast throughput that can be attained with newer instruments. Similarly, although the Division has a reasonable number of HPLC unit's apart from two, the remainder are 10-15 years old.

7. A large portion of the financial resource of the Division comes from the IAG with the NTP which nominates compounds for study. The fee structure is on a per project basis which does not provide the financial resources required to replace aging instrumentation and other infrastructure needs. It is recommended that the NCTR leadership re-negotiate the IAG with NTP so that adequate funding is available to maintain core facilities.

8. The Division has both a mass spectrometric resource and an analytical chemistry resource. It is not clear why these need to be separate. They could be both directed by a single senior leader in analytical chemistry.

9. It was noted that the NCTR was conducting research that was in competition with academically based research funded by federal agencies. In some instances the research is duplicative. A more concerted effort should be made to identify academic partners to prevent duplication of effort and unnecessary expenditure of federal tax dollars. Thus when choosing a research problem due diligence should be conducted in study design.

10. Professional interactions with the University of Arkansas should be reassessed with the goal to increasing interactions in several different areas. The number of division members holding Adjunct Faculty appointments appear to have ebbed in recent years. Opportunities to enhance this interaction should be examined. Additional interactions should be explored such as the possibility of externships to provide graduate students in pharmacology and toxicology exposure to the research of NCTR. This could be used by participating Universities to strengthen their own graduate programs and training grant applications. In the present funding climate funding agencies are attracted to innovative training programs.

Structure of review and review document:
The research programs of the Division of Biochemical Toxicology were reviewed during a visit by the Site Visit Team on April 29-30 2008. Since the last review of this Division was more than 10 year ago, the review included progress in this extended time period, recent progress and plans for new efforts that are currently being developed. The following review comments are based on presentation made during the site visit as well as review of written material that was sent to the site visit team in advance. The order of the following review statements is the same as the order of oral presentation of material which is different than the order in the written review document. The titles of the projects listed below are consistent with the titles in the written document. Prior to the review of the individual projects listed below, Dr. Beland (Director, Division of Biochemical Toxicology) provided an overview of the Division outlining the focus of the Division, the personnel and other resources available in the Division and the publication productivity of the Division.

NTP studies at NCTR and phototoxicology, photocarcinogenesis, and mechanisms of toxicity of pigments (Dr. Paul Howard)
This part of the Division's effort is very extensive and diverse and will be addressed under the several specific projects.

Overview: NTP studies at NCTR
Summary
The NCTR has become increasingly involved in the design and performance of studies that are managed through the National Toxicology Program (NTP) that is administered by NIEHS in Research Triangle Park NC. NCTR is well incorporated into the activities of the NTP and fully participates in the selection of agents for submission for consideration of evaluation under the NTP system thereby providing an important avenue to focus a portion of NTP effort on molecules and issues relevant to the responsibility of FDA. When a molecule of interest to FDA is selected for NTP evaluation, the NCTR staff and NTP staff work closely to design the study appropriate to meet the needs of the FDA Product Centers. The NCTR staff involved in the study has ongoing interaction with the NTP staff during the course of the study and the interpretation of the observations. Release of study results follows the NTP system with the NCTR staff preparing the material in the form of a NTP Technical Report which is subsequently reviewed through the NTP system. Publication of study results in the peer reviewed literature may occur providing another avenue for public release of data or more extensive release of data. Research activities that are part of the NTP effort are financed by the NTP through an established Interagency Agreement (IAG) that has been in place since 1992. This agreement also outlines the working relationship of NCTR and the NTP

Critique
To date, 10 Technical Reports have been published on studies conducted under the IAG while numerous other studies are currently in progress in the laboratory or in the final phases of data interpretation or report preparation. These studies include numerous compounds that can be identified under the following broad categories:

- Multigenerational studies, endocrine active agents (Methoxyclor, Genistein, Nonylphenol, Vinclozolin, Ethinyl estradiol)
- Dietary supplement (Riddelliine, Aloe vera, Bitter orange/citrus aurantium, Usnic acid, Glucosamine/chondroitin sulfate)

- AIDS therapeutics (Combination of zidovudine, lamivudine, nevirapine, , nelfinavir and efavirenz)
- Pediatric (Ketamine, Diethyl-hexyl phthalate)
- Drug interaction (Chloral hydrate, Chloral hydrate diet restricted)
- Phototoxicity (α- & β- hydroxy acids, Aloe vera, topical, Lemon & lime oil furocoumarins, Retinyl palmitate, Permanent make-up pigments)
- Nanoscale materials (Titanium dioxide & zinc oxide, Titanium dioxide &Tg.Ac transgenic mice, Nanoscale silver, Nanoscale gold)
- Food contaminants and food safety (Fumonison, Urethane ± ethanol, Acrylamide, Malachite green)

It is clear that the molecules selected for evaluation are indeed important to the agency and represent the interest of multiple FDA Product Centers. It is also clear that decision on continuing evaluation of a specific molecule is being based on data that is developed with effort on some molecules ending based on the results from short term studies.

Recommendation

NCTR and the Division should continue interacting with both the FDA Product Centers and the NTP to assure that the best scientific approaches and appropriate resources of the NTP are utilized to address specific needs of FDA.

The funding approach defined by the IAG should be reexamined and renegotiated. While the funds coming to NCTR through the IAG are apparently adequate to cover the cost of the performance of the individual studies, it is also apparent that this funding does not provide funding for NCTR to support an adequate infrastructure particularly in the area of analytical instrumentation. Funding through the IAG should adequately support the NCTR infrastructure that is required for the NTP related activities.

As Dr. Paul Howard assumes greater responsibility as the Deputy Director of the Office of Science Coordination with the responsibilities of coordinating the IAG between NTP and NCTR, it is essential that other NCTR staff assume greater responsibility for the oversite of programs and laboratories that Dr. Howard currently leads.

Overview: Phototoxicology, photocarcinogenesis and mechanisms of toxicity of pigments

Project 1: Development and continued operation of the NTP Center for Phototoxicology (S0615) (Dr. Paul Howard)

Summary

The Phototoxicology Laboratory is an NTP resource that is located in and managed by the NCTR and is one of only 2 facilities in the world that can expose large numbers of mice to simulated solar light. Dr. Paul Howard has been primarily responsible for the development of this facility. With his assumption of additional duties as the Deputy Director of the Office of Science Coordination, Dr. Howard's efforts will be reduced in the area of photoxicology. He will continue with the responsibility of Director of NTP Center for Phototoxicology but Dr. Boudreau will be the Manger of the facility and will oversee the day to day operations of the laboratory.

Critique

There is no doubt that the Phototoxicology Laboratory is an important facility to support the mission of the FDA. Since there are limited capabilities throughout the world and since skin care products do not require pre marketing assessment, it is imperative that the FDA have the capability to assess products where special concern may develop. The laboratory has been very methodically developed over a number of years and has proven capable of performing well defined studies which require the specialty expertise that has been developed at NCTR. Specifically, the laboratory has supported 3 major protocols on photocarcinogenesis of test articles: 1) α- and β- hydroxy acids 2) topically applied aloe vera 3) topically applied retinyl palmitate. In short the laboratory has established a proven "track record". Future plans include improving the methodology for calculating and reporting dose. While SKH-1 hairless mice have been primarily used in the past, the use of specific transgenic mice will be incorporated into future studies. Plans are underway to evaluate a potential short term in vivo assay to replace the currently used 1 year photocarcinogenesis study.

Recommendations

The phtototoxcicology laboratory should be well supported by both NTP and NCTR since it provides a physical facility and intellectual expertise that can not be provided by other organizations. It is in a unique position to address issues that are of great importance to meeting the mission of FDA.

Project 2: Photocarcinogenicity of topically applied α- & β- hydroxy acids (E2131, E2137) (Dr. Paul Howard)

Summary

α-hydroxy and β-hydroxy acids are found in topical creams for removing the stratum corneum and restructuring the underlying collagen. These molecules were nominated by CFSAN for evaluation by the NTP due to concern of possible deleterious effects. The objectives were to determine the impact on cell proliferation and to determine the photocarcinogenicity of topically applied hydroxyl acids exposed to simulated sunlight.

Critique

The studies have been successfully completed. The hydroxy acids (glycolic and salicylic) caused a wave of cell proliferation at 12 to 16 hours after administration. The subsequent photocarcinogenicity study utilized doses of light that were significantly less than the doses required to cause sunburn in mice allowing evaluation of the effects of the hydroxy acids at light doses that did not induce toxicity or damage to the skin. Due to the complexity of study design (evaluating 2 different molecules with and without light and light at 2 different doses) the study included 40 groups of mice. There was no consistent effect of glycolic acid on skin carcinogenesis with or without light. Salicylic acid had a protective effect on light induced skin tumors. The NTP Technical Report has been peer reviewed and published in 1996. The phototoxicology laboratory is commended for developing such a thorough study design and for successfully completing the complex study.

Recommendation

No recommendations are provided since this project has been completed.

Project 3: Development of mouse model for tattooing (E7105) (Dr. Paul Howard)

Summary

Animal models for evaluation of effects of tattoo inks were not available. Therefore, the NCTR developed a wide ranging program for the evaluation of the effects of tattoos

since the practice of tattooing is wide spread and little data is available on health effects. The data being generated will enable the FDA to make science based decisions regarding the safety of tattoo risks. The specific aims of this project were to:(1) develop a mouse model for tattooing; (2) determine the biological impact of tattoo pigments in mice; (3) determine the photostability of tattoo pigments; (4) determine if tattoo inks are metabolically activated; (5) determine the recovery of tattoo inks from tattooed skin; (6) determine the biodistribution of a tattoo pigment in mice; and (7) determine the photocarcinogenicity of tattoo inks in mice exposed to simulated solar light.

Critique

Excellent progress has been made in developing and evaluating testing systems needed to address important biological questions related to human health. Hairless mice [SKH-1 (hr^{-}/hr^{-})] have been successfully tattooed using commercial tattooing needles. In the presence of simulated solar light, the evaluation of Pigment Red 22, Pigment Yellow 74 and CdS as a positive control identified no severe toxicity with the exception of CdS. Gene and protein expression indicated that sustained changes were present in the skin and lymph nodes for 15 weeks following tattooing suggesting that tattooing may result in permanent gene expression changes in the skin and lymph nodes. Photodecomposition of several tattoo pigments has been demonstrated indicating that fading tattoos may be releasing ink fragments of unknown toxic and carcinogenic potential. Based on in vitro evaluation of metabolism, some tattoo inks can be metabolized resulting in genotoxic products. The photodecomposition process is being evaluated for the production of reactive oxygen species. The biodistribution of tattoo ink is being characterized with deposition identified in the local lymph nodes. Tritiated Pigment Yellow 74 has been demonstrated in the liver, gall bladder and kidney in addition to the local lymph node with a large percentage excreted into the intestinal tract.

