Report of a Workshop to Assess the Modes of Action of Lung Tumors in Mice from Exposures to Styrene Ethylbenzene, and Naphthalene

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Introduction

"A Workshop to Assess the Modes of Action of Lung Tumors in Mice from Exposures to Styrene Ethylbenzene, and Naphthalene" was held on September 17, 2013 at the Northern Kentucky University METS Center near Cincinnati Ohio. The workshop was organized by the Styrene Information & Research Center (SIRC), who invited four independent experts to serve as a panel to (a) evaluate the data for consistency, quality, and relevance to the mode of action (MOA); (b) critique the MOA hypothesis; (c) identify issues for further consideration and research; and (d) develop an independent report on the workshop. The panel members were:

- Dr. Michael L. Dourson, Toxicology Excellence for Risk Assessment
- Dr. William Farland, Colorado State University
- Dr. David R. Mattie, Air Force Research Laboratory (AFRL)
- Dr. M.E. (Bette) Meek, University of Ottawa

These panelists were provided with travel support and an honorarium for their participation[1]. SIRC contracted with Toxicology Excellence for Risk Assessment (TERA) to assist the panel by taking notes of the presentations and discussions and preparing a first draft of their report. SIRC also asked Dr. Michael Dourson of TERA to facilitate the panel discussions. Prior to the workshop, SIRC provided key references to the panel: Cruzan et al. (2009, 2012, 2013), Carlson (2012), and Collins, Bodner and Bus (2013), as well as a list of suggested discussion questions. The panel members served as individuals on this panel, representing their own personal scientific opinions. They did not represent their companies, agencies, funding organizations, or other entities with which they are associated. Their opinions should not be construed to represent the opinions of their employers or those with whom they are affiliated.

SIRC invited four other experts who are engaged in research on the proposed MOA and styrene, ethylbenzene and/or naphthalene toxicity. Much of this research has been supported by SIRC, other industry groups and companies. These experts made presentations and were available to provide answers and information to the panel during the discussion period. The experts and their presentations are listed below and the full agenda is found in Appendix A.

- Dr. George Cruzan: Potential modes of action for chemicals that cause lung tumors in mice, but not rats, that are metabolized by CYP2F2
- Dr. Laura Van Winkle: Pathology and MOA data for naphthalene
- Dr. B. Bhaskar Gollapudi: Alternative hypothesis: genotoxicity
- Dr. James Bus: Human Relevance: Analysis of the MOA data using the human relevance framework

The workshop was open to the public and over 40 people attended in-person or via live webcast. In-person and webinar observers were invited to ask questions and make comments periodically throughout the workshop.

TERA drafted this report and it was reviewed by the panel members and finalized to reflect their individual and collective opinions. The presenters reviewed the summaries of their presentations and statements attributed to them to insure their statements were accurately captured.

1 Dr. David Mattie of the AFRL was not provided travel, an honorarium, or any other compensation for his participation.

Presentations

Mr. Jack Snyder of SIRC opened the meeting and welcomed the experts, presenters and observers. He noted that the workshop was being broadcast live via webinar and a recording will be available for a limited time after the workshop. He briefly explained the background and objectives of the workshop (see above).

Presentation 1: Potential modes of action for chemicals that cause lung tumors in mice, but not rats, that are metabolized by CYP2F2, George Cruzan

Dr. Cruzan's talk focused on the styrene experimental animal studies and data that are relevant to the hypothesized MOA. He also briefly discussed the more limited data available on ethylbenzene. Slides from his presentation are found in Appendix B.

Dr. Cruzan briefly summarized the results of studies on styrene that have been conducted in rats (gavage, drinking water, and inhalation) and in mice (gavage and inhalation). Lung tumors are seen in studies with mice, but not rats; and the key question is "Are humans like mice or rats?" He discussed the progression of toxicity in mice and proposed the following:

> *Lung metabolism of styrene by CYP2F2 produces ring-oxidized metabolites that are toxic to Clara cells. Continued exposure results in continual cell replication, leading to hyperplasia. Newly formed Clara cells lack production of Clara Cell Secretory Protein (CCSP) (CC10), which reduces resistance to lung tumors.*

Dr. Cruzan presented several lines of evidence to support this hypothesized MOA. He reviewed the evidence for this CYP2F2 MOA in mice, including studies utilizing wild type and CYP2F2 knockout (KO) mice, bromodeoxyuridine (BrdU) labeling, and analysis of bronchoalveolar lavage fluid (BALF). The data demonstrate that lung toxicity from styrene and ethylbenzene require metabolism by CYP2F2 and the toxic metabolites are ring-oxidized. He discussed and presented arguments to refute two other MOA hypotheses: (1) that the styrene-7,8-oxide (SO) is the toxic metabolite for styrene and (2) that toxicity is seen primarily in tissues that produce high levels of R-styrene oxide (R-SO) enantiomer and that the toxic metabolite must be R-SO or be derived from R-SO. Dr. Cruzan believes that the side-chain only oxidized metabolites are not toxic; styrene-7,8-oxide is not the toxic metabolite from styrene; and 1-phenylethanol is not the toxic metabolite from ethylbenzene. He summarized the relevant mouse lung tumor information:

- Mouse lung tumors are not related to SO - there are no lung tumors from exposure to SO alone
- No lung tumors are seen in the absence of ring oxidation – 3-methyl-styrene or 4-methyl-styrene
- 4-Hydroxystyrene is toxic at much lower dose than styrene or SO
- Some 4-Hydroxystyrene toxicity is seen even in the absence of CYP2F2 metabolism
- CYP2F2 creates ring-oxidized metabolites that are toxic to Clara cells

Dr. Cruzan then presented information on human relevance of the CYP2F2 MOA. The human isoform of CYP2F2 is CYP2F1. He summarized results of studies on styrene and SO done by SIRC using a CYP2F1 transgenic (TG) mouse model, which showed no lung toxicity from either styrene or SO in the TG mice and some toxicity from 4-Hydroxy-styrene in the TG mice. He noted that levels of CYP2F1 in the transgenic mouse are lower than CYP2F2 levels in the wild type mouse, but higher than in humans.

For styrene, Dr. Cruzan concluded:

- Mouse lung tumors from styrene exposure are likely caused by ring-oxidized metabolites produced by CYP2F2 metabolism
- Styrene oxide is not the toxic metabolite
- Human CYP2F1 does not produce sufficient metabolites to cause toxicity or tumors
- Mouse lung tumors from styrene do not indicate human risk of cancer from styrene

Dr. Cruzan noted that ethylbenzene has been found to be less potent than styrene and while there are less data for ethylbenzene, the data available demonstrate parallels to styrene. He noted that ethylbenzene was found to be negative in a genotoxicity battery, but produced lung, liver and kidney tumors in a National Toxicology Program (NTP) 2-year bioassay. Metabolism of ethylbenzene in the lung is by CYP2E1 to 1-phenylethanol (1-PE), but 1-PE was not tumorigenic in a 2-year bioassay nor does it cause increased BrdU labeling in mouse lung. Therefore, 1-PE is not the toxic metabolite and mouse lung tumors are not related to main metabolism by CYP2E1. Active ethylbenzene metabolites are produced in the mouse lung and there is evidence that CYP2F2 is involved.

Clarifying Questions

A panel member asked Dr. Cruzan whether anyone has tried to a match the amount of metabolism for the chemicals and studies listed in Table 1 of Cruzan et al. (2009) to get a tissue dose and then correlate to the tumors. Dr. Cruzan answered that this has not been done and it would be difficult because of the large number of different types of cells involved in the whole lung. One would have to isolate and examine those cells and the methods to do this are difficult and time-consuming (e.g., isolate micro-dissected airways or dissect Clara cells) and results may no longer reflect the exposure.

Panel members asked Dr. Cruzan to review the pharmacokinetics of styrene and styrene oxide in mice and rats again. Dr. Cruzan explained that 75% of styrene is metabolized to SO in rats and mice regardless of route. In rats about 50% is metabolized by mandelic acid and 25% by glutathione conjugation. In mice it is about 35% of each. The primary difference in mice is greater metabolism to phenylglyoxylic acid and more ring oxidation. In humans, 95% is metabolized by mandelic acid with much less glutathione conjugation and ring oxidation. He also noted in response to another question that they have not compared the levels of ring oxidized metabolites in the urine of wild type (WT) and knockout (KO) mice.

In the Lijinsky (1986) gavage study, mice had forestomach but not lung tumors after administration of SO. A panel member asked whether one would expect to see lung tumors. Dr. Cruzan agreed this is a legitimate question. If SO is the tumorigenic agent and enough of an oral dose of SO reaches the lung, it should cause lung tumors, but such tumors did not occur.

George Woodall, an observer from the U.S. Environmental Protection Agency (EPA), noted that SO is fairly reactive and asked if SO were given by gavage, how much will actually get to the lung. Dr. Cruzan answered that kinetics studies and a physiologically-based pharmacokinetic model (PBPK) model allowed them to estimate that 95% of SO gets metabolized in the stomach. While a lot of SO is reacted within the stomach and does not get to the lung, they were able to use the PBPK model to estimate that the amount of SO that got to the lung from an oral exposure of SO is equivalent to the amount of SO metabolized from styrene.

Presentation 2: Pathology and MOA Data for Naphthalene, Dr. Laura Van Winkle

Dr Van Winkle presented on pathology and MOA for naphthalene. Slides from her presentation are found in Appendix B. She noted that a National Toxicology Program (NTP) 2-year bioassay of naphthalene found some evidence of alveolar/bronchiolar adenomas of the lung increased in the high dose group in female mice, but no evidence of carcinogenic activity in male mice. However, both male and female rats showed clear evidence of carcinogenesis with adenomas of the respiratory epithelium and neuroblastomas of the olfactory epithelium in the nose, but no lung tumors. In experimental animal studies, naphthalene injures Clara cells in mice regardless of the route of exposure. Distribution of Clara cells differ among species; in the mouse up to 75% of the epithelial lining is Clara cells. The terminal bronchioles, which contain approximately 75% Clara cells, are less than 5% of the lung by volume. In primates there are fewer Clara cells and they have a more restricted distribution being most abundant in the respiratory bronchiole. The site of naphthalene damage varies by species but no lung toxicity is seen even at high doses in rats. There are also differences in cell proliferation between male and female mice.

Dr. Van Winkle described the cycle for Clara cell injury and repair, noting that it is similar for naphthalene, styrene and ethylbenzene. The injured cells squamate (becoming flattened), divide and proliferate, and then re-equilibrate by migration to a more regular appearance. Following a single exposure, the cells develop markers of differentiated cells and return to normal; however after repeated exposure the differentiation does not occur. Repeated inhalation, or i.p. injection, of naphthalene causes "tolerance." Repeated exposure results in cells that are unlike the normal, unexposed Clara cells in terms of: differentiation markers such as CCSP, the presence of focal regions of cellular hyperplasia, and resistance to additional injury. Dr. Van Winkle noted that it is unclear what role the tolerant conducting airway epithelium (or alveolar epithelium) plays in formation of bronchiolar and alveolar adenomas as described in the NTP chronic bioassay.

Dr. Van Winkle summarized the key information and sequence of events for naphthalene toxicity:

- Glutathione depletion –a necessary, but not sufficient event for toxicity
- P450 mediated metabolism
- Protein binding of reactive metabolites
- Metabolism of naphthalene is more robust in sensitive species and in specific respiratory tract target regions
- Naphthalene epoxide and downstream metabolites are toxic to Clara cells
- CYP2F2 contributes to mouse lung Clara cell toxicity

The kinetics of metabolism are important; without conversion to the intermediate epoxide, naphthalene is toxicologically inert. Dr. Van Winkle identified several possible reasons why mice, rats, humans, and other primates differ, including differences in the catalytic activity of P450 2F, differences in the amounts of protein expressed in the airways, differences in the amounts of P450/cell (hot spots in the lung), and contribution of other P450s.

In summarizing metabolite toxicity, Dr. Van Winkle noted that in a variety of model systems (perfused lung, hepatocytes, and Clara cells) naphthalene, naphthalene epoxide and naphthoquinones are cytotoxic. Naphthalene oxide is a more potent toxicant in Clara cells and isolated perfused lung than are the quinones. There is no lag in the binding of radioactivity from 3H-naphthalene oxide suggesting that the epoxide may be the key step in this process. She noted:

- CYP2F2 (mouse) and CYP2F4 (rat) show high catalytic turnover of naphthalene (>100 min-1)
- Mouse – rat differences in rates of airway metabolism are due to substantial differences in the amounts of CYP2F protein present
- CYP2F1 (human) metabolizes naphthalene poorly
- Mouse CYP2F has much higher catalytic activities (higher Vmax and lower Km) than any of the recombinant human proteins tested
- CYP2F2 null mice have a 160-fold decrease in naphthalene metabolism

Dr. Van Winkle concluded that it is likely that CYP2F2 metabolism of naphthalene contributes to tumor formation in the mouse based on the following evidence:

- P450 activation is required for toxicity
- Sites of toxicity in the lung correlate to sites of tumor formation
- CYP2F2 in the mouse metabolizes naphthalene extremely well
- Knockout of CYP2F2 diminishes toxicity

Clarifying Questions

A panelist asked about the sex difference in mouse lung tumors development – if females do not develop tolerance as well, why do they not get lung tumors? Dr. Van Winkle answered that they know the extent of injury in female mice is greater and a balance of events in females makes them more susceptible. She believes it has something to do with the amount of initial damage. She also noted that P450 levels are lower in tolerant mice.

Another panel member sought clarification on how the term tolerance is being used, noting that Dr. Van Winkle described tolerance as associated with a change in architecture and biochemistry. The panel member thought that if the injury is such that it can be repaired and look like normal tissue after one dose, then that would not be called tolerance. Tolerance is really dependent on the undifferentiated state of Clara cells. Dr. Van Winkle responded that it takes two weeks for the cells to recover. In most tolerating regimens the dosing is daily and so the recovery process is stalled and the cells do not differentiate fully. A single exposure to naphthalene does not result in tolerance.

Panel members asked several questions about the ring oxidized metabolites. In response, Dr. Van Winkle said they believe that the ring-oxidized metabolite (the epoxide) is more damaging, but she thinks more evidence is needed. They currently have a study underway for all naphthalene metabolites; but they think the metabolites other than the ring-oxidized are minor. The naphthalene epoxide is short-lived but stable in blood. Dr. Cruzan added that naphthalene oxide is ring-activated because it has no side chain. Dr. Van Winkle's laboratory recently received a grant from the National Institutes of Health (NIH) to do naphthalene studies in KO animals; it would be interesting to see how naphthalene oxide performs in such a system.

Dr. Gollapudi asked whether there is any potential for some of the ring-oxidated metabolites to be estrogen receptor (ER) agonists. Dr. Van Winkle did not know, but said unpublished studies looking at naphthalene in a complete mouse did not see an ER gene expression response.

David Adenuga, an observer from Exxon Mobil Biomedical Sciences, asked if the presenters could comment on what it means that lung tumors are seen in males but not females with ethylbenzene and for naphthalene the opposite is the case. Dr. Van Winkle noted that there is a definite sex difference in naphthalene injury and repair response between the sexes and thought this could be influenced by the female estrous cycle. Dr. Cruzan added that for styrene the response is a mixed case. Tumors are seen in both sexes, although the response is stronger in males; however, in females the tumors are seen at a lower dose.

Christopher Bevan, an observer from CJB Consulting LLC, asked a question regarding P450 expression. He asked, if acute exposure of mice to naphthalene is cytotoxic to Clara cells, and the cells that replace the Clara cells are not the same, does the expression of P450 in the "new" cells differ? Dr. Van Winkle answered that this is poorly understood. During post natal development P450 expression and Clara cells develop in parallel. She did not know if this was examined in tolerant animals. Dr. Bevan added that metabolism after single and repeated exposure is different and has not been well-studied.

Presentation 3: Alternative Hypothesis: Genotoxicity, Bhaskar Gollapudi

Dr. Gollapudi reviewed the available data and evidence for a mutagenic MOA for the three chemicals – styrene, ethylbenzene, and naphthalene. Slides from his presentation are found in Appendix B. He started his presentation by noting his personal belief that if one conducts enough genotoxicity assays, there is a good chance of getting a positive response in at least one assay. Likewise if the experimental conditions are changed enough, a positive response will likely occur. He also believes that DNA adducts are a biomarker of exposure and not an indicator of mutagenicity *per se*; there is a background incidence of DNA adducts and cells have mechanisms to deal with them. And lastly he does not believe binning chemicals into just two bins – mutagenic or non-mutagenic -- is appropriate and one must look at dose response, which is an important consideration for this group of chemicals.

For styrene, Dr. Gollapudi identified the key events for the hypothesized mutagenic MOA and focused on the first three events:

1. Metabolism of styrene to reactive metabolite(s) leading to DNA adduct formation. Dr. Gollapudi noted that styrene induces low levels of DNA adducts in mouse lung and has an extremely weak covalent binding index. The small number of adducts is unlikely to contribute to increased mutation burden owing to cellular DNA repair capabilities and the styrene oxide metabolite will be efficiently detoxified in lung tissue.
2. Ring oxidized metabolites of styrene induce cytotoxicity and compensatory cell proliferation. Dr. Cruzan explained that this has been shown to be mouse specific (Cruzan et al., 2012, 2013).
3. Cell proliferation leads to fixation of DNA adducts into mutations. Dr. Gollapudi summarized the data and concluded that the weight of the evidence indicates that styrene is a weak mutagen *in vivo*; there is no increase in chromosomal aberrations and a questionable increase in sister chromatid exchange in tumor target tissues. He added that there is no convincing evidence of genotoxicity in humans other than DNA adducts, which are indicators of exposure.
4. Sustained cell proliferation leading to amplification of induced/spontaneous mutations and hyperplasia.
5. Progression of hyperplasia into adenomas and carcinomas.

For styrene, Dr. Gollapudi therefore concluded that the weight of evidence (WOE) suggests that styrene is not likely an *in vivo* mutagen, including in tumor target tissue; that mutagenicity is not likely responsible for lung tumors due to efficient detoxification of SO; and that SO itself is not a lung tumorigen. He thought confidence in this assessment can be further enhanced by evaluating dose response and temporality for mutations induced in a neutral gene (e.g., *cII*) and an oncogene (e.g., *K-ras*) in mouse lung.

For ethylbenzene Dr. Gollapudi presented a summary of genetic toxicology testing that showed few positive outcomes. He concluded that the WOE suggests that ethylbenzene is not a mutagen and that it is unlikely that mutagenicity is an early and influential key event in the etiology of mouse lung tumors. He did note that lack of mutagenicity data in tumor target tissue is a potential data gap, but that he does not consider this critical given the WOE based on the available data.

Dr. Gollapudi also discussed the genotoxicity data for naphthalene and cited several recent reviews that report generally negative results. He noted the following:

- No activity in gene mutation assays with bacterial and mammalian cell cultures.
- Negative results from in vitro and in vivo unscheduled DNA synthesis (UDS).
- Non-genotoxic in rodent bone marrow micronucleus tests.
- Low or no activity in sister chromatid exchange (SCE) tests
- Some evidence for DNA reactivity of metabolites,
  – in vitro (naked DNA), ex vivo (nasal epithelium), and in vivo (skin, non-tumor target),
  – no in vivo data following systemic exposure
- Clastogenic to cells in culture.

He shared dose response information demonstrating thresholds from a naphthalene *in vitro* micronucleus assay (Recio et al., 2012) that used flow cytometry to increase the assay power to detect small differences. For naphthalene, Dr. Gollapudi concluded that the weight of evidence suggests that it is not an *in vivo* mutagen and that mutation is not an initial key event in the etiology of naphthalene-induced lung tumors. He noted that he has moderate confidence in this assessment and that it also could be enhanced by evaluating dose response and temporality for DNA adducts and mutations in a neutral gene (e.g., *cll*) and an oncogene (e.g., *K-ras*) in mouse lung.

Clarifying Questions

A panel member noted that when looking at mutagenicity for MOA, one needs to take dose response into account. The panelist asked whether the presenters have looked at the genetic activity profiles (GAP) for these compounds or the "patterns" in the data in relation to phylogenetic order and dose-response. Often, when this information is taken into account, it is quite complementary to the hypothesized MOA (e.g., clastogenicity at cytotoxic doses is completely consistent with a non-mutagenic MOA). The panelist was aware of the GAP for ethylbenzene and noted that with few exceptions, it is consistent with a non-mutagenic MOA. Dr. Gollapudi explained that not all of the compounds have data in the tumor target tissues and he would be more comfortable with dose-response data in the target tissue rather than comparing to *in vitro* concentrations without modeling the data to predict *in vivo* exposures. The panelist agreed that while it is best to have data in the target tissue at relevant dose levels, this is not always possible and that some information on "patterns" of response can be gleaned based on dose-response information from *in vitro* assays.

A panel member asked why the naphthalene micronucleus test was positive *in vitro*, but negative *in vivo*. Dr. Gollapudi explained that the *in vivo* assay used bone marrow – the concentration of the genotoxic metabolite(s) at this target tissue might not have been high enough to induce an effect. For example, efficient detoxification through glutathione (GSH) conjugation would minimize bone marrow exposure to the reactive metabolites of naphthalene. This can easily be tested by modeling the *in vitro* dose metric to extrapolate to *in vivo* concentrations required to induce an effect.

An observer, David Dankovic from the National Institute of Occupational Health Sciences (NIOSH), asked about the role of metabolic activation in the *in vitro* assays and how many were done with exogenous metabolizing systems. Dr. Gollapudi responded that the studies done *in vitro* used S9 activation. Since the Phase I and Phase II enzymes are mixed together, it is difficult to separate metabolic activation (e.g., through p450) from detoxification (e.g., through GSH conjugation).

Another observer, George Woodall of the U.S. Environmental Protection Agency (EPA), noted that there are GAPs for all three compounds available on the IARC website, but that they were last updated around the year 2000. Additionally, he noted that many assays were not performed for several of the chemicals being discussed, and that a lack of data from those unperformed assays was not the same as absence of an effect.

Presentation 4: Human Relevance: Analysis of the MOA Data Using the Human Relevance Framework; James Bus

Dr. James Bus of Exponent presented an analysis of the MOA data, based upon the Cruzan et al. (2009) publication on the CYP2F2-mediated MOA for mouse lung tumors, for which he was an author, and additional recent studies and analyses. Slides from his presentation are found in Appendix B. Dr. Bus used the International Programme for Chemical Safety (IPCS) human relevance framework (Boobis et al., 2006, 2008) to provide a structured approach to evaluating a hypothesized MOA.

Cruzan et al. (2009) presented the following as a hypothesized MOA:

> "Metabolism of several chemicals by CYP2F2 in terminal bronchiolar Clara cells in mice results in the localized generation of cytotoxic metabolites and subsequent reparative cell proliferation. These metabolites generally are hydroxylated-benzene-ring derivatives.
>
> …On continued exposure, the increased cell replication leads to cellular crowding and then to hyperplasia in the terminal bronchioles. As the hyperplasia continues, it expands into the surrounding alveolar ducts. Some of these hyperplasias proceed to form adenomas in the mouse lung. Depending on the severity of the stress, a few of the adenomas may progress to carcinomas.
>
> …The analogous CYP2F4 in rats may be as capable of forming these cytotoxic metabolites as mouse CYP2F2; however, rats have much lower levels of CYP2F4 in terminal bronchioles and do not produce sufficient levels of these metabolites to cause cytotoxicity, hyperplasia, or lung tumors" (Page 2-3)

Dr. Bus discussed the key events, noting that data from several chemicals and species are combined to support these key events and contribute to the specificity and biological plausibility of the MOA. The following key events follow delivery of the chemical to the respiratory system – from inhalation exposures and via systemic circulation following oral absorption:

1. Metabolism in the lung by CYP2F2 in the Clara cells. Since publication of Cruzan et al. (2009) additional data in studies with CYP2F2 knock out and CYP2F1 humanized mice add additional support for the essentiality of this key event. Thus, CYP2F2-mediated generation of cytotoxic metabolites represents the key metabolic gateway responsible for lung toxicity and postulated tumorigenicity; that is, if the metabolic gateway is closed (CYP2F2 KO and CYP2F1 humanized mice), lung toxicity is completely abated.
2. Cytotoxicity mediated by reactive metabolites formed from CYP2F2 metabolism - leading to hyperplasia; increased cell replication only in Clara cells; replication is sustained, and cytotoxic events (e.g., GSH depletion) demonstrated.
3. Sustained reparative hyperplasia – sustained cell replication leads to late occurring lung tumors, this is consistent with epigenetic events. The lack of genotoxic response also supports an epigenetic MOA.
4. Progression to late-developing tumors.

