Naphthalene Research Plan Outline

submitted to

Naphthalene State-of-the-Science Symposium (NS^3)

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by the

ad hoc Naphthalene Coalition

American Association of Railroads
American Coke and Coal Chemicals Institute
American Petroleum Institute
Asphalt Institute
Naphthalene Council
National Petrochemical Refiners Association
Utilities Solid Waste Activities Group

Introduction

Naphthalene is a constituent of petroleum and coal and is produced naturally whenever organic materials – such as wood – are burned. Thus naphthalene is found in gasoline, diesel, jet fuel, kerosene, and home heating oil as well as in coal furnaces and smoke from forest fires. Naphthalene is also distilled from petroleum and coal tar for uses as familiar as mothballs. The principle use of distilled naphthalene is as an intermediate, used to make other chemicals.

Recently, a study of laboratory rats indicated that naphthalene exposure can cause tumors in the noses of rats forced to breathe naphthalene-contaminated air for two years. Previous studies of mice exposed to naphthalene had resulted in equivocal evidence of carcinogenicity. The relevance of these studies to the assessment of cancer risks to humans has been controversial.

The *ad hoc* Naphthalene Coalition (Coalition) has evaluated the existing data and identified some key uncertainties and potential research options to address these uncertainties (see tables listed below). The Coalition would like to share this with the NS^3 participants, as we believe this is a useful reference.

The Coalition hopes that participants in NS^3 will consider, comment on, and contribute insights to this outline of a potential research plan so that data can be collected to support a robust assessment of naphthalene risks.

The submission consists of the following:

Table 1	Summary of Existing Data, Uncertainties and Potential Research Matrix,
Table 2	Comparison of Naphthalene with Chloroacetanilide (CAN) Pesticides Mode of Action (MOA) Research Program,

Table 3	Some Existing Uncertainties Related to Naphthalene Carcinogenicity Risk Assessment,
Table 4	Exposure, Epidemiology, Cancer Incidence References,
Appendix A	Review of Naphthalene Metabolism and Toxicity in Rodents and Primates and Implications for Risk Assessment: Summary of Existing Data (Through 2005), and
Appendix B	Toxicology/Mechanism of Action Potential Research Plan.

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Table 1. Summary of Existing Data, Uncertainties and Potential Research Matrix

Research Area/Existing Data	Potential Research: Short Term Time Frame	Potential Research: Longer Term Time Frame	Comments on Uncertainty
Exposure - Occupational • Air Force JP-8 jet fuel study[1-6] ➢ Median in low/moderate exposed: 0.0004 / 0.002 ppm (4-hr sampling time)[1] ➢ Median in high exposed: 0.09 ppm (4-hr sampling time)[1] ➢ Range: <0.0001 - 0.746 ppm (4-hr sampling time)[1] • Air National Guard study[7] ➢ No air levels reported • Industries with naphthalene-containing streams[8, 9] o Petroleum/refining ➢ ~0.001 ppm (8-hr) o Asphalt ➢ ~0.001 ppm (8-hr) o Creosote ➢ ~0.2 - 0.5 ppm (8-hr) o Other industries (*e.g.*, coking) ➢ ~0.03 - 0.25 ppm (8-hr)	Explore existing studies for data	Additional analyses of stored Air Force JP-8 study[1] blood & urine samples	Most appropriate biomarker of exposure ➢ -1- vs. 2-naphthol vs. other potential exposure biomarkers (*e.g.* de-icing agent in JP-8 at constant ratio to naphthalene)? ➢ impact of other chemical exposures on above biomarkers?
Exposure - Ambient & Consumer • General ambient air levels[8-10] ➢ ~≤0.0001 - 0.004 ppm • Air in homes w/o smokers[8] ➢ ~0.0001 - 0.305 ppm • Biomonitoring[8, 11] ➢ Geometric mean 1-napthol (creatinine-adjusted) 1.9 µmol/m (slightly higher levels for 2-napthol)	Analyze ambient air data from Canada to assist in 'validating' cancer potency estimates (compare observed vs predicted cancers in population)	Use population biomonitoring[8, 11] & human biomarker study data (*e.g.*, AF & National Guard studies[1-7]) to estimate population exposure levels	Some uncertainty related to whether consumer exposure data exist ➢ Uncertainty relative to occupational & ambient levels; some key sources declining in U.S. [*e.g.*, mothballs]

Table 1. Summary of Existing Data, Uncertainties and Potential Research Matrix (Cont.)

