Naphthalene

5 Year Research Program
(2007 – 2011)
First Year Results & Plan for the Next Four Years

March 31, 2008

Research Program Funded by Participants in the Naphthalene Coalition:
Association of American Railroads
American Petroleum Institute
Asphalt Institute
Electric Power Research Institute
Naphthalene Council
Utilities Solid Waste Activities Group

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5 Year Research Program
(2007 – 2011)

INTRODUCTION

A consortium of associations representing diverse industries is funding a five-year research program to further understanding of the potential human cancer effects that could be associated with exposure to naphthalene to better inform risk assessment for naphthalene. Industries supporting the research program include naphthalene manufacturers, petroleum refiners and marketers and industries that rely on organic fuels (including petroleum, coal and biomass based fuels).

The US National Toxicology Program conducted two-year bioassay studies of naphthalene inhaled by mice (NTP 1992) and by rats (NTP 2000). The results of these studies have been reviewed in the context of many other *in vitro* and *in vivo* studies of naphthalene exposure in experimental animals as well as the context of the absence of evidence of carcinogenicity of naphthalene in humans by government sponsored scientific review bodies that have reached different conclusions. The United Nation's International Agency for Research on Cancer (IARC 2002) concluded that results of NTP's mouse study (NTP 1992) are not relevant for human risk assessment, that naphthalene is not genotoxic, and that the relevance of NTP's rat study (NTP 2000) is of uncertain relevance for human risk assessment. In its naphthalene risk assessment, the European Union reached similar conclusions (EU 2003). Thus, both IARC and the EU recognize naphthalene as carcinogenic in experimental animals with uncertain relevance to human carcinogenicity. These same NTP data have, however, been used in attempts to develop quantitative risk assessments (e.g., California (2005) and a withdrawn EPA draft assessment) that extrapolate the high-dose effects in observed in laboratory rats and mice to human cancer risk using multiple default assumptions, resulting in a cancer risk calculated to be 10 to 40 greater than that estimated for benzene.

Efforts to quantitate potential naphthalene risks require multiple default assumptions that additional research may be able to refine. Thus, interested industry groups joined together in a coalition to identify research relevant to evaluating the potential carcinogenicity to humans of exposure to naphthalene and to fund a research program to carry out the identified research. This document contains an outline of research to be conducted over a five year span beginning in 2007 and continuing through 2011. Documents describing the development and implementation of the research program are included here as follows:

- Some results of sponsored research have already been made public through abstracts included here, presented at the American College of Toxicology 2007 annual meeting (November 2007) or to be presented at the upcoming Society of Toxicology 2008 annual meeting.
- The US Environmental Protection Agency's Office of Pesticide Programs recently asked Dr. Harvey Clewell to present results of his Physiologically-based Pharmacokinetic

Model (PBPK) for naphthalene. Dr. Clewell's presentation slides are included in this document.

- The research program was developed using input of scientists who participated in the independently conducted Naphthalene State-of-the-Science Symposium (NS^3), which was organized and implemented by an independent not-for-profit group (Regulatory Checkbook) and jointly funded by the US Environmental Protection Agency and the Naphthalene Coalition. NS^3 deliberations are detailed in six papers about to be published in a refereed journal, all of which are included here.
- In 2004, the Naphthalene Coalition commissioned Dr. Byron Butterworth to conduct an independent review of the scientific literature concerning the genotoxicity of naphthalene. Dr. Butterworth's expert opinion is included in this document

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Naphthalene Research Program: 5 Year Plan

ITEM			2007	2008	2009	2010	2011
Laboratory/Computation Toxicology Studies	**Investigator(s)**	**Facility**					
Parameters for Naphthalene-specific metabolism factors	A. Buckpitt	UC Davis					
Upper Respiratory Tract Uptake of Naphthalene	J.B. Morris	Univ. Connecticut	Rat	Mouse			
PBPK Model	H. Clewell	Hamner/CIIT					
P450 Cross-species modeling	D. Lewis	Univ. Surrey (UK)					
1-day (acute) dose-response study	D. Dodd						
5-Day Dose-Response Study	D. Dodd	CIIT	Exposures	Pathology			
Quality Assurance/Quality Control	(J. Baldwin)	--					
Ongoing Uncertainty Analysis	(L. Rhomberg)						
Annual Research Planning Meeting	as needed	--		june 24-25			
Mouse lung MOA colony support (program support by SIRC)	X. Ding	SUNY Albany					
Analysis of uninary metabolites in 5 day study	TBD	TBD					
90-day Dose-response Study - F344 Rat	TBD	TBD					
Metabolism: nasal effects vs. metabolic differences at different doses	TBD	TBD					
Primate studies: nasal lesion induction	A. Buckpitt	UC Davis					
AF study blood & urine samples	TBD	TBD					
Quinone-specific/epoxide studies	TBD	TBD					
Glutathione studies	TBD	TBD					
Metabolism: follow-on metabolic mechanistic studies (if warranted by results of previous study)	TBD	TBD					
Primate dose-response studies (if indicated by results of previous study)	A. Buckpitt	UC Davis					
Human ADME studies	TBD	TBD					

Color Key:

- Research initiated; abstract available &/or manuscript in preparation
- Study design in place; project funding expected to commence in 2008
- Study design to be developed; year indicates projected start of funding
- Research program support

Nasal Epithelial Lesions in F344 and SD Rats Following Acute Exposure to Naphthalene Vapor

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Introduction

Chronic inhalation exposure to naphthalene (NP) vapor at 10 ppm (the lowest concentration studied) resulted in respiratory adenomas of the nasal epithelium of male rats, olfactory epithelial neuroblastomas in female rats, and additional non-neoplastic nasal lesions (NTP, 2000). Nasal cavity metabolism of NP (via P450-dependent monooxygenases) appears to be an important factor in cytotoxicity and location of NP-induced injury (Lee et al., 2005). Strain differences in nasal cavity P450 expression may impact potential differences in NP-induced low dose effects in the nasal cavity. The purpose of this study was to establish an exposure-response relationship and threshold for nasal epithelial effects in male and female F344 and Sprague Dawley (SD) rats following an acute (single six hour) inhalation exposure to 0 (control), 0.1, 0.3, 1, 10, and 30 ppm NP.

Methods

Inhalation Exposures:

- Naphthalene vapors: heat solid naphthalene, vapor carried by nitrogen gas into exposure chamber inlet.
- Small generators used for 0.1, 0.3, and 1 ppm exposure chambers.
- Small generators placed in oven to control temperature.
- Large generation system used for 10 and 30 ppm exposure chambers.
- Large glass system wrapped with heat tape to sublimate the naphthalene.
- Concentrations in the chambers controlled by adjusting heating temperature and nitrogen flow rate through generators.

Naphthalene Concentration Measurements:

- Naphthalene concentration measured using a gas chromatograph (GC, Model 5890 Series II, Hewlett Packard, Palo Alto, CA).
- Fused silica film, 15 m x 0.53 mm id capillary column.
- Flame ionization detector (FID).

Exposures

Single whole body exposure, 6 hours

	Target	Male Rat exposure	Female Rat Exposure
	ppm	ppm	ppm
Exposure Chamber	0.0	0.0	0.0
	0.1	0.09 ± 0.03[1]	0.10 ± 0.02[1]
	0.3	0.30 ± 0.05	0.28 ± 0.04
	1.0	1.10 ± 0.09	1.07 ± 0.14
	10.0	11.6 ± 1.2	12.3 ± 1.4
	30.0	28.5 ± 5.2	30.7 ± 6.7

[1]mean ± standard deviation

Histopathology

- The day after the exposure rats were anesthetized with sodium pentobarbital and killed by exsanguination.
- Noses were fixed in 10% neutral buffered formalin, decalcified, trimmed into six standard sections, and processed to hematoxylin and eosin stained slides for histological evaluation.

Results

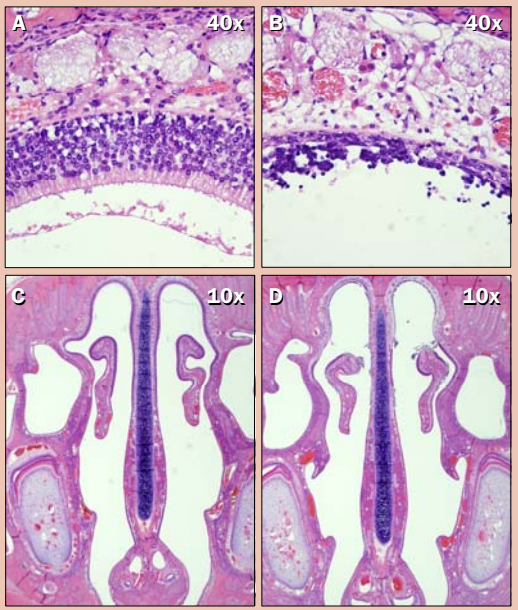
Pathology Incidence Table, Female SD Rat

	Control	100 ppb	300ppb	1ppm	10ppm	30ppm
Nose, Tip						
Necrosis, Respiratory Epithelium (RE)	0	0	0	0	2	2
Nose, Level I						
Necrosis (RE)	0	0	0	0	5	5
Nose, Level II						
Foreign Body Granuloma	0	0	0	0	0	0
Necrosis, Olfactory Epithelium (OE)	0	0	0	0	5	5
Nose, Level III						
Necrosis (OE)	1	1	2	4	5	5
Nose, Level IV						
Necrosis (OE)	0	0	0	0	1	5
Nose, Level V						
Necrosis (OE)	0	0	0	0	0	0

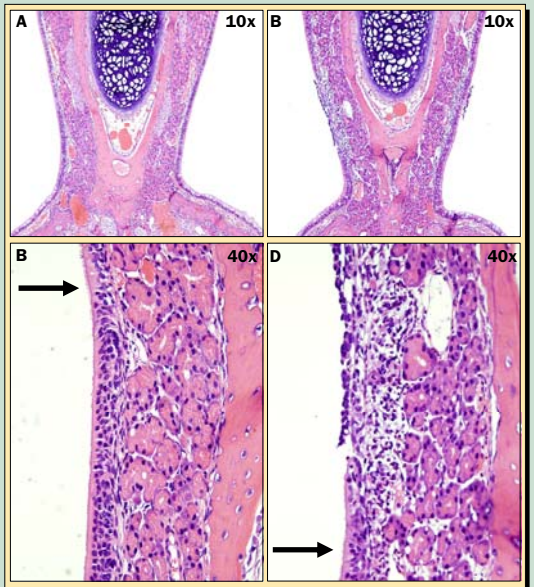
Pathology Incidence Table, Male SD Rat

	Control	100 ppb	300ppb	1ppm	10ppm	30ppm
Nose, Tip						
Necrosis (RE)	0	0	0	0	0	0
Nose, Level I						
Necrosis (RE)	0	0	0	1	5	5
Nose, Level II						
Foreign Body Granuloma	0	1	1	0	0	0
Necrosis (OE)	0	0	0	1	5	5
Nose, Level III						
Necrosis (OE)	0	2	3	4	5	5
Nose, Level IV						
Necrosis (OE)	0	0	1	0	5	5
Nose, Level V						
Necrosis (OE)	0	0	0	0	1	1

n=5



Olfactory epithelium of the dorsal meatus and ethmoid turbinates (Level III) of SD male rats to show control (A & C) and 30 ppm naphthalene treated (B & D). Note that the olfactory necrosis is bilateral (D) and moderately severe.



Photomicrographs of male F344 rat septal olfactory organ (level III) to show control (A & C) and 10 ppm naphthalene treated (B & D) tissues. Note the normal respiratory epithelium (arrow) above and below the olfactory patch in both treated and control. The olfactory necrosis is moderate.

Pathology Incidence Table, Female F344 Rat

	Control	100 ppb	300ppb	1ppm	10ppm	30ppm
Nose, Tip						
Necrosis (RE)	0	0	0	0	5	5
Nose, Level I						*
Inflammation (RE)	0	0	0	0	0	0
Necrosis (RE)	0	0	0	0	5	4
Nose, Level II						
Necrosis (OE)	0	0	0	0	5	5
Necrosis (RE)	0	0	0	0	2	0
Nose, Level III						
Necrosis (OE)	0	0	0	4	5	5
Nose, Level IV						
Necrosis (OE)	0	0	0	0	0	3
Nose, Level V						
Necrosis (OE)	0	0	0	0	0	0

Pathology Incidence Table, Male F344 Rat

	Control	100 ppb	300ppb	1ppm	10ppm	30ppm
Nose, Tip						
Necrosis (RE)	0	0	0	0	1	1
Nose, Level I						
Inflammation (RE)	2	1	0	0	0	0
Necrosis (RE)	0	0	0	0	5	5
Nose, Level II						
Necrosis (OE)	0	0	0	0	5	5
Necrosis (RE)	0	0	0	0	0	0
Nose, Level III						
Necrosis (OE)	0	0	0	5	5	5
Nose, Level IV						
Necrosis (OE)	0	0	0	0	4	5
Nose, Level V						
Necrosis (OE)	0	0	0	0	0	0

n=5 (*, n=4)

Conclusions

- Lesions related to exposure to NP were observed in some of the respiratory epithelial locations but were seen predominately as necrosis in the olfactory epithelium.
- Severity of lesions was exposure concentration dependent and ranged from minimal to marked.
- The necrosis was characterized by cytoplasmic vacuolation, loss of proper epithelial orientation, condensation of the cytoplasm, pyknotic and karyorrhectic nuclei, and sloughing of the necrotic epithelium.
- Female SD rats exposed to 10 and 30 ppm have lesions roughly similar in severity, incidence and distribution to male SD rats, and male and female F344 rats.
- Clear minimal effects of exposure to NP were observed in all rats at 1ppm.
- While clear NP-induced necrosis was seen at 1 ppm in most rats, the severity was minimal.
- The difference in response between the two rat strains may be due to a difference in the level of P450 (and their isoforms) located in the olfactory and respiratory epithelium at the various nasal levels.

Future Directions

- A five day study with recovery groups is underway to address the further interpretation of this lesion.
- For this study, lesion mapping of 5 day treated and 5 day treated followed by a 14 day recovery will be conducted.

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Acknowledgements

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THE HAMNER INSTITUTES FOR HEALTH SCIENCES
WHERE GREAT MINDS & MEDICINE MEET

Nasal Uptake of Naphthalene in the F344 Rat

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TOXICOLOGY PROGRAM, UNIVERSITY OF CONNECTICUT, STORRS, CT AND DEPT MOLEC BIOSCI, UCDAVIS

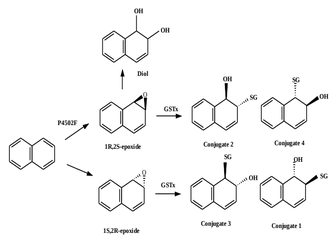
ABSTRACT

Naphthalene is a nasal toxicant and carcinogen in the rat. Information on inhalation dosimetry is needed to support development of a quantitative inhalation risk assessment for this compound. In the current study, upper respiratory tract (URT) uptake of naphthalene was measured in the male and female F344 rat at exposure concentrations of 3, 10 or 30 ppm under constant velocity inspiratory flow conditions at flow rates of 150 or 300 ml/min. To assess the potential importance of nasal CYP metabolism of naphthalene, groups of rats were pretreated with the CYP inhibitor 5-phenyl-1-pentyne (100 mg/kg, ip). In vitro metabolism of naphthalene was reduced by over 80% by the inhibitor. Nasal uptake of naphthalene in female rats was concentration dependent with uptake efficiencies (flow 150 ml/min) of 40, 34 and 28% being observed at inspired concentrations of 3, 10 and 30 ppm, respectively. A similar effect was observed in male rats (flow 150 ml/min) with uptake efficiencies of 53, 38 and 42% being observed at 3, 10 and 30 ppm, respectively. Uptake was more efficient in the male than female rat, likely do the larger size (220 g vs 150 g) of the male rats used in the study. Pretreatment with the inhibitor did not alter URT uptake of a non-metabolized vapor (acetone). In contrast, uptake of naphthalene was significantly reduced by pretreatment with the effect being greater at the lower inspired concentrations. Specifically, in pretreated female rats (150 ml/min) URT uptake averaged 25, 29 and 26%, at inspired concentrations of 3, 10 and 30 ppm, respectively. Thus, the concentration dependence of uptake was abolished by 5-phenyl-1-pentyne pretreatment. These results provide evidence that nasal CYP metabolism of naphthalene contributes to nasal scrubbing of this vapor and is also involved in the concentration dependence of uptake that is observed.

INTRODUCTION

Naphthalene is a nasal cytotoxicant and carcinogen in the rodent. Chronic (2 yr) exposure to naphthalene at concentrations of 10 , 30 or 60 ppm results in marked inflammation and cytotoxicity in both the respiratory and olfactory epithelium of the male and female rat. Increased incidence of respiratory ademonas was observed in the male rats; in female rats an increased incidence of repiratory adenomas was observed as well as an increased incidence of olfactory neuroblastomas (Long et al., 2003), The mechanisms responsible for the gender difference are not known.

Shown below is the metabolic pathways for naphthalene. The first step is thought to be catalyzed in the rodent nose by CYP2F2. Metabolic activation is critical to naphthalene' toxicity (Buckpitt et al., 2002; Genter et al., 2006; Bogen et al., 2008) but the contribution of each metabolite to the cytotoxicity and/or the gender difference is not fully understood. A suicide inhibitor of this CYP (5-phenyl-1-pentyne) was used in this study to examine the importance of metabolism in nasal dosimetry.



Knowledge of inhalation dosimetry is integral to quantitative risk assessment of airborne materials. The current experiments were designed to define to role of nasal metabolism in nasal dosimetry of naphthalene. Specifically these experiments were designed to:

1) Characterize naphthalene metabolism in the male and female rat and the effect of 5-PP on same.

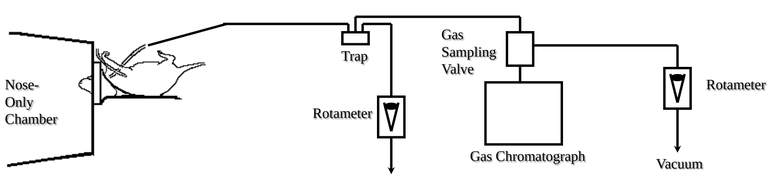
2) Measure nasal naphthalene uptake in control and PP-treated rats of both genders.

Such data will define the contribution of local metabolism to inhalation dosimetry and provide insights on potential mechanisms for the gender difference in induction of olfactory neuroblastomas by inspired naphthalene.

MATERIALS AND METHODS

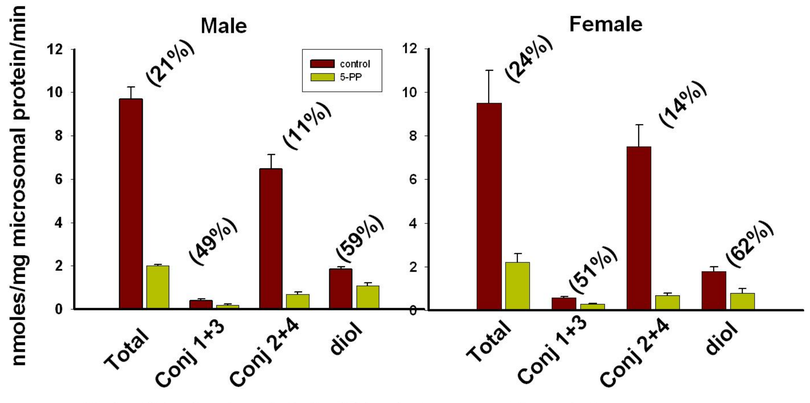
In Vitro Studies:
Olfactory and respiratory mucosa were obtained from control or 5-phenyl-1-pentyne pretreated rats (100 mg/kg, ip) and microsomes prepared. Microsomes were incubated with 0.25 mM naphthalene in the presence of optimal amounts of glutathione (as a metabolite trap) and the formation of metabolites determined by hplc (Shultz et al, 1999) .

In vivo studies:
Uptake of inspired naphthalene was measured in the upper respiratory tract (URT) of male and female rats by established techniques (Morris, 1999). This entailed isolation of the URT by insertion of an endotracheal tube in urethane-anesthetized animals and placement of the animal in a nose-only exposure chamber (see schematic). Concentration of naphthalene in air entering and exiting the isolated URT was determined by gas chromatography. Uptake efficiency was calculated from these data and was measured in control and 5-phenyl-1-pentyne pretreated rats (100 mg/kg ip) at inspiratory flow rates of 150 and 300 ml/min.



RESULTS

IP Administration of 100 mg/kg 5-Phenyl-1-Pentyne Decreases Nasal Olfactory Microsomal Metabolism of Naphthalene in Male and Female Rats

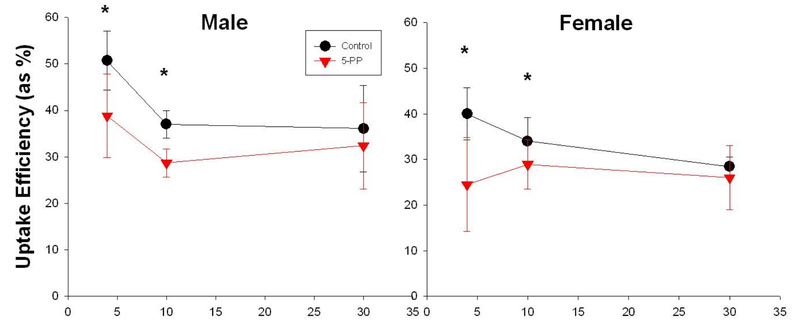


(Note: (%) refers to activity in PP treated compared to control
Conj 1+3 derived from 1S,2R-oxide; Conj 2+4 derived from 1R,2S-oxide)

--5-PP inhibits nasal metabolism of naphthalene by ~80%
--Inhibition is likely isoform selective (based on the formation of rates if 1R,2S vs 1S,2R oxides)
--No significant differences apparent between male and female rat olfactory mucosa
--Identical patterns were observed in respiratory mucosa but total activity was 4-fold lower than in olfactory mucosa

RESULTS, CONT

IP Administration of 100 mg/kg 5-Phenyl-1-Pentyne Decreases Uptake Efficiency of Inspired Naphthalene in the Male and Female Rat URT



(Note: * Indicates uptake efficiency significantly lower in 5-PP compared to control rats
Uptake measured at 150 ml/min, similar results were obtained at 300 ml/min)

--The same patterns were observed in male and female rats
--Uptake was concentration dependent in control rats with lower uptake efficiencies being observed at the higher concentrations.
--The concentration dependence was absent 5-PP treated rats suggesting saturation and/or capacity limitation of naphthalene metabolism at the higher concentrations was responsible for this phenomenon.

CONCLUSION

Naphthalene is scrubbed with moderate efficiency in the URT. Metabolism contributes to the uptake process, therefore, species and high-to-low dose extrapolations would most properly include consideration of appropriate metabolic parameters.

Nasal dosimetry and metabolism appear similar in both male and female rats as evidenced by similar metabolic and URT uptake profiles suggesting pharmacokinetic differences are unlikely to account for the gender differences in nasal toxicity.

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Assessing Nasal Tissue Dosimetry of Naphthalene with a Hybrid CFD-PBPK Model

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[3]Pharmaceutical Sciences, University of Connecticut, Storrs, CT, USA.

Abstract

Naphthalene is an important intermediate in chemical synthesis of phthalic anhydrides, carbamate insecticides, resins and tanning agents; it is a component of petroleum fuels and is used as a moth repellent. Chronic inhalation of naphthalene leads to nasal inflammation, hyperplasia of the respiratory epithelium and metaplasia of the olfactory epithelium in rats at concentrations >10 ppm, the lowest concentration tested. Increased incidences of adenoma in respiratory epithelium (male rat) and neuroblastoma in the olfactory epithelium (female rat) were also noted. Differences between the rodent and human nose in anatomy and biochemistry are important considerations in conducting interspecies extrapolations for a naphthalene risk assessment. As a first step, a physiologically-based pharmacokinetic model was constructed to assess the localized uptake and metabolism of naphthalene in the nasal respiratory and olfactory epithelium. Metabolic constants were calibrated to upper respiratory tract extraction data for naphthalene. Vmax for nasal oxidation was estimated to be 34.4 nmol/min/mL with a Km of 3.0 μM. Steady state naphthalene concentrations were predicted to be 266, 796, 2403 μM in the rat olfactory epithelium and 286, 850, 2557 μM in the rat ventral respiratory epithelium at the cytotoxic (3.4 ppm), and the cytotoxic/tumorigenic exposure concentrations of 10 and 30 ppm, respectively. At 3 ppm and 150 ml/min, the percent extraction for naphthalene in the upper respiratory tract is ≈ 40%. In the absence of naphthalene metabolism, the model predicts that the percent extraction in the URT is reduced to ≈ 23 %. In contrast, the difference in extraction with and without naphthalene metabolism is predicted to be less than 2% at 30 ppm. This model will be extended to the human to provide the basis for rodent to human extrapolation to assess risk from naphthalene exposure.

Introduction

Naphthalene is a component in petroleum-based fuels, and is the active ingredient in moth balls. Exposure to naphthalene vapor results in toxicity in the lungs of mice and in the nasal epithelium of both mice and rats (NTP 1992, 2000). The toxicity of naphthalene appears to result from the production of reactive metabolites by oxidative metabolism (Warren et al. 1982; Buckpitt et al. 2002), in particular by CYP2F isoforms (Baldwin et al., 2004; 2005). Human risk assessments for inhaled naphthalene that are based on its nasal toxicity in rodents require the calculation of a Human Equivalent Concentration (HEC); that is, the continuous exposure concentration in the human that would produce a tissue exposure at the site of toxicity equivalent to that at the NOAEL or LOAEL in the animal. In the absence of a chemical-specific dosimetry model, the HEC can be calculated using the default cross-speices dosimetry approaches described in the EPA's RfC dosimetry guidelines (USEPA 1994). For an extrathoracic effect such as the nasal toxicity of naphthalene, the category 1 (reactive) gas default would apply. Typical values of the Regional Gas Dose Ratio (RGDR) obtained from this default calculation when comparing rat and human are on the order of 0.2. That is, the HEC would be roughly 5-fold lower than the time-weighted average animal exposure concentration. However, recent developments in the area of computational fluid dynamic (CFD) and physiologically based pharmacokinetic (PBPK) modeling of the nose have made it possible to provide chemical-specific RGDRs for many category 1 gases (Morris et al. 1993; Kimbell et al. 1997; Plowchalk et al. 1997; Frederick et al. 1998). In some cases, chemical-specific analyses for category 1 gases have resulted in RGDRs closer to unity than the default approach (Andersen et al. 1999). The model described here was developed to provide chemical-specific dosimetry in support of risk assessments for naphthalene based on its nasal toxicity in the rat.

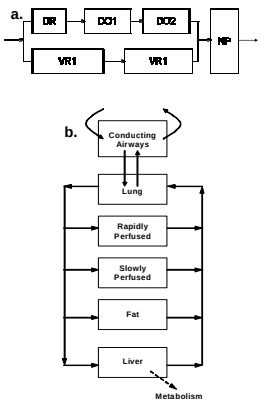

Figure 1. CFD (a) and PBPK (b) model structures.

Methods

Table 1. Physiological parameters for rat and human.

Parameter	Rat	Human	Source
Flows Parameters			
Cardiac Output (ml/min/g$^{0.75}$)	1.756	1.208	(Brown *et al.* 1997)
Nasal Cavity Blood Flow (% CO)	0.15	0.25	(Rat: Teegarden *et al.* 2007, Morris *et al.* 1993; Human: Holmberg 1989, Paulsson *et al.* 1985)
Minute Ventilation (ml/min/g$^{0.75}$)	1.909	1.743	(Brown *et al.* 1997)
Tissue Blood Flows (% CO)			
Liver	25	23	(Brown *et al.* 1997)
Fat	7	6	(Brown *et al.* 1997)
Poorly Perfused	100-sum of other	100-sum of other	
Rapidly Perfused	50	44	(Brown *et al.* 1997)
Tissue Volumes (% BW)			
Liver	3.7	2.57	(Brown *et al.* 1997)
Fat	6.5	13.6	(Brown *et al.* 1997)
Poorly Perfused	69	55	(Brown *et al.* 1997)
Rapidly Perfused	91-sum of other	91-sum of other	
Partition Coefficient			
Blood/Air	571	571	(NTP, 2000; Morris, personal communication)
Tissue/Blood	4	4	(NTP, 2000)
Liver/Blood	7	7	(NTP, 2000)
Fat/Blood	160	160	(NTP, 2000)
Richly Perfused/Blood	4	4	Set to Tissue/Blood
Poorly Perfused/Blood	4	4	Set to Tissue/Blood

- Based on the inhalation-route vinyl acetate and acetaldehyde models (Bogdanffy *et al.* 1999a; Teegarden *et al.* 2007)
- Nasal tissue divided into 3 layered structure (lumen, an epithelial cell layer, and a submucosal tissue layer).
- Human model reduces to a single dorsal olfactory compartment due to limited size.
- Nasal compartment Vmax initially fit to experimental nasal uptake data (Morris, personal communication), the rate for the DR and DO was set to twice the value and the VR was set to half the value to obtain a relative capacity ratio on the order of 4:1 for the two tissues (Buckpitt et al., 1992).
- HECs evaluated for critical studies for short-, intermediate- and long-term exposure using the respective points of departure (POD, NOAEL or LOAEL) along with uncertainty factors.
- Two approaches were taken for HEC estimation: 1) Simulate rodent study POD (6 h/day, 5 days/wk) and then estimating continuous exposure tissue conc. at steady state for human (without nasal metabolism); 2) Simulate rodent study and then apply uncertainty factors to tissue concentration before estimating HEC.
- Human nasal metabolism was set to zero for both approaches (conservative as metabolism would reduce tissue concentration in human and increase the HEC).

Table 2. Model parameters for nasal compartments.

Parameter	Rat	Human	Source
Tissue Surface Areas (cm^2)			
Dorsal Respiratory	0.2	10.1	(Bogdanffy *et al.* 1999)
Anterior Dorsal Olfactory	0.42	13.2	(Bogdanffy *et al.* 1999)
Posterior Dorsal Olfactory	6.33	-	(Bogdanffy *et al.* 1999)
Anterior Ventral Respiratory	1.8	42.1	(Bogdanffy *et al.* 1999)
Posterior Ventral Respiratory	4.5	72.3	(Bogdanffy *et al.* 1999)
Mucus Thickness (cm)	0.001	0.001	(Plowchalk et al. 1997)
Epithelial Tissue Thickness (cm)			
Dorsal Respiratory	0.005	0.005	(Bogdanffy *et al.* 1999)
Anterior Dorsal Olfactory	0.005	-	(Bogdanffy *et al.* 1999)
Posterior Dorsal Olfactory	0.005	0.004	(Bogdanffy *et al.* 1999)
Anterior Ventral Respiratory	0.005	0.005	(Bogdanffy *et al.* 1999)
Posterior Ventral Respiratory	0.005	0.005	(Bogdanffy *et al.* 1999)
Submucosa Thickness (cm)			
Dorsal Respiratory	0.002	0.002	(Plowchalk *et al.* 1997)
Anterior Dorsal Olfactory	0.002	-	(Plowchalk *et al.* 1997)
Posterior Dorsal Olfactory	0.002	0.002	(Plowchalk *et al.* 1997)
Anterior Ventral Respiratory	0.002	0.002	(Plowchalk *et al.* 1997)
Posterior Ventral Respiratory	0.002	0.002	(Plowchalk *et al.* 1997)
Lumen Volumes (cm^3)			
Dorsal Respiratory	0.004	0.74	(Bogdanffy *et al.* 1999)
Anterior Dorsal Olfactory	0.012	0.56	(Bogdanffy *et al.* 1999)
Posterior Dorsal Olfactory	0.054	-	(Bogdanffy *et al.* 1999)
Anterior Ventral Respiratory	0.09	3.5	(Bogdanffy *et al.* 1999)
Posterior Ventral Respiratory	0.09	5.16	(Bogdanffy *et al.* 1999)
Air-phase Mass Transfer Coefficients (cm/min)			
Dorsal Respiratory	1864	180	(Frederick *et al.* 1998)
Anterior Dorsal Olfactory	1637	821	(Frederick *et al.* 1998)
Posterior Dorsal Olfactory	1908	-	(Frederick *et al.* 1998)
Anterior Ventral Respiratory	1941	147	(Frederick *et al.* 1998)
Posterior Ventral Respiratory	2033	177	(Frederick *et al.* 1998)
Diffusivity Constant (cm^2/min)	0.00045	0.00045	(USEPA 1996)

Table 3. Metabolic parameters for naphthalene.

Parameter	Value	Source
Vmax (nmol/min/mg protein)		
Liver	16.5	(Willems *et al.*, 2001)
Lung	16.5	(Willems *et al.*, 2001)
Dorsal Respiratory	34.4	(Buckpitt, personal comm.)
Dorsal Olfactory	34.4	(Buckpitt, personal comm.)
Ventral Respiratory	8.6	(Buckpitt, personal comm.)
Km (nmol/ml)		
Liver	6	(Willems *et al.*, 2001)
Lung	6	(Willems *et al.*, 2001)
Dorsal Respiratory	3	(Buckpitt *et al.*, 2002)
Dorsal Olfactory	3	(Buckpitt *et al.*, 2002)
Ventral Respiratory	3	(Buckpitt *et al.*, 2002)

Results

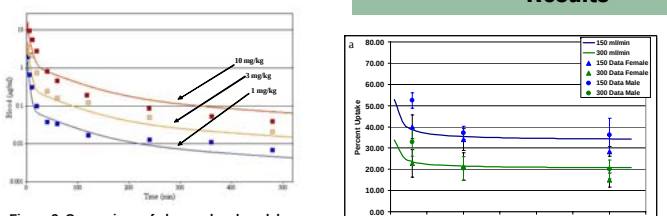

Figure 2. Comparison of observed and model prediction of rat blood time-course concentration after a single iv dose of naphthalene (data from Willems *et al.* 2001).

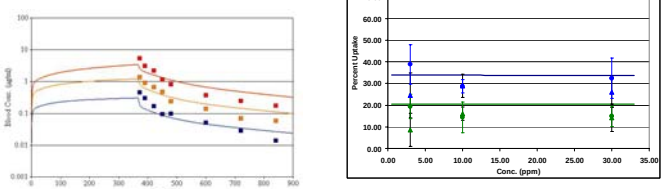

Figure 3. Comparison of observed and model prediction of rat blood time-course concentration after a single 6 hour inhalation exposure to naphthalene (data from Willems *et al.* 2001).

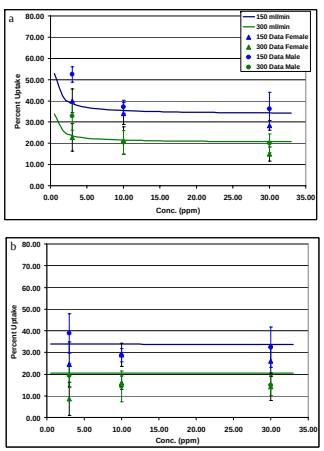

Figure 4. Comparison of the observed and model predicted extraction of naphthalene in the rat upper respiratory tract. Panel a shows the comparison for naïve rats and panel b shows the comparison for rats pretreated with 5-phenyl pentene to assess uptake without metabolism (Data from John Morris, personal communication).

Table 4. Model predicted human equivalent concentration (HEC, unadjusted) for naphthalene at the points of departure for the rat. The HEC was estimated as a continuous human exposure concentration resulting in equal tissue concentrations of the average for the rodent exposure studies (6 hous/day, 5 days/week).

Exposure concentration for Critical Endpoint (ppm)		Predicted Steady State Tissue Concentration in Rodent (nmol/ml)		Model Predicted Human Continuous Exposure (ppm)	
Chamber	Time Weighted	Proximal DO	Proximal VR	Dorsal Olfactory	Ventral Respiratory
3.0	0.54	38.3	44.0	0.587	0.592
1.0	0.18	11.6	14.5	0.178	0.198
10.0	1.8	132	147	2.027	2.007

Table 5. Model predicted human equivalent concentration (adjusted) for naphthalene at the points of departure. The predicted nasal tissue concentration for the rat was adjusted by the uncertainty factor prior simulations for human and the HEC was estimated as a continuous human exposure concentration resulting in equal tissue concentrations on average for the rodent exposure studies (6 hous/day, 5 days/week).