Future activities will evaluate photodecomposition of tattoo inks focusing on the spectra used for laser based removal of tattoos. In addition, future effort will focus on the potential immunotoxicity of tattoo inks (See next project)

Recommendation

Due to the wide spread practice of tattooing in humans, the NCTR is encouraged to support this project at the highest possible level.

Project 4: Immunogenicity of permanent make-up inks (E2161) (Dr. Paul Howard)

Summary

In 2004, over 50 adverse events related to a specific permanent make-up product line were reported to the FDA. These events, primarily disfiguring inflammatory reactions, increased the concern regarding the lack of toxicological data and potential for human adverse health effects related to permanent make-up inks. The specific aim of this project was to use a modification of the mouse local lymph node assay (LLNA) to determine if the inks related to adverse human effects induce an immunologic response. Therefore a modified LLNA was developed to accommodate test articles with poor solubility and poor penetration. Three recalled pigments induced lymph node proliferation while color matched pigments from another source did not cause a similar response. This result indicates that the inks associated with the disfiguring effects contain an immune sensitizing ingredient that is contained in the insoluble fraction of the ink.

Critique

This is a relatively new project that has met the first objective of determining if the specific make up inks are immunogenic. The second goal to be addressed is to determine the immunogenic ingredient so the FDA can notify manufacturers of the immunogen to avoid inclusion in future product lines. The approach to assess for bacteria is appropriate. If bacteria are not identified, the insoluble fraction of the ink will be fractionated and the various fractions evaluated for immunogenicity in the LLNA. The fraction containing immunogenicity will be characterized using co-chromatography on HPLC or if appropriate using mass spectrometry. This approach is a thoughtful and measured approach to the identification of the fraction that caused the original health issues. During the presentation, more extensive plans were outlined for assessing protein expression in lymph nodes although the objective of these studies is unclear.

Recommendation

Since the potential health effects of tattoo inks are a very important issue, the NCTR and FDA are encouraged to continue to support this program. The identification of the immunogenic component of the ink is important and should be expedited. The value of the evaluation of the effects on lymph node proteins is unclear. This approach should be reconsidered or a rationale should be developed for the approach. If this program and/other programs are going to continue to address the mechanisms of immunotoxicity, recruitment of an immunotoxicologist should be given serious consideration.

Division of Biochemical Toxicology Chemistry Support Group: Analytical chemistry support for NCTR GLP and NTP studies (Dr. Paul Siitonen)

Summary

The Chemistry Support Group has been located within the Division of Biochemical Toxicology since October of 2004. This group provides analytical chemistry support for multiple ongoing experiments being conducted at the NCTR, many of which require adherence to FDA's Good Laboratory Practices (GLPs). Prior to animal studies, all compounds scheduled for GLP toxicological evaluation must undergo characterization analyses to determine identity and chemical purity, and to detect possible deleterious contaminants. The group supports analysis of test substances in support of acute and chronic NTP toxicity studies. Following study completion summary reports are prepared for all NTP projects for inclusion in final reports. Analytical procedures utilize multiple disciplines and instrumentation located within the Division including: gas chromatography, high performance liquid chromatography, LC/MS, GC/MS, 13C and 1H NMR, and inductively coupled plasma (ICP)/MS.

The Chemistry Support Group is also responsible for evaluating the feed, bedding, and water used in animal studies. It performs analyses for essential nutrients in animal diets as well as for contaminants in diets, bedding, and water. Nutrient analyses are performed for percentage of fat and protein, vitamins A, B1, and E, and selenium, as well as for the percentage of moisture in the diets. Contaminants evaluated include acrylamide, aflatoxins B1, B2, G1, and G2, arsenic, cadmium, fumonisin (total), lead, mercury, PCBs, and pesticides. Bedding is evaluated for particle size distribution, moisture content, PCBs, and pesticides. Water is evaluated for suspended solids, pesticides, pH, and trace metals content.

Critique:

Mr. Siitonen has 26 years of post-baccalaureate experience in analytical chemistry and has risen through the ranks of the NCTR. In 2006, he became the Lead Chemist/NTP Team Leader in the Division. A total of 25 publications are listed. He directs the work of

6 full-time chemists. This facility core provides essential chemistry support for the Division of Biochemical Toxicology and is ably led. However, it is unclear why this support group has a separate leadership and structure to the mass-spectrometry group; they could be combined. Resources are an issue. While there seems to be a sufficient number of personnel there is only one-in house chemist for the synthesis of heavy istopically labeled internal standards (Dr. da Costa). Although a reasonable number of HPLC units are listed only two modern HPLC units using the Waters alliance technology are available the other units are 10-15 years old which corresponds to their functional life in a laboratory setting. In the present management structure the NTP through the IAG only funds individual projects but does not set aside resources for infrastructure to replace aging instrumentation. By contrast the ICP/MS for nanoparticle detection and heavy metal detection is considered state-of-the-art. Plans to move forward with nantoxicological studies seem well thought out; however, progress may be affected by the lack of immunotoxicologists within the Division.

Recommendations

The Division Director in consultation with NCTR leadership should develop a plan to modernize much of the HPLC equipment. This plan should address the IAG with the NTP which seems to fail to realize that individual projects also require investment in infrastructure. The Division should also rationalize why it feels necessary to separate the MS support group from the analytical chemistry group when they could be combined under a single senior leader. The need for more synthetic chemistry support is also apparent. The instrumentation seems to be in place for nantoxicology studies but resources should be made available to hire an immunotoxicologist to the Division.

Project 1: Equipment calibration and maintenance for mass spectrometry (S0069) (Dr. Goncalo Gamboa da Costa)

Project 2: Analytical mass spectrometry collaborations (S0634) (Dr. Goncalo Gamboa da Costa)

Summary

The Mass Spectrometry Laboratory is responsible for the mass spectral analyses of all the test articles, test animal feed, and water used in experimental protocols conducted in NCTR. These analyses aim to provide identification of the test articles by a combination of procedures that involve comparison of the experimental data with spectral data published by the National Institute of Standards and Technology, comparison with standards, and interpretation of fragmentation patterns. Attempts are also made to detect possible contaminants in the test articles, within the limitations of the analytical methodologies used. Analyses are conducted at or above the regulatory requirements specified in each protocol. For example, all test articles used in NCTR protocols are handled according to GLP standards, regardless of the individual protocol requirements. Furthermore, attempts are always made to conduct the analyses of any test article by at least two independent methodologies [*e.g.* HPLC-ESI-MS and GCMS (EI) or GC under CI and EI].

A second group of analyses is conducted in support and/or collaboration with a number of ongoing projects in NCTR, other governmental agencies, and national and foreign research institutions and universities. As a direct result of their collaborations, members of the mass spectrometry group co-authored a total of 72 publications in peer reviewed journals.

The Mass Spectrometry Group currently maintains three instruments, ensuring a wide range of analytical methodologies that involve a variety of ionization techniques (EI, CI, ESI, APCI) and introduction techniques (probe, GC, LC, flow injection) using both MS and MS/MS capabilities.

Critique

Dr. Gamboa da Costa is a Visiting Scientist which implies that the appointment is temporary raising concerns about sustainability in leadership. It was explained at the site-visit that the Visiting Scientist category is used for appointees who are neither permanent residents or US citizens since this prevents permanent employment. A change in visa status of Dr. da Costa should be actively pursued to sustain leadership in this position. While Dr. da Costa seems very capable for the task in hand the appointment of a more senior investigator in this leadership role as Director of such an important facility core for the NCTR mission should be considered.

Apart from a ThermoFinnigan TSQ Quantum Ultra which is 5 years old; the remaining two instruments the TSQ7000 and the ThemroFinnigan Voyager are approaching obsolescence. Purchase of a single new triple quadrupole MS with UPLC has occurred. There is concern that this valuable facility is under resourced for its work. The facility has averaged in excess of 600 analytical runs during the last year which is quite modest. The publication record of 72 papers over a 10 year period is considered outstanding. It was unclear how many man hours were used for analytical methods development. Attempts should be made to log this use so that a more realistic estimate of this core use and its essential contribution can be made. This could strengthen the case for more resources.

Recommendations

The Division Director in consultation with NCTR leadership should consider the appropriate resourcing of this facility core with respect to instrumentation, leadership and personnel.

Overview: Assessment of the nephrotoxic effects of a combined exposure to melamine and cyanuric acid (Dr. Goncalo Gamboa da Costa)

Project 1. Assessment of the nephrotoxic effect of a combined exposure to melamine and cyanuric acid (C080041) (Dr. Goncalo Gamboa da Costa)

Summary

This project was initiated by the FDA to assess the renal toxicity of melamine and cyanuric acid when used in combination. Both substances were found to be high in wheat-gluten used in the preparation of pet food. Consumption of the adulterated pet food resulted in kidney failure and deaths of household pets.

The goals of the assessment were to: (1) investigate the PK and determine the NOAEL of combined exposure in a rodent model; (2) investigate occurrence of metabolomic and proteomic early biomarkers of melamine and cyanuric acid nephrotoxicity; and (3) investigate the PK and determine the NOAEL of combined exposure in a model animal for kidney anatomy and physiology (swine kidney) similar to humans.

Accomplishments to date have been to develop heavy isotopically labeled [$^{13}C_3$]-cyanuric acid and [$^{15}N_3$]-melamine as internal standards for rat urine and blood measurements and develop a stable isotope dilution LC/MS method for their quantitation. This is ongoing.

Critique
The synthesis of [^{13}C]-cynauric and [^{15}N]-melamine provides valuable internal standards for stable isotope dilution LC-MS/MS methodologies to quantitate their levels in plasma and urine with precision and will be invaluable to investigators in monitoring exposure to these agents. It was surprising to learn that Dr. da Costa not only synthesized these standards himself but in addition he is the only one with this expertise in the facility core. This questions whether the core is under resourced in synthetic chemistry. It is noted that the Division of Chemistry at NCTR was disbandoned awhile ago. It was also described that the synergistic toxicity seen with melamine and cyanuric acid was due to co-exposure to "scrap"-melamine which contains other components. It was pointed out that other components in the scrap melamine could contribute to the nephrotoxicity. Until this is resolved, it was felt that PK studies in the swine kidney model were premature.

Recommendations
This is an important project and it is recommended that a complete ADME profile be developed following the administration of ratios of melamine and cynauric acid in the rodent model. This study should be completed before plans are put into place to continue with this project in the swine kidney. The presentation also revealed a possible shortage of synthetic expertise in the core that should be addressed.