Research Area/Existing Data	Potential Research: Short Term Time Frame	Potential Research: Longer Term Time Frame	Comments on Uncertainty
Mortality / Cancer Incidence Epidemiology Studies • Air Force nested case-control study (cancer risk in JP-8 exposed jobs vs. unexposed jobs)[12, 13] • Industries with naphthalene-containing streams[8] o Petroleum/refining[14] o Asphalt o Creosote[15] • Population-based case-series and other studies[16-18]	Attempt to further develop existing data sets (*e.g.*, define / develop exposure estimates?)	Enhance Air Force nested case-control study with additional data collection (*e.g.*, environmental sampling, improved case ascertainment, earlier onset outcome, etc) Assess feasibility of using military and Veteran's Administration mortality/cancer incidence data sources, perhaps supplemented by DoD Medical Surveillance System's stored serum (collected on all military personnel). Evaluate using DoD's ongoing Millenium Cohort Study as the research platform for such.	Relevant tumor type to evaluate in humans unclear (nasal/sinus, all respiratory, others??) All existing studies have no exposure estimates specific to naphthalene; multiple potential confounding exposures (*e.g.*, PAHs) -thus etiology of any observed increases unclear, potential dilution of no/low exposed Statistical power for certain tumor types -nasal/sinus (rare outcome) QA/QC issues associated with stored DoD surveillance system serum

Table 1. Summary of Existing Data, Uncertainties and Potential Research Matrix (Cont.)

Research Area/Existing Data	Potential Research: Short Term Time Frame	Potential Research: Longer Term Time Frame	Comments on Uncertainty
Molecular Epidemiology Studies • Multiple human exposure biomarker studies in occupational[1-7] and environmental settings[8, 11, 19] • No human studies of carcinogenicity-related biomarkers of effect (*e.g.* DNA damage) in relation to biomarkers of exposure (*e.g.*, 2-naphthol)	Examine stored blood samples from Air Force JP-8 study[1] for genotoxicity endpoints in relation to stored urinary samples with biomarkers of exposure (*e.g.*, 2-naphthol) Update / publish B Butterworth white paper on review of genotoxicity	Explore adding data collection for blood/urine to planned (2007) military study of JP-8 exposed workers & neurological endpoints ➢ Add genotoxicity & exposure biomarkers & potentially nasal swab measures (i.e., endpoints to parallel those examined in animal studies)	No data on potential genotoxicity in humans ➢ Most appropriate biomarker of exposure? ➢ Most appropriate genotoxicity measure? Relevance of non-genotoxic mechanisms to assessing human risk ➢ Most appropriate nongenotoxic measures? Relevance of population biomonitoring data to estimating cancer risk

Table 1. Summary of Existing Data, Uncertainties and Potential Research Matrix (Cont.)

Research Area/Existing Data	Potential Research: Short Term Time Frame	Potential Research: Longer Term Time Frame	Comments on Uncertainty
Mechanism/ Mode of Action ****See Appendix A for summary of existing data and Appendix B for detailed potential research plan	Pilot studies to determine the appropriate methods for generation of adequate and accurate naphthalene exposure atmospheres. Single inhalation exposure studies to evaluate dose response for nasal epithelial lesions and establish threshold (no effect) for the lesions. Acute oral dose correlative study to evaluate induction and character of nasal lesions with this route of exposure. Short term inhalation studies (4 week to 13 week) to evaluate dose response for histologic effects in the rat nasal epithelia and determine the threshold for the effects.	Intermediate term inhalation studies to evaluate the interaction between naphthalene metabolism in nasal epithelial tissues and tissue responses, *i.e.*, do metabolites form and sequester in nasal epithelial tissues and what are the metabolites. This may be achieved by use of radiolabeled material to characterize tissue distribution (whole body radiography) and identity of metabolites. Single and intermediate term inhalation studies to evaluate alterations in gene expression and formation of adducts to cellular proteins and/or DNA in nasal olfactory tissues of rats in relationship to metabolism.	Metabolism kinetics (1,2-naphthol excretion conversion) Site concordance (human target tissue?)