Exposure Concentration for Critical Endpoint (ppm)		Uncertainty Factor	Tissue Concentration after Safety Factor Adjustment (nmol/ml)		Model Predicted Human Exposure (ppm)	
Chamber	Time Weighted		Proximal DO	Proximal VR	Dorsal Olfactory	Ventral Respiratory
3.0	0.54	30	0.733	1.36	0.0112	0.0183
1.0	0.18	30	0.225	0.44	0.00329	0.00593
10.0	1.8	300	0.215	0.44	0.00329	0.00593

Conclusions

- Incorporation of CFD to a previously published PBPK model for naphthalene was able to describe the blood time-course for both iv bolus and inhalation exposures to naphthalene.
- Model predicted uptake of naphthalene in the upper respiratory tract was within 1 SD of nearly all concentration/flow rate combinations.
- The adjusted short duration HEC was estimated to be 0.011 ppm for dorsal olfactory and 0.018 ppm for ventral respiratory
- The adjusted HECs for the intermediate and long duration were identical with 0.00329 ppm for the dorsal olfactory and 0.00593 ppm for the ventral respiratory.
- The RGDRs obtained with the model were slightly greater than one. This is roughly a factor of 5 higher (less conservative) than RGDRs obtained by USEPA/OPP using the default approach for a category 1 gas (≈ 0.2).

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Nasal Epithelial Lesions in Rats Following an Acute Inhalation Exposure to Naphthalene Vapor at Low Concentrations

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Abstract

Inhalation exposures of F344 and SD rats to naphthalene (NP) vapor have resulted in nasal lesions. In order to examine the differences in NP-induced effects in the nasal cavity between F344 and SD strains, and to study the exposure-response relationships for nasal epithelial effects at low NP concentrations, a system to generate and characterize NP vapor at 30, 10, 1, 0.3, and 0.1 ppm was developed and tested. NP vapor was generated by diluting sublimed solid NP into the exposure chamber air supply. NP concentration was measured by gas chromatography. Aerosol testing was performed to verify that solid naphthalene particles (potentially formed by the condensation of naphthalene) were not present. The next day after a single 6-hour whole body exposure, groups of male and female F344 and SD rats were killed and nasal tissues were taken for histopathological analysis. Respiratory and olfactory necrosis was observed at the 10 and 30 ppm exposure concentrations in male and female, F344 and SD rats. Olfactory epithelium necrosis was observed in SD and F344 rats at 1 ppm, whereas the SD rats appeared to be more sensitive, with minimal olfactory epithelium damage in some animals as low as 0.1 to 0.3 ppm.

Introduction

- Chronic naphthalene (NP) exposure (10 ppm) resulted in respiratory adenomas of the nasal epithelium (male rats), and olfactory epithelial neuroblastomas (female rats) (NTP, 2000).
- Nasal cavity metabolism of NP (via P450-dependent monooxygenases) may be an important factor in cytotoxicity and location-induced injury (Lee et al., 2005).
- The purpose of this study was to establish an exposure-response relationship and threshold for nasal epithelial effects in male and female F344 and Sprague Dawley (SD) rats following an acute inhalation exposure to 0, 0.1, 0.3, 1, 10, and 30 ppm NP.

Methods

Inhalation Exposures

- Naphthalene vapor generation: heat solid naphthalene, vapor carried by nitrogen gas into exposure chamber inlet. (see diagram)
- Small generators used for 0.1, 0.3, and 1 ppm exposure chambers.
- Small generators placed in oven to control temperature.
- Large generation system used for 10 and 30 ppm exposure chambers.
- Large glass system wrapped with heat tape to sublimate the naphthalene.
- Concentrations in the chambers controlled by adjusting heating temperature and nitrogen flow rate through generators.

Naphthalene Concentration Measurements

- Naphthalene concentration measured using a gas chromatograph (Model 5890 Series II, Hewlett Packard, Palo Alto, CA).
- Fused silica film, 15 m x 0.53 mm id capillary column.
- Flame ionization detector (FID).

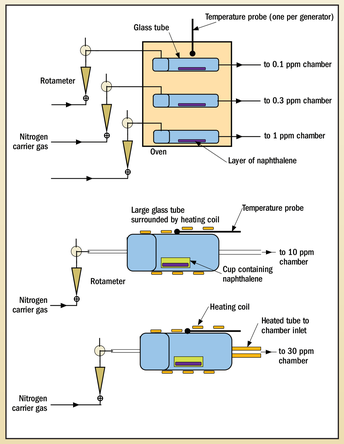
Exposures Single whole body exposure, 6 hours

Histopathology

- The day after exposure rats were anesthetized with sodium pentobarbital and killed by exsanguination.
- Noses were fixed in 10% neutral buffered formalin, decalcified, trimmed into six standard sections, and processed to hematoxylin and eosin stained slides for histological evaluation.

Results

Naphthalene Generation System



Naphthalene Generator Temperatures and Flow Rates

Napthalene Concentration (ppm)	Generator Temperature (°C)	Generator Flow Rate (L/min)
0.0	not applicable	not applicable
.10	40.1 – 43.7[a]	0.25[a]
.30	40.0 – 43.8	0.34 – 0.35
1	36.4 – 43.0	1.2
10	46.4 – 48.3	14.0 – 15.3
30	48.8 – 53.4	20.0 – 23.2

[a] Values are ranges of ± 1SD of the mean

Aerosol Concentration Measurements

Napthalene Concentration (ppm)	Total Particle Concentration (#/cm3)	Particle Size (µm)
Room air[a]	564 ± 22 [b]	0.14 (1.9) [c]
0.0	59 ± 18	0.21 (1.5)
.10	67 ± 20	0.20 (1.7)
.30	60 ± 12	0.21 (1.6)
1	82 ± 9	0.20 (1.8)
10	63 ± 12	0.20 (1.8)
30	55 ± 18	0.23 (1.5)

[a] Outside of inhalation exposure chambers
[b] Mean ± SD
[c] CMD (GSD)

Measured Exposure Concentrations

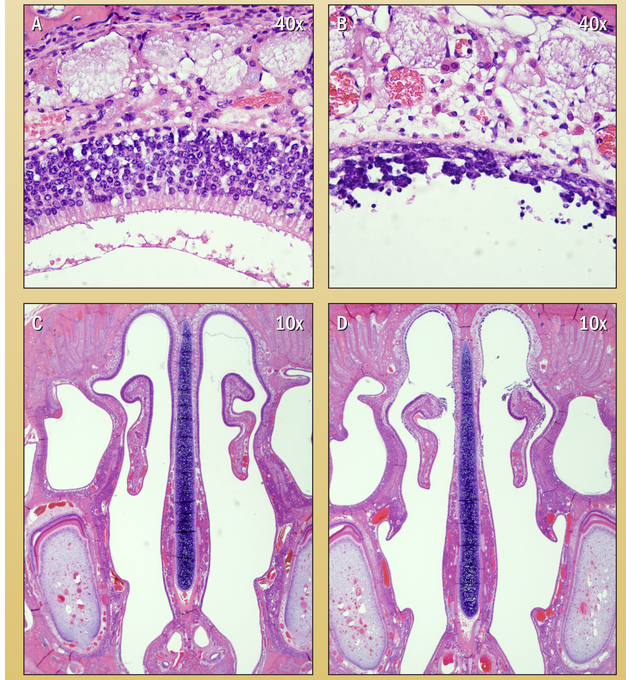
Exposure Chamber	Target Napthalene Concentration (ppm)	Male Rat Chamber Actual Concentration (ppm)	Female Rat Chamber Actual Concentration (ppm)
	0.0	0.0	0.0
	.10	0.09 ± 0.03[a]	0.10 ± 0.02
	.30	0.03 ± 0.05	0.28 ± 0.04
	1	1.10 ± 0.09	1.07 ± 0.14
	10	11.6 ± 1.2	12.3 ± 1.4
	30	28.5 ± 5.2	30.7 ± 6.7
QC Samples			
	Bag Standard[b]	0.56 ± 0.07	0.55 ± 0.12
	Dynacalibrator[c]	1.79 ± 0.12	1.80 ± 0.16

[a] Mean ± SD
[b] Bag prepared from certified cylinder
[c] Model 340, VICI Metronics

Body Weights (g) at Necropsy

Napthalene Concentration (ppm)	F344 Males	F344 Females	SD Males	SD Females
0.0	119 ± 5[a]	103 ± 7	167 ± 26	154 ± 4
.10	107 ± 13	103 ± 5	182 ± 19	138 ± 16
.30	121 ± 8	102 ± 4	180 ± 20	158 ± 8
1	117 ± 7	107 ± 1	185 ± 13	149 ± 4
10	113 ± 5	99 ± 5	161 ± 25	154 ± 12
30	113 ± 10	101 ± 3	176 ± 18	144 ± 21

[a] mean ± SD, n=5



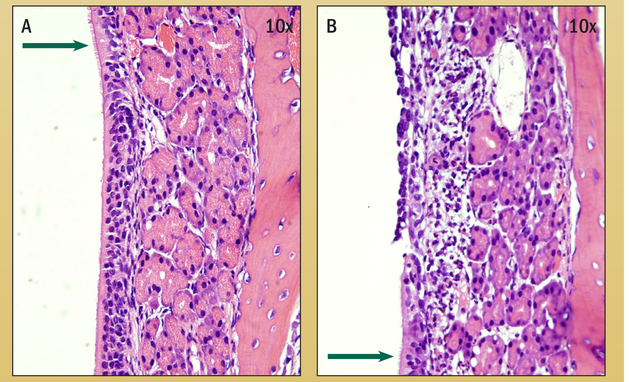
Olfactory epithelium of the dorsal meatus and ethmoid turbinates (Level III) of SD male rats to show control (A & C) and 30 ppm naphthalene treated (B & D). Note that the olfactory necrosis is bilateral (D) and moderately severe.

Incidence of Nasal Olfactory Necrosis

Exposure Concentration ppm	F344 Males	F344 Females	SD Males	SD Females
0	0/5	0/5	0/5	1/5
.10	0/5	0/5	2/5	1/5
.30	0/5	0/5	3/5	2/5
1	5/5	4/5	4/5	4/5
10	5/5	5/5	5/5	5/5
30	5/5	5/5	5/5	5/5

Incidence of Nasal Respiratory Necrosis

Exposure Concentration ppm	F344 Males	F344 Females	SD Males	SD Females
0	0/5	0/5	0/5	0/5
.10	0/5	0/5	0/5	0/5
.30	0/5	0/5	0/5	0/5
1	0/5	0/5	1/5	0/5
10	5/5	5/5	5/5	5/5
30	5/5	5/5	5/5	5/5



Photomicrographs of male F344 rat septal olfactory organ (level III) to show control (A) and 10 ppm naphthalene treated (B) tissues. Note the normal respiratory epithelium (arrow) above and below the olfactory patch in both treated and control. The olfactory necrosis is moderate.

Conclusions

- A NP inhalation exposure system was developed that generated stable concentrations as low as 0.1 ppm.
- No aerosol was detected in the inhalation exposure chambers at concentrations as high as 30 ppm.
- Lesions related to exposure to NP were observed in some of the respiratory epithelial locations but were seen predominately as necrosis in the olfactory epithelium.
- Severity of lesions was exposure concentration dependent and ranged from minimal to marked.
- The necrosis was characterized by cytoplasmic vacuolation, loss of proper epithelial orientation, condensation of the cytoplasm, pyknotic and karyorrhectic nuclei, and sloughing of the necrotic epithelium.
- Clear minimal effects of exposure to NP were observed in rats at 1ppm.
- Minimal olfactory necrosis was observed in some SD rats below 1ppm.
- Differences in the level of P450 (and their isoforms) located in the olfactory and respiratory epithelium may explain differences in cell response, stain response and location of effect.

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Future Directions

- A five day study with recovery groups is underway to address the further interpretation of these results.
- For this study, lesion mapping of 5 day treated and 5 day treated followed by a 14 day recovery will be conducted.

Acknowledgements

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RAT STRAIN DIFFERENCES IN NASAL TISSUE RESPONSE AND METABOLISM

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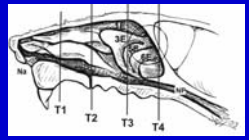


Cross-section views of nasal passages (T1 to T4)

ABSTRACT

While there are extensive investigations of species differences in toxic response, surveys of intra-strain differences in tissues other than liver are lacking. As rare nasal tumors have been observed in NTP Fisher rat cancer bioassays associated with cytotoxicity, a literature review of cytogenic and tumor responses in the nasal regions of rat strains was carried out for naphthalene, propylene oxide and formaldehyde to determine the most appropriate strain to use in assessing cancer risk to humans. A compilation of available data from studies employing Sprague Dawley (SD), Fisher 344 (F344), Wistar (WT), and other rat strains allowed for a diagrammatic mapping and comparison of abnormal cellular responses and key enzymes involved in metabolism. The distributions of these responses at various test concentrations and durations are presented and critical data gaps are highlighted. Bioactivation by CYP is associated with nasal injury / toxicity; olfactory nasal regions show highest P450 concentrations whereas non-olfactory regions have about 50 times lower metabolic activity. Detoxifying pathways include glutathione-S-transferase (GST) a epoxide hydrolase (EH); in Long Evans rats, EH is not reported to be localized in the dorsal meatus, a nasal area associated with injury for some chemicals. Distribution of metabolic enzymes rather than absolute level of an enzyme may dictate lesion distribution for bioactivated toxicants. However, while differences in metabolism have been documented between SD, F344 and WT in tissues such as liver or lung, those for nasal tissue require further research.

INTRODUCTION

A recent symposium on naphthalene carcinogenicity (Belzer *et al., in press*; North *et al., in press*) identified the relevance of observed nasal tumors in a Fisher rat bioassay (NTP, 2000) as a key uncertainty in developing valid and reliable scientific estimates of the human carcinogenic risk associated with environmental exposure to naphthalene. While an extensive research program is underway to investigate the potential inter-species differences in response for naphthalene, (The Naphthalene Coalition, 2007) this paper addresses possible intra-strain differences in the rat for nasal tumors; such a comparative assessment has hitherto not been conducted. As nasal tumors have been observed in rat cancer bioassays for agents other than naphthalene and have been associated with cytotoxicity and genotoxicity (NTP, 1985; Kerns *et al.*, 1983), potential intra-strain differences have implications for human health chemical risk assessment. Specifically, further understanding of relative susceptibilities between rat strains may aid in determining the most appropriate strain for assessing chemical risks to humans.

To address this question, we performed a qualitative assessment of the potential relevance of nasal tumors in rats to humans using naphthalene and several other 'model' chemical compounds. The assessment included identification and comparison of factors such as nasal irritation, metabolism, and genotoxicity involved in nasal-related health effects in rats and humans. The objectives were to 1) mine available data and highlight potential differences in rat strain susceptibilities, 2) identify critical data needed to improve upon the assessment, and 3) place qualitatively the naphthalene rat nasal tumors findings into perspective with regard to human relevance.

MATERIALS AND METHODS

1. General Approach

The overall approach was to compare and contrast data available on biological endpoints and metabolic enzymes relevant to chemical induced nasal lesions in three rat strains. Data in the published literature were compiled from studies employing Sprague Dawley (SD), Fisher 344 (F344), and Wistar (WT) rat strains. Metabolic enzymes reviewed include bioactivation - cytochrome P450 and detoxification - glutathione S-transferase (GST), epoxide hydrolase (EH), formaldehyde dehydrogenase (FDH), aldehyde dehydrogenase (ADH). The nasal lesions evaluated included abnormal cellular responses including hyperplasia, necrosis and tumors.

The above information allowed for a diagrammatic mapping and comparison of abnormal cellular responses and key enzymes involved in metabolism in the nasal region. This mapping comparison was done by illustrating the location and severity of endpoints and distribution of metabolic enzymes by nasal region. The distributions of these responses at various test concentrations and durations were also arrayed, and critical data gaps were highlighted. Naphthalene metabolism was of particular focus in the rat strain review.

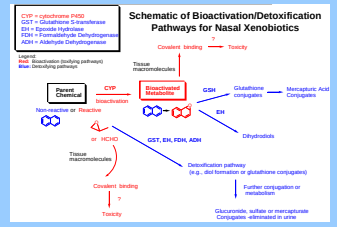
2. Rationale for chemical selection

Selection of 'model' chemicals for this assessment included biological responses consistent with nasal tumors, such as nasal irritation and/or genotoxicity, but also included chemicals with similar detoxification pathways [e.g., propylene oxide, epoxide metabolite of naphthalene] and/or different metabolism pathways.

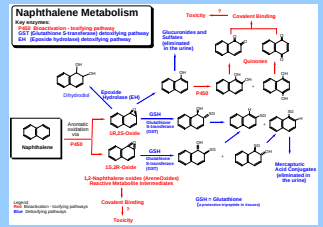
3. Literature search methods (e.g., databases searched, terms, time period, etc)

The information was obtained from literature searches using a number of databases. The specific databases utilized included EMBASE/PASCAL/BIOSIS, Medline/Toxfile/NTIS, and PubMed/Google. The search terms used were "nasal" + "rat:strain" + "chemical".

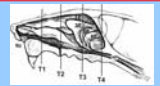
Metabolism Enzyme Systems in Nasal Tissue and Evaluation of Potential Rat Strain Differences



Dahl and Hadley, 1991; Reed, 1993; Morgan, 1995; Thornton-Manning and Dahl, 1997; Xing and Dahl, 2003



Buckpitt *et al.*, 2002 and 2006 and references therein.



Cross-section views of nasal passages (T1 to T4)

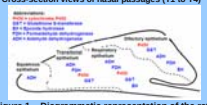


Figure 1. **Diagrammatic representation of the rat nose showing the distribution of selected metabolic enzymes in the airway lining the epithelia.** Bioactivation enzymes in red. Detoxification enzymes in blue. Diagram modified from Morgan, 1995 and including GST data from Robinson *et al.*, 2003.

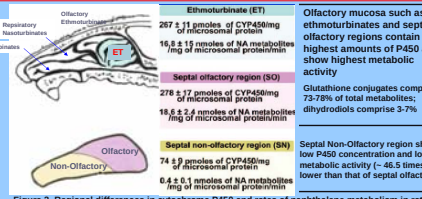


Figure 2. **Regional differences in cytochrome P450 and rates of naphthalene metabolism in rat nasal mucosa.** Nasal metabolism has been extensively studied in SD rats. Data above from Lee *et al.*, 2005a. There are little data reported for the nasal metabolism of naphthalene in F344 for comparison. Rates of naphthalene metabolism (microsomes) were obtained from the sum of rates of formation of both glutathione conjugates and dihydrodiol metabolites. Lee MG *et al.*, 2005a also reported that nasal airflow pattern may determine pattern of olfactory mucosal injury from inhaled naphthalene.

Olfactory mucosa such as ethmoturbinates and septal olfactory regions contain highest amounts of P450 and show highest metabolic activity

Glutathione conjugates comprise 73-78% of total metabolites; dihydrodiols comprise 3-7%

Septal Non-Olfactory region show low P450 concentration and low metabolic activity (~ 46.5 times lower than that of septal olfactory)

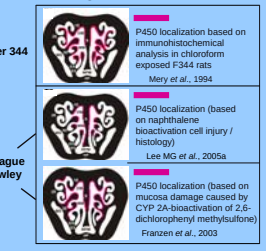


Figure 3. **Distribution of P450 Enzymes in the Rat Nasal Cavity, Level T3.** Location of P450 enzymes (Pink color) primarily in rat nasal olfactory mucosa in SD and F344. Nasal regions showing cell injury/histopathology have been associated with P450 bioactivation enzymes.

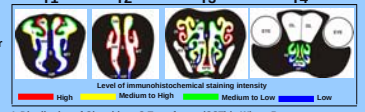


Figure 4. **Distribution of Glutathione S-Transferase (GST) in Wistar Rat.** GSH conjugation may be a predominant detoxification pathway in rat nasal cavity tissues. Nasal tissue GSH/GST levels protect against reactive metabolite(s) or oxidative damage until GSH stores are depleted. (Robinson *et al.*, 2003; Potter *et al.*, 1995; Lee MS *et al.*, 2005b)

Figure 5. **Distribution of Epoxide Hydrolase (EH) in Long Evans Rat.** EH is reportedly absent in the dorsal meatus (DM) region of nasal cavity in Long Evans rat. Dorsal meatus is the site of nasal injury for some chemicals. Speculation that reactive epoxide metabolites formed *in situ* in the olfactory mucosa of the DM area are poorly detoxified (e.g., absence of EH or if not by GSH-GST). Distribution of metabolic enzymes, rather than the absolute level of an enzyme in the tissue may dictate lesion distribution in the case of toxicants which are bioactivated in the target tissue. (Genter *et al.*, 1995)

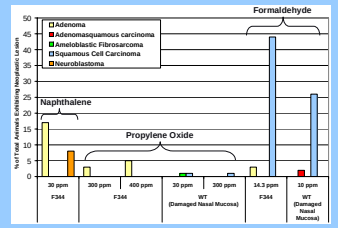


Figure 7. **Incidence of Naphthalene, Propylene Oxide and Formaldehyde-Induced Neoplastic Lesions in F344 and WT Rats.** Two year treatment with naphthalene, propylene oxide or formaldehyde induced both benign and malignant lesions in the nasal region of F344 rats. Only malignant lesions were observed in response to propylene oxide and formaldehyde in WT rats. Electrocoagulation was used to damage the nasal mucosa of WT rats prior to exposure. Squamous cell carcinoma was observed in a single WT rat with undamaged mucosa (data not shown). F344 = Fisher 344; WT = Wistar. (Long *et al.*, 2003; Renne *et al.*, 1986; Kuper *et al.*, 1988; Kerns *et al.*, 1983; Woutersen *et al.*, 1989)

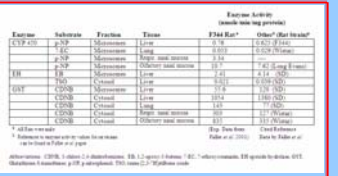


Figure 6. **Table taken from Faller *et al.*, 2001 summarizing Measured Enzyme Activities for Nasal, Pulmonary and Hepatic Cell Fractions Isolated from F344 Rats toward reference substrates and their comparison with literature values. Mouse and human values were not included so as to focus on rat data and potential strain differences.**

As part of a study to investigate interspecies differences in nasal, lung and liver metabolism of propylene oxide (PO), various control F344 rat cytosolic or microsomal fractions including those from respiratory and olfactory nasal mucosa were assayed for specific metabolic activity toward reference substrates. Comparison with literature values for other rat strains using the same ref substrate indicated some apparent differences (~2.5 times) in GST metabolic activity between F344 and Wistar rats in the nasal mucosa cytosolic fractions. Further work to confirm these findings with other rat strains and with different substrates are needed (side-by-side experiments).

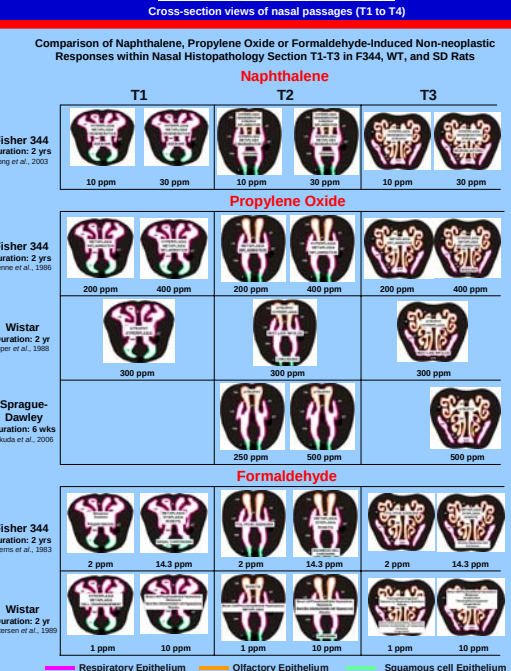
The metabolism of propylene oxide has been extensively studied in F344 rat for interspecies comparison with mouse and human in various tissues including nasal mucosa (Faller *et al.*, 2001; Csanady and Filser, 2007). Direct comparative metabolism studies with other rat strains however were not carried out in those studies.

Examples of Rat Strain Difference in Metabolism have been reported in liver, lung, kidney between SD, F344, Wistar or other strains. see Stott *et al.*, 2004; Oesch *et al.*, 1977; Yoshikawa *et al.*, 1982; Mendrala *et al.*, 1993; Hissink *et al.*, 1996, 1997; Newton *et al.*, 1983; Longo *et al.*, 1988; van der Laan *et al.*, 1993. There is a paucity of studies reported for rat strain differences in nasal metabolism although Longo *et al.* 1988 have alluded that there might be potential variation among different strains in this tissue.

Other Relevant References on Rat Strain Differences

See also Kacew et al., 1995; Kacew and Feating, 1996; and Kacew, 2001 for reviews on role of rat strain in differential sensitivity or responsiveness to chemicals and pharmaceuticals.

Comparison of Naphthalene, Propylene Oxide or Formaldehyde-Induced Non-neoplastic Responses within Nasal Histopathology Section T1-T3 in F344, WT, and SD Rats



SUMMARY AND CONCLUSIONS

METABOLISM

- Nasal metabolism of naphthalene has been extensively studied in SD rats with regard to P450 bioactivation and GST and EH detoxification. Comparative studies in F344 or Wistar rats have not been reported for naphthalene.
- Rat strain differences between SD, F344 or WT have been reported for the metabolism of some chemicals in the liver and lungs. However, evaluation of rat strain differences in nasal tissue metabolism requires further research. .
- Regional distributions of P450, GST and EH enzymes have been reported in rat nasal tissues but have not been characterized for all different strains for comparison. Possible rat strain differences in nasal airflow patterns, regional dosimetry and tissue susceptibility, GSH stores, PK-ADME and other factors are topics for further investigation.
- Species differences in metabolism have been studied for naphthalene and propylene oxide. Interspecies dosimetry modeling of inhaled formaldehyde in the nasal passages has been reported (Kimbell *et al.*, 2001). Rat strain differences in nasal metabolism and regional target tissue susceptibility remain an area for further research

NASAL TISSUE RESPONSE

- Observed non-neoplastic lesions induced by naphthalene, propylene oxide or formaldehyde vary across strains and the degree of susceptibility of each strain to chemical-induced nasal damage is not apparent.
- The underlying mode of action for consistent development of adenomas in response to chemical exposure may be an indicator of relative susceptibility by F344 in comparison to WT and SD
- Considerations of both species and strain differences should be included as part of an effective and rational approach to human risk assessment

Development of a Physiologically Based Pharmacokinetic (PBPK) Model of Naphthalene to Support Cross-Species Dosimetry

Harvey Clewell

Director, Center for Human Health Assessment
The Hamner Institutes for Health Sciences

Naphthalene PBPK Model

Goal

- Provide alternative to Category 1 gas default approach for cross-species dosimetry
  - Current default dosimetry for respiratory tract effects (Category 1 gases) is not appropriate for compounds like naphthalene, whose uptake is mediated by partitioning and local metabolism
  - Example: acetaldehyde
    - Cat 1 Default RGDR: 0.18
    - PBPK-based RGDR: 1.4
- New data
  - Upper respiratory tract uptake data (Morris, 2007)
  - Nasal metabolism measurements (Buckpitt, 2007)
  - Blood:air partition coefficient (Morris, 2007)

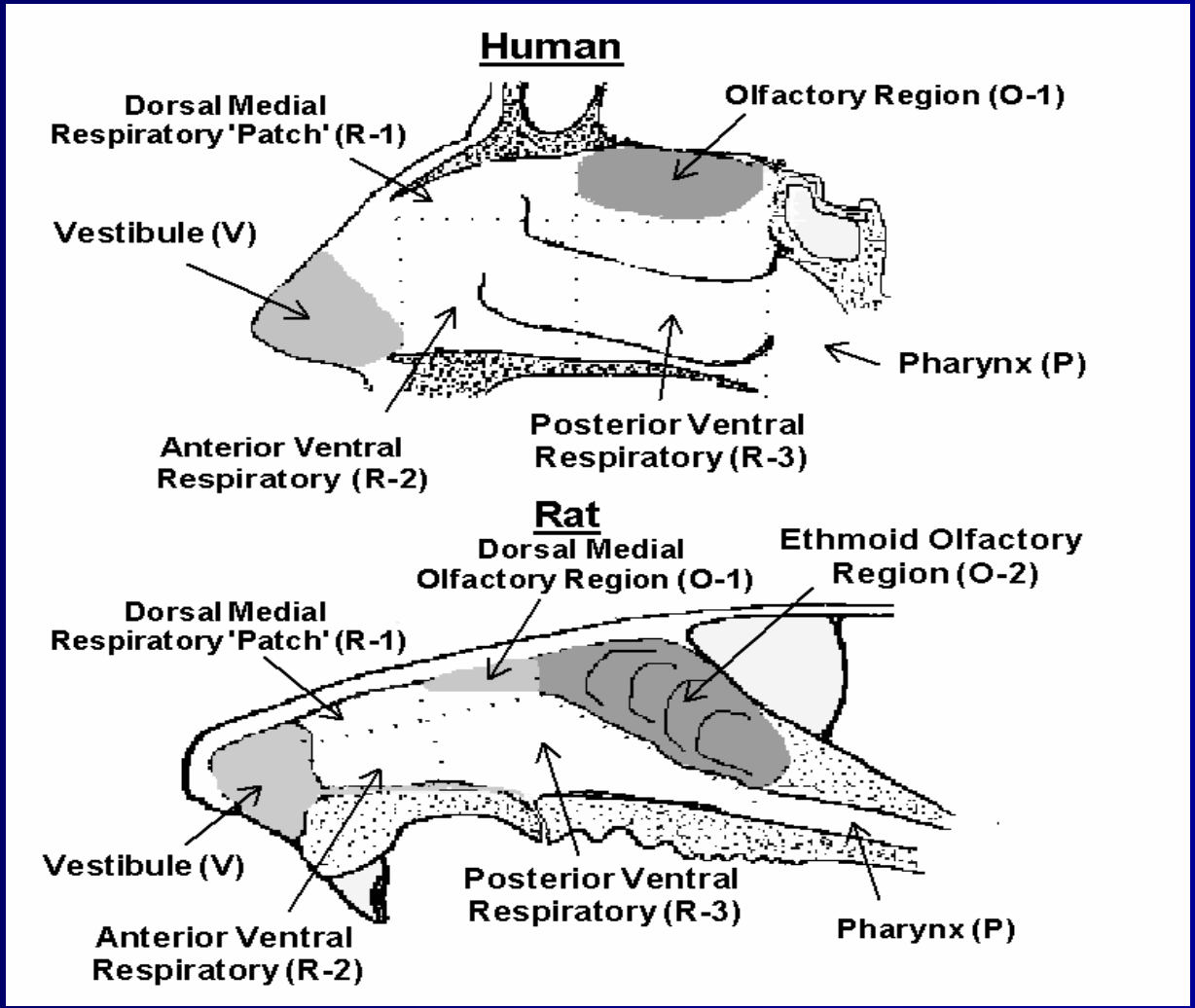
Naphthalene PBPK Model

Approach

- Starting point:
  - PBPK model for acetaldehyde (Teeguarden et al., 2007)
  - Naphthalene-specific parameters from existing systemic PBPK models (Willems et al., 2001)
- New data
  - Upper respiratory tract uptake data (Morris, 2007)
  - Nasal metabolism measurements (Buckpitt, 2007)
  - Blood:air partition coefficient (Morris, 2007)

Nasal Dosimetry Modeling

Hybrid PBPK/CFD Approach



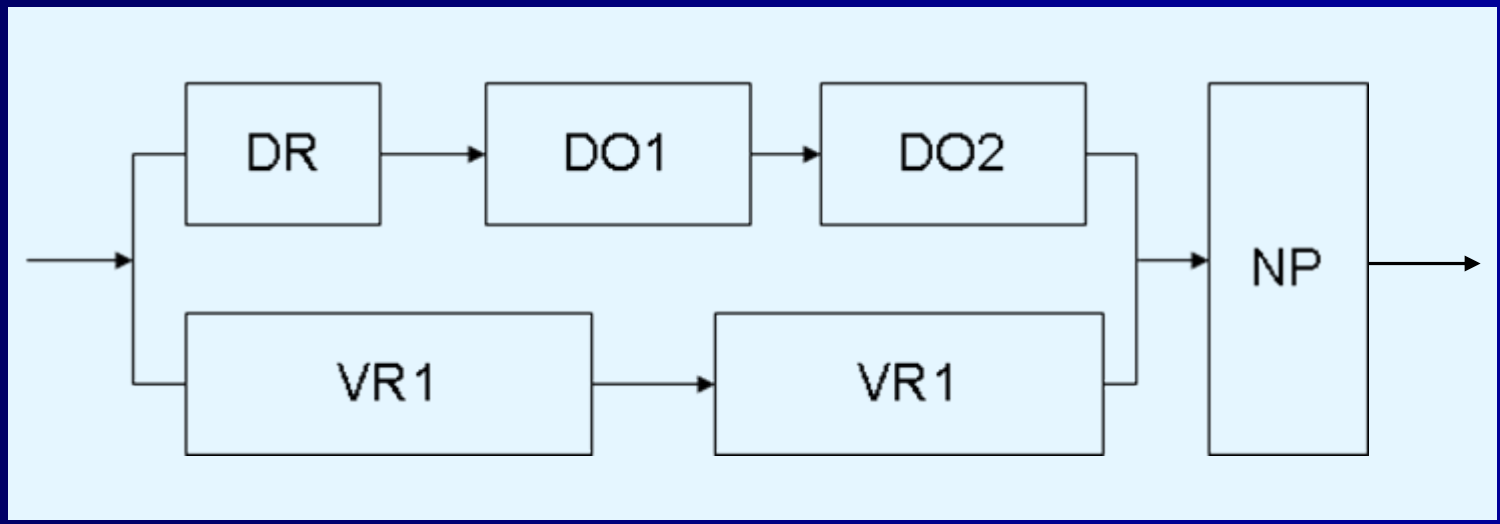
Nasal Dosimetry Modeling

Alternative Approaches

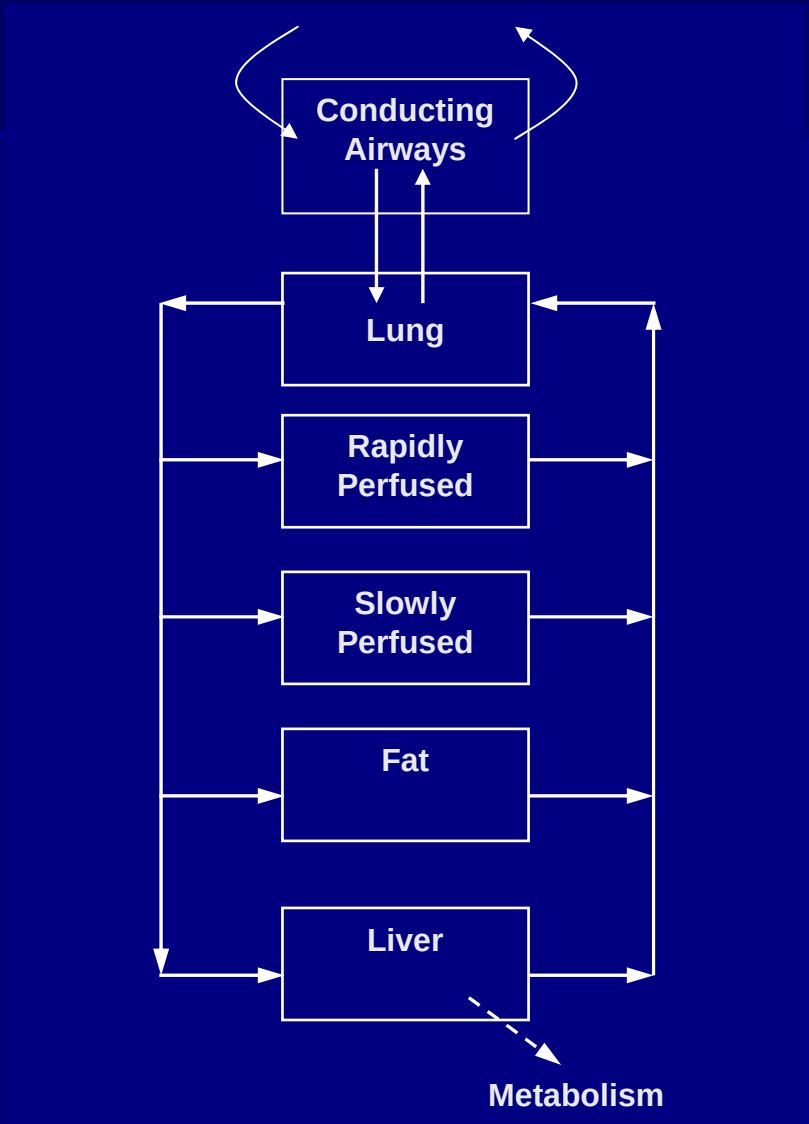
- Spatially resolved CFD modeling
  - Example: formaldehyde (Kimbell et al. 1993)
  - Can add saturable boundary condition to describe metabolism
- Hybrid PBPK/CFD
  - Examples: vinyl acetate, acrylic acid
  - Multicompartment PBPK model of nasal kinetics (Morris et al, 1993)
  - Kg's (mass transfer coefficients) from CFD (Frederick et al., 1998)