Overview: Bioterrorism, food safety, food science, and animal models for human melanoma (Dr. William Tolleson).

PROJECT 1 (E7089): Tyr-HRAS+ Ink4a/Arf transgenic mice as an animal model for cutaneous and ocular melanoma (Dr. W.H. Tolleson).

Summary (provided by investigator):
Characterization of a laboratory animal model suitable for fundamental and applied melanoma research benefits public and private research efforts by addressing the etiology and treatment of this aggressive form of cancer. Research performed by this laboratory associates exposure to UVA and UVB radiation with photochemical damage in skin, occurrence of cutaneous melanoma, and altered gene expression characteristic of melanoma. The transgenic animal system used in these studies produces intraocular melanomas arising in the posterior uveal tract, the most common site for human ocular melanomas. Melanomas of the anterior eye or ocular tumors observed in other transgenic animal models of ocular neoplasia are typically mixed with components from the retinal pigment epithelium. Therefore, Tyr-HRAS+ Ink4a/Arf transgenic mice provide a useful animal model for this devastating disease – one for which relatively few effective therapeutic options are available, emphasizing the need for an appropriate animal model for vigorous medical research.

Critique:
This project is consistent with NCTR expertise in phototoxicity and represents a useful animal model system that can be used to explore efficacy of regulated sunscreens. No further work is planned and a method is being published for potential consideration of technology transfer to other interested laboratories.

Recommendations:
No recommendations necessary in that no further project work in expected.

Project 2 (E7291): Laboratory studies in melamine and cyanuric acid biochemical toxicology (W.F. Tolleson)

Summary (provided by investigator):
Numerous pet deaths caused by renal failure were associated with the national pet food recall of 2007. Chemical analyses of affected pet food products revealed contamination with melamine and cyanuric acid, nitrogen-rich industrial chemicals used as a flame retardant and in plastics manufacturing. The significant enhancement in melamine-induced kidney failure, when co-exposed to cyanuric acid, compelled the Agency to request additional animal studies in rats and miniature pigs to determine the No Observed Adverse Effect Level (NOAEL) for the combination of melamine and cyanuric acid. The research in this area determined whether melamine or melamine cyanurate exhibit measurable cytotoxicity and investigated biochemical properties of these agents relevant to renal failure and toxicity.

Critique:
The primary test system to evaluate potential melamine, melamine cyanurate and cyanuric acid interactions was an in vitro RAW264.7 mouse macrophage cell culture. Although the experimental system provided some insights into mode of action in these cells, it was not clear if the responses, or lack thereof, reflected likely mode(s) of action functioning in vivo. However, further experimentation revealed that the test system exhibited formation of internal spherical structures that resembled pathology associated with renal failure, and that these structures were potential protein-chemical complexes. Thus, this system may prove useful for evaluation of mode of action of substances that have been linked to renal failure in pet foods.

Recommendations:
Further characterization of the biochemical/physical nature of the renal calculi isolated from animals exhibiting renal failure with that of the spherical structures in the cell systems will further confirm the usefulness of the model system to predict in vivo toxicity.

Project 3 (P0682) Thermal stability of ricin in fruit juices; (P0683) Effect of primary yogurt fermentation on the cytotoxic activity of the bioterrorism agents ricin and abrin; and (P0684) Real-time PCR assays for ricin and related potential bioterrorism agents in foods (W.F. Tolleson).

Recommendations:
Projects P0682 and 0683 are completed and no further work is planned. P0684 is on hold until further materials are received from collaborators. These projects have demonstrated functionality of a method to detect broad classes of food toxins, and provides a useful mechanism to evaluate potential impacts of food processing on toxin reduction.

Overview: Mechanistic studies of pyrrolizidine alkaloid carcinogenesis and evaluation of metabolic pathways associated with PAH carcinogenesis (**Dr. Peter P. Fu)**
The goals of this research program are to elucidate mechanisms of genotoxicity in food contaminants pyrrolizidine alkaloids (PA) and polycyclic aromatic hydrocarbons (PAHs) and the phototoxicity of cosmetics (retinyl palmitate and aloe vera). Results from this work fit into the broad mission of the regulatory concerns of the FDA and EPA.

PROJECT 1: A study of genotoxic mechanisms of riddelliine carcinogenesis (E2133) (**Dr. Peter P. Fu)**

Summary

The principal objective of this protocol was to develop methodologies to detect and quantify the riddelliine-modified DNA adducts contained in the target (liver) tissues of rats treated with riddelliine *in vivo*. To study the mechanisms by which riddelliine induces hepatocellular tumors, determine the metabolic activation pathways of riddelliine and identify its activated metabolites and the riddelliine-derived DNA adducts *in vivo.* The dehydroretronecine (DHR)-modified DNA adduct was a DNA adduct that could potentially form from riddelliine as well as from other tumorigenic macrocyclic pyrrolizidine alkaloids, such as retrorsine and monocrotaline, *in vivo*. It was proposed to study whether or not DHR-DNA adducts are commonly formed from animals treated with other tumorigenic macrocyclic pyrrolizidine alkaloids.

It was found that metabolism of pyrrolizidine alkaloids *in vivo* and *in vitro* generates dehydroretronecine (DHR) as a common reactive metabolite irrespective of pyrrolidizine alkaloid class suggesting a common mechanism of genotoxicity. DHR gives rise to DHR-DNA adducts using rat and human liver microsomes for bioactivation of pyrrolizidine alkaloids. These studies suggest that the same genotoxic mechanism functions in humans as well. A [^{32}P]-postlabeling/HPLC method for detection of (i) two DHR-3'-dGMP and four DHR-3'-dAMP adducts, and (ii) a set of eight DHR-derived DNA adducts *in vitro* and *in vivo* was established. The approach involved (i) synthesis of DHR-3'-dGMP, DHR-3'-dAMP, and DHR-3',5'-dG-bisphosphate standards, and characterization of their structures by mass and 1H-NMR spectral analyses; (ii) development of optimal conditions for enzymatic DNA digestion, adduct enrichment, and [^{32}P]-postlabeling; and (iii) development of optimal HPLC conditions. Using this methodology, eight DHR-derived DNA adducts, including the two epimeric DHR-3',5'-dG-bisphosphate adducts resulting, from metabolism of riddelliine by rat liver microsomes in the presence calf thymus DNA were detected. Identical adducts were seen in livers of rats dosed with riddelliine.

Structural analysis using LC-ES/MS/MS showed that DHR bound covalently to both 3'- and 5'-guanine, -adenine, and -thymine bases (but not cytosine) of dinucleotides to produce two or more isomers of each DHR-dinucleotide adduct. By comparing adduct formation at dissimilar bases within individual dinucleotides, the relative reactivity of DHR with individual bases was determined to be: guanine > adenine ~ thymine. Identification of the entire set of DHR-derived DNA adducts further validated the conclusion that riddelliine is a genotoxic carcinogen and enhances the applicability of these biomarkers for assessing carcinogenic risks from exposure to pyrrolizidine alkaloids. These studies are complete and no additional studies are planned

Critique

A [^{32}P]-post-labeling method for the detection of two DHR-3'-GMP and four DHR-3'-dAMP adducts and a set of eight DHR-derived adducts formed *in vitro* and *in vivo* was established. The method was used to detect these adducts during the bioactivation of riddelliine with rat liver microsomes and trapping the reactive intermediates with calf-thymus DNA. Experiments were also replicated in human liver microsomes and identical adducts were observed suggesting that this pyrrolizidine alkaloid will be genotoxic humans. The adducts were also observed in blood (leukocyte-DNA) of animals fed riddelliine by gavage. Together these studies make a strong case for gentoxicity in

riddelliine. The next step would be to validate adduct measurements by stable isotope dilution LC-MS approaches and this is proposed as part of Project 3. These studies resulted in 10 papers and no further studies are planned.

Recommendation
No recommendation is required since these comprehensive studies are considered complete**.**

PROJECT 2: A study of genotoxic mechanisms of carcinogenic pyrrolizidine alkaloids and pyrrolizidine alkaloid *N*-oxides (E7104) (**Dr. Peter P. Fu)**

Summary
Tumorigenic pyrrolizidine alkaloids belong to one of three chemical classes: retronecine-type, otonecine-type, or heliotridine-type. Subsequent mechanistic studies were performed on tumorigenic pyrrolizidine alkaloids of the different structural classes to determine whether they work through the same mechanism as riddellliine.

Accomplishments include demonstration that F334 rat liver microsomes metabolize clivorine a tumorigenic otonecine type pyrrolizidine alkaloid to reactive intermediates that produce the DHP-DNA adduct with calf thymus DNA. This suggests that the principle pathway of metabolic activation involves oxidative N-demethylation of the necine base, transannular ring closure and dehydration. Subsequently the same DNA adducts were observed with heliotridine type pyrrolizidine alkaloids establishing the DHP-DNA adduct as the common genotoxic adduct. Studies were expanded to include two additional retronecine alkaloids (monocrataline and retrorsine). N-oxide metabolism of these pyrrolizidine alkaloids also resulted in the common DHP-DNA adducts. Bioactivation of monocrotaline and retrorsine was almost completely abolished by triacetyleandomycin, a P450 3A4 enzyme inhibitor, phenotyping this enzyme as being responsible. Metabolism of monocrotaline in the presence of calf thymus DNA resulted in a set of eight DHPderived DNA adducts identified as two epimeric DHP-3'-dGMPs and six DHPderived dinucleotide adducts. These DNA adducts were identical to those formed from metabolism of riddelliine *in vivo* and *in vitro* in the presence of calf thymus DNA. A similar DNA adduct profile was detected in the livers of female F344 rats fed monocrotaline. Metabolism of retrorsine produced similar results. The ability to form the common DHP-DNA adducts irrespective of pyrrolidizine compound class suggest a common genotoxic mechanism, and the possibility that DHP-DNA adducts can be biomarkers of exposure to tumorigenic pyrrolidine alkaloids.

The group also determined that the livers of female F344 rats gavaged with one of the three dietary supplements (i) comfrey root extract, (ii) comfrey compound oil, or (iii) coltsfoot root extract, or an extract of a Chinese herbal plant, *Flos farfara* (Kuan Tong Hua), contained DHP-derived DNA adducts, although at lower levels than that observed with riddelliine. DHP-derived DNA adducts were not detected from the commercial comfrey leaves (in tablet), comfrey leaves (in pepsin), comfrey consoude, or coltsfoot tussilage. *In vitro* rat liver microsomal metabolism of these commercial dietary supplements and herbal plant extracts was conducted and similar results were obtained. These results suggest that these exogenous DHP-derived DNA adducts can be potentially employed to detect genotoxic and tumorigenic pyrrolizidine alkaloids in dietary supplements and herbal medicinal plants, and are potential biomarkers of pyrrolizidine alkaloid tumorigenicity and exposure. This project resulted in 15 publications.