Table 1. Summary of Existing Data, Uncertainties and Potential Research Matrix (Cont.)

Research Area/Existing Data	Potential Research: Short Term Time Frame	Potential Research: Longer Term Time Frame	Comments on Uncertainty
Mechanism/Mode of Action (Cont.)	Short term inhalation studies (concentrations established above) in rats to evaluate if naphthalene induces cell proliferation in the nasal epithelial tissues and if the histologic effects and cell proliferation are reversible.	Correlative inhalation studies in primates and mice to evaluate similarities or differences in nasal epithelial tissue responses that were seen in rats. This work may be expanded to evaluate lung tissues as well.	
Toxicology ****See Appendix A for summary of existing data and Appendix B for detailed potential research plan	--	Evaluate existing mixture study data (*e.g.* jet fuel) in rodents as surrogate for low-dose naphthalene.	NOEL Chronic inhalation study w/ mixture containing naphthalene - CDC biomonitoring data Potential flaws in NTP study? (cytotoxicity at all doses indicates MTD exceeded)

Table 2. Comparison of Naphthalene with Chloroacetanilide (CAN) Pesticides MOA Research Program

End Point	CAN Data	CAN Research	Naphthalene	Comments on Naphthalene Uncertainty
Genotoxicity	Weight of evidence indicates not genotoxic	--	Weight of evidence indicates not genotoxic	Seven well conducted *in vivo* studies and most *in vitro* studies of naphthalene *per se* warrant a high degree of confidence that naphthalene is not genotoxic *in vivo*. Metabolism data exist to suggest that genotoxic naphthoquinones, which are secondary metabolites in rodent systems formed via CYP2F, may be of little significance in primates.
Target Tissue (site concordance)	1.Nasal turbinate tumors in male and female rats in nasal tissues. 2. Tissue distribution studies demonstrate that CANs or their metabolites distribute to the nasal	1.Nasal tumors in male and female rats were reported for 3 of the 5 CANs in chronic oral dosed studies. The tumor response was dose responsive. No nasal tumors were reported in mice. 2. Oral dosing studies with radiolabeled CANs using whole body	1. Tumors in the nasal epithelial tissues were seen following chronic exposure of male and female rats. No nasal tumors seen in mice but lung tumors seen in mice. 2. Tissue distribution studies not done but metabolism studies show that the nasal tissues in	1. Inhalation and oral studies demonstrate toxicity in rat nose. Relationship between metabolism in nasal tissues and cellular interactions resulting in toxicity not demonstrated. 2. Site concordance could be demonstrated through conduct of tissue distribution studies, which will distinguish tissue-specific effects from those due to systemic action.

Table 2. Comparison of Naphthalene with Chloroacetanilide (CAN) Pesticides MOA Research Program (Cont.)

End Point	CAN Data	CAN Research	Naphthalene	Comments on Naphthalene Uncertainty
Target Tissue (site concordance) (Cont.)	turbinates.	radiograph showed that the CANs inducing nasal tumors are distributed to the nasal turbinates. Studies in mice, hamsters and squirrel-monkeys demonstrate no nasal distribution in these species.	rats metabolize naphthalene.	
	3. Metabolism studies with CANs show that the CANs producing nasal tumors have a common reactive metabolite.	3. The major metabolite seen in CANs metabolism studies is a reactive dialkyl benzoquinone imines that have been associated with production of nasal tumors in rats.	3. Metabolites have been identified for rat nasal tissues and mouse long tissues.	3. Data exist to demonstrate relationship between metabolism and mouse lung tumors. Further data in rats are required.

Table 2. Comparison of Naphthalene with Chloroacetanilide (CAN) Pesticides MOA Research Program (Cont.)