Nasal Dosimetry Modeling

Hybrid PBPK/CFD Approach

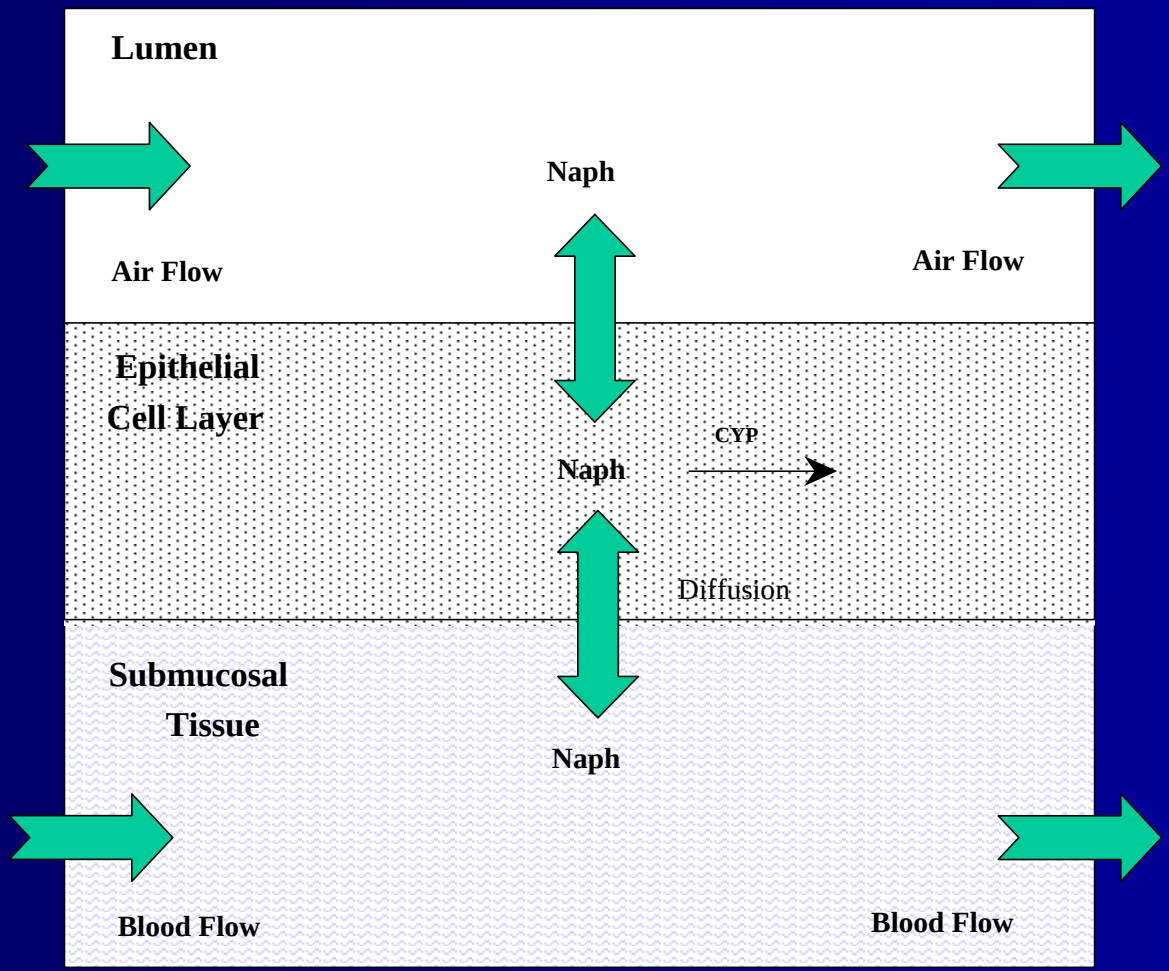


Hybrid CFD/PBPK Model Structure cont'd



Nasal Dosimetry Modeling

A Three-Layer Nasal Tissue Model for Naphthalene



Physiological Parameters

Parameter	Rat	Human	Reference
Flows Parameters			
Cardiac Output (ml/min/$g^{0.75}$)	1.756	1.208	(Brown *et al.* 1997)
Nasal Cavity Blood Flow (% CO)	0.15	0.25	(Rat: Teegarden *et al.* 2007, Morris *et al.* 1993; Human: Holmberg *et al.* 1989, Paulsson *et al.* 1985)
Minute Ventilation (ml/min/$g^{0.75}$)	1.909	1.743	(Brown *et al.* 1997)
Tissue Blood Flows (% CO)			
Liver	25	23	(Brown *et al.* 1997)
Fat	7	6	(Brown *et al.* 1997)
Poorly Perfused	100-sum of other	100-sum of other	
Rapidly Perfused	50	44	(Brown *et al.* 1997)
Tissue Volumes (% BW)			
Liver	3.7	2.57	(Brown *et al.* 1997)
Fat	6.5	13.6	(Brown *et al.* 1997)
Poorly Perfused	69	55	(Brown *et al.* 1997)
Rapidly Perfused	91-sum of other	91-sum of other	
Partition Coefficient			
Blood/Air	571	571	(NTP, 2000)
Tissue/Blood	4	4	(NTP, 2000)
Liver/Blood	7	7	(NTP, 2000)
Fat/Blood	160	160	(NTP, 2000)
Richly Perfused/Blood	4	4	Set to Tissue/Blood
Poorly Perfused/Blood	4	4	Set to Tissue/Blood

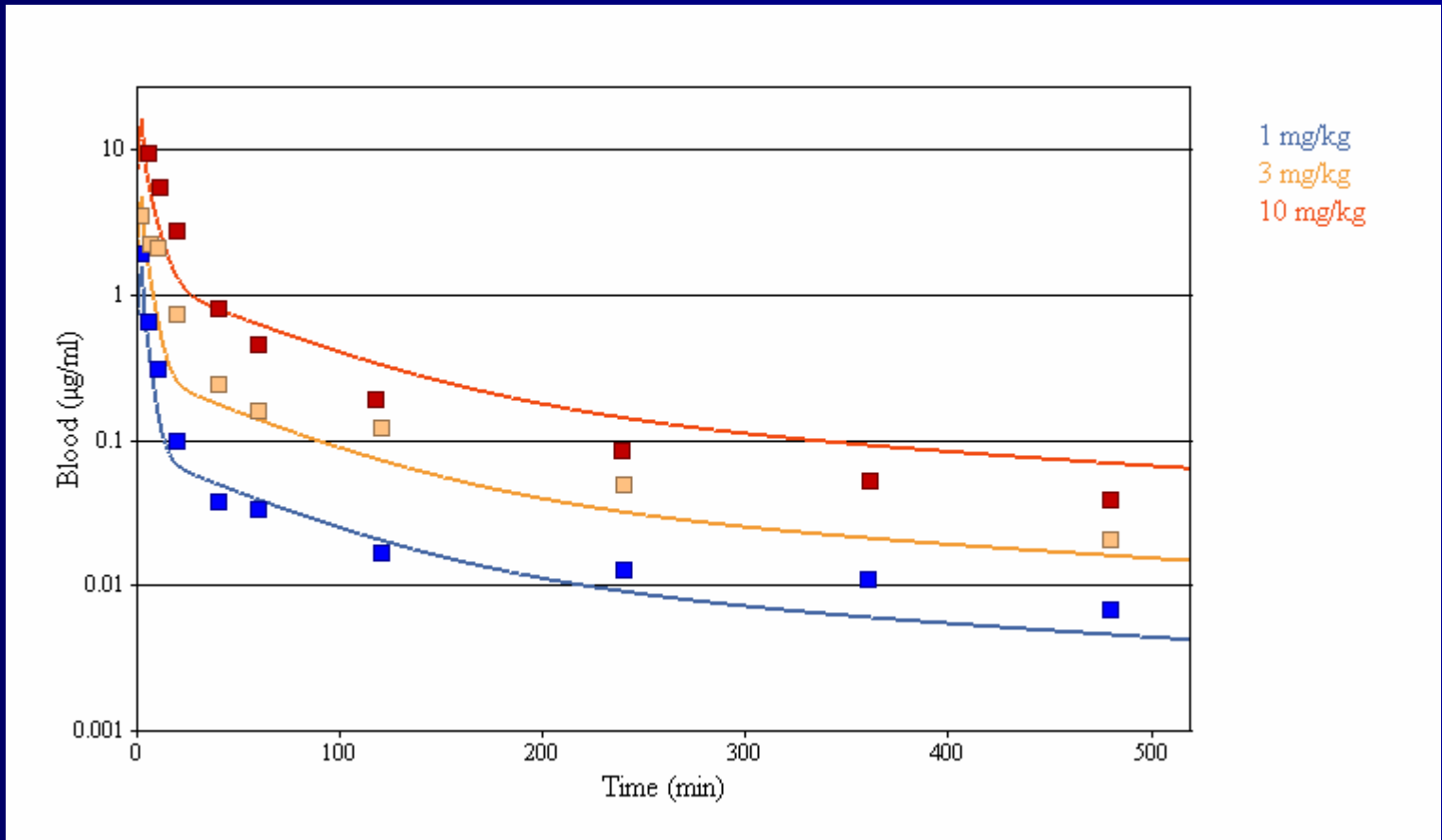
Physiological Parameters

Parameter	Rat	Human	Reference
Tissue Surface Areas (cm^2)			
Dorsal Respiratory	0.2	10.1	(Bogdanffy *et al.* 1999)
Anterior Dorsal Olfactory	0.42	13.2	(Bogdanffy *et al.* 1999)
Posterior Dorsal Olfactory	6.33	-	(Bogdanffy *et al.* 1999)
Anterior Ventral Respiratory	1.8	42.1	(Bogdanffy *et al.* 1999)
Posterior Ventral Respiratory	4.5	72.3	(Bogdanffy *et al.* 1999)
Mucus Thickness (cm)[7]	0.001	0.001	(Plowchalk *et al*. 1997)
Epithelial Tissue Thickness (cm)			
Dorsal Respiratory	0.005	0.005	(Bogdanffy *et al.* 1999)
Anterior Dorsal Olfactory	0.005	-	(Bogdanffy *et al.* 1999)
Posterior Dorsal Olfactory	0.005	0.004	(Bogdanffy *et al.* 1999)
Anterior Ventral Respiratory	0.005	0.005	(Bogdanffy *et al.* 1999)
Posterior Ventral Respiratory	0.005	0.005	(Bogdanffy *et al.* 1999)
Submucosa Thickness (cm)			
Dorsal Respiratory	0.002	0.002	(Plowchalk *et al*. 1997)
Anterior Dorsal Olfactory	0.002	-	(Plowchalk *et al*. 1997)
Posterior Dorsal Olfactory	0.002	0.002	(Plowchalk *et al*. 1997)
Anterior Ventral Respiratory	0.002	0.002	(Plowchalk *et al*. 1997)
Posterior Ventral Respiratory	0.002	0.002	(Plowchalk *et al*. 1997)
Lumen Volumes (cm^3)			
Dorsal Respiratory	0.004	0.74	(Bogdanffy *et al.* 1999)
Anterior Dorsal Olfactory	0.012	0.56	(Bogdanffy *et al.* 1999)
Posterior Dorsal Olfactory	0.054	-	(Bogdanffy *et al.* 1999)
Anterior Ventral Respiratory	0.09	3.5	(Bogdanffy *et al.* 1999)
Posterior Ventral Respiratory	0.09	5.16	(Bogdanffy *et al.* 1999)
Air-phase Mass Transfer Coefficients (cm/min)			
Dorsal Respiratory	1864	180	(Frederick *et al.* 1998)
Anterior Dorsal Olfactory	1637	821	(Frederick *et al.* 1998)
Posterior Dorsal Olfactory	1908	-	(Frederick *et al.* 1998)
Anterior Ventral Respiratory	1941	147	(Frederick *et al.* 1998)
Posterior Ventral Respiratory	2033	177	(Frederick *et al.* 1998)
Diffusivity Constants (cm^2/min)	0.00045	0.00045	(U.S. EPA, Soil Screening Guidance)

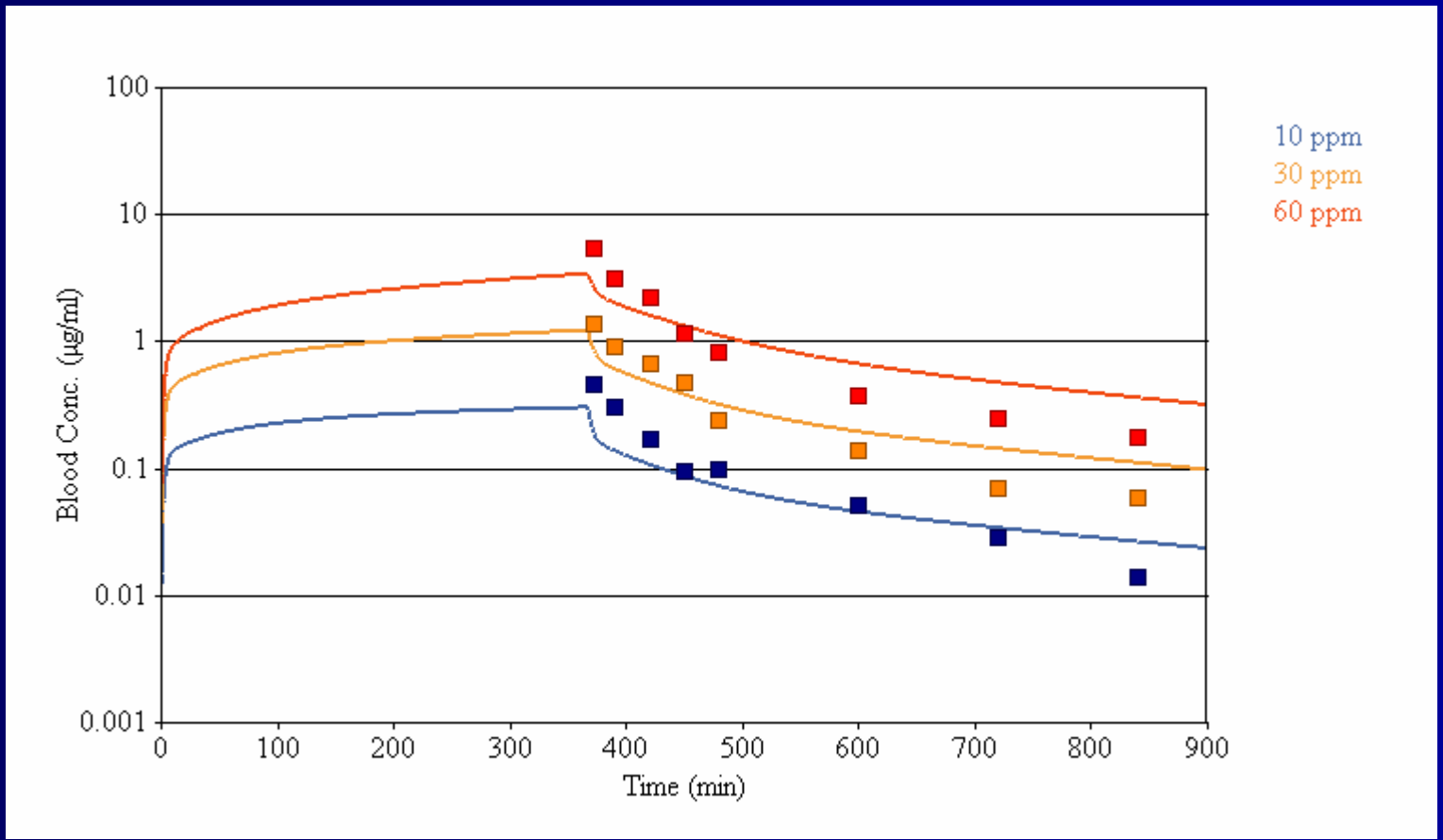
Metabolic Parameters

Parameter	Lung	Source
Vmax (nmol/mg protein/min)		
Liver	16.5	(Willems et al., 2001)
Lung	16.5	(Willems et al., 2001)
Dorsal Respiratory	34.4	(Buckpitt, personal comm.)
Dorsal Olfactory	34.4	(Buckpitt, personal comm.)
Ventral Respiratory	8.6	(Buckpitt, personal comm.)
Km (nmol/ml)		
Liver	6.0	(Willems et al., 2001)
Lung	6.0	(Willems et al., 2001)
Dorsal Respiratory	3.0	(Buckpitt et al., 1995)
Dorsal Olfactory	3.0	(Buckpitt et al., 1995)
Ventral Respiratory	3.0	(Buckpitt et al., 1995)

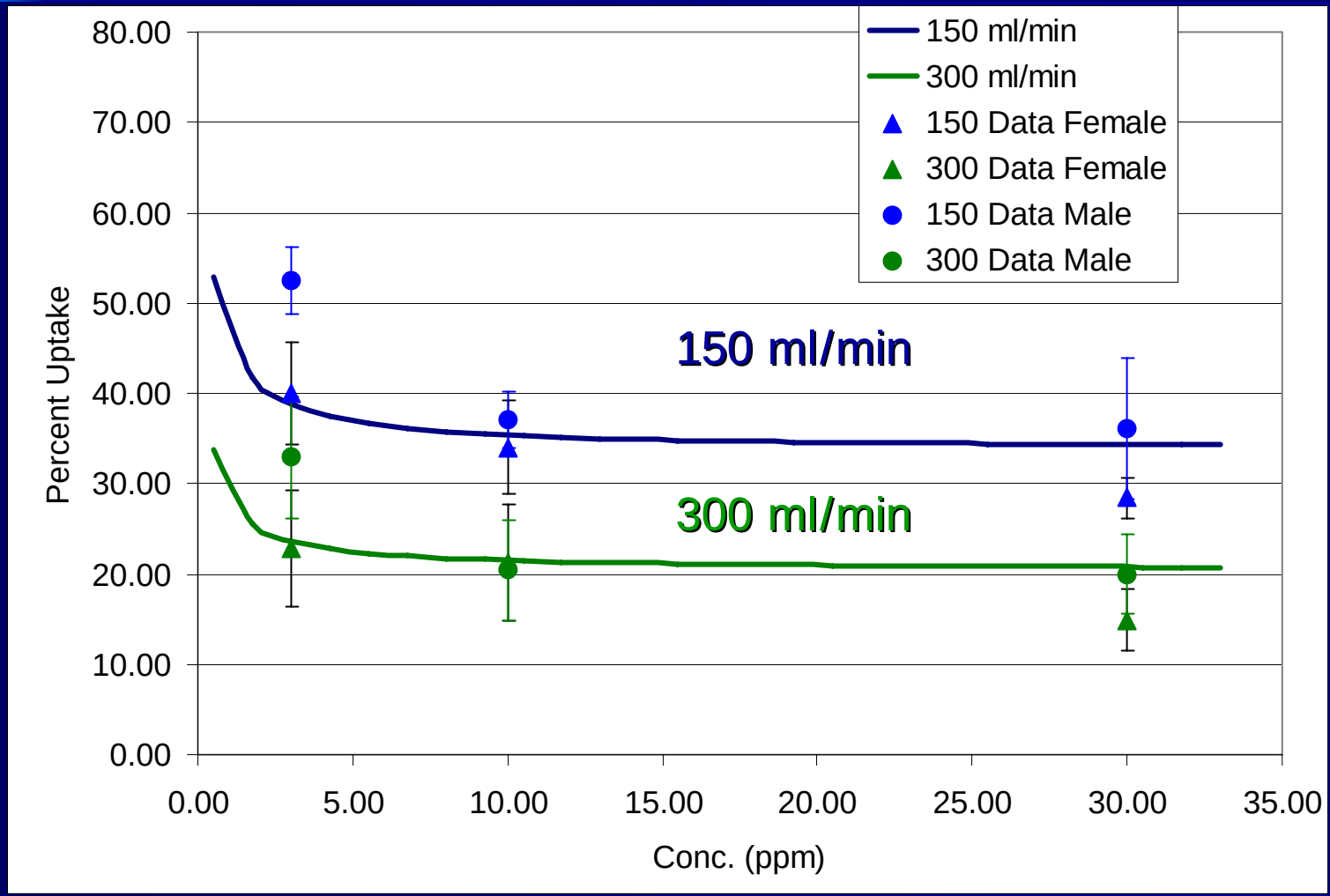
Naphthalene iv dosing study in male rats (NIEHS, NTP Task No. Chem 000646, 1995)



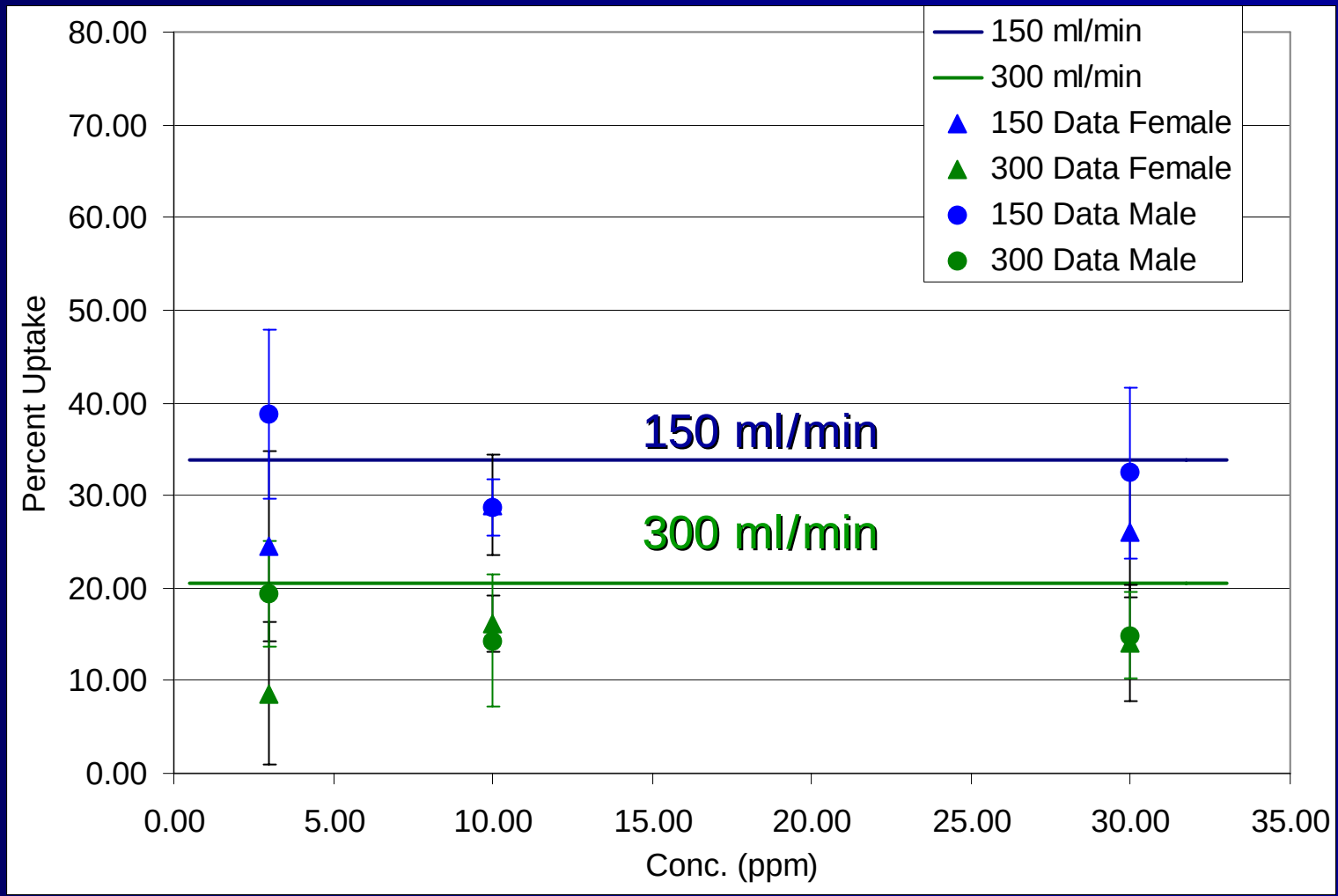
Naphthalene in blood of male rats after 6 hr inhalation exposure (NTP, 2000)



Model predicted URT extraction of naphthalene in male and female rats



Model predicted URT extraction of naphthalene in male and female rats after exposure to 5-phenyl pentene



Predicted Nasal Epithelial Naphthalene Concentration

Rat			Human		
Inhaled Concentration (ppm)	Dorsal Olfactory 1 (µM)	Ventral Respiratory 1 (µM)	Inhaled Concentration (TWA - continuous exposure) (ppm)	Dorsal Olfactory (µM)	Ventral Respiratory (µM)
0.1	0.733	1.36	0.018	1.16	1.32
1.0	11.6	14.5	0.18	11.6	13.2
10.0	132	147	1.8	116	132
30.0	402	443	5.4	348	397
60.0	807	887	10.7	690	786

Rat: - 14 day exposure (6 h/day 5 days/week)
– average concentration for day 8 – 14

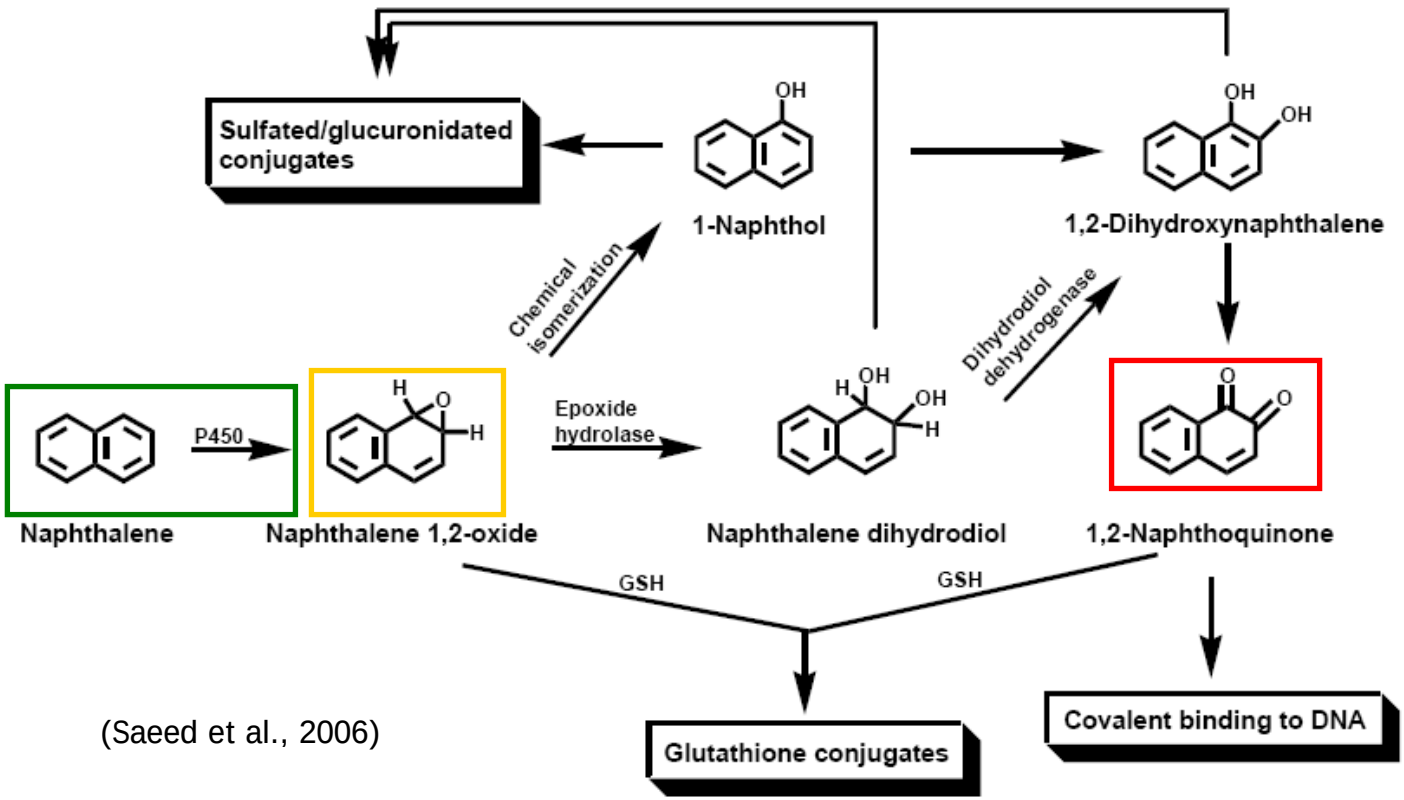
Human: - continuous exposure at TWA for rat inhalation concentration
- assuming no nasal metabolism (conservative assumption)

Naphthalene PBPK Model

Future Directions

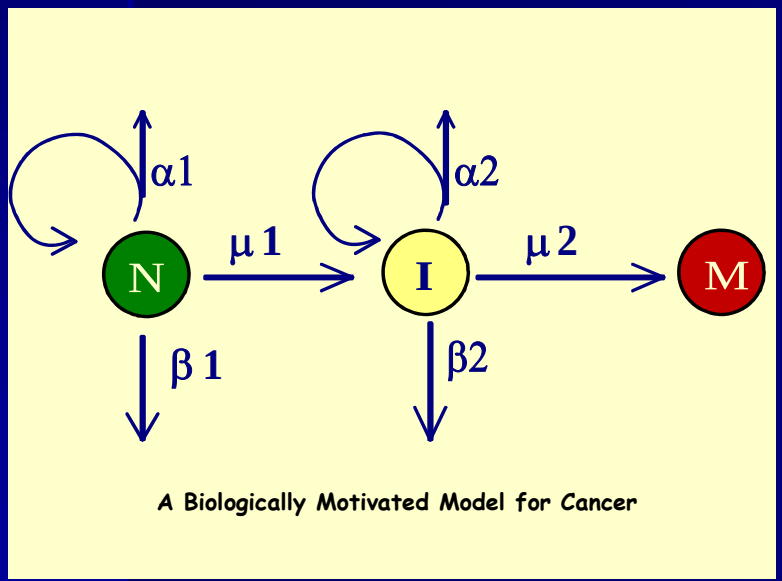
- Evaluate dosimetry for production of naphthalene oxide
  - Cyp 2F protein in nasal olfactory tissue (Buckpitt):
    - Mouse:Rat:Monkey = 600:300:20
  - Maximum Cyp 2F metabolism (Buckpitt):
    - Mouse:Human = 100:0.035
  - Primary human isoform: Cyp 2e1
- Evaluate other dose metrics
  - Concentration of naphthalene oxide
  - Concentration of 1,2-napthoquinone

Alternative Naphthalene Dose Metrics



(Saeed et al., 2006)

Beyond PBPK: Development of a Biologically Based Dose-Response Model



Key Capability of BBDR: Describes the Interaction of Mutation and Cell Division

Acknowledgements

- Jerry Campbell (The Hamner)
  - Created the hybrid CFD/PBPK model for naphthalene
- Justin Teeguarden (PNL)
  - Provided the hybrid CFD/PBPK model for acetaldehyde
- John Morris (U Conn)
  - URT extraction data
  - Blood:air partition
- Allan Buckpitt (UC Davis)
  - Metabolism data

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NAPHTHALENE STATE-OF-THE-SCIENCE SYMPOSIUM (NS^3)

PUBLICATIONS

Belzer, Richard B., James S. Bus, Ercole L. Cavalieri, Steven C. Lewis, D. Warner North and Richard C. Pleus. The naphthalene state of the science symposium: Objectives, organization, structure, and charge. Regul. Toxicol. Pharmacol. (2008), doi:10.1016/j.yrtph.2007.10.017.

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The naphthalene state of the science symposium: Objectives, organization, structure, and charge

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Abstract

This report provides a summary of the objectives, organization, structure and charge for the naphthalene state of the science symposium (NS^3), Monterey, CA, October 9–12, 2006. A 1-day preliminary conference was held followed by a 3-day state of the science symposium covering four topics judged by the Planning Committee to be crucial for developing valid and reliable scientific estimates of low-dose human cancer risk from naphthalene. The Planning Committee reviewed the relevant scientific literature to identify singularly knowledgeable researchers and a pool of scientists qualified to serve as expert panelists. In two cases, independent scientists were commissioned to develop comprehensive reviews of the relevant science in a specific area for which no leading researcher could be identified. Researchers and expert panelists alike were screened for conflicts of interest. All policy issues related to risk assessment practices and risk management were scrupulously excluded. NS^3 was novel in several ways and provides an innovative model for the effective use of peer review to identify scientific uncertainties and propose research strategies for reducing or eliminating them prior to the conduct of risk assessment.

Keywords: Peer review; Peer review organization; Peer review objectives; Peer review structure; Peer review charge; Risk assessment; Information quality; Naphthalene

1. Introduction

Human health risk assessment has historically contained a mix of science and judgment (NRC, 1983), but the application of judgment has stirred repeated controversy because the boundaries between science and judgment have not always been clear (OMB, 1990; NRC, 1994). Peer review, the primary quality assurance tool used in scholarly settings, has been applied by some federal government agencies (EPA, 2000, 2006) and recently extended to the entire Executive branch (OMB, 2005). The purpose is to maximize the quality of scientific information disseminated by the government and used for decision-making (OMB, 2002).

In federal practice, peer review typically occurs late in the risk assessment process when scientific and policy issues have become intertwined. The peer review model set forth and applied here is novel because it is designed to be

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implemented at a much earlier stage, preferably prior to the development and dissemination of human health risk assessment.

The goal of human cancer risk assessment is to assess human cancer risk. That is, risk assessment is an input to risk management decision-making by individuals authorized by statute to make choices on behalf of the public. Risk management decisions also take account of other scientific and technical information, such as economics and engineering, and public values and preferences (NRC, 1983, 1994). In part because scientific knowledge is always incomplete and uncertain, however, cancer risk assessment historically has contained numerous inference guidelines (NRC, 1983) or default values (NRC, 1994) to accommodate uncertainty, which are regarded as "public health-protective policy" (i.e., risk management-driven) but replaced with scientific information as it becomes available (EPA, 2005). The goals of the symposium were to (1) determine whether adequate scientific information existed to replace certain defaults now; (2) identify uncertainties that could be significantly reduced or resolved by scientific research; and (3) obtain expert scientific judgment concerning uncertainties that probably could not be resolved by scientific inquiry.

2. Objectives

This alternative peer review model is appealing in cases where scientific uncertainty is great, and the resolution of uncertainty by carefully targeted research has significant potential social value. Naphthalene was selected because it met these criteria. A recent whole animal bioassay suggested the possibility that exposure could be tumorigenic (NTP, 2000; Abdo et al., 2001). This study led to one final (OEHHA, 2004a,b) and one external review draft (EPA, 2004) risk assessment that included quantitative predictions of low-dose human cancer risk, and an IARC monograph (2002) classifying naphthalene as a Group 2B carcinogen ("possibly carcinogenic to humans").

The symposium had three objectives. First, multiple expert panels were convened to review the relevant primary scientific literature and develop a consensus view of the state of the science in each area. Secondary and other derivative scientific literature was generally excluded, but expert panels retained (and frequently exercised) the right to consider any scientific information they wished. To ensure that the symposium retained a scientific focus, policy-related literature was excluded and expert panels were actively discouraged from bringing it in. The Planning Committee encountered no resistance to that effort.

Second, expert panels were asked to identify gaps in scientific knowledge, discern which of these gaps were crucial to resolve for estimating low-dose human cancer risk, and propose targeted research projects that could be performed in a timely manner to significantly reduce or eliminate these scientific uncertainties.

Third, expert panels were asked to confront scientific uncertainties that could not be resolved through targeted research and provide thoughtful science- (not policy-) based judgments. The Planning Committee hoped to apply the lessons from experiments such as those by Evans et al. (1994) and the advice given by the National Research Council in its recent review of dioxin (NRC, 2006) concerning the acknowledgment and incorporation of uncertainty into risk assessment.

The symposium explicitly excluded from its set of objectives all policy matters related to the management of human cancer risk.

3. Organization

The Planning Committee was constructed to have expertise in multiple areas including toxicology, pharmacology, and decision sciences. One member of the committee, Dr. William O. Berndt, professor emeritus and former chairman of the Department of Pharmacology at the University of Nebraska Medical Center, died before the symposium was held. The participation of a senior Environmental Protection Agency scientist was sought but had to be dropped to accommodate EPA grant restrictions, which prohibit federal employees from serving. Regulatory Checkbook and the University of Nebraska Medical Center collaborated as co-organizers of the symposium, with additional non-financial sponsorship from the Society for Risk Analysis.