It was proposed to study two tumorigenic otonecine-type pyrrolizidine alkaloids, senkirkine and clivorine, in order to establish the generality of the metabolic activation pathway leading to the formation of DHR-derived DNA adducts. To date only clivorine has been examined; therefore, future studies will focus on senkirkine. Since senkirkine is not commercially available, it has been purchased in the senkirkine-containing Chinese herbal plant, *Flos farfara* obtained from the Chinatown district of San Francisco. The senkirkine is being extracted and purified for metabolism and DNA adduct experiments. This project will be terminated on September 30, 2008 and the study will be continued by a new project (E7289) entitled "Detection and Quantification of Hepatotoxic and Tumorigenic Pyrrolizidine Alkaloids in Chinese Herbal Plants, Herbal Dietary Supplements, and Humans."

Critique

The group has provided compelling evidence that the same reactive intermediate is responsible for DHP-DNA adduct formation irrespective of pyrrolizidine alkaloid chemical class. Irrespective of the class of alkaloid not all herbal supplements containing these alkaloids produce DNA-adducts when given by gavage, suggesting that differences in the complex mixtures and bioavailability may affect the genotoxic profile observed. This work is important considering the widespread use of these herbal supplements, and the work has been done extraordinarily well. The issue remains as to how much of this work should be continued if supplement use is to be federally regulated.

Recommendation

This work has been comprehensively done and it is uncertain how much more will be learned by examining additional pyrrolidizine alkaloids for genotoxicity. With the established-DNA adduct detection methods in place the issue becomes whether these should be used in population based studies to conduct biomonitoring and risk assessment. Also, next steps would have to be aimed at determining why different herbal plant extracts give DHP-adducts and some do not. It is likely that this will be accomplished with the E7289 project**.**

PROJECT 3: Detection and quantification of hepatotoxic and tumorigenic pyrrolizidine alkaloids in Chinese herbal plants, herbal dietary supplements, and humans (E7289) (**Dr. Peter P. Fu)**

Summary

This new project has the following aims:

1. Develop an LC/ES/MS/MS method for detection and quantification of DHP-derived DNA adducts in rodents and humans.

In order to develop an LC/ES/MS/MS method for detection and quantification of DHP-derived DNA adducts in rodents and humans, the group will (a) synthesize non-labeled and [^{15}N]-labeled DHP-derived dG, dA, and DNA adduct standards; (b) develop an LC/ES/MS/MS method for detection and quantification of DHP-derived DNA adducts from metabolism of individual pure pyrrolizidine alklaloids including riddelliine and lasiocarpine, *in vitro* and *in vivo*; (c) Use the developed LC/ES/MS/MS methodology to quantify the levels of DHP-derived DNA adducts in livers of rats dosed with different amounts of riddelliine, and to correlate these adducts with tumorigenicity reported by NTP; (d) Use the developed LC/ES/MS/MS methodology to quantify the levels of DHP-derived DNA adducts in liver and leukocytes of rats dosed with different amounts of herbal plants, including comfrey, *Flos farfara*, and coltsfoot, that contain pyrrolizidine alkaloids; and (e) Use the developed LC/ES/MS/MS methodology to quantify the levels

of DHP-derived DNA adducts in human leukocytes in individuals who take Chinese herbal medicine.

2. Develop an LC/ES/MS/MS method for detection and quantification of DHP-derived hemoglobin adducts in rodents and humans.

In order to develop an LC/ES/MS/MS method for detection and quantification of DHP-derived hemoglobin (Hb) adducts in rodents and humans, the following experiments are to be conducted: (a). Synthesize labeled and unlabeled DHP-derived Hb-*N*-terminal valine adduct standards and pentafluorophenyl isothiocyanate (PEPITC) derivatives; (b). Develop an LC/ES/MS/MS method for detection and quantification of DHP-derived Hb adducts from metabolism of individual pyrrolizidine alkaloids, including riddelliine and lasiocarpine, *in vitro* and *in vivo*.; (c). Use the developed LC/ES/MS/MS methodology to quantify the levels of DHP-derived Hb adducts in blood of rats dosed with different amounts of herbal plants, including comfrey, *Flos farfara*, and coltsfoot, that contain pyrrolizidine alkaloids; and (d) Use the developed LC/ES/MS/MS methodology to quantify the levels of DHP-derived Hb adducts in blood of humans who take Chinese herbal medicine.

3. Develop an LC/ES/MS/MS method for detection and quantification of genotoxic pyrrolizidine alkaloids in herbal plants and herbal dietary supplements.

In order to quantitate the genotoxic/tumorigenic pyrrolizidine alkaloids in herbal products LC/ES/MS/MS, multiple reaction monitoring measurements will be made on Chinese herbal extracts using one or more structurally related pyrrolizidine alkaloids of known quantity as an external standard. This method has been developed to identify the protonated molecular ion [M+H]+ of individual pyrrolizidine alklaoids and to determine two specific fragment ions for reteonecine, otonecine, and platynecine-type pyrrolizidine alkaloids. Using this methodology five different pyrrolidizine alkaloids were identified in one Chinese herbal medicine, Qianliguang. This project resulted in the publication of two papers.

<u>Critique</u>
Studies planned in this project are a logical extension of completed work in Projects 1 and 2. The need to develop a stable-isotope dilution LC-MS method to detect DHP-DNA adducts *in vitro* and *in vivo* is supported by the [^{32}P]-post-labeling studies and can be applied to humans to detect DNA-adducts in leukocytes. These studies seem to be the correct next step and there is high enthusiasm for them to proceed as a new direction of effort will be put into developing analytical methods to detect Hb-DHP adducts. This is high-risk in the absence of evidence that Hb-DHP adducts form in the first place. It is uncertain how these adducts would be synthesized *in vitro*. The studies on the LC-MS/MS detection and quantitation of pyrrolizidine alakloids in Chinese herbal extracts could absorb a large amount of resource and effort when it is considered that the number of different herbal extracts that require analysis could be overwhelming.

<u>Recommendations</u>
There is high enthusiasm to develop the stable-isotope dilution LC-MS methods for DHP-DNA adduct detection. This method would validate the *in vitro* and *in vivo* [^{32}P]-post-labeling performed and would provide the necessary assay to detect these adducts in human leukocytes in individuals taking these dietary supplements. The work on DHP-Hb adducts seems premature until the DNA work is complete. While there is a high

degree of confidence that the methods are already in place to measure pyrrolizidine alkaloids in different Chinese herbal extracts, this work should only be continued if there is a plan which will prioritize the analysis to be performed.

PROJECT 4: Determination of PAH metabolic activation pathways leading to tumorigenicity *in vivo* by LC-ES-MS/MS methodologies (E7237) (**Dr. Peter P. Fu)**

Summary

There are three main metabolic pathways leading to the activation of polycyclic aromatic hydrocarbons (PAHs) to derivatives that can bind to DNA and initiate tumor formation: (i) CYP catalyzed monooxygenation of PAH *trans*-dihydrodiols to produce *bay*-region diol epoxides; (ii) one-electron oxidation of parent PAHs to yield reactive radical cations, and (iii) aldo-keto reductase-catalyzed oxidation of PAH *trans*-dihydrodiols to generate *ortho*-quinones. The goals of this study were to determine the major route of metabolic activation by measuring the DNA-adducts that come from each pathway using state-of-the art stable-isotope dilution LC-MS methods. The work was performed collaboratively with Jeffrey Ross at the EPA (from Dr. Stephen Nesnow's group). The aims of the project were to:

1. To utilize previously developed HPLC-ES-MS/MS methodologies with modification, to detect and quantify the exogenous benzo[*a*]pyrene (BP)- and benz[*a*]anthracene (BA)-derived DNA adducts and the endogenous oxidative DNA adducts resulting from these three metabolic pathways.

2. To determine the levels of stable BP-DNA adducts (*i.e.*, dG-*N2*-BPDE), unstable depurinating BP-DNA adducts (*i.e.*, Gua-N7-BP and Ade-N7-BP), BP 7,8-dione adducts, and oxidative DNA adducts (*i.e.*, 1,*N6*-etheno-dA, *3,N4*-etheno-dC, M1-dG, and 8-oxo-dG) in the lungs of A/J mice treated with various levels of BP for different lengths of time.

3. To extend the study to determine the levels of BA-DNA adducts formed through these three activation pathways under similar experimental conditions.

4. To determine the relative contribution of each pathway in PAH-mediated carcinogenesis by measuring all postulated DNA adducts using a common LC-ES/MS/MS analytical procedure. Results from BP and BA should provide sufficient data to obtain a definitive conclusion. If not, more tumorigenic PAHs, such as 7,12-dimethylbenz[*a*]anthracene (DMBA) will be examined.

The group synthesized unlabeled and stable-isotopically labeled BP- radical cation DNA adducts of BP e.g., BP-6-N7-dGua, BP-6-N7-Gua, and BP-6-C8-Gua for stable isotope dilution LC-MS. They also prepared the stable BP-7,8-dione-dG and dA adducts. They also established LC-MS methods to detect these adducts as well as the lipid peroxidation induced etheno-DNA adducts and the dG-*N2*-BPDE adduct in the same DNA sample with a sensitivity of 1 adduct per 10^8 nucleotides. They observed significant changes in adduct levels in lung from A/J mice treated with BP vs. vehicle-treated mice for BPDE-dG, but not BP-7,8-dione-DNA adducts, etheno-DNA adducts, or any of the depurinating adducts. These observations provide no evidence for one-electron oxidation of BP in mouse lung, but do not preclude formation and loss of depurinating adducts during DNA isolation. Future studies using whole organ extractions may address this possibility.

Future studies are also aimed at detecting BA-3,4-dione, BA-8,9-dione and BA-10,11-dione derived DNA adducts since their corresponding *trans*-dihydrodiols are produced metabolically. The group has already synthesized the stable adducts with BA-3,4-dione. Studies on the radical cation pathway will be extended to DMBA due to the possibility of metabolic activation of methyl groups at C7 and C12.