End Point	CAN Data	CAN Research	Naphthalene	Comments on Naphthalene Uncertainty
Target Tissue (site concordance) (Cont.)	4. Tissue binding potential of CANs metabolites were investigated. 5. Cell proliferation in nasal tissues was evaluated following repeated exposures.	4. The dialkyl benzoquinone imines were shown to form protein adducts in nasal epithelial tissues leading to cell death. 5. Increased cell proliferation was seen in nasal tissues for the 3 CANs that induced nasal tumors. The cell proliferation effect was dose responsive.		

Table 2. Comparison of Naphthalene with Chloroacetanilide (CAN) Pesticides MOA Research Program (Cont.)

End Point	CAN Data	CAN Research	Naphthalene	Comments on Naphthalene Uncertainty
Key Event(s)	Alkyl benzoquinone imines are formed in the nasal tissues. These reactive metabolites form protein adducts leading to cell death which triggers regenerative cell proliferation. If sustained, the increased cell proliferation leads to nasal tumor formation.	The mode of tumorigenic action for the CANs is supported by dose-response concordance between the induction of cell proliferation in nasal tissues and occurrence of nasal turbinate tumors. No tumors were observed at doses in which there was no increased cell proliferation, demonstrating a threshold for the tumorigenic process.	NTP rat study was conducted at high doses, thus no NOEC has been developed.	Absence of a NOEL/NOEC prevents identification of a threshold/dose-response concordance. Further studies to evaluate dose response for induction of nasal lesions as well as other related "key event" markers are needed.

Table 3
SOME EXISTING UNCERTAINTIES RELATED TO NAPHTHALENE CARCINOGENICITY RISK ASSESSMENT

- What cancer endpoint(s) is (are) relevant to an evaluation of human cancer risk and exposure to naphthalene (*e.g.*, nasal/sinus, all respiratory, lymphohematopoietic cancers, other tumor types?)?
- Does particle absorption pay a role in naphthalene exposure?
- Did naphthalene condensation occur during NTP's rat study?
- How do the monkey and human respiratory tract differ?
- Is the naphthaquinone pathway specific to rodents? Is naphthaquinone produced via a primary or a secondary metabolic pathway? If the naphthaquinone pathway is secondary, under what circumstances is the pathway activated? For example, is the naphthaquinone pathway active only at high naphthalene concentrations?
- Are glutathione depletion and effects on DNA repair mechanisms the reasons why toxicity and tumors are seen when high levels of naphthaquinone or other reactive metabolites are formed? Glutathione conjugation would tie up the reactive metabolites. If the glutathione is depleted, there is reactive metabolite that could react with cellular components (proteins, DNA) forming adducts which could lead to cell death and other changes. With DNA adducts, effects on DNA repair mechanisms or overwhelming the capacity of the cell for DNA repair could lead to a tumor response.
- Free radical production through local inflammatory process/cytotoxicity induced by naphthalene could result in oxidative damage. This itself may result in tumor response or exacerbate the above metabolite-mediated response. Could naphthalene-induced rodent tumors be mediated through an epigenetic mechanism?
- The working hypothesis is that CYP2F2-mediated metabolism of naphthalene in rat nasal tissues and mouse lung tissues produces reactive metabolites. Data are believed to exist that demonstrate that CYP2F2 enzymes are insignificant in primate (monkey) nose and lung tissues. Are humans similar to monkeys with regard to CYP2F2 enzymes in nose and lung tissues? Are there other organs within humans that should be studied for potential production of reactive naphthalene metabolites?
- Can naphthalene exposure levels be estimated/calculated for the JP-8 jet fuel exposure studies?
- Is the anti-icing agent that is present in JP-8 at constant ratio concentrations to naphthalene a potential biomarker of exposure?
- Is 2-naphthol a unique biomarker for naphthalene exposure? Does exposure to substituted naphthalenes or other substances also result in urinary 2-naphthol?

Table 3 (Cont.)
SOME EXISTING UNCERTAINTIES RELATED TO NAPHTHALENE CARCINOGENICITY RISK ASSESSMENT

• Given potential low statistical power for certain cancer outcomes (*e.g.*, nasal/sinus cancer) in existing epidemiology studies that evaluated mortality and/or cancer incidence in industries with naphthalene-containing streams, do these studies reveal anything significant about potential cancer risk in relation to exposure to naphthalene or naphthalene-containing substances?