4. Structure

The Planning Committee reviewed the literature and identified the top four scientific issues to be addressed:

(a) Whole animal bioassays conducted on behalf of the National Toxicology Program.
(b) Human exposure, epidemiology, and cancer incidence.
(c) Cytotoxicity as a mode of action for observed carcinogenesis in animals.
(d) Genotoxicity as a mode of action for observed carcinogenesis in animals.

The selection of cancer extrapolation models was excluded in the ground that it was not a strictly scientific enterprise.

For each issue, a module was constructed consisting of one or more extended scientific presentations delivered by recognized research experts in the field. Where such experts could not be identified, the Planning Committee commissioned scientists recognized in the subject matter to prepare and deliver research papers (Price and Jayjock, 2008; Brusick, 2008).

To ensure fidelity to established peer review guidelines (OMB, 2005) and credibility within the larger scientific community, the Planning Committee sought expert panelists who met the following criteria: (1) widely recognized expertise in the underlying scientific sub-discipline, with

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or without extensive familiarity with naphthalene; (2) a prior research publication history that would be unlikely to impair the ability to fairly review competing or novel scientific views; (3) acceptance of the symposium structure and organization; and (4) willingness to limit review to scientific matters and to refrain from infusing scientific debate with policy considerations. In addition, the Planning Committee decided to err on the side of having too many expert panelists rather than too few, in the belief that a multiplicity of voices and perspectives would be the most effective instrument for diluting any particular expert panelist's inherent biases.

The NS^3 structure is unique, novel and valuable as a peer review tool for several reasons. First, NS^3 relied on primary researchers instead of secondary research interpreters as the leading source of scientific information. Conventional government peer review practice places secondary interpreters between primary researchers and peer reviewers. This creates manifold opportunities for confusion, misunderstanding, and the introduction of information of a non-scientific nature into scientific peer review.

Second, the role of peer reviewers at NS^3 was fundamentally different. Conventional government peer review is an evaluation of the completeness and adequacy of a secondary scientific synthesis. Expert panelists at NS^3 were not asked to evaluate completeness or adequacy of a single document, but instead to synthesize relevant scientific knowledge and identify promising avenues for research that could inform risk assessment.

Third, a conventional government peer review panel might have (say) 15 members responsible for evaluating all material scientific aspects of a draft risk assessment, but only two or three members might be qualified to examine a particular detailed area. In contrast, NS^3 focused on just the four most salient scientific issues and assembled no less than four and as many as eight qualified experts for each issue. Whereas spreading expertise too thinly can dilute the effectiveness of conventional government peer review, the NS^3 model ensures that critical scientific issues are addressed by a significantly larger number of qualified experts. This approach significantly increases intellectual diversity in peer review and better assures that scientific credibility is maintained.

Fourth, NS^3 explicitly and transparently restricted its review to scientific matters and refrained from debating policy judgments. The line distinguishing science from policy is not well defined (NRC, 1983, 1994), but excursions across it are increasingly easy to discern and formally discouraged as a matter of government policy (OMB, 2002).

Fifth, NS^3 explicitly provided extensive opportunities for public interaction with both research speakers and expert panelists. Conventional government peer review severely restricts public participation, and often regards public contact with peer reviewers as unethical. At NS^3, for every hour devoted to presentations by distinguished research scientists, approximately an hour was made available for open public discussion. Session moderators were empowered and encouraged to permit all nature of relevant scientific discussion but prevent these discussions from straying into policy matters.

5. Selection and compensation

The Planning Committee selected research speakers and expert panelists in a collaborative process. Actual procedures for recruitment varied somewhat, but in general, they received formal letters of invitation either from the University of Nebraska or Regulatory Checkbook. Three invited research speakers declined; one decline proved to be non-essential, and the other two declines led to the commissioned paper by Brusick (2008), which after the fact the Planning Committee concluded should have been part of the original design. All expert panelists and commissioned speakers were vetted to identify and disclose conflicts of interest such as, but not limited to, a financial relationship to one of the sponsors. One research speaker (Abdo) was a retired government employee judged by the Planning Committee to have essential knowledge about relevant primary research. One research speaker employed by EPA was invited but declined; prospective expert panelists employed by EPA and California EPA also were invited but declined.

All research speakers, expert panelists, and facilitators were offered honoraria for their service; for various reasons, some declined. All were offered reimbursement of their travel expenses; for various reasons, some declined. Authors of the commissioned papers (Brusick, 2008; Price and Jayjock, 2008) were also compensated, and Brusick was compensated for converting the Module D report into publishable form.

6. Charge

Each panel was given roughly the same charge, albeit tailored slightly where necessary to accommodate differences in subject matter.

(a) Knowledge and uncertainty:
- What scientific statements can be made with a high degree of confidence?
- What scientific statements cannot be made with a high degree of confidence?

(b) Of those scientific statements that cannot be made with a high degree of confidence, which are quintessential uncertainties for human cancer risk assessment?

(c) Considering quintessential scientific uncertainties:
- What specific research projects could be undertaken promptly and cost-effectively that would resolve them?
- How should results from such studies be interpreted?

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(d) What constitutes best scientific judgment about quintessential scientific uncertainties that cannot be addressed by science promptly and cost-effectively?

Fig. 1 was developed to focus research speaker presentations, public discussion, and expert panel deliberations. Panel (a) established at the outset that only science would be discussed. The primary responsibility of the research speakers was to synthesize relevant scientific knowledge and guide expert panelists to distinguish between knowledge and uncertainty, as shown in Panel (b). Expert panelists then sifted through the uncertainties to distinguish those that were quintessential from those which were not, as shown in Panel (c). Finally, as shown in Panel (d), they were asked to propose targeted, cost-effective research projects that could be completed in a timely manner to address those quintessential uncertainties resolvable through science.

An expert in decision sciences facilitated each expert panel. In addition to moderating discussion and debate, they had substantive responsibility for assisting the subject matter experts in distinguishing among types of uncertainty so that quintessential uncertainties could be identified and research projects devised. A commonly used strategy was to ask experts to explain how the data would resolve uncertainty assuming that they had it in hand today.

In many cases, the expert panels struggled to orient their thinking along these lines. As research scientists, all had a great deal of experience proposing and evaluating research projects, but the criteria normally used to evaluate intellectual merit are different from those which the Planning Committee asked be applied. Few expert panelists had prior experience with the application of value-of-information principles for ranking such proposals. The NS^3 process yielded many valuable insights on specific research. Nevertheless, there remains considerable room for improvement in devising more effective strategies for eliciting expert scientific judgment in ways that target scarce research resources to resolve the most pressing scientific uncertainties.

7. Sponsorship funding

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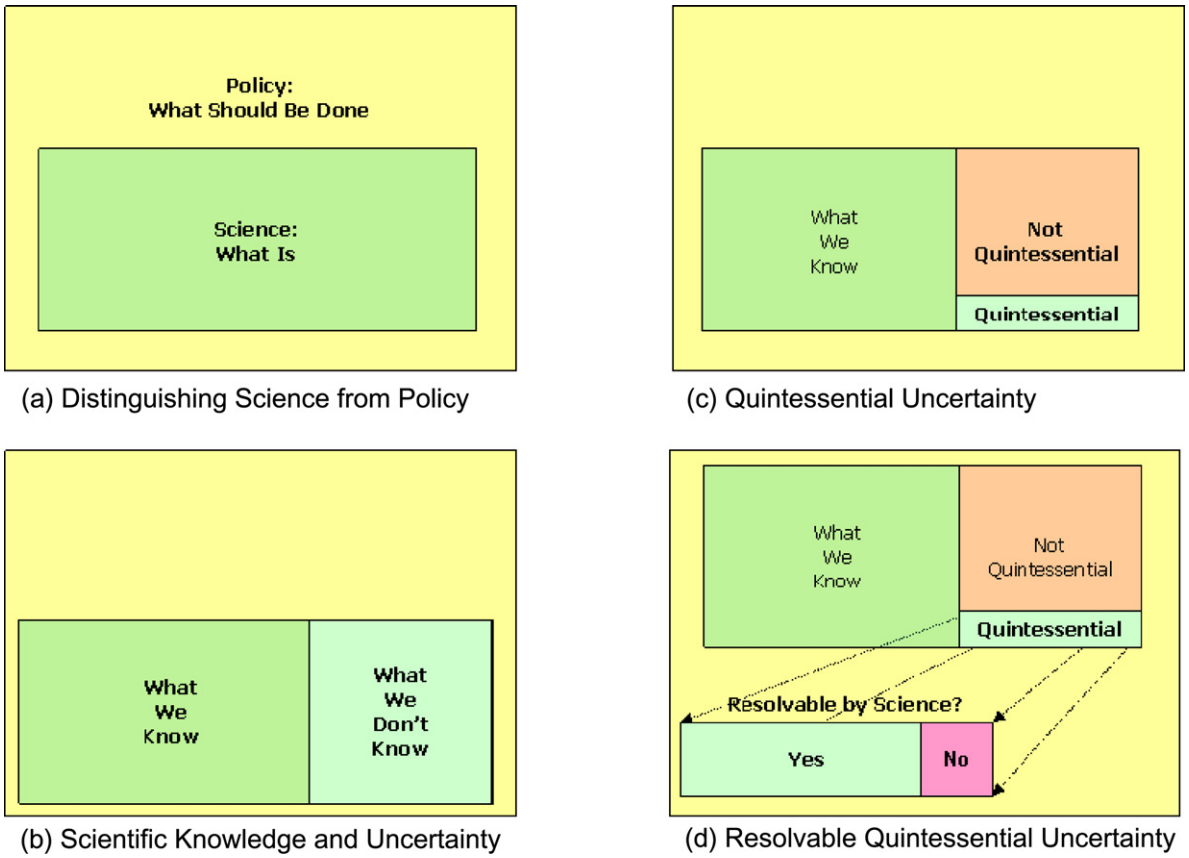

Fig. 1. The NS^3 model for identifying and resolving scientific uncertainty.

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501(c)(3) non-profit organization. The Society for Risk Analysis was a non-financial sponsor.

Under the terms of the restricted grants, the Planning Committee retained total substantive and managerial control over the content, structure, and charge; the selection of research speakers and the commissioning of research papers by Price and Jayjock (2008) and Brusick (2008); the selection of expert panelists; the submission of final reports; and the provision of compensation including honoraria to research speakers, expert panelists, and facilitators. While the Planning Committee welcomed input from all, and received valuable advice from EPA and industry sponsors, at no time did the Planning Committee cede control to any sponsor. The Planning Committee alone had sole responsibility for executing NS^3, but the expert panelists and facilitators are responsible for the content of the commissioned papers (Price and Jayjock, 2008; Brusick, 2008) and module papers (North et al., 2008; Griego et al., 2008; Bogen et al., 2008; Brusick et al., 2008). In two of the four modules (North et al., 2008; Brusick et al., 2008), research speakers were invited by the expert panelists to contribute to the development of the expert panel report; accordingly, they are listed as co-authors.

Conflict of interest disclosure statement

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A Review of Whole Animal Bioassays of the Carcinogenic Potential of Naphthalene

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A Review of Whole Animal Bioassays of the Carcinogenic Potential of Naphthalene

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Abstract

This report provides a summary of deliberations conducted under the charge for members of Module A participating in the Naphthalene State-of-the-Science Symposium (NS3), Monterey, CA, October 9-12, 2006. Whole animal bioassays have been performed by the National Toxicology Program in mice and rats to ascertain the carcinogenic potential of naphthalene by inhalation exposure. A statistically significant increased incidence of pulmonary alveolar/bronchiolar adenoma (a benign lesion), was observed among female mice; an observed increase among the males did not reach statistical significance. No nasal tumors were observed in either sex. A tumorigenic response was observed in both sexes of rats, in males an increased incidence of nasal respiratory epithelium adenoma (a benign rather than malignant lesion) and in females, olfactory epithelial neuroblastoma. Interpretations of these studies vary. On the one hand, evidence of extensive non-neoplastic response in both sexes of both species indicates cytotoxicity occurred at all doses, and strongly suggests that cytotoxicity played a significant role in the tumor responses observed in the target tissues. On the other hand, olfactory epithelial neuroblastoma has rarely been observed in NTP bioassays. This review seeks to develop a consensus understanding of the scientific evidence provided by these studies, taking into account that they have been used as the basis for quantitative human cancer risk assessment, and suggests scientific studies that, if performed, could resolve scientific uncertainties.

1. Introduction

Two whole animal bioassays performed by the National Toxicology Program (NTP) were reviewed. In the B6C3F1 mouse bioassay (NTP 1992, Abdo et al. 1992), a statistically significant increased incidence of pulmonary alveolar/bronchiolar adenoma (a benign lesion), was observed in the females; an observed increase in the males did not reach statistical significance. No nasal tumors were observed in either sex of mice. In the F344 rat study (NTP 2000, Abdo et al. 2001), a tumorigenic response was observed in both sexes, in males an increased incidence of nasal respiratory epithelium adenoma (a benign rather than malignant lesion) and in females, olfactory epithelial neuroblastoma. The latter is a type of malignant tumor that has rarely been observed in National Toxicology Program bioassays.

The primary purpose of conducting this state-of-the-science review is to develop a consensus understanding of the scientific inferences that can (or cannot) be drawn from these studies, taking into account that they have been used as the basis for quantitative human cancer risk assessment. A second purpose is: to determine what critical scientific issues these bioassays did not (or perhaps could not) resolve; to parse these scientific issues into those that are critical to resolve for human cancer risk assessment and those that are not; and to suggest scientific studies that, if performed, could resolve these questions. Details about objectives, organization, structure and charge are reported elsewhere (Belzer et al. 2007, in this issue).

From these bioassay results, no information is available on either the cytotoxic and carcinogenic responses in these two species following naphthalene exposures below 10 ppm. Many opportunities exist for obtaining such information, primarily through the application of short-term tests to determine the mode(s) of toxic and/or tumorigenic action. Specific proposals for such investigations are presented in the reports by Bogen et al. (2007) and Brusick et al. (2007), both in this issue. While additional bioassays on naphthalene could be carried out to investigate possible carcinogenic response at doses below those

causing extensive cytotoxicity, the opportunity to contribute significant high-value information from additional whole animal bioassays appears to be limited. The expert panel expressed unanimous support for less expensive --and probably more informative --shorter-term tests such as those discussed by Bogen et al (2007, in this issue). A majority of the expert panel felt that the next phase of research should focus on the mechanisms for carcinogenicity, most specifically, on the role of cytotoxicity in the processes leading to the tumor responses that have already been observed. Determining dose levels for the onset of cytotoxic effects may facilitate understanding of the mechanisms leading to the carcinogenic responses. Ultimately, whole animal tests may be useful for confirming hypotheses about the mechanisms leading to carcinogenicity and the dose ranges in which these mechanisms operate.

The National Toxicology Program (NTP) 2-year inhalation toxicology and carcinogenicity studies of naphthalene were conducted because previous studies (Schmahl, 1955, Knake, 1956) regarding the carcinogenicity of naphthalene were considered inadequate (e.g., appropriate controls not included, small number of animals used) and because low-level human exposure was assumed to be widespread. Additionally, a bioassay for carcinogenicity of chemicals using the A/J strain of mice gave equivocal results with naphthalene. It caused an increase in the multiplicity of lung adenomas, but not in the numbers of mice bearing the tumor (Adkins et al., 1986). The whole body inhalation route of exposure was chosen by the NTP because it was believed to best represent the primary route of human exposure. A complete account for materials and methods of the NTP 2-year studies can be found in the Technical Reports (NTP 1992, 2000).

2. Mouse Bioassay

(a) Brief description of study design.

Groups of male and female B6C3F1 were exposed to atmospheres containing 0 ppm (75 mice per sex), 10 ppm (75 mice per sex), or 30 ppm (150 mice per sex) of naphthalene 6 hours daily, 5 days per

week for 103 weeks. Twice as many animals were exposed to the highest concentration of naphthalene, because of lack of information on the survival from the long-term toxic effects of inhaled naphthalene at 30 ppm. Survival and body weight were recorded, and complete histopathological evaluations were performed on all mice.

Naphthalene-exposed animals were reported to be huddled together in their cages during exposure. At the experimental exposure levels, naphthalene appeared to be sufficiently irritating that animals responded with reduced breathing rates.

(b) Results.

Survival of control males was significantly lower than that of exposed males: 37% versus 75% (10 ppm) and 89 % (30 ppm). The lower survival of controls was attributed to infighting among group-housed controls that resulted in wounds and secondary infection. Infighting did not occur among group-housed exposed males. There was no difference in survival between control and exposed females. Average weight for male controls declined from 36 g at week 36 to 33 g at week 40, and did not fully recover until week 52. Growth and survival curves are provided in NTP (2001, pp. 24, 26).

Lung tumor incidences are shown in Table 1. Although there was a positive trend, pairwise comparison of tumor incidence between exposed and control groups did not reach statistical significance at the 5% level. There was a significant positive trend in the incidence of alveolar/bronchiolar adenoma in females, but this response in the control and 10 ppm groups was lower than in historical controls. The incidence of adenomas in females exposed to 10 ppm was below that in the control group, so only the response at 30 ppm in the females reached statistical significance. There was only one female with alveolar/bronchiolar carcinoma (in the 30 ppm exposure group), and no carcinomas were observed in the control and 10 ppm groups. The incidence of alveolar/bronchiolar adenoma and combined alveolar/bronchiolar adenoma and carcinoma in the 30 ppm exposure group (22%) was significantly

greater than that of the control (8.6%). This incidence also was greater than the NTP historical control range (39/466 or 8.4%; range (0-10%), and judged by NTP to be related to exposure.

Incidences of non-neoplastic lung lesions among male and female mice are shown in Table 2. Incidences of lymphocytic infiltration and chronic and granulomatous inflammation were significantly increased in both exposed groups (10 and 30 ppm) for both males and females, compared to unexposed controls. Incidences of inflammation of the glandular epithelium were significantly increased in the 30 ppm exposure group of females only. Incidences of all other of the reported lung lesions were statistically significantly increased at both doses in both males and females.

Incidences of nasal lesions, including inflammation and olfactory epithelium metaplasia and respiratory epithelium hyperplasia, are shown in Table 3. They are statistically significant for both sexes at both doses, and for all inflammation types.

No nasal tumors were observed in the mouse.

(c) Discussion

In the judgment of the expert panel, naphthalene concentrations used in the NTP mouse study exceeded the maximum tolerated dose (MTD). The incidences of inflammation were at or near 100% at both doses in both sexes and clearly compromised their well-being (NRC 1993; p. 16). Extrapolation of results obtained above the MTD to humans has long been problematic (NRC 1993; pp. 17-18, 47, 49-51). Concentrations used in the bioassay were several thousand times greater than environmental concentrations experienced by humans (Griego et al. in this issue).

3. Rat Bioassay

(a) Brief description of study design.

Groups of 49 male and 49 female Fisher 344 rats were exposed to 0, 10 ppm, 30 ppm, or 60 ppm of naphthalene for 6 hours per day, 5 days per week for 105 weeks. The lowest exposure concentration was equal to the TLV (ACGIH 1990), and the highest exposure concentration was the highest concentration that could be achieved without condensation of naphthalene in the inhalation chamber. Survival and body weights were recorded, and complete histopathology evaluations were performed on all rats.

(b) Results.

Survival and mean body weights of exposed and control animals were similar. See Abdo et al. (2001) for more detail.

The incidence of nasal tumors is shown in Table 4. A statistically significant increase in respiratory epithelial adenoma (a benign lesion) was found at all doses in males but not in females. A statistically significant increase in olfactory epithelial neuroblastoma (a malignant lesion) was found only in the 60 ppm dose in females, but not in males. Because this increase was statistically significant and had not been seen in previous NTP studies (Haseman and Hailey, 1997), it was considered exposure related.

The incidences of non-neoplastic lesions in the nasal regions are shown in Tables 5 (males) and 6 (females). The increased incidences of tissue injuries following naphthalene exposure were statistically significant for both sexes at all doses for all effects save one (squamous metaplasia was elevated, but not significantly so, at the 10 ppm dose in both sexes). In addition, the incidence of non-neoplastic lesions was very high, and near 100% in some cases. Severity of the olfactory epithelial and glandular lesions increased with increasing exposure concentration of naphthalene (Abdo et al. 2001). Growth and survival curves are provided in NTP (1992, pp. 32, 37).

(c) Discussion.

For the same reasons as stated with respect to the mouse bioassay, the panel concludes that naphthalene concentrations used in the NTP rat study exceeded the MTD. The incidences of inflammation were at or near 100% at both doses in both sexes; this clearly compromised their well-being (NRC 1993; p. 16). Evidence of extensive nonneoplastic response in both sexes of both species indicates cytotoxicity occurred at all doses, and strongly suggests that cytotoxicity played a significant role in the tumor responses observed in the target tissues (female mouse lung, rat nose). The exposure levels used in the NTP 2-year inhalation study of naphthalene exceeded the minimum level to cause extensive cytotoxicity.

Dose selection was not preceded by short-term toxicity testing (NRC 1993; p. 16), and though the lowest dose (10 ppm) was selected to be equal to the TLV, it was also at least 3,000-fold greater than levels measured in ambient air (Price, 2007; Griego et al., both in this issue). As in the case of the mouse bioassay, interpreting these bioassay results for estimating low-dose human cancer is highly problematic.

4. Challenges Interpreting and Utilizing the NTP Bioassays

In the past four decades many hundreds of chemical agents have been tested for carcinogenicity through standardized bioassays (NRC 1993; p. 17). A substantial fraction of these bioassays have been carried out through the National Toxicology Program (the rat study for naphthalene was the 500th). Most have followed a similar design as the bioassays for naphthalene, using the same species of mouse and rat over most of the lifetime of these animals, usually a period of two years. Tests are carried out at the MTD and fractions thereof. The MTD is usually established through subchronic tests on the animals, often 90 days, and the MTD is defined as the highest dose the test animals can tolerate without experiencing more than 10% decrement in weight gain or observable toxicity, and does not alter the animals' longevity or well-being from non-cancer effects (NRC 1993; p. 16, 21). In the case of these two NTP studies the highest dose was selected based on engineering criteria.

Issues concerning extrapolation to humans of effects obtained in animal tests at MTD or greater exposures were the subject of a National Research Council review (NRC, 1993). In its report, the NRC committee concluded – even more forcefully – that whole animal bioassay has limited information value for human cancer risk assessment:

> Without accompanying information on mechanisms or results at low doses, animal bioassay results alone (i.e., without parallel data on mechanisms and dose-response relationships) do not add greatly to our ability to make regulatory decisions because of the uncertainty about the human implications of positive results in animal bioassays (p. 58).

The committee also concluded that the primary value of whole animal bioassay is the qualitative determination of whether a chemical has human carcinogenic potential, not whether it causes cancer in humans:

> The committee concludes that the MTD bioassay as currently conducted in rodents is most useful as a qualitative screen to determine whether a chemical has the potential to induce cancer. It also provides information on the carcinogenic potential of a substance at high doses and some information about the dose-response curve. It does not provide (nor was it intended to provide) all the information useful for low-dose human risk assessment. In most cases, additional information is likely to be needed to determine the extent to which the induction of cancer in rodents adequately predicts the human response and how the results of the relatively high dose assay can best be used to make inferences about the expected effects at low doses. Some of the required information might be obtainable from study of tissues from animals subjected to long-term bioassay or from ancillary studies incorporated in the rodent bioassay. Future bioassays should be designed to reveal the overall toxic responses induced by the test chemical and not just the

carcinogenic response. But in general, other information needs will require other studies (pp. 62-63).

The NRC committee developed majority and minority recommendations concerning how future whole animal bioassays ought to be better planned and conducted. Both views are helpful in the context of naphthalene:

Majority:

> **If [a] bioassay conducted with the MTD is positive, additional studies should be performed to reduce uncertainties in predicting human responses to the test material and to assist in performing quantitative risk assessments** (p. 64, bold in original).

Minority:

> **Bioassay doses should be selected by a panel of experts on the basis of a careful evaluation of studies conducted before the bioassay is initiated. Information gathered before the bioassay is conducted would include information about mechanisms of toxicity in test animals and an elucidation of the dose-response relationship for such toxicity. The [highest dose tested] should be chosen as the highest dose that can be expected to yield results relevant to humans, not the highest dose that can be administered to animals without causing early mortality from causes other than cancer** (p. 64, bold in original).

These issues and recommendations are especially important for judging the relevance of the results of the NTP bioassays of naphthalene for the purpose of human health hazard identification. First, both NTP studies utilized doses that exceeded the MTD (op. cit., Sections 2 and 3). Thus, they have interpretative problems beyond those in which the MTD is the highest dose. Second, frank cytotoxicity

was observed in most species/sex/dose combinations, and it occurred in the same target tissues as did the tumors.

Further evidence supporting a cytotoxic mechanism can be inferred from Brusick (2007, in this issue), who provided persuasive evidence that the very limited mutagenic/genotoxic effects of naphthalene observed in vitro and in vivo are mediated by cytotoxicity. This is discussed in greater detail in Bogen et al. (2007, in this issue).

5. Research Directions

Predicted tumor rates based on extrapolation from the NTP bioassays could be compared to observed rates in humans. Observed rates are likely to be substantially below the predicted rates. According to most of the available information, it can be concluded with some reasonable certainty that naphthalene does not represent a classical genotoxic carcinogen. If additional animal bioassays were performed using lower dose levels (care being taken to include at least one group that tries to duplicate the NTP results as a positive control), nonlinearities in dose-response would likely be evident. Although this might address the question of whether a linear extrapolation from the NTP bioassays reasonably accurately predicts (or closely approximates) low-dose human cancer risk, the results from such a study would not illuminate the cause of carcinogenesis observed in rats at high doses. For that reason, mechanistic research is preferred to further bioassay.

(a) Cytotoxicity.

Mechanisms other than DNA damage might account for the observed tumor responses in the NTP bioassays. The classic examples are formaldehyde, alpha-2μ globulin nephropathy and trichloroethylene, where either a species- and sex- specific mechanisms or a non-genotoxic event can be assumed to be the driving force in the eventual tumor development (NRC 1994). In general, a mechanistically oriented

research program and some additional literature review might yield a refined interpretation of the tumor data.

The most prudent and useful scientific approach to further clarify the pathogenesis of the tumors observed in rodents from supra-MTD doses, and its relationship to the potential effects of naphthalene on exposed humans, is to examine in more depth the relationship among cytotoxicity, cell proliferation, and carcinogenicity of the target cells in the respiratory tract. The cytotoxicity observed at all dose levels in the NTP bioassays implies that the bioassay results should be interpreted with much greater than usual caution as a basis for calculation of human cancer risk.

Species differences in the site of toxicity resulting from exposure to naphthalene have been observed by others. West et al. (2001) exposed mice and rats to vapor at concentration of 0-110 ppm for 4 hours. They found that inhaled naphthalene caused lung proximal airway injury in mice at concentration well below the TLV and current OSHA standard. In rats, no injury was observed in the lung at concentration as high as 100 ppm. Brown (1990) and Brown et al., (1991) observed that rats are more susceptible to epithelial tumors of the nose than are mice. In addition, the tendency of rats but not of mice to develop nasal tumors following exposure to naphthalene may be related in part to anatomic differences in the nasal passages in these two species. These differences could lead to differences in dose delivered at this site (Swenberg et al., 1985). Rates of production and clearance of the active metabolite of naphthalene by nasal epithelia and lung could also account for species differences in sites of tumors. Another plausible explanation for species difference in sites of naphthalene toxicity and carcinogenicity comes from the results of a study by Baldwin et al. (2004). They compared pulmonary and nasal expression levels of the cytochrome P450 enzyme, CYP2F, in mice, rats and monkeys. Naphthalene is metabolized by CYP2F to the active metabolite 1,2-epoxide, which may be the moiety responsible for cytotoxicity. Mice expressed 4-8-fold greater CYP2F in lung than rats. The nasal olfactory epithelium contained the highest amount of

P450 of all tissues studied in rats. Of all tissues studied in monkeys only the nasal ethmoturbinates contained quantifiable amounts of CYP2F, which was roughly 1/10th to 1/20th of the corresponding tissues in rats and mice, respectively. These issues are discussed in detail in Bogen et al. (2007, in this issue).

(b) Pharmacokinetic, biochemical and gene expression research.

Pharmacokinetic data are needed for rodents and humans. Butadiene is one case where differences in metabolism among species suggests strongly that the murine sensitivity results from high production of the diepoxide compared to humans and rats. The butadiene literature should be consulted for relevant information that may provide insight into inter-species differences among various tissues that carry significant capacity to metabolize naphthalene (Henderson et al., 1996, 1999, 2001; Dahl and Henderson, 2000; Thornton-Manning, et al. 1996, 1997a, 1997b, 1998). Determining isoforms of P450 involved may provide insight as to interspecies differences in sensitivity. The investigators should be aware that the dose/effect curve could be one where lower doses are relatively more potent. This can happen, for example, if two activation steps are required, and the procarcinogen competes for the enzymes that carry out the second step.

Toxicity and mutagenicity of the individual naphthalene oxide stereoisomers should receive further study to determine if different tissues produce and metabolize them at different rates. Such information could be used to determine tissue origin of metabolites in vivo.

Microarrays should be used at various stages of toxicity and carcinogenicity to determine which genes are being affected. The investigation should look for epigenetic mechanisms on changed gene expressions as appropriate. Lung preneoplastic lesions should be examined for gene expression changes.

(c) Determining doses that do not cause cytotoxicity.

Further research on naphthalene toxicity/carcinogenicity could include a series of short-term *in vivo* studies to determine the concentration of inhaled naphthalene that clearly does not induce histologic evidence of cytotoxicity in the respiratory tract of laboratory animals. A practical approach would be to begin with short-term inhalation studies concentrating on morphologic changes in the nasal mucosa of rats and the airway epithelium of mice, then use data from these studies to perform similar short-term studies in nonhuman primates. An important initial challenge will be the development and validation of an inhalation exposure generation and monitoring system which accurately and consistently generates and monitors exposure chamber atmospheres at naphthalene concentrations above and below the levels at which cytotoxicity is detectable in various species.

The initial rodent studies should emphasize characterization of subtle morphologic changes in the respiratory and olfactory nasal epithelium of naphthalene-exposed versus air control rats at the light microscopic level, and similar determinations for effects on the airway epithelium of naphthalene-exposed mice with emphasis on effects on Clara cells.

The importance from a regulatory as well as a scientific standpoint of the olfactory neuroblastomas induced in rats in the chronic naphthalene bioassay suggests that a valuable early step would be to more clearly identify the cell of origin of these tumors and to clarify the pathogenesis of the process from cytotoxicity to neoplasia of the olfactory epithelium. There is information in the literature indicating that the sustentacular cells, not sensory neuroepithelial cells, are the immediate target cells for the toxic effect of some chemicals on the olfactory mucosa, and that the horizontally oriented “dark” basal cell is the stem cell responsible for regeneration of sensory cells as well as sustentacular cells (Moulton, 1974; Graziadei and Graziadei, 1979). Primary vacuolation and degeneration of sustentacular cells followed by loss of sensory cells was demonstrated in single oral dose studies with a Type IV phosphodiesterase inhibitor candidate drug (Pino et al., 1999). Loss of sensory cells was followed by basal cell hyperplasia by 48

hours post-exposure. This same drug, when given via inhalation in a 2-year study, induced tumors classified as neuroblastomas in the nasal cavity of rats. These authors described the neuroblastomas as being present in a background of atypical basal cell hyperplasia and other lesions similar to those elegantly described and illustrated by Long et al. (2003) in the naphthalene rat inhalation bioassay performed by NTP.

The illustrations and descriptions in the papers by Pino et al. and in the NTP chronic study by Long et al. suggest that the cells undergoing atypical hyperplasia in the olfactory epithelium in both these studies are the "dark" basal cells, precursors of sustentacular and light basal cells. It would be informative to do short term studies on naphthalene in rats, initially via oral exposure as was done in Pino's work, then by inhalation, to more clearly identify a range of exposure concentrations and durations to naphthalene that induces these sustentacular cell lesions followed by sensory cell degeneration. These data will provide valuable information in setting target concentrations for longer-term studies to determine a range of exposure concentrations that do and do not induce cytotoxicity in the form of sustentacular degeneration followed by sensory loss and basal cell hyperplasia. These same initial inhalation exposures would provide tissues to study the earliest stages of metabolic activation, gene expression, transcriptomics, and other tools using laser capture microdissection and other techniques.

Once the critical determination of no cytotoxic effect (NOEL, "no observed effect level") naphthalene concentrations for these sites are determined, the next steps should involve utilization of the most valuable research tools described and proposed at the symposium to compare and contrast the morphologic, biochemical, and molecular characteristics of cells and tissues at the target sites from naphthalene-exposed animals with unexposed controls at various concentrations above and below the no-effect level, at various durations of exposure and following recovery from exposure.

(d) Animal studies.

As discussed above in Section 4, the NTP bioassays were conducted with airborne naphthalene concentrations that exceeded the MTD. The available data also do not allow establishing a NOAEL for non-carcinogenic endpoints. Following further research as discussed above in this Section, a 40-52 week study in F344/N rats could be performed at atmospheres of naphthalene containing 0, 0.01, 0.1, 1.0, and 10 ppm of naphthalene, with interim monthly sacrifices of 5-10 rats per exposure group and 25 rats at termination. Nasal tissue should be examined microscopically for the presence of atypical olfactory hyperplasia, and tissue samples might be made available for other researchers to investigate for biomarkers of toxicity and carcinogenicity of naphthalene, such as DNA adducts or metabolites of naphthalene, as well as for changes in DNA of affected cells and inhibition of apoptosis in the olfactory epithelium.

The highest concentration recommended is the same as the lowest concentration used in the NTP studies and is included to determine whether the NTP results observed at this level are reproducible. The lowest level recommended is approximately that of high levels of naphthalene in ambient air: in indoor air from consumer products such as mothballs, and in some occupational settings.

The objectives of this study are to generate possibly critical whole-animal data to inform and validate in whole animals the mechanistic studies proposed elsewhere (Bogen et. al., 2007; Brusick et al., 2007). For example, it would be useful to know the dose-response, including the no-observable effect level (NOEL) in the rat nose for biomarkers of effect and sensitivity that are thought to be determining events in the tumor development that was observed at exposure levels exceeding the MTD. Given the fact that there are potentially important differences in naphthalene metabolism and toxicity between rodents and primates (Bogen et al. 2007; in this issue), data from a limited number of primate models would be useful for calibrating these species differences. Elucidation of interspecies differences in cytotoxicity and the pharmacokinetics and pharmacodynamics of the naphthalene metabolites responsible for cytotoxicity and

for lesions will be needed for understanding how responses in rodents can be used for quantitative assessment of the tumor responses in primates, including humans.

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Table 1:

Lung Tumor Incidence in Mice in the 2-Year Inhalation Study of Naphthalene (National Toxicology Program (NTP), 1992)

Dose	0 ppm	10 ppm	30 ppm
MALES			
Alveolar/Bronchiolar Adenoma [a]	7/70 (10%)	15/69 (22%)	27/135 (20%)
Alveolar/Bronchiolar Carcinoma [a]	0/70 (0%)	3/69 (4%)	7/135 (5%)
Alveolar/Bronchiolar Adenoma or Carcinoma [a]	7/70 (10%)	17/69 (25%)	31/135 (23%)
FEMALES			
Alveolar/Bronchiolar Adenoma [a]	5/69 (7%)	2/65 (3%)	28/134 (21%)**
Alveolar/Bronchiolar Carcinoma	0/69 (0%)	0/65 (0%)	1/134 (1%)
Alveolar/Bronchiolar Adenoma or Carcinoma [a]	5/69 (7%)	2/65 (3%)	29/134 (22%)**

Incidence expressed as number of animals with the lesion divided by number of animals examined.

[a] Positive trend ($p < 0.001$)
** Greater than control ($p < 0.01$)

5-year historical rate for all lung tumors combined in NTP bioassays:
males: 94/478 (20%; range 10 % - 30%);
females: 39/466 (8%;'range 0% -10%)

Table 2:

Non-neoplastic Lung Lesion Incidence in Mice in the 2-Year Inhalation Study of Naphthalene (National Toxicology Program (NTP), 1992)

Dose	0 ppm	10 ppm	30 ppm
MALES			
Infiltration cellular, histiocyte [a]	1/70 (1%)	12/69 (17%)**	16/135 (12%)**
Inflammation, chronic [a]	0/70 (0%)	21/69 (30%)**	56/135 (41%)**
Inflammation, granulomatous	0/70 (0%)	19/69 (28%)**	15/135 (11%)**
FEMALES			
Infiltration cellular, lymphocyte [a]	11/69 (16%)	21/65 (33%)*	46/134 (34%)**
Inflammation, chronic [a]	3/69 (4%)	13/65 (20%)**	52/134 (39%)**
Inflammation, granulomatous [a]	0/69 (0%)	38/65 (59%)**	42/134 (31%)**
Glands, inflammation [a]	1/69 (1%)	3/65 (5%)	15/134 (11%)**

Incidence expressed as number of animals with the lesion divided by number of animals examined.