Critique

This project was initiated as a collaborative effort with the EPA. Both Dr. Fu and investigators at the EPA had knowledge that identical studies on DNA-adduct synthesis detection and quantitation were being performed by Dr. Penning's group at the University of Pennsylvania that are funded by the NCI. In fact the majority of the literature cited is from Dr. Penning's group. It is remarkable that no attempt was made to collaborate with Dr. Penning on this study to prevent duplication of effort when resources are scarce. The adduct work performed is of high standard, however, a number of problems exist. First, not all the structures of the stable-BP-7,8-dione-dG and dA adducts were presented but because this is a collaboration with EPA they are no doubt identical to those reported by Balu et al. These adducts are formed in 50% DMFA at 55 ^{o}C and the hydrated and cyclized adducts that result may be artifacts of the reaction conditions and may not be found *in vivo*. Second, as a measure of the oxidative lesions that arise from the aldo-keto reductase pathway only the etheno-dG adduct was measured. It is clear that the most mutagenic lesion formed from this pathway is 8-oxo-dGuo but no attempt was made to measure this lesion *in vitro* or *in vivo*. Third, failure to take these points into account may lead to the measurement of the wrong adducts in the A/J mice and this could have been avoided if consultation had been sought. To extend this work to BA seems undesirable since this is a weak carcinogen. Also no evidence exists that aldo-keto reductases will oxidize BA-8,9-*trans*-dihydrodiol or BA-10,11-*trans*-dihydrodiol to the corresponding quinones. Thus adduct work on these diones seems premature. To extend this work to DMBA also seems inappropriate when this is a synthetic carcinogen. Studies would be more relevant if they were extended to carcinogenic PAH for which there is concern about human exposure.

Recommendation

This project has an important goal since if the mechanism of PAH activation in humans was understood this could lead to effective biomonitoring and risk-assessment. If this project is to continue the correct adducts should be measured and consultation sought. There is no enthusiasm for extending this work to BA and DMBA since these seem the incorrect choices.

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Overview: Evaluating the toxicology of nanoscale material (Dr. Neera V. Gopee)

Nanoscale materials are being incorporated into a wide variety of commercial products, including topically applied sunscreens, with unknown human toxicity. It has been suggested that the toxicity of nanoscale materials may differ from their "bulk"-sized counterparts based on size, chemistry and surface properties. Dermal transport and associated toxicity of nanoparticles in this small size range are relatively unexplored, and is the focus of the following projects.

Project 1: Skin penetration, phototoxicity, and photocarcinogenicity of nanoscale oxides of titanium and zinc (E2156, E2158, P00686) (Dr. Neera V. Gopee)

Summary:

Nanoscale materials are being incorporated into a wide variety of commercial products, including topically applied sunscreens. In order to help address these safety concerns regarding dermal penetration of nanoscale materials in topically applied products, the NTP requested investigating whether nano-scale TiO2 could penetrate skin and the consequential adverse biological consequences as part of the National Nanotechnology Initiative (NNI) risk assessment. The project is divided into four studies:

(1) Skin penetration, phototoxicity, and photocarcinogenicity of nanoscale oxides of titanium and zinc: quantum dots as surrogates.
(2) Skin penetration, phototoxicity, and photocarcinogenicity of nanoscale oxides of titanium and zinc
(3) Preliminary and range-finding studies on the tumorigenicity of photoactive nanoscale titanium dioxide in Tg.AC transgenic mice
(4) Dermal penetration of micron- and nano-scale titanium dioxide in mini-pigs following topical application

Critique:
The project is a collaboration among investigators from NCTR, NIEHS, CFSAN, CDER and Rice University, and involves a very rigorous study of dermal penetration of metal oxides. The nanoparticles are applied topically or using intradermal injection; the latter as a worse case scenario of damaged skin. The investigative team has examined the penetration and subsequent biodistribution of the particles using multiple skin models, including normal and tape-stripped mouse, ex vivo samples from human, and in a mini-pig model. The results consistently indicate that there is no increase in the level of TiO2 in lymph nodes and liver of the treated animals. The investigators conclude that TiO2 does not penetrate skin or become biodistributed systemically to sentinel organs.

Recommendation:
The site visit team remarked that the project involved “proving a negative” and concurred that the investigators had generated sufficient data, by multiple models, to conclude that TiO2 did not penetrate normal skin. The investigators should limit expenditure of further resources on the initial question, and make their findings available to NNI and the rest of the nanotech community. The reviewers cautioned against using surrogates such as quantum dots for other nanoparticles.

Project 2: NTP nomination evaluating the toxicological potential of colloidal nanogold (proposed) (Dr. Neera V. Gopee)

Summary:
Human drug exposure to gold nanoparticles (nAu) may occur through its incorporation into dental and bone implants, through ingestion of colloidal gold as dietary supplement, by inhalation through the handling of nanoscale gold powders, and via dermal application of nanocrystalline powders. This nomination focuses on nAu because adequate risk research of colloidal gold is lacking and is critical in understanding the potential toxicity of this novel group of nanomaterials. The first objective of this nomination will be to adequately evaluate the effect of particle hydrodynamic radius and particle coating on the pharmacokinetic profile of nAu following an acute single exposure to nAu via oral and i.v. routes in rodents. The second aim will be to evaluate the effect of particle hydrodynamic size and surface coating on the toxicological profile of nAu following sub-acute and sub-chronic oral exposure in rodents.

Critique:

This project was recently nominated for study and is still in the planning phase. As presently formulated, the objective involves the elucidation of how physicochemical properties of gold colloids (e.g. size, surface coatings) impact the disposition, metabolism, elimination and toxicity of the nanoscale gold.

Recommendation:
The site visit team urged the investigators to avoid general structure-activity studies, but rather to address specific toxicities. The experimental design should reflect the FDA requirements that generated the nomination. The NCTR is particularly well-suited to conduct long-term toxicity studies, given the lack of this data in the literature.

Overview: The nutritional toxicology of dietary supplements, the photobiology and phototoxicology of cosmetic ingredients and the toxicological potential of nanoscale materials (Dr. Mary D. Boudreau)
Dr Boudreau joined the Division of Biochemical Toxicology in 2000, and is principal investigator on projects focused on assessing the biological toxicity and potential carcinogenesis of dietary supplements and evaluating the photobiology and photococarcinogenicity of cosmetic ingredients.

Project 1: Bioassays in the F344 rat and the B6C3F1 mouse administered Aloe vera plant constituents in the drinking water (E2142) (Dr. Mary D. Boudreau)
Summary
Botanicals, such as aloe vera, are commonly used as dietary supplements -- with minimum standards of safety. Aloe vera is ingested as a laxitive, but is also credited with anti-tumor, anti-arthritic, anti-diabetic, and anti-rheumatoid properties; these claims are largely unsubstantiated by scientific studies. Because of it's widespread use as an alternative medicine, Aloe vera was nominated by the National Cancer Institute and selected by the NTP as a high priority agent for oral studies at the NCTR. The goal of the study is to examine the orally-administered toxicity of the plant extract, primarily by histopathological methods. The project also examines the effect of aloe vera extracts on changes in intestinal microflora, since mammals cannot degrade the □-bonds present in this cellulose-based supplement.

Critique
Sub-chronic (14-day) dosed water and mechanistic studies on the Aloe vera whole leaf extract were conducted in rat and mouse models at concentrations of 1%, 2%, and 3%. The results showed that at concentrations greater than 1% caused a significant reduction in body weight gains and in gastrointestinal transit times in the rat model, but not in mice. Histopathology of the mucosa of the large intestine of rats showed dose-related hyperplastic changes, attributed to proliferation of goblet cells within the mucosa.

A two-year carcinogenesis bioassays of Aloe vera whole leaf extract were then conducted, with concentrations of 0.5%, 1%, and 1.5%. Histopathology evaluations are currently incomplete for this project; however, gross examination of organs and tissues indicated that more severe lesions were observed in the gastrointestinal tract than previously observed in animals from the sub-chronic studies. Using an in vitro system, Dr. Boudreau's research team also concluded that whole leaf extract of aloe vera has a profound effect on the growth of bacteria in mixed cell cultures, and on their production of short-chain fatty acids.

Recommendation

The Principal Investigator has published her findings in two manuscripts, and is currently preparing the project's final report – which is scheduled for completion before the end of the year. The site visit team concurs that Dr. Boudreau has identified pathologies due to orally delivered aloe vera extract, and has potentially established a mechanism of toxicity with the altered production of short-chain fatty acids. Once finalized, the pathology report will fulfill the objectives of the original nomination.

Project 2: Effects of Topical Exposure to Aloe vera test substances on the photocarcinogenicity of simulated solar light in SKH-1 mice (E2140) (Dr. Mary D. Boudreau)

Summary

Aloe vera is incorporated into a myriad of skin care and cosmetic ingredients. The National Cancer Institute nominated Aloe vera for topical studies based on its widespread use in skin care and cosmetic products, the lack of toxicological information on the effects of its chronic use, and that chronic use may promote skin tumor formation. The focus of this project is to evaluate whether or not the topical application of creams containing Aloe vera plant extracts enhance the photocarcinogenicity of simulated solar light (SSL). The applications of aloe vera creams and the exposures of Crl:SKH-1 (hr-/hr-) hairless mice to SSL were conducted 5 days a week for a period of 40 weeks and were followed by a 12-week observation period by digital photography. At necropsy, gross skin lesions were noted and trimmed for histopathologic evaluations. Non-neoplastic and neoplastic skin lesions were assessed for incidence and multiplicity. *In vitro* studies of Aloe vera products were also conducted to elucidate possible mechanisms of toxicity. Following exposure to ultraviolet light, the production of lipid peroxides and the generation of reactive oxygen species were evaluated with electron spintrapping techniques.

Critique

The data analyses showed that the topical treatment of mice with the Aloe vera plant extracts or aloe-emodin did not significantly affect the survival, body weights, or the in-life determinations of skin lesion onset, incidence, or multiplicity. Aloe vera test substances and aloe-emodin produced consistent, yet quite similar, weak photocarcinogenesis-enhancing effects, especially in female mice. These effects included increased incidence and multiplicity of squamous cell neoplasms. Under in vitro conditions, aloe vera products and extracts were found to generate reactive oxygen species, which resulted from the formation of lipid peroxides. The technical report for this project is currently in its final revision.

Recommendation

The project is responsive to the original nomination and has identified a weak photocarcinogenic effect of aloe products. The site review team encourages the researchers to further investigate/determine whether onset or multiplicity is the more sensitive indicator of photocarcinogenesis. The review team recommends a cautious approach, however, to the proposed "evaluation of differentially expressed genes as markers of skin cancer" -- due to the large variability in gene-based methods, and the complexity of validation.

