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**Peer Consultation Workshop on Research Needs
Related to the IRIS Draft Toxicological Review of Naphthalene**

FINAL REPORT

**National Center for Environmental Assessment
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NOTICE

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This report was prepared by the Oak Ridge Institute of Science and Education (ORISE), Department of Energy, under an Interagency Agreement (No. DW-89939822-01-0, Project 04-12) with EPA's National Center for Environmental Assessment (NCEA), Office of Research and Development, as a general record of discussion of the peer consultation meeting. This report captures the main points of presentations and highlights discussions among the participants. This report does not contain a verbatim transcript of all issues discussed during the peer consultation, and it does not embellish, interpret, or enlarge upon matters that were incomplete or unclear. Except as specifically noted, no statements in this report represent analyses by or positions of EPA or ORISE.

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1.0 INTRODUCTION

1.1 Workshop Purpose

Naphthalene has been recently characterized as a likely human carcinogen by the U.S. Environmental Protection Agency's (EPA) Integrated Risk Information System (IRIS) Program in a draft Toxicological Review of Naphthalene (U.S. EPA, 2000), based on new information from a two-year inhalation rat bioassay conducted by the National Toxicology Program (NTP, 2000). In the NTP study, positive trend increases in the incidences of two rare nasal tumors, olfactory neuroblastomas in males and females and adenomas of the respiratory epithelium in males, were observed. EPA derived an inhalation unit risk was derived from these findings. The draft assessment was reviewed by an independent external peer review panel in July 2004. Among comments made by the external peer review panel was the desirability of future research to characterize naphthalene's carcinogenic mode of action. To further discuss this comment, EPA decided to sponsor a one-day peer consultation workshop, inviting experts in naphthalene toxicology and chemistry, inhalation toxicology, genetic toxicology, and risk assessment to discuss the specific types of studies that would improve characterization of the mode of action of nasal tumor formation and provide estimates of required research time and resources.

The expert opinions and recommendations from this workshop will be considered by EPA in determining a course of action for the development of a scientifically defensible human risk assessment for naphthalene inhalation carcinogenicity.

1.2 Workshop Participants

The workshop was organized and conducted by the Oak Ridge Institute of Science and Education (ORISE), Department of Energy, under an Interagency Agreement with EPA's National Center for Environmental Assessment (NCEA), Office of Research and Development. The workshop was held on April 7, 2005, at the Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania. Eight experts (four from the original external peer review panel, and four additional experts selected by EPA) were invited to present their views and recommendations. Dr. Bernard Goldstein, Dean of the Graduate School of Public Health at the University of Pittsburgh, chaired the workshop. The participants were asked to present their views in response to five charge questions provided by EPA. The final workshop report represents a summary of the discussion that occurred at the meeting. The report has been reviewed and accepted by the peer consultation panel as representative of the discussion.

A list of the eight panel participants and their affiliations can be found in Appendix A. The meeting was attended by approximately 20 observers, who are listed in Appendix B. The meeting agenda is shown in Appendix C. Overheads presented by panelists are reproduced in Appendix D.

1.3 Charge Questions

The panel was asked to focus the discussion on the following five charge questions:

(1) What specific studies would clarify whether naphthalene induces olfactory epithelial and respiratory epithelial tumors in rats through a genotoxic mechanism? Discuss specific issues related to these studies (e.g., if metabolite formation is needed, how would this be accomplished; if Ames tests are proposed, then what strains and tissue fractions would be best?).

(2) Which studies would be the most critical for elucidating whether a genotoxic mode of action is operating?

(3) What resources (level of effort, funds, time) would be required to perform the suggested studies?

(4) If the critical studies identified above show that genotoxicity is not likely under conditions that lead to tumors *in vivo*, what critical studies or evaluations could be used to see if effects on cell cycling/proliferation (including apoptosis) or cytotoxicity might play a role in tumor formation?

(5) What resources (level of effort, funds, time) would be required to perform the suggested studies?

2.0 SUMMARY OF OPENING REMARKS AND PRESENTATIONS

2.1 Welcome: Introduction and Charge

Dr. Goldstein welcomed the panelists and observers, and opened the meeting with brief introductory remarks on the objectives of the workshop. It was suggested by Dr. Goldstein that the workshop discussion begin with a focus on areas of concern associated with naphthalene (NP) inhalation carcinogenicity, identified from the previous comments of the external peer reviewers. Dr. Goldstein briefly reviewed the comments from the external peer review that were related to the mode of action of naphthalene carcinogenicity (See Appendix D-1 for a copy of the overheads. See <http://www.epa.gov/IRIS/whatsnew.htm> for the complete external peer review report). The goal was to expand the external peer review discussion within the context of the charge questions for the present workshop. Dr. Goldstein presented the charge questions and noted that the key concerns were the roles of genotoxicity versus cytotoxicity in the induction of naphthalene carcinogenicity, identification of studies that might be conducted to resolve these issues, and the level of resources needed.

2.2 Overview of the Genetic Toxicology of Naphthalene and Its Metabolites

Following Dr. Goldstein's introductory remarks, Dr. Eastmond provided an overview of the large body of data on the mutagenicity and genotoxicity of NP and its major metabolites (See Appendix D-2 for a copy of the overheads).

2.2.1 Bacterial gene mutation assays

In *Salmonella typhimurium*, all assays were negative except one for 1,2-naphthoquinone (1,2-NQ). SOS response and SOS chromotest were also negative, as was the PolA or rec assay. The Mutatox test was positive with S9; however, given the numerous negative studies, Dr. Eastmond considered this result to be an anomaly.

2.2.2 *In vitro* eukaryotic gene mutation, cytogenetic, or DNA damage assays

In studies identifying mutations at the HPRT and TK loci, the results were negative at the TK locus and equivocal at the HPRT locus for both NP and 1,4-naphthoquinone (1,4-NQ). In the *in vitro* micronucleus test, NP was weakly positive (some chromosomal breakage) in MCL5 cells, a cell line transfected with the genes for several cytochrome P450 isoforms and epoxide hydrolase which likely have bioactivating properties. In the same test, 1,4-NQ was modestly positive, inducing chromosomal breakage and loss.

NP was strongly positive in the NTP (2000) study with cultured Chinese hamster ovary (CHO) cells, inducing structural chromosomal aberrations with S9 and significant increases in sister chromatid exchanges (SCE), with and without metabolic activation. In rat hepatocytes tested *in vitro*, NP was negative under conditions of alkaline elution, and NP, 1-naphthol, and 2-naphthol were also negative for unscheduled DNA synthesis (UDS). NP was also negative in five cell transformation assays using various cell lines. In contrast, in preimplanted whole mouse embryo treated *in vitro* with either NP, 1,2-NQ or 1,4-NQ, structural chromosomal changes in the presence of S9 were observed.

2.2.3 *In vivo* eukaryotic gene mutation, cytogenetic, or DNA damage assays

In recombination assays using *Drosophila melanogaster*, NP induced somatic mutations. Micronuclei were also induced in the erythrocytes of NP-exposed salamander larvae (*Pleurodeles waltl*). Alkaline elution (measuring single strand breakage) and UDS in rat hepatocytes treated *in vivo* were negative. No increases in micronuclei were observed in the bone marrow of male ICR Swiss mice and male and female CD-1 mice. Dr. Eastmond noted that the focus of these micronucleus assays was on benzene, and these tests were conducted at a time when guidelines for conducting genotoxicity studies were changing. Therefore, it is important to evaluate technical aspects of the individual studies when assessing the findings. Micronuclei were also induced in the erythrocytes of NP-exposed when assessing the findings. In other rodent studies, NP induced DNA fragmentation in a variety of tissues. For example, in a study involving normal and p53 heterozygote transgenic mice, NP at moderate doses (≥ 32 mg/kg) induced DNA fragmentation in mouse liver and brain. DNA fragmentation was also seen in rats administered NP at a higher dose (110 mg/kg) for 120 days. However, all these studies have some limitations especially those using the higher doses; genotoxic chromosomal breakage may have occurred as well as breakage associated with cytotoxicity.

Recently, studies using the Comet assay have been conducted assaying DNA damage in the white blood cells (WBC) of exposed workers. Although these studies focused on multiple

types of polycyclic aromatic hydrocarbons (PAH), increases in WBC of PAH-exposed humans were positively correlated with NP and phenanthrene. The findings were statistically significant; however, there were numerous confounders in these assays, making the results difficult to interpret.

2.2.4. Summary of Genetic Toxicity

In *in vitro* studies, NP appears to be a clastogen, not a mutagen. Results are mixed in *in vivo* studies. No genotoxicity studies have been conducted in the target organs of carcinogenicity. This data gap is particularly important because NP appears to exhibit tissue-specific metabolism and effects. In target tissues, NP is metabolically activated to reactive intermediates such as quinones and epoxides. Inhalation exposure also may be critical for NP toxicity. Dr. Eastmond noted that there are some similarities in the genotoxicity profile of NP with that of benzene, a compound on which he has worked extensively.