[a] Significant positive trend ($p < 0.01$)
* Greater than control ($p < 0.05$)
** Greater than control ($p < 0.01$

Table 3:
Nasal Inflammation Incidence in Mice in the 2-Year Inhalation Study of Naphthalene (National Toxicology Program (NTP), 1992)

Dose	0 ppm	10 ppm	30 ppm
MALES			
Inflammation [a]	0/70 (0%)	67/69 (97%)**	133/135 (99%)**
Olfactory epithelium, metaplasia [a]	0/70 (0%)	66/69 (96)**	134/135 (99%)**
Respiratory epithelium, hyperplasia [a]	0/70 (0%)	66/69 (96%)**	134/135 (99%)**
FEMALES			
Inflammation [a]	1/69 (1%)	65/65 (100%)**	134/134 (100%)**
Olfactory epithelium, metaplasia [a]	0/69 (0%)	65/65 (100%)**	134/134 (100%)**
Respiratory epithelium, hyperplasia [a]	0/69 (0%)	64/65 (100%)**	134/134 (100%)**

Incidence expressed as number of animals with the lesion divided by number of animals examined.

[a] Significant positive trend ($p < 0.001$)
** Greater than control ($p < 0.001$)

Table 4:
Nasal Tumor Incidence in Rats in the 2-Year Inhalation Study of Naphthalene (National Toxicology Program (NTP), 2000)

Dose	0 ppm	10 ppm	30 ppm	60 ppm
MALES				
Respiratory Epithelial Adenoma [a]	0/49 (0%)	6/49 (12%)**	8/48 (17%)**	15/48 (31%)**
Olfactory Epithelial Neuroblastoma [b, c]	0/49 (0%)	0/49 (0%)	4/48 (8%)	3/48 (6%)
FEMALES				
Respiratory Epithelial Adenoma	0/49 (0%)	0/49 (0%)	4/49 (8%)	2/49 (4%)
Olfactory Epithelial Neuroblastoma [a, c]	0/49 (0%)	2/49 (4%)	3/49 (6%)	12/49 (24%)**

Incidence expressed as number of animals with the lesion divided by number of animals examined.

[a] Significant positive trend ($p < 0.001$)
[b] Significant positive trend ($p = 0.027$)
[c] 5-year historical rate in controls: 0/299.
** Greater than control ($p \leq 0.013$)

Table 5:

Non-neoplastic Nasal Lesion Incidence in Male Rats in the 2-Year Inhalation Study of Naphthalene (National Toxicology Program (NTP), 2000)

Dose	0 ppm	10 ppm	30 ppm	60 ppm
Olfactory Epithelium				
Hyperplasia, atypical	0/49 (0%)	48/49 (98%)**	45/48 (94%)**	46/48 (96%)**
Atrophy	3/49 (6%)	49/49 (100)**	48/48 (100)**	47/48 (98%)**
Inflammation, chronic	0/49 (0%)	49/49 (100%)**	48/48 (100)**	48/48 (100%)**
Respiratory Epithelium				
Hyperplasia	3/49 (6%)	21/49 (43%)**	29/48 (60%)**	29/48 (60%)**
Metaplasia, squamous	0/49 (0%)	15/49 (31%)**	23/48 (48%)**	18/48 (38%)**
Goblet cell, hyperplasia	0/49 (0%)	49/49 (100%)**	29/48 (60%)**	26/48 (54%)**
Glands				
Hyperplasia	1/49 (2%)	49/49 (100%)**	48/48 (100%)**	48/48 (100%)**
Metaplasia, squamous	0/49 (0%)	3/49 (6%)	14/48 (29%)**	26/48 (54%)**

Incidence expressed as number of animals with the lesion divided by number of animals examined.

** Significantly greater than control ($p < 0.01$).

Table 6:

Non-neoplastic Nasal Lesion Incidence in Female Rats the 2-Year Inhalation Study of Naphthalene (National Toxicology Program (NTP), 2000)

Dose	0 ppm	10 ppm	30 ppm	60 ppm
Olfactory Epithelium				
Hyperplasia, atypical	0/49 (0%)	48/49 (98%)**	48/49 (98%)**	43/49 (88%)**
Atrophy	0/49 (0%)	49/49 (100%)**	49/49 (100%)**	47/49 (96%)**
Inflammation, chronic	0/49 (0%)	47/49 (96%)**	47/49 (96%)**	45/49 (92%)**
Respiratory Epithelium				
Hyperplasia	0/49 (0%)	18/49 (37%)**	22/49 (45%)**	23/49 (47%)**
Metaplasia, squamous	0/49 (0%)	21/49 (43%)**	17/49 (35%)**	15/49 (31%)**
Goblet Cell, hyperplasia	0/49 (0%)	16/49 (33%)**	29/49 (59%)**	20/49 (41%)**
Glands				
Hyperplasia	0/49 (0%)	48/49 (98%)**	48/49 (98%)**	42/49 (86%)**
Metaplasia, squamous	0/49 (0%)	2/49 (4%)	20/49 (41%)**	20/49 (41%)**

Note: incidence express as No. of animals with the lesion/No. of animals examined.

** Greater than control ($p < 0.01$)

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Available data on naphthalene exposures: Strengths and limitations ☆

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Abstract

Exposures to naphthalene occur from a number of sources. Data on these exposures have been reported by a number of researchers; however, these data have not been organized into a consistent framework. In addition, while naphthalene has been identified as an animal inhalation carcinogen there has been no attempt to define the ranges of long-term average inhalation exposures for the general population or sub populations that are relevant to the assessment of the risk of the occurrence of chronic effects. This paper attempts to organize the available data on naphthalene levels in air and provide ranges for the long-term average concentrations experienced by different populations. The paper also discusses the limitations of available data and identifies areas for future research.

Keywords: Naphthalene; Exposure; Epidemiology; Cancer incidence; Exposure sources; Consumer products; Industrial products; Pesticidal products; Cigarettes; Long-term average exposure concentrations

1. Background

Naphthalene has been the subject of a number of exposure and risk assessments over the last 26 years (EPA, 1980,1982,1984,1988a,1988b,2002; ATSDR, 2005, and Preuss et al., 2003). The purpose of this document is to summarize the findings of these assessments and other recent publications and present a description of what is known and unknown about human exposure to naphthalene. The exposures estimates developed in this report focus on the chronic inhalation exposure levels to naphthalene that are relevant to the evaluation of chronic effects and give less attention to dermal and oral exposures and acute inhalation exposures. The assessment considers occupational exposures and exposure from environmentally mediated sources, indoor sources, and consumer products. (This report does not review data on exposures from the abuse of naphthalene such as sniffing or ingesting mothballs.)

The approach used in this report is to use the readily available data to characterize the sources of exposure that affect the general U.S. population and then to evaluate the sources that affect specific sub-populations. This approach is used in the Voluntary Children's Chemical Evaluation Program (VCCEP) for the chemicals acetone, benzene, and xylenes (ACC,2003,2005,2006). Within this framework the available data are evaluated and where possible, conclusions are generated on the range of chronic exposures to naphthalene that occur in the general populations and the identified sub-populations. The limitations of the data are discussed and areas where additional research could be performed are identified.

2. Naphthalene's relevant chemical properties

Naphthalene is a white solid that readily sublimes at room temperature. Its chemical structure consists of two fused benzene rings. Naphthalene is the simplest member of the polycyclic aromatic hydrocarbons (PAHs). A summary of the compound's properties is given in Table 1.

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Table 1
Select properties of naphthalene

Molecular weight	128.19 g/mole
Color	White
Physical state	Solid
Melting point	80.5 °C
Boiling point	218 °C
Density at 20 °C	1.145 g/mL
Odor	Strong (tar or mothballs)
Odor threshold	
Water	0.021 mg/L
Air	440 μg/m^3
Solubility	
Water at 25 °C	31.7 mg/L
Organic solvents	Soluble
Partition coefficients	
Log Kow	3.29
Log Koc	2.97
Vapor pressure	0.087 mmHg

Source: ATSDR (2005).

As these properties indicate, the compound is a semivolatile with a saturated air concentration of 600,000 μg/m^3. In most indoor and outdoor environments the compound is encountered as a vapor. In cigarette smoke the compound is found in the particulate phase. In indoor environments naphthalene tends to partition to indoor surfaces. This partitioning tends to lower peak concentrations and increase the duration of exposure.

Naphthalene is rapidly removed from air by reaction with hydroxyl radicals. The half life in air is 8 h (Bernhard and Simonich, 2000; Lu et al., 2005). Naphthalene has a short half life (<7 days) in surface waters and soils due to biodegradation and photolysis (ATSDR, 2005).

Naphthalene has been found to occur in ground water under anaerobic conditions where there is an on-going source such as the presence of petroleum products in the aquifer. Naphthalene does not bioaccumulate to any extent due to the ability of organisms to metabolize the compound. Naphthalene is found in certain foods at low levels and higher levels can occur in foods that are smoked or charbroiled. Given the low levels in food EPA (2002) has stated that inhalation exposure is the dominant route of exposure for naphthalene for the average individual. Given the rapid removal of Naphthalene from air, exposures to naphthalene occur at locations that are proximate to the sources of release in both time and space.

3. Sources of naphthalene exposures

Naphthalene is a product of incomplete combustion of biomass and petroleum products Preuss et al., 2003; Schauer et al., 2001. The compound is also found in wood smoke, cigarette smoke, and is generated during the grilling or charbroiling of meats (ATSDR, 2005). Naphthalene is also found in a number of commercial and consumer products. The following Table 2 presents the levels of naphthalene in petroleum products. As the table indicates, the naphthalene occurs in at levels of roughly 0.25% in many petroleum products and at higher levels in jet fuels. Naphthalene is also a component of asphalt (ATSDR, 2005). Use of asphalt on roads results in long-term sources of release for naphthalene (Lu et al., 2005).

Table 2
Concentration of naphthalene in petroleum products

Petroleum product	Range in weight %	Average weight %
Gasoline	0.15–0.36	0.25
Diesel fuels (#2)	0.01–0.80	0.26
Kerosene	0.15–0.46	0.31
Jet Fuel-4	0.25–0.50	0.25
Jet Fuel-5	*	0.57
Jet Fuel-7	*	0.72
Jet Fuel-8	*	1.1
No. 2 Fuel oil	0.009–0.40	0.22
No. 6 Fuel oil	0.0021–0.015	0.0042
Lubricating and motor oils	0.00005–0.25	0.059

* Single data point. Source: Potter and Simmons (1998).

Table 3 presents a summary of information on naphthalene in consumer products. The products fall into two categories. The first group includes products where naphthalene is a component of a hydrocarbon mixture used as a fuel additive or as a carrier in pesticides. These products would not be anticipated to contribute significantly to indoor air levels since they are used outside of the house and have a low potential for dermal contact.

The second group is the pesticide products in which naphthalene is an active ingredient to control insects that damage clothing or to repel pests. In addition, the Internet is replete with guidance recommending the use of naphthalene mothballs to repel skunks and raccoons from attics, basements, crawlspaces, and gardens.[1]

Naphthalene is released into the atmosphere from a number of industries including coking, petroleum refining, aluminum production, wood treatment (creosote), industries that use asphalt (paving and roofing), chemical manufacturing plants that use naphthalene as a raw ingredient, and industries using petroleum products (in particular jet fuels).

4. Measurement of naphthalene in air

ATSDR, 2005 and Preuss et al., 2003 have summarized the majority of air data available before 2003. In this report we have taken these data and organized the information into the following framework.

Ambient levels:

- Naturally occurring levels (pristine areas);
- Levels in suburban/urban areas

[1] See http://www.ghorganics.com/page6.html, http://www.canr.uconn.edu/ces/ipm/homegrnd/htms/8racoon.htm, http://solutionsforyourlife.ufl.edu/hot_topics/environment/nuisance_wildlife.html (Web pages accessed October, 2006).

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Table 3
Levels of naphthalene in various consumer products

Brand	Category	Form	Percent
STP gas treatment	Auto products	Liquid	1–2
STP octane performance booster	Auto products	Liquid	1–2
STP oxygenated gas treatment	Auto products	Liquid	0–3
STP super concentrated fuel injector cleaner	Auto products	Liquid	0–2
STP gas treatment-08/03/2000	Auto products	Liquid	0–2
STP complete fuel system cleaner	Auto products	Liquid	21–42
STP fuel injector/carburetor cleaner	Auto products	Liquid	0–2
STP fuel injector/carburetor cleaner-08/07/1998	Auto products	Liquid	0–2
STP gas treatment-09/30/1998	Auto products	Liquid	0–2
STP octane performance booster-11/13/1998	Auto products	Liquid	0–2
STP oxygenated gas treatment-12/24/1998	Auto products	Liquid	1–2
STP super concentrated intake valve cleaner	Auto products	Liquid	0–2
STP fuel injector/carburetor cleaner-01/18/2000	Auto products	Liquid	0–2
STP fuel injector and carburetor treatment 12 Fl. Oz.	Auto products	Liquid	0–2
Spectracide brush killer concentrate	Pesticides	Liquid	0.7
Enoz Old Fashioned Mothballs	Pesticides	Solid	99.9
Repel pet and stray repellent	Pesticides	Powder	16.0
Snake away	Pesticides	Granules	7.0
Bonide mosquito beater granules	Pesticides	Granules	4.50
Bonide shotgun rabbit & dog repellent	Pesticides	powder	4.50
Monsanto Lasso Herbicide (agricultural)	Pesticides	Liquid	0.09–0.15

NLM household products database and packaging information for specific products.

Indoor air levels:

- Homes
- Homes with naphthalene products

Occupational Exposure Levels:
Low exposure industries

- Chemical industry
- Refineries
- Asphalt
- Aluminum production

High exposure industries

- Wood treatment (creosote)
- Coke production
- Jet fuel related

For each of these categories of exposure sources, a range for the long-term average levels is proposed based on available data. As discussed below, the available data has a number of limitations that preclude the estimation of upper bounds of exposure for each source of estimates or the fraction of the exposed populations receiving a specific dose.

5. Estimating long-term average exposure concentrations

In this section, a range of exposures are proposed for each of the sources. This range is intended to describe both the uncertainty and the interindividual variation in the long-term average concentrations to which individuals in the relevant populations are exposed. The range is described using a log scale of concentration in units of ½ of log 10 $\mu g/m^3$ (1, 3, 10, 3, 100, ...). The goal in this effort is capture the sense of the available data and to place the data into an ''order of magnitude'' framework to present each source of exposure's relative importance clearly. As new data are developed these assigned ranges may change.

5.1. Levels of naphthalene in pristine air

A number of studies have reported naphthalene levels in locations other than urban areas. Naphthalene is expected to occur at low levels in pristine air because of natural sources (biomass burning). Based on data from White and Hardy (1994) and Hoff and Chan (1987) a range of 0.0001–0.003 $\mu g/m^3$ is proposed for a long-term average concentration in pristine air.

5.2. Levels of naphthalene in suburban and urban air

Studies of naphthalene in urban air have reported that levels have high spatial and temporal variability (Reisen, 2003 and Reisen and Arey, 2005). Elevated levels are directly related to traffic with the highest levels being reported in highway tunnels and areas with high vehicular traffic (Lu et al., 2005; ATSDR, 2005). Most of the data in the literature on levels in the U.S. predate current controls on vehicles and gasoline reformulation and may be overestimates of current exposures. Lu et al., reports modeling of naphthalene levels in the Los Angeles basin that range from 0.0 to 0.5 $\mu g/m^3$ in the summer and 0–1.0 $\mu g/m^3$ in winter. These predictions were shown to be reasonably consistent with recent measurements. Because of the lim-

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ited relevant monitoring data for areas outside of Southern California, it is difficult to provide an estimate of current range of annual averages of naphthalene in the different suburban and urban areas of the U.S. However, because Southern California is known to be representative of urban areas with air pollution problems, the levels reported by Lu et al. can be taken as a conservative estimate for the U.S. urban and suburban air concentrations. A range of 0.001–1 μg/m^3 is proposed for levels in suburban and urban air.

5.3. Levels of naphthalene in vehicles

Naphthalene levels in vehicles can be elevated by the vehicle's or other vehicles' exhaust entering the cabin. Lu et al. (2005) cites Batterman et al. (2002) that naphthalene concentration of 1.2 μg/m^3 in buses in Detroit, Michigan. ATSDR cites an average level from Löfgren et al., 1991 of 4.5 μg/m^3 (ATSDR, 2005). Lu et al. has suggested that based upon data on the elevation of benzene and other aromatics in vehicles, vehicle air levels for naphthalene be set at three times the ambient levels. Based on this recommendation, vehicle levels are estimated to range from 0.003 to 3 μg/m^3.

5.4. Levels of naphthalene in indoor air

Very limited data are available on naphthalene in indoor air in the US. The data are at most 24-h average samples and may not be reflective of long-term average exposures. However, the data clearly indicate that residential indoor air levels are higher than ambient levels. This finding is reasonable since a number of activities can result in releases of naphthalene to the indoor environment. These include:

- Smoking;
- Kerosene space heaters;
- Wood stoves;
- Emissions from vehicles and stored petroleum products in attached garages;
- Cooking; and
- Use of products containing naphthalene (mothballs).

The available data suggest that indoor air levels are likely to fall into the range of 0.1–10 μg/m^3. Appendix A of this report presents a one-compartment model of residential air concentrations. This model demonstrates that modest releases (0.2–20 g/y) from these sources of naphthalene are capable of causing levels in this range.

5.5. Levels of naphthalene in indoor air from use of naphthalene products

The list of products in Table 3 are all intended to be used in a garage or outside of the home with the exception of products designed to control insect damage (mothballs). Mothballs are designed to be used in enclosed spaces (boxes, bags, or closets) and in these closed spaces the vapor builds up and suppresses insects. However such containers are not vapor-proof and act as continuous sources of naphthalene in indoor air. Zhu et al. (2003) reported that levels of 0.3–13 μg/m^3 in bedrooms adjacent to closets containing mothballs. Such products can also cause exposures by both dermal contact and elevating levels in the individuals' breathing zone when wearing clothing or using bedding that was treated with mothballs. The levels of exposures from these long-term sources are not well documented.

There is also evidence of instances where mothballs are used as area fumigants to control a wide range of pests. Such off-label uses have the potential to cause higher exposures since large amounts are placed on open trays in attics or other portions of the home. ATSDR cites Linick (1983) as reporting a measurement of 100 μg/m^3 in one home where mothballs were misused. This level was measured after remediation and likely underestimates actual exposures. Based on the available data and the estimates provided in Appendix A, homes using mothballs are estimated to have long-term levels that range from 1 to 100 μg/m^3 for typical use and 10–300 ug/m^3 for misuse scenarios.

5.6. Levels of naphthalene in other indoor environments

Data on naphthalene in day care centers were reported by Wilson et al. (1999). The mean level measure in 9 day care samples was 0.2 μg/m^3. No other data were identified for other indoor environments. Because of this limited data no estimates were made for other indoor environments.

5.7. Occupational levels of naphthalene

Elevated levels of naphthalene in the workplace have been shown to occur in a number of industries. In general the amount of data in various industries is not large and varies in quality. In many instances the data are based on short term grab samples.

The level of exposures varies greatly from industry to industry. Preuss et al. (2005) reported that 3 hr grab samples in coking and coal tar industries ranged from <1 to 703 μg/m^3, with higher levels occurring in the coal tar and coking industries than in the product of refractory materials or graphite electrodes. Data on exposure to naphthalene in military personnel exposure to jet fuel was reported by Egeghy et al. (2003). Levels for task involving a high potential for exposure ranged from 12 to 3900 μg/m^3. Preuss et al. (2003) presented a summary table for occupation exposure information in the U.S. and Europe.

Based on these data the daily exposures to naphthalene appear to fall into two ranges. For individuals in the following industries, daily exposures (8 h TWA) fall into a range of 10–300 μg/m^3. Converting this data to 24 h seven day a week exposures drops the range by a factor of roughly 3. Thus the chronic exposures would be expected to range from 3 to 100 μg/m^3.

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- Refining and petroleum industries;
- Asphalt industries paving and roofing;
- Industries that use pitch to manufacture refractory materials or graphite electrodes;

Levels in the following industries' daily exposures fall into a higher range of 100 to 3000. Converting this data to 24 h seven day a week exposures drops the range by a factor of roughly 3. Thus the chronic exposures would be expected to range from 30 to 1000 μg/m^3.

- Creosote production and use;
- Workers exposed to jet fuels;
- Coal tar and coke industries;
- Production of naphthalene from coal tar;
- Production of mothballs; and
- Chemical industries that use naphthalene as a raw material.

6. Summary of existing data and general conclusions

Fig. 1 in Griego et al. (2007), presents a summary of the levels of naphthalene that the general U.S. population experiences outdoors, indoors, and in vehicles and the levels experienced by specific sub-populations. The exposure sources in the first four categories in the chart affect the general population. The remaining categories present levels that affect only a small portion of the U.S. population. Fig. 1 intentionally uses a symbol of a cloud to emphasize the uncertainty in the range of levels for the different sources and the limitations in the available data.

Fig. 1 also includes the range of annual average air concentrations that correspond to the exposure levels used in the NCI bioassays. The bioassays exposed mice and rats to levels of 10–60 ppm for 6 h/d and 5 d/week. This corresponds to a 24 h 7 d average concentrations of 9000–56,000 μg/m^3.

A number of general observations can be made on the patterns of exposure to naphthalene in the U.S. population.

1. Background exposures to naphthalene for most individuals are a function of both outdoor and indoor exposures with indoor sources making a greater contribution for at least some individuals.
2. Mothballs are a poorly characterized but significant source of exposure. This source has the greatest potential to result in exposures greater than 10 μg/m^3 for children and for adults outside of the workplace.
3. Occupation exposures are the dominate source of exposure for workers in affected industries.
4. Occupational exposures are 10- to 100-fold lower than the air concentrations used in the animal bioassays. Exposures to indoor and outdoor sources are 3000 to 10,000-fold lower than the air concentrations in the bioassays.
5. Biomonitoring studies need to recognize that dermal and oral sources of naphthalene may contribute to total body burdens in individuals with low inhalation exposures (Wilson et al., 1999).
6. Exposures to environmental sources of naphthalene are variable over a day and may have a strong seasonal variation (Lu et al., 2005). Low level dose-response models may need to take this variation into consideration.

7. Cigarettes and naphthalene exposure

Direct exposure to naphthalene occurs from the use of tobacco products. Estimates of the inhalation dose per cigarette are reported to range from 0.3 to 4 ug (Preuss et al., 2003). For a smoker consuming a pack a day this would correspond to a dose of 6–80 μg. A 24 h exposure to air levels of 0.3–4 μg/m^3 would produce a similar dose range in adults. This suggests the cigarette smokers receive doses that are similar in magnitude to the doses that they receive from both outdoor and residential exposures. While no studies have been made for naphthalene, Wallace (1996) concluded that the dominant source of exposure to benzene for smokers is direct exposure rather than environmental tobacco smoke. A similar finding would be expected for naphthalene. The effects from exposure to naphthalene in cigarette smoke may be different than exposures to naphthalene from other sources since naphthalene in cigarette smoke is reported to be bound to particles and does not occur as a free vapor.

8. Potential areas for exposure research

Data on naphthalene exposures are limited. Prior to the findings of carcinogenic activity in rodents, naphthalene was regarded as a widespread contaminant in air that was of minimal concern. As a result naphthalene was rarely the focus of specific studies. Naphthalene was included in surveys as a compound that was detectable with broad spectrum analytical techniques, as one of the components of petroleum products, or as one of the PAHs. In many instances data collected on naphthalene were not thoroughly analyzed and were included only for the sake of completeness[2]. This has led to a relatively small amount of information on the compound in the published literature and few systematic attempts to evaluate the range and variation in chronic exposures in the U.S. populations.

Second, naphthalene levels are highly variable over time and space. Variation in sampling protocols (duration of the sampling, exact location in the home or in the city where the air sample is taken and the time of day the sample is taken) can influence the findings of a survey. The air monitoring data for naphthalene often consists of grab samples

[2] See the lack of analysis of the naphthalene data in Chuang et al., 1999 and Wilson et al., 1999.

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of varying durations analyzed using different analytical methods.

Third, the ranges of values reported by individual studies are not likely to be representative of the ranges of long-term average exposures of individuals in the sampled environments. The reason for this is that variation in long-term average exposures are driven by factors such as variation in duties over a career in an industry, job tenure, population mobility, and long-term patterns in consumer behavior that are not captured by short term samples. For these reasons the range and variation in the reported measurements should not be used as an indication of the range of chronic exposures to naphthalene in the general population or in any of the sub populations. However, the central tendency of the data set for the different sources maybe appropriate for making inferences on the relative magnitude of the typical chronic exposures.

A final problem with the data on suburban and urban levels of naphthalene is that older studies are likely to overestimate current exposures. Air levels in urban and suburban areas are believed to be driven in large part by vehicle emissions. Since naphthalene is an aromatic compound it contributes to photochemical smog and ozone formation. Programs to reduce emissions of aromatics such as the new vehicle standards and use of reformulated gasoline in many cities have resulted in a reduction in aromatics such as benzene and naphthalene levels in the last 10 years (ACC, 2006; Lu et al., 2005). Evidence of the impact of such programs can be seen in Shauer et al. (2002) who reported that current catalytic converters decreased levels of naphthalene by a factor of 50 in automobile exhaust. Because of these changes, levels of naphthalene reported before 2000 may not be representative of current U.S. levels (Lu et al., 2005).

Because of these issues the following areas for exposure research are suggested:

1. Identify, collect, and organize existing data on naphthalene. These data should be used to confirm where data gaps exist for the compound and how the future studies, surveys, or modeling projects should be designed.
2. Establish regular surveys of levels in major urban areas to determine what are the current levels of exposures.
3. Perform surveys of the use of consumer products containing naphthalene and cooking and heating practices that release naphthalene into indoor air.
4. Include naphthalene in future surveys of indoor air pollutants and develop targeted surveys for homes that use naphthalene containing products.
5. Consider the development of chronic residential exposure models based on the models used for residential pesticides.
6. Perform additional occupational monitoring surveys for naphthalene related industries.
7. Consider developing a PBPK model in humans that would allow the determination of the doses associated with biomarker values.

The above areas are critical for both the evaluation of the risks posed by naphthalene and the design of risk management strategies for the various sources and populations. The importance of specific research projects; however, will be dependent on the hazard findings for the compound. For example, if chronic exposures to levels below 10 ug/m^3 are deemed to be of low concern, then research related to exposures from outdoor sources may not be important.

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Appendix A. Modeling residential levels of naphthalene in indoor air

Approximate long-term average indoor air levels can be used to estimate the strength of sources in indoor air. The relationship between the mass of a compound released to indoor air and average levels can be approximated by modeling the house as a single compartment. Using this assumption the indoor air concentration can be determined by the following equation:

Indoor Air Concentrations = Average Outdoor Air Concentration + S/(Q * V * 24) where S is the mass of the compound emitted into the residences air from a source in a day, Q is the hourly air exchange rate, and V is the volume house.

In this assessment the value of Q and V are taken from EPA's Exposure Factors Handbook estimates for the average U.S. residence.

$$Q = 0.63\ \mathrm{h}^{-1}$$

$$V = 369\ \mathrm{m}^3$$

Using this model the amount of naphthalene required to increase indoor air concentrations by various amounts can be calculated. Table A1 presents the amounts associated with different indoor air concentrations.

A single box of moth balls (Enoz Old Fashioned Mothballs) contains 396 g of naphthalene. Thus one box of mothballs evenly and completely released is sufficient to raise the indoor air concentration to an average level of approximately 200 μg/m^3 in a residence for a period of one year. In smaller homes or homes with lower air exchange rates the amounts required to produce this concentration would be less.

Table A1
Naphthalene source strength and average indoor air levels

Outdoor air concentration μg/m^3	Indoor air concentrations μg/m^3	Source μg/d	Source g/yr
0.1	0.3	1100	0.4
0.1	1	5000	2
0.1	3	16000	6
0.1	10	55000	20
0.1	30	170000	60
0.1	100	550000	200
0.1	200	1660000	400

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Naphthalene metabolism in relation to target tissue anatomy, physiology, cytotoxicity and tumorigenic mechanism of action

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Abstract

This report provides a summary of deliberations conducted under the charge for members of Module C Panel participating in the Naphthalene State-of-the-Science Symposium (NS^3), Monterey, CA, October 9–12, 2006. The panel was charged with reviewing the current state of knowledge and uncertainty about naphthalene metabolism in relation to anatomy, physiology and cytotoxicity in tissues observed to have elevated tumor incidence in these rodent bioassays. Major conclusions reached concerning scientific claims of high confidence were that: (1) rat nasal tumor occurrence was greatly enhanced, if not enabled, by adjacent, histologically related focal cellular proliferation; (2) elevated incidence of mouse lung tumors occurred at a concentration (30 ppm) cytotoxic to the same lung region at which tumors occurred, but not at a lower and less cytotoxic concentration (tumorigenesis NOAEL = 10 ppm); (3) naphthalene cytotoxicity requires metabolic activation (unmetabolized naphthalene is not a proximate cause of observed toxicity or tumors); (4) there are clear regional and species differences in naphthalene bioactivation; and (5) target tissue anatomy and physiology is sufficiently well understood for rodents, non-human primates and humans to parameterize species-specific physiologically based pharmacokinetic (PBPK) models for nasal and lung effects. Critical areas of uncertainty requiring resolution to enable improved human cancer risk assessment were considered to be that: (1) cytotoxic naphthalene metabolites, their modes of cytotoxic action, and detailed low-dose dose–response need to be clarified, including in primate and human tissues, and neonatal tissues; (2) mouse, rat, and monkey inhalation studies are needed to better define in vivo naphthalene uptake and metabolism in the upper respiratory tract; (3) in vivo validation studies are needed for a PBPK model for monkeys exposed to naphthalene by inhalation, coupled to cytotoxicity studies referred to above; and (4) in vivo studies are needed to validate a human PBPK model for naphthalene. To address these uncertainties, the Panel proposed specific research studies that should be feasible to complete relatively promptly. Concerning residual uncertainty far less easy to resolve, the Panel concluded that environmental, non-cytotoxic exposure levels of naphthalene do not induce tumors at rates that can be predicted meaningfully by simple linear extrapolation from those observed in rodents chronically exposed to far greater, cytotoxic naphthalene concentrations.

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1. Introduction

The current state of knowledge and uncertainty about naphthalene metabolism in relation to anatomy, physiology and cytotoxicity in tissues observed to have elevated tumor incidence in rodent bioassays was the focus of the review for Panel C of the Naphthalene State of the Science Symposium (NS^3) (Belzer et al., 2007, in this issue). As discussed by North et al. (2007, this issue), these bioassays found increased incidences of nasal respiratory epithelial adenomas and of rare nasal olfactory epithelial neuroblastomas in female rats, and of nasal respiratory epithelial adenomas in male rats exposed to naphthalene vapor concentrations of 10, 30, or 60 ppm for 2 years (NTP, 2000; Abdo et al., 2001; Long et al., 2003). There were increased incidences of alveolar/bronchiolar adenomas or carcinoma in female (but not male) B6C3F1 mice exposed to 30 (but not to 10) ppm naphthalene for 2 years (NTP, 1992; Abdo et al., 1992). A previous study found increased tumor multiplicity in tumor-bearing A/J strain mice exposed to 10 or 30 ppm for 6 months (Adkins et al., 1986). The few case reports of cancer in naphthalene-exposed humans involve four laryngeal cancer cases among smokers in workers at an East German naphthalene purification plant (Kup, 1978; Wolf, 1976,1978), and a series of 11 colorectal carcinoma patients 18 to 30 years old admitted to a Nigerian hospital, ''half'' of whom ''gave a definitive history of ingesting'' the ''naphthalene compound'' *kafura* used as part of ''local indigenous treatment for 'piles' or any anorectal problem'' (Ajao et al., 1988). These case reports have been viewed as insufficient to evaluate human carcinogenicity of naphthalene (EPA, 1998,2004,2002).

The proposed U.S. Environmental Protection Agency (EPA) reclassification of potential human carcinogenicity of naphthalene from ''possible'' to ''likely'' and accompanying linear no-threshold risk extrapolations (EPA, 2004), follows a classification of naphthalene by the International Agency for Cancer Research (IARC, 2002) as a 2B carcinogen (possibly carcinogenic to humans), based on the same bioassay data. The EPA proposal was based primarily on the proposition that combined data from lifetime bioassays of cancer induced in B6C3F1 mice and F344/N rats exposed chronically to naphthalene by inhalation (NTP, 1992,2000) are plausibly consistent with an exclusively or predominantly genotoxic mode of action. The EPA (1998) had previously classified naphthalene as a possible human carcinogen (Group C, inadequate human and limited animal data), concluding that its human carcinogenic potential could not be determined from available suggestive rodent tumor data, and that ''it appears unlikely that naphthalene represents a genotoxic carcinogen.'' The proposed EPA (2004) reclassification of naphthalene as a likely human carcinogen with a plausibly genotoxic mode of action thus hinged pivotally on interpretation of recent NTP (2000) bioassay data of naphthalene in rats, further supported by evidence that key naphthalene metabolites, such as 1,2- and/or 1,4-naphthoquinone, showed apparent genotoxic and/or mutagenic activity in a relatively small subset of prokaryotic and eukaryotic in vitro studies (Arfsten et al., 1994; Flowers-Geary et al., 1996; NTP, 1992; Wilson et al., 1996; Yu et al., 2002; Schreiner, 2003; and Brusick, 2007, this issue). No consideration, however, was given by the EPA to dose–response relationships observed in genotoxicity assays interpreted as ''positive.'' Specifically, the EPA (2004) considered neither whether cytotoxic oxidative damage and attendant DNA fragmentation may have occurred in ''positive'' genotoxicity assays observed for naphthalene or its metabolites, nor whether these endpoints occurred with similar and substantially non-linear low-dose dose–response relationships consistent with a cytotoxic mechanism of apparent ''genotoxic'' action.

Current bioassay data clearly establish that chronic inhalation of naphthalene can induce respiratory tract tumors. Classification of naphthalene ''carcinogenicity,'' per se, begs the critical scientific question of whether at very low environmental concentrations this compound presents a cancer risk either to humans or to rodents. Major conclusions reached by Panel C on factual issues quintessential for assessing cancer risk from low-level environmental naphthalene exposures are summarized below. These conclusions address the Panel's charge to summarize conclusions it reached concerning: (1) scientific claims considered to be of high confidence, (2) key uncertainties that could be addressed by cost-effective research feasible to complete relatively promptly and how corresponding research results should be interpreted, and (3) the Panel's best scientific judgments concerning those quintessential uncertainties not resolvable by prompt, cost-effective research.

2. Scientific claims with high confidence

2.1. NTP rat nasal tumor occurrence was greatly enhanced, if not enabled, by adjacent, histologically related focal cellular proliferation

Histopathology findings obtained from the NTP (2000) bioassay strongly suggest that nasal tumor formation in rats chronically exposed to naphthalene by inhalation was associated with, if not enabled solely, by chronic tissue damage and associated regenerative and focal hyperplasia. The extent of chronic nasal cytotoxicity and hyperplasia detected in nearly 100% of all exposed animals (regardless

of dose group) was described by Long et al. (2003) as follows (bold added):

> **Neuroblastomas occurred amid a complex spectrum of non-neoplastic lesions of the olfactory epithelium**. The principal non-neoplastic proliferative lesion was atypical hyperplasia, which… consisted of proliferating nests of dysplastic olfactory epithelial cells… and/or **multifocal nodular proliferations** of basal cells extending into the submucosa… The hyperplastic cells were **deeply basophilic and, in many areas, continuous with the neoplasms. Such continuity was most clearly observed in association with small neuroblastomas**. Atrophy of olfactory epithelium was characterized by… loss of epithelial cells… **there was also loss of olfactory neurons. The most severe lesions had complete loss of** sustentacular cells and **neurons, leaving only basal epithelial cells**.
>
> **Respiratory epithelial adenomas also occurred amid a spectrum of non-neoplastic lesions** of the respiratory epithelium and the submucosal glandular epithelium. …In a few animals, **focal proliferation of hyperplastic** cuboidal respiratory **epithelium resembled early adenoma formation**.
>
> …Although the incidence and severity of these non-neoplastic lesions frequently increase in an exposure-dependent manner, they commonly occur with no evidence of nasal carcinogenicity, indicating that factors other than the extent of tissue injury from chronic nasal toxicity contribute to nasal carcinogenesis… **Atypical hyperplasia of the olfactory basal cells** occurred at very high frequencies in all male and female groups exposed to naphthalene. This **was considered an unusual proliferative lesion**, because it had not been reported in previous NTP inhalation studies. **Morphologically, these cells were similar to, and frequently formed a continuum with, those of the neuroblastomas**. This appearance suggests that the **atypical hyperplasia may represent a precursor for nasal olfactory carcinogenesis**. In addition, a few **animals had localized proliferative changes of the respiratory epithelium that were morphologically similar to respiratory epithelial adenomas**.

