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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

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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.

Keywords

Naphthalene; Cancer; Carcinogenicity; Tumorigenesis; Mode-of-action; Mechanism of action; Cytotoxicity; Olfactory epithelial neuroblastoma; Respiratory epithelial adenoma; Inflammation; Metabolism; Cytochrome P450; 1,2-Naphthoquinone; 1,4-Naphthoquinone; 1,2-Epoxide; Glutathione depletion; Species differences; Age-dependent differences; Gender differences; PBPK modeling

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., 2008). As discussed by North et al. (2008), 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 life-time 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, 2008). 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, multi-focal 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 ≥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 metabolically 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 ≤0.1 mM but was substantial (≥63%) at naphthalene concentrations ≥0.5 mM, and the latter cytotoxic 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 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. (2008) 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 informed by results of experiments proposed by North et al. (2008) 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, 2008), 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., 2008 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., ≥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.

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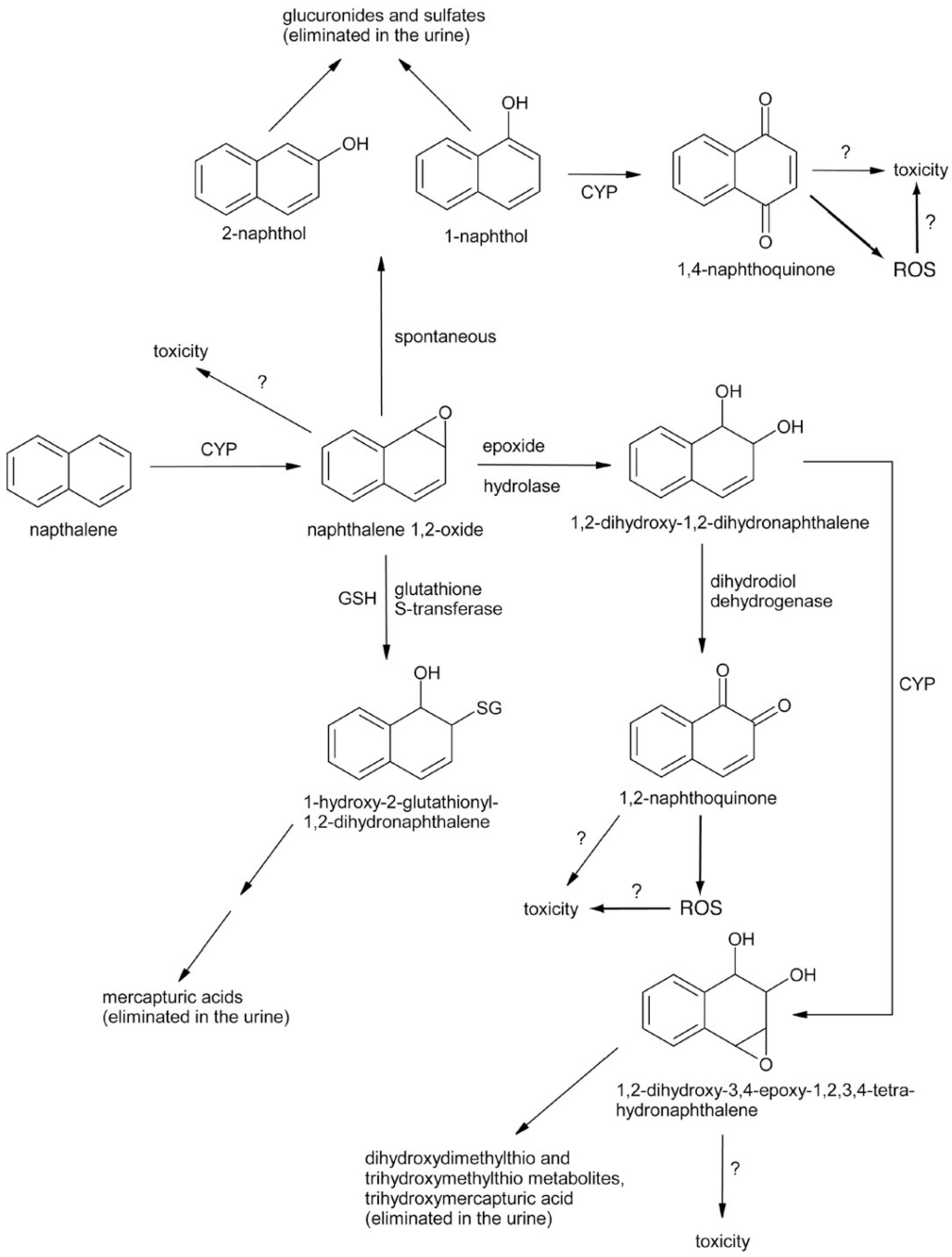

Fig. 1.

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.

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	≥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 ≥ 4 concentrations spanning those in each species that show toxicity and those corresponding to the no e.ect level for respiratory tract lesions, including ≥ 2 intermediate concentrations allowing quantitative characterizations of cytotoxic dose–response.