Project 3: Effect of topically applied skin creams containing retinyl palmitate on the photocarcinogenicity of simulated solar light in SKH-1 mice (E2143) (Dr. Mary D. Boudreau)

Summary

Skin care products that contain various retinoids are becoming an increasing source of cutaneous vitamin A. Retinyl esters, especially retinyl palmitate (RP), are the preferred retinoid for incorporation into skin care products because they are more chemically and thermally stable than retinol. The focus of this project was twofold: (1) To test the hypothesis that the topical application of creams containing RP enhances the phototoxicity and photocarcinogenicity of SSL or that of specific components of the ultraviolet spectrum, namely UVA and UVB. (2). To determine the mechanisms of phototoxicity/photococarcinogenicity of RP. Research aim 2 is further divided into four sub aims to examine:

- Photodecomposition of RP in ethanol by UVA light – formation of photodecomposition products
- UVA photoirradiation of RP – formation of singlet oxygen and superoxide, and their role in induction of lipid peroxidation
- Photo-induced DNA damage and photocytotoxicity of RP and its photodecomposition products
- Photomutagenicity of retinyl palmitate, anhydroretinol, and retinyl palmitate 5,6-epoxide by UVA irradiation in mouse lymphoma cells
- Levels of retinyl palmitate and retinol in the skin of SKH-1 mice topically treated with RP and concomitant exposure to simulated solar light for thirteen weeks

Critique

This project is a collaboration among NCTR, NIEHS and CFSAN, and has been underway for approximately seven years – resulting in over a dozen manuscripts. The investigators use Crl:SKH-1 (hr-/hr-) hairless mice irradiated with SSL, UVA, or UVB for their in vivo model. Data from mice receiving chronic (40-week) topical applications of control cream or creams containing RP (0.1%, 0.5%,1.0%, and 2.0%) or retinoic acid (0.001%) are now being analyzed. The final pathology report notes a mild increase in squamous cell neoplasms for RP application, independent of photoirradiation. The final technical report is scheduled for release in early summer.

Recommendation

As presented to the site visit team, the investigators have established that retinyl palmitate is a mild carcinogen independent of photoirradiation. Upon UVA exposure, the research has also established that RP generates reactive oxygen species and induces lipid peroxidation under in vitro conditions. This latter body of work certainly contributes to the understanding of potential mechanisms of phototoxicity of RP, as evidenced by the scientific literature generated by this project. However, the lack of a clear difference between irradiated and control mice for the RP-treated chronic study suggests that the in vitro results are not aligned with the in vivo observations. The principal investigator should re-evaluate the need for further in vitro mechanistic studies if the pathology report and final analysis support the null hypothesis for focus #1 above -- i.e. if there is not a statistically significant difference between the SSL/UVA/UVB and untreated mice.

Project 4: Toxicological risks associated with nanoscale materials – nanoscale silver. (Proposed) (Dr. Mary D. Boudreau)

Summary:

Silver nanoparticles are found in an increasing number of consumer products, such as water purification, food packaging, odor resistant textiles, personal hygiene, household appliances, and medical devices, including wound dressings, catheters, prostheses, and surgical appliances. There is a paucity of published research that addresses the accumulation, distribution, metabolism, and excretion (ADME) of silver nanoparticles in mammalian systems. The nomination of silver nanoparticles by the FDA recognizes the possible toxicity concerns due to the lack of understanding of various aspects of silver nanoparticles and recommends studies to determine the potential for silver nanoparticles to interact with tissues, cells, and molecules. The specific aims of this project will be to conduct rodent studies using oral and intravenous routes to determine ADME properties of silver nanoparticles

Critique:
Nanosized silver is now commonly used as an anti-microbial additive for numerous products within CFSAN's regulatory purview. The work proposed by Boudreau and her colleagues will help increase our understanding of the biocompatibility and/or potential toxicities or nanosized silver. The research will utilize both in vitro and in vivo studies to elucidate mechanisms of silver nanoparticle toxicity, initially focusing on reactive oxygen species generation in a cell culture system. The effect of nanoparticle size and morphology on toxicity will also be determined. General ADME parameters will be characterized in support of this project. Laboratory experiments for the study have not yet been initiated, and Dr. Boudreau will coordinate with FDA Centers prior to the start of work.

Recommendation:
The SAB recommends that the proposed studies be pursued based on the presence of silver nanoparticles in a wide variety of commercial products. The SAB urges Dr. Boudreau and her colleagues to focus on a specific target of toxicity, and to avoid more general structure-activity studies. The site visit team also recommends that she coordinate with CDRH, due to overlap of ongoing efforts, and with the EPA, which has regulatory jurisdiction over non-medicinal antimicrobials and pesticides.

Overview: Effect of endocrine disruptors on reproductive organ structure and function and carcinogenesis and dietary modulation of toxicity (Dr. K. Barry Delclos)
The following 5 projects represent components of an ongoing effort to address issues related to hormonally active agents or endocrine disruptors. The endocrine disruptor effort has spanned several years so the projects represent essentially completed projects as well as projects that are currently under way or are proposed. Early studies with several potential endocrine disruptor agents were done in collaboration with the NTP with results published through NTP technical documents. These studies frequently focused on the evaluation of potential mutigenerational reproductive effects with differing exposure windows across generations. The effects of genistein and ethinyl estradiol were evaluated in previous years while the evaluation of nonylphenol is currently being completed (see Project 1 below)

Project 1: Di(2ethyhexyl)phthalate (DEHP) toxicokinetics in neonatal male rhesus monkeys following intravenous and oral dosing (E2160) (Dr. K. Barry Delclos)
Summary

This project is designed to provide information for the design of a future study to evaluate the potential testicular effects of DEHP in rhesus monkeys. The neonatal time period for exposure was selected to cover the time period when the hypothalamic-pituitary-testicular axis in neonatal monkeys and when metabolic capabilities of the animals are expected to be changing. This exposure scenario was selected to mimic the DEHP exposure in human infants receiving DEHP though intravenous systems in neonatal intensive care units. The project has 2 primary goals: The first goal is to quantify the toxicokinetics of intravenous and oral doses of DEHP administered to male rhesus monkeys during the first 12 postnatal weeks. The second goal is to utilize blood and testicular tissue to establish methods to be used in a subsequent subchronic study and to estimate variability of specific parameters to assist in determining numbers of animals per group in the future study.

Critique

The approaches being used in this pilot/method development study are appropriate. The information obtained should provide important information for the design of subsequent studies. The study has been initiated but no data was available at the time of review. The data from this study will be used to design a larger study in conjunction with CBER and CDRH. The definitive study, to be initiated in late 2008 or early 2009, is anticipated to include daily dosing of DEHP over the first 10 to 12 weeks of life using a dose range of 0.5 to 500 mg/kg/day. Endpoints will include a number of hormonal evaluations as well as extensive histopathology with a particular focus on the testis. Current plans are to have evaluation at the end of the dosing period and not to hold animals until puberty for a more complete assessment of reproductive toxicity.

Recommendation

The use of a well designed pilot study to evaluate multiple parameters including toxicokinetics is strongly endorsed and supported by the reviewers. No specific recommendations can be made at this time.

Project 2: Effect of sedatives on the metabolism of di(2-ethylhexyl)phthalate (DEHP) administered by intravenous injection and the relationship of DEHP metabolism to biological effects in neonatal rats (E2162; pending) (Dr. K. Barry Delclos)

Summary

In comparison to the previous project in neonatal primates, this project will assess the metabolism of DEHP in neonatal rats with special emphasis on the effects of ketamine on DEHP metabolism. As in the corresponding monkey study, the relationship of DEHP metabolism to biological effects will be assessed in this neonatal rodent study. The effect of ketamine on metabolism has been selected for evaluation in this study since ketamine will be repeatedly used as the sedative in the monkey study and because ketamine is used as a sedative in human neonatal intensive care units. There is no existing data on the effect of ketamine on DEHP metabolism.

The results of a pervious neonatal rat study have been used by the FDA to set tolerable intake levels of DEHP by the intravenous route. However, the previous study did not include toxicokinetics of DEHP or DEHP metabolites. Therefore the present study will provide information on toxicokinetic parameters in relation to toxicity endpoints (testicular weight and seminiferous tubule diameter) that were described as modified in the previous study since these endpoints will be evaluated in the currently planned study.

Critique

The evaluation of toxicokinetics of DEHP and DEHP metabolites is appropriate and will provide important new information relative to DEHP effects in rodents. The current attempt to confirm previously descried testicular effects in neonatal rats exposed to DEHP is warranted. The combination of confirmation of previous findings in the context of exposure data should provide important information for reevaluation of tolerable daily DEHP intakes by the intravenous route in humans. From the material presented, it is unclear how the rodent data on metabolism of DEHP in conjunction with ketamine administration will provide information important to study design or data evaluation in the monkey study.

Recommendation

The evaluation of toxicokinetics in rodents in relation to testicular effects in the neonatal animal is endorsed by the review group. A clearly articulated rational for assessing the effects of ketamine on DEHP metabolism should be developed. This rationale should clearly indicate how the data will be used in cross species extrapolation either impacting the design or result interpretation in the monkey study or impacting the assessment of human risk.

Project 3: Dietary modulation on the renal and testicular toxicities of p-nonylphenol and di(2-ethylhexyl)phthalate (DEHP) (E7142) (Dr. K. Barry Delclos)

Summary

This study appears to have originated from the observation of polycystic kidney disease (PKD) in animals treated with nonylphenol (NP) in a dose range finding study that used a special diet designed to have low isoflavone levels. Since the kidney effect was not anticipated, the investigators hypothesized that the special diet had exacerbated the kidney effect. Therefore, a subsequent study evaluated the effect of NP using 4 different diets several with reduced isoflavones (based on reduction or exclusion of soy from the diet) compared to the effect when a standard rodent diet (containing soy) was used. The results indicated that diet significantly modulated the development of PKD induced by NP in rats and that soy components, as well as complex dietary factors, may account for protection again PKD that was noted in the standard rodent diet group. Subsequent studies in young rats demonstrated that NP induced apoptosis in the renal tubules prior to cyst formation and importantly that apoptosis was significantly lower in animals receiving standard rodent diet. Gene expression studies demonstrated NP effects are modified by diet.

Based on the results from the NP studies, dietary modulation of renal and testicular effects related to DEHP administration was evaluated. Several DEHP effects such a delay of preputial separation and hormonal changes were evident at either a lower dose or at an earlier age in animals receiving low soy based diet. However, there was no significant effect of diet alteration on renal cyst induction based on histopathology examination.

Stated future plans focus on a further evaluation of gene expression including the development of methodology for collecting Leydig cells for gene expression studies. The methods developed will be applicable to other projects at NCTR.

Critique

The use of multiple diets to alter isoflavone to assess the impact on PKD in rats represented a thoughtful and appropriate approach to addressing the unexpected finding

of PKD associated with NP administration. The resultant studies have demonstrated the impact of diet on PKD and on DEHP alterations of reproductive parameters. While these are interesting findings and worthy of publication, the future direction of the project is unclear. The stated future plans appear to be a wrap up of previous efforts with emphasis on the gene expression evaluation. The project description does not provide a clear statement of how the effort meets a priority need of the FDA. The project appears to have been developed as a logical extension to address an unexpected observation of PKD but not highly focused on an agency need.