This description of cytotoxicity and hyperplasia in direct association with both tumor types (nasal neuroblastomas and nasal respiratory epithelial adenomas) is similar to descriptions of multistep neoplastic transformation from focal hyperplastic tissue to nodular hyperplasia to adenomas or carcinomas observed in chemically induced or promoted carcinogenesis in the rodent liver and gastrointestinal tract (Farber, 1984; Farber and Cameron, 1984; Pitot et al., 2000). While initiation with a genotoxic agent is typically used in these experimental rodent carcinogenesis systems in order to generate observable tumor rates, subsequent promotion involving either enhanced cell proliferation, or just oxidative stress associated with additional genotoxic or cytotoxic exposure(s) (Sanchez-Perez et al., 2005), can be required to elevate these rates to observable levels. Naphthalene was clearly cytotoxic to epithelial and neural cells in nasal tissue of exposed NTP (2000) bioassay rats. The cytotoxic damage and regenerative hyperplasia strongly support the notion that these effects likely amplified the incidence of tumor occurrence in that study, through clonal expansion of premalignant cell populations that then became available for subsequent malignant transformation. A partly genotoxic mode of action cannot be ruled out, as may be indicated by absence of nasal tumors in mice chronically exposed to 10 or 30 ppm naphthalene, despite evidence of nasal irritation, nasal respiratory epithelium hyperplasia, and nasal olfactory-epithelium metaplasia in these mice (NTP, 1992; Abdo et al., 1992). However, in contrast to the spectrum of non-neoplastic nasal lesions including atypical, strongly basophilic, multifocal and/or "unusual" types—some involving moderate to severe atrophy—detected in naphthalene-exposed rats (Long et al., 2003) discussed above, average severity scores reported for non-neoplastic nasal lesions that occurred in similarly exposed male and female mice all fell within a "minimal" to "mild" range, below scores $\geqslant 3$ associated with "moderate" or "severe" toxicity (Abdo et al., 1992). The combined rodent bioassay evidence therefore indicates a likely predominant cytotoxic mode of action contributing to the nasal tumors observed in naphthalene-exposed rats. Consequently, cancer risk associated with a plausible genotoxic mode-of-action component cannot meaningfully be either estimated at bioassay doses, or extrapolated to low environmental doses, from the NTP (2000) rat nasal tumor data.

2.2. Elevated incidence of mouse lung tumors occurred at a concentration (30 ppm) cytotoxic to the same lung region at which tumors occurred, but not at a lower and less cytotoxic concentration (tumorigenesis NOAEL = 10 ppm)

The conclusion regarding mode-of-action for nasal tumors in naphthalene-exposed rats is also appropriate for alveolar/bronchiolar adenomas (and one carcinoma) in female B6C3F1 mice, which tumors were observed after chronic exposure to 30 ppm but not 10 ppm naphthalene (NTP, 1992,2002; Abdo et al., 1992). The respective chronic naphthalene exposures produced dose-dependent cytotoxicity in the same (distal-bronchial/alveolar) lung region in mice, but not in rats (West et al., 2001). Non-ciliated Clara cells exhibited relatively high susceptibility to naphthalene-induced cytotoxicity. These cells also demonstrate the greatest capacity to metabolize naphthalene (see below).

2.3. Naphthalene cytotoxicity requires metabolic activation; unmetabolized naphthalene is not a proximate cause of observed toxicity or tumors

Fairly detailed pathways of naphthalene metabolism have been identified (reviewed in Buckpitt and Franklin, 1989; Buckpitt et al., 2002; Waidyanatha et al., 2002; EPA, 2004; ATSDR, 2005). Briefly, naphthalene is meta-

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bolically activated by one or more forms of cytochrome P450 (CYP) to a chiral 1,2-epoxide, which can react directly to form covalent adducts with cellular nucleophiles such as DNA and protein, or undergo subsequent transformation to other reactive metabolites or to detoxified intermediates that are excreted, primarily in urine (Fig. 1). Glutathione-*S*-transferase (GST) appears to play a key detoxification role in ameliorating the cytotoxicity associated with the naphthalene 1,2-epoxide derivative, as glutathione depletion before naphthalene exposure enhances acute naphthalene-induced injury in mouse-lung Clara cells, cells that are likely targeted by naphthalene metabolites during the initiation process of mouse lung tumorigenesis (Warren et al., 1982; West et al., 2000; Plopper et al., 2001; Phimister et al., 2004). In isolated murine Clara cells, decreased cell viability was non-detectable at naphthalene concentrations $\leqslant 0.1$ mM but was substantial ($\geqslant 63\%$) at naphthalene concentrations $\geqslant 0.5$ mM, and the latter cyto-

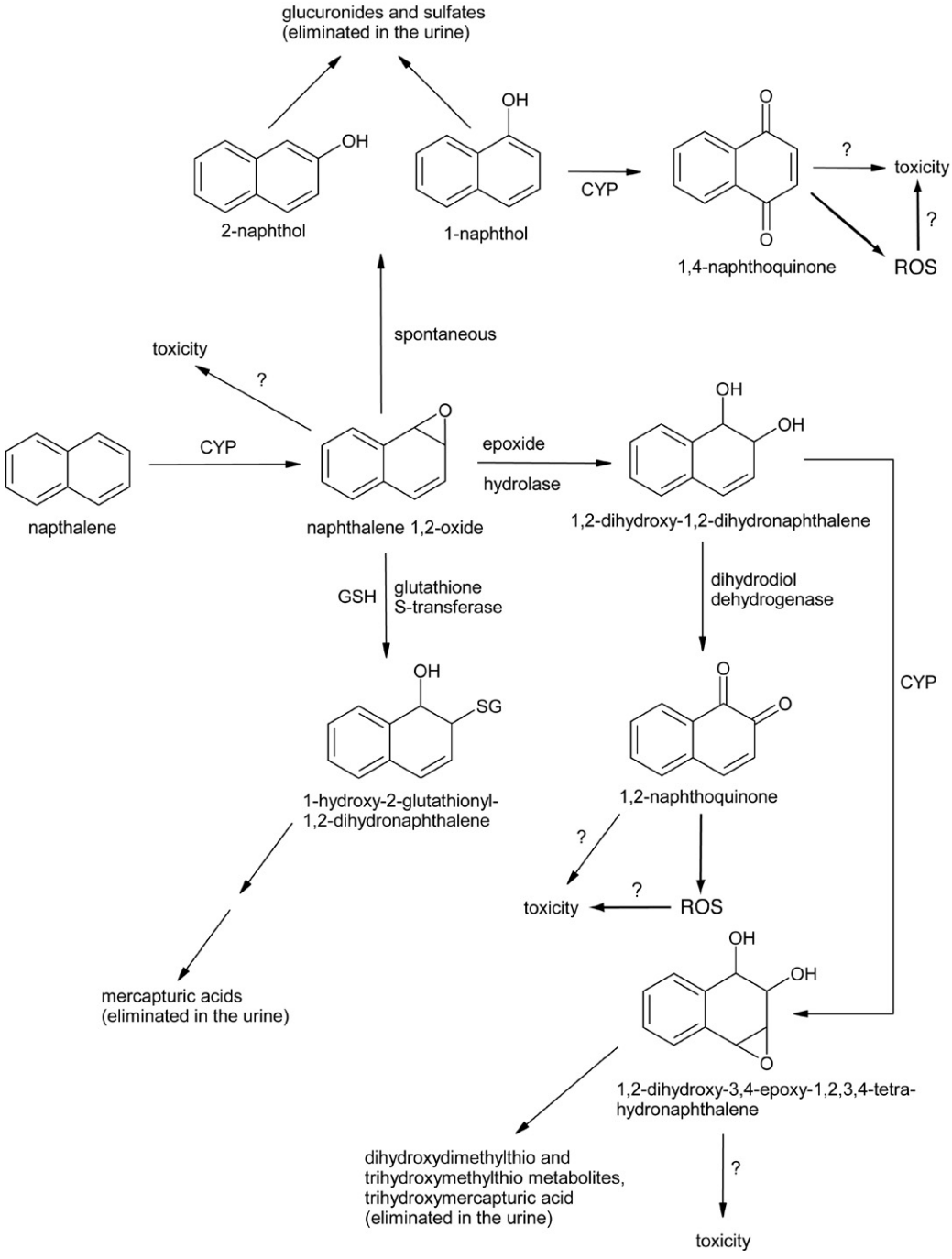

Fig. 1. Scheme for naphthalene metabolism and formation of multiple reactive metabolites that may be involved in naphthalene toxicity. Adapted from Buckpitt et al., 2002; Waidyanatha et al., 2002; ATSDR, 2005; CYP, cytochrome P450 enzymes(s) including (but perhaps not limited to) CYP2F; SG, glutathionyl moiety; GSH, reduced glutathione; ROS, reactive oxygen species.

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toxic effects were blocked by preincubation with the CYP inhibitor, piperonyl butoxide (Chichester et al., 1993). In those isolated cells, incubation with 0.5 mM 1,2-dihydrodioxy-1,2-dihydronaphthalene (henceforth referred to as dihydrodiol), 1-naphthol, or 1,2-naphthoquinone decreased cell viability approximately as effectively as 0.5 mM naphthalene, whereas incubation with 0.5 M naphthalene oxide or 1,4-naphthoquinone significantly decreased viability more than the same concentration of parent compound; moreover, naphthalene-oxide-induced cytotoxicity was not blocked with piperonyl butoxide (Chichester et al., 1993). In isolated mouse-lung Clara cells exposed to naphthalene, 1,2-naphthoquinone was one of two detected types of covalent, naphthalene-related protein adduct (Zheng et al., 1997). Incubation of isolated murine hepatocytes with the (1*S*,2*R*)-naphthalene-oxide enantiomer—which is converted much more slowly to the dihydrodiol than the (1*R*,2*S*)-epoxide—resulted in nearly complete loss of cell viability, whereas incubation with the (1*R*,2*S*) enantiomer with a shorter half life had almost no effect on cell viability (Buonarati et al., 1989).

Naphthalene did not activate transcription mediated by aryl hydrocarbon receptor (AHR) in an in vitro luciferase reporter assay, and while knockout mice homozygous for deficiency in AHR, in CYP1A1 or in CYP1A2 dosed with naphthalene—regardless of pretreatment with the CYP2F inhibitor 5-phenyl-1-pentyne—all exhibited olfactory toxicity, CYP1A1- and CYP1A2-null mice pretreated with that CYP2F inhibitor exhibited no naphthalene olfactory toxicity (Genter et al., 2006). These studies demonstrated that CYP1A and CYP1A2 genes, which are inducible by AHR in the mouse respiratory tract, do not function to influence naphthalene-induced toxicity, and confirm previous observations (Phimister et al., 2004) that CYP2F2 bioactivates naphthalene in that target tissue to one or more reactive metabolites that induce cytotoxicity after depleting glutathione.

Pooled human liver microsomes (pHLMs) were found to metabolize naphthalene to trans-1,2-dihydro-1,2-naphthalenediol (dihydrodiol), 1-naphthol, and 2-naphthol, with corresponding kinetics characterized by K_m values of 23, 40, and 116 μM and V_{max} values of 2860, 268, and 22 pmol/mg protein/min, respectively (Cho et al., 2006). CYP isoform screening in this study identified CYP1A2 as the most efficient isoform for producing dihydrodiol and 1-naphthol, and CYP3A4 as the most effective for 2-naphthol production. Whereas further pHLM metabolism of 2-naphthol was found to produce 2,6- and 1,7-dihydroxynaphthalene, dihydrodiol and 1-naphthol were not efficiently metabolized by pHLMs (Cho et al., 2006). CYP1A2 and 2D6*1 were identified as the most active isoforms for producing 1,4-naphthoquinone, and CYP3A4 and CYP2A6 the most active at metabolizing dihydrodiol, though at rates less than those at which 1-naphthol was observed to be metabolized (Cho et al., 2006).

Necrosis of bronchial epithelial (Clara) cells in mice (O'Brien et al., 1985, 1989; Tong et al., 1981) and necrosis of olfactory epithelial cells in mice, rats and hamsters (Plopper et al., 1992) following intraperitoneal injection of naphthalene strongly indicate that metabolic activation in target tissues plays a dominant, and possibly exclusive, role in site-specific naphthalene cytotoxicity. There is no evidence that unmetabolized naphthalene is cytotoxic, or that unmetabolized naphthalene is genotoxic at non-cytotoxic concentrations. No tumors were observed in tissues where naphthalene was not cytotoxic.

2.4. There are clear regional and species differences in naphthalene bioactivation

Naphthalene bioactivation varies considerably among species and among different anatomical regions of the respiratory tract (Buckpitt and Bahnson, 1986; Buckpitt et al., 1992,1995,2002; Thornton-Manning and Dahl, 1997; Baldwin et al., 2004,2005; Boland et al., 2004). Relative to microsomal preparations made from mouse, those derived from hamster, rat, and rhesus macaque lung tissue were observed to metabolize naphthalene to dihydrodiol and glutathione conjugates at relative rates of 37%, 12%, and 1%, respectively. The enantiomeric form of the metabolites also vary among organs. For example, the mean (±1SD) of lung-to-liver ratios for values of the ratio between 1*R*,2*S*- and 1*S*,2*R*-epoxide enantiomers formed by organ-specific mouse microsomal preparations was measured to be 7.9 ± 2.0 at substrate concentrations ranging from 0.06 to 1 mM naphthalene, which contrasts with a lung-to-liver ratio of 1.3 ± 0.3 observed for the total rate of metabolic activation over this range of substrate concentrations (calculated from Table 1 of Buckpitt et al., 1992). Studies investigating naphthalene bioactivation in lung to dihydrodiol and glutathione conjugates demonstrated that the distal bronchiole region possesses the greatest activity in mice, rats and hamsters (Buckpitt et al., 1995). The total metabolic activity in this region was about 10-fold greater in mice than in rats. In contrast, GST levels varied only about 2-fold across species or lung tissue region (Buckpitt et al., 1995).

In the nose, naphthalene bioactivation using microsomal preparations made from the olfactory region of the mouse, hamster, rat nasal mucosa was 2- to 3-fold greater than from septum or lateral-wall regions (Buckpitt et al., 1992). The 1*R*,2*S*-epoxide was the predominant enantiomer formed in all three species (Buckpitt et al., 1992).

Rates of formation of naphthalene 1*R*,2*S*-oxide in mouse, rat, and hamster airway explant incubations correlate well with immunolocalization of CYP2F2, but not with CYP2B4 that also is found in pulmonary Clara cells; and CYP2B4 inhibition does not block naphthalene metabolism by mouse lung microsomal enzymes (Buckpitt et al., 1995; Roberts et al., 1993). A subsequent immunolocalization study (complemented by peptide mass fingerprinting, and RT-PCR analysis of CYP2F mRNA expression) failed to detect CYP2F in rhesus macaque tissue of any kind studied other than nasal ethmoturbinates, where levels

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Table 1
Summary of proposed studies of uptake and metabolism in rodents and monkeys

Species	Endpoint[a]	Number (n) of animals per time point	Proposed sacrifice times	Comments[a]
Rodents (B6C3F1 mice, F344/Nrats)	URT uptake	8 to 10 per group	1 h	2 or 3 inspiratory flow rates, in both normal and metabolically (CYP450-) inhibited animals
	Initial body burden	4	0 h	
	Tissue distribution and clearance	3	0.5, 1, 4, 8, 24, 48, and 72 h	
	Excretion	$\geqslant 5$	7 days	Adjust n as needed to provide sufficient sample for use by collaborating scientists biomarkers
Non-human primates	Initial body burden	3	0 h	
	URT uptake	3	0 h	2 or 3 inspiratory flow rates in each animal
	Tissue distribution and clearance	3	1, 8, 24, 48 and 72 h	Modify as needed based on rodent study results
	Excretion	5	7 days	Make samples available to co-investigators of parallel studies

[a] Each study should use $\geqslant 4$ concentrations spanning those in each species that show toxicity and those corresponding to the no effect level for respiratory tract lesions, including $\geqslant 2$ intermediate concentrations allowing quantitative characterizations of cytotoxic dose–response.

were 10- and 20-fold lower than in corresponding rat and mouse tissue, respectively (Baldwin et al., 2004). However, human liver microsomes convert naphthalene to its dihydrodiol intermediate at faster rates than mouse and rat liver microsomes (Kitteringham et al., 1996).

The human enzyme that is orthologous to the mouse CYP2F2 enzyme is CYP2F1. The CYP2F1 mRNA has been identified in human respiratory tissues by a number of different laboratories (see Raunio et al., 1999; Ding and Kaminsky, 2003). The CYP2F1 enzyme was expressed in lymphoblastoid cells and shown to metabolize naphthalene to its epoxide, albeit at very low rates (Lanza et al., 1999). This enzyme is highly unstable (Baldwin et al., 2005), but it has been over-expressed in a human bronchial epithelial cell line (Nichols et al., 2003), and used to evaluate the mechanisms of cytotoxicity of 3-methylindole, a prototypical pneumotoxicant, and the bioactivation of benzene (Sheets et al., 2004). This cell line could be used for cytotoxicity and mutagenesis studies with naphthalene.

Because naphthalene is not considered capable of inducing cytotoxicity (or genotoxicity) without metabolic activation, and because there are clear regional and species differences in naphthalene bioactivation, estimation of potential human cancer risk associated with naphthalene exposure—regardless of assumed mode of action—cannot be done meaningfully based on rodent bioassay results characterized simply in terms of bioassay-administered naphthalene doses or concentrations. Administered naphthalene doses or concentrations must first be converted to an estimated biologically effective dose at the target tissue. Estimation of an average dose in the entire target organ (e.g., rate of metabolism of naphthalene in the lung per gram of lung tissue) would be less satisfactory than an estimate for the region in which toxicity is observed (e.g., the nasal olfactory region or the terminal bronchiolar region of the lung). A number of different surrogates for the biologically effective dose could be considered, depending on the richness of the data available on the metabolism of naphthalene and its metabolites in the target tissues of both the experimental animal and the human. At a minimum, administered dose or concentration should be replaced with peak or average daily rate of metabolism in the target tissue (but not total metabolism in the body, which would be dominated by the liver). If sufficient data are available, a preferred surrogate closer to the ultimate toxic form could be used, such as mean or peak intracellular concentration of (total, or only 1*R*,2*S* enantiomeric) naphthalene-oxide, or of 1,2-naphthoquinone in the target tissue. Physiologically based pharmacokinetic (PBPK) models developed for naphthalene (e.g., Sweeney et al., 1996; Quick and Shuler, 1999; Ghanem and Shuler, 2000; Willems et al., 2001) demonstrate a reasonable approach to estimate such reasonably plausible measures of surrogate biologically effective dose as a function of bioassay-administered dose. However, the current models are only capable of estimating doses for the total lung. Additional elaboration of these models would be required to extend dosimetry to the nose as well as to support regional dosimetry within the nose and lung.

2.5. Target tissue anatomy and physiology is sufficiently well understood for rodents, non-human primates and humans to parameterize species-specific PBPK models for nasal and lung effects

Based on animal carcinogenicity and cytotoxicity data, the target tissue of primary concern for potential human cancer risk posed by environmental naphthalene exposure is respiratory tract (including nasal) epithelium. Current understanding of the anatomy and physiology of nasal/respiratory tissues in rodents, primates and humans is adequate to extend the PBPK models developed for naphthalene nasal and lung effects (e.g., Sweeney et al., 1996; Quick and Shuler, 1999; Ghanem and Shuler, 2000; Willems et al., 2001) to include a more detailed description of target tissues. An existing model for a similar compound, styrene

(Sarangapani et al., 2002), would help to inform this effort. Further improvements would result from additional data, particularly on (i) blood:air and tissue:air partition coefficients for naphthalene; (ii) the concentration- and airflow-dependence of naphthalene uptake in the upper respiratory tract (URT) in rodents and primates; (iii) region-specific metabolism of naphthalene in rodent, primate and human tissues; and (iv) relative toxicities of naphthalene and its metabolites in different regions of the nose and lungs of rodents and non-human primates. The human bronchial epithelial cell line that over-expresses CYP2F1 could be used to evaluate metabolism and cellular effects in a relevant human cell line.

3. Uncertainties quintessential for human cancer risk assessment, and feasible experiments that could be done to reduce or eliminate these uncertainties

3.1. Cytotoxic naphthalene metabolites, their modes of cytotoxic action, and detailed low-dose dose–response need to be clarified, including in rodent, primate and human tissues, and in neonatal tissues

The possibility that low-dose linear risk extrapolation of tumor risk from data gathered at high, cytotoxic doses may introduce profound conservative bias hinges on the assumption that cytotoxicity and regenerative hyperplasia have a quasi-threshold type of dose–response. In vitro experiments can quantify and characterize the low-dose dose–response for cell killing in relevant target tissues and species, allowing meaningful inter-species and dose extrapolation for this critical endpoint.

A **series of studies over 1 to 2 years** could apply short-term in vitro assays to quantify naphthalene- or metabolite-induced reduction in target-cell viability in B6C3F1 mouse, F344/N rat, primate and human explants of regionally defined respiratory and nasal olfactory epithelia. Selection of particular regions and target cell types for focused investigation should be informed by results of experiments proposed in this issue by North et al. (2007) concerning species differences in acute and subchronic toxicity to inhaled naphthalene. Alternative measures of putative biologically effective dose (BED) should be characterized, such as protein binding, DNA adducts, abasic DNA sites, GST levels, and oxidative stress, measured using F-2 isoprostanes in exposed tissue and/or in culture media or by other means. The F2-isoprostanes are viewed as the most reliable, sensitive, and specific biomarkers of oxidative stress (Montuschi et al., 2004; Morrow, 2005). Parallel experiments done using CYP-isozyme-specific enzyme inhibitors (see, e.g., Hynes et al., 1999; Born et al., 2002), by suppressing enzymatic activity through iRNA, or by using explant tissue derived from gene-knockout strains, could provide straightforward tests of alternative metabolic pathway assumptions. The tissue-specific activity or concentration determined to correlate best with reduced cell viability will define the best available corresponding BED metric for that observed cytotoxicity. The quantitative dose–response relation between naphthalene concentration and the BED metric(s) identified will provide key information for corresponding species-specific PBPK models (discussed further in Sections 3.3 and 3.4 below), and are likely to generate testable hypotheses concerning the existence of a non-genotoxic mode of action for naphthalene-induced tumorigenesis. A non-genotoxic mode of action would be supported by evidence that induced cell killing has a sigmoidal, substantially non-linear dose–response relationship with either administered naphthalene concentration or corresponding BED, coupled with evidence that any naphthalene-induced genotoxicity is undetectable at non-cytotoxic naphthalene concentrations.

It will be important to include neonatal tissues among test explants to be examined, in order to investigate potential age-dependent differences in susceptibility to naphthalene-mediated cytotoxicity or developmental toxicity. If feasible, to more reliably characterize potential on developmental variation in rate-limiting metabolic activities governing naphthalene activation and deactivation pathways, remaining questions concerning the ontogeny of CYP2F enzymes should also be investigated (Choudhary et al., 2003,2005). Additional studies may be required to characterize other enzymes associated with the production and clearance of the key metabolites of naphthalene, including naphthalene-oxide (both enantiomers) and 1,2-naphthoquinone, as indicated by the evidence regarding the most appropriate measure of BED. These additional enzyme studies should be performed in rodent, primate, and human tissues.

3.2. Mouse, rat, and monkey inhalation studies are needed to better define in vivo naphthalene uptake and metabolism in the upper respiratory tract (URT)

Better understanding of comparative URT uptake and in situ metabolism of naphthalene is required to interpret observed species differences in URT cytotoxicity. **A study requiring approximately 1 year** to complete could apply acute (and, as feasible, repeated acute-dose) in vivo assays to quantify and compare URT uptake and metabolism of naphthalene administered by inhalation, as outlined in Table 1. Parallel studies with and without CYP inhibition will allow confirmation of hypotheses generated using more detailed in vitro explant studies. These in vivo studies should be done using different naphthalene concentrations expected (based on in vitro studies and in vivo inhalation studies) to induce a range of cytotoxic severity, again focusing on concentrations expected to shed the greatest light on low-dose dose–response relations (but also including 10 and 30 ppm naphthalene as bioassay-related reference points). Tissue- and region-specific CYP enzymes and their relative activities should be identified and quantified in these studies. This study should be coupled to the PBPK-model validation study proposed below. Selection of specific concentrations for focused investigation should be

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informed by results of experiments proposed by North et al. (2007) this issue) concerning species differences in acute and subchronic toxicity to inhaled naphthalene.

3.3. In vivo validation studies are needed for a PBPK model for monkeys exposed to naphthalene by inhalation, coupled to cytotoxicity studies referred to above

A monkey PBPK model needs to be validated in order to link data gathered from naphthalene cytotoxicity and DNA-damage studies in naphthalene-exposed monkeys and rodents to improved understanding of cancer bioassay results, and thereby to improve biologically based human risk prediction. **A study requiring approximately 1 year** to complete could be performed to collect blood and tissue data on naphthalene, naphthalene-oxide, urinary adducts, and related measures from naphthalene-exposed monkeys. In these studies, monkeys in metabolism cages would be exposed to ^{14}C-radiolabeled naphthalene for one or repeated 6-h periods. Tissues would be obtained for analysis upon serial sacrifice. Tissue- and region-specific P450 enzymes and their relative activities should be identified and quantified in these studies. Such data are needed to obtain reliable parameter estimates for a PBPK model of naphthalene in monkeys, similar to models cited above already developed for rodents. Such a study should include determinations of blood:air and tissue:air partition coefficients for naphthalene for monkey, rat and mouse, as well as the human blood:air partition coefficient for naphthalene.

3.4. In vivo studies are needed to validate a human PBPK model for naphthalene

A human PBPK model needs to be validated in order to link data gathered from proposed studies of naphthalene cytotoxicity, and of naphthalene DNA-damage (Brusick, 2007, this issue), in naphthalene-exposed monkeys and rodents to improved understanding of cancer bioassay results, and thereby to improved biologically based human risk prediction. Validation in this context would focus on estimation of key parameters that are feasible to measure in a **1-year time-frame in a series of short-term experiments** using human volunteers under an IRB- and USEPA-HSRB-approved study protocol. These experiments would involve controlled administration of approximately 1 part per trillion of ^{14}C-radiolabeled naphthalene in air (i.e., a concentration that is a fraction of ambient levels of naphthalene typically found in indoor air, as discussed in Griego et al., 2007 (this issue) by inhalation for 6 h. Naphthalene and metabolites in blood and urine at these exposure levels can be analyzed readily by accelerator mass spectrometry (AMS) (see, e.g., Bogen et al., 1998; Dingley et al., 1998; Williams et al., 2002; Cupid et al., 2004). Measured samples should include at least hourly blood samples. Interindividual variation in metabolic capacity can be assessed in vivo by experiments involving a larger number of (e.g., $\geqslant$40) subjects exposed for just one hour. To assess possible saturation-related non-linearity in naphthalene metabolism or adduct formation, results from the acute 6-h study done at very low concentration should be compared to results obtained using the same administered concentration of ^{14}C-radiolabeled naphthalene diluted in a substantially larger concentration of unlabeled naphthalene (e.g., 0.1 or 1 ppm in air from a mothball that is partly unwrapped to expose a precisely defined uncovered surface area).

4. Best scientific judgment about quintessential uncertainty not resolvable by prompt cost-effective research

4.1. Could naphthalene induce tumors at environmental, non-cytotoxic exposure levels at rates predictable from currently available data?

The panel's unanimous opinion is that, based on currently available data, it is extremely improbable that environmental, non-cytotoxic exposure levels of naphthalene induce tumors at rates that can be predicted meaningfully by simple linear extrapolation from those observed in rodents chronically exposed to far greater, cytotoxic naphthalene concentrations. Results from studies proposed in Section 2 are required to confirm this hypothesis beyond a reasonable doubt.

Conflict of interest disclosure

The authors declare that they have no conflicts of interest. Each received an honorarium from Regulatory Checkbook, PO Box 319, Mt. Vernon, VA 22121, for service as set forward in Belzer et al. (2007), (in this issue).

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Possible genotoxic modes of action for naphthalene ☆

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Abstract

This report provides a summary of deliberations conducted under the charge for members of Module D participating in the Naphthalene State-of-the-Science Symposium (NS^3), Monterey, CA, October 9–12, 2006. The charge directed the panel to ascertain to the best of its ability a consensus judgment of the state-of-the-science concerning the potential for a genotoxic mode of action for naphthalene and its metabolites, with implications for low-dose extrapolations of cancer risk estimates for exposed populations. Where scientific uncertainties remained, the panel was asked to identify which scientific uncertainties (if any) could be resolved through targeted, timely, cost-effective research. The report provides a brief summary of naphthalene genotoxicity; identifies those areas where there is a general scientific consensus regarding the effects of naphthalene; identifies areas of uncertainty regarding the effects of naphthalene; and key questions that currently limit our ability to assess the genotoxic risks of naphthalene. The report also outlines a set of six studies that could resolve some of these key uncertainties.

Keywords: Naphthalene; Cancer; Carcinogenicity; Mutagenicity; Mode of action; Mechanism of action; Genotoxicity; Metabolism; 1,2-Naphthoquinone; DNA adducts; Stable adducts; Depurinating adducts; Biomarkers

1. Introduction

Naphthalene, a bicyclic aromatic compound, can be found environmentally as a constituent of coal tar, crude oil, and cigarette smoke. It is also used in chemical manufacturing as a chemical intermediate for many commercial products ranging from pesticides to plastics. Because of its widespread human exposure (see Griego et al., in this issue), its toxicological properties have been the subject of numerous assessments. Of particular interest was an evaluation for the potential to induce tumors.

Studies conducted by the National Toxicology Program (NTP) are discussed in North et al. (in this issue) The concentrations of naphthalene to which the mice and rats were exposed in the bioassays were judged to exceed the maximum tolerated dose (MTD) and were about 5000 times higher than ambient air concentrations, estimated at 0.0002 ppm by Griego et al. (in this issue).

The findings that naphthalene can produce respiratory tract tumors in mice and rats raises the question of whether humans, at environmental or workplace concentrations of

☆ The authors declare that they have no conflicts of interest.
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naphthalene, are at risk for cancer. In order to address these concerns, a better understanding of the mode(s) of action of naphthalene will be needed.

Relevance of the rodent cancer bioassay data to humans has been contested for a number of reasons but has been particularly focused on the fact that tumors occurred at high dose levels associated with substantial target site toxicity (North et al., 2007). Areas of uncertainty are related to the differences in airway anatomy and metabolism between rodents and humans (Bogen et al., 2007). Finally, an understanding of the DNA reactivity of naphthalene will be critical to extrapolating effects found at high concentrations to lower, environmental concentrations.

2. Naphthalene genotoxicity

The available database for naphthalene genotoxicity is extensive and has been reviewed recently by IARC (2002), Schreiner (2003), and preliminarily by the US EPA (2004a,b). The IARC monograph concludes that naphthalene is not mutagenic in the most commonly used tests to measure genotoxicity. Schreiner suggests that at reasonable MTD concentration, naphthalene is not mutagenic to bacteria or mammalian cells in culture, but does produce toxicity-dependent secondary damage to DNA at excessive concentrations. Evidence for the secondary damage is seen as DNA fragmentation in vivo, as well as sister chromatid exchange (SCE) and chromosome breakage in vitro. Schreiner concludes that the DNA fragmentation and chromosome breakage events are consistent with a threshold-related cytotoxicity mechanism of tumor induction, driven by cytotoxicity and cell regeneration leading eventually to tumor development.

In its review, the US EPA summarizes essentially the same set of data but reaches a different conclusion. In the external review draft IRIS report (EPA, 2004b) the Agency proposed that "a possible genotoxic mode of carcinogenic action cannot be discounted as there are some data indicating genotoxicity of 1,2-napthoquinone." This reference to the mutagenicity of 1,2-naphtoquinone was derived from a paper by Flowers-Geary et al. (1996) in which a small increase in the reversion counts was seen with strain TA104, a Salmonella strain believed to respond to oxidative DNA damage.

A critical assessment of the published genotoxicity database was presented at the NS^3 Symposium (Brusick, in this issue), which concluded naphthalene does not appear to bind to DNA and that the vast majority, if not all, of the legitimate positive genotoxicity could be explained by cytotoxicity. However, a presentation by Cavalieri (in preparation) argued that studies in his laboratories demonstrated that naphthalene (or some of its metabolites) binds to DNA producing a class of transitory "depurinating adducts" and therefore should be considered a true (albeit extremely weak) genotoxic carcinogen. Resolution of the mode of action for the genotoxic activity of naphthalene and, more importantly, its relative potency may be essential to the development of an accurate risk assessment of this chemical.

The panel prepared a series of statements defining what it believed were facts with a high degree of certainty regarding the genotoxicity of naphthalene as well as some statements with a high degree of uncertainty. The group outlined a set of research studies that could be undertaken to resolve some of the critical uncertainties regarding the role of naphthalene genotoxicity in rodent respiratory cancer.

3. Scientific statements with a high degree of certainty

Under conditions employed in the NTP bioassays, naphthalene induces respiratory tumors in rodents only at sites showing signs of significant cytotoxicity.

The tumors in both species were strongly associated with chronic inflammation, hyperplasia, metastatic changes, and cell proliferation, all signs indicative of cytotoxicity. Reviews of genotoxicity studies conducted with naphthalene demonstrate that most, if not all, of the positive findings can be explained by cytotoxicity and not direct interaction of the chemical with DNA. Assays such as the Ames test, which measure gene mutation from base change mutations produced by DNA adduction, were reported to show no evidence of mutagenic activity when subjected to naphthalene (EPA, 2004b).

(a) Naphthalene metabolites have been shown to produce stable DNA adducts, rapidly depurinating adducts and oxidative DNA damage.

Studies by Saeed et al. (2007) have reacted metabolites of naphthalene with DNA and isolated adducts. From these studies, they hypothesize that naphthalene metabolite 1,2-naphthoquinone interacts with purine bases to form depurinating adducts. They also hypothesize that these adducts are mutagenic and may be responsible for tumor initiation. Apurinic sites formed by depurinating adducts may be converted to mutations by error-prone base excision repair (Chakravarti et al., 2000, 2007). Before this information can be built into a carcinogenic mode of action for naphthalene, additional work must be conducted to demonstrate (a) that the metabolic steps necessary to produce depurinating adducts occur in target tissues in vivo, (b) the susceptibility of the damaged sites to reversal by normal DNA repair, and (c) the expression of base change mutations in relevant oncogenes or tumor suppressor gene in pre-cancerous or tumor tissues. Finally, it will be necessary to determine if target tissue toxicity plays a necessary role in the progression of mutations produced by depurinating adducts to tumor cell initiation.

4. Scientific statements with a high degree of uncertainty the resolution of which is quintessential for human risk assessment

(b) Does naphthalene have characteristics of a genotoxic carcinogen?

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What characteristics are needed to define a chemical as a genotoxic carcinogen? In general, a chemical may be defined as a genotoxic carcinogen if it, or its metabolites, are shown to bind covalently to DNA producing chemical-specific adducts in the tissue(s) associated with tumor induction. The number DNA adducts may be directly proportional to dose over a wide range of exposure levels. These adducts should be linked to the induction of mutation in cells associated with tumor target sites. Reaction of a few naphthalene metabolites with pure DNA in vitro have produced adducts with adenine and guanine (Saeed et al., 2007).

(c) Will biomarkers of naphthalene interactions with DNA be generated in animals or humans following environmental and/or occupational exposures?

An important element for understanding the magnitude of carcinogenic risk from environmental or occupational naphthalene exposures would be the ability to find and measure relevant markers of exposure (Markushin et al., 2006). Identification of abasic sites in tumor site tissues or the presence of depurinating DNA adducts in the urine or plasma of animals exposed to carcinogenic levels of naphthalene would provide valuable information regarding the role of DNA binding and tumor initiation from naphthalene and could serve as biomarkers of likely carcinogenic risk to humans.

(d) Do specific molecular lesions in target cells determine the progression from pre-neoplastic tissue to tumors in rodents exposed to carcinogenic levels of naphthalene?