Table 4
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APPENDIX A

REVIEW OF NAPHTHALENE METABOLISM AND TOXICITY IN RODENTS AND PRIMATES AND IMPLICATIONS FOR RISK ASSESSMENT: SUMMARY OF EXISTING DATA (THROUGH 2005)

Appendix A: Review of Naphthalene Metabolism and Toxicity in Rodents and Primates and Implications for Risk Assessment: Summary of Existing Data (Through 2005)

The Cancer Risk Assessment for naphthalene was based on results from long-term inhalation toxicity studies in rats and mice conducted by the National Toxicology Program (NTP). In the mouse study, a statistically significant increase in the incidence of alveolar/bronchiolar adenomas in the lung was observed in female mice exposed to 30 ppm naphthalene (NTP, 1992). No other treatment-related tumors were seen in mice. The tumorigenic response in rats was limited to nasal tissues (NTP, 2000). A statistically significantly increased incidence of respiratory epithelial adenomas was seen in male rats at all exposure levels (10, 30 and 60 ppm). In female rats, the incidence of olfactory epithelial neuroblastoma was statistically significantly higher than control for the 60 ppm exposed group.

Following naphthalene exposure, similar species- and site-specific patterns of cytotoxicity are seen. In mice, naphthalene exposure results in cellular swelling, vacuolation and necrosis of lung Clara cells but these findings are not seen in naphthalene-exposed rats. In the nasal tissues, naphthalene-induced cytotoxicity in the olfactory epithelium was noted in both rats and mice. However, nasal olfactory epithelial cytotoxicity was only seen at very high exposure concentrations in mice compared to rats suggesting a greater sensitivity for this tissue in rats. Both cytotoxic responses in the mouse lung epithelial tissue and mouse and rat nasal epithelial tissue are thought to be related to the inherent electrophilicity of reactive metabolic intermediates or the propensity of the metabolites to generate reactive oxygen species. The induction of alveolar/bronchiolar adenomas and cytotoxicity in the mouse lung have been extensively evaluated by a number of researchers and have been correlated with higher rates of formation of specific enantomeric epoxide derivatives of naphthalene in mice as compared to rats.

The literature related to naphthalene-induced nasal epithelial cytotoxicity and tumor formation in rats is limited. Based on existing literature, a number of mechanisms have been proposed. These mechanisms include anatomical and physiological differences, differences in metabolizing enzymes and metabolites and, differing rates of metabolism forming either more toxic or less toxic metabolites. Recent publications add significant knowledge to the relationship of site-specific metabolism in lung and nasal tissues from rodents and primates and are discussed below. The list of references is included in this document.

The key question in conducting human risk assessment or hazard characterization is the relevance of observed toxicity in rodents to man. In the recent publications, primates were evaluated as a human surrogate to determine if primates (and man) metabolize naphthalene at similar rates and similar metabolic profiles as rodents with the presumption that such metabolism would lead to cytoxicity and tumorigenicity in target tissues. The converse is that if primates do not metabolize naphthalene in target tissues, cytotoxicity or a tumorigenic response would not be seen.

The vast majority of research to date has been related to bioactivation of chemicals by cytochrome P450 monooxygenases in rodent species. The importance of cytochrome P450s in the metabolic activation of a number of relatively nontoxic chemicals to cytotoxic or carcinogenic intermediates is well accepted in rodent species (for review, see Yost, 2001). A number of studies have demonstrated the importance of various cytochrome P450s in generating reactive metabolites that produce Clara cell injury in the rodent lung (for review, see Buckpitt et. al., 2002). The correlation between species- and site-selective susceptibility of naphthalene cytotoxicity and the rate of naphthalene metabolism and total protein-bound metabolites in different airway subcompartments from rodent lungs have been reported by several investigators (Buckpitt et. al., 1995, Cho et. al., 1995).