Dr. Bus concluded that without CYP2F2 metabolism in the lung, the animal is not vulnerable to lung toxicity and tumor progression. The lung tumors in mice are late developing and most are seen at the highest tested concentrations; the available data consistently show that cytotoxicity precedes tumors. In the tumorigenic dose range, ring-oxidized metabolites drive cytotoxicity.

A mutagenic MOA must be considered as an alternative. Dr. Bus noted that styrene oxide (SO) is hypothesized as a putative genotoxic and cytotoxic agent; however, SO is not toxic to the lung in CYP2F2 KO or CYP2F1-humanized mice. Ethylbenzene cannot form alkyl-epoxide metabolites, yet it still produces lung tumors. A number of key uncertainties, inconsistencies and data gaps were presented. Dr. Bus concluded by providing answers to the key human relevance framework questions for the CYP2F2 MOA:

- Is the weight of evidence sufficient to establish the MOA in animals? Yes.
- Are the key events qualitatively plausible in humans? A qualified No. Humans are qualitatively different, with no significant formation of the cytotoxic metabolite as demonstrated in a humanized mouse model. However, there may be some other P450 that might produce some small levels of metabolism leading to the other possible key events. Importantly, if such alternative event(s) are present, they did not manifest in highly sensitive cell proliferation assessments in CYP2F2 KO or humanized mice experiments, suggesting a low probability of occurrence.
- Taking into account kinetic and dynamic factors, are the key events in the animal MOA plausible in humans? No. If sufficient concentrations of active metabolites are produced, this is highly unlikely to occur given the cross-compound evidence of the central role of mouse-specific CYP2F2 in mediating cytotoxicity. Thus, the hypothesized MOA suggests these chemicals are not expected to cause lung tumors in humans.

Clarifying Questions

A panel member asked about temporality of key events and the difference in time span between early and late key events, explaining that this type of information should be included with the dose-response data in a dose-response/temporal concordance table. Dr. Bus noted that evidence is available for styrene - BrdU labeling studies have shown clear evidence of early onset of cell proliferation for some of the chemicals. Ethylbenzene studies run out to 28 days still see increased cell proliferation with BrdU labeling; while longer dosing does not see cell proliferation in classical measures, one can see pathologically evidence. Some bioassays only had terminal sacrifice, so do not have intervening observations.

An observer, George Woodall of EPA, asked about tissue concordance – are the same or similar effects as seen in the mouse lung also possible in other tissues in humans, perhaps lymphocytes (i.e., what is the likelihood of hypothesized MOA to be active in another tissue?). Dr. Bus responded that CYP2F2 predominates in the mouse lung tissue. The observation that styrene lung toxicity is totally absent in CYP2F1 humanized mice over-expressing CYP2F1 relative to humans, strongly indicates that expression of CYP2F1 in any other human tissue is unlikely to be adequate to result in toxicity in other organs/tissues. In other words, if styrene cannot produce lung toxicity in CYP2F1 humanized mice (the organ in which CYP2F1 predominates), it is highly unlikely CYP2F1-mediated metabolism in other human tissues/organs would result in toxicity. There is no evidence that CYP2F1 is present in human lymphocytes.

Dr. Dourson read a question that was submitted by a webinar observer, Mark Stelljes of SLR Consulting, via email: "It is the opinion of Cal EPA (not my opinion) that tumors can occur in humans at sites that are different than where tumors occur in mice (e.g., neuroblastomas from naphthalene). Therefore, studies showing a lack of relevance between mouse tumors and tumors in the same tissues in humans does not mean the chemical (e.g., naphthalene) cannot cause cancer in humans." As this question was addressed by some of the previous discussion, Dr. Dourson further added that until about 10 years ago we would ask about relevance of tumors to humans. However, now we use the MOA framework to evaluate the mode of action in animals and ask a different series of questions: first, is the MOA known in experimental animals, next, is it qualitatively relevant to humans, and finally, is it quantitatively relevant to humans. Tissue concordance is assumed between the experimental animals and humans in the absence of information to answer these questions. Alpha-2u-globulin rat kidney tumors were an early example of tumors judged not relevant to humans or indicative of human carcinogenicity based on this kind of MOA thinking.

Workshop Discussion

Discussion questions were provided to the panel to help frame the discussion. However, the panel members were invited to raise any additional issues or comments they thought relevant to the discussion.

Panel members sought clarification on the primary question to be addressed in the workshop. They asked whether SIRC was seeking to a) determine if CYP2F2-mediated cytotoxic metabolism is a reasonably hypothesized MOA for the chemicals being discussed, or b) what would need to be shown for a new chemical to support this MOA? Dr. Cruzan clarified that they are seeking input from the experts on both questions, they would like the panel to evaluate the existing data for these chemicals collectively (discussion questions 1-5) and then if there is time, discuss what studies and data would be needed for a new chemical to be evaluated for this MOA (discussion question 6).

Workshop Discussion Questions

1. Is the CYP2F2-mediated mode of action proposed for mouse lung toxicity and tumors adequately defined in animals? If not, what data are necessary, or useful, to enhance confidence in the MOA?
2. Are the data on the three chemicals mutually consistent with the hypothesized MOA, or are there discordances that diminish the plausibility of the MOA?
3. Do the data indicate whether the hypothesized MOA, if established in animals, is likely to be qualitatively irrelevant to humans?
4. If the hypothesized MOA is qualitatively relevant for humans, is it characterized as quantitatively irrelevant (or relevant) to humans?
5. If necessary, what new data might further clarify the question of human relevance?
6. Is there a set of criteria, based on short-term experimental protocols (≤90 days) that can be applied to other chemicals to determine if they operate by the same mode of action as that presented?

Discussion Question 1: Is the CYP2F2-mediated mode of action proposed for mouse lung toxicity and tumors adequately defined in animals? If not, what data are necessary, or useful, to enhance confidence in the MOA?

Panel members sought clarification regarding which of the chemicals discussed in the Cruzan et al., (2009) paper should be considered for evaluation of the hypothesized mode of action. Panel members agreed that evaluating weight of evidence for the hypothesized MOA of cytotoxicity following CYP2F2 metabolism, based principally upon the data for those chemicals where it has been most fully investigated (namely styrene, naphthalene and ethylbenzene), is appropriate. The panel agreed that the data on these chemicals should be considered collectively and where data on patterns of, for example, dose-response and temporality for hypothesized key events for individual chemicals are not consistent, these should be identified, as well as the impact of differences between them (e.g., metabolism).

For the unified MOA, the panel discussions focused on a number of issues, in particular the temporal sequence and dose-response concordance, presentation of data in a comprehensive template, key events, and the role of potential genotoxicity.

Temporal and Dose Sequence

Panel members emphasized the importance of arraying the data related to dose-response and temporal sequence of events. A panelist suggested presenting information on the dose and temporal relationships in a specific format and offered to share an example template with the presenters. The template helps organize the evidence at different levels of biological organization, including how to interpret the data, to more clearly communicate the information. Other panelists agreed and noted that the changes in cellular architecture and changes in the Clara cells, including lack of differentiation, need to be better articulated in relation to the temporal sequence of events that lead to mouse lung tumors. They also noted that there is little information about what is happening between metabolism and cytotoxicity.

Panelists asked if there is any additional information beyond histopathology at the end stages that could help better define the temporal sequence. Dr. Van Winkle explained that for naphthalene both the

CYP2F2 metabolism and cytotoxicity occur after just a single exposure. Cells swell and then form vacuoles about three hours after injury; at six hours there is membrane permeability leading to cell death. This continues for 24 hours. Glutathione depletion is seen within two hours after exposure. These types of morphological data are not available for other mouse lung toxicants such as styrene or ethylbenzene; however, short-term dosing with these substances results in terminal bronchiole cell cytotoxicity as evidenced by increased BrdU cell proliferation. Dr. Bus added, for example, that for ethylbenzene repeated exposure (5 days a week at tumorigenic dose levels), cell replication measured by BrdU shows a wave of cell proliferation at one week and a drop off at four weeks. Dr. Bus also noted that bronchoalveolar lung lavage fluid contained elevated levels of lactate dehydrogenase, protein, and cells following a single exposure to styrene, a clear indicator of an immediate cytotoxic response.

A panel member thought that that more emphasis should be given to the rearrangement of cellular architecture, which was a response to the initial cytotoxic response. There is a set of cells that are potentially tolerant with regard to cytotoxicity, but fundamentally different than Clara cells in normal mouse or rat; these Clara cells proliferate and give rise to tumors. The panelist wanted to learn more about those cells, as their ability to function under different cellular conditions after the first cytotoxic response may be a basis for the proliferative response.

Dr. Bus agreed this question is also on the table – do these cells resume normal epithelial cell characteristics, including function of P450 enzymes. Or, are they replaced with a different type of epithelium? At any point do the cells regain full Clara cell maturity to the point where the cells are vulnerable to a new wave of cell proliferation? He noted that it might be challenging to identify when one would look for this in the experiments. The panelist hypothesized that a single exposure might lead to cytotoxicity, reparative hyperplasia with differentiation, and healing. Chronic exposures would be different with potential proliferation of undifferentiated cells.[2]

Dr. Cruzan explained that it is well understood that when there is an acute insult the lumen fills up with CCSP and the cells die. Regenerated cells do not produce as much CCSP. There is a CCSP KO mouse and he noted that without exposure to naphthalene or styrene, CCSP KO mice develop lung tumors by four months and die by six months of age. The role of CCSP may be very important, but how does it fit into the MOA is a question. There is no KO mouse that lacks both CYP2F2 and CCSP; the CCSP KO mice are very sensitive, but also have CYP2F2. Dr. Bus noted that there is a fine line between MOA and mechanism of action. For the MOA, an observation of CYP2F2 metabolism and hydroxylated ring activated metabolites causing reparative cell proliferation and a change in cellular architecture is needed. These observations represent a mode but not a precise mechanism of toxic action. How these observed cellular changes progress to tumors is uncertain.

A panel member suggested that if one were to look at histopathology after the initial cytotoxic response for these chemicals; one would see the flattened cells and change in morphology of the Clara cells. Dr. Van Winkle explained that those kinds of changes are hard to see in traditional paraffin stains, her work on naphthalene used high resolution histopathology.

A panel member noted that if the threshold response characteristic of the mouse that is mediated by CYP2F2 is cytotoxicity in the Clara cell architecture and if data from naphthalene are characteristic for others with this MOA, then it is the Clara cell architecture changes that make the mouse unique among species and allows for the late stage carcinogenicity. That is, the mouse cells will respond to continued exposure in a proliferative state; in rats the Clara cells maintain their classic character and do not have a proliferative response. Therefore, one needs to tease out what is going on at the biochemical level; although another panelist noted that one should consider what the quantitative impact of this information would be on the risk assessment. Dr. Bus noted that the difference is that the rat cannot go through the CYP2F2 gate and a panelist voiced agreement that the chain of events starts with the CYP2F2 metabolism.

2 Post-workshop, Dr. Bus added the following note – The course of the discussion here was in part driven by the question of whether the epithelial cells replacing those damaged by chemical cytotoxicity were histologically or functionally different from original Clara cell epithelial cells. It turns out this question has been explored in West et al., (2002) for naphthalene tolerance – the replacement cells appear phenotypically normal but do appear to have an enhanced ability to support cellular GSH status. Although this paper was not discussed during the workshop, it appears to clarify some of the queries raised in these paragraphs.

The panel agreed that there is a good level of support for the CYP2F2-mediated mode of action proposed for mouse lung toxicity and tumors, although additional evaluation and presentation of the data will enhance confidence in the MOA. For example, the presenters could line up the considerations for WOE in a prioritized context: biological concordance, essentiality of key events, and concordance of empirical evidence. More detailed data with regard to earlier cellular changes for styrene and ethylbenzene (like that available for naphthalene) would contribute to confidence in the hypothesized MOA.

Temporal/Dose Concordance Template

Because this is such a complex data set and involves three chemicals, a panelist suggested first looking at biological concordance, then counterfactual evidence (i.e., essentiality of the key events), and then concordance of empirical evidence (e.g., concordance of dose-response, temporal and incidence aspects), followed by consistency (i.e., whether the pattern of effects across species/strains/organs/test systems is what would be expected based on the hypothesized MOA) and analogy (whether the mode of action would be anticipated based on broader chemical specific knowledge). The panel recommended that the SIRC team lay out the empirical evidence for hypothesized key events data to more fully consider concordance of temporality and dose-response, but noted that they would not expect consistency of all data. Using a more complete temporal/dose concordance template (than that presented in the Cruzan et al., 2009 publication) one could capture additional key information (species, sex, strains, and organs) that can help identify data gaps and outliers. This will allow for evaluating consistency amongst the chemicals, more clearly delineate the uncertainties, and provide support for the comparison of the extent of the WOE for this MOA with other accepted MOAs.

A panelist suggested reframing the original "Yes/No" qualitative human relevance question to ask, consistent with evolution of the MOA/HR framework: "Is there an event precluded in human relevance by biology?" For the quantitative differences, one is really looking for what are the critical or rate limiting steps for the hypothesized sequence that can inform interspecies and human variability, but particularly the latter.

If the MOA cannot be precluded in humans, one then needs to think about quantitative differences between species and how to use the available information to take into account quantitative impact for dose-response in humans. For example, lining up the three compounds, one could ask "what is the potency for these three in terms of MOA and quantitative scaling to humans?" The SIRC team responded to this question, noting that naphthalene and styrene show fairly similar potency in mouse studies, with no tumors seen at 10 ppm (naphthalene) but tumors are seen at 30 ppm (naphthalene, females only), 20 ppm (styrene females) and 40 ppm (styrene males). Ethylbenzene is much less potent than the other two. In humanized mice exposed to styrene no lung toxicity was seen and so humans would therefore be much less sensitive than mice for lung tumors via this MOA.

Key Events

With agreement that the postulated MOA is adequately defined in animals, the panel discussed the key events. Looking at the hypothesized MOA as described by Figure 2 of Cruzan et al. (2009) and the list of key events from Dr. Bus' presentation, a panel member noted that the cytotoxic response is mediated through metabolism by CYP2F2 in the mouse lung. Metabolism by CYP2F2 leading to cytotoxicity is clear, but what happens between cytotoxicity and the apical endpoint of late-stage tumors is not well understood. Dr. Bus agreed that there is somewhat of a black box between cytotoxicity and tumors, but noted that this is common with other MOAs, including alpha 2u-globulin and the MOA for chloroform (i.e., cytotoxicity followed by regenerative cell proliferation). He noted that there is ample evidence for biological plausibility that sustained cellular proliferation leads to tumor progression regardless of the site of tumor. Dr. Cruzan noted that this evidence includes BrdU labeling studies of animals exposed to styrene for up to 90 days that demonstrate progression to hyperplasia; data on the other chemicals could also be integrated.

Key Events

- Metabolism in the lung by CYP2F2
- Cytotoxicity mediated by reactive metabolites formed from CYP2F2 metabolism
- Sustained reparative hyperplasia
- Progression to late-developing tumors

A panelist asked what is known about the cells in the BrdU studies. Drs. Bus and Van Winkle explained that these cells are in the terminal bronchioles and are the replacement cells made within the last five days. They are Clara cells but that may not be what they looked like five days earlier; there was no labeling in alveolar cells. The panelist explained that one would gain greater confidence by understanding the sequelae of the cytotoxic response; that is a real change in the tissue that makes it susceptible to the events that lead to tumors. Dr. Bus noted that a bioassay with styrene in knockout and transgenic animals will prove this if no tumors were seen. Other panel members noted that the presenters provided enough information on the essentiality of the key events to define the hypothesized MOA at this time, and compared to other MOAs such as that for chloroform, the available data are sufficient. Additional empirical observations would help increase or decrease confidence, but the additional information is not essential. One panel member explained that the extent of counterfactual evidence presented (i.e., demonstrating the essentiality of key events) is significant and could be best demonstrated by presenting the data in the context of rank ordered Bradford Hill considerations for WOE of hypothesized MOAs and comparative WOE (see suggested template in Appendix C). This panelist thought that the case for this MOA would be relatively strong, based on comparison with that for other chemicals with which the panelist is familiar.

Role of Genotoxicity in the MOA

The panel discussed the genotoxicity data and how or whether mutagenicity plays a role. George Woodall, a workshop observer from EPA, asked if there is any evidence of mutagenicity in Clara cells and whether mutagenicity is occurring in the cells from the spontaneous mutation background or as a result of the chemical exposure. He also asked whether both mechanisms might be working in a unified MOA. A panelist suggested that the information for the CYP2F2 and mutagenic MOAs could be lined up side-by-side and the relative weight of evidence for each compared, but also suggested that looking at temporality may help bring the two together and determine if mutation is a secondary or initiating event. If mutagenicity is only seen at higher doses, then it cannot be a key event and is unlikely to be an initiating versus a secondary key event. Dr. Bus noted that one can assume that there is some potential for genotoxicity in that CYP2E1 produces reactive epoxide metabolites, e.g., styrene oxide, capable of forming very low levels of DNA adducts. But the question is whether the genotoxicity is a necessary catalyst of lung tumors because if it were so, then why are tumors not seen in the rats despite higher levels of epoxide metabolites relative to mice? He stated these findings point to cell proliferation as the key driver of tumorigenicity. Without the cell proliferation driver, there is no progression to tumors; otherwise rats, which do not exhibit cell proliferation but do have DNA adducts, would show a tumorigenic response. Thus, if there is a very weak mutagenic component, it is not contributing to the tumor response in the absence of cell proliferation. What the rats lack is cell proliferation driven by CYP2F2 metabolism. A panel member expressed agreement with what Dr. Bus said and thought that for the cytotoxic response a yes/no answer is not needed on genotoxicity. There is enough information to say it does not look like a “classical” genotoxic/mutagenic MOA response. One cannot answer if there is a genotoxic component at the level of response from proliferating cells. Rats have a lot of protein and GSH giving them protection. The mouse cells are proliferating, but perhaps they have a different balance of CYPs and/or GSH depletion that could allow a trickle of genotoxicity to contribute, but the genotoxicity is not necessary for the mouse tumors. Dr. Gollapudi agreed that one cannot ever rule out mutagenicity, but it does not appear to be an initial key event.

Dr. Cruzan added that there are some genotoxicity data derived from the mouse lung. A tumor initiation assay in Strain A mice was negative; there was no increase in chromosomal aberrations in lung cells of mice exposed to 125-500 ppm styrene for 2 weeks and in a study yet to be published, there were no increases in *Salmonella* revertants from styrene using S9 from mouse lung. A genomics study to be initiated in October will add a lot of information on gene pathways affected in mouse lung and whether they are affected in the absence of CYP2F2 metabolism. Panelists noted that the new study results will have to be very carefully evaluated to see if mutation is an early initiating key event and that other pieces of ancillary information from ethylbenzene and styrene oxide could be included. Otherwise, the evidence to date suggests that genotoxicity is not an early and influential key event in the progression to tumors.

Discussion Question 2: Are the data on the three chemicals mutually consistent with the hypothesized MOA, or are there discordances that diminish the plausibility of the MOA?

Panel members agreed that the available data presented for the three chemicals appear consistent with the hypothesized MOA. The panel did not see much discordance among the chemicals in the information provided that would diminish the plausibility of the MOA. They reflected on the evidence of sex differences in the various study results, but thought these differences all fall within the realm of biological variability and therefore did not think this discordant. The panel thought that there are opportunities to strengthen the presentation of information and thereby increase understanding of uncertainty and confidence. One panel member noted that while it does not appear that there are outliers in the data set (i.e., data not entirely consistent with the hypothesis), this is not what one would expect unless the mechanistic data were collected concurrently within the bioassays. Generally there are data that do not "line up" with the expected pattern given normal biological variation and such outliers should be identified and explained. This includes, for example, documentation of temporal and dose-response concordance which is best addressed using benchmark doses for comparisons.

Discussion Question 3: Do the data indicate whether the hypothesized MOA, if established in animals, is likely to be qualitatively irrelevant to humans?

A panel member noted that the wording of this question does not reflect experience in application of the MOA/Human Relevance framework. The key question to address is "ultimately is there a difference between animals and humans that precludes the effect occurring in humans, based on variations in biology for the key events in the hypothesized MOA?" The panel members agreed that the answer to this question is "no" and the discussion highlighted a number of reasons. There is an analog of CYP2F2 in humans (CYP2F1), but it is not very active in terms of metabolizing to the important cytotoxic compounds, nor is there a proliferative response in human cells *in vitro*. Assuming that this is the relevant gene, the question is whether there is a small number of humans that will respond like mice. In addition, Dr. Bus noted that Clara cells, which contain the majority of P450 metabolizing activity in rodents, represent a significantly smaller proportion of the respiratory epithelium in humans relative to mice and rats, thus providing further biological plausibility to observations of very low human metabolism of these compounds. Human lung preparations (from multiple surgery patients and cadavers across all three compounds) consistently exhibit very limited microsomal metabolism, including that to the hypothesized reactive ring oxidized metabolites. Importantly, Dr. Bus also noted that the level of gene expression of CYP2F1 in the humanized mouse is higher than what has been measured in humans; so, even though no lung toxicity was observed in humanized mice, this mouse model nonetheless likely represents a case of a possible "sensitive" human polymorphism. Thus, he concluded, the lack of lung toxicity in humanized mice over-expressing CYP2F1 is entirely consistent with the conclusion that the hypothesized CYP2F2 MOA in mice is qualitatively not relevant to humans. Dr. Cruzan noted that there may be other chemicals metabolized by CYP2F1 in humans. A panelist, noting that the Collins et al. (2013) paper discounted the lung tumors due to smoking, asked whether the human epidemiology data are relevant to what is happening in the mice. Dr. Bus explained that lung cancer mortality was inversely related to cumulative exposure and duration of exposure, which does not fit with what one would expect unless one considers more intensive smoking in workers with shorter work experiences with styrene (i.e., smoking was more predominant among short term workers). An observer, Dr. Woodall, noted that the Collins et al. smoking explanation may be possible, but is not necessarily proven, and peak rather than cumulative exposure may be more important, or there may be a healthy worker effect. A panel member noted that Table 4 of Collins et al. shows the data for peak exposures and the tumor effect is not found. Dr. Cruzan disagreed with the possibility of a healthy worker effect, given the causes of death. Another panel member pointed out that these epidemiology studies were not designed to inform the MOA unless one understands the power of the study to detect an effect in relation to the tumor of interest.

The panel agreed that there is no evidence to preclude that this MOA is not potentially qualitatively relevant in humans. There is an analog to CYP2F2 in humans, although it is not very active and no proliferative responses have been seen in tissues of workers exposed to up to 100 ppm styrene.

Discussion Question 4: If the hypothesized MOA is qualitatively relevant for humans, is it characterized as quantitatively irrelevant (or relevant) to humans?

Given that the MOA is qualitatively relevant to humans, the next question in the original MOA framework is whether it is quantitatively relevant or not to humans. The panel thought that practically speaking the MOA is not likely relevant to humans based on quantitative differences and discussed the data and evidence for this.

One panelist noted the lack of response at comparable dose levels in the Cruzan et al. (2013) humanized mouse study and asked how much less sensitive would humans be. Another, looking at Table 2 of the Cruzan paper, noted that the differences in response between the wild type and transgenic mice were dramatic, with no evidence of the effect in the transgenic mice. Dr. Bus suggested looking at the overall WOE and asking how it would be quantitatively relevant to humans. Studies of lifetime exposures with rats show no evidence of lung tumors. Compared microsomally, rats have significantly less capacity to metabolize these chemicals through the CYP2F2 pathway in the lung. Therefore, for this MOA to be relevant to humans, humans would have to have more metabolism than the rat. If humans were comparable to rats, they would not have had a tumor response, considering the power of the rat studies to detect a response.

A panel member agreed that the key question for this MOA is if it is likely lung tumors would be seen in humans based on quantitative differences. The answer is not "Yes" or "No", but rather the question is whether there is there large enough difference between mice and humans that the MOA is not relevant to humans. Phenobarbital is an early example of a MOA that was determined to be qualitatively relevant, but quantitatively not relevant. One panelist pointed out that this type of analysis could be considered "quantitative bounding" based on characterization of likely differences in sensitivity related to hazard. This can be distinguished from subsequent dose-response analysis, where interspecies differences and human variability are more robustly addressed quantitatively. Most importantly, one needs to transparently describe the determinants of likely variations between animals and humans in dose-response based on the documented hypothesized MOA as a basis to predict risk in the population of interest. If this relates to kinetic differences, relevant information can be collected as a basis to model potential impact for humans.