Elucidating such a progression is essential for the construction of an accurate cancer risk assessment. The current level of knowledge about the carcinogenic mode of action of naphthalene in rodents does not allow one to identify steps that drive the process from those that occur incidental to the process (see North et al., 2007). Elucidating those that drive the process will likely lead to an appreciation of why respiratory tissues are targeted and the mode of action underlying the development of tumors found there.

5. What specific research projects can be undertaken to resolve or reduce the levels of uncertainty for those issues quintessential for human risk assessment?

A set of six studies that could resolve some of these key uncertainties was proposed. Basic designs are provided for each of the six studies along with major inferences that may be derived from alternative outcomes.

5.1. Study I: A short-term rat inhalation bioassay of molecular dosimetry of DNA adducts and cell proliferation studies in olfactory mucosa

5.1.1. Rationale

This study would involve a rat inhalation study of molecular dosimetry of DNA adducts and corresponding cell proliferation in olfactory mucosa. The study would measure stable adducts and abasic sites in target cells, and depurinating adducts in urine. Naphthalene inhalation exposure would be conducted in a manner similar to NTP bioassays, using exposures from the previously observed carcinogenic dose of 10 ppm, down to 3 and 1 ppm. Sacrifices would be made at 1, 5, and 20 days.

This study should be conducted in coordination with the rat (and possibly mouse) bioassays proposed by other groups, looking for the same indicators and outcomes using the methods indicated above.

Additional exposure groups at lower concentrations (below 1 ppm) should be considered to identify possible effects at these doses and to better determine low-dose extrapolation functions and parameters.

5.1.2. Major inferences

Stable DNA adducts in target tissue. If *stable* DNA adducts of naphthalene metabolites are found in the exposed rat olfactory mucosa, it would support the hypothesis that there is a genotoxic mechanism for naphthalene and support the hypothesis that naphthalene could cause cancer in humans through such a mechanism. Finding such adducts would not address whether naphthalene exposure at environmental levels could cause cancer.

A failure to find stable adducts from naphthalene metabolites in the exposed rat olfactory mucosa would provide possible evidence that naphthalene does not have a genotoxic mechanism and possible evidence that naphthalene does not cause cancer in humans through such a mechanism.

Depurinating DNA adducts in urine. Although naphthalene forms the depurinating adducts in the exposed tissue, they are not retained by the tissue. Rather, these adducts are released form the cellular DNA, shed into the bloodstream and finally excreted by urine. Therefore, urine analysis provides an easy and non-invasive test for detecting naphthalene–DNA adducts. This type of urine analysis is not a new technology; its value has been previously demonstrated in studies with PAH and estrogens (Chakravarti et al., 2001; Rogan et al., 1990; Todorovic et al., 2001). If *depurinating* DNA adducts of naphthalene metabolites are found in the urine of exposed rats, it could support a genotoxic mechanism for naphthalene. In addition, it would provide strong evidence that animals exposed to naphthalene can exhibit biomarkers of genotoxic exposure (i.e., depurinating DNA adducts, or possibly excision repaired stable adducts) in their urine (and possibly plasma).

A failure to find depurinating DNA adducts of naphthalene metabolites in the urine of exposed rats would provide possible evidence that naphthalene is not genotoxic and possible evidence that naphthalene that naphthalene does not cause cancer in humans through such a mechanism. The absence of depurinating DNA adducts also would raise doubt as to whether animals exposed to naphthalene exhibit biomarkers of genotoxic exposure that can be measured.

Presence of abasic sites in olfactory mucosal DNA. Increased naphthalene exposure to olfactory tissue should

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show increased formation of depurinating adducts and a corresponding increase in abasic sites in DNA. Although abasic sites can be measured, if olfactory tissue has the ability to rapidly repair abasic sites, a dose–response relationship may not be observed. A finding that both depurinating adducts and abasic sites are increased in olfactory DNA would support the hypothesis that rapidly depurinating DNA adducts are a genotoxic lesion associated with naphthalene exposure and provide strong evidence that naphthalene has a genotoxic mechanism, but provide only weak evidence that naphthalene could cause cancer in humans through such a mechanism. A finding that depurinating adducts are increased but abasic sites are not increased in olfactory DNA would not support the hypothesis that abasic sites can be measured for assessing genotoxicity associated with naphthalene exposure, and provide weak evidence that does not have a genotoxic mechanism.

A finding that increased numbers of naphthalene-induced abasic sites in target tissues are dose-related and can be fit to a dose–response function. This would provide moderate support for informing the selection of a naphthalene dose–response model and a science-based low-dose extrapolation. A finding that the numbers of naphthalene-induced abasic sites in target tissues are not dose-related and cannot be fit to a dose–response function provides possible support for the conclusion that meaningful low-dose extrapolations cannot be made from this abasic site data or depurinating DNA adducts.

Dose relationship in measured effects. A finding that the DNA adduct concentrations (stable in target cells, depurinating in urine) are dose-related and can be fit to a dose–response function could enable dose–response (though additional, lower dose experiments might be needed to accomplish this) can enable the selection and fitting of a naphthalene dose–response model. The exposure–response of increased abasic sites would provide vital information distinguishing between linear and nonlinear models. A finding that the DNA adduct concentrations are not dose-related and cannot be fit to a dose–response function will preclude the development of a supportable low-dose extrapolation.

Cell proliferation results. The identification of elevated cell proliferation in the target cells exhibiting elevated DNA adducts supports the hypothesis that cytotoxic effects of naphthalene are prerequisite for genotoxicity. Failure to find elevated cell proliferation in the target cells exhibiting elevated DNA adducts provide some evidence that cytotoxic effects of naphthalene are not such a prerequisite.

5.2. Study II: A mechanistic tumor initiation study in mouse skin measuring possible enzyme(s) for metabolic activation of naphthalene, stable and depurinating adducts, abasic sites and analysis of H-ras mutations in preneoplastic skin and tumors (if they develop)

5.2.1. Rationale

DNA adduct studies in mouse skin treated with naphthalene or some of its metabolites revealed that the two depurinating adducts, 1,2-dihydroxynaphthalene (1,2-DHN)-4-N3Ade and 1,2-DHN-4-N7Gua are formed by 1,2-naphthoquinone (1,2-NQ) (Cavalieri and Rogan, 2006). In addition, some stable adducts are formed by 1,2-NQ. When mouse skin was treated with naphthalene itself, additional stable adducts are formed that presumably derive from naphthalene-1,2-oxide. These results suggest that the key enzyme(s) for metabolic activation of naphthalene are present in mouse skin. Therefore, this organ could be a target for carcinogenic activity. Furthermore, detailed studies of stable and depurinating adducts formed by treatment of SENCAR mouse skin with naphthalene, coupled with analysis of H-*ras* mutations in preneoplastic skin and tumors (if they develop), will provide key information on the mechanism of metabolic activation that generates tumor-initiating mutations. The SENCAR mouse skin model is capable of showing mutagenesis by weak carcinogens such as estrogens (Chakravarti et al., 2001). As shown in recent studies, H-*ras* mutations in preneoplastic skin of this mouse show excellent correlation with DNA adducts (Chakravarti et al., 2000, 2007).

5.2.2. Experimental

The proposed dose–response study will be conducted in the skin of female SENCAR mice. Groups of mice will be topically treated with naphthalene at doses of 300, 100, or 30 nmol per treatment three times a week for 40 weeks. A control group will be treated with the vehicle (50 μl acetone). At various time-points during the experiment, skin from a few mice will be analyzed for abasic sites, depurinating and stable DNA adducts and the presence of H-*ras* mutations. Tumor formation in the skin will be observed throughout the length of the experiment, 52 weeks. If tumors arise, they will be analyzed for H-*ras* mutations.

5.2.3. Major inferences

Integrated information concerning the metabolic activation of naphthalene is expected to be obtained concerning the mutational ability of abasic sites, the DNA adducts formed and, the possible weak tumorigenic activity of naphthalene in mouse skin.

Depurinating DNA adducts in urine. The identification of depurinating DNA adducts of naphthalene metabolites in the urine of exposed mice would support the hypothesis that naphthalene has a genotoxic mechanism, provide some support for the hypothesis that naphthalene may cause cancer through a genotoxic mechanism, and provide strong evidence that animals exposed to naphthalene can exhibit biomarkers of genotoxic exposure, i.e., depurinating DNA adducts (or possibly excision repaired stable adducts) in their urine (and possibly plasma) particularly if they are also associated with increased abasic sites in skin. A failure to identify abasic sites in DNA and depurinating DNA adducts of naphthalene and its metabolites in the urine of the mice would provide possible evidence that naphthalene does not have a genotoxic mechanism, possible evidence that naphthalene does not cause cancer through a genotoxic carcino-

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gen, and raise doubt as to whether animals exposed to naphthalene with skin treatments exhibit biomarkers of genotoxic exposure that can be measured.

Depurinating DNA adducts in target skin cells. A finding of naphthalene-derived depurinating DNA adducts in the target cells would support the hypothesis that naphthalene has a genotoxic mechanism. If there is a corresponding increase in abasic sites in skin DNA, the conclusion will be stronger and this provide some support for the hypothesis that naphthalene can cause cancer through a genotoxic mechanism (depending on the occurrence of tumors in the target cells). A failure to find naphthalene-derived depurinating DNA adducts in the target cells will provide some support for the hypothesis that naphthalene does not have a genotoxic mechanism.

Mutations in the Harvey ras oncogene. A finding of exposure-related increases in mutations in the Harvey ras oncogene would support the hypothesis that naphthalene has a genotoxic mechanism and support the hypothesis that naphthalene can cause cancer through a genotoxic mechanism. A failure to find exposure-related increases in mutations in the Harvey ras oncogene would support the hypothesis that naphthalene does not cause cancer through a genotoxic mechanism.

5.3. Study III: Measurement of DNA adducts of naphthalene in urine of exposed human populations for use as a possible biomarker

5.3.1. Rationale

There is an urgent need for biomarkers of human exposure to naphthalene. A possible measure of human exposure to naphthalene may be provided by analysis of the depurinating 1,2-DHN-4-N3Ade and 1,2-DHN-4-N7Gua adducts in urine. This approach is suggested by the successful analysis of depurinating estrogen-DNA adducts in the urine of men with prostate cancer (Markushin et al., 2006) and women with breast cancer (Gaikwad et al., 2007).

Two millilitres aliquots of urine will be partially purified by solid-phase extraction and then analyzed for naphthalene metabolites and depurinating DNA adducts by ultra-performance liquid chromatography/tandem mass spectrometry. Because there may be serious legal issues with analysis of urine from workers, these studies will begin with urine samples from heavy smokers (Casale et al., 2001). Naphthalene is an important component of the tar and vapor phases of organic smoke, including cigarette smoke. Heavy cigarette smokers may provide a useful model to study depurinating naphthalene adducts. If permission is granted, these studies will be extended to workers occupationally-exposed to naphthalene. These studies should be done in a blinded fashion, with exposure information only known after urinary data are completed.

5.3.2. Major inferences

The identification of targeted DNA adducts in urine would provide evidence that people exposed to naphthalene exhibit potential biomarkers of genotoxic exposure. The failure to identify targeted DNA adducts in urine would indicate that people exposed to naphthalene may not exhibit measurable biomarkers of genotoxic exposure.

5.4. Study IV: Examination of pathways of DNA damage response caused by naphthalene and its metabolites to identify the mode of action for naphthalene carcinogenicity

5.4.1. Rationale

This study addresses the pathways of DNA damage response caused by naphthalene and its metabolites to identify whether naphthalene is a mutagenic or non-mutagenic carcinogen in mice. A series of isogenic cell lines deficient in various DNA metabolism pathways will be utilized to characterize the DNA damage responses caused by test compounds. Based on the results from the cultured cells, mice deficient in specific DNA damage responses (e.g., nucleotide excision repair (NER), responsible for stable bulky adducts) will be exposed to naphthalene.

5.4.2. Major inferences

DNA damage responses in a series of isogenic cells deficient in various DNA metabolism pathways. Identification of DNA damage responses caused by naphthalene metabolites would support the hypothesis that naphthalene has a genotoxic mechanism. Also, it would show which kind of DNA lesions (stable adducts, depurinating adducts, or abasic sites) significantly impact cells exposed to naphthalene or its metabolites. Failure to identify DNA damage responses caused by naphthalene metabolites would provide some evidence that naphthalene does not have a genotoxic mechanism.

Stable DNA adducts in target tissue. Identification of increased numbers of stable DNA adducts from naphthalene in the exposed DNA repair (NER) deficient mouse lung versus wild-type mouse lung would support the hypothesis that naphthalene has a genotoxic mechanism and support the hypothesis that naphthalene could cause cancer through a genotoxic mechanism. Failure to identify increased numbers of DNA adducts from naphthalene in exposed DNA repair deficient mouse lung would provide some evidence that naphthalene does not have a is not genotoxic when compared to its effect on wild-type mouse lung, and provide some evidence that naphthalene is not a genotoxic carcinogen when compared to its effect on wild-type mouse lung.

Clara cell toxicity in mouse lung. Identification of more extensive Clara cell toxicity in the naphthalene-exposed DNA repair (NER) deficient mouse lung versus wild-type mouse lung would support the hypothesis that Clara cell toxicity is associated with the existence of DNA damage. Failure to identify acceleration of Clara cell toxicity in the naphthalene-exposed DNA repair (NER) deficient mouse lung compared to wild-type mouse lung would provide strong evidence that cell toxicity is independent of DNA damage.

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Mouse lung tumor (long-term project). Identification of an increased incidence of lung tumors in the naphthalene-exposed DNA repair (NER) deficient mouse compared to that in the wild-type mouse would support the hypothesis that naphthalene is a genotoxic mouse lung carcinogen. Failure to identify acceleration of Clara cell toxicity in the naphthalene-exposed DNA repair (NER) deficient mouse lung compared to wild-type mouse lung would support the hypothesis that naphthalene is a non-mutagenic mouse lung carcinogen.

5.5. Study V. An evaluation of the enzymology of naphthalene metabolism, with implications for the likelihood that specific metabolic pathways will occur in different species

This project has two parts.

5.5.1. Part A

Based on results from a preliminary study of naphthalene in mouse skin presented by Cavalieri (in preparation), it is possible that naphthalene and its metabolites 1-naphthol, naphthalene-1,2-dihydrodiol and 1,2-dihydroxynaphthalene are converted to 1,2-naphthoquinone by CYP1B1. This is suggested by similar studies conducted on the metabolism of estradiol to estradiol-3,4-quinone (Belous et al., 2007). To demonstrate whether CYP1B1 is involved, we propose to use recombinant human CYP1B1 to activate the various metabolites, 1-naphthol, naphthalene-1,2-dihydrodiol and 1,2-dihydroxynaphthalene, to 1,2-naphthoquinone in the presence of DNA. Formation of the 1,2-DHN-4-N3Ade and 1,2-DHN-4-N7Gua adducts will be the endpoint for the studies.

If warranted, the ability of other human recombinant CYP, such as CYP1A1, to activate naphthalene and its metabolites to 1,2-NQ will also be analyzed.

5.5.2. Part B

It is also proposed to use a CYP2F1-transfected human lung epithelial cell line (BEAS-2B) developed by Dr. Garold Yost (University of Utah) for studying mutagenesis by naphthalene. These transfected BEAS-2B cells express the human CYP2F1 under a CMV promoter. The parental BEAS-2B cells may be a useful model for the human lung Clara cells, as they retain the expression of several cell surface markers. These cells have only a background level of activity of metabolizing genotoxic compounds. Studies in the Yost laboratory indicate that the residual activity is related to CYP1A1. Comparison of naphthalene mutagenesis in BEAS-2B and BEAS-2B-CYP2F1 cells will address some of the important questions about naphthalene genotoxicity in human lung.

The results of these two types of study will provide insight into how humans metabolize naphthalene. A finding that CYP 1B1 is the enzyme that catalyzes 1,2-dihydroxynaphthalene to 1,2-naphthoquinone would help to elucidate a key metabolic pathway, with implications for the likelihood that this pathway will occur in different species. A failure to find that CYP 1B1 is the enzyme that catalyzes 1,2-dihydroxynaphthalene to 1,2-naphthoquinone would leave metabolic pathways and species extrapolation more uncertain.

5.6. Study VI. Bioassays with knockout and "humanized" mice to determine key enzymes in the initial metabolic activation of naphthalene in mice and study mediation processes for carcinogenicity in the liver or lung

The tissue-selective tumorigenicity of naphthalene in rats and mice is likely related to the occurrence of highly active P450 enzymes in the target tissues.

Available mouse models for these studies include *Cyp1b1*-knockout, liver-specific P450 reductase (*Cpr*) knockout, lung-specific P450 reductase (*Cpr*) knockout, and CYP2F1-transgenic mice. *Cyp2f2*-knockout mice are expected to be available in the near future. Loss of P450 reductase will lead to loss of all microsomal P450 activities. These mice will be exposed to naphthalene under conditions used in the NTP study.

Transgenic mouse models, which are either already available or will be available in the near future, can be used to answer the following questions:

(a) Is the lung tumorigenicity of naphthalene in mice mediated by metabolic activation in the liver or in the lung?

If lung tumors are not found in mice lacking liver metabolic activation activity, or if a specific decrease in lung metabolic activation does not lead to a decrease in lung tumor incidence, then this indicates that hepatic metabolic activation plays an important role in lung tumor formation in mice. Consequently, risk prediction for humans would have to consider human hepatic P450 activity since the stability of the reactive metabolites is likely the same in mice and humans.

If lung tumors are found in mice lacking liver metabolic activation activity, or if a specific decrease in lung metabolic activation does lead to a decrease in lung tumor incidence, then this indicates that hepatic metabolic activation does not play a critical role in lung tumor formation in mice. Consequently, risk prediction for humans would primarily consider human lung P450 activity.

(b) Can naphthalene induce lung tumors in mice lacking CYP2F2, which is believed to be the key enzyme in the initial metabolic activation of naphthalene in mice?

If lung tumors are not found in *Cyp2f2*-null mice, then this indicates that P450s other than CYP2F2 are not critical in naphthalene-induced tumorigenesis in mice.

If lung tumors are found in the *Cyp2f2*-null mice, then this indicates that other P450s are equally important or more important than CYP2F2 in tumorigenicity.

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(c) Can naphthalene induce lung tumors in mice lacking CYP2F2, but expressing human CYP2F1 (i.e., CYP2F-humanized mice)? CYP2F1 is believed to differ from rodent CYP2F enzymes in having a very low activity toward naphthalene.

If tumors are not found in CYP2F-humanized mice, then this suggests that human CYP2F1 may not be able to mediate naphthalene-induced lung cancer.

If tumors are found in CYP2F-humanized mice, and the tumor numbers are greater in the humanized mice than in *Cyp2f2*-null mice, then this indicates that human CYP2F1 is capable of mediating naphthalene-induced lung cancer, and, consequently, naphthalene could be a human carcinogen. Future efforts would then focus on identifying factors that influence CYP2F1 expression and functions in human tissues.

(d) Can naphthalene induce lung tumors in mice lacking CYP1B1, which has been proposed to mediate a critical pathway to genotoxicity?

If lung tumors are not found in *Cyp1b1*-null mice, then this indicates that CYP1B1 plays an important role in the mechanisms of naphthalene carcinogenicity, and the data would support the proposed role of CYP1B1 in the mechanism of naphthalene genotoxicity.

If a difference in lung tumor incidence is not found between the *Cyp1b1*-null and wild-type mice, then CYP1B1 is unlikely important for naphthalene genotoxicity and tumorigenicity.

In parallel with these bioassays, stable DNA adducts in isolated Clara cells and depurinating adducts in the urine can be measured, and toxicokinetics of plasma naphthalene and its metabolites can be determined, for the various transgenic mice. The results will validate and help to explain the findings from the bioassays.

6. Summary

This report provides a summary of the current state-of-the-science concerning the role played by genetic toxicity and DNA damage in the mode of action for naphthalene-induced tumors in rats and mice and defines specific areas of uncertainty that must be addressed to improve the state of knowledge. A series of possible studies are outlined that address the critical areas of uncertainty. The study outlines provide a level of detail that makes them useful as guides, but they are not intended to be final definitive research protocols.

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Critical assessment of the genetic toxicity of naphthalene ☆

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Abstract

Studies demonstrating that naphthalene produces respiratory tract tumors in mice and rats raised the question of whether humans are at risk for cancer, at environmental or workplace concentrations of naphthalene. Arguments in favor of a threshold-dependent mode of action for tumor induction have been based on the facts that naphthalene does not appear to bind to DNA *in vivo* and that the rodent tumors occurred at high dose levels associated with substantial target site toxicity. A summary of more than 45 publications describing results for naphthalene in genetic toxicology test methods shows that 80% of the studies reported found no evidence of genotoxicity for naphthalene and that some of the studies which reported positive finding were technically unsuited to study this class of chemicals and, therefore, generated unreliable data. The remaining positive findings for naphthalene were all consistent with secondary DNA effects produced by toxicity from naphthalene alone or one of its metabolites. Based on the data reviewed in this report, it is not apparent that genetic lesions produced by naphthalene or any of its metabolites drive the tumorigenic activity.

Keywords: Naphthalene; Naphthoquinone; Genotoxicity; Cancer; Mode of action; Clastogenicity; Cytotoxicity; Mutation; Adducts

1. Introduction

Naphthalene, a bicyclic aromatic compound can be found environmentally as a constituent of coal tar, crude oil and cigarette smoke. It is also used in chemical manufacturing as a chemical intermediate for many commercial products ranging from pesticides to plastics. Because of extensive human exposure, the toxicological properties of naphthalene have been the subject of numerous assessments. Of particular interest to regulators has been an evaluation of naphthalene's potential to induce tumors.

Studies conducted by the National Toxicology Program (NTP) established that naphthalene was carcinogenic to both mice and rats in life-time inhalation exposures (Abdo et al., 1992, 2001). Significant increases in tumors were found in both species at concentrations near 10 ppm. The primary site of tumors in the mice was lung and the primary site in rats was the nose. The concentrations of naphthalene to which the mice and rats were exposed in the bioassays were considerably higher than the typical human ambient air concentrations estimated at 0.0002 ppm.

The findings that naphthalene can produce respiratory tract tumors in mice and rats raised the question of whether humans, at environmental or workplace concentrations of naphthalene, are at risk for cancer. In order to address these concerns, a better understanding of the mode(s) of action of naphthalene in the rodent models will be needed.

Relevance of the naphthalene rodent cancer bioassay data to humans has been contested for a number of reasons. Arguments in favor of a threshold-dependent mechanism have been based on the facts that naphthalene does not appear to bind to DNA *in vivo* and rodent tumors occurred at high dose levels associated with substantial target site toxicity (Buckpitt et al., 2002). Additional arguments are related to the differences in airway architecture and metabolism between rodents and humans.

The available database for naphthalene genotoxicity is extensive and has been reviewed recently by IARC (2002), Schreiner (2003), the US EPA (2004a,b) and Butter-

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worth (2004). For the most part, the reviews consisted of compiled lists of assay responses without any critical assessments of the studies or interpretation of the complete data set. The reviews by Schreiner and Butterworth provided data interpretations and both authors suggested that naphthalene is not mutagenic to bacteria or mammalian cells in culture, but does produce toxicity-dependent secondary damage to DNA at excessive concentrations. They hypothesized an indirect mechanism for naphthalene genotoxicity citing excessive toxicity. Evidence for the secondary damage was evident in the DNA fragmentation responses *in vivo*, as well as sister chromatid exchange (SCE) and chromosome breakage *in vitro*. Schreiner (2003) concluded that the DNA fragmentation and chromosome breakage events are consistent with a threshold-related cytotoxicity mechanism of tumor induction, driven by cytotoxicity and cell regeneration leading eventually to tumor development. Butterworth's interpretation of the genetic toxicology data was similar and also indicated that while there may be *in vitro* studies suggesting that naphthoquinone metabolites bind to DNA (McCoull et al., 1999), the majority of the interactions between naphthalene metabolites and macromolecules *in vivo* is with proteins (Waidyanatha et al., 2002) resulting in cytotoxic rather than genotoxic events.

The US EPA summarized essentially the same set of data, but formed a different conclusion. In the IRIS report (US EPA, 2004b) the agency concludes that "a possible genotoxic mode of carcinogenic action cannot be discounted as there are some data indicating genotoxicity of 1,2-napthoquinone". This reference to the mutagenicity of 1,2-naphtoquinone was derived from a paper by Flowers-Geary et al. (1996) in which a small increase in the reversion counts was seen with several tester strains including TA104, a Salmonella strain believed to respond to oxidative DNA damage. A recent publication by Saeed et al. (2007), reported the chemical formation of purine adducts and concluded that naphthalene should be considered a genotoxic carcinogen. However, no studies demonstrating the formation of DNA adducts or DNA binding *in vivo* have been published.

Resolution of the genotoxic mechanism(s) of naphthalene is essential for the development of an accurate risk assessment of this chemical for humans. The following critical assessment is based primarily on the published genotoxicity database provided in the ATSDR report (2005). This database includes the information assessed by Schreiner (2003), US EPA (2004a,b) and Butterworth (2004).

2. Critical assessment: naphthalene

Is it necessary that the database for naphthalene have all negative studies in order to be classified as a non-genotoxic carcinogens? An extensive investigation of the test responses of chemicals having large, heterogeneous databases conducted by a working group of the International Commission for Protection Against Environmental Mutagens and Carcinogens showed that genotoxicity profiles of most non-carcinogens and non-genotoxic carcinogens contain at least one positive response (Mendelsohn et al., 1992). Consequently, a chemical believed to be a non-genotoxic carcinogen need not have all tests for DNA reactivity exhibit negative findings. False positive responses occur for several reasons including spurious (non-repeatable) positives and positives due to secondary treatment-related factors such as pH, osmolarity and cytotoxicity (Brusick, 1987; Muller and Sofuni, 2000). To develop a reliable interpretation of heterogeneous genetic toxicology data, such as that associated with naphthalene, one must provide a critical assessment of all studies and then inspect the weight-of-evidence from the valid studies.

2.1. The majority of all genotoxicity studies failed to show any genotoxicity

A summary of more than 45 publications describing results for naphthalene in genetic toxicology test methods shows that 80% of the studies conducted reported no evidence of genotoxicity for naphthalene (Schreiner, 2003; Butterworth, 2004; ATSDR, 2005). Related compounds 1- and 2-methylnaphalene are also without genotoxic activity in either the Ames test or tests for SCEs in cultured human cells (Florin et al., 1980; Kulka et al., 1988). A gross examination of this data would suggest that the weight-of-evidence favors an interpretation that naphthalene is not a genotoxic carcinogen. However, the US EPA (2004b) suggested that the subgroup of studies reporting positive effects should be given significant weight in developing a mode of action for the carcinogenic activity of naphthalene. A critical assessment of the studies used by the US EPA to form this opinion reveal a number of technical issues that raise concerns about the reliability of the studies.

2.2. Reliability of responses found in the naphthalene genotoxicity database

A critical inspection of studies compiled from the naphthalene published genetic toxicology literature indicates that the database for this chemical is typical of those found for other extensively tested chemicals. There is a substantial amount of redundancy in the tests conducted, particularly the Ames test, but there are a small number of relatively esoteric and obscure methods employed that are not adequately validated to always appreciate the significance of positive or negative responses in them. The following set of concerns point out some of the issues addressed in the critical assessment of the database.

- Responses with the naphthalene or metabolites in the same genetic endpoint, at comparable dose levels, were conflicting in several of the tests. Because some of these assays are known to produce spurious responses (Kirkland and Dean, 1994), the validity of some of the data was difficult to assess.

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- Some tests used to evaluate naphthalene or metabolites were known to have high sensitivity and low specificity for this class of compounds (e.g., Drosophila wing spot test). As a consequence, positive results for naphthalene in these tests should be viewed with skepticism.
- Some of the endpoints selected for assessment (e.g., DNA fragmentation) are known to be susceptible to induction by secondary effects as well as direct DNA damage.
- Naphthalene is known to be metabolized by rat microsomes *in vitro* to toxic naphthoquinones, the hypothesized reactive intermediates, and tests known to produce false positive results from cytotoxicity, such as *in vitro* clastogenicity, did show positive responses with S9 (NTP, 1992; Gollahon et al., 1990). None of the assays measuring specific gene mutations were positive when S9 mix was employed (Mortelmans et al., 1986; Kaden et al., 1979; Sasaki et al., 1997).

2.3. Studies in which naphthalene failed to show genotoxicity

The genetic toxicology studies which found no evidence of genotoxic activity cover a broad range of genetic endpoints and phylogenetic levels and are summarized in Table 1. Confirmatory responses in the same or similar tests and genetic endpoints were evident in the database and support the absence of activity.

2.4. An analysis of the positive findings for naphthalene

Table 2 summarizes those studies in which positive finding were reported by the authors. Two of the studies should not be considered reliable for technical reasons. The "Mutatox Test" is reported to be a test measuring reverse mutation in *Vibrio fischeri* (strain M169). The mutant strain is a non-luminescent variant which can be reverted to the luminescent form as measured via a light detector and has been viewed as a possible alternative to the Ames test. Scientists comparing the results of the Mutatox test with results of the Ames test (Jarvis et al., 1996) suggest that any positive in this assay should be confirmed using an Ames test. All 15 of the standard Ames tests with naphthalene were reported to be negative raising serious questions about the reliability of the Mutatox assay.

The second study considered to be unreliable is the Drosophila wing test; it cannot be used to define naphthalene as a genotoxic carcinogen as this test appears to be uniquely sensitive to this class of chemicals but cannot differentiate between carcinogens and non-carcinogens. In addition to naphthalene, two other non-carcinogens anthracene and phenanthrene were reported to be positive in this assay (Delgado-Rodriguez et al., 1995).

The NTP study of sister chromatid exchange (SCE) indicated a questionable positive response in CHO cells (NTP, 1992). A study of the ability of naphthalene to induce SCE in human leukocytes supplemented with human liver microsomes by Wilson et al. (1996) was reported to be negative raising some concern about the reproducibility of this genetic lesion in mammalian cells. CHO cells are believed to be more sensitive to secondary mechanisms of genotoxicity than primary human cells, and use of human cells and human S9 would appear to provide a more relevant response (Kirkland and Dean, 1994).

Positive *in vitro* clastogenic positive responses with naphthalene either required (NTP, 1992) or were amplified with addition of S9 (Gollahon et al., 1990), suggesting that the highly reactive naphthoquinones produce cytotoxic effects through reaction with chromosomal proteins. The failure to find gene mutation under similar treatment conditions supports the hypothesis that the chromosomal damage was a secondary response to cytotoxicity.

The *in vivo* DNA fragmentation events identified by Bagchi et al. (1998, 2002) would be expected to lead to induction of either DNA repair (UDS) or clastogenic lesions such as micronuclei if naphthalene was genotoxic. Neither UDS nor clastogenic activity was observed in mice or rat exposed to much larger dose levels of naphthalene than those shown to induce DNA fragmentation. The fragmentation they detected is more likely the result of a secondary effect produced by release of lysosomal enzymes (linked to toxicity) that would degrade DNA during sample preparation.

Table 1
A summary of the studies with naphthalene finding no evidence of genotoxicity

- Gene (or point mutation) tests in bacteria, cultured mammalian cells with and without S9 mix (17 studies). These studies included gene mutation in the Ames test, a test for forward mutation in *S. typhimurium* strain TM677 and at the *tk* and *hprt* loci of cultured human lymphoblastoid cells
- Chromosome breakage *in vivo* (2 studies)
- DNA repair synthesis induction in rat liver cells either *in vitro* or *in vivo* (4 studies)
- Six bacterial tests measuring induction of repairable DNA damage (e.g., Rec Assay, SOS chromotest)
- Morphological cell transformation assays employing hamster, mouse, rat and cultured human cells *in vitro* (5 studies)

Table 2
Studies reporting positive findings with naphthalene

1. Reversion of bioluminescent bacteria "the Mutatox test" (Arfsten et al. 1994)
2. Drosophila wing spot test (Delgado-Rodriguez et al., 1995)
3. Micronuclei induction in amphibian larvae (Djomo et al., 1995)
4. Chromosome damage in preimplantation embryos *in vitro* with the addition of S9 (Gollahon et al., 1990)
5. Chromosome breaks in CHO cells *in vitro* (NTP, 1992)
6. Weak induction of SCEs (NTP, 1992)
7. Induction of micronuclei *in vitro* in human lymphoblasts (Sasaki et al., 1997)
8. DNA fragmentation in rat liver and brain tissues following subchronic exposure to naphthalene (Bagchi et al., 2002)
9. DNA fragmentation in mouse liver and brain tissues following single doses of naphthalene (Bagchi et al., 1998)

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3. Critical assessment: naphthalene metabolites

3.1. DNA binding studies

Naphthalene appears to have no specific affinity for binding directly to DNA; however, an early study by McCoull et al. (1999) indicated possible DNA binding from metabolites. More recently Saeed et al. (2007) reported that 1,2-naphthoquinone reacts with the purine adenine and guanine *in vitro* to form adducts at the N3 and N7 positions of the two molecules, respectively. Based upon previous research with estrogenic chemicals and benzene, Saeed et al. hypothesized that the naphthoquinone adducts produced by naphthalene metabolites would produce depurinated sites in the DNA that could lead to mutation and tumors (Chakravarti et al., 2000; Markushin et al., 2006). While DNA binding studies provide evidence that the necessary reactivity to produce DNA adducts is possible, there have been no studies in living organism that confirm such adducts are, in fact, produced. This means that a mechanistic understanding of the genotoxic effects seen from naphthalene or its metabolites must be derived from conventional genetic toxicology methods.

3.2. Genetic test results of naphthalene metabolites

Some of the major naphthalene metabolites were tested as pure compounds. The major metabolite of naphthalene is 1,2-arene oxide, which is unstable leading ultimately to the production of 1,4- and 1,2-naphthoquinones. Other metabolites include 1-naphthol and 1,2-dihydroxynaphthalene. Table 3 summarizes the result of genetic toxicology studies on naphthalene metabolites. Positive responses for 1,4-naphthoquinone were seen in clastogenicity and SCE tests previously identified as susceptible to secondary effects from cytotoxicity related to the depletion of intracellular glutathione (Wilson et al., 1996). When 1,4-naphthalene was evaluated in tests that measure specific gene mutation it was consistently absent of any genotoxicity. This response profile would be characteristic of chemical with an indirect mechanism of genotoxicity. The 1-naphthol, the epoxide and the 1,2-arene oxide metabolites were not genotoxic, but were only subjected to one test each.

Flowers-Geary et al. (1996) used the pre-incubation modification of the Ames test to evaluate a series of quione compounds derived from polycyclic aromatic hydrocarbons. Among the chemicals tested was 1,2-naphthoquinone. The tester strains used in the study were TA98, TA97a, TA100 and TA104. TA97a and TA98 both detect frameshift mutagens. Frameshift mutagens, particularly those active in strain TA97a, act through intercalation (non-covalent DNA interactions). Strains TA100 and TA104 typically respond to chemicals that bind to DNA forming adducts and produce base-pair substitution mutations. With 1,2-naphthoquinone, the most responsive strain based on fold-increase over the control and mutagenic efficiency was strain TA100 with a mutagenic efficiency value of 19.4. It was followed by TA97a (mutagenic efficiency of 18.9) and then by TA104 (mutagenic efficiency of 13.1). The response in strain TA98 was less than two times the spontaneous amount and did not achieve positive response criteria. All tests were conducted without the addition of S9 as the quinone is assumed to be the terminal metabolite form and the addition of S9 proteins would probably only suppress any mutagenic response. The results in their study suggest that if naphthalene was metabolized by S9 to 1,2-naphthoquinone, that the tester strain most sensitive to the mutagenic activity of the metabolite would be strain TA100. The naphthalene database contains more than 10 independent Ames tests employing strain TA100 plus S9 mix and none showed any indication of mutagenic activity. The negative results in those assays do not override the data of Flowers-Geary et al. but do show that it would require a considerable amount of naphthalene (certainly an amount that would be completely cytotoxic to bacteria) to generate sufficient 1,2-naphthoquinone to induce mutation in a strain of *Salmonella typhimurium* that is highly modified to enhance sensitivity.

Table 3
Studies reporting positive/negative findings with naphthalene metabolites

Positive studies

1. 1,4-Naphthoquinone induced chromosome breakage *in vitro* in human lymphoblastoid cell line MCL-5 (Sasaki et al., 1997)
2. 1,2- and 1,4-naphthoquinone induced SCE in human lymphocyte cultures (Wilson et al., 1996)
3. 1,2-Naphthoquinone induced reversion of *S. typhimurium* strains TA100* and TA104* in the absence of S9 (Flowers-Geary et al., 1996).