2.3 Discussion of the Genetic Toxicology of Naphthalene and Its Metabolites

Considerable discussion followed Dr. Eastmond's presentation. Dr. Penning noted that many of the mutagenicity studies were flawed and thus, the data were limited for assessment of mutagenicity. His laboratory has conducted numerous studies with polycyclic aromatic *ortho*-quinones using p53 cDNA in *in vitro* mutagenesis assays. Although 1,2-NQ is an electrophilic metabolite, it is also redox active. When quinones are allowed to redox-cycle to generate reactive oxygen species (ROS) they were found to be more mutagenic than the parent quinone. Therefore, he cautioned that mutagenicity studies should be designed so that mutation in the presence and absence of redox-cycling could be assessed. NP or its metabolites may not be the toxic moiety; toxicity may occur via the production of ROS.

A question was raised regarding the certainty with which NP could be classified as a non-mutagen. Dr. Penning noted that the "jury is still out" on this question, and Dr. Eastmond concurred, adding that although NP appeared to be a clastogen, a potential for mutagenicity cannot be ruled out. Oxidative damage or stress may lead to oxidation of DNA leading to 8-oxo-2-deoxyguanosine, a potentially mutagenic lesion. For example, quinones are highly redox active molecules and can undergo redox cycling, leading to the formation of numerous ROS. ROS can cause severe intracellular oxidative stress through the formation of oxidized macromolecules. Dr. Morris noted that a number of compounds are oxidants but are not carcinogens; for example, chlorine is a profound oxidant but it is not a nasal carcinogen. Dr. Genter stated that the major limitation of most of the currently available assays is that they are conducted using standard protocols, with and without liver S9 activation. As some metabolic enzymes present in NP target tissues are not present in the S9 fraction, mutagenicity associated with these enzymes cannot be evaluated. Dr. Genter suggested that whole-animal *in vivo* mutagenesis studies would be useful in elucidating target-specific mechanisms. One can assume in this case that metabolism and the resulting mutagenicity occurs near the point of contact; however, one cannot rule out the fact that metabolites generated in the liver may recirculate to the nasal passages.

Dr. Van Winkle agreed with these comments. ROS generated by metabolism can cause DNA damage, and this may be the mechanism of action for NP toxicity and mutagenicity. Dr. Eastmond noted that both NP metabolites, 1,4-NQ and 1,2-NQ, are potential redox-cyclers. Dr. Fanucchi added that NP induces two tumor types. With acute NP exposure, frank cytotoxicity is observed in olfactory epithelial tissue, but no cytotoxicity is evident in respiratory epithelial cells. Dr. Genter stated that these findings occur with a single exposure that induces acute effects. The effects of repeated exposures are not known. Dr. Penning added that there are many metabolites that could cause mutagenicity/genotoxicity (e.g., NP-1,2-oxide, 1,2-NQ, 1,4-NQ). There may also be secondary effects associated with lipid peroxidation. What is needed is a return to basic biology. Little is known about either the enzymology and kinetics of NP metabolism and the same is true of its downstream metabolites. He suggested that a basic approach would be to: (1) list target tissues and gender differences among target tissues; and (2) assess metabolic profiles of the various metabolites in order to identify putative candidates for the toxic mode of action evaluation across gender.

3.0 SUMMARY OF DISCUSSION ON CHARGE QUESTIONS

3.1 Charge Question #1: What specific studies would clarify whether naphthalene induces olfactory epithelial and respiratory epithelial tumors in rats through a genotoxic mechanism?

3.1.1 Initial Studies

What are the first steps to elucidating whether NP-induced tumorigenesis is mediated by a genotoxic mode of action? Dr. Goldstein asked Dr. Eastmond to begin this discussion.

Dr. Eastmond suggested the performance of a series of specialized genotoxicity studies to answer this question.

Determine NP genotoxicity in the rat olfactory and respiratory epithelia and in the mouse lung by measuring covalent binding when NP is administered via inhalation.

The following methods were recommended to measure covalent binding in nasal and lung tissues: (I) liquid scintillation counting (LSC); (II) ³²P-postlabeling; or (III) accelerator mass spectrometry (AMS). AMS is the most sensitive method for quantitation. This method detects very low levels of binding but does not necessarily result in the identification of the binding species.

Examine mutations in target tissues.

Can NP be metabolized into covalent binding metabolites, forming lesions that can be converted into mutations during the repair process? Dr. Eastmond suggested that one *in vivo* rat model that might be used to examine mutations is the Big Blue (BB), a transgenic mouse with the *lacI* construct as a transgene reporter. The insertion of this construct into the genome allows for the detection of point mutations or small deletions in any target tissue. Chromosomal

damage, measured by the formation of micronuclei, and cell proliferation in target tissues can also be measured. The BB mouse, or Mutamouse, can also measure mutations in target tissues of the mouse and the BB rat could be used to detect mutations in rat tissues. He noted that the use of the micronucleus assay is limited if the cells being examined do not contain adequate amounts of cytoplasm. The sensitivity of the micronucleus assay can be increased by measuring the incorporation of the 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog; this approach can be used to distinguish replicating from non-replicating cells.

To assess DNA damage in target tissues, Dr. Eastmond recommended the Comet assay, using single cell electrophoresis that can be modified to detect single strand breakages, double strand breakages, and oxidative lesions. These types of genotoxic effects can be repaired *in vivo*; a limitation of this assay is that it does not give information about whether the observed lesions are heritable or pre-mutagenic.

Other secondary assays that might be employed for NP genotoxicity testing include (1) the Ames test with nasal or lung microsomes; (2) lymphoblastoid cells expressing various CYP isoforms; and (3) measurement of 8-oxo-2-deoxyguanosine (8-oxo-dG). The 8-oxo-dG test assesses the formation of oxidative DNA lesions. This test is informative if oxidative damage is considered to be a critical event in the formation of tumors and might become the primary test for this end point. Dr. Eastmond noted that establishing whether a chemical compound is not genotoxic is difficult because it requires a high burden of proof. There are numerous genotoxic mechanisms that might be occurring in target tissues or via circulating metabolite(s). Determination of lack of genotoxicity requires a large number of negative tests and a weight-of-evidence approach.

For investigation of the non-genotoxic mechanisms of action, Dr. Eastmond recommended modeling toxicity and cell proliferation in the target tissues. This approach would provide biomarkers that could be useful for identifying the concentrations or doses needed to induce the carcinogenic effects observed in the NTP (2000) bioassay.

Dr. Genter agreed with Dr. Eastmond that the optimal method for determining whether NP induces olfactory epithelial and respiratory epithelial tumors in rats through a genotoxic mechanism is to use the BB *in vivo* mutagenesis assay. An *in vivo* assay is preferable to one *in vitro* because the metabolism of the whole animal is considered, not just that of a single tissue. She has used the BB assay in a repeated-dose (dietary) study with alachlor, an herbicide with complex metabolism involving both the liver and olfactory mucosa. An increase in mutant frequency was seen in the olfactory mucosa (target organ for alachlor carcinogenicity), but no increases were detected in either respiratory mucosa or liver (neither are target organs). Dr. Genter added that in this study, an increase in thyroid mutant frequency was also not observed. This is an important finding because the weight-of-evidence for alachlor-induced thyroid tumors indicates that the mechanism of action is non-genotoxic, and one would not expect to find an increase in mutant frequency in the thyroid gland. Thus, the BB is a powerful rodent model system.

3.1.2 Discussion

In the discussion that followed these test recommendations, Dr. Goldstein observed that the BB may be a good model for initial testing, given time and cost issues. However, Dr. Penning disagreed, noting that the most important end point for assessment of genotoxicity associated with tumor induction is information on the formation of DNA adducts that can be correlated with tumor incidences. He described a classic experiment in which a linear positive relationship was observed between the formation of O⁶-methyl guanine DNA adducts and the incidence of mouse lung adenomas in AJ mice treated with NNK (a nicotine derived nitrosamine ketone). The number of tumors increased with increasing adduction. This strongly suggests that there is a causal relationship between the adduct and the tumor. Dr. Goldstein noted that many different DNA adducts may be formed due to NP administration. The discussion focused on the following topics shown in italics.

Does one need to test for all putative adducts?

Dr. Penning replied that it was necessary to have a candidate list, of reactive metabolites which should be followed up by testing to identify the subset that produces the dominant DNA adducts. Using Liquid Chromatography/Mass Spectrometry (LC/MS) would be a good approach as it has high sensitivity. In order to optimize performance, basic information is needed on adduct chemistry, identification and quantitation. Quantitation would be best achieved using a stable isotope dilution strategy and would require the synthesis of the isotopically labeled standards.

Is there enough information on candidate species?

Dr. Penning replied that there are insufficient data and more would be needed. For example, the glutathione conjugates of 1,2-NQ and 1,4-NQ may be more electrophilic and more redox active than the unconjugated compounds. However, Dr. Fanucchi noted that nasal tissue does not appear to contain significantly more glutathione than pulmonary tissue. It was agreed that more information on possible candidate species for adduct formation was needed.