The panel and presenters discussed what data and analyses might be useful to estimate the species differences. The available data show a substantial variation in metabolism between mice and rats, and humans are likely to be even less sensitive. This information is helpful to provide at least "bounding" of the size of the quantitative difference. The relative number of Clara cells amongst these species and the amount of active metabolite in these cells can also contribute to a quantitative estimate of this difference. One might also compare the dose in the transgenic mice at which no BrdU labeling is seen, to the lowest dose in wild type mice with increased cell proliferation.

Dr. Bus suggested that the rat data might be used to further bound differences for the sensitive human. The humanized mouse represents a sensitive human because it has more CYP2F1 than is expressed in humans, but the rat, which has an even higher level of metabolism than the humanized mouse, exhibited no tumorigenic responses even at styrene exposures up to 1000 ppm. Importantly, studies have confirmed that CYP2F1 expressed in humanized mice is functional, though its endogenous substrates are unknown. A panelist asked how much more sensitive is this humanized mouse compared with humans? Dr. Cruzan said there are limited data on how much CYP2F1 is in humans, but Dr. Van Winkle's laboratory will be looking into this. Other panelists agreed that there appear to be considerable quantitative differences between the mouse, rat and humans to propose that the CYP2F2 MOA for mouse lung tumors is not relevant to humans. Although it is difficult to estimate the quantitative difference at this time, Dr. Van Winkle noted that the difference between mice and humans is about 100-fold for metabolism on rate of formation of toxic metabolites while the difference between the mouse and rat is less than 100-fold.

The panel noted that this issue is similar to that recently discussed in an update of the Peroxisome proliferator-activated receptor alpha (PPARα) MOA (Corton et al., 2014) and discussed how best to communicate uncertainty about quantitative interspecies variations. They agreed that the wording used by Cruzan et al. (2009) accurately describes the situation for exposures at environmental levels:

> "Therefore, while this mode of action is theoretically possible in humans if sufficient concentrations of active metabolites are produced, this is highly unlikely to occur given the cross-compound evidence of the central role of mouse-specific CYP2F2 in mediating cytotoxicity. Thus, the hypothesized MOA developed from this cross-compound analysis suggests these chemicals are not expected to cause lung tumors in humans" (page 12)

David Adenuga, an observer from Exxon Mobil Biomedical Sciences asked about the potential that there are other CYPs in humans that could potentially metabolize these chemicals in the lung. Dr. Cruzan said it is a possibility, though three different laboratories have failed to measure metabolism of styrene in human lung tissue.

David Dankovic, an observer from the National Institute of Occupational Safety and Health (NIOSH), asked the panel to put the expected differences between humans and mice in context and discuss how the results compare to what would be expected. Panel members noted that generally metabolic rate between rodents and humans scales to the ¾ power with body weight. They thought that ultimately the question Dr. Dankovic is asking relates to the implications of these kinetic differences based on assumptions one would normally make.

The panel concluded that this MOA may be quantitatively unlikely to be relevant to humans (and practically speaking irrelevant), though it cannot be precluded. The data suggest that the quantitative differences between mice and humans are probably at least 100. To the extent possible, these differences should be additionally quantified. Relevant considerations include differences in the number of Clara cells, the amount and effectiveness of the CYP2F1 analog in rats compared to humans, and the rates and extent of metabolism among the species.

The panel also agreed with the conclusion from the Cruzan et al. (2009) publication:

> "Therefore, while this mode of action is theoretically possible in humans if sufficient concentrations of active metabolites are produced, this is highly unlikely to occur given the cross-compound evidence of the central role of mouse-specific CYP2F2 in mediating cytotoxicity. Thus, the hypothesized MOA developed from this cross-compound analysis suggests these chemicals are not expected to cause lung tumors in humans" (page 12)

One of the expert panel members briefly presented some recent updates to the MOA/Human Relevance framework that might be of assistance to the SIRC team in moving forward. The panelist explained that the current thinking is to build the MOA from the bottom up and think in a more predictive context. Problem formulation is critical at the front end. The extent of the analysis is dependent on the purpose of the assessment – whether adverse effects are observed or hypothesized. The heavy reliance on Yes/No answers is gone; rather the emphasis is on assessing qualitative and quantitative human concordance and level of confidence and identification of critical data gaps. One is also encouraged to look at comparative weight of evidence and how much of one case will be relevant to others, as well as potentially competing MOA hypotheses. New templates have been developed for weight of evidence including dose response and temporality. Consideration of dose-response concordance in WOE analysis for MOA emphasizes kinetic and dynamic data early on and the implications of these data for risk analysis. The goal of continuing refinement is to build on experience in application, increasing transparency in a systematic approach, as a basis to clearly separate personal and policy judgments. The panelist thought that the SIRC team has addressed much of this already; the comparative WOE relates to looking at alternative modes and more work is needed regarding the temporality data as discussed earlier. The panelist shared additional suggestions with the SIRC team authors post-workshop on how to present the dose-response and temporal information. The panelist suggested that the data for the three best-studied compounds be visually aligned in tables either vertically or horizontally and provided some ideas for formatting (see Appendix C). Careful thought will be needed to determine how best to compare/contrast doses and associated effects for each of the studied chemicals.

Discussion Question 5: If necessary, what new data might further clarify the question of human relevance?

Earlier in the workshop discussions, the panel members recommended that the SIRC team take an integrated look across compounds to show the level of support for the hypothesized MOA using updated approaches and templates for the MOA framework (see Discussion Question 1). With this updated and complete analysis, the need for additional data can then be better evaluated.

A panel member thought that further studies in humanized mice could get a better handle on variation in gene function and explore whether polymorphisms in CYP2F1 exist in humans and compare to CYP2F2 in the mouse. Another panel member asked about the validity of the transgenic mouse model. The SIRC team explained that knockout and transgenic mouse models in general have been used for over a decade; the U.S. Food and Drug Administration (FDA) accepts them for making drug decisions. This TG mouse model has been used for two or three chemicals and the SIRC team has investigated the model. SIRC is holding 100 male and female KO and TG mice for up to 108 weeks of age. Most of the KO mice have reached this age and several of the TG mice have. There is no difference in survival of the KO and TG mice compared to WT mice. Dr. Ding, the TG mouse model developer, has reported that the level of other CYPs in the KO and TG mice are not different from the level in WT mice. Dr. Cruzan thought it may be better to have a TG humanized mouse with just the CYP2F1 gene, and not the two other transgenes (but such a mouse does not currently exist), but noted that the CYP2A13 is probably good to include (equivalent to CYP2A5 in mouse) as it is contributing to nasal toxicity. A panel member noted that the developers of this TG mouse have published papers on their construct and thought that the information regarding the TG mouse that the SIRC team presented, along with the fact that the developers have looked at activity of the three genes in the TG mouse, is good evidences that it is a good tool.

Dr. Cruzan reviewed the current studies underway or planned for styrene. He explained that a genomics study with styrene will start shortly at The Hamner Institutes for Health Sciences, where they will look at early and late changes in gene expression in whole mouse lungs to identify gene pathways affected by styrene in WT, KO and TG mice exposed at 40 or 120 ppm styrene at one week and four weeks. Dr. Bus explained that if the study shows what they anticipate, the genomic study would help in the evaluation of future chemicals for early events. The Hamner will compare the styrene results with what they previously found for naphthalene.

SIRC has also been discussing with EPA a two-year inhalation study with styrene to look at production of lung tumors in WT, TG, and KO mice. SIRC has the breeding colony, but it will take six months to get the colony up to the necessary size. The hypothesis to be tested is that there will be no tumors in the KO or TG mice. They asked the panel if they have suggestions for additional endpoints and one panel member suggested including intermediate time points if possible, and high-resolution microscopy like that done for naphthalene, to solidify the naphthalene/styrene connection. A panel member encouraged SIRC to consider the necessary use of animals and what will be gained by taking the animals out to two years. Another panel member thought that between these two studies, they may address the variation in gene function and explore polymorphisms for humans. A third panel member asked whether there is possibility in the studies to additionally inform about what is happening in these animals between cytotoxicity and tumor production.

Dr. Van Winkle described planned studies for naphthalene. They plan to look at the mass balance of naphthalene metabolites in mouse airways and are using monkey lung tissue to determine whether the metabolic pathways, and relative amounts of metabolite formed, are the same or different between these two species. They are also planning to test naphthalene toxicity in TG humanized mouse, human lung tissue, and evaluate the potential role of liver metabolism in lung toxicity.

David Adenuga (an observer with Exxon Mobil Biomedical Sciences) asked if there is any way to clarify that the strain difference is key, and there is not something else going on. Dr. Cruzan was not sure that much further would be gained from sorting out the strain differences, rates of metabolism and susceptibility issues. In CD1 mice, about 50% of them are more susceptible than the other 50% for lung tumors. The CD1 mice are more susceptible in general, for lung tumors, than the C57BL/6 mice and differences are not related to metabolism. The comparative study among three strains (CD1, C57BL/6 and B6C3F1) showed that C57BL/6 was the most sensitive strain to styrene lung toxicity over five days, based on BrdU labeling.

Discussion Question 6: Is there a set of criteria, based on short-term experimental protocols (≤90 days) that can be applied to other chemicals to determine if they operate by the same mode of action as that presented

Panel members discussed a number of criteria that would be useful to test other chemicals for this mode of action. They proposed that the following evaluations and criteria would be necessary to demonstrate this MOA in other compounds.

1. Evaluate the ring oxidation potential of the chemical's structure, looking for demonstration of ring-oxidized metabolites, including *in vitro* CYP2F2 metabolism studies
2. Look at the GAPs, to determine if mutation is an early and influential key event in the mode of action
3. Look for evidence of acute cytotoxicity in mice and rats (*in vivo*)
4. If the cytotoxicity response is specific to mice (and not rats), then use CYP2F2 knockout mouse to demonstrate that the response is dependent upon CYP2F2 metabolism
5. Lastly, test in the humanized TG mouse to confirm humans will not metabolize sufficient compound via CYP2F1 to produce lung tumors in a "susceptible" system

Panel members and others suggested that numbers 4 and 5 might be combined into a single study (thereby reducing animal usage with a common set of control animals).

Drs. Bus and Cruzan noted that not all compounds would need ring oxidation for this MOA to be relevant, but panel members recommended restricting it in this way for the time being because this is a common criterion for the three model compounds; with more chemicals evaluated the ring oxidation requirement could be reevaluated. Panel members also recommended very carefully considering alternative MOAs based on genotoxicity or hormonal pathways.

A webinar observer, Christine Palermo of Exxon Mobil Biomedical Sciences, asked why, if there is already a fairly extensive amount of counterfactual evidence to support the involvement of CYP2F2 metabolism, that two-year cancer bioassays in TG and KO mice are needed for styrene. She further asked what question these studies are designed to answer. Dr. Bus responded that the initial thinking is that there may be a weakly active secondary MOA that may express itself over a chronic timeframe. There is also motivation for genomic analysis in shorter term studies that support this thinking and approach. Panel members agreed with the observer's concern, but noted that this needs to be done as a proof of concept with at least one of the model chemicals in order to forgo these studies for other compounds in the future. More information and data are always needed at the outset, in order for a new MOA to gain acceptance by the scientific risk community. While alternative MOAs need to be explored, the extent of investment in their consideration should be proportional to the extent of their plausibility based on supporting evidence and contrasted with that required for default. Dr. Cruzan noted that the 2-year study as designed right now with one exposure level in each of the three strains and using males only is estimated to cost $1 million.

Panel members suggested that for the three model chemicals, the potential to act via a mutagenic mode of action should be considered in a comparative analysis, taking into consideration more robust GAP analysis for dose response. The available IARC GAP profiles could be considered.

Two additional comments from observers were presented. David Adenuga suggested including *in vitro* CYP2F2 metabolism studies, prior to a knockout or transgenic mouse study. A webinar observer, Ruth Lunn of the National Institute of Environmental Health Sciences (NIEHS), submitted a question via email. She noted that although styrene causes lung toxicity in C57BL mice, styrene did not cause lung tumors in the C57BL mice in a gavage study by Ponomarkov and Tomatis (1978). She asked if this C57BL is the same strain as the wild type mice used in the styrene studies. Dr. Cruzan replied that it is the same strain and explained that the Ponomarkov and Tomatis study used a low dose of 300 mg/kg-day for one day per week (equivalent to 60 mg/kg/day 5 days/week) and its results are not surprising. NCI administered 175 mg/kg-day to B6C3F1 mice and did not find increased lung tumors. In the NCI bioassay, 350 mg/kg-day administered 5 days a week, resulted in lung tumors that were statistically significantly increased compared to controls, but within the laboratory historical control range.

Summary

The panel agreed that there is a good level of support for the CYP2F2-mediated MOA proposed for mouse lung toxicity and tumors, although additional evaluation and presentation of the data will enhance confidence in this MOA. They suggested lining up the considerations for WOE in a prioritized context: biological concordance, essentiality of key events, and concordance of empirical evidence. They thought that more detailed data with regard to earlier cellular changes for styrene and ethylbenzene (like that available for naphthalene) would contribute to confidence in the hypothesized MOA. The panel agreed that the cytotoxic response is mediated through metabolism by CYP2F2 in the mouse lung. However, while metabolism in the lung by CYP2F2 leading to cytotoxicity is clear, what happens between cytotoxicity and the apical endpoint of late-stage tumors is not well understood.

The evidence to date suggests that genotoxicity is not an early and influential key event in the progression to tumors. However, the potential to act via a mutagenic MOA should be considered in a comparative analysis, taking into consideration more robust GAP analysis for dose response. The available IARC GAP profiles could be considered.

Panel members agreed that evaluating weight of evidence for the hypothesized MOA of cytotoxicity following CYP2F2 metabolism, based principally upon the data for those chemicals where it has been most fully investigated (namely styrene, naphthalene and ethylbenzene), is appropriate. The panel agreed that the data on these chemicals should be considered collectively and where data on patterns of, for example, dose-response and temporality for hypothesized key events for individual chemicals are not consistent, these should be identified, as well as the impact of differences between them (e.g., metabolism). Panel members agreed that the available data presented for the three chemicals appear consistent with the hypothesized MOA and did not see much discordance among the chemicals in the information provided that would diminish the plausibility of the MOA.

The panel concluded that this MOA may be quantitatively unlikely relevant (and practically speaking irrelevant) to humans, although it cannot be precluded. The data suggest that the quantitative differences between mice and humans are probably at least 100. To the extent possible, these differences should be additionally quantified. Relevant considerations include differences in the number of Clara cells, the amount and effectiveness of the CYP2F1 analog in rats compared to humans, and the rates and extent of metabolism among the species.

The panel discussed the considerable quantitative differences between the mouse, rat, and humans to propose that the CYP2F2 MOA for mouse lung tumors is not relevant to humans and noted that this is similar to a recent update of the PPARα MOA (Corton et al., 2014). They agreed that the wording used by Cruzan et al. (2009) accurately communicates the uncertainty about quantitative interspecies variations:

> "Therefore, while this mode of action is theoretically possible in humans if sufficient concentrations of active metabolites are produced, this is highly unlikely to occur given the cross-compound evidence of the central role of mouse-specific CYP2F2 in mediating cytotoxicity. Thus, the hypothesized MOA developed from this cross-compound analysis suggests these chemicals are not expected to cause lung tumors in humans" (page 12)

Panel members discussed a number of criteria that would be useful to test other chemicals for this MOA. They agreed that the following evaluations and criteria would be necessary to demonstrate this MOA in other compounds.

1. Evaluate the ring oxidation potential of the chemical's structure, looking for demonstration of ring-oxidized metabolites, including *in vitro* CYP2F2 metabolism studies
2. Look at the GAPs, to determine if mutation is an early and influential key event in the MOA
3. Look for evidence of acute cytotoxicity in mice and rats (*in vivo*) If the cytotoxicity response is specific to mice (and not rats), then use CYP2F2 knockout mouse to demonstrate that the response is dependent upon CYP2F2 metabolism
4. Lastly, test in the humanized TG mouse to confirm humans will not metabolize sufficient compound via CYP2F1 to produce lung tumors in a "susceptible" system

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Appendix A: Workshop Agenda

WORKSHOP TO ASSESS THE MODES OF ACTION OF LUNG TUMORS IN MICE
From Exposure to Styrene, Ethylbenzene, & Naphthalene

AGENDA

9:00 Welcome (J. Snyder)

A. Introductions

B. Welcome and review purpose and goal of workshop

9:15 Introduction (G. Cruzan): Potential Modes of Action for Chemicals that cause lung tumors in mice, but not rats, that are metabolized by CYP2F2

A. Cytotoxicity and/or mitogenesis from CYP2F2 metabolism

B. Genotoxicity from these compounds or their metabolites

9:30 Presentation 1 (G. Cruzan): Cytotoxicity from CYP2F2 Metabolism of styrene and ethylbenzene

Description of toxicity, necessity of CYP2F2 metabolism – pharmacologic inhibitors, knockout mice (2E1 and 2F2), metabolites produced by CYP2F2 and relationship to major pathways, non-tumorigenic

10:00 Presentation 2 (L. Van Winkle): Pathology and MOA data for Naphthalene

Lung toxicity and proliferation patterns, evidence for CYP2F2 metabolism (inhibitors, Km, knockout mice). Summary of evidence for metabolites in relation to toxicity and genotoxicity.

10:30 Break

10:45 Presentation 3 (B. Gollipudi): Alternative Hypothesis: Genotoxicity

11:15 Presentation 4 (J. Bus): Human Relevance

Analysis of the MOA data using the Human Relevance Framework

12:00 Lunch

1:00 Panel Discussion (Moderator: M. Dourson)

1. Is the CYP2F2--mediated mode of action proposed for mouse lung toxicity and tumors adequately defined in animals? If not, what data are necessary, or useful, to enhance confidence in the MOA?

2. Are the data on the three chemicals mutually consistent with the hypothesized MOA, or are there discordances that diminish the plausibility of the MOA?

3. Do the data indicate whether the hypothesized MOA, if established in animals, is likely to be qualitatively irrelevant to humans?

4. If the hypothesized MOA is qualitatively relevant for humans, is it characterized as quantitatively irrelevant (or relevant) to human?

5. If necessary, what new data might further clarify the question of human relevance?

6. Is there a set of criteria, based on short--term experimental protocols (≤ 90 days) that can be applied to other chemicals to determine if they operate by the same mode of action as that presented?

3:45 Wrap up and Next Steps

Appendix B: Presentation Slides

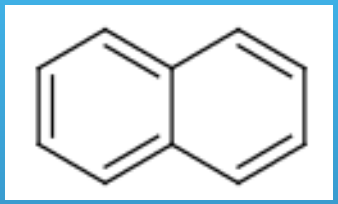
Styrene Tumor Mode of Action

George Cruzan, PhD, DABT
ToxWorks

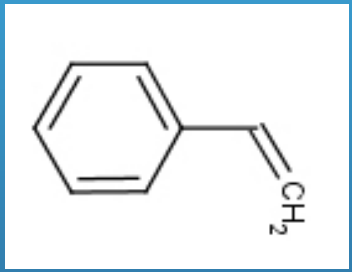
This research was sponsored by the Styrene Information and Research Center, Washington, DC

The chemicals

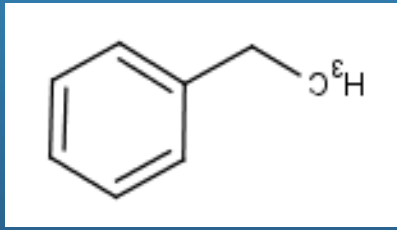
- Naphthalene



- Styrene



- Ethylbenzene



Human Cancer Data

- Ethylbenzene – no studies
- Naphthalene – limited data; no increase in lung tumors reported
- Styrene – 13 cohort studies; no styrene-related increases in lung tumors. One study (Wong, 1990; Wong et al., 1994; Collins et al, 2013) increased lung tumors, inverse dose-response, attributed to smoking

Rat Studies

- 4 Gavage studies – no increases in tumors
  - NCI, 1979a, b, Ponomarkov and Tomatis, 1978, Conti et al., 1988
- 1 Drinking water study - no increases in tumors
  - Beliles et al., 1985

Rat Studies

- 3 Inhalation studies
  - Conti et al., 1988 - Increased malignant mammary tumors 25-300 ppm 1 year exposure
  - Jersey et al., 1978 - Increased malignant mammary tumors, but within historical control range at 600 ppm, not at 1000 ppm – 2 years
  - Cruzan et al., 1998 – No increase in mammary tumors at 50 and 200 ppm 2 years; decreased malignant mammary tumors at 500 and 1000 ppm

Mouse Studies

- 4 Gavage studies
  - 2- Suggestive increase in lung tumors - Ponomarkov and Tomatis, 1984; NCI, 1979a
  - 2- No increase in tumors - Ponomarkov and Tomatis, 1984; NCI, 1979b
- 1 Inhalation Study
  - Increase in lung tumors at 40, 80 and 160 ppm in males, 20, 40, 160 females - Cruzan et al., 2001

Mode of Action

- Why Mice and Not Rats?

- Are Humans Like Mice or Like Rats?

Progression of Toxicity in Mice

- Single exposure – inc. LDH, Cells and protein in BALF
- Up to 2 weeks – inc. BrdU labeling, dec. CCSP
- 3 months – decreased staining of Clara cells; cellular crowding in terminal bronchioles
- 12 months – hyperplasia in terminal bronchioles
- 18 months – hyperplasia in terminal bronchioles extending into alveolar ducts
- 24 months – lung tumors, mostly benign

Proposed MOA

- Lung metabolism of styrene by CYP2F2 produces ring-oxidized metabolites that are toxic to Clara cells. Continued exposure results in continual cell replication, leading to hyperplasia. Newly formed Clara cells lack production of CCSP(CC10), which reduces resistance to lung tumors.

Evidence for CYP2F2 MOA

- Inhibit 2F2 (5P1P), reduce toxicity - (BALF, BrdU)
- Eliminate 2F2 (knockout mouse), eliminate toxicity from styrene – (BALF, BrdU)
  - Normal levels of BrdU labeled cells and CCSP positive cells
- Eliminate 2F2, eliminate toxicity from SO

Labeling index (% of cells labeled with BrdU) in terminal bronchioles of wild type and CYP2F2(-/-) mice exposed to styrene, or styrene oxide, for 5 days.

Treatment (mg/kg/day)	CYP2F2(+/+) [WT]		CYP2F2(-/-) [KO]	
	Males	Females	Males	Females
Control - 0	5.2	5.7	3.8	4.6
Styrene - 200	47.2	34.9	2.9	4.6
S-SO - 200	37.8	46.6	4.2	4.5
R-SO - 200	39.0	43.9	4.4	4.7

- Cruzan et al., 2012

Role of Ring Oxidation

- 6-10% of styrene urinary metabolites in mice are ring-oxidized; 0.5% in rats
- No lung tumors in mice from 3-methyl-styrene or 4-methylstyrene (no ring oxidation possible)
- 4-Hydroxystyrene (4HS, 4-vinylphenol) more toxic than styrene or SO (NOAEL 2 mg/kg/day over 2 weeks compared to 100 mg/kg/day for styrene)

Role of Ring Oxidation

- WT mice produce 4HS, 3,4-dihydroxystyrene and 4-hydroxystyrene oxide from styrene in lung
- Limited ring oxidation of styrene in 2F2-KO mice
- Limited ring oxidation of 4HS in 2F2-KO mice
- Some toxicity from 4HS in 2F2-KO mice

BrdU Labeling index in terminal bronchioles of WT, and KO mice exposed to 4-hydroxystyrene for 5 days

Treatment	Males	Females
WT Controls	5.1(0.5)	5.8(1.1)
WT (105 mg/kg/day)	**48.0** (1.7)	**51.7** (1.4)
KO Controls	4.5(0.5)	4.2 (0.5)
KO (60 mg/kg/day)	6.3 (0.8)	8.4 (0.6)
KO (105 mg/kg/day)	6.0 (0.5)	13.4 (1.4)

Default MOA Hypothesis

- Styrene is genotoxic carcinogen via metabolism to SO.

Alternative MOA of genotoxicity via SO will be discussed later.

Hypothesis that SO is toxic metabolite

- Styrene metabolized to styrene-7,8-oxide (SO) primarily by CYP2E1
- Level of SO from liver through blood to lung much greater in mice than in rats at same exposure concentration
- Level of SO in mouse lung much greater than in rat lung at same exposure concentration
- Administration of SO causes lung toxicity in mice

Data Do Not Support Hypothesis

- Mice exposed to SO did not get lung tumors
- Blood level of SO in rats at 1000 ppm (no tumors) is 100 fold higher than in mice at 40 ppm (lung tumors)
- Level of SO in perfusate from isolated lungs in rats exposed to 1000 ppm is 8 fold higher than in mice at 40 ppm.
- No reduction in lung toxicity of styrene from inhibition of CYP2E1 or in CYP2E1-knockout mice.