Negative studies

1. 1,4-Napthoquinone and 1-napthol were not mutagenic in the standard Ames test (Sakai et al., 1985; McCann et al., 1975)
2. 1,4-Naphthoquinone was not mutagenic in human lymphoblastoid cells at either the *tk* or *hprt* loci (Sasaki et al., 1997)
3. 1,2-Arene oxide was did not induce SCEs in cultured human lymphocytes (Wilson et al., 1996)

4. Summary and conclusions

The database of published studies for naphthalene can be characterized as a large, heterogeneous set of data from a broad range of model systems including microbial, insect, *in vitro* cell culture including human cells and rodent *in vivo* models. The responses of naphthalene in the majority of these tests indicated the compound is not DNA reactive and is non-genotoxic. Some of the studies which reported positive finding were clearly technically unsuited to study this class of chemicals and generated unreliable data. The remaining positive finding for naphthalene were all consistent with secondary effects produced by toxicity from naphthalene alone or one of its metabolites. High dose cytotoxicity is well-documented cause of chromosome damage in mammalian cells (Galloway, 2000).

Research has shown that under controlled chemical conditions naphthoquinones may form covalent adducts with

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guanine or adenine bases. The only that indication that such reactions occur in living organisms is the study by Flowers-Geary et al. (1996) reporting that 1,2-naphthoquinone is directly mutagenic in Ames strains TA100, TA97a and TA104, although similar activity cannot be produced *in vitro* by the addition of metabolic enzymes to naphthalene.

Based on the data reviewed in this report, it is not apparent that genetic lesions produced by naphthalene or any of its metabolites drive the tumorigenic activity of this compound exhibited in the rodent studies reported by NTP. A set of events consisting of target site toxicity and the induction of cellular regeneration appears to be the "driving" mode of action for naphthalene.

Conflict of interest statement

The author declares that he has no conflicts of interest. Each received an honorarium from Regulatory Checkbook, P.O. Box 319, Mt. Vernon, VA 22121, for service as set forward in Belzer, et al. (2007, in this issue).

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INDEPENDENT EXPERT OPINION ON THE GENOTOXIC POTENTIAL OF NAPHTHALENE COMMISSIONED BY THE NAPHTHALENE COALITION FOR SUBMISSION TO THE US ENVIRONMENTAL PROTECTION AGENCY

Expert Opinion on the Genotoxicity and Carcinogenicity of Naphthalene

Byron E. Butterworth, Ph.D.

July 23, 2004

EXPERT OPINION ON THE GENOTOXICITY AND CARCINOGENICITY OF NAPHTHALENE

Byron E. Butterworth, Ph.D., Fellow ATS

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EXECUTIVE SUMMARY

The issue addressed in this document is whether direct genotoxic activity played a role in naphthalene-induced tumors in the respiratory track of mice and the nose of rats or whether the tumor development was the result of activity associated with the cytotoxicity and inflammation at the tissue target sites. In situations with important compounds such as naphthalene, the database on genotoxic potential grows extremely large and there is always a scattering of reported positive responses, even for clearly nongenotoxic compounds. A critical review of the broad database is required to resolve the inevitable conflict between those focusing on positive vs. negative responses. In this independent review I critically examined the published studies on naphthalene relative to its carcinogenic and genotoxic potential. Emphasis was placed on the relevance of the endpoint measured; whether an assay defined effects in the whole animal; the degree of validation, specificity, and reliability of the test system; whether cell culture effects translated into analogous activity in the whole animal; the internal consistency of similar tests; and the quality of the experimentation.

- Naphthalene failed to produce liver tumors in either B6C3F_1 mice or F344 rats in the NTP bioassays. Liver is metabolically competent and B6C3F_1 mice are genetically predisposed and extremely susceptible to induced liver cancer. The lack of liver tumors in this strain is a strong indication of the lack of genotoxic activity of naphthalene.

- Naphthalene tested negative in seven well-validated and meaningful in vivo genotoxicity and/or cancer assays providing solid evidence that naphthalene is without genotoxic activity in a range of tissues in the whole animal.

- Numerous widely accepted in vitro assays confirmed the lack of genotoxic activity of naphthalene. Some evidence of induced cytogenetic effects was seen in vitro, but these did not translate into activity in the whole animal.

- The few positive responses reported were noted only with assay systems with known low specificity, or lacked significant validation, or used questionable procedures.

The weight of evidence strongly supports the conclusion that naphthalene is not genotoxic and that tumor formation was likely via a nongenotoxic-cytotoxic mode of action. Thus, protecting individuals from naphthalene exposures likely to induce cytotoxicity, will also be protective against an increased risk of cancer.

INTRODUCTION

Naphthalene induced an increase in alveolar/bronchiolar adenomas and one carcinoma in female B6C3F_1 mice in a 2 year inhalation bioassay (NTP 1992). Naphthalene also induced an increase in adenomas of the nasal respiratory epithelium and an increase in neuroblastomas of the olfactory epithelium of male and female F344/N rats in a separate 2 year inhalation bioassay (NTP, 2000). A critical question is whether direct genotoxic activity played a role in tumor formation or whether the tumor development was the result of activity associated with the cytotoxicity and inflammation at the tissue target sites. Appropriate risk assessment and safety considerations are quite different depending on the manner in which the tumors were formed.

Because of the large literature and differing views on the carcinogenic potential of naphthalene, I was asked by the Naphthalene Coalition to provide an independent expert opinion regarding the genotoxic potential of naphthalene and probable mode of action of naphthalene induced rodent tumors. That Association was helpful in providing me with a broad spectrum of published articles and reviews of as well as compensating me for my time. The opinions presented are mine alone and were not influenced by the Sponsor.

By way of introduction, I have been actively involved in research in genetic toxicology and chemical carcinogenesis for over 30 years. I have served as Chief of the Molecular Biology Section at DuPont's Haskell Toxicology Laboratory. I have held various positions at the Chemical Industry Institute of Toxicology including Head of the Department of Genetic Toxicology and Director of the Chemical Carcinogenesis Program. I am currently President of Butterworth Consulting providing expertise on toxicological issues. I have served as President of the Carcinogenesis Specialty Section of the Society of Toxicology, and am a fellow of The Academy of Toxicological Sciences. The *in vivo* hepatocyte DNA repair assay was developed in my laboratory. I have directed research studies on the mechanism of action of numerous carcinogens and played a leading role in defining how knowledge of mode of action might guide the risk assessment process (Butterworth *et al.*, 1995; Butterworth and Bogdanffy, 1999).

Arriving at an appropriate carcinogen classification and risk assessment approach for naphthalene is complicated because of the unusually large amount of experimentation that has been conducted with this compound. As such, differing viewpoints of mechanism and cancer risk can be justified by uncritically picking which published studies to emphasize. The goal of this report is to provide a fresh, comprehensive perspective and critical assessment of the science. In some cases rather than listing all published papers here, I will refer to summaries and conclusions from review articles. Review articles that were particularly comprehensive and well written include those by Buckpitt et al. (2002), Schreiner (2003), and IARC (2003).

MODE OF ACTION

Cancer is a complex multistep process involving sequential mutations in growth control genes and clonal expansion of precancerous and cancerous cell populations. Chemical carcinogens may interact with cells and biological systems in various ways that directly or indirectly result in the mutational and clonal growth events associated with carcinogenesis. It is valuable to consider the manner in which mutations are induced or the way in which clonal growth of preneoplastic cells is affected by the

chemical agent of concern. The term “mechanism of action” is often used in this context, that term implies a too simplistic view of an exceedingly complex process. The broader term “mode of action” is more appropriate. Mode of action is defined as the identification of a fundamental obligatory step in the carcinogenic process (Butterworth et al., 1995). Examples include genotoxic, nongenotoxic-cytotoxic, and nongenotoxic-mitogenic modes of action.

Genotoxic carcinogens are direct acting compounds that are DNA reactive or have DNA reactive metabolites, directly act to alter chromosome structure or number, and induce mutational events at the base-pair or chromosomal level. Genotoxic chemicals are reproducibly and broadly active in assays that measure activity such as reactivity with DNA, induction of mutations, induction of DNA repair, or cytogenetic effects. Risk assessments models for genotoxic carcinogens such as the linearized multistage (LMS) model generally assume a linear response that goes through the origin at low doses.

Nongenotoxic chemicals are those that lack DNA reactivity or direct chromosome-altering activity as a primary biological effect. Nongenotoxic-cytotoxic carcinogens exhibit sustained toxicity to the target tissue. Mutational and promotional biological activities occur as a result of the continual toxicity and cell death . Examples include cytotoxicity and inflammation releasing nucleases and generating DNA reactive oxygen radicals, induced regenerative cell proliferation increasing the probability of mutational events as well as greatly increasing the effective mutation rate of any endogenous or exogenous DNA adducts, and release of growth factors providing a selective growth advantage to precancerous or cancerous cells. Because the driving forces for tumor formation all result from preceding toxicity, doses that are not toxic present no increased risk of induced cancer.

Nongenotoxic-mitogenic carcinogens induce direct stimulation of growth and appear to provide a selective growth advantage to initiated precancerous cells. These

agents usually induce promotional activity. Doses below which induced cell growth and promotion are not seen are assumed not to present an increased risk of cancer

The naphthalene-induced pulmonary alveolar/bronchiolar adenomas and a carcinoma in female mice were associated with chronic inflammation in the lung. Chronic inflammation, metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium were also seen in the nose. (NTP, 1992). The naphthalene-induced respiratory epithelial adenomas and olfactory epithelial neuroblastomas of the nose in male and female F344 rats were associated with atypical hyperplasia, atrophy, chronic inflammation, and hyaline degeneration of the olfactory epithelium; hyperplasia, squamous metaplasia, hyaline degeneration and goblet cell hyperplasia of the respiratory epithelium, and glandular hyperplasia and squamous metaplasia (NTP, 2000). In no case was tumor induction observed without preceding cytotoxicity. Clearly, events associated with cytotoxicity played a primary role in the induction of tumors by naphthalene. The critical question addressed in this paper is whether a genotoxic component was also involved.

Carcinogens acting by a genotoxic mode of action tend to exhibit carcinogenic activity across target organs, gender, and species (Ashby and Tennant 1991). More potent genotoxic carcinogens also exhibit shortened time to tumors. The only tumors induced by naphthalene were highly tissue-specific and were associated with site- and species-specific patterns of naphthalene metabolism and resulting cytotoxicity (reviewed by Buckpitt et al., 2002). This pattern indicates that naphthalene is not a classical multi-tissue genotoxicant.

In general, a substantial degree of sustained cytotoxicity, regenerative cell proliferation, and associated events are required for tumor formation by a nongenotoxic-cytotoxic mode of action (Butterworth and Bogdanffy, 1999). In contrast, the combination of forced cell turnover in the presence of a DNA reactive genotoxic mutagen is an exceedingly potent means of tumor induction. In that case, DNA adducts are present in high numbers and cell replication results in their conversion to irreversible

mutations before DNA repair can occur. Some potent mutagens when given at a cytotoxic dose that also induces necrosis and cell turnover can actually be single dose carcinogens. Induction of cell division is always a component of experimental models that are designed to maximize the effectiveness of mutagens (Tsuda et al., 1980; Columbano et al., 1981; LaVoie et al., 1988). Contrasting the nasal tumor responses for formaldehyde and naphthalene suggests that direct genotoxicity was not involved in the tumorigenic process for naphthalene. Formaldehyde is both a direct acting mutagen and a nasal cell toxicant. When the airborne concentration of formaldehyde was increased to the level that there was induced toxicity and cell proliferation, tumors were also produced at that level (Kerns et al., 1983; Monticello and Morgan, 1990). The toxicity and tumor dose-response curves rose in parallel because the genotoxic and cytotoxic activities were such a potent combination (Morgan et al. 1986). In contrast to formaldehyde, naphthalene did not show this tight correlation with toxicity. Naphthalene induced chronic inflammation, metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium of the nose and chronic inflammation of the lung at both the low and high concentrations in both sexes of mice (NTP, 1992). Yet, the only tumors seen were a modest incidence of lung tumors at the high dose in the female mice (NTP, 1992). If naphthalene had been genotoxic as well as cytotoxic, it is probable that tumors would have been seen at all sites, at both doses, and in both sexes of the mice. Cytotoxic and carcinogenic effects were more closely linked in the rat naphthalene inhalation bioassay (NTP, 2000).

REVIEW OF THE WHOLE ANIMAL AND IN VITRO GENOTOXICITY LITERATURE

Because of the sensitive nature of many cell culture assays used to screen for potential genotoxic activity, even nongenotoxic compounds will invariably be reported as positive in the odd test when subjected to a large number of assays in many laboratories. In such cases model systems and experimental details must be critically reviewed with more emphasis placed on whole animal assays and test systems proven by large validation studies. This critical weight of the evidence approach is mandatory in arriving at any meaningful conclusion for compounds such as naphthalene. Genetic toxicology assays are organized in a tiered manner. Testing usually begins with short-

term inexpensive bacterial or cell cultures assays. Positive results are then verified in *in vivo* genotoxicity assays to determine whether those effects translate into activity in the whole animal. This is critical because of the substantially more complex nature of processes such as uptake, distribution, activation, detoxification, and elimination that occur in the treated animal. In this section the genotoxic activity of naphthalene is contrasted in test systems beginning with the most relevant whole animal assays and ending with cell culture screening tests and poorly validated systems.

Significance of the Lack of a Tumor Response in the B6C3F$_1$ Mouse Liver

The B6C3F$_1$ mouse is genetically predisposed to formation of liver tumors and as such constitutes a particularly sensitive rodent cancer model (Drinkwater, 1994). A common criticism of the B6C3F$_1$ mouse is that it is uncommonly susceptible to the induction of liver cancer, and thus is an inappropriately sensitive system in trying to realistically predict carcinogenic potential in humans. For example, it is common for a chemical to induce cancer in the mouse liver, but not the rat liver or other target organs (Ashby and Tennant, 1988). Liver tumors are readily induced in the B6C3F$_1$ mouse by chemical carcinogens acting through different processes including genotoxic, nongenotoxic-cytotoxic, and nongenotoxic-mitogenic modes of action (Butterworth et al., 1995; Ashby and Tennant, 1988). Further, liver is the most prevalent target tissue site for a broad spectrum of chemical carcinogens (Ashby and Tennant, 1988, 1991). Because mutation induction is cumulative and irreversible, cancer bioassays are meaningful long term tests for genotoxic activity, and this is particularly true for the induction of B6C3F$_1$ mouse liver tumors. Thus, the lack of a liver tumor response in the naphthalene B6C3F$_1$ mouse and F344 rat cancer bioassays constitutes very strong scientific evidence that no genotoxic activity was occurring in either the mouse or rat liver in response to naphthalene. This lack of genotoxic activity is in spite of the fact that naphthalene is metabolized to naphthalene-1,2-epoxide, 1-naphthol, and 1,2-naphthalenediol by liver CYP enzymes (Buonarati et al., 1989; Buckpitt et al., 2002).

In several instances noted below patterns of cytotoxicity or genotoxicity are ascribed to metabolites of naphthalene. As examples, naphthalene induced

chromosomal aberrations in CHO cells in culture with liver S9 (NTP, 1992). The putative liver metabolites 1,2- and 1,4-naphthoquinone induced sister chromatid exchanges (SCE) in human peripheral mononuclear leukocytes (MNL) (Wilson et al., 1996). A redox cycle producing active oxygen species has been proposed for metabolites of naphthalene as a toxic pathway in isolated hepatocytes (D'Arcy Doherty et al., 1984). Naphthalene induced DNA fragmentation has been reported in the livers of treated mice (Bagchi et al., 2000). Results such as these have been cited as evidence that naphthalene is acting via a genotoxic mode of action. A common feature of these reports is the use of liver preparations, liver cells, or liver tissue. Yet, the lack of liver tumors in the F344 rat and particularly in the $B6C3F_1$ mouse bioassays quite conclusively show that no such genotoxic processes were occurring in the liver with continual 2-year exposures to naphthalene (NTP, 1992; NTP, 2000). This is perhaps the strongest evidence of all that these proposed pathways are not operational.

A/J Mouse Lung Cancer Model

The A/J mouse lung cancer model is an intermediate-term cancer bioassay designed to screen for potential lung carcinogens (Stoner, 1991). A/J mice are genetically predisposed to be susceptible to the formation of lung tumors. Concern with this assay is that it tends to be somewhat oversensitive. A/J mice were exposed for up to 30 ppm of naphthalene for 6 hours per day, 5 days per week, for six months (Adkins et al., 1986). Naphthalene did not induce an increase the incidence of lung adenomas relative to concurrent controls. The authors of the study state: "Exposure to naphthalene was without significant effect on tumor frequency, but was significant when the incidence of tumor formation was compared with the pooled, negative control response." That is, in fact, a misstatement. Naphthalene did not induce an increase in the incidence of lung adenomas relative to concurrent controls. The increase in the tumors per tumor-bearing mouse was slightly elevated only because the control for the naphthalene group was *unusually low* compared to all the other groups. No increase in the tumor incidence was seen at all. When compared to the pooled negative control response, naphthalene was clearly negative by any measure.

The A/J mouse lung cancer assay is sensitive to genotoxic chemicals including polycyclic hydrocarbons, nitrosamines, nitrosoureas, carbamates, aflatoxin, metals, and hydrazines (Stoner, 1991). Thus, if naphthalene had exhibited genotoxic activity, it almost surely would have induced lung tumors in this bioassay. The negative result in this study is a strong indication that naphthalene does not express genotoxic activity in the mouse lung. This evidence supports the view that the lung tumors induced in the $B6C3F_1$ mouse inhalation study with naphthalene were driven by a nongenotoxic-cytotoxic mode of action (NTP, 1992).

Whole Animal Initiation Promotion Assays

Several assay systems have been defined to measure the ability of the test chemical to either induce mutations that will lead to cancer (initiation) or upon repeated exposure will enhance the clonal growth of precancerous and cancerous cells (promotion). In one model in order to test for genotoxic potential the chemical is given to partially hepatectomized F344 rats so that the liver cells are actively dividing (Tsuda et al., 1980). If the test chemical or its liver metabolites have genotoxic potential, then the genotoxic effect is greatly enhanced by the fact that the DNA is replicating and thus forming irreversible mutations. Precancerous foci of cells expressing gamma-glutamyl transpeptidase (GGTP) are then evaluated as a measure of genotoxic activity relevant to the initiation of cancer. Significantly, naphthalene tested negative in this assay (Tsuda et al., 1980).

Neonatal Mouse Model

The neonatal mouse model is an intermediate-term cancer bioassay to evaluate the ability of the test chemical to induce mutations relevant to cancer induction. Cells are rapidly dividing in very young animals. This high degree of cell proliferation dramatically enhances the effectiveness of any potential mutagen because DNA adducts and damaged DNA are rapidly converted to irreversible mutations. Male and female CD-1 mice were given intraperitoneal injections of naphthalene on days 1, 8, and 15 of life. The mice were then held for one year to allow tumor formation. No

naphthalene induced increase in the incidence of tumors was observed (LaVoie et al., 1988).

In Vivo Micronucleus Assay

The in vivo micronucleus assay examines the ability of the test article, to induce chromosomal damage as assessed by the induction of micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of treated mice. Naphthalene tested negative in two separate mouse micronuclei assays (Schreiner 2003; IARC, 2002). This observation is important because the micronucleus assay correlates with and is a measure of chromosomal damage (Shelby and Witt, 1995). The micronucleus assay thus serves as an indication as to whether activity in cell culture chromosomal assays translate into activity in the whole animal. While positive activity was seen with naphthalene in some cell culture chromosomal assays (see below) the response in the whole animal micronucleus assays was negative, indicating that the response is a cell culture phenomenon.

DNA Repair in Hepatocytes In Vitro and In Vivo

Hepatocytes are particularly important cells in which to assess genotoxic activity because they have a breadth of metabolic activation capability. Chemicals or their metabolites that bind to the DNA of hepatocytes induce a process of excision repair, in which the adducted or damaged DNA bases are removed. This involves unscheduled DNA synthesis (UDS), which can be quantitated by autoradiographic analysis of the incorporation of tritiated thymidine in to the nucleus. DNA repair can be measured either in cultures of primary hepatocytes or in hepatocytes in which the animal was treated with the test chemical (Butterworth et al., 1987a, 1987b). Naphthalene was negative in both the in vitro and in vivo hepatocyte DNA repair assays (Schreiner 2003).

DNA Breakage Assays

DNA that has been damaged and has an increased incidence of DNA single-stranded breaks will elute under alkaline conditions through a filter at a faster rate than will undamaged DNA. This is the operating principal of the hepatocyte alkaline elution

assay. Use of hepatocytes has the advantage of employing metabolically competent cells. Consistent with the negative hepatocyte DNA repair assays, naphthalene tested negative in the primary rat hepatocyte alkaline elution assay both in vitro and in vivo (reviewed by Schreiner, 2003).

Bagchi et al. (1998a) reported that incubation of the J774A.1 macrophage cell line without added metabolic activation with naphthalene at concentrations above 100 μM and up to 500 μM resulted in increased lipid peroxidation, cytochrome c reduction, hydroxyl radical production, modulation of intracellular oxidized states, and DNA fragmentation. Of particular concern with this study is the fact that assays that measure DNA fragmentation are very prone to artifacts associated with toxicity. Such concerns apply to all assays based on DNA integrity including the alkaline elution assay noted above. Reaching a toxic dose with even a nongenotoxic chemical will result in the release of lysosomal enzymes and other events that can damage the DNA and yield a false positive response. Thus, extreme care must be taken to assure that studies are not conducted at the toxic end of the concentration range. Validation toxicity vs. elution dose-response relationships must first be established when using this endpoint. In this study, the technique used to measure DNA fragmentation relies on the separation of DNA fragments from intact chromatin by centrifugation at 27,000 x g for 20 minutes (Bagchi et al., 1998a). No validation studies are referenced for this technique to demonstrate that it can distinguish genotoxic from nongenotoxic chemicals and the degree to which toxicity alone begins to result in DNA fragmentation. In fact, in these studies increases in DNA fragmentation increased in parallel with increased toxicity (decreased viability). These results are also contradicted by a rat hepatocyte alkaline elution assays in which naphthalene tested negative for DNA fragmentation (Sina et al. 1983).

Similar DNA fragmentation studies using the same centrifugation technique have been extended to mice and rats treated with naphthalene. Wild type and p53 deficient C57BL/6NT mice were given a single unspecified dose of naphthalene and 24 hours later liver and brain tissues were harvested and stored frozen. DNA fragmentation was

assessed by the centrifugation technique noted above (Bagchi et al., 1998a). Increased DNA fragmentation was reported in both the liver and brain (Bagchi et al., 2000). Female Sprague-Dawley rats were given naphthalene at 110 mg/kg/day, p.o. in corn oil, for 120 consecutive days. Liver and brain samples were stored frozen and subsequently analyzed for DNA fragmentation as noted above. A time related increase in DNA fragmentation was noted in both the liver and brain (Bagchi et al., 1998b). Issues of sample handling and toxicity are critical in DNA breakage assays. No toxicity data were given for either of these two assays. Any mechanical action, such as freezing and thawing the samples can greatly affect results. Again, it is not clear that the techniques used in these experiments had been validated. Further, these data are inconsistent with the negative in vivo and in vitro hepatocyte DNA repair assays for naphthalene (Schreiner, 2003) as well as a negative hepatocyte alkaline elution assay performed in vivo (Kitchen et al., 1992, 1994).

Cell Transformation Assays

Cell transformation assays measure the ability of the test chemical to transform cells in culture from a relatively normal state to one that has attributes of cancer cells. For example, such transformed cells will produce tumors when transplanted into immunosuppressed mice. Some transformation assays use metabolically competent cells while others employ S9 for metabolic activation. Naphthalene tested negative in four separate cell transformation assays including Fischer rat embryo cells, Syrian baby hamster kidney cells, human diploid fibroblasts and mouse whole mammary gland cultures (reviewed by Schreiner 2003 and IARC, 2003). This uniform negative response is strong evidence of lack of mutagenic or promotional biological activity of naphthalene.

Gene Mutation Assays in Mammalian Cells

Knowledge of mutagenic potential in mammalian cells is valuable because this is a critical endpoint. Mammalian cell culture systems are somewhat more relevant than bacterial mutagenicity assays. Naphthalene was negative in a human B-lymphoblastoid

cell line mutagenicity assay (Sasaki et al., 1997). The naphthalene metabolite 1,4-naphthoquinone tested negative in the same study.

Bacterial Mutagenicity Assays

The Ames bacterial mutagenicity assay performed in selected strains of Salmonella and E. coli is perhaps the most well validated and widely used screen for mutagenic activity and potential carcinogenicity. Strengths of the assay are that it includes a metabolic activation component in the form of S9, it is particularly sensitive to DNA reactive mutagens, and has actual induction of mutations as an endpoint. Naphthalene has uniformly tested negative in numerous Salmonella reverse mutation assays, SOS response bacterial assays, the E. coli rec assay, and E. coli pol assay (reviewed by Schreiner, 2003 and IARC, 2003). Further, the metabolites 1-naphthol, and naphthoquinone tested negative as well. These data strongly support the conclusion that neither naphthalene nor its S9 metabolites are DNA reactive mutagens. This conclusion is significant in that the primary metabolite of naphthalene is naphthalene-1,2-epoxide, and many epoxides are mutagens.

There is one report that 1,2-dihydroxy 1,2-dihydronaphthalene is weakly positive in some strains in the Ames test (Bolton et al., 2000). This is a review article and no experimental details or control data are presented. The article states that the data are taken from two other papers (Creek et al., 1997 and Smith, 1996). Yet, neither of those papers contain any mutagenicity data at all. It appears that this metabolite is either not produced by the metabolic activation systems in the numerous genotoxicity assays conducted with naphthalene, or is actually a non-mutagen. Without more specific data and confirmatory studies, no conclusion can be drawn at this time.

Extensive validation studies by the National Institute for Environmental Health Sciences (NIEHS) have established that the combination of the Salmonella mutagenicity assay and the mouse micronucleus assay is currently the most effective primary screen for chemicals that present a genotoxic carcinogenic hazard to humans

(Shelby, 1988). At this point it is important to note that naphthalene is negative in both of these assays.

Mammalian Cell Culture Chromosomal Aberration Assays

Measurement of the ability to induce chromosomal damage is an important predictor of carcinogenic activity. There is, however, concern in the genetic toxicology community, supported by the published literature, that conducting *in vitro* cytogenetic assays, such as the CHO assay, under current testing guidelines produces a high proportion of false positive responses (Galloway, 2000; Muller and Kasper, 2000; Muller and Sofuni, 2000). The basis for this conclusion is the lack of confirming genotoxic activity in other assay systems for a high proportion of these positive responses. These authors suggest that much of the problem is that the current guidelines require testing at high concentrations that produce excessive toxicity leading to false positive responses and lack of biological relevance.

Naphthalene tested positive in the CHO chromosomal aberration assay only in the presence of a rat liver microsomal fraction (S9) (NTP, 1992). This was a well conducted assay and the responses were reproducible and were seen over a wide range of doses. Therefore, the activity appears to be significant and is not the result of the high-toxicity artifact noted above. Sasaki et al. (1997) reported that naphthalene induced chromosome breakage-type micronuclei. Chromosomal damage has been reported in a mouse embryo culture system, but only in abstract form (Collahon et al., 1990). Thus there is some evidence that a metabolite of naphthalene exhibits clastogenic activity in cell culture systems.

It is somewhat unusual for a compound to be metabolized to a clastogen by S9 in a cell culture assay but to show no activity with S9 in bacterial assays. That, however, appears to be the case with naphthalene. The uniform negative response of naphthalene in the whole animal assays noted above, however, indicates that this cell culture clastogenic activity is not manifested in the whole animal.

Mammalian Cell Culture Sister Chromatid Exchange (SCE) Assays

SCE assays assess the exchange of like segments of genetic material between sister chromatid strands. A major concern with this assay is the very high false positive rate seen with cell culture SCE assays. A major validation study examined the ability of the Ames test, the mouse lymphoma mutagenicity assay, the CHO chromosomal aberration test and the CHO SCE assay to predict carcinogenic potential across a large number of compounds (Tennant et al., 1987). One significant finding was that the CHO SCE has a specificity of only 45%. That is, well over half of the time, non-carcinogens produced a positive response in this assay. Because of this high false positive rate, this assay is seldom used in current testing strategies and any positive result must be viewed skeptically.

Naphthalene was reported to induce an SCE response in the CHO SCE assay with and without S9 (NTP 1992). Schreiner (2003) noted: “A question of biological relevance of the SCE results from this NTP assay has been raised because the effect was seen only in the second of two trials and the statistical significance of the increased relative SCE/chromosome ratio appeared dependent on lower control values in the second trial. The UK Health and Safety Executive (HSE), when considering this assay for the ECC human health risk assessment report on naphthalene... considered the overall result to be negative.” Further, the report of activity without S9 in this SCE assay would indicate the ability of the parent compound to directly damage DNA, which is inconsistent with numerous other studies. In contrast. naphthalene tested negative for induction of SCE in cultures of human MNL with and without S9 (Wilson et al., 1995). The naphthalene metabolite naphthalene-1,2-epoxide was negative for the induction of SCE in MNL cells (Wilson et al., 1996). The weight of evidence indicates that neither naphthalene nor its S9 metabolites induce SCE in culture.

Non-Mammalian Assays

Naphthalene has been reported to exhibit some activity in a few non-mammalian test systems such as wing somatic mutation and recombination in the fruit fly (reviewed by Schreiner, 2003 and IARC, 2003). These assays have not been extensively

validated and the predictability and relevance to mammals has not been established. The exceptionally large whole animal genotoxicity database for naphthalene noted above is without exception negative and supercedes these less conventional systems,

Conclusions from the Genotoxicity Studies

Seven well validated and well conducted whole animal assays with naphthalene produced negative responses. These assays included different strains of rats and mice, different target organs and different genotoxic endpoints. These studies constitute strong evidence that naphthalene is not genotoxic in the whole animal.

Critical evaluation of the numerous cell culture assays with naphthalene also indicate an overall lack of genotoxic activity. Scattered positive responses were noted in a few assays using poorly validated systems or questionable procedures. Three cell culture assays indicated induction of clastogenic activity by naphthalene. However, such activity did not translate to cytogenetic effects in the whole animal test systems employed.

The IARC review of naphthalene stated: "There is no evidence for mutagenic activity of naphthalene in the most widely used genotoxicity assays" (IARC, 2002). Similarly, the Schreiner (2003) review concluded: "Taken together, results of available genetic toxicity assays and the association between cell damage and tumors at target sites suggest that naphthalene carcinogenesis involves cytotoxicity rather than mutagenesis as the primary event, with tissue regeneration and possible chromosomal changes occurring thereafter, consistent with a threshold-related model of action." My assessment is that naphthalene does not exhibit genotoxic activity in the treated animal and that the tumors induced in the NTP inhalation bioassay were driven by a nongenotoxic-cytotoxic mode of action.

OXIDATIVE STRESS AND MACROMOLECULAR BINDING

Wilson et al. (1996) have proposed that the cytotoxicity and putative genotoxicity of naphthalene may be associated with the formation of 1,2-naphthoquinone and 1,4-

naphthoquinone from 1-naphthol rather than the naphthalene-1,2-epoxide. They and others have speculated that these naphthoquinones induce cytotoxicity and DNA breakage by binding to cellular macromolecules or by a redox cycle process with molecular oxygen that generates active oxygen species (D'Arcy Doherty et al., 1984; Wilson et al., 1996). Concerns with this hypothesis include inconsistencies with the tissue-specific patterns of naphthalene induced toxicity and cancer, and the low specificity and potential for artifacts of the genetic toxicity tests employed.

Naphthalene did not induce SCE in human MNL cells with or without addition of a metabolic activation system of human liver microsomes (Wilson et al., 1995). In contrast, 1,2-naphthoquinone and 1,4-naphthoquinone, but not naphthalene-1,2-epoxide, did induce SCE in this assay system (Wilson et al., 1996). Because microsomes increased the toxicity of 1-naphthol to human MNL cells, these authors suggested that the metabolites produced were the naphthoquinones. Metabolic production of the naphthoquinones, however, was never demonstrated. Rather, the authors cited the D'Arcy Doherty et al. (1984) paper as having demonstrated that 1-naphthol is metabolized to the naphthoquinones. In fact, the D'Arcy Doherty et al. (1984) paper states: "However, we have been unable to detect naphthoquinone formation from 1-naphthol in either hepatocytes or rat liver microsomes in the presence of NADPH, using high pressure liquid chromatography." Interpretation of these data are limited in that the SCE has a 55% rate of false positives (Tennant et al., 1987), and it is not clear how the concentrations inducing toxicity in the cell culture studies relate to physiological concentrations. Further, these results are contradicted by a mutagenicity study in human B-lymphoblastoid cells in which 1,4-naphthoquinone tested negative (Sasaki et al., 1997).

D'Arcy Doherty et al. (1984) found that 1-naphthol was metabolized in primary hepatocyte cultures predominantly to its glucuronic acid and sulphate ester conjugates, but small amounts of covalently bound and potentially toxic products were also seen. Based on the observation of increased hepatocyte toxicity of 1-naphthol, 1,2-naphthoquinone, and 1,4-naphthoquinone in the presence of dicoumarol, these

investigators suggested that 1-naphthol might be metabolized to the naphthoquinones and that these species may be inducing toxicity via a redox cycle that generates active oxygen species (D'Arcy Doherty et al., 1984). Dicoumarol inhibits DT-diaphorase which catalyses the reduction of quinones to diols via a non-toxic pathway rather than the toxic redox cycle pathway. One concern with this study is that the covalent binding of 1-naphthol to cellular components was very small and in one of the two concentrations examined, the degree of binding correlated with decreased toxicity. Further, dicoumarol also inhibited the Phase II detoxification of 1-naphthol to the 1-naphthol sulphate and 1-naphthyl-beta-D-glucuronide conjugates. It is possible that this inhibition, rather than the increased redox cycle proposal, was the reason that increased toxicity was seen with dicoumarol.

A chemical synthesis of the DNA base adduct 7-(naphthalene- 1,2-dion-4-yl)guanine was carried out in a solution of 1:1 acetic acid in water by reacting naphthalene- 1,2-dione with 5 equivalents of 2'-deoxyguanosine (McCoull et al., 1999). This compound was produced as a standard that might be used to identify such adducts in vivo. Although no such in vivo studies have been conducted. This paper is often cited as evidence that naphthalene produces DNA adducts in vivo.

Of concern with all of the cell culture studies noted above is the fact that the concentrations used have not been compared to the concentrations that are likely to occur in the whole animal or in people following environmental exposures. Until such comparisons are made, extrapolation to effects in animals or people must be viewed with caution.

The clearest evidence of binding of naphthalene metabolites to cellular proteins are studies in which cysteinal adducts were quantitated in blood hemoglobin and albumin from F344 rats 24 hours after a single oral doses of naphthalene (Waidyanatha et al., 2002). Dose-dependent increases in naphthalene-1,2-oxide, 1,2-naphthoquinone, and 1,4-naphthoquinone protein adducts were readily measured in the blood of treated animals.

Conclusions Regarding Oxidative Stress and Macromolecular Binding

The data from the in vivo protein binding studies clearly indicate the formation of 1,2-naphthoquinone and 1,4-naphthoquinone as well as binding to protein of these metabolites and naphthalene-1,2-oxide (Waidyanatha et al., 2002). This binding would appear to be specific to protein and not to take place to a biologically-significant degree with DNA because of the large body of evidence indicating that naphthalene is not genotoxic. Thus, such covalent binding is likely to result in cytotoxic rather than genotoxic events. The degree to which these metabolites participate in a redox cycle pathway producing oxidative stress remains somewhat speculative.

POTENTIAL SITE SPECIFIC GENOTOXICITY

A critical review of the extremely large genetic toxicology literature for naphthalene clearly shows that the compound is not a classical multi-tissue genotoxicant. In agreement with this conclusion is the highly selective site-specific pattern of tumor formation. Although it is unlikely, it remains possible that in addition to cytotoxicity, genotoxic metabolites may have been produced in an extremely localized manner. At this time no information is available from either tissue specific genetic toxicology assays or in vitro assays using microsomal preparations from rat or mouse respiratory epithelium, nasal epithelium, or CYP2F2 as the metabolic activation portion of the assay.

RISK ASSESSMENT

The weight of the evidence strongly supports the conclusion that naphthalene is not genotoxic. Tumor formation in the mice and rats both appear to be driven primarily, and probably exclusively, by biological activity associated with chronic inflammation, cytotoxicity, hyperplasia and metaplasia (NTP, 1992; NTP 2002). Thus, protecting individuals from naphthalene exposures likely to induce cytotoxicity, will also be protective against an increased risk of cancer.

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