Recommendation
The reviewers assume that this project will end with the completion of the gene expression studies. The reviewers endorse ending this study and redirection of resources to other activities. If this project is to continue, a clear rationale for application to the FDA mission and needs must be developed.

Project 4: p-Nonylphenol: Evaluation of reproductive effects over multiple generations and summary of overall endocrine disruptor research results (E2135) (Dr. K. Barry Delclos)

Summary
This is a very large project conducted over multiple years under the Interagency Agreement with the NTP. The effort was designed to address a basic question related to the "endocrine disruptor" hypothesis and specifically designed to determine whether estrogenic effects would be magnified, sustained, diminished or reversed in subsequent generations or would carry over into unexposed generations or would lead to chronic toxicities including neoplasms. Using the conditions tested (low phytoestrogen diet), nonylphenol (NP) showed little activity other than induction of renal cysts under continuous exposure conditions in contrast to effects noted with ethinyl estradiol and genistein in other studies outside this project. NP did cause a modest increase in male mammary hyperplasia at the highest dose but did not accelerate the onset of aberrant estrous cycles in females as demonstrated with ethinyl estradiol and did not have effects on female mammary neoplasms as noted in the genistein study.

Critique
The evaluation process using multiple generations as described in the presentation provides a particularly thorough assessment of reproductive effects of NP. The technical report and several manuscripts require completion. There is a plan to synthesize the results of the genistein, ethinyl estradiol and NP studies to determine if there are points learned from these studies that should impact standard testing designs in the future.

Recommendation
The planned effort to evaluate the several studies assessing "endocrine disruptors" to determine if there are points learned based on the evaluation of multiple compounds is strongly supported. It is important to obtain as much information from these extensive studies as possible including the identification of common versus compound unique responses from this pharmacological class of molecules. No other recommendation is provided since this project is ending.

Project 5: Influence of prenatal estrogen exposure on mammary gland and prostrate susceptibility to carcinogenesis (Proposed) (Dr. K. Barry Delclos)

Summary

While not apparent from the project title, this project is focused on bisphenol A (BPA) which has previously been demonstrated to cause alterations of prostate and mammary gland development and estrogen sensitivity, prostatic intraepithelial neoplasia and preneoplastic mammary gland lesions. However, the human relevance of these previous study results has been questioned due to use of the parenteral route of administration and use of DMSO. The studies to be proposed will have several goals: 1) to examine the rat strain and tissue variations in the expression of the potential BPA targets mentioned above and their modulation by diet; 2) to examine the effects of developmental exposure to BPA on proliferative lesions and in target organs (prostate and male and female mammary glands); 3) to examine the effects of developmental exposure to BPA on the expression of specific genes and gene products reported in the literature to be altered by BPA and/or estrogen exposure (e.g. hormone and growth factor receptors); 4) to conduct a dose range finding study, with oral dosing, for a transplacental chronic toxicity study in rats; and 5) to conduct a transplacental chronic toxicity study in rats.

Critique

The plans for this project are very vague since the proposed project is essentially a concept at this time. There is certainly a need to provide additional data on bisphenol A since there appears to be many unanswered questions based on recent reviews of the toxicity of the molecule.

Recommendation

It is imperative that the specific plans for this project be focused on the critical issues related to human risk assessment. While there may be numerous questions and issues regarding bisphenol A, they are probably not of equal importance in terms of assessing human risk. The NCTR/NTP is encouraged to develop specific plans and make them available for public review and comment before proceeding. This effort should start with the identification of specific data gaps followed by definition of specific research approaches.

Overview: Investigation of toxicological mechanisms (Dr. Daniel Doerge)

This research program seeks to define biochemical mechanisms for toxicity and has relied on modern mass spectrometric methodology to facilitate comprehensive investigations of chemicals that impact public health due topresence in FDA-regulated products, especially food and dietary supplements. Major ongoing projects have focused on: 1) acrylamide (AA), a likely human carcinogen produced at ppm levels during cooking of many starchy foods; and 2) soy-derived phytoestrogens. MS-based investigations of AA in rodent species include quantification of AA-derived DNA and hemoglobin adducts, pharmacokinetic evaluations of AA and metabolites in blood and urine, and combining such quantitative information into a physiologically based pharmacokinetic (PBPK) model to permit extrapolation of human internal exposures and DNA damage using available biomonitoring data. The goal of such efforts is to reduce the uncertainty in estimating human risks for cancer and neurotoxicity resulting from AA exposure through food. Similarly, MS-based investigations of soy phytoestrogens in rodent models have produced metabolic and pharmacokinetic data to understand better the role of soy foods and supplements in disease processes important to aging women, including cognition, obesity, and estrogen-dependent breast cancer. In addition, high throughput MS-based assays have been applied to large human epidemiological investigations to understand better potential translational aspects of soy consumption as

it may relate to diet-drug and diet-disease interactions. (overview provided by investigator)

PROJECT 1 (E7212): Development of a PBPK/PD model for acrylamide

Summary (provided by investigator):

Acrylamide (AA) is an important industrial chemical with annual worldwide production estimated at >200 million kg. Concern about human toxicity from AA arises from the observations that AA is neurotoxic in experimental animals and in humans, is mutagenic to male germ cells, shows a variety of evidence for genotoxicity *in vitro* and *in vivo*, and is carcinogenic in several organs of experimental animals following chronic exposure. Several international regulatory bodies (IARC, WHO/FAO, U.S. EPA) have concluded that AA is a probable human carcinogen.

The goal of this research is to develop, using toxicokinetic and biomarker data collected in B6C3F1 mice and F344 rats, a physiologically based pharmacokinetic-pharmacodynamic (PBPK/PD) model for AA and GA from which tissue levels of parent compound, its genotoxic metabolite, and their disposition can be simulated across species, including the human. Biomarkers of exposure including hemoglobin adducts of AA and GA and GA-derived DNA adducts will provide a pharmacodynamic link with measures of genotoxic damage. The ultimate goal is to develop a PBPK model that can predict concentrations of AA and GA in human tissues along with the resultant DNA and brain damage for use in assessing human cancer and neurotoxicity risks from consuming AA in the diet.

Critique:

This is a highly productive project which is effectively leveraged with several internal and external collaborators through a CRADA. Using acrylamide as the model compound, this project addresses an important health issue of how to address health risks associated with natural, genotoxic toxicants present in the food supply. The findings of this study may provide generic insights into this very important and significant issue. The project appropriately recognizes the complexity that acrylamide represents only one of potentially hundreds if not thousands of natural toxicants in the food supply, and that measures taken simply to reduce acylamide exposures may result in corresponding increases in other natural toxicants that may present even greater heatlh risks. The development of PBPK models and associated biological markers is an effective means to facilitate these complex interactions. The project clearly exploits the strengths of the laboratory's analytical expertise.

Recommendations:

Investigators should consider conducting studies which characterize internal dosimetry of acyrlamide and its metabolites/biomarkers under conditions of dietary exposure that are relevant to toxicity tests conducted with this substance. Such information will be useful to further validate their PBPK models. This project also presents the opportunity to examine and challenge some of the fundamental assumptions driving chemical risk assessments, e.g., genotoxic carcinogens must be assumed to present a linear, no-threshold risk. Characterization of the dosimetry and associated toxicology of the many genotoxic natural chemicals in foods that are otherwise regarded as healthy may allow the validity of this current risk *assumption* default to be supplanted by new science. Examining these types of broader risk implications should become a major future focus of this project. The external collaborations used in this project are applauded, and

present opportunities to engage investigators and technologies that are not available within NCTR.

PROJECT 2 (E7210): Phytoestrogens and aging: dose, timing, tissue

Summary (provided by investigators):

The estrogenic nature of soy isoflavones has been known for fifty years. In the past ten years, research on isoflavones has focused mainly on certain biological activities thought to provide health benefits for reduction of chronic diseases of aging, such as endocrine-dependent cancers (breast and prostate), cardiovascular disease, and osteoporosis. Many of these potentially beneficial effects of isoflavones are associated with their estrogen agonist action. This presents a paradox, because dietary estrogens, like endogenous estrogens and estrogens used in hormone-replacement therapy (HRT), are likely to give rise to a complex set of benefits, as well as risks. The recent results from the Women's Health Initiative highlight this dilemma. In this large, randomized control trial, HRT reduced the risk of colorectal cancers and hip fractures, but at the same time increased the risk for coronary heart disease and invasive breast cancer.

The overall goal of the collaborative project is to conduct highly interactive investigations of the effects of phytoestrogen dietary exposure using well established animal models for the study of: breast cancer progression to hormone-independence, obesity and the risk of diabetes, and cognitive function. This goal will be achieved through four complementary synergistic projects conducted at the University of Illinois, Urbana-Champaign (UIUC): 1) Genistein and Endocrine Resistance in Breast Tumors; 2) Dietary Phytoestrogens and Adipocyte Development; 3) Dietary Phytoestrogens and Cognitive Function During Aging; and 4) Phytoestrogen Action Through Estrogen Receptors α and β.

Critique:

This project represents an important research effort to characterize the potential health effects of soy isoflavones which are major natural constituents in many foods and dietary supplements. Understanding risk implications of these compounds is important given the relatively high human exposures to these substances. The research has been highly productive and resulted in a large number of peer reviewed publications in well respected journals. The studies use the strengths of the NCTR laboratories effectively to address key experimental hypotheses. Planned studies addressing impacts of phytoestrogens on aging (NIA P01) creatively tap high level external research collaborators to provide expertise not present within NCTR. These studies will effectively integrate biological responses with excellent characterization of dosimetry, a key element needed for realistic interpretation of potential human health risks and benefits. The mouse MCF-7 xenograft model appears to be well suited to explore hypotheses aimed at clarifying impacts of soy isoflavones on breast cancer.

Recommendations:

The proposed research strategy is well defined and supported by appropriate internal and external collaborations. This project will likely lead to significant research outcomes that will inform critical decisions on natural chemicals with significant human exposures.

Overview: Carcinogen DNA interactions (Dr. Frederick Beland)

The major emphasis of Dr. Beland's laboratory has been to understand the relationship between DNA adduct formation and the subsequent mutagenic and carcinogenic

responses. On those lines, the projects presented here cover an ample spectrum of compounds either food contaminants, therapeutic agents and potential liver toxicants.