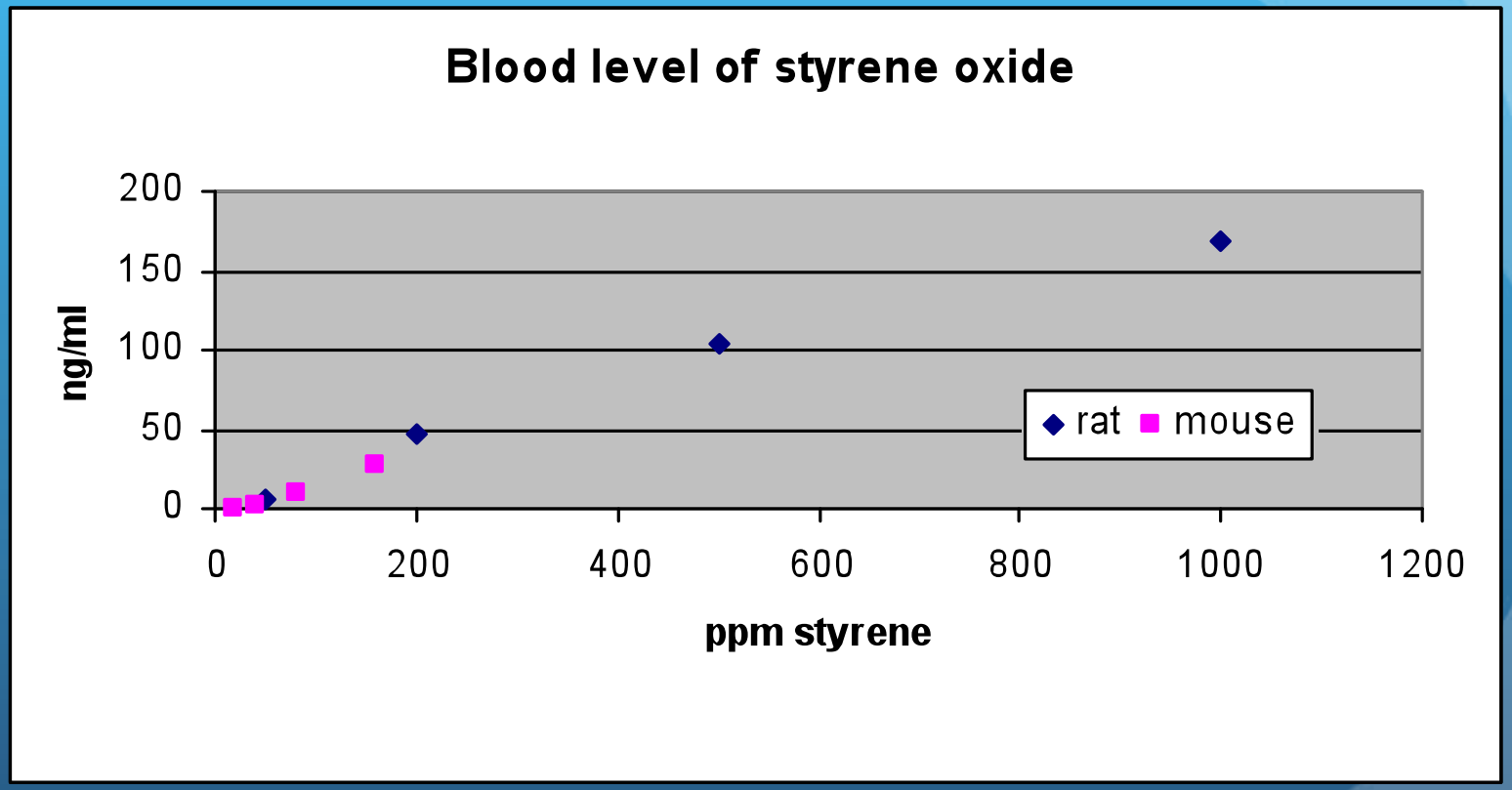
Styrene Oxide

- 2 rat studies – forestomach necrosis and tumors
- 1 mouse study – forestomach necrosis and tumors

  liver tumors low dose males

- DNA adducts $<1/10^6$ in forestomach
- Increased cell replication parallels tumors in forestomach

Styrene oxide at end of 6-hour exposure during chronic inhalation studies



SO in perfusate of isolated lungs exposed to styrene through trachea

- **SO level in rat lung:**

Styrene ppm:	50	160	475	985
SO (ng/ml)	.06	.50	1.05	2.05

- **SO level in mouse lung**

Styrene ppm:	42	164	407
SO (ng/ml)	.25	1.83	3.16

- From Hofmann *et al.*, 2005, whole lung explants

Alternative Hypothesis

- Toxicity is seen primarily in tissues that produce high levels of R-SO (rat and mouse nasal olfactory epithelium and mouse lung terminal bronchioles;
- Therefore, R-SO is toxic metabolite.

Hypothesis 2: R-SO is toxic metabolite

- SO contains a chiral carbon at α position
- In rats R:S-SO ratio is slightly < than 1
- In mouse liver R:S ratio is slightly > than 1
- In mouse lung R:S ratio is about 3
- R-SO in mouse lung is estimated to be about 15-fold higher than in rat lung
- Therefore, the toxic metabolite must be R-SO or derived from R-SO

Data Do Not Support This MOA

- No difference in toxicity between R and S following single exposure (BALF – Carlson)
- No difference in toxicity between R and S following 5 days of exposure (BrdU – Cruzan et al., 2012)

Summary of Mouse Lung Tumor MOA

- Mouse lung tumors not related to SO – No lung tumors from SO
- No lung tumors in absence of ring oxidation – 3-,4-MeS
- 4HS toxic at much lower dose than styrene or SO
- Some 4HS toxicity even in absence of CYP2F2 metabolism
- CYP2F2 creates ring-oxidized metabolites that are toxic to Clara cells

Human Relevance of CYP2F2 MOA

- Human isoform is CYP2F1
- 2F2 and 2F1 shown to metabolize 3-methylindole; 2F1 metabolizes naphthalene about 1% of 2F2 rate; preliminary indication styrene not metabolized by 2F1
- Developed transgenic mouse to study 2F1 ability to produce toxic metabolites

CYP2F1 Transgenic Mouse

- Inserted transgene containing DNA for CYP2F1 (lung and nasal), 2A13 (lung and nasal), and 2B6 (liver) into CYP2F2-KO mice
- 2F1 and 2A13 protein expressed in lung and nasal tissue of these mice
- CYP2F1 present in TG mouse lung at 1/40th level of 2F2 in WT mice (greater than amount in human Clara cells)
- Lung microsomes from TG mice metabolize naphthalene (Ding) and 3-methylindole (Yost)

CYP2F1 Transgenic Mouse

- No lung toxicity from styrene or SO in TG mice
- Some toxicity from 4HS in TG mice

Labeling index (% of cells labeled with BrdU) in terminal bronchioles of wild type and CYP2F2(-/-), 2F1-tg mice exposed to styrene for 5 days.

	WT Mice		TG Mice	
	Males	Females	Males	Females
Control	4.7 (2.0)	7.2 (2.7)	5.2 (2.1)	7.5 (3.0)
Styrene - 200	35.4 (9.6)	50.0 (8.9)	2.7 (1.1)	3.5 (0.9)
R-SO - 200	35.6 (2.5)	42.0 (8.5)	6.3 (2.2)	6.0 (2.9)
S-SO - 200	27.5 (17.0)	42.3 (1.1)	4.3 (3.0)	6.8 (2.0)
4HS - 105	48.0 (1.7)	51.7 (1.4)	12.3 (6.6)	22.9 (8.3)

Conclusions

- Mouse lung tumors from styrene exposure caused by ring-oxidized metabolites produced by CYP2F2 metabolism
- Styrene oxide is NOT the toxic metabolite
- Human CYP2F1 does not produce sufficient metabolites to cause toxicity or tumors
- Mouse lung tumors from styrene do not indicate human risk of cancer from styrene

Ethylbenzene

EB Cancer Data

- NTP developed series of genotoxicity tests to be surrogate for 2-yr bioassay
- Performed 2-yr bioassays on some + chemicals to show accuracy
- Performed 2-yr bioassays on – chemicals to show specificity
- EB negative in gene tox battery; expected to be – in 2-yr bioassay - NOT

EB Cancer Data

- Increase in lung tumors in male mice and liver tumors in female mice at 750 ppm
- Increase in kidney tumors in male and female rats and testicular tumors in male rats at 750 ppm
- No increases at 250 ppm

EB Lung MOA Data

- Decreased staining of Clara cells; hyperplasia of terminal bronchioles extending into alveolar ducts (referred to as bronchiolar metaplasia of alveolar ducts in NTP report)
- Increased cell proliferation in males exposed to 750 ppm

Active EB Metabolites in Lung

- Primary metabolism of EB is by CYP2E1 to 1-phenylethanol (1-PE)
- 1-PE not tumorigenic in 2-yr bioassay
- 1-PE does not cause increased BrdU labeling in mouse lung

Active EB Metabolites in Lung

- Lung metabolism produces 2- and 4-ethylphenol (2- and 4-hydroxyethylbenzene)
- Increased cell proliferation from 4-ethylphenol (unpublished BASF report)
- EB lung cell proliferation inhibited by 5P1P

  (evidence of CYP2F2)

EB Conclusion

- Mouse lung tumors not related to main metabolism (CYP2E1)
- EB less potent than styrene (750 ppm vs 40 ppm)
- MOA data limited, but parallels styrene

Pathology and Mode of Action for Naphthalene

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Mouse lung Tumor workshop
Sept 2013

Mouse lung tumors and naphthalene exposure

NTP 2 yr inhalation bioassay

Female mice – some evidence – alveolar/bronchiolar adenomas of the lung increased in high dose group.

Male mice - no evidence of carcinogenic activity.

Male and Female rats – clear evidence of carcinogenesis: adenomas of the respiratory epithelium and neuroblastomas of the olfactory epithelium in the nose. No lung tumors

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Additional study by Adkins found increased tumors in A/J mice exposed to 10 or 30 ppm for 6 mos.

Toxicopathology

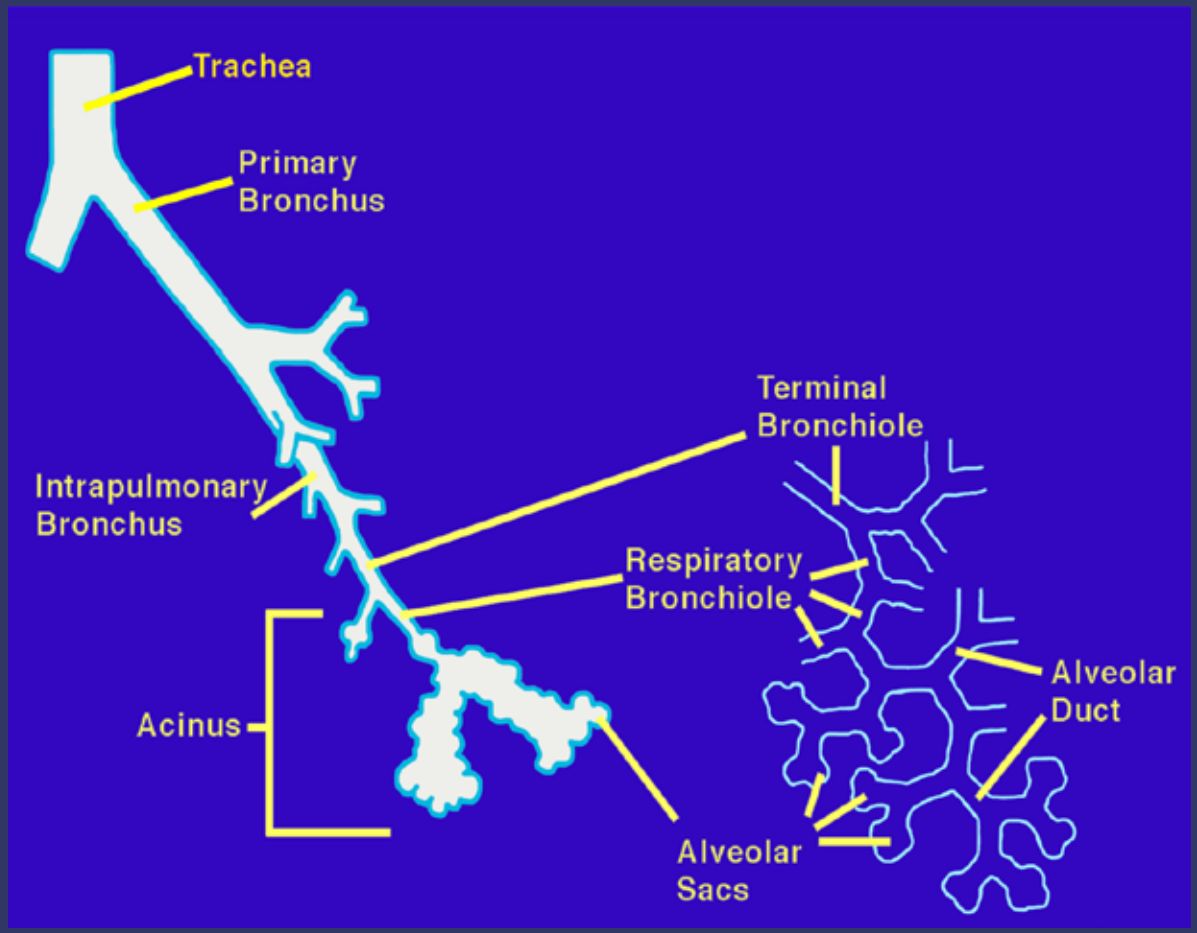
In general, tumors are found at sites of toxicity: What do we know about the toxicopathology of NA injury?

NA injures Clara cells regardless of route of exposure

Toxicity Distribution in the airway depends on

- route of exposure
- dose
- duration (acute vs chronic)
- sex and age of the animal

Location, Location, Location



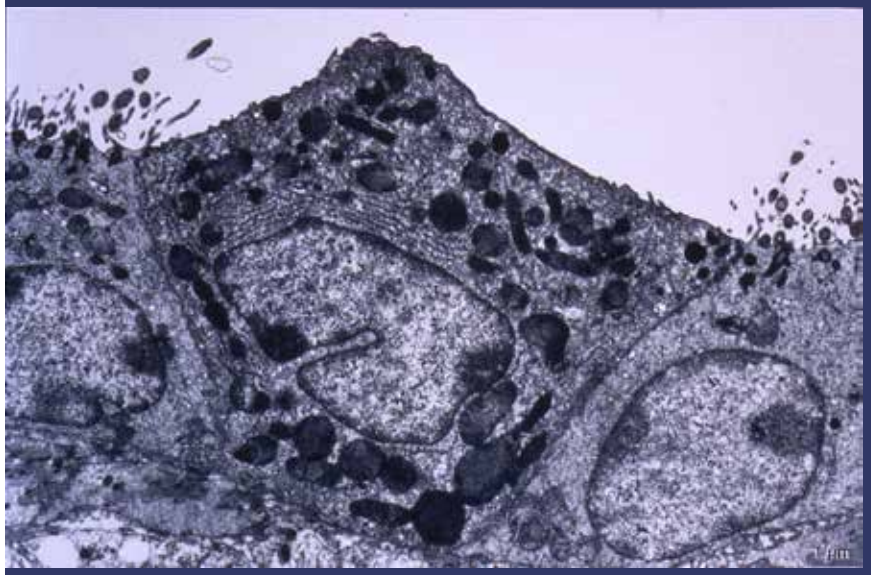
-Distribution of target/ sensitive cells

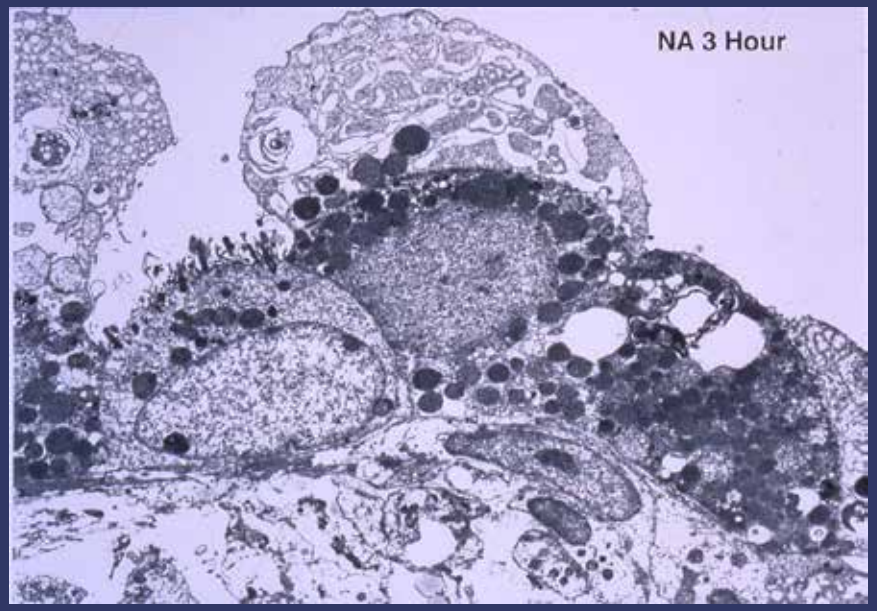
- capability to repair following injury

Mouse Clara cells and ciliated cells in all intra and extrapulmonary airways. Up to 70-85% Clara cells.

Primates: Clara cells have a more restricted distribution

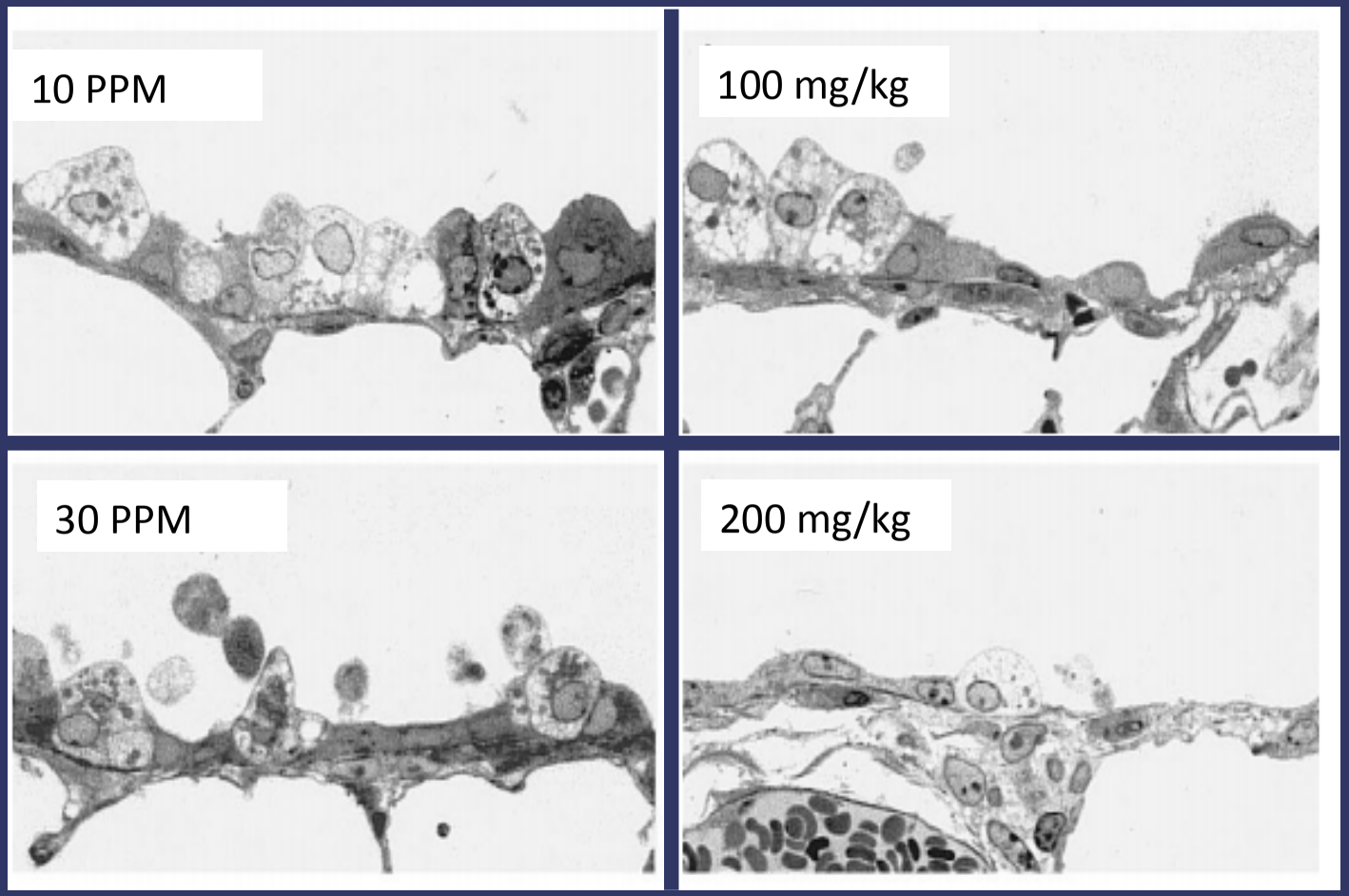
Naphthalene is toxic to mouse Clara cells





Images from Van Winkle et al 1999

and terminal bronchiolar Clara cells



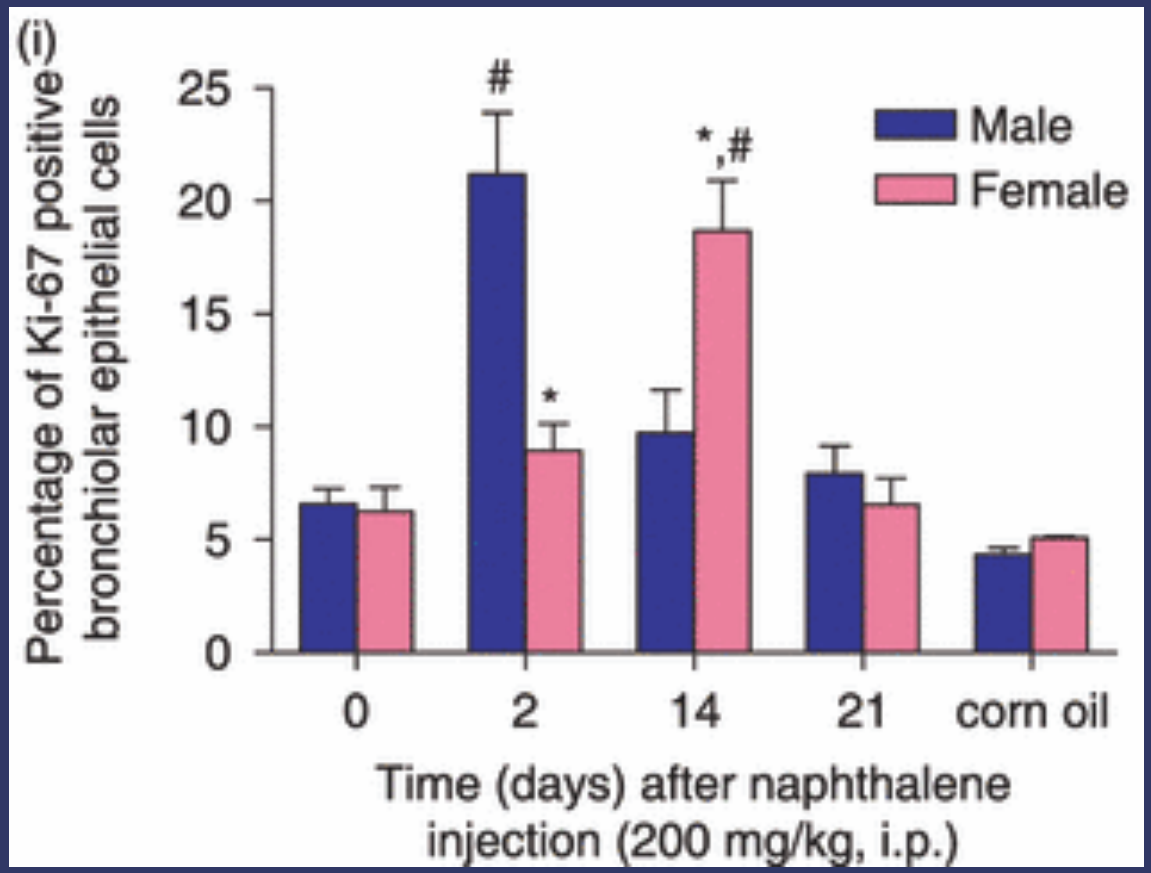
From West et al 2001

Species and Site Selective Toxicity of Naphthalene

			Distal		Nasal	Epithelium
Species	Dose	Trachea	Bronchiole	Parenchyma	Olfactory	Respiratory
Mouse	50	0	+	0	0	0
	100	0	++	0	0	0
LD_{50}=380 mg/kg	200	+	+++	0	0	0
	400	++	++++	0	++	0
inhalation	2-5 ppm	+			+	
	10 ppm	+++	++		++	++
Rat	200	0	0	0	++	0
	400	0	0	0	+++	0
LD_{50}=1600 mg/kg	800	0	0	0	+++	0
	1600	0	0	0	+++	0
inhalation	3.4 ppm	0			++++	
	100 ppm	0				