In order to identify whether or not adducts are produced by putative candidates, assays must be very sensitive. Dr. Buckpitt stated that he has conducted an experiment with tritium-labeled NP, looking at the lung. At the time, the nose had not been identified as a target organ of NP carcinogenicity. Mice were administered 5-10 mCi of radiolabel, and DNA was isolated. The findings were very clear; no radioactivity was observed in the DNA. If sensitivity can be as low as 1 adduct/10⁸, one can then make the assumption that adducts are either present or absent. The limitation of this approach is that it does not detect other candidate species, such as those which are depurinated and those which result from ROS-derived adducts.

Dr. Buckpitt suggested a tiered approach. First, does binding occur? Second, what species bind? There are a number of species that do not bind; this is one of the limitations of the BB model. Where it works, it is a very good system. However, the BB model only detects a subset of the mutations in the universe of mutations that can cause cancer.

What is known about extrapolation from rodents to primates?

Dr. Buckpitt noted that species differences, specifically extrapolation from rodents to primates, is an important issue in mutagenicity and target tissues. Dr. Goldstein stated that interspecies extrapolation was not part of the charge to the panel. He suggested that species-specificity of effects and mechanisms of action is a dose issue, not a hazard characterization one. Other panelists disagreed, noting that human relevance is now being considered as part of hazard characterization. Dr. Morris stated that if metabolic profiles differ significantly across species (and there was general agreement that they do), then interspecies extrapolation is an important concern. He commented that the charge was too “black and white”. There are significant differences between rats and mice; a comprehensive comparison between these two rodent species would be both interesting and informative. Dr. Penning concurred with Dr. Morris, noting that charge question #1 was very specific with regard to target tissues and metabolism and that these issues are species-specific. Dr. Fanucchi noted that if findings in rodents might possibly occur in humans, then they need to be considered. However, Dr. Buckpitt stated that the concern is not with the mouse-and-rat world, but with humans.

Could a tiered approach to genotoxicity testing be developed?

Discussion reverted to basic issues concerning the continuum from genotoxicity to cytotoxicity. Dr. Van Winkle noted that protein adducts may not be important in downstream events leading to carcinogenicity if they are easily and quickly repaired. Dr. Penning stated that many covalent protein adducts are irreversible, such as those formed with protein kinase C isoforms. Dr. Eastmond proposed consideration of three endpoints: (1) oxidative damage; (2) covalent DNA adducts, and (3) interference with enzymes involved in DNA repair and maintenance, as topoisomerases. Dr. Eastmond mentioned that NQ metabolites had been reported to inhibit isolated topoisomerase II in isolated systems. Dr. Morris added that tumors are produced in two separate tissues, an important consideration. He suggested the performance of *in vitro* studies, then acute *in vivo* studies followed by repeated-exposure studies. It was noted that there are no repeated inhalation exposure studies investigating putative mechanisms of toxicity/carcinogenicity in the nose.

Dr. Goldstein agreed with Dr. Morris that the tiered approach was a reasonable one, if genotoxicity was occurring. What types of studies would detect genotoxicity and with what degree of confidence?

Two sets of studies were suggested by some panelists:

- (1) Repeated inhalation exposure with olfactory and nasal epithelia as target tissues, and measurement of the time course of occurrence of cytotoxicity/toxicity; this type of experiment would require cell proliferation data (see Charge Question #4); and
- (2) Characterization of NP effects in target tissue, followed by detailed investigation of the causes of these effects; this could be accomplished by mutagenicity assays in target tissues in conjunction with *in vivo* mouse micronucleus and BB transgenic studies. This combination of

studies would detect mutagenic and clastogenic effects including large-scale chromosomal aberrations, oxidative damage, protein binding, and DNA adduct formation.

With regard to reactive metabolites, Dr. Buckpitt noted that Dr. Penning is identifying their DNA adducts, and is developing standards for their quantitation using LC/MS, and that these data would be very useful for assessment of genotoxicity. Dr. Penning presented his list of putative candidate metabolites which bind covalently to DNA, to form stable and depurinating adducts, and those that induce oxidative damage [See Appendix D-3 for a copy of the overhead]. It was noted that some of these adducts have large numbers of stereoisomers that would also have to be measured.

Dr. Goldstein questioned whether studies typically utilized in regulatory testing might be informative. Dr. Morris replied that the Ames test with nasal S-9 would be particularly useful. If this assay yields a positive result, then one can conclude that mutagenicity is occurring and further testing would be unnecessary. Dr. Fanucchi suggested that the Ames test with respiratory and olfactory S9 from rats and lung S9 from mice would provide a comprehensive assessment. Dr. Morris recommended that all 3 tissue-specific microsomal activating fractions be tested in the Ames assay in both rats and mice. None of these studies have been done.

A simple workup of tiered testing, including cost considerations, followed. It was agreed by some panel members that the Ames assay should be conducted initially, as it is cost effective. Inhalation studies should be next, as route of exposure data are crucial to assessment of *in vivo* genotoxicity. Dr. Penning noted that all possible candidates would have to be considered; this is a long list. For example, there are 4 stereoisomers of the NP diol epoxide which can give rise to 16-stereoisomeric adducts. There are also metabolites that form depurinating adducts, and several that can induce oxidative damage. Dr. Morris stated, that if all assays were negative, the weight-of-evidence against genotoxicity would be very strong. However, the problem with negative data is that one can always say that other genotoxic candidates may exist.

It was agreed that a simplified approach would be to use S9 nasal fraction and S9 respiratory fraction in the Ames assays. However, even if these results were to be negative, lack of genotoxicity could not be assumed. Dr. Penning noted that the inclusion of control mutagens was important. Therefore, quantitation could be specified down to a level of sensitivity; for example, a candidate species is not mutagenic down to a level of mutation frequency of 10^{-8} to 10^{-9} nucleotides. However, even under these conditions, it is difficult to conclude that no genotoxicity is present.

What level of evidence is needed to conclude that NP is not genotoxic?

Dr. Goldstein stated that if one could conclude that NP is reasonably unlikely to be genotoxic, based on the weight-of-evidence of negative *in vitro* data, are *in vivo* studies still necessary? Dr. Fanucchi replied that circulating metabolites (e.g., from the liver) are unlikely, but cannot be ruled out in the absence of *in vivo* data. Dr. Penning noted that many metabolites

are themselves reactive and interpretation of data would be confusing if only *in vitro* systems were used.

Dr. Morris stated, that based on what we know about the nasal toxicology of other compounds, the mechanism of action of NP-induced nasal carcinogenicity has to be genotoxic. Numerous compounds produce nasal toxicity but not tumors. Therefore, negative genotoxic data are suspect and difficult to accept. Otherwise, one would have to conclude that NP is “totally unique”.

Dr. Eastmond noted that there are numerous genotoxic mechanisms. The Ames assays identifies some, but not all, mechanisms. Is mutagenicity the key challenge leading to downstream events? Is mutagenicity a primary or secondary effect? In order to cover the spectrum of possibilities, he recommended that the Ames assay be conducted initially. Positive results would give some indication of possible genotoxicity. However, if NP is compared with benzene, genotoxicity may not be detected in the Ames assays, because it may be occurring via clastogenic mechanisms. Therefore, *in vivo* data are also needed. He commented that the NP situation is very similar to that of benzene about twenty years ago.

3.1.3 Additional Studies

3.1.3.1 In vitro studies

A discussion of other *in vitro* tests that might be useful in the first tier of studies followed.

Use of human or non-human primate S9 fraction from olfactory and respiratory nasal epithelia.

In support of this approach, it was noted that biopsies are not expensive and could be conducted repeatedly with non-human primates if more tissue is needed. However, Dr. Buckpitt noted that nonhuman primates will give no information if mutagenesis is S9-activated. The necessary CYP isoforms are uncommon in non-human primate nasal tissues, occurring in approximately 1 out of 8 animals. Dr. Morris commented that the ultimate objective of quantitative risk assessment was to estimate potential human risks. The rat model is usually a good human model in that it targets the tissues of concern for humans. However, rat nasal pathways are very different from those of humans and thus may have limited human relevance. Nonetheless, in order to conduct whole-animal *in vivo* studies, one needs to know which metabolites are active. The rat model itself does not provide all the answers; however, it does reduce uncertainty.

Use of p53 in vitro mutagenicity assays

Dr. Penning disagreed with the initial approach suggested in the previous discussion (Section 3.1.2) to conduct further testing in the Ames assay. In his judgment, sufficient testing in this system has already been conducted. Further, the Ames assay does not detect mutagenicity occurring as a result of ROS generation or downstream metabolic events with ease. In his

laboratory, the p53 *in vitro* mutagenicity assay can distinguish between direct acting mutagens and those that require redox-cycling. He emphasized that the spectrum of metabolites from the nasal system differs from that of the liver. The p53 *in vitro* system gives information similar to that obtained in the Ames assay and also detects ROS derived mutations. Therefore, this model is preferable. Dr. Eastmond added that the Ames test strains TA102 and 104 were sensitive to oxidative stress.

It was agreed that the p53 *in vitro* mutagenicity assay could be considered, using the list of reactive metabolites to form the DNA adducts being studied by Dr. Penning.

Use of accelerator mass spectrometry (AMS) with in vitro radiolabeling

Although AMS is an excellent system, testing is expensive and there are only a limited number of such systems worldwide, primarily at Lawrence Livermore National Laboratory.

Limitations of in vitro approaches

Dr. Fanucchi stated that live olfactory epithelial cells were difficult to maintain in culture; she has never been able to culture them successfully. However, mouse lung tissue can be maintained in culture. Otherwise, one needs to go into *in vivo* inhalation studies, which have different concerns including issues of anatomy and distribution of gas.

3.1.3.2 In vivo Studies

Discussion of *in vivo* tests that could be useful to study genotoxicity ensued. Dr. Penning suggested that an *in vivo* model could be used to detect adducts, utilizing a sequential series of standards such as the ones that he is developing. However, he also noted that a cell-based system could be used to measure these end points.

Use of BB transgenic model(s)

The use of the *in vivo* BB transgenic model was recommended. Dr. Genter noted that tissues in this system can be dissected and examined separately. A limitation of this model is that it can identify point mutations and small deletions but will not pick up large deletions or chromosomal breaks. Dr. Eastmond added that the BB was a very valuable tool but was not particularly sensitive. If the results were positive, then this model system would be useful. However, it only measures a subset of the spectrum of possible genotoxic effects, and another test would be needed to measure chromosomal damage. Further, Dr. Eastmond noted that although the BB is well accepted as a model, its utility in examining NP genotoxicity raises several questions. How practical is this model for assessment of genotoxicity in nasal tissues? What would be the appropriate parameters for an inhalation experiment? What is the appropriate exposure duration? What is the appropriate expression period?

In response to Dr. Eastmond's questions, Dr. Genter recommended an appropriate exposure duration for the BB model of 1 week if NP is considered to be a direct-acting

carcinogen, and 3 months if NP is considered to be an indirect-acting carcinogen, such as alachlor which she is currently testing for mechanisms of action. However she added that little is currently known about possible effects of repeated inhalation exposure to NP. What are the critical pre-tumorigenic lesions? What is the level of confidence that these lesions will lead to tumors?

Dr. Fanucchi added that interspecies differences have to be considered in development of appropriate parameters for *in vivo* inhalation testing. For example, the level of metabolic capacity in rats and mice differs, and this difference would affect time to mutagenesis.

What are relevant end points to be measured in in vivo testing?

Dr. Buckpitt noted that Dr. Penning has generated analytic methods to look at a number of metabolites; these methods could be used to investigate *in vitro* adduct formation using relevant rodent tissues. Identification of metabolites that form adducts would provide appropriate end points for measurement in *in vivo* studies.

The panelists agreed that oxidative damage should be considered as an end point. Dr. Penning concurred with measurement of this end point but noted that he had concerns about cross-platform comparisons (interlaboratory differences). He suggested that each DNA adduct should be studied by a single laboratory to avoid the limitations associated with comparison of results for the same end point across different laboratories.

Dr. Goldstein expressed concerns about examination of only end points that are associated with oxidative damage/stress, noting that ozone has been studied since 1967 and has been repeatedly shown to induce oxidative damage but no tumors. Therefore, the use of only these end points would not provide a comprehensive and complete assessment. In response, Dr. Penning replied that evidence for correlation of adducts and tumors would be a necessary followup step to demonstrate a causal association of adduct formation with tumor development, as was done with the AJ mice lung adenoma data described previously.

Additional concerns regarding end points associated with oxidative damage were expressed. Dr. Eastmond noted that measurement of an end point such as an increase in 8-oxo-2-deoxyguanosine adducts is indicative of the occurrence of oxidative stress but does not tell whether the oxidative stress is primary (i.e., induced directly by NP administration) or secondary (i.e., due to other cytotoxic mechanisms or upstream effects). This test generally does not discriminate between primary or secondary effects. Further, it was agreed that performance of this assay was difficult to do well, and “easy to do badly”. Dr. Eastmond suggested that the Comet assay as a test with utility for assessment of DNA breakage and oxidative lesions. As this test does not distinguish between repairable and irreparable lesions *in vivo*, it would be important to look at responses in this assay at both early and late time points following *in vivo* exposure in order to assess the degree of repair.

Other assays

Dr. Eastmond noted that none of the above-mentioned assays gives a good indication of the permanence or heritability of the DNA damage/adduct formation. Dr. Genter stated that this question would be addressed by focusing on the lesioned tissues, i.e, target cells. Dr. Eastmond agreed and suggested the use of the Comet assay with target cells following *in vivo* exposure.

3.1.3.3 Preliminary Research Needs Prior to Performance of Extensive Studies

Dr. Penning stated that in his judgment, a significant amount of developmental work was necessary. If one assumes that covalent adducts are not active in lesion formation, what types of adducts/metabolites can induce oxidative damage? The most important research need is to develop analytical methods with a specified level of sensitivity for other potential adducts/metabolites. Dr. Penning estimated that this would take approximately 2 years. Following development of these methods, multiple studies could be performed simultaneously, with different laboratories working with different adducts to address cross-platform concerns. He estimated that performance of these studies would take about 1 year. Therefore, a minimum of 3 years would be needed to conduct this work.

Dr. Morris also noted that there are several research needs with regard to nasal tumorigenesis. Tumor areas could be dissected and tested *in vitro* following *in vivo* inhalation exposure. However, there was uncertainty with regard to the degree of differentiation of tumor sites and the localization of tumorigenic regions within the nasal pathways. Dr. Genter stated that lesions appeared to be very regional with regard to location and proposed that selected regions be identified and that regional counts be done to address these issues.

What level of sensitivity would be needed? How many mg of wet weight tissue would be needed? If the level of sensitivity was to 10 µg DNA from 10 mg wet weight tissue, one would need to pool tissues from mice. In contrast, this level of sensitivity would be easy to attain in a single rat.

It would also be useful to look at urinary levels of DNA adducts in humans; however, it was not known whether any assays that are currently being developed are also applicable to human populations.

3.2 Charge Questions #2 and #3: Which studies would be the most critical for elucidating whether a genotoxic mode of action is operating? What resources (level of effort, funds, time) would be required to perform the suggested studies?

Two testing strategies, both involving some degree of tiered testing, were put forth to elucidate whether a genotoxic mode of action induces NP carcinogenicity. Estimated resource (time and cost) requirements were also presented.

Test strategy # 1

Dr. Penning recommended the following test strategy:

- I. Expression profiling of enzyme isoforms to identify metabolic candidates for testing. Resources: 1.5 months and \$200 K.
- II. Mutagenicity assay testing, BB rat model testing. Resources: 2 years and \$300 K.
- III. DNA methods development. Resources: 2 years and \$300 K.

He noted that mutagenicity testing could be conducted concurrently with DNA adduct methods development, and that additional costs, approximating \$150 K would be needed for testing these adducts.

Test strategy # 2

Dr. Eastmond recommended the following test strategy:

- I. Mutagenicity testing, using the Ames, crude liquid scintillation counting (LSC), ³²P-labeling, and accelerator mass spectrometry (AMS) assays. Estimated resources for these tests were 0.5 years and \$25 - 40K for crude LSC; \$50 - 60K for ³²P-labeling; and ≥ \$75 K for AMS (not including inhalation costs).

He noted that the cost for AMS testing would depend on the complexity of the experiment including the duration of exposure, the number of samples tested at a given time point, and the number of different time points sampled.

- II. Target tissue analysis using the BB transgenic model. Resources: 1 - 1.5 years and \$150K (minimal BB assessment) to \$400K (whole animal assessment).
- III. Comet Assay to assess DNA damage, using single tissue and multiple doses, and micronucleus test. Resources: 0.5 year and \$40-50K for Comet assay and \$50 K for micronucleus test (not including inhalation costs).

Limitations of cost estimates

It was noted that these estimates were for university-based laboratories and that cost estimates for contract laboratories using GLP might be considerably higher, as much as 4-5 fold higher, including direct and indirect cost sharing.

Further, these estimates did not reflect costs associated with the conduct of an inhalation exposure study. Costs estimates were direct, with the assumption that the inhalation exposure study would be conducted elsewhere. Dr. Eastmond noted that an inhalation study might cost up

to \$200 K for a contract laboratory with GLP procedures. Panelists agreed that all these estimates may be at the low-end, or underestimates, of actual costs.

3.3 Charge Question #4: If the critical studies identified above show that genotoxicity is not likely under conditions that lead to tumors *in vivo*, what critical studies or evaluations could be used to see if effects on cell cycling/proliferation (including apoptosis) or cytotoxicity might play a role in tumor formation?

Panelists engaged in broad discussion on a large number of issues regarding testing of nongenotoxic endpoints. A number of concerns were related to: (1) the need for tissue-specificity in this type of testing; (2) the paucity of data on the basic biology of the nasal pathways, and the need for more research in this area; (3) the effects of tissue-specific biological factors on design of non-genotoxic experiments for NP; and (4) the timing of performance of non-genotoxicity testing with regard to genotoxicity testing (i.e., should genotoxicity studies be done first, followed by non-genotoxicity studies or should some or all of these studies be conducted simultaneously).

3.3.1 Issues Associated with Non-genotoxicity Testing of Naphthalene

Development of tolerance in the lung to repeated NP dosing

Dr. Buckpitt noted that the mouse lung is a sensitive tissue, in which proximal through distal toxicity is observed following acute NP administration. In the rat, no lung lesions occur following a range of acute NP concentrations given either parenterally or via inhalation exposure; however, lesions are formed in the rat nasal olfactory mucosal following a single acute 4-hour inhalation exposure of 3.4 ppm.

With repeated dosing in the mouse, exposure tolerance is evident because lung toxicity disappears. If NP is administered either intraperitoneally or via inhalation at toxic concentrations (200 mg/kg) for 7 days, mouse lung tissue examined at this time point looks fairly normal. Further, if a challenge test is done on Day 8, using a high NP dose (300 mg/kg), there is minimum cytotoxicity on Day 9 that occurs mainly in the Clara cells of the distal lung. Dr. Van Winkle noted that similar findings have been observed when tolerance is expressed *in vivo* (via either acute or repeated dosing) and subsequently challenged *in vitro*. The development of tolerance appears to be related to induction of glutathione synthesis via induction of gamma-glutamyl cysteine synthetase, an essential enzyme required for glutathione synthesis. Induced glutathione confers protection against target cell injury in the lung. Whether this occurs in the nose is not known. Further, these findings do not address issues pertaining to the possible existence of a circulating metabolite that may be acting on lung tissue. It was also noted that NP administered intermittently may induce greater tissue injury as compared with continuous exposure because of the development of tolerance under the latter conditions. A similar pattern of effects has been suggested to occur with benzene.

Age and gender differences in NP-induced lung injury

Acute exposure studies have been conducted with both adults and neonates, and tissue injury in the lung assessed. Dr. Fanucchi noted that in neonates, damage was not restricted to the distal airways but was also seen in the lobar bronchi; observed histopathology included findings of hyperplastic nodules and mucous cell metaplasia. These findings also showed gender differences, with the female mouse being more sensitive.

Design of repeated dose studies for assessment of cytotoxicity

Prior to conducting repeated-dose inhalation studies for the assessment of toxicity, some basic studies would need to be done to address issues relevant to study design. The most basic question concerns the time points at which relevant target cells should be harvested. In order to identify these time points, characterization of the repair system in the nose is required; this system is reasonably well known for the mouse lung but not for the nasal passages. Repair systems are complex, and it is uncertain if the repair phase in the rat nose is likely to take more or less time than that in the mouse lung. Further, the extent of repair is also not known. It was noted that with methyl bromide, histological repair occurs with continuous exposure. The target tissue looks normal; however, metabolic expression is very different if examined immunologically or histochemically following exposure.

Dr. Morris noted that the dose-response curve for NP toxicity is not known, except at high doses. Dr. Genter stated that data from Dr. Buckpitt's laboratory demonstrates nasal injury specific to the dorsal meatus at 3.4 ppm following a single acute dose. At higher doses, lesions occur in the same location, suggesting that the site of the lesion is airflow-driven.

Potential differences in rat strain response

Concerns were expressed with regard to rat strain differences in repair systems and tissue injury. A number of experiments have been done with Sprague-Dawley rats, whereas the BB model is F344-based and probably not notably different from the strain used in the NTP (2000) bioassay. There are very large mouse strain differences and it is likely that rat strains exhibit similar differences.

Should cytotoxicity studies precede genotoxicity studies?

Dr. Goldstein questioned whether existing data suggest that cytotoxicity studies should precede genotoxicity studies. Dr. Genter noted that it was important to first identify the time points at which lesions occur, where they occur, and whether they are reversible or irreversible. It may be possible that cytotoxicity is driving genotoxicity. Some panel members agreed that cytotoxicity was necessary but not likely to be sufficient for tumor formation, because cytotoxicity is induced by other compounds that do not produce tumors (e.g., ozone).

Dr. Penning stated that cytotoxicity cannot be separated from genotoxicity. NP may have both properties, depending on the exposure concentration, target cells examined, and tissue-

specific modes of oxidative injury repair. There is no vacuum of knowledge with regard to the dose-response for cytotoxicity. Formaldehyde exhibits a classic dose-response curve in the form of the shape of a hockey stick. In contrast, NP shows a flat dose-response cytotoxicity curve, whereas the dose-response for nasal lesions shows a positive trend. Further, under conditions of acute exposure, CYP concentrations decrease and glutathione is depleted whether or not cytotoxicity is observed.

Mapping of cytotoxic lesions

Dr. Morris stated that it was important to map the distribution of lesions as well as tumors. For lesions, this can be done with repeated-dose, time-concentration studies that examine the time-course of occurrence of lesions and their distribution. From these data, a NOAEL could be determined for lesion formation. Subsequent studies could then be conducted to assess whether cell proliferation occurs at the NOAEL for lesion formation. Dr. Genter noted that lesions are much more extensive than tumor formation; therefore, studies must be designed carefully to ensure that the results can be interpreted clearly. Dr. Van Winkle stated that NP dosing increases cell proliferation in the lung for several days following cessation of exposure and a similar situation might occur in the nose. Therefore, the timing of cell harvesting is important and would need to be worked out.

Route of exposure

Route of exposure was also identified as an issue. Intraperitoneal injection is less relevant than inhalation exposure, and may also induce more NP metabolites. Therefore, the appropriate route of exposure is via inhalation.

Lack of information on target cells in the nasal passages

It was noted that there was less information available on the nose than on other organ systems. For example, the identification of the target cells is unclear. Dr. Genter suggested that stem cells of neuronal origin may be the target cells. Stem cells are undifferentiated, and olfactory neuroblastomas may be derived directly from the stem cells. The respiratory epithelium has a basal layer although the histology of this epithelium varies, depending on location. For example, there is no clear basal cell population in the respiratory epithelium of the turbinates. Further, the repair system in the respiratory epithelium is not well characterized; cell regeneration occurs rapidly and there appears to be a high level of plasticity. Stem cells are not needed for regeneration. Flat basal epithelial cells may divide into daughter cells that mature and slough off within 30 days. Alternatively, cells may divide into pluripotent cells. The nature of regeneration is dependent on regional localization.

Summary of Concerns Regarding Studies of Cytotoxicity

Studies linking cytotoxicity (including cell proliferation and apoptosis) to nasal tumor formation cannot be conducted until some basic information regarding the nasal pathways is obtained. There is a paucity of data on nasal characteristics, including lack of information on

regional differences in cell types and contents of the respiratory and olfactory epithelia, and on repair processes. There may also be large rat strain differences. Further, it is not known how these variables are affected by NP exposures. Other concerns include time points at which to harvest cells for cytotoxicity assessment, mapping of nasal lesions and tumors, time-course determination of lesion formation, reversibility/irreversibility of nasal lesions, and the possibility of development of tolerance to NP in nasal tissues with repeated dosing, as occurs in the lung.

3.3.2 Human Relevance of Naphthalene Effects in Rat Nasal Pathways

Questions were raised regarding the human relevance of NP effects in the nasal pathways of rats. Do we need more information on non-human primates and on nasal tissues in humans? This topic was extensively discussed. It was agreed that human relevance was an important issue. Large differences in nasal anatomy and air-flow characteristics exist between rodents and humans; further, nasal tumors have been observed in rats but not mice, indicating significant species differences among rodents.

Dr. Goldstein noted that there are regulatory guidelines and default assumptions for interpreting animal data with regard to human relevance, and that existing rodent data should be interpreted in this context. It was important to get as much information as possible by designing focused animal experiments to identify mechanisms of nasal tumor formation in rats. Then, humans or nonhuman primates can be studied and similarities and differences among species examined. Dr. Goldstein also added that the charge questions were not directed toward assessment of human relevance and thus this topic was beyond the scope of the workshop objectives.

3.3.3 Design of Rodent Cytotoxicity Studies

Panelists agreed that a tiered approach was optimal, beginning with acute inhalation studies.

Acute inhalation study design

This study design would involve testing of single exposure concentrations over a range of concentrations to determine the time course for induction of nasal histopathology in the F344 rat. It was noted that the F344 rat was most relevant to assessment of cytotoxicity because NTP (2000) used this strain for the carcinogenesis bioassay. Dr. Morris suggested that single exposure concentration-response experiments also be conducted in the mouse; comparisons between the rat and mouse provide information that is important for assessment of human relevance.

Further, it was important to establish a range of NP concentrations that induces rat nasal effects, with the lowest dose being < 1 ppm. There was some concern about whether it was possible to get a dose as low as < 1 ppm; however, Dr. Buckpitt noted that this could be done relatively easily by increasing compound dilution. Suggestions were also made to examine a

subset of blood and/or urinary metabolites in order to identify potential animal biomarkers that could be correlated with human biomarkers.

Repeated inhalation exposure studies in rats

Panelists agreed that the concentration-response data collected from the acute inhalation exposure studies should then be used to select 3 exposure concentrations for a repeated inhalation exposure study of 3-6 months duration, with a number of interim sacrifices in order to examine the time course of histopathologic effects under conditions of repeated exposure.

What dosing patterns should be employed in this study? There was general agreement that exposure concentrations should be compatible with existing bioassay data, and some panelists noted that the highest dose should not be greater than 10 ppm. Discussion ensued regarding the frequency of interim sacrifices. One suggestion was to conduct daily interim sacrifices, using the BrdU assay for determination of cell proliferation. An alternate suggestion was to first obtain time-course data for lesion formation, followed by the BrdU assay at relevant time points. Dr. Goldstein suggested that mapping be conducted, assuming a standard dose-response curve is obtained (i.e., increasing response with increasing concentration), and that blocks of tissue be saved and stored in order to subsequently perform mapping studies. The objectives of these studies would be to localize the occurrence of lesions and then to correlate sites of lesion formation with those in which cytotoxicity is occurring.

Dr. Eastmond emphasized that this study was a substantial one as it included repeated dosing, two sexes, multiple animals per dose group, and numerous interim sacrifices to plot the time course of cytotoxicity and lesion formation. Further, there were a large number of end points to be assessed, including multiple metabolites. Dr. Goldstein suggested that at least a subset of these animals also be tested later for genotoxicity. Retrospective tissue analysis could be conducted using genotoxicity assays such as the Comet, the micronucleus test, and DNA adduct formation tests.

Dr. Genter suggested that another end point, DNA methylation, be evaluated. DNA methylation bridges the gap between genotoxicity and cytotoxicity because this is an effect on DNA that is traditionally considered to be nongenotoxic. This type of study can also be done with very small amounts of DNA.

Acute and repeated exposure inhalation studies in mice

Assuming that similar tiered studies will be conducted with mice, what strain of mouse should be used? One suggestion was to use the CYP2F2 knockout mouse. Cell cycling/proliferation could be examined with BrdU assays. Apoptosis may be able to be assessed via histopathologic examination. Specific apoptosis tests were not suggested. The importance of apoptosis was noted; specifically apoptosis was likely to be involved in differential selection of cell populations, thus leading to a promoting rather than initiating effect. Dr. Genter noted that apoptosis would be difficult to quantitate in nasal tissue because it occurs at a very low rate in the nose. Dr. Penning suggested the use of knockout mouse models for

PKC, p53, and p21 to examine non-genomic events mediated by NP and its metabolites. However, he noted that mice only developed lung tumors, not nasal tumors. Dr. Buckpitt stated that mice did exhibit a weak nasal response in the NTP (2000) bioassay. Had higher concentrations been used, such as 60 ppm, olfactory lesions may have been observed.

If such studies were to be performed, what level of confidence could be applied to the results?

Dr. Penning noted that this type of study should be testing a hypothesis: specifically, does NP or its metabolites disrupt cell-cycle check points? It was generally agreed by the panelists that affirmation of this hypothesis would indicate a non-genotoxic response, but would not necessarily be conclusive in terms of non-genotoxicity versus genotoxicity because cytotoxicity may be only a part of the mode of tumorigenic action. Other mechanisms, including genotoxic mechanisms, may also be involved.

Other tests: use of a cell-based model to assess effects of NP on cell-cycling mechanisms

Dr. Penning noted that the hypothesis to be tested is one of basic science, and could also be tested in a cell-based model using cells from either mouse lung and/or rat nose. Specifically, can NP and/or its metabolites affect cell-cycling mechanisms that can be interpreted as indicative of a promoting capacity, such as interference with protein kinase C activities? Very little information is known on whether NP has any promoting properties. However, even if this mode of action were to be observed, one could not conclude that it was relevant to effects occurring in target tissues. Dr. Morris noted that this type of experiment could be conducted in a single day, and would provide important non-genotoxic information.

Dr. Buckpitt noted that this type of study could be done in 24 hours, with the use of single dose and single target zone. If the nasal lesion is present, one can conclude that the lesion formation is driven by metabolic activity. If the lesion is not present, difficulties arise in the interpretation of results. For example, is there delivery of NP to the nasal system? Is NP active at the selected target site?

With regard to the use of the CYP2F knockout mouse, Dr. Buckpitt stated that NP inhibits isoforms other than 2F. If findings in this knockout strain were negative, no conclusions could be reached. If the findings were positive, they would suggest an inhibitory effect on cell metabolism and could be correlated with results in nonhuman primates. Studies of metabolic inhibition, including effects on quinone reductase and aldo-keto reductase, could then be conducted.

Dr. Van Winkle emphasized the importance of characterizing broad-based metabolic inhibition in nasal tissues. It is not known which CYP might be involved, whether more than one CYP2F isozyme might be involved, and whether other isoforms and not CYP2F might be involved.

It was suggested that CYP isoforms may also be inducible in nasal tissues. However, Dr. Genter disagreed, stating that many people have tried to induce CYP in nasal tissue, with great difficulty and little success, although induction does occur in the liver.

Could various inducers or hepatectomy be utilized to increase hepatic metabolism or assess whether active metabolite(s) are transported from the liver?

Dr. Eastmond questioned whether a hepatectomy would provide useful information to assess whether an active metabolite was transported from the liver. Dr. Buckpitt stated that this type of experiment was not informative, because performance of a hepatectomy alters kinetics; thus, one would not know if the results were due to kinetic changes involving the parent compound or involving liver metabolites. The question of whether inducers of hepatic metabolism might be useful was discussed. Dr. Buckpitt noted that this type of experiment was very difficult to interpret; for example, an experiment has been conducted in which animals were given phenobarbital prior to the lung toxicant 4-ipomeanol. Phenobarbital-induced animals showed decreased lung toxicity because the liver rapidly cleared this compound by conjugation. Dr. Morris suggested that induction of liver metabolism may also change CYP kinetics in the nose.

Should cytotoxicity studies focus on the lung rather than on nasal tissues?

The rationale for this approach was presented, mainly that (1) single dose exposures cause a very predictable time course of tissue injury in the Clara cells of mice, and (2) age and gender differences have been demonstrated with young and female mice being more susceptible. Therefore, more data are available on NP-induced toxicity in the mouse lung relative to the rat nose.

Some panelists noted that it was not clear whether cytotoxicity studies using mouse lung tissue would yield informative data. If a single NP intraperitoneal injection is administered, examination of lung cell repair following epithelial sloughing demonstrates the occurrence of widespread cell proliferation, including proliferation of cells that are not in the target zone. With repeated NP exposures, the pattern of proliferation is much more confusing; there is no single spike. Thus, overlapping repair processes co-occur with processes involved in confirmed tissue injury. There are also a number of data gaps with regard to lung cytotoxicity. For example, do female mice develop tolerance with repeated exposures? Existing data on tolerance are for males, not females.

It was noted that the key questions for cytotoxicity involve issues regarding selection of dose and end points with which to perform metabolic studies. These studies can be conducted with both lung and nasal tissues; distal airways and nasal tissue are easy to dissect. *Ex vivo* experiments are also relatively simple to do. One can manipulate parameters in cell types of interest in both lung and nasal epithelium.

Dr. Morris agreed that studies should be performed with target tissues in both species. He emphasized that his interest was in comparing and contrasting responses between mice and rats.

In rats, there is nasal but not lung toxicity, whereas the reverse occurs in mice. These data support the conclusion that different mechanistic patterns are occurring between the two species and thus it was important to study both target tissues.

Additional discussion on the use of transgenic mouse models

Panelists agreed that transgenic mouse studies should be conducted and that the following enzymes should be monitored: (1) epoxide hydrolase; (2) GST; and (3) reductases. Dr. Fanucchi reiterated that there was a paucity of data in target tissues, especially in the nose. Limited information is available on CYP and glutathione. Changes in enzyme profiles associated with NP treatment were not well characterized. Expression profiles for some proteins are available and are more useful than mRNA expression profiles because proteins are functional. Further, she noted that existing data are for males, and that nothing is known about females. Dr. Penning agreed that expression profiles for protein were important; however, he also thought that mRNA expression profiles would be useful information with regard to genotyping.

Dr. Buckpitt added that CYP2F in the mouse has an extreme affinity for NP and that development of this knockout mouse, if none is currently available, would provide information on whether this CYP isoform is associated with observed changes in target tissues. Dr. Penning suggested that reductases were also important to assess, particularly dihydrodiol dehydrogenases, isoforms of the aldo-keto reductase superfamily, and recommended the use of a transgenic knockout mouse model for their study.

3.4 Charge Question # 5: What resources (level of effort, funds, time) would be required to perform the suggested (cytotoxicity) studies?

Resources required for acute exposure inhalation studies

A discussion about the general costs of conducting acute inhalation studies ensued following the recommendations for acute cytotoxicity testing. No specific cost estimates were offered. Panelists agreed that inhalation exposure is the most expensive testing route; however, it is also the most relevant. Histopathologic assessment of nasal pathways is also expensive. Multiple rats per dose group are needed to reliably determine the time course for occurrence of nasal histopathology. Nonetheless, these studies are important for determination of the NP concentration-response curve in the rat.

Resources required for repeated exposure inhalation studies

Assuming the use of GLP for the repeated inhalation exposure studies in the rat and the mouse, Dr. Goldstein asked for an estimated cost for these studies. None of the panelists offered cost estimates. Nonetheless, Dr. Goldstein noted that it was important to provide enough information to design a “Cadillac” study, as if money were no object.

Panelists agreed that these types of studies would be very expensive, approaching the standard two-year animal bioassays conducted by NTP (2000) which cost approximately \$6 million. Addition of transgenic studies would accelerate the costs, possibly exponentially.

4.0 SUMMARY OF DISCUSSION ON OTHER RELATED TOPICS

4.1 Comparison of Rodent and Non-Human Primate Studies

Dr. Van Winkle noted that Dr. Buckpitt had done some work with NP in monkeys. In monkeys, NP metabolism is a lot slower than in either rodent species. However, metabolism to reactive metabolites which become bound covalently to protein appears to be similar to that of the rat. It is not known whether these effects are associated with cytotoxicity or genotoxicity. Dr. Buckpitt thought that this question could be answered by developing biomarkers not of exposure but of events driving the cytotoxic (or carcinogenic) response. Correlation of a specific protein adduct (or adducts) with cytotoxicity would allow investigators the ability to measure these across species and in exposed humans to determine relative sensitivity. Similarly, if naphthalene metabolites generate DNA adducts, these may be able to be measured in biofluids of exposed populations. In this case animal correlations would be essential. A high rate of adduction occurs, in the order of 0.7 μg adducts/nanomole. This suggests that there are multiple mechanisms of adduct formation and that a number of proteins are adducted, as indicated by protein folding.

Would a short-term inhalation toxicity study in primates provide useful information?

Dr. Buckpitt stated that he thought that this type of study would be very useful. If NP exposure concentrations up to 60 ppm are administered to monkeys and no effects are observed, strong support would be provided for the conclusion that marked species differences occur, most likely through differences in the rate of naphthalene bioactivation. Glutathione depletion could potentially be used to monitor this. He added that this type of study could not be done following intraperitoneal injection of NP and would have to be conducted via inhalation exposure.

Dr. Penning noted that 1,2-NQ and 1,4-NQ are redox active compounds. Could mechanisms be discerned by measurement of these compounds alone in experimental studies? These metabolites are bi-functional: they are electrophilic and will generate ROS. It would be useful to identify biomarkers that measure the consequence of electrophile or ROS formation following NP administration in non-human primates. Dr. Morris agreed with this approach. However, Dr. Goldstein disagreed, noting that primate studies should not be conducted without more information on relevant end points and metabolism in rodents. He suggested that the approach used with butadiene might be suitable for NP. However, given the paucity of data on metabolic pathways and end points, this approach does not appear to be useful at this time. Dr. Goldstein added that it might be worthwhile to conduct non-human primate studies to inform the issue of interspecies extrapolation. However, again he did not think that sufficient information on metabolic pathways and end points were currently available to obtain useful information in non-human primates.

Dr. Penning noted that quinones appear to cause oxidative stress and if this can be confirmed, it would be a good end point to measure in non-human primates. A short-term primate study could demonstrate that in the absence of quinone generation, downstream metabolites do not form. Does quinone formation, which occurs in rodents, also occur in primates? Dr. Buckpitt noted that in one of his primate studies, quinones were not generated following NP administration. However, this end point was not the focus of the study; therefore, the reliability of the finding is uncertain. It was agreed that the first metabolic step is essential in rodents for the cascade of downstream events induced by NP exposures. If it does not occur in primates, then this finding would be indicative of marked species differences. Dr. Eastmond expressed concerns that quinone generation in non-human primates might occur at very low levels or below the limit of detection. Dr. Buckpitt replied that quinone generation can be measured at very low levels and via the use of radiolabeled material. Dr. Eastmond noted that there were regulatory guidelines for interpretation of findings with regard to determination of a threshold versus non-threshold (low-dose linear) response, and that it would be important to consider these guidelines in designing a primate study.

Are there any data on naphthalene cytotoxicity in humans?

Dr. Goldstein asked if there were any human studies available following NP exposure. Dr. Fanucchi noted that there were no human studies of which she was aware. Dr. Genter added that a human biopsy would be difficult to obtain in the relevant region of the olfactory pathways because this region is too close to the brain, unless there was a medical condition necessitating removal of the whole turbinates. It would be difficult, but possible, to obtain human respiratory epithelium. Dr. Eastmond stated that human biomonitoring was neither useful nor heuristic until there were better precursor or biomarker data. He suggested epoxide or quinone adducts of proteins as potential biomarkers and noted that one investigator, Stephen Rappaport, has looked at these biomarkers as end points for benzene toxicity in humans.

4.2 Relative Timing of Performance of Cytotoxicity and Genotoxicity Studies

Among cytotoxicity experiments previously suggested, which ones are really needed prior to performance of genotoxicity studies? Which of these studies could be done simultaneously with genotoxicity studies and which could be done following genotoxicity studies?

Dr. Goldstein stated that at this time, none of the suggested studies would conclusively differentiate between cytotoxicity and genotoxicity. How might this fact affect the ordering of data collection? Dr. Goldstein added that NP may not become fully genotoxic unless a high level of cell proliferation occurs. Therefore, defining the conditions under which cell proliferation occurs and the extent of cell proliferation in target tissues are important data to generate prior to assessment of genotoxicity. This type of information has been determined for formaldehyde and acetaldehyde in *in vivo* studies. Dr. Eastmond suggested that cell proliferation may occur prior to or concurrent with genotoxicity; an adduct or other pre-mutagenic lesion would become fixed into a mutation during cell replication. For heritable chromosomal damage to occur, the cell would ordinarily need to go through a mitosis.

Therefore, the observed genotoxic effects could be due to a combination of cytotoxicity and direct DNA damage. Alternatively, cytotoxicity may be the key event in the absence of genotoxicity. Although DNA adducts may occur in target tissues, if there is no cell replication, fixation of damage is less likely to occur. Therefore, performance of cytotoxicity studies prior to genotoxicity studies would be beneficial.

APPENDIX A

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APPENDIX B

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APPENDIX C

Peer Consultation Workshop on Research Needs Related to the IRIS Draft Toxicological Review of Naphthalene

University of Pittsburgh
Pittsburgh, PA

April 7, 2005

MEETING AGENDA

Location: Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA

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| 8:30 am | Registration |
| 9:00 | Orientation, Instructions and Conflict of Interest Discussion
(Brian Herndon, ORISE) |
| 9:15 | Introduction to the Workshop (Dr. Goldstein, Chair) |
| 9:30 | Panel Chair's Introduction and Overview of Charge |
| 9:45 | Charge Question #1 (Panel) |
| 11:00 | Charge Question #2 (Panel) |
| 12:00 noon | Lunch |
| 1:00 pm | Charge Question #3 (Panel) |
| 2:00 | Charge Question #4 (Panel) |
| 3:30 | Charge Question #5 (Panel) |
| 4:30 | Conclude Meeting (Dr. Goldstein, Chair) |

APPENDIX D

Panelists' Overheads

APPENDIX D-1

Dr. Bernard Goldstein

Comments from the external peer review (July 2004) that were related to the mode of action of naphthalene carcinogenicity:

Charge Question:

4. *Inhalation Carcinogenicity of Naphthalene*

4.a An assumption has been made that the nasal tumors in rats and lung tumors in mice are relevant to human carcinogenesis. Has this assumption been transparently and objectively described?

Laura Van Winkle:

...However, it would be nice to have some human data that shows an increase in tumors after NA exposure, or barring that, data in nonhuman primates on NA metabolism and cytotoxicity.

Charge Question:

4. b Naphthalene is described as likely to be carcinogenic to humans via the inhalation route of exposure based on the U.S. EPA 1999 Draft Revised Cancer Guidelines (www.epa.gov/ncea). Do the available data support this statement?

James Chen:

...I suggest adding a statement that the overall evidence for naphthalene in the group of agents designed as likely human carcinogens is at the low end.

Michael Dourson:

I am comfortable with this statement and agree with several panel members that the supporting data fall at the animal end of the range of data in this definition. I may or may not agree with adding a statement that “naphthalene is likely to be carcinogenic to humans *at doses that exceed a threshold for cytotoxicity*”, depending on further suggested work as shown in the answer to question 3.

John Morris:

...In my view, the absence of in vitro genotoxicity tests that include a target organ (e.g. nasal S9) activating system represents a critical data gap that might be highlighted.

Laura Van Winkle:

...The current data appear to be at the minimal end of fulfilling the requirement for NA to be considered as likely to be carcinogenic to humans because there is no convincing human data. Further information, not available at this time, regarding NA toxicity or carcinogenicity in humans and nonhuman primates might well justify reclassification of

NA to “suggestive”, particularly if it is shown that the rodent carcinogenicity and MOA are not relevant to humans and/or nonhuman primates. In the absence of this information we must assume that the animal carcinogenicity and mode of action are relevant to humans, but this is not known.

Charge Question:

4.c An inhalation unit risk has been derived utilizing benchmark dose modeling to define the point of departure of 10% extra risk followed by linear low-dose extrapolation below the point of departure.

4.c.1 The inhalation dosimetry equations used in the calculation of the human equivalent concentrations are for a category 1 gas (U.S. EPA, 1994). Is the explanation for the dosimetry choice in the derivation of the inhalation unit risk scientifically justified and transparently described?

Michael Dourson:

I do not agree with EPA’s use of a category 1 RGDR, nor with observer comments on the use of a category 3 RGDR. Naphthalene is a category 2 gas. EPA needs to do its homework here and parse out the proportion of dose expected to arrive at the target tissues by way of systemic circulation and direct absorption. If the data are truly not helpful in making a reasonable guess, then I would be comfortable with a 50/50 split...

Mary Beth Genter:

Based on the discussions and definitions of Category 1, 2, and 3 gases, naphthalene best fits the description of a Category 2 gas, not Category 1.

John Morris:

...A careful application of the RfC methodology would suggest that naphthalene is in fact best modeled as a category 2 gas.

...As is documented for other compounds (e.g. acetaldehyde) the relative quantitative importance of metabolism may differ dramatically at high compared to low exposure concentrations. This represents another factor that should be considered relative to the inhalation dosimetry.

Charge Question:

4.c.2 Has support for the use of linear low dose extrapolation been objectively and transparently presented? Are there other modeling approaches that should have been considered instead of or in addition to the low dose linear extrapolation approach?

Michael Dourson:

I am not comfortable with the support for the linear case being made by EPA, especially in light of the public comments, which show little support for naphthalene’s supposed mutagenicity. Even EPA acknowledges this. EPA needs to define what data would cause it to move from this linear default, and not just state that an understanding of

naphthalene's MOA is not sufficient. Although modeling a noncancer cytotoxicity precursor is unlikely to yield credible results, since the incidence of these noncancer lesions is so high at low dose and the dose response behavior is so flat, it would be enlightening to model both a linear and nonlinear curve at the same time---like a hockey stick, but with no threshold. Consistent with this dual approach is the fact that tumors are only found with extensive noncancer toxicity, and that no genotoxicity is found except with some metabolites that make up an unknown, but suspected to be small, fraction of the overall metabolites.

Michelle Fanucchi:

The support is clearly presented. This appears to be the appropriate modeling approach.

David Gaylor:

Low dose linear extrapolation is justified, but likely to be overly conservative...

John Morris:

The rationale for the linear low dose extrapolation was clearly and objectively described in my view. It is my belief that a significant advance would be made by reliance on a dosimetrically-based PBPK model for low dose extrapolation. The state-of-the-art is sufficiently advanced to allow inclusion of inhalation pharmacokinetic considerations in the risk assessment process. Greater clarity and objectivity would be provided by text that lays out the alternative risk assessment approaches (cytotoxicity driven non-linear, or mixed mode of action), and also explicitly indicates the basis for selection of the approach that was utilized.

Charge Question:

4.c.3 The inhalation unit risk is based upon the summed risks of developing olfactory neuroblastomas and respiratory epithelial adenomas in male rats derived from a time-to-tumor analysis. Is this approach scientifically justified? Are there other modeling approaches that should have been considered instead of or in addition to the approach taken? Has the best data set been chosen for derivation of the inhalation unit risk? Has the modeling been accurately and transparently described?

Michael Dourson:

...After I think that I understood it, my first question was why not add up all the nonsignificant tumors in males? Why just stop with neuroblastomas for males and epithelial adenomas for females? Why not take the approach adding both the male and female responses for individual tumor types?...

It appears that the answers to these questions might be that EPA is trying to maximize the cancer potency factor. I have no conceptual problem with this, but if this is the case, EPA needs to clearly state it.

Michelle Fanucchi:

This approach is very conservative, but given the lack of complete information, it appears to be justified...

David Gaylor:

...The summed risks correctly estimate the risk of an olfactory neuroblastoma **or** a respiratory epithelial adenoma. This is the most conservative approach. However, if it is desired to estimate the risk that an **individual** develops either type of tumor (the more usual calculation), then the risk of an individual producing both types of tumors must be subtracted from the sum to avoid double counting of individuals with both types. For rare tumors, as is the case here, this is a minor adjustment...

Mary Beth Genter:

I believe that the approach is valid, although this tumor type is very unusual...

Laura Van Winkle:

The inhalation unit risk is based upon the summed risks of developing olfactory neuroblastomas and respiratory epithelial adenomas in male rats derived from a time-to-tumor analysis. *Is this approach scientifically justified?* Yes from a “choice of models” point of view. It is a strength of this modeling approach that it allows different tumor types to contribute to the estimate of risk...

APPENDIX D-2

Dr. David Eastmond

Research Recommendations to determine whether naphthalene is genotoxic in the rat olfactory epithelial and respiratory epithelial tissues. Similar studies could/should be done for the female mouse lung. Exposures should be by inhalation

Covalent binding to nasal (and/or lung) tissues

LSC

³²P

AMS

Mutation in target tissues (or elsewhere)

Rat-Big Blue

Mouse-Big Blue or Mutamouse

Chromosomal damage and cell proliferation in the target

Micronucleus assay

BrdU incorporation

DNA damage in target tissue

Comet assay-DS vs SS breaks, oxidative lesions

Secondary assays-

Ames with nasal or lung microsomes

Cell assays with lymphoblastoid cells expressing specific Cyp isoforms

8-hydroxy dG studies

To convincingly establish that something is non genotoxic requires a fairly high standard of proof, in part because there are a number of genotoxic mechanisms and because one is trying to prove a negative

For non genotoxic mechanisms of action, I would recommend modeling toxicity and cell proliferation in the target tissues. These could be used as biomarkers to provide insights into the concentrations/doses where effects are likely to be seen in the cancer bioassay.

APPENDIX D-3

Dr. Trevor Penning

Potential DNA-Adducts that Arise from Naphthalene Metabolism

Covalent-Bulky Stable Adducts

NP-1,2-oxide-N²-dGuo (2 trans-opened and 2 cis-opened adducts)-4 total

NP-1,2-oxide-N⁶-dAdo (2 trans-opened and 2 cis-opened adducts)-4 total

NP-1,2-dione-N²-dGuo (1,4- and 1,6-addition possible) 2-total

NP-1,2-dione-N⁶-dAdo (1,4- and 1,6-addition possible) 2-total

(+)-trans-anti-NPDE (diol epoxide)-N²-dGuo

(-)-trans-anti-NPDE (diol epoxide)-N²-dGuo

(+)-cis-anti-NPDE (diol epoxide)-N²-dGuo

(-)-cis-anti-NPDE (diol epoxide)-N²-dGuo

(+)-trans-anti-NPDE (diol epoxide)-N⁶-dAdo

(-)-trans-anti-NPDE (diol epoxide)-N⁶-dAdo

(+)-cis-anti-NPDE (diol epoxide)-N⁶-dAdo

(-)-cis-anti-NPDE (diol epoxide)-N⁶-dAdo

(8-total diol-epoxide adducts which become 16 if the syn diol epoxide is formed instead of the anti diol epoxide)

Covalent-Depurinating Adducts

NP-1,2-dione-N7-Gua (1,4- and 1,6-addition possible) 2-total

NP-1,2-dione-N7-Ade (1,4- and 1,6-addition possible) 2-total

NP-1,4-dione-N7-Gua (1,4- and 1,6-addition possible) 2-total

NP-1,4-dione-N7-Ade (1,4- and 1,6-addition possible) 2-total

Oxidative DNA Adducts

8-oxo-dGuo

M1-dG (malondialdehyde)

4-hydroxy-2-nonenal propano-dGuo

4-oxo-2-nonenal etheno-dGuo, etheno-dAdo and etheno-dCyd

[dGuo, 2'-deoxyguanosine; dAdo, 2'-deoxyadenosine; dCyd, 2'-deoxycytidine; Gua, guanine; and Ade, adenine]