The literature shows that there is a strong link between CYP450 metabolism of naphthalene and induction of cellular injury in rodent lung. In rats, the olfactory epithelium contains the greatest amount of CYP protein of all tissues studies in this species, thus, the olfactory epithelium has high metabolic capacity. Recently, Lee et. al. (2005) evaluated the relationship between olfactory region specific injury and CYP450 metabolism in rats following acute intraperitoneal injection or 4-hour inhalation exposure of naphthalene. After exposure, microsomal incubations from the olfactory mucosa of the nasal septum (high air flow), the ethmoturbinates from both sides of the nasal passage and lined with olfactory mucosa (low air flow) and nonolfactory region of the nasal septum lined with nonolfactory mucosa (high air flow) were evaluated. This research showed that severe injury to the nasal mucosa following either intraperitoneal dosing or inhalation exposure was only seen in those areas capable of metabolizing naphthalene. Under both exposure regimens, the nasal tissue injury was cell-type specific limited to the olfactory mucosa. Following inhalation exposure only, the mucosal injury patterns were region-specific (along the medial meatus) and predominantly seen in regions of highest air flow. Mucosal injury following systemic exposure was widespread through the nasal mucosa and included the medial meatus and the ethmoturbinates. These findings lead the study authors to surmise that the acute injury from inhaled naphthalene results from *in situ* metabolism of naphthalene rather than resulting from naphthalene metabolites delivered from extranasal tissues.

In general, the P450 activities and amount of P450 protein present of both microsomal and intact airway preparations from lungs of nonhuman primates or humans are 10- to 100-fold lower than those in rodents (for review, see Hukkanen et.al., 2002). Studies with microsomal preparations derived from specific lung subcompartments from rats and rhesus monkeys have shown that P450 activities were lower for primates in all cases but that the differences were 2- to 3-fold rather than 10- to 100-fold. More importantly, it was noted that the distribution of P450 isoform activities were different between the two species (Lee et.al., 1998). Boland et. al. (2004) evaluated CYP450 metabolism and metabolite formation of naphthalene and 1-nitronaphthalene in various subcompartments of the monkey lung including the trachea, proximal, midlevel, distal airways and parenchyma. The distribution of naphthalene metabolism in the lung was relatively homogeneous with slightly higher activities seen in the parenchyma and possibly

midlevel airways. The predominant water soluble metabolite seen in all compartments was naphthalene dihydrodiol and the levels of the dihydrodiol were approximately 100-fold lower than those previously reported in rodents. A high percentage of overall naphthalene metabolites bound to proteins were seen in the monkey at 2- to 3-fold lower than rodents. The study data clearly demonstrated that there are dramatic rodent-primate differences in P450 metabolism of naphthalene and 1-nitronaphthalene and the distribution of metabolites are very different between rodents and primates. The study authors opined that "these differences (P450 metabolism and metabolite distribution) raise important questions about the use of data obtained in rodent bioassays for chemicals such as naphthalene which seem to be very weak respiratory tract carcinogens".

Extensive research has shown that there is a strong association between CYP2F expression levels and tissue susceptibility to naphthalene cytotoxicity (Buckpitt et. al., 2002). Baldwin et. al.(2004) evaluated CYP2F expression levels in pulmonary and nasal tissues from rodents and primates. Evaluations included:

> Immunoblotting of rat and mouse lung airway segments, kidney, liver and nasal tissues.
>
> Immunomapping of CYP2F in the rhesus in monkey airway segments (trachea, proximal and medial conducting airways, respiratory bronchioles and parenchyma) and nasal subcompartments (septum, maxilloturbinates, nasoturbinates, and ethmoturbinates.
>
> Electrophoresis and immunoblotting of ethmoturbinate microsomes from mice rats and monkeys.
>
> Identification of ethmoturbinate microsomal proteins by peptide mass fingerprinting.