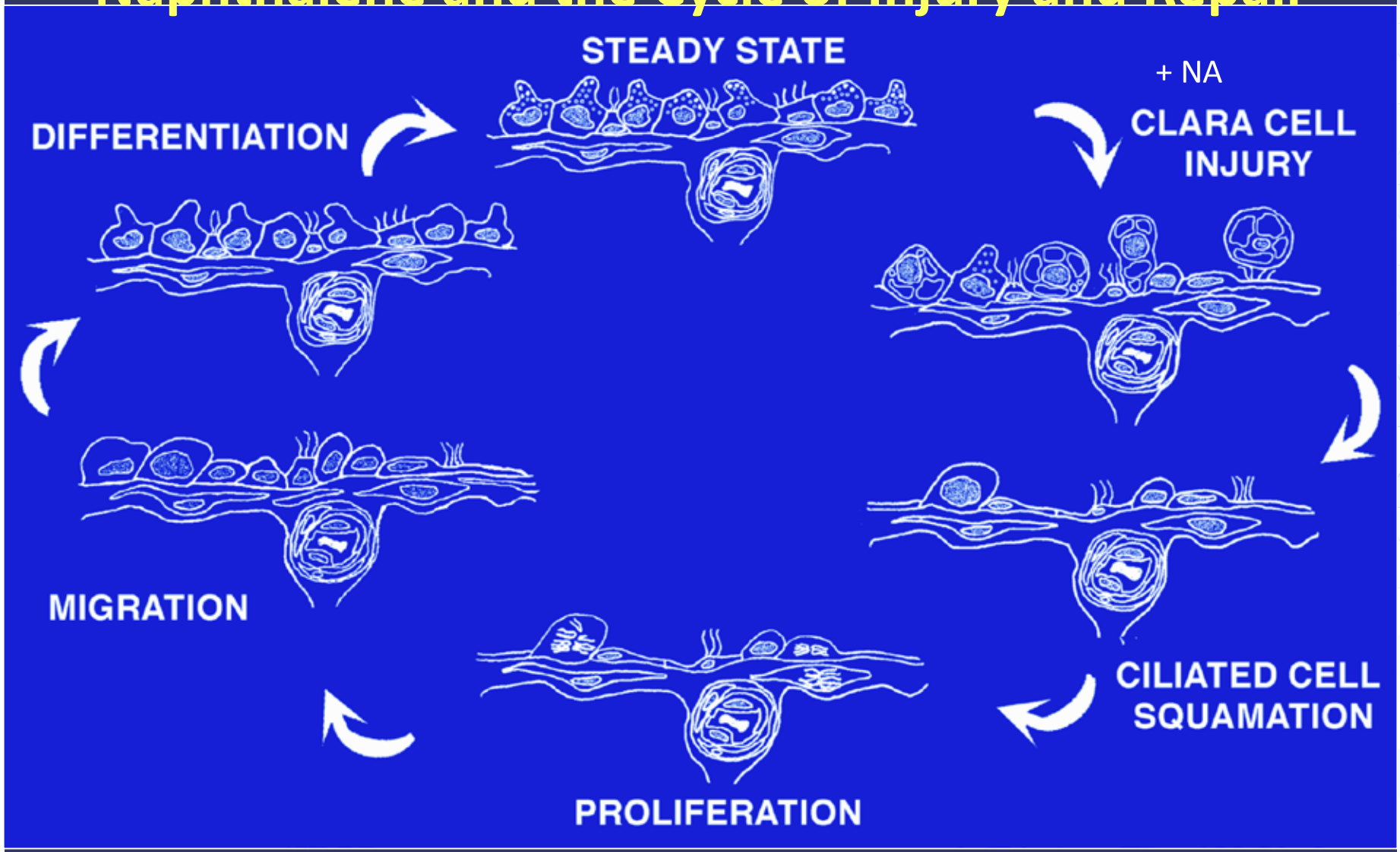
Plopper et al., 1992; 1993; West et al, 2001; Lee et al., 2005

Cell Proliferation Male vs Female Mice



Oliver et al 2009

Naphthalene and the Cycle of Injury and Repair



from Van Winkle et al . Am J Pathol. 151 (443-459) 1997

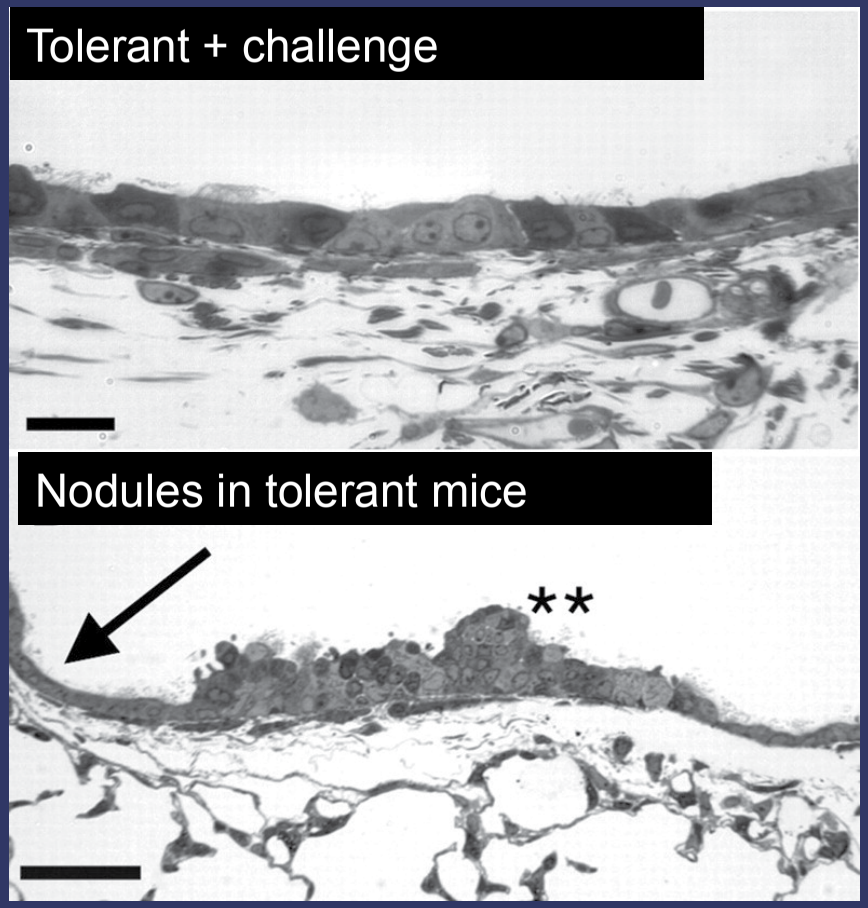
Repeated Inhalation or Injection of Naphthalene causes “Tolerance”

Tolerance is resistance to a high challenge dose following a week or more of exposure to repeated exposures

-NA i.p. tolerance Lakritz et al 1996; O’Brien et al 1989

-NA inhalation tolerance West et al 2003

Tolerance to inhaled NA



West J A A et al. Toxicol. Sci. 2003;75:161-168

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TOXICOLOGICAL SCIENCES

CCSP Expression in Tolerant Mice

Control

Tolerant

Lakritz et al 1996

Repeated Exposure

In female mice, tolerance is incomplete following i.p. exposures. Sutherland et al 2012

Tolerance is facilitated by induction of gamma GCS. West et al 2002.

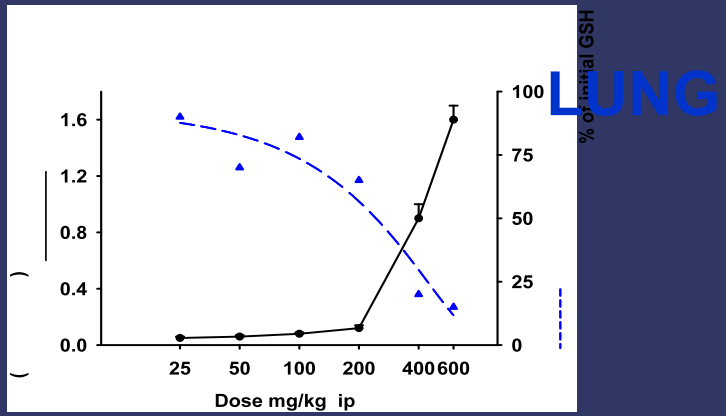
Repeated exposure results in cells that are unlike the normal steady state in terms of differentiation markers such as CCSP, the presence of focal regions of cellular hyperplasia and resistance to additional injury.

It is unclear what role the tolerant conducting airway epithelium (or alveolar epithelium) plays in formation of bronchiolar and alveolar adenomas as described in the NTP chronic bioassay.

How does NA cause toxicity?

- Glutathione depletion
- P450 mediated metabolism
- Protein binding of reactive metabolites
- Metabolism of NA is more robust in sensitive species and in specific respiratory tract target regions
- Naphthalene epoxide and downstream metabolites are toxic to Clara cells
- CYP2F2 contributes to mouse lung Clara cell toxicity-lessons from the knockout mouse
- What do kinetics and metabolism tell us about species specific toxicity in relation to the mouse

Formation of reactive naphthalene metabolites in vivo-dose threshold-mouse



LUNG

Warren et al., 1982

Evidence for the involvement of P450 generated reactive, glutathione depleting metabolites in toxicity

Pretreatment	Airway epithelial Injury	Whole Lung Covalent Binding
None	--	--
Piperonyl Butoxide	↓↓↓↓	↓↓↓↓
Diethyl maleate	↑↑↑↑	↑↑↑↑

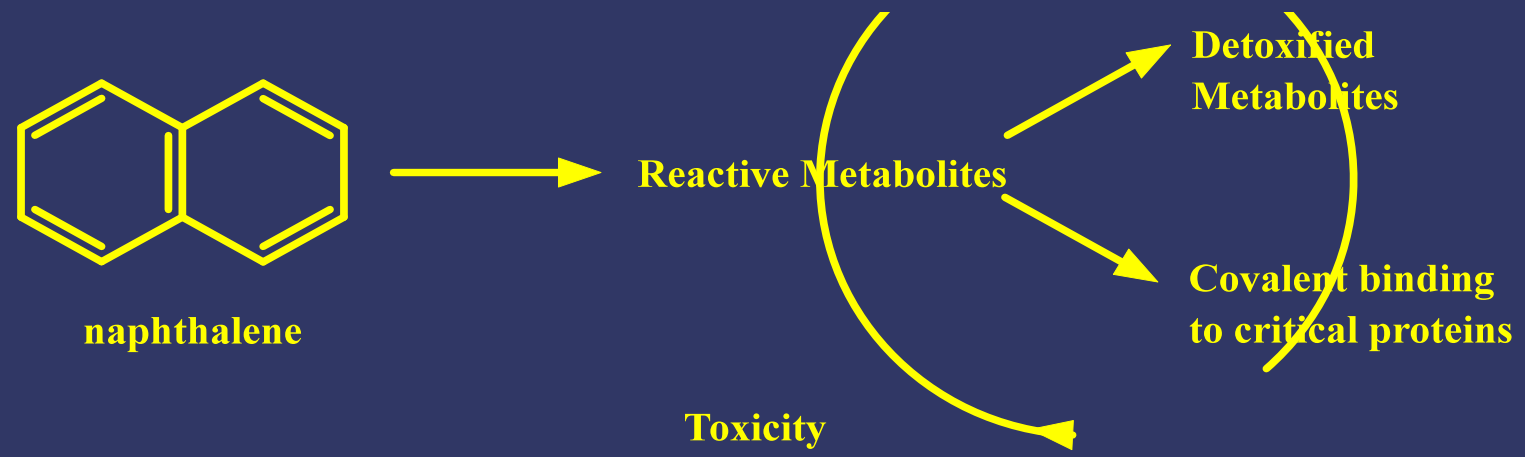
Warren et al., 1982; DEM finding confirmed for inhalation of 15ppm NA in Phimister et al 2004

Possible sequence of events leading to cytotoxicity

Cell is left without normal protective mechanisms protein thiol oxidation ensues leading to protein unfolding. The cell attempts to correct this but critical proteins involved in protein folding are adducted. → Toxicity

Glutathione depletion is a necessary but not sufficient event for toxicity

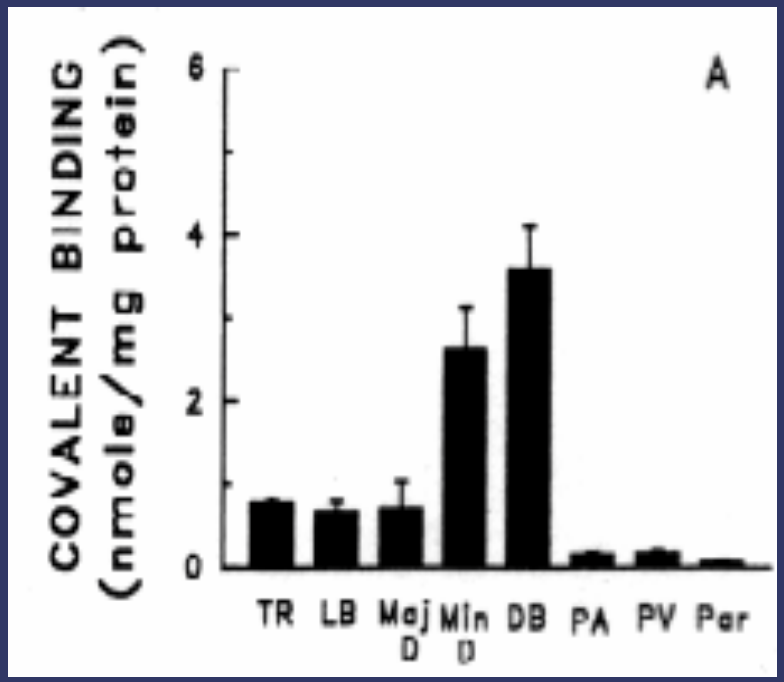
Importance of Understanding Reactive Metabolite Protein Binding in the Mechanism of Toxicity



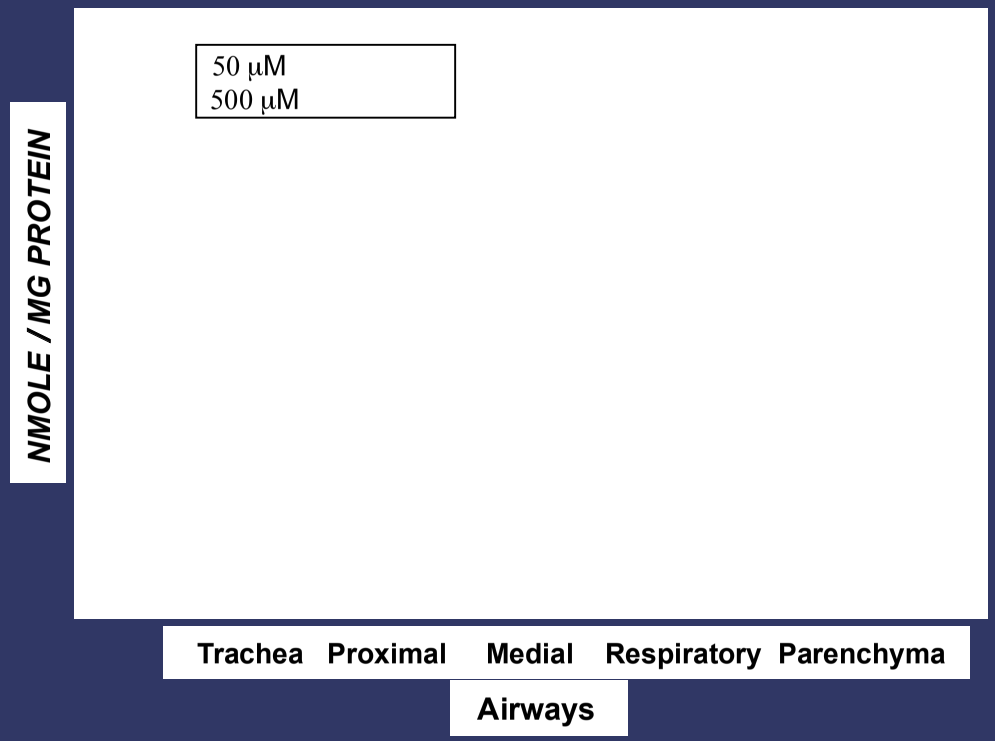
- Reactive metabolite formation precedes earliest signs of toxicity and is dose dependent
- Reactive metabolite binding much higher in airway cell proteins than whole lung
- Reactive metabolite binding generally coincides with changes in toxicity

Total reactive metabolite formation in dissected airways: mice vs. rhesus macaques

1 : ~ 0.3

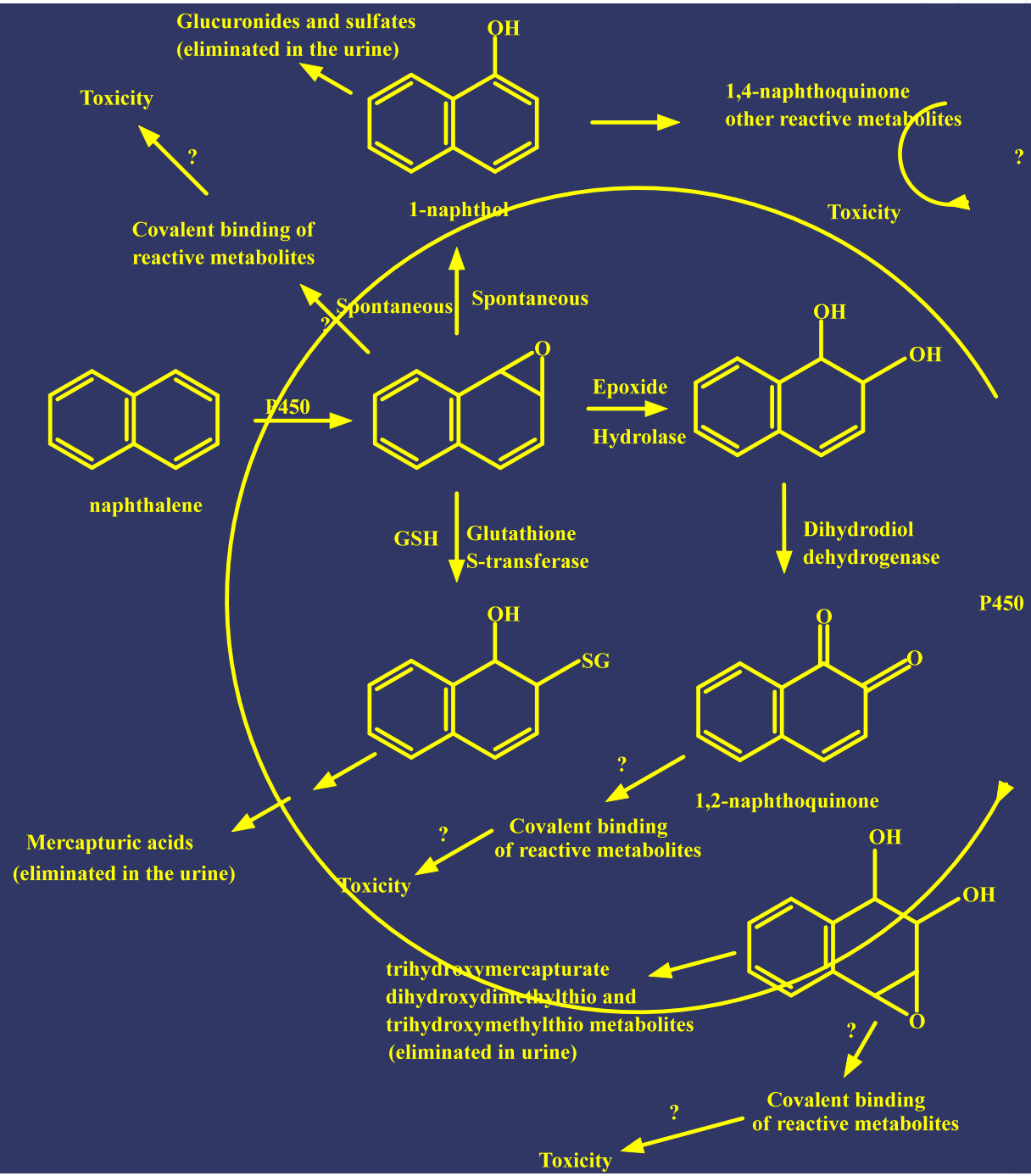


A. Total reactive metabolite formation in dissected mouse airways incubated with NA (500 μM). Cho et al., 1994

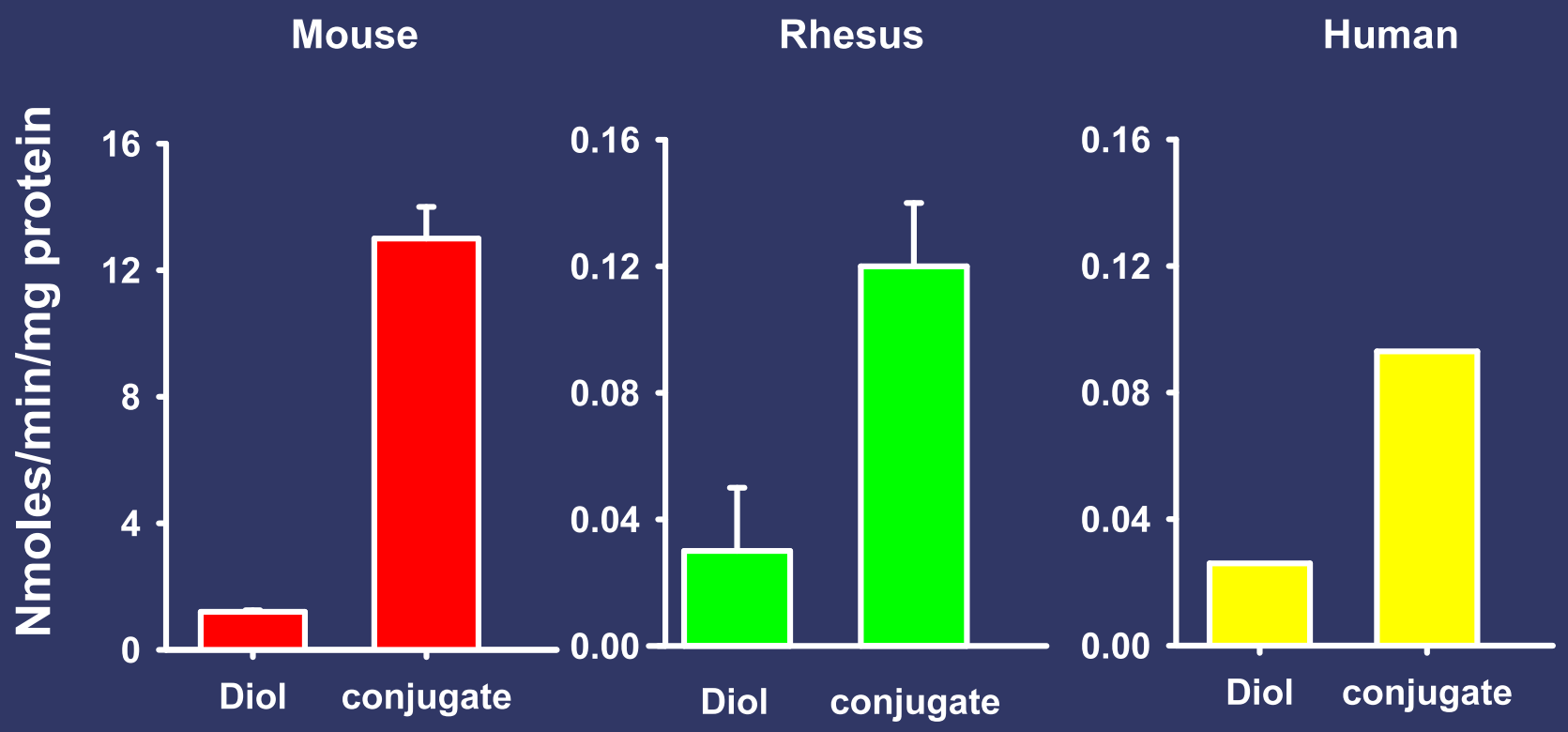


Total reactive metabolite formation in dissected monkey airways incubated with NA (500 μM and 50 μM).

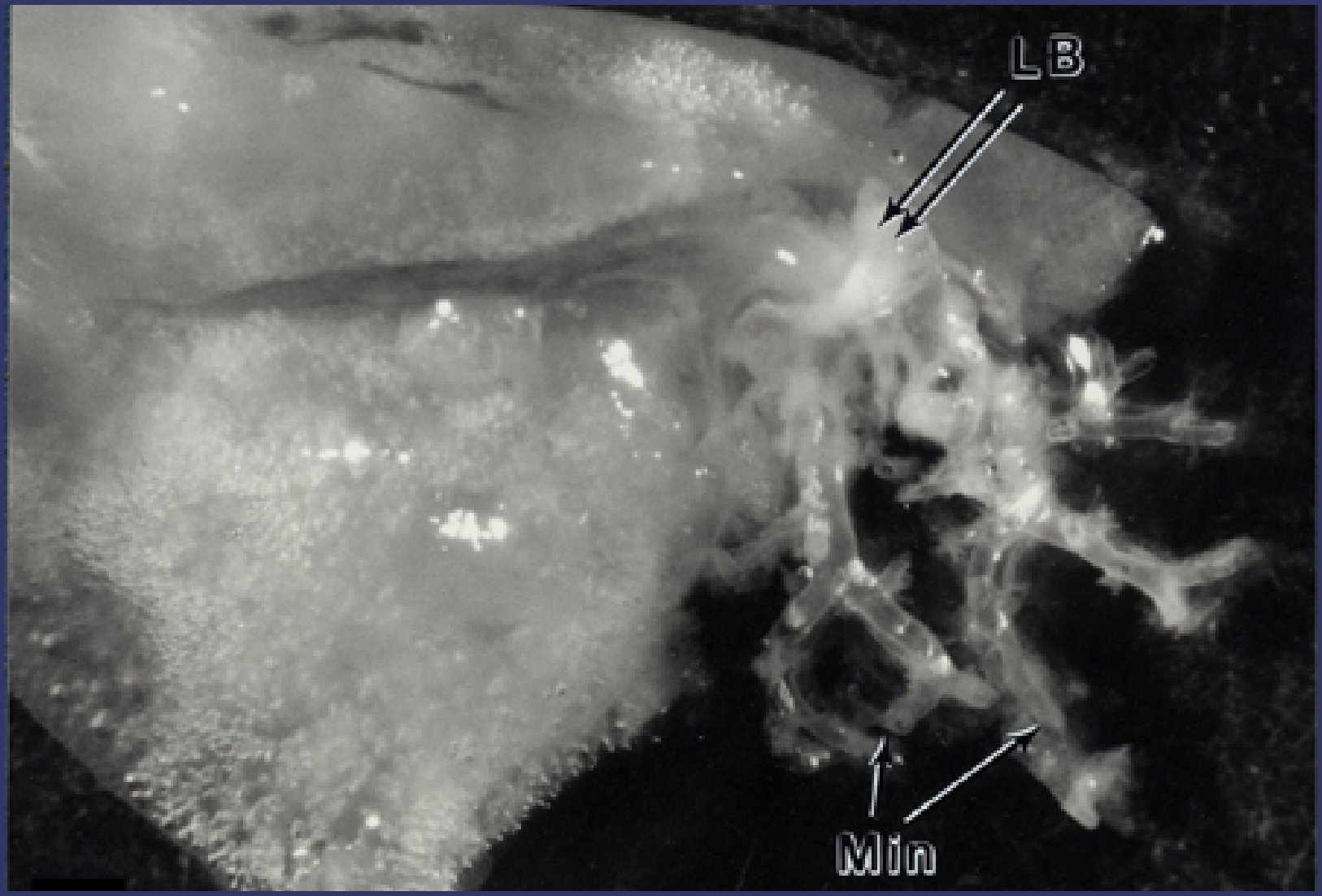
Naphthalene Metabolism



Species Comparisons of Rates of Naphthalene Metabolism in Whole Lung Microsomal Incubations-

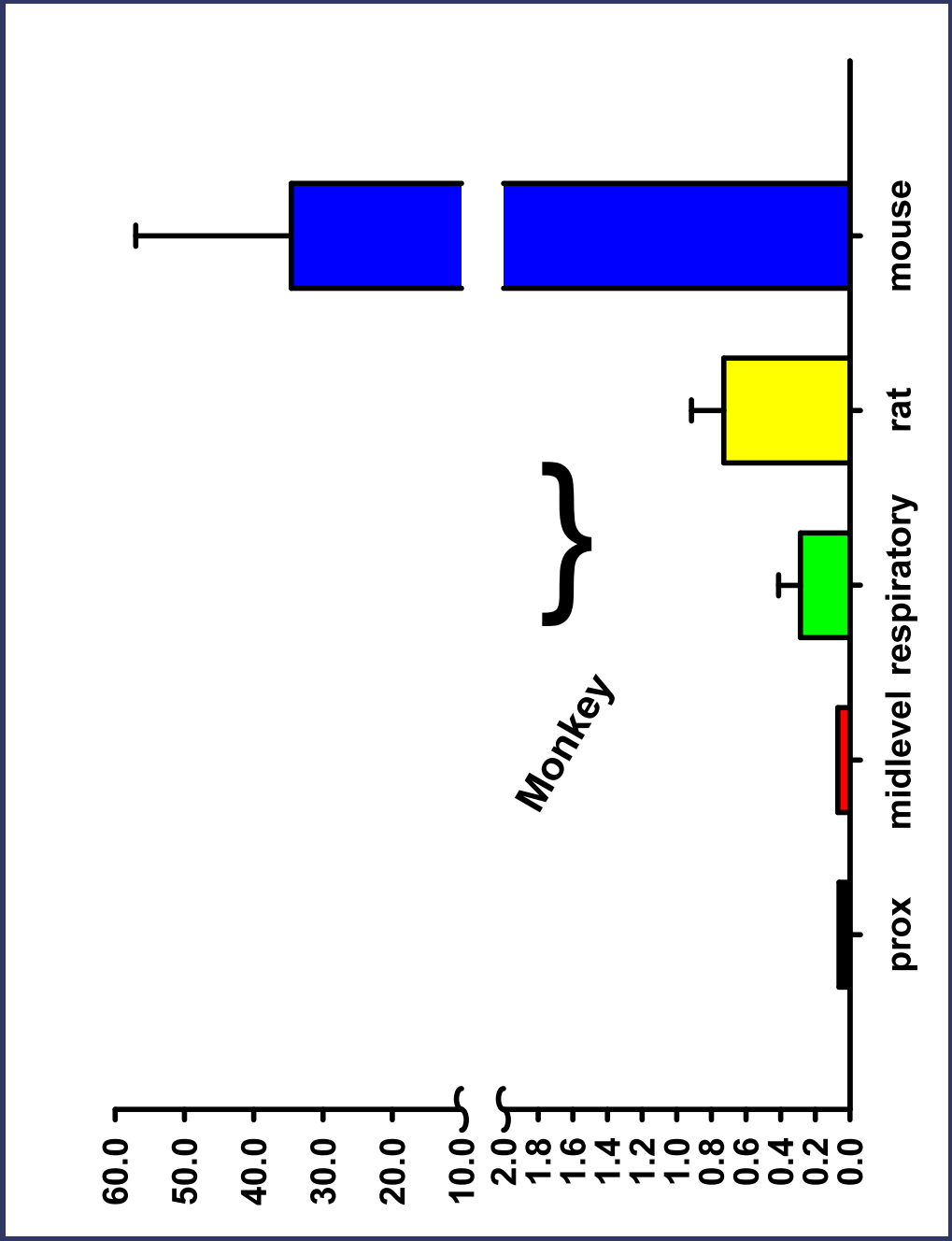


Airway microdissection



Plopper et al 1991; Van Winkle et al 1996

Total Metabolites



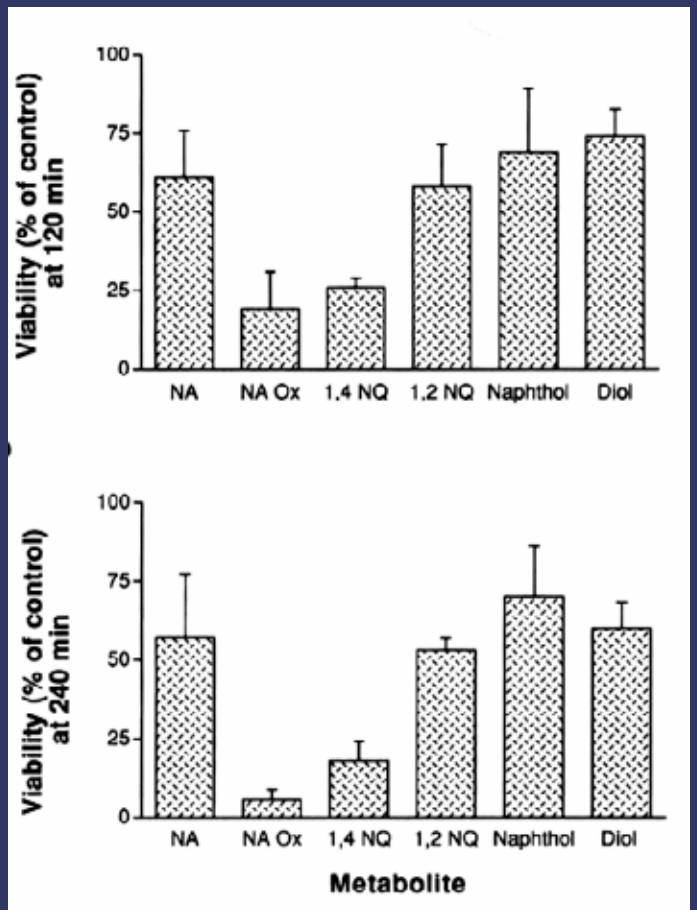
Which Metabolites are Toxic?

Evidence that the Epoxide causes Toxicity

naphthalene → Naphthalene oxide → naphthol → naphthoquinone

Toxicity of naphthalene and Metabolites in isolated mouse Clara Cells

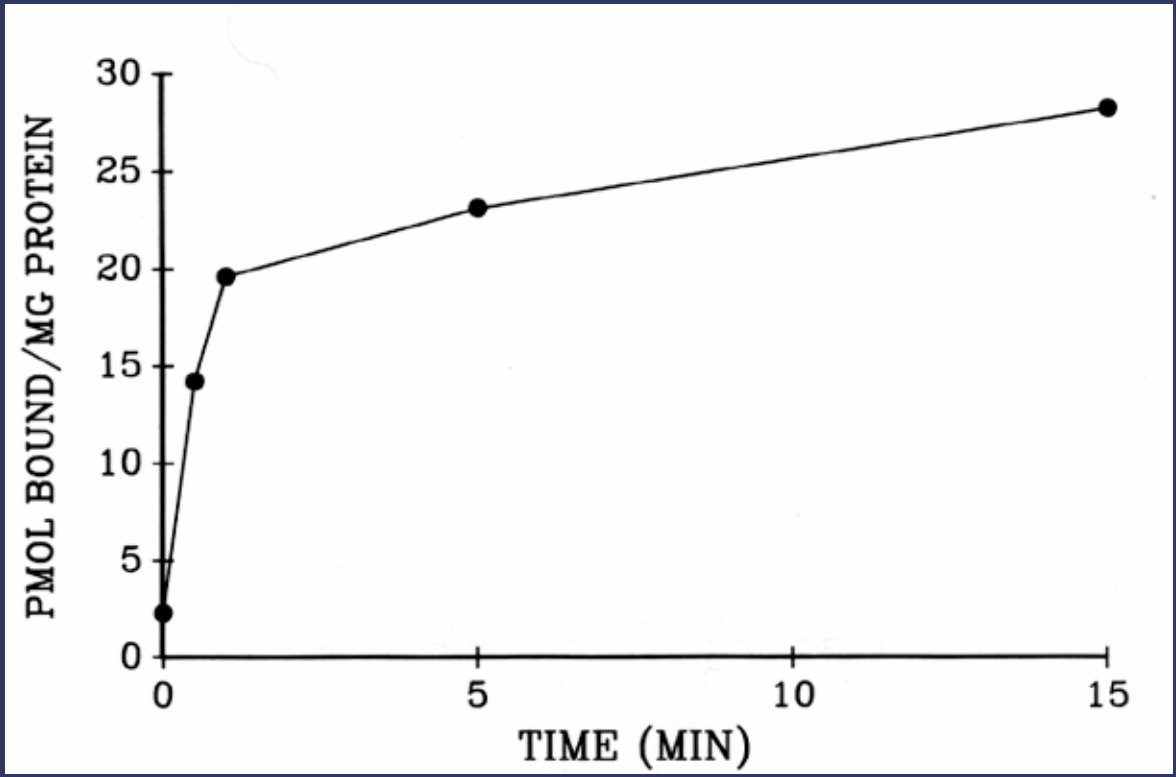
- May not reflect local concentrations
- Quinones may not have crossed membrane



Data from Chichester et al., 1994

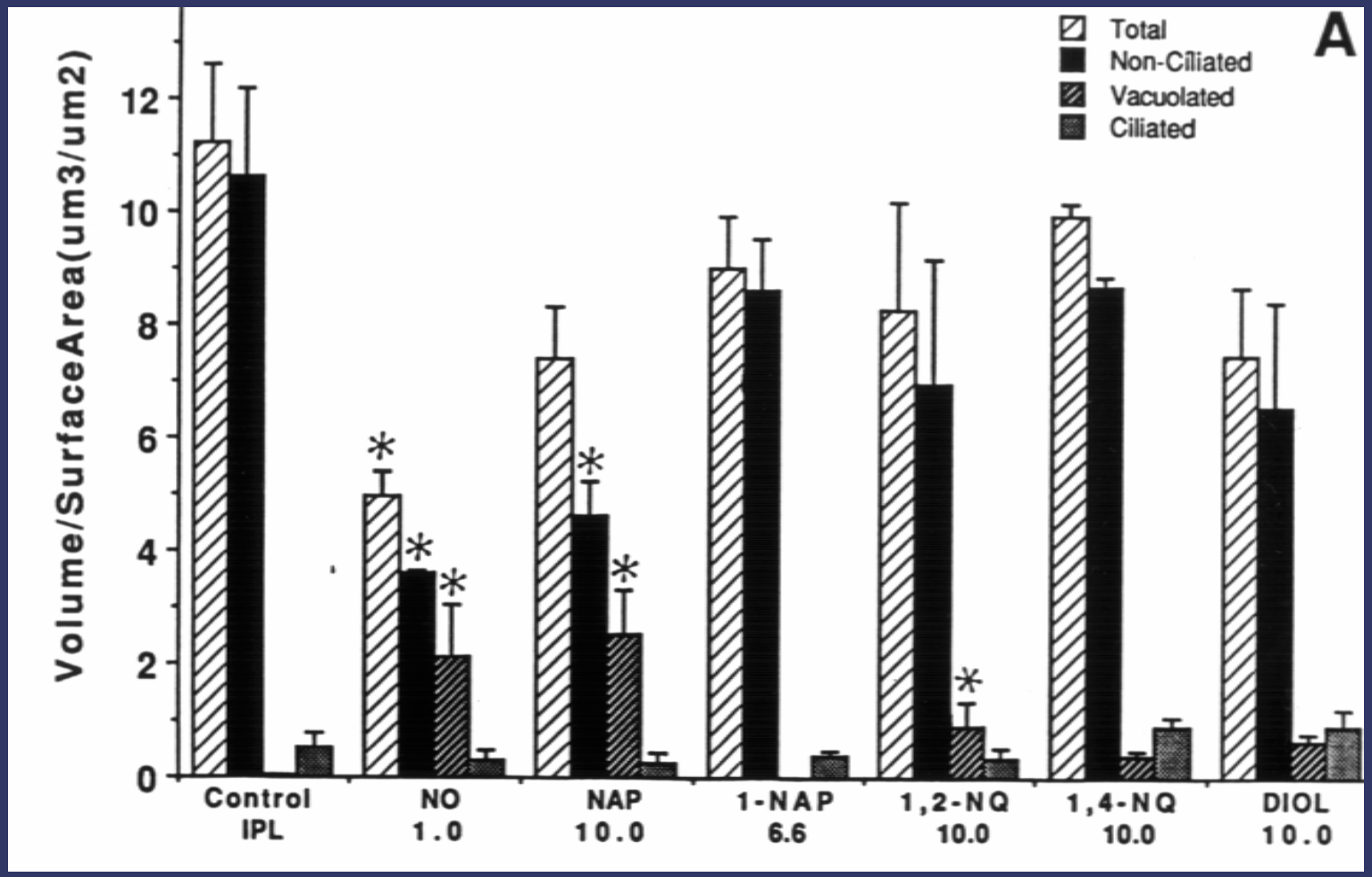
Covalent Binding of Radioactivity from ^{3}H-Naphthalene Oxide is rapid

naphthalene → naphthalene oxide → naphthol → naphthoquinone



Buonarati et al., 1989

Morphometric alterations in the airway epithelium of the perfused mouse lung in response to naphthalene or metabolites



Kanekal et al., 1991. Concentrations were infused over 60 min followed by 4 hrs with Waymouth's medium

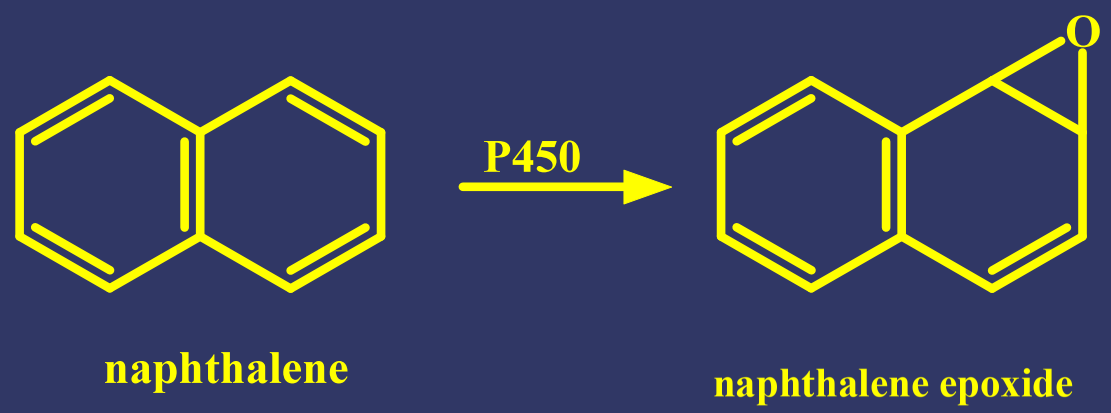
Summary

- In a variety of model systems (perfused lung, hepatocytes, Clara cells) naphthalene, naphthalene epoxide and naphthoquinones are cytotoxic
- Naphthalene oxide is a more potent toxicant in Clara cells and isolated perfused lung than are the quinones.
- There is no lag in the binding of radioactivity from ^{3}H-naphthalene oxide suggesting that the epoxide may be the key step in this process

Mouse/rat/human/primate differences may be related to:

- Differences in the catalytic activity of P450 2F
- Differences in the amounts of protein expressed in the airways
- Differences in the amounts of P450/cell (hot spots in the lung)
- Contribution of other P450

Importance of Kinetics of Metabolism



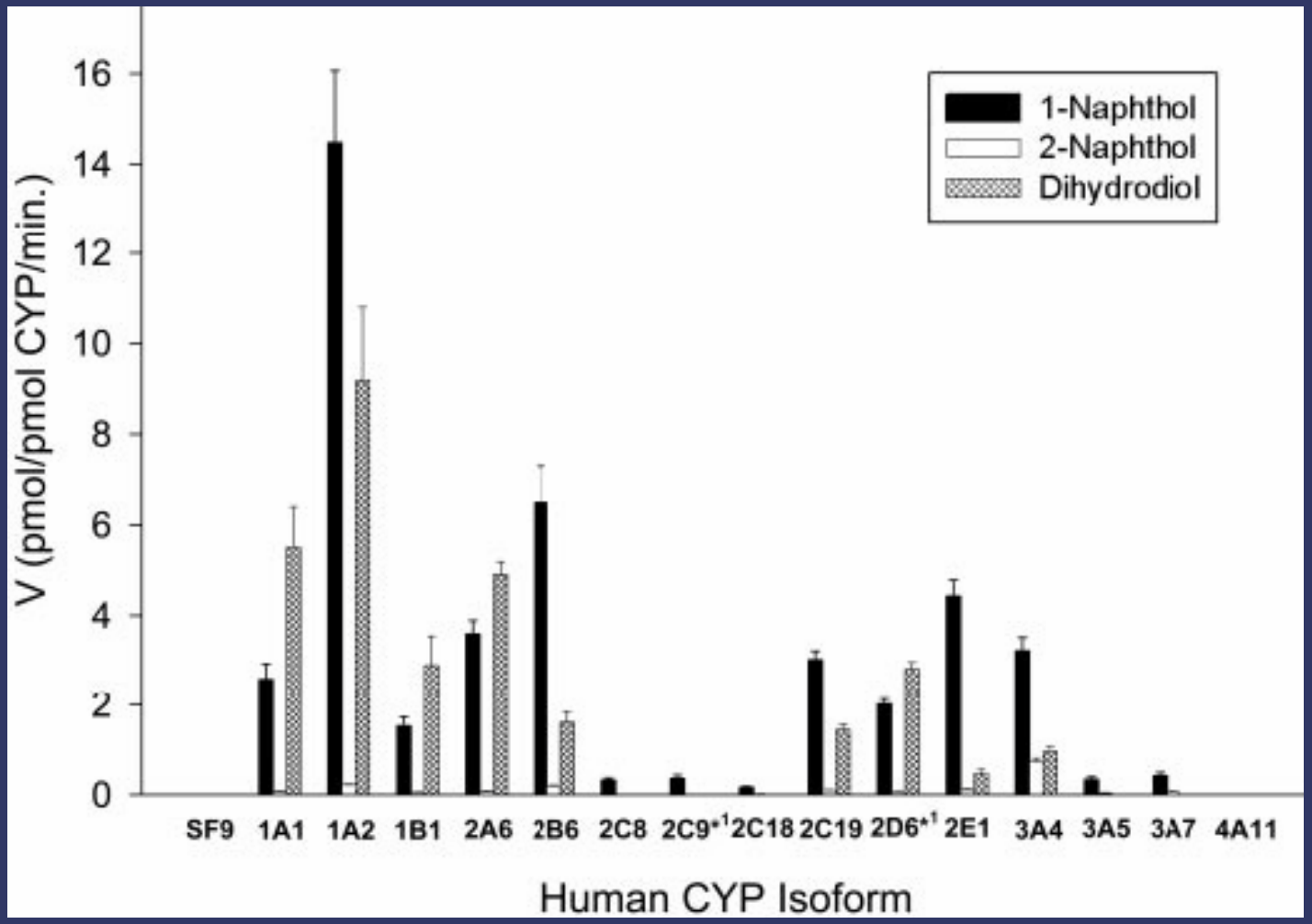
- Without conversion to the intermediate epoxide, naphthalene is toxicologically inert
- Overall P450 levels in primate lung are low-therefore high catalytic efficiency is essential if enzyme is to be important
- Amounts of catalytically active protein present per cell are important

Comparison of Recombinant P450s from Mice, Rats and Monkeys

Species/ CYP	Sequence similarity to human	K_M	V_{max}
Mouse CYP2F2	80	3 μM	104 min^{-1}
Rat CYP2F4	81	3 μM	107 min^{-1}
Monkey CYP2F5	97	No detectable P450	

Shultz et al., 1999; 2001; Baldwin et al, 2005

Comparison of catalytic activities of human recombinant P450 proteins



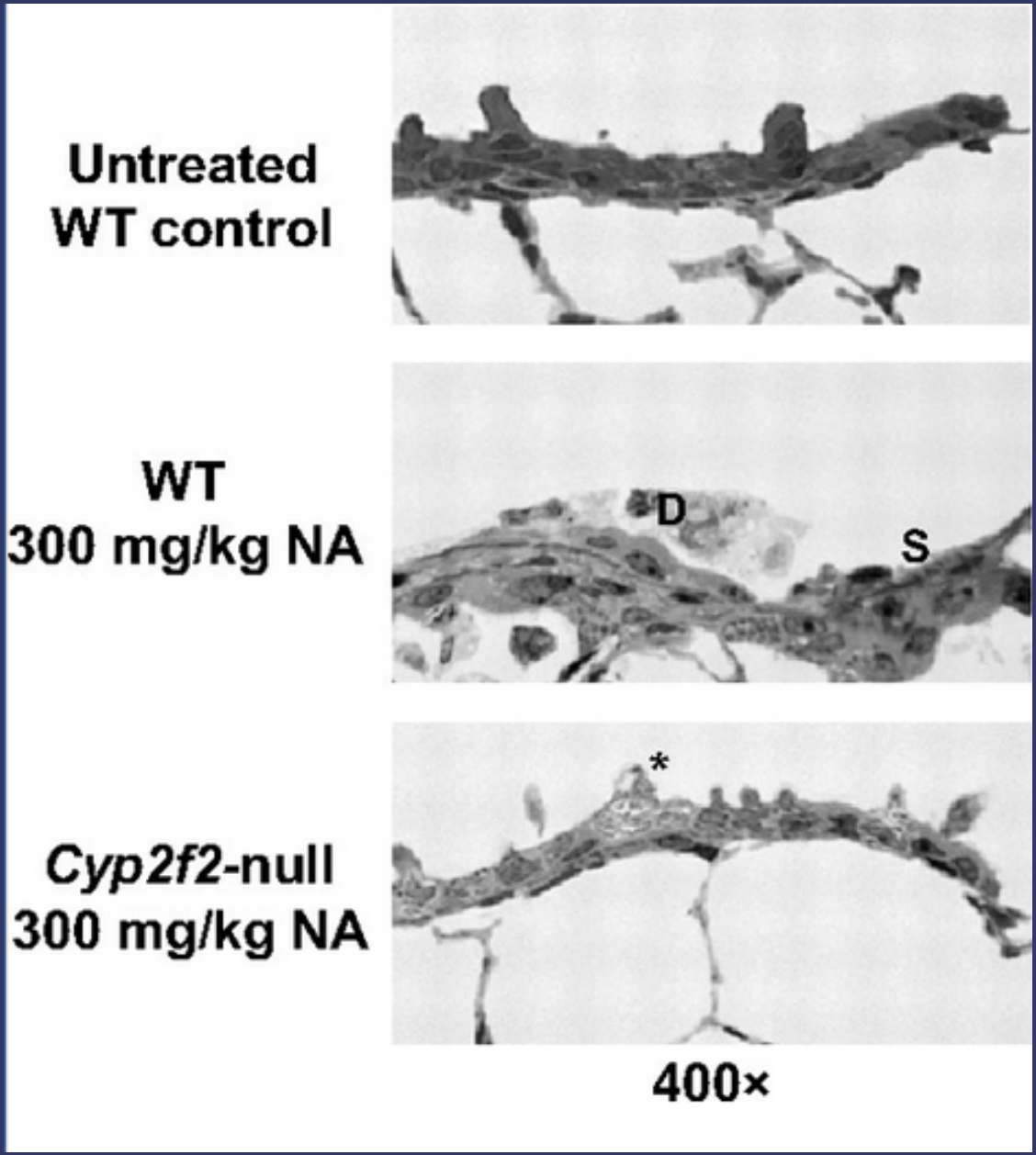
Cho, Rose, Hodgson: Drug Metab. Dispo, 2006

Comparison of kinetics of naphthalene metabolism in recombinant human P450s

P450 isoform	V_{max} pmoles/ pmoles/min	K_m µM
1A1	9.1	111
1A2	35.8	73
2B6	20.2	58.6
2E1	8.4	10.1
3A4	8.1	60.7
2F2 (mouse)	107	3

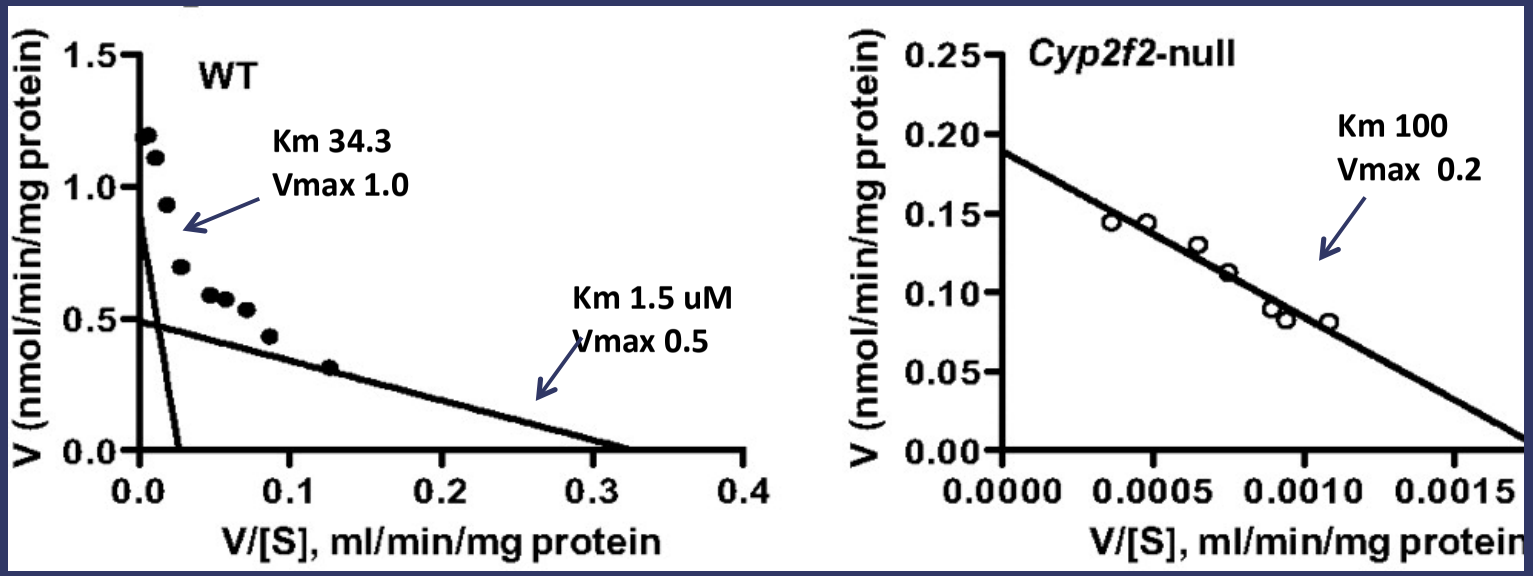
Cho, Rose and Hodgson, DMD, 2006

CYP2F2 Knockout mice


Untreated
WT control
WT
300 mg/kg NA
D
S
*
Cyp2f2-null
300 mg/kg NA
400×

Li et al 2011

Eadie-Hofstee plots for in vitro NA-GSH formation by lung microsomes from WT and Cyp2F2-null



***Cyp2f2*-null mice, compared with WT mice, have much (up to ~160-fold) lower catalytic efficiency toward NA.**

Summary of Metabolite Toxicity

- In a variety of model systems (perfused lung, hepatocytes, Clara cells) naphthalene, naphthalene epoxide and naphthoquinones are cytotoxic
- Naphthalene oxide is a more potent toxicant in Clara cells and isolated perfused lung than are the quinones
- There is no lag in the binding of radioactivity from ^{3}H-naphthalene oxide suggesting that the epoxide is a key step in this process

Summary

- CYP2F2 (mouse) and CYP 2F4 (rat) show high catalytic turnover of naphthalene (>100 min^{-1})
- Mouse – rat differences in rates of airway metabolism are due to substantial differences of the amounts of CYP2F protein present
- CYP2F1 (human) metabolizes naphthalene poorly.
- Mouse CYP2F has much higher catalytic activities (higher V_{max} and lower K_m) than any of the recombinant human proteins tested.
- Cyp2F2 null mice have a 160 fold decrease in NA metabolism

Genotoxicity?

Short term mutagenesis assays for naphthalene are mostly negative

No in vivo genotoxicity assays that have either positive or negative results for lung.

1, 2 NQ causes reverse mutations in S typhimurium testing

1, 2 NQ and 1, 4 NQ cause sister chromatid exchanges in vitro.

1, 2 NQ forms DNA adducts and causes DNA strand scission.

Recent studies (Recio et al 2013) have shown that NA increases micronucleus formation in human lymphocytes in vitro with an added S9 fraction. Further chromosomal changes in lymphocytes children have been correlated with exposure to naphthalene (Orjuela et al 2012)

However, GSH levels and lung cell detoxification capabilities are quite robust, so the relevance of these findings to lung tumors is unknown

Conclusion re: mouse tumors

P450 activation is required for toxicity

Sites of toxicity in the lung correlate to sites of tumor formation

Cyp2F2 in the mouse metabolizes NA extremely well

Knockout of CYP2F2 diminishes toxicity

It is likely that Cyp2F2 metabolism of NA contributes to tumor formation in the mouse

Acknowledgements

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Van Winkle Lab

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CARB- Calif. Air Resources Board
API- American Petroleum Institute

environmental • failure analysis & prevention • health • technology development

Exponent®

Consideration of Mutagenic MoA for Lung Tumors Induced by Styrene, Ethyl Benzene, and Naphthalene

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Senior Managing Scientist
Exponent®
Midland, MI

17 September 2013

Workshop On
Modes of Action of Lung Tumors in Mice From Exposure to Styrene, Ethylbenzene & Naphthalene

Foreword

- ***"The determination that a chemical carcinogen can induce mutation in one of a number of mutation assays is not sufficient to conclude that it causes specific tumors by a mutagenic MOA or that mutation is the only key event in the pathway to tumor induction"*** **(EPA Draft MMOA Framework, 2007*).***
- **DNA Adduct(s) ≠ Mutations. They are markers of exposure.**

TABLE 1
Steady-State Amounts of Selected endogenous DNA Damage

Endogenous DNA lesions	Number per cell
Abasic sites	30,000
N7-(2-Hydroxyethyl)guanine (7HEG)	3000
8-Hydroxyguanine	2400
7-(2-Oxoethyl)guanine	1500
Formaldehyde adducts	960
Acrolein-deoxyguanosine	120
Malondialdehyde-deoxyguanosine	60
N^2,3-Ethenoguanine (εG)	36
1,N^2-Ethenodeoxyguanosine (1,N^2-εdG)	30
1,N^6-Ethenodeoxyadenosine (1,N^6-εdA)	12
Total	38,118

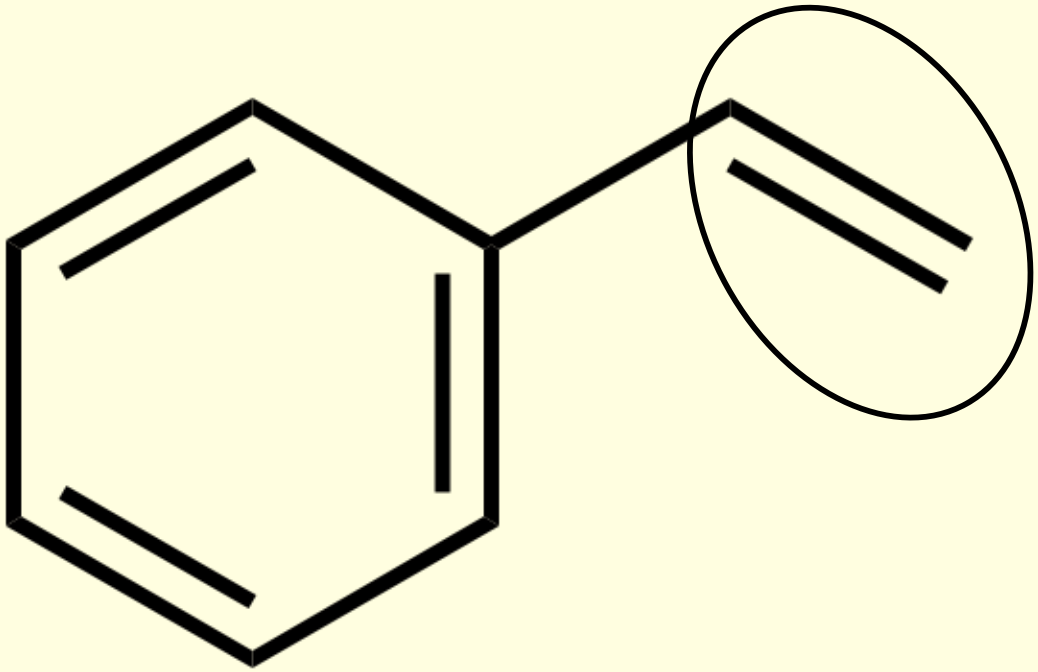
Swenberg *et al.*, 2011

Environmental and Molecular Mutagenesis 54:8–18 (2013)

Quantitative Approaches for Assessing Dose–Response Relationships in Genetic Toxicology Studies

B.B. Gollapudi,[1] G.E. Johnson,[2] L.G. Hernandez,[3] L.H. Pottenger,[1] K.L. Dearfield,[4] A.M. Jeffrey,[5] E. Julien,[6] J.H. Kim,[7*] D.P. Lovell,[8] J.T. MacGregor,[9] M.M. Moore,[10] J. van Benthem,[3] P.A. White,[11] E. Zeiger,[12] and V. Thybaud[13]

Styrene



Hypothesized Mutagenic Mode of Action for Styrene

Styrene is activated to reactive metabolites by mouse lung Clara cells. Some of these metabolites induce cytotoxicity while other(s) produce a very low frequency of DNA adducts. In the presence of regenerative cell proliferation, some of the DNA adducts will be fixed into mutations. Sustained cell proliferation leads to amplification of both spontaneous and induced mutations in critical genes, hyperplasia and neoplasia. However, this is a species-specific phenomenon that is not applicable to humans due to quantitative differences in the activity of the cytochrome P450 orthologs responsible for the production of the cytotoxic metabolite(s).

Key Events in the Mutagenic MoA

1. Metabolism of styrene to reactive metabolite(s) leading to DNA adduct formation.
2. Ring oxidized metabolites of styrene induce cytotoxicity and compensatory cell proliferation.
3. Cell proliferation leads to fixation of DNA adducts into mutations.
4. Sustained cell proliferation leading to amplification of induced/ spontaneous mutations and hyperplasia.
5. Progression of hyperplasia into adenomas and carcinomas.

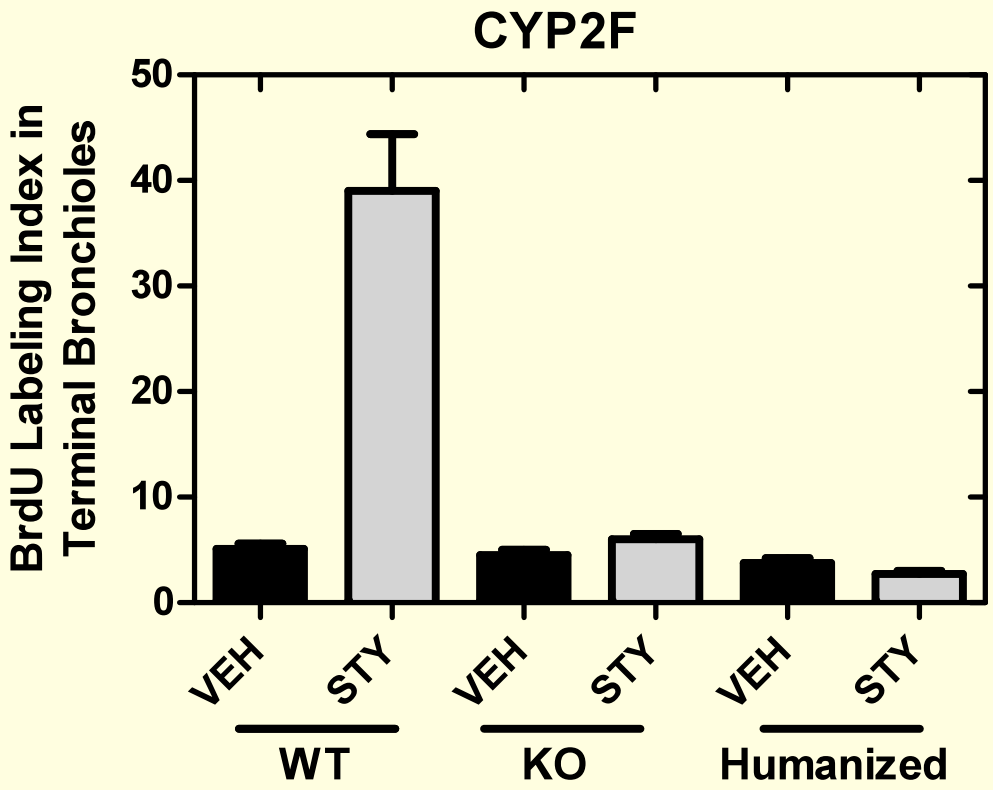
KE 1: Metabolism of styrene to reactive metabolites leading to DNA adduct formation

- Styrene (S) metabolized to styrene oxide by Clara cells
- S induces low levels of DNA adducts in mouse lung.
- Extremely weak covalent binding index for lung: < 1
- N7 and O6 guanine adducts are most frequent.

WoE: SO from S efficiently detoxified in lung tissue

KE 2: Cytotoxicity from ring oxidized metabolites

Cruzan et al., 2012 & 2013



KE 3: Fixation of DNA Adducts into Mutations - Summary of Literature (modified from NTP)

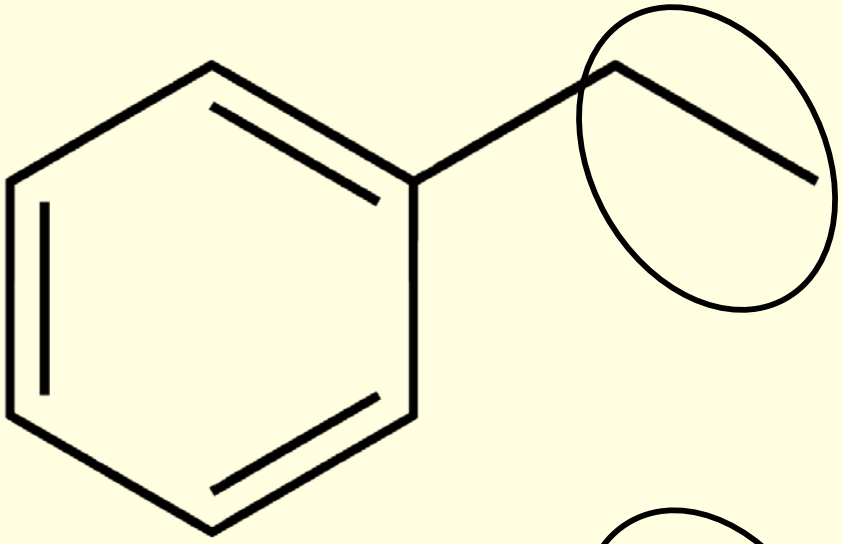
Effect	In vitro	In vivo	
		Rodents	Humans
DNA adducts	NSR[a]	+	+
DNA damage[b]	+[1]	(+)	+
Mutations		NS	(+)
bacteria	–		
lower eukaryotes	±		
mammalian cells	±		
Chromosomal aberrations	+	–	(+)
Sister chromatid exchange	(+)	+	±
Micronuclei	+	–	±
Aneuploidy or polyploidy	±	+[1]	–

+ = predominantly positive results; +[1] = positive results in the only study reviewed; ± = similar number of positive and negative results or multiple studies with positive and negative results; (+) = weakly positive results; – = predominantly negative results.
NA = not applicable; NSR = no studies reviewed.
[a] Studies with styrene-7,8-oxide did cause DNA adducts *in vitro*.
[b] Includes alkali-labile sites and single-strand breaks.

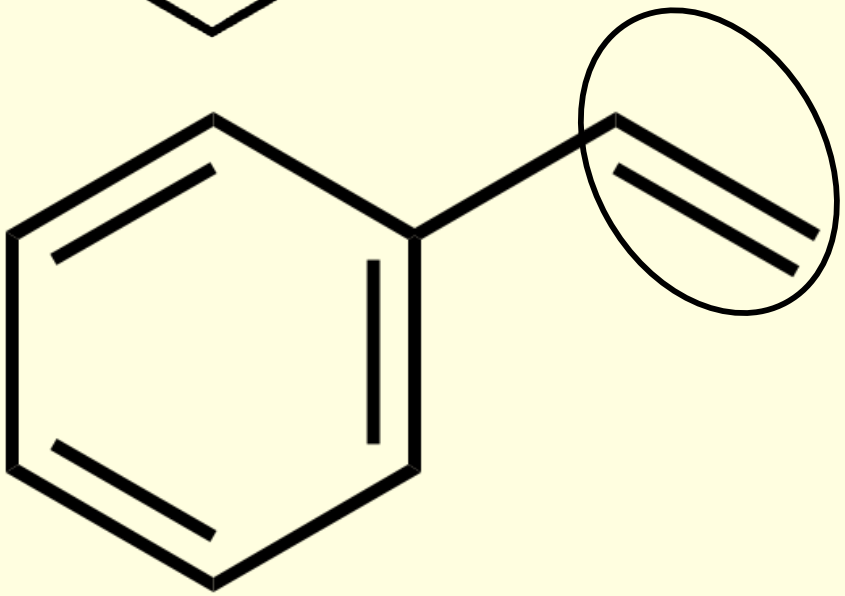
WoE: weak mutagenicity *in vivo;* no ↑ increase in CA & questionable ↑ in SCE in tumor target tissue

Conclusions for Styrene

- **Weight of evidence suggests that S is not likely an *in vivo* mutagen, including in tumor target tissue.**
- **Mutagenicity not likely responsible for lung tumors**
  - Efficient detoxification of SO
  - SO itself is not a lung tumorigen
- **Confidence in this assessment can be further enhanced by evaluating dose response and temporality for mutations induced in a neutral gene (e.g., cII) and an oncogene (e.g., K-ras) in mouse lung.**



Ethylbenzene



Styrene

Mutagenicity of Ethylbenzene

(modified from Henderson *et al*., 2007)

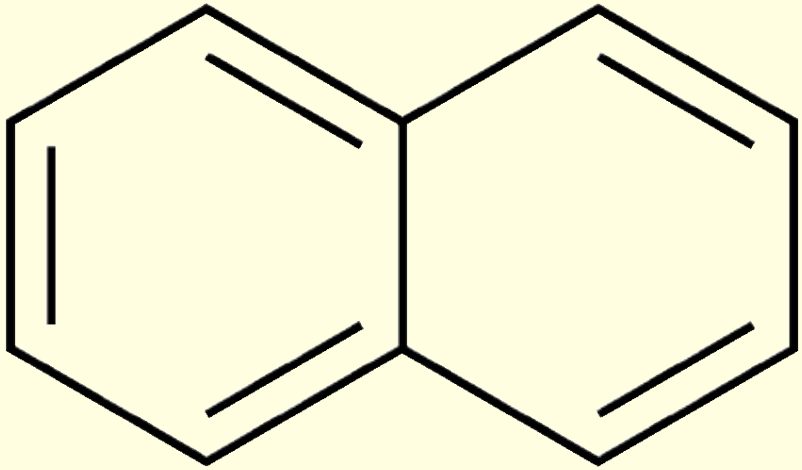
Table 1
Summary of genetic toxicology testing of ethylbenzene evaluated

Endpoint	Outcome
Bacteria and yeast	
Bacterial reverse mutation	—
	—
	—
	—
Gene conversion and mutation in yeast	—
Gene conversion in yeast	—
Mammalian cell assays	
Mouse lymphoma gene mutation assay	(+)
SCEs in cultured human lymphocytes	—
	—
Chromosome aberrations CHO cells	—
	—
Micronucler in SHE cells	+
In vivo assays	
Mouse bone marrow micronucleus (intraperitoneal administration)	—
Mouse peripheral blood micronucleus	—
Mouse liver UDS assay	—

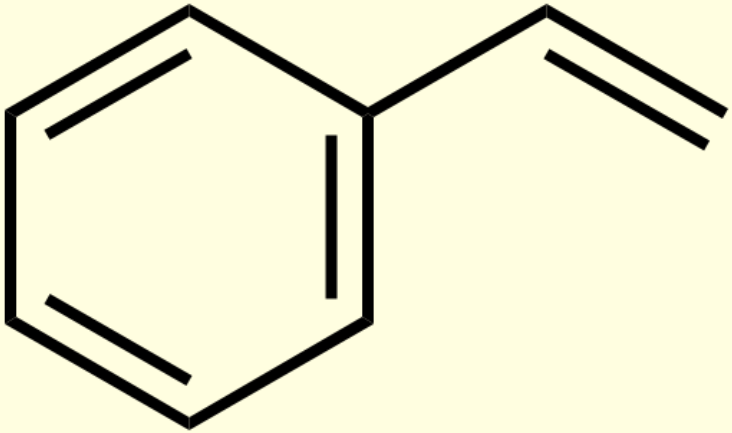
Oxidative DNA Damage *In Vitro* +

Ethylbenzene: Mutagenic MoA

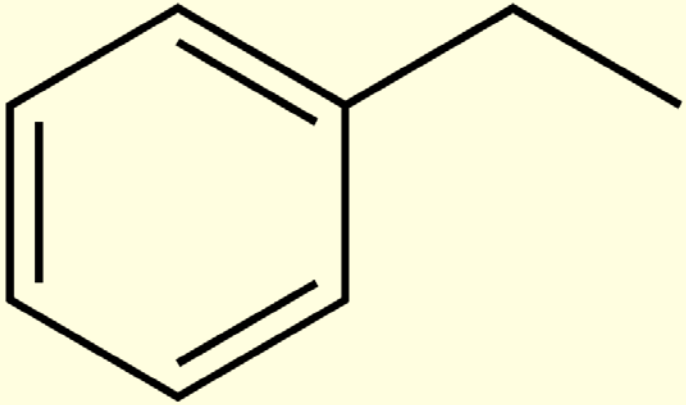
- **WoE suggests that ethylbenzene is not a mutagen.**
- **It is unlikely that mutagenicity as an early key event in the etiology of mouse lung tumors.**
- **The confidence in this assessment is very high.**
- **Lack of mutagenicity data in tumor target tissue is a potential data gap,**
  - not considered to be critical given the WoE based on available mutagenicity data.



Naphthalene



Styrene



Ethylbenzene

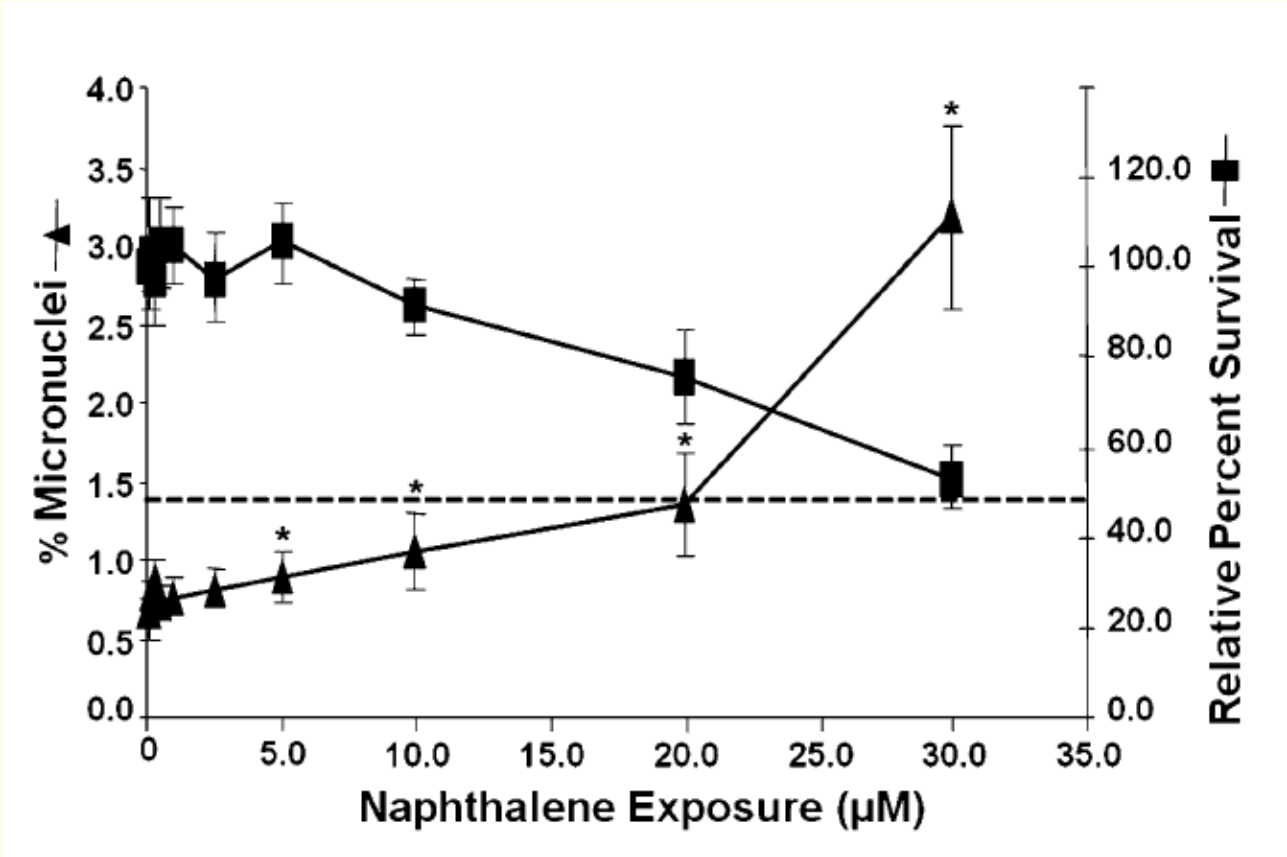
Reviews on Genotoxicity on Naphthalene

- Schreiner (2003): "*Results of standard genetic toxicity assays suggest that naphthalene is not likely to be genotoxic in vivo.*"
- EU Risk Assessment (2003): "*Overall, the balance of evidence indicates that naphthalene is not genotoxic.*"
- Flowers *et al.* (2006): "*Naphthalene has produced negative results in most genotoxicity and mutagenicity tests; however, the role of genotoxicity in the MOA has not been characterized in target tissues*"
- Brusick (2008): "……. *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..*"

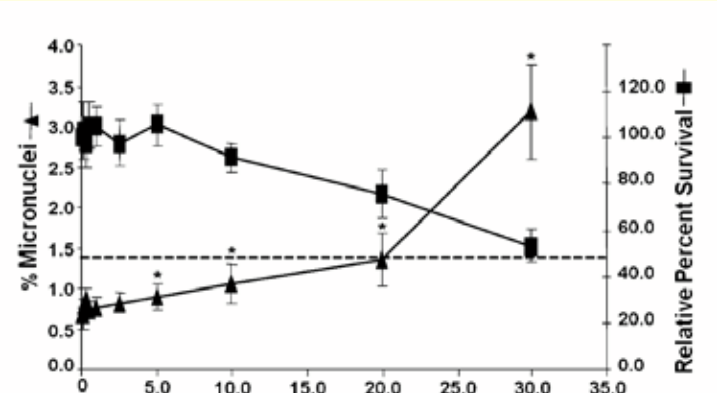
Naphthalene Genotoxicity Highlights

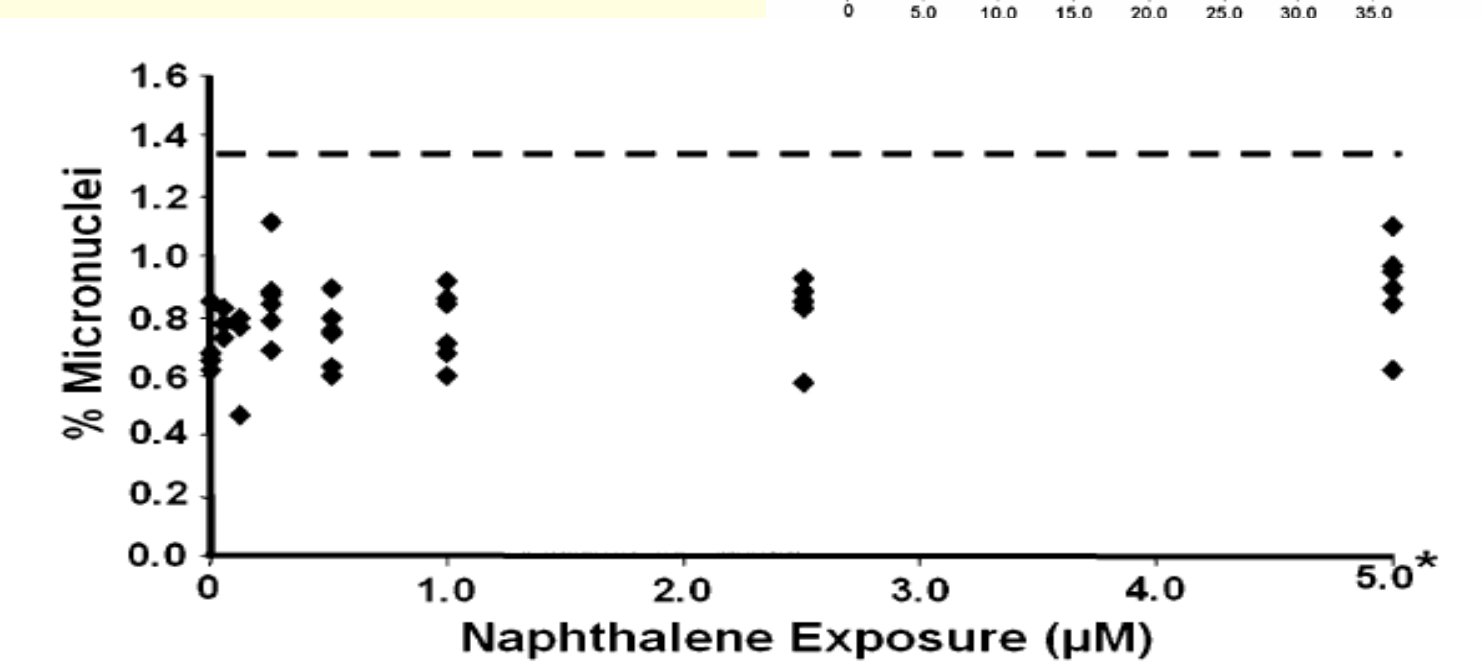
- **No activity in gene mutation assays with bacterial and mammalian cell cultures.**
- **Negative results from *in vitro* and *in vivo* UDS.**
- **Non-genotoxic in rodent bone marrow micronucleus tests.**
- **Low or no activity in SCE tests**
- **Some evidence for DNA reactivity of metabolites,**
  - *in vitro* (naked DNA), *ex vivo* (nasal epithelium), and *in vivo* (skin, non-tumor target),
  - no *in vivo* data following systemic exposure
- **Clastogenic to cells in culture.**

Naphthalene (+S9): Dose Response for MN in TK6 Cells (Recio *et al*., 2012)

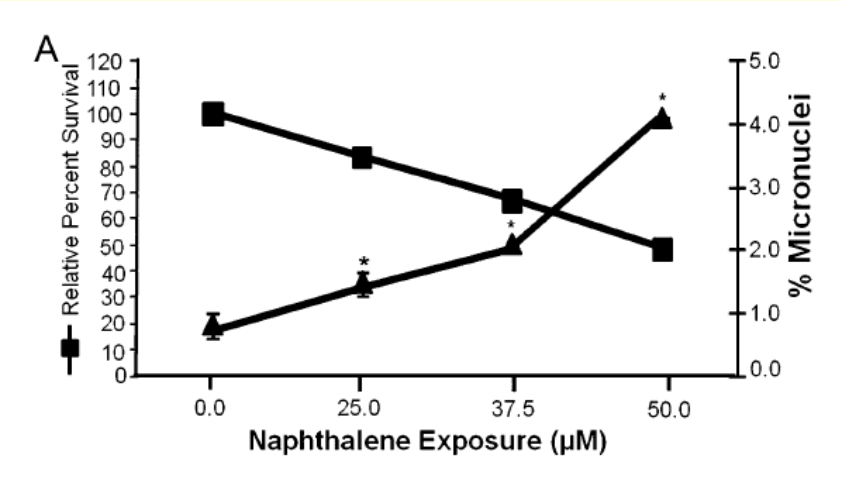


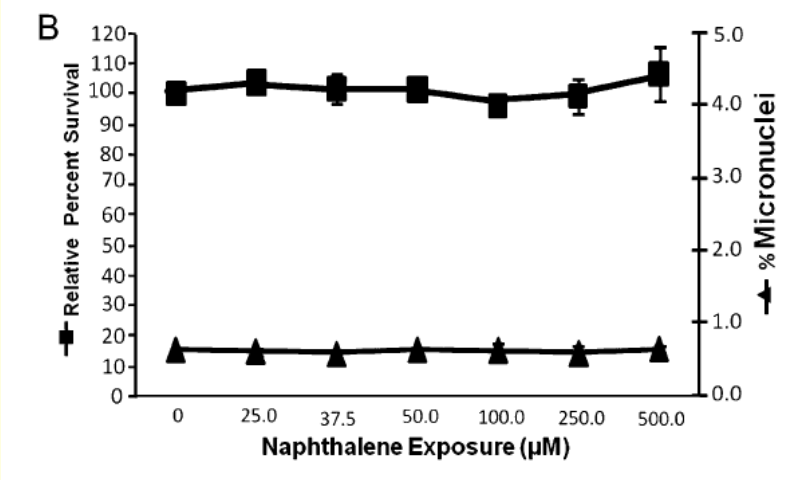
Naphthalene (+S9): Dose Response for MN in TK6 Cells (Recio *et al*., 2012)





Naphthalene (+S9): Dose Response for MN in without (A) and with (B) 5 mM GSH Supplementation (Recio *et al*., 2012)





Conclusions for Naphthalene

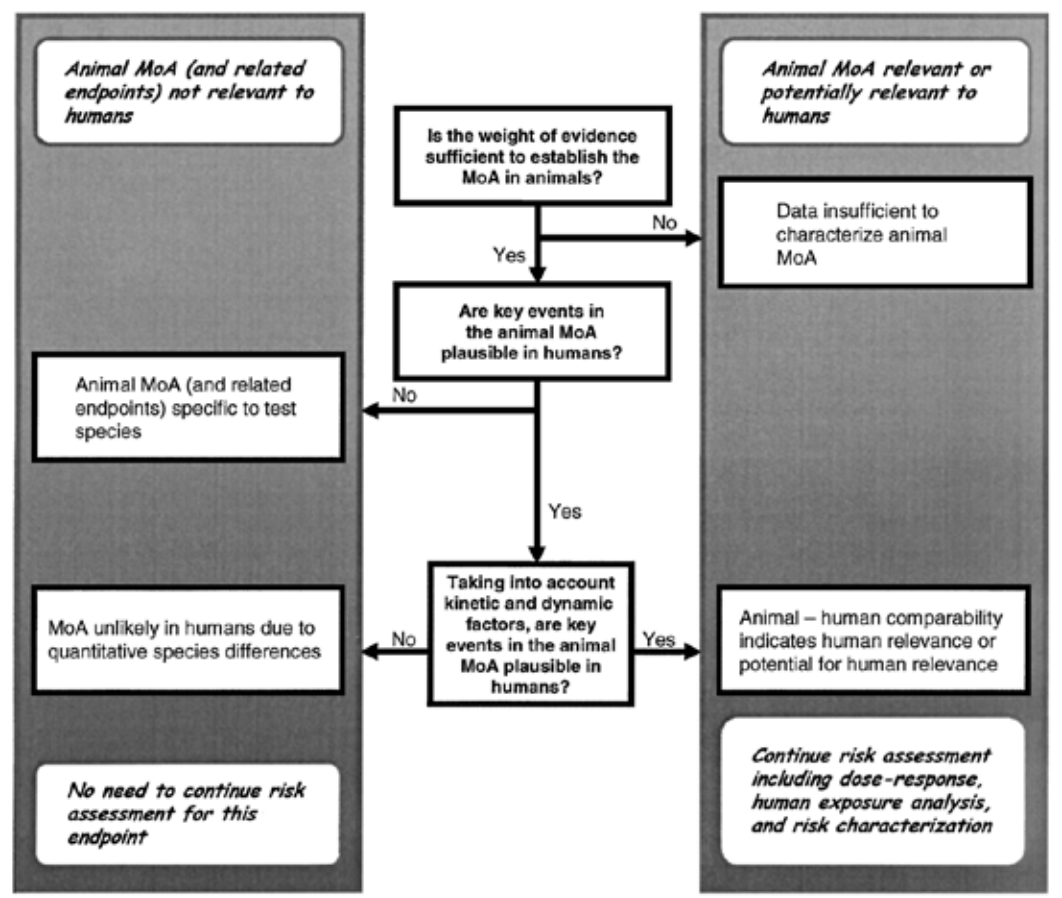
- **Weight of evidence suggests that naphthalene is not an in vivo mutagen.**
- **Mutagenicity is not an initial key event in the etiology of naphthalene-induced lung tumors.**
- **Confidence in this assessment is moderate.**
  - can be enhanced by evaluating dose response and temporality for DNA adducts and mutations in a neutral gene (e.g., cII) and an oncogene (e.g., K-ras) in mouse lung.

Human Relevance: Analysis of the MoA Data using the Human Relevance Framework

James S. Bus PhD, DABT, ATS
Exponent, Inc.

"Workshop to Assess the Modes of Action of Lung Tumors in Mice"
Cincinnati, OH,
September 17, 2013

Human Relevance Framework: Key Questions



From: Cohen *et al.*, Crit. Rev. Toxicol. 33: 581-589, 2003

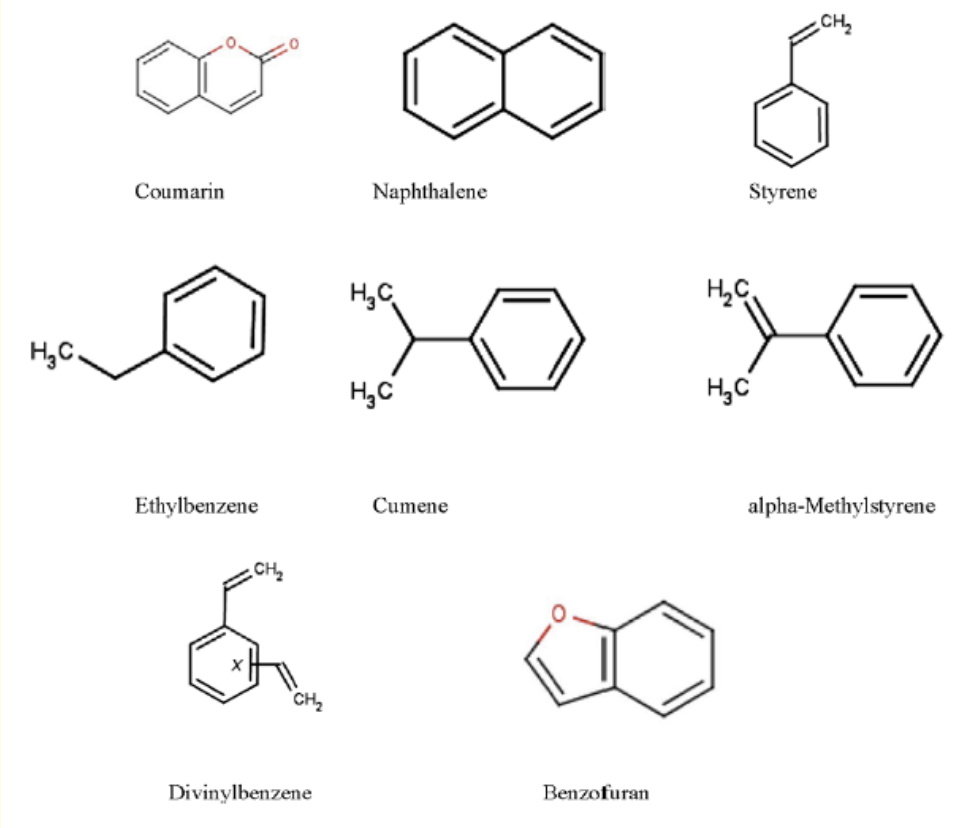
Answering Framework Questions: A Structured Approach

- Postulated MoA
- Key events
- Dose-response and temporal associations
- Strength, consistency, and specificity of association of tumor response with key events
  - Weight of evidence linking key event, precursor lesions, and tumor response
- Biological plausibility and coherence
- Alternative MoA(s)
- Uncertainties, inconsistencies, and data gaps
- Assessment of MoA – level of confidence

Mouse Lung Tumors Mediated through CYP2F2 Mouse Lung Specific Metabolism

- **Most MoA analyses are chemical specific**
- **Accumulating body of evidence *from multiple chemicals* mutually supportive of CYP2F2-mediated mouse lung specific toxicity and tumorigenicity**
- **Hypothesized MoA reviewed in:**
  - **Cruzan *et al.*, Mouse specific lung tumors from CYP2F2-mediated cytotoxic metabolism: An endpoint/toxic response where data from multiple chemicals converge to support a mode of action. Regul. Toxicol. Pharmacol. 55: 205-218, 2009.**

Chemicals Potentially in MoA Class



From: Cruzan *et al., 2009*

Mouse *vs* Rat Lung Tumor Development

Examples of chemicals that cause mouse, but not rat, lung bronchiolo-alveolar adenomas or carcinomas.

Chemical	Exposure route and dose (strain) to mice	Lung tumors in male mice	Lung tumors in female mice
Coumarin	Oral: 0, 50, 100, 200 mg/k/day (B6C3F1)	14/50; 8/50; 14/50; 25/50	2/51; 5/49; 7/49; 27/51
Naphthalene	Inhalation: 0, 10, 30 ppm (B6C3F1)	7/70; 17/69; 31/135	5/69; 2/65; 28/135
Styrene (ethenylbenzene)	Oral: 0, 150, 300 mg/kg/day (B6C3F1)	0/20; 6/50; 9/50	0/20; 1/50; 3/50
	Inhalation: 0, 20, 40, 80, 160 ppm (CD-1)	17/50; 24/50; 36/50; 30/50; 36/50	6/50; 16/50; 17/50; 11/50; 27/50
Ethylbenzene	Inhalation: 0, 75, 225, 750 ppm (B6C3F1)	7/50; 10/50; 15/50; 19/50	4/50; 6/50; 5/50; 8/50
α-Methylstyrene (isopropenylbenzene)	Inhalation: 0, 100, 300, 600 ppm (B6C3F1)	13/50; 6/50; 9/50; 9/50	2/50; 7/50; 5/50; 6/50
Cumene (isopropylbenzene)	Inhalation: 0, 250, 500, 1000 ppm (B6C3F1)	19/50; 38/50; 42/50; 43/50	4/50; 31/50; 42/50; 46/50
Divinylbenzene	Inhalation: 0, 10,30, 100 ppm (B6C3F1)	16/50; 10/50; 8/50; 20/50	6/50; 12/50; 8/50; 13/50
Benzofuran	Oral: 0, 30, 60 mg/kg/day (B6C3F1)	10/50; 9/40; 19/50	2/50; 9/50; 14/50
	Exposure route and dose (strain) to rats	Lung tumors in male rats	Lung tumors in female rats
Coumarin	0, 25, 50, 100 mg/kg/day	1/50; 2/50; 0/50; 1/50	0/50; 0/50; 0/50; 1/50
Naphthalene	0,10, 30, 60 ppm	2/50; 3/50; 1/50; 0/50	1/50; 0/50; 0/50; 0/50
Styrene (ethenylbenzene)	Oral: 0, 500, 1000, 2000 mg/kg/day	1/40; 1/50; 1/50; 0/50	0/40; 1/50; 0/50; 0/50
	Inhalation: 0, 50, 200, 500, 1000 ppm	0/60; 0/60; 1/60; 0/60; 0/54	0/60; 0/60; 0/60; 0/60; 0/60
Ethylbenzene	0, 75, 250, 750 ppm	3/50; 1/50; 0/50; 1/50	1/50; 1/50; 1/50; 0/50
α-Methylstyrene (isopropenylbenzene)	0, 100, 300, 1000 ppm	1/50; 1/50; 0/50; 2/50	0/50; 0/50; 0/50; 1/50
Cumene (isopropylbenzene)	0, 250, 500, 1000 ppm	1/50; 2/50; 2/50; 450	1/50; 0/50; 1/50; 1/50
Divinylbenzene	0, 100, 200, 400 ppm	1/50; 1/50; 1/50; 1/50	0/50; 0/50; 0/50; 1/50
Benzofuran	0, 30, 60, 120 mg/kg/day	1/50; 2/50; 3/50; NT	0/50; NT 2/50; 3/50

Cruzan *et al.*, 2009

Hypothesized MoA (Cruzan *et al.*, 2009)

"Metabolism of several chemicals by CYP2F2 in terminal bronchiolar Clara cells in mice results in the localized generation of cytotoxic metabolites and subsequent reparative cell proliferation. These metabolites generally are hydroxylated-benzene-ring derivatives.