PROJECT 1: DNA adducts of tamoxifen (E7011) (Dr. Frederick Beland)

Summary

Tamoxifen is used as adjuvant therapy for early-stage breast cancer, it reduces the chance that the original breast cancer will come back in the same breast or elsewhere. It also reduces the risk of developing new cancers in the other breast. Additionally, it is used for at least 5 years to prevent breast cancer in women who have never been diagnosed with breast cancer but who are at increased risk of developing the disease. Despite being beneficial for breast cancer treatment and prevention, tamoxifen is known to increase the risk of endometrial cancer probably by the induction of tamoxifen-DNA adducts in human endometrium. The focus of this project was to characterize DNA adducts from suspected tamoxifen metabolites, and develop methods for their detection and quantitation.

Critique

Dr. Beland's laboratory has made significant contributions in the area of tamoxifen-DNA adducts characterization, and the proposed experiments with the tamoxifen analogues toremifene and GW5638 constitute a reasonable extension of his long record of accomplishments in the field.

Recommendation

The SAB recommends completion of this project by characterization of the DNA adducts induced by toremifene and GW5638.

PROJECT 2: Perinatal carcinogenicity of drug combinations used to prevent mother-to-child transmission of HIV (E2141) (Dr. Frederick Beland)

Summary

Mother-to-child transmission of HIV-1 can be reduced substantially by the use of antiretroviral drugs. Based upon the success of antiretroviral regimens in reducing mother-to-child transmission of HIV-1, increased attention has been paid to the possible long-term effects of these drugs in uninfected children. The goal of this NIEHS/NTP-funded project was to determine the carcinogenicity, mutagenicity, DNA incorporation, and metabolism of combinations of antiretroviral drugs administered transplacentally to pregnant C57BL/6N mice, perinatally to their B6C3F1 offspring, and neonatally to B6C3F1 mice.

Critique

This lab has demonstrated to be well equipped to conduct the type of experiments described under this project. Transplacental studies as well as neonatal studies with various combinations of NRTIs seem to confirm other results published in the literature. Additionally, a comprehensive NTP report is being finalized. The group showed that AZT, but not 3TC, is genotoxic in neonatal B6C3F1/*Tk* mice. Additionally the lab showed that ddI was not mutagenic in neonatal B6C3F1/*Tk+/-* mice and that it did not potentiate the mutagenicity of AZT. Their conclusion that AZT, 3TC, and the combination of AZT and 3TC are transplacental mutagens agrees with other studies. Importantly the lab is addressing a relevant problem regarding other compounds used in the anti-HIV/AIDS therapy. Studies are also being conducted to determine whether or not the NVP-DNA adducts characterized in the lab are formed *in vivo*.

Recommendation
The SAB recommends the continuation of the characterization and detection of NVP adducts in vivo.

PROJECT 3: Genotoxicity and carcinogenicity of acrylamide and its metabolite, glycidamide, in rodents: two-year chronic carcinogenicity study (E2150) (Dr. Frederick Beland)
PROJECT 4: Genotoxicity and carcinogenicity of acrylamide and its metabolite, glycidamide, in rodents: neonatal mouse bioassay (E7185) (Dr. Frederick Beland)
Summary
Acrylamide, a water-soluble α,β-unsaturated amide, is a contaminant in baked and fried starchy food, including French fries, potato chips, and bread, as a result of Maillard reactions involving asparagine and reducing sugars. This lab pursued the hypothesis that acrylamide is a genotoxic carcinogen as a result of metabolic conversion to glycidamide, which reacts with DNA. Since the metabolic conversion of acrylamide differs in mice and rats, the group proposed to compare the extent and types of tumors in B6C3F1 mice and F344 rats treated chronically with acrylamide and glycidamide. In addition, since neonatal mice are very sensitive to genotoxic carcinogens, newborn mice will be treated with acrylamide and glycidamide to obtain information regarding the mechanisms of acrylamide tumorigenesis.

Critique
The observation that neonatal mice are very sensitive to genotoxic carcinogens, and the suggested experiments in newborn mice with acrylamide and glycidamide, could provide important information on the mechanisms of acrylamide tumorigenesis.

Recommendation
The SAB considers of great importance the continuation and elucidation of the mechanism of carcinogenesis induced by acrylamide.

PROJECT 5: Liver Toxicity Biomarkers Study: Phase 1, Entacapone and Tolcapone (E7266) (Dr. Frederick Beland)
Summary
The goal of this project is to identify molecular biomarkers that can be used in preclinical and clinical studies to predict potentially harmful effects of drugs in humans. Rats will be exposed to 2 different compounds with and without liver toxicity. The following will be the tests performed on samples obtained after treatment:

- Urine will be collected for metabolomic analysis by NMR and mass spectrometry,
- Blood will be collected for clinical chemistry, proteomic, metabolomic, and gene expression analyses
- Liver samples will be collected
- DNA methylation and global histone modification status of the livers will also be measured.

Critique
As suggested by Dr. Beland the utility of this approach cannot be established by the examination of a single pair of drugs. However the availability of funds to pursue similar studies with multiple compounds in uncertain.

Recommendation

Given the ambitious characteristics of this project, this Site Visit Team recommends completing the results from the analysis of these two compounds and then consider the continuation only with a defined collaboration as established in the CRADA.

Overview: Toxicities of nucleoside reverse transcriptase inhibitors (Dr. Jia-Long Fang)

Dr. Fang's laboratory seeks to develop and validate quantitative biomarkers of NRTI toxicities. The strategy proposed to achieve that goal is to integrate signaling pathways for cell proliferation, apoptosis and DNA repair with the analysis of NRTI-induced DNA damage.

PROJECT 1: Toxicities of AZT and 3TC (E2141) (Dr. Jia-Long Fang)

Summary

The current project is designed to explore the relationships between cell proliferation and apoptosis, cell cycle progression, telomerase activity, senescence, the incorporation of AZT into DNA, and repair of AZT-induced DNA damage in cultured cells continuously treated with these drugs for prolonged periods of time, and to examine the differences in the response between tumorigenic and non-tumorigenic cell lines exposed to AZT to understand the mechanisms of AZT-induced toxicities.

Critique

This project wants to elucidate an array of endpoints that have been reported previously by many authors. (Incorporation of AZT into DNA, interference of cell cycle progression, increase in apoptosis and a decrease in telomerase activity, ROS induced genotoxicity, methylation and the use of the comet assay to assess DN A strand breaks).

Recommendation

The Site Visit Team recommends refocusing the investigation towards areas that have not been covered by other laboratories and need more exploration. The area of DNA repair merit more attention. It would be relevant to generate results in non-transformed human cells to obtain data relevant to the human situation. The experiments with THLE2 cells should be pursued. The status and activity of the enzyme TK1 should be confirmed in the ^{32}P system to detect incorporation. Furthermore, rather than a multiple endpoint approach the Site Visit Team recommends definition of a clear objective to pursue.

PROJECT 2: Mitochondrial toxicity of AZT (E2141) (Dr. Jia-Long Fang)

Summary

This project seeks to evaluate the effects of AZT on the levels of the subunits in mitochondrial respiratory chain complex, the activity of respiratory chain complex, mitochondrial membrane potential, the cellular levels of ATP and lactate, and mitochondrial protein profiles. The ultimate goal is to develop biomarkers for therapeutic monitoring of mitochondrial toxicity in patients being treated with NRTIs.

Critique

A different response in tumorigenic vs non tumorigenic cells was observed. NIH3T3 cells exposed to AZT had a significant dose-dependent decrease in mitochondrial membrane potential. In contrast, HepG2 cells exposed to AZT had a significant dose dependent increase in mitochondrial membrane potential. In contrast, no differences were observed in the activities of respiratory chain complex II, III, IV, and V liver, heart and skeletal muscle of B6C3F1/*TK+/-* and B6C3F1/*TK+/+* mouse pups on postnatally.

Recommendation
Many of the endpoints analyzed in this study confirm results from other laboratories. There is ample record in the literature of the effect of AZT in mitochondria. Since the objective of the project is to develop biomarkers of exposure and mono-therapy is not longer the recommended therapy; the Site Visit Team recommends focus towards a combination study were the evaluation of multiple nucleoside analogs is explored.

Overview (E7181): Epigenetic alterations: A common mechanism involved in genotoxic and non-genotoxic rat (Dr. Igor Pogribny)

Summary (provided by investigator)

The major goal is to understand and elucidate the role of epigenetic changes in the mechanism of carcinogenesis, with the ultimate goal to identify biological markers for the early detection and prevention of cancer. The primary research focus is to elucidate the biochemical and molecular mechanisms that induce and promote carcinogenesis through the use of several different models of rodent carcinogenesis that are highly relevant to humans. The development and progression of cancer in humans is a multistep and longterm process. Investigating the molecular mechanisms of this multistep process in humans is frequently impractical and, in most cases, unethical. Relevant rodent models provide a unique opportunity to study the role of etiological factors and mechanisms of tumor development.

Findings since the last SAB visit indicate that stable hypomethylation of DNA and repetitive elements and altered gene-specific methylation associated with gene silencing are key events in neoplastic cell transformation induced by methyl deficiency. Additionally, these alterations in cytosine methylation are accompanied by a progressive decrease in global and gene-specific trimethylation of histone H4 lysine 20 (H4K20me3). More importantly, the alterations in the DNA and histone methylation patterns are specific only to carcinogenesis in target liver tissue and do not occur in the nontarget tissues, such as kidneys, spleen, pancreas, and brain.

These findings, particularly the early appearance of epigenetic changes during carcinogenesis and the target organ specificity, have led us to suggest that epigenetic changes may be sensitive and useful indicators of carcinogenic process. Additionally, they may be predictive biomarkers for a toxicity and carcinogenicity assessment, because one of the many needs in cancer research is rapid identification of human carcinogens before their dissemination into society. This has a special significance for non-genotoxic carcinogens, considering the fact that most carcinogens presented to regulatory agencies today are not genotoxic carcinogens.

Critique:

This project investigates a rapidly emerging area of toxicological research and interest, the potential for epigenetic events to drive cancer outcomes. The project continues to exhibit research productivity, and interactions with both internal and external collaborators have added to the effectiveness of the research. The investigator is using state-of-art technologies to identify potential epigenetic biomarkers of cancer outcomes which may prove useful for early identification of breast cancer as well as provide tools to examine efficacy of various chemopreventative therapies and populations which may be susceptible to cancer outcomes. Exploitation of rodent strain-specific differential responses to epigenetic alterations is an effective research strategy to develop

reasonable hypotheses to explore the implications of such changes to potential human health.

Recommendations:
Overall this project is well constructed and led. Efforts should be made to assure that the strategies of this project are coordinated with ongoing research in the genotoxicity division of NCTR. The emphasis on exploring the implications of nutritional status on epigenetic events driving cancer responses is encouraged and should be extremely important.