In the monkey, immunoblot analysis failed to detect any trace of immunoreactive protein in all airway levels, the nasal and maxilloturbinates and 2 of 3 septal samples. Readily detectable and quantifiable levels of a single immunoreactive protein with a molecular mass identical to rCYP2F4 was see in protein samples from the primate ethmoturbinates only When compared to identical evaluations in rodents, the amounts of immunoreactive proteins seen in the primate ethmoturbinates were 10- and 20-fold less than that from the ethmoturbinates from rats and mice, respectively. Peptide mass fingerprinting of the ethmoturbinate microsomal proteins showed two immunoreactive bands in the mouse and rat and a single band in the monkey. For both mice and rats, the predominant P450 proteins observed in the upper band were CYP2A and CYP2G1. The lower band was found to contain CYP2F with some signal associated with CYP2A. For monkeys, the ethmoturbinate microsomes contained CYP2A with suggestion of the presence of CYP2J2 and CYP2F. The study authors concluded that the data "demonstrating no detectable CYP2F in any of the lung subcompartments tested, suggest the rhesus macaque to be refractory to naphthalene-induced pulmonary toxicity. Even though

assessment of nasal susceptibility is less clear, the magnitude of the rhesus-to-rodent differential in olfactory CYP2F expression would also suggest a lack of susceptibility for the monkey."

The literature shows that cytotoxicity in Clara cells and possibly the tumorigenic response in the mouse lung are linked to naphthalene metabolism in that tissue. Indirect evidence suggests that cytotoxicity in nasal tissues may also be related to tissue specific metabolism. The recent publications discussed above clearly demonstrate that there are distinct differences in naphthalene metabolism in pulmonary and nasal tissues between primates and rodents. Collectively, these papers show that primate lung had nasal tissue have very low metabolic activity as compared to rodents and the metabolism of naphthalene in target tissues is minimal, at best. The metabolic differences strongly suggest that primate and human lung and nasal tissues may not be susceptible naphthalene induced cytotoxicity as a result of its site specific metabolism.

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APPENDIX B

TOXICOLOGY / MECHANISM OF ACTION POTENTIAL RESEARCH PLAN

CANCER RISK ASSESSMENT

The USEPA published Cancer Risk Assessment guidelines that provide a framework for assessing nongenotoxic modes of tumorigenic action. In those guidelines, the required lines of evidence for such mechanisms are:

1. Identification of Key Events
2. Strength. Consistency and Specificity of Association
3. Genotoxicity
4. Dose Response Concordance
5. Temporal Relationships
6. Biological Plausibility and Coherence

Each of these items is explained in the guidelines. The pesticide and chemical industries have been conducting mechanistic for various classes of chemicals since the publication of the draft Cancer Risk Assessment guidelines were published in the mid 1990's. A recent evaluation is pertinent to the induction of nasal tumors upon long term naphthalene exposure and is discussed below.

CASE STUDY FOR NASAL TUMORS AND MODE OF TUMORIGENIC ACTION ASSESSMENT.

Recently, USEPA evaluated the cumulative toxicity of several chloracetanilide (CAN) pesticides. Chronic oral exposure to the CANs (acetochlor, alachlor and butachlor) produced nasal turbinate tumors in male and female rats. The CAN registrants conducted a number of studies with the various products to evaluate the underlying mechanism leading to the tumor responses

The mechanistic data for the CANs can be summarized as follows:

1. In general, the CANs were not genotoxic.
2. Radiographic studies following oral dosing showed that the CANs or their metabolites distribute and sequester in the nasal tissues.
3. CANs are metabolized to benzoquinone imines which form protein adducts in the nasal tissues leading to cell death and regenerative cell proliferation.
4. Studies in rats have shown dose-response concordance in the induction of cell proliferation in nasal tissues and nasal turbinate tumors.
5. Cell proliferation is reversible upon cessation of treatment.

Based on the above data, it was postulated that mechanism of toxicity postulated for the formation of these tumors was regenerative cell proliferation of the nasal epithelium leading to neoplasia upon sustained exposure. It was proposed that the toxicity in the nasal epithelium was due to metabolism at that site or sequestering of toxic metabolites by the nasal tissues. USEPA agreed with this mechanism in its assessment of the CANs.

NAPHTHALENE

The Cancer Risk Assessment guidelines and mechanistic studies on the CANS provide a basis to evaluate available mechanism-related naphthalene data and to assess research priorities to further evaluate the mode of tumorigenic action.

Naphthalene has been evaluated in a large number of mutagenicity studies both *in vitro* and *in vivo*. The weight-of-evidence for these studies clearly shows that naphthalene is not genotoxic. The lack of genotoxicity is the key hurdle for moving forward with evaluation of a nongenotoxic mechanism of tumorigenicity.

The NTP chronic bioassay in rats with naphthalene clearly showed that the nasal epithelium was the primary target organ for naphthalene toxicity. Nasal toxicity was characterized by cell death and regenerative cell proliferation. An oral-dosed study also demonstrated toxicity in nasal tissues. Metabolism studies in rats show that significant naphthalene metabolism occurs in rat olfactory tissues and the metabolite profile has been elucidated. The induction of nasal lesions following oral administration coupled with metabolism in nasal tissues suggests that nasal toxicity may be related to sequestering and direct toxicity in nasal tissues or *in situ* metabolism to a toxic metabolite in this tissue. However, a relationship between metabolism and cellular interactions resulting in toxicity has not been demonstrated. This relationship can be evaluated by the following:

1. Evaluation of alterations in gene expression in the nasal epithelia and airways of mice and rats following single and multiple exposures to naphthalene via inhalation. A marker of oxidative DNA injury, *e.g.*, 8-hydroxy-2-guanosine would be evaluated in target and nontarget tissues of rats and mice and see if this correlates with the sites of tumorigenesis. In the same studies, alterations in the transcriptome could be assessed to see if there are changes related to target tissue responses, i.e., are DNA repair genes down regulated in the target tissues but not in the nontarget tissues. Determine if there are differences that relate to whether the tissue is a target tissue for the species by evaluation of the gene array pattern. Determine the NOAEL/LOAEL for findings and compare to the exposure levels inducing pathological changes/tumors. If divergences are seen between rats and mice, the research may be expanded to primates as a surrogate for humans.

2. Evaluate metabolite binding to cellular proteins. If reactive metabolites such as quinones bind proteins in target cells and parent naphthalene does not, then the relationship between tissue specific metabolism, toxicity and tumorigenicity would be supported.

The test concentrations used in the NTP study were based on the highest concentration that could be generated and dilutions of that concentration. Thus, three relatively high concentrations of naphthalene were tested. As a result of these high concentrations, nasal toxicity was seen at all exposure concentrations in the NTP rat inhalation study. The lack of a no observed effect concentration (NOEC) is critical problem as a linkage between

"key events" leading to tumorigenicity and the tumorigenic response can not be determined. Further, demonstration that the effects are threshold-based is a key component of tumor mechanistic evaluation and can only be determined when a clear NOEC has been shown. The NTP study does not permit assessment of dose response concordance for nonneoplastic findings (key events) that lead to tumorigenic response.

Assessment of key events and dose concordance requires the conduct of additional *in vivo* studies. The primary studies would be inhalation studies in rats evaluating several parameters. It may be necessary to conduct several studies of varying durations to get enough information to submit credible data for mode of tumorigenic action. The basic approach would be conducting a acute inhalation studies to establish appropriate exposure parameters and concentrations for longer term studies. Short term (8-13 week) inhalation studies would be conducted to:

1. Evaluate dose response relationships and time course for inducing histologic changes in the nasal epithelia and identification of a No Observed Effect Concentration (NOEC). A NOEC is essential is establishing dose concordance of effects.

   This evaluation would require sacrifice of animals at various intervals during the study with complete histopathologic evaluation (cytoxicity, hyperplasia, metaplasia, hypertrophy) of the nasal epithelia.

2. Evaluate cell proliferation in the nasal epithelia.

   This would be done by either special stains or by BrdU perfusion and staining. It will be important to evaluate the time course of cell proliferation to see if the response is early or short term as compared to continuous.

3. Evaluate reversibility of histologic changes and cell proliferation.

   This is achieved by inclusion of recovery group animals that would be sacrificed at various period following exposure.

Collectively, the above studies should provide sufficient information for establishing a credible basis for the mode of tumorigenic action for naphthalene in rat nasal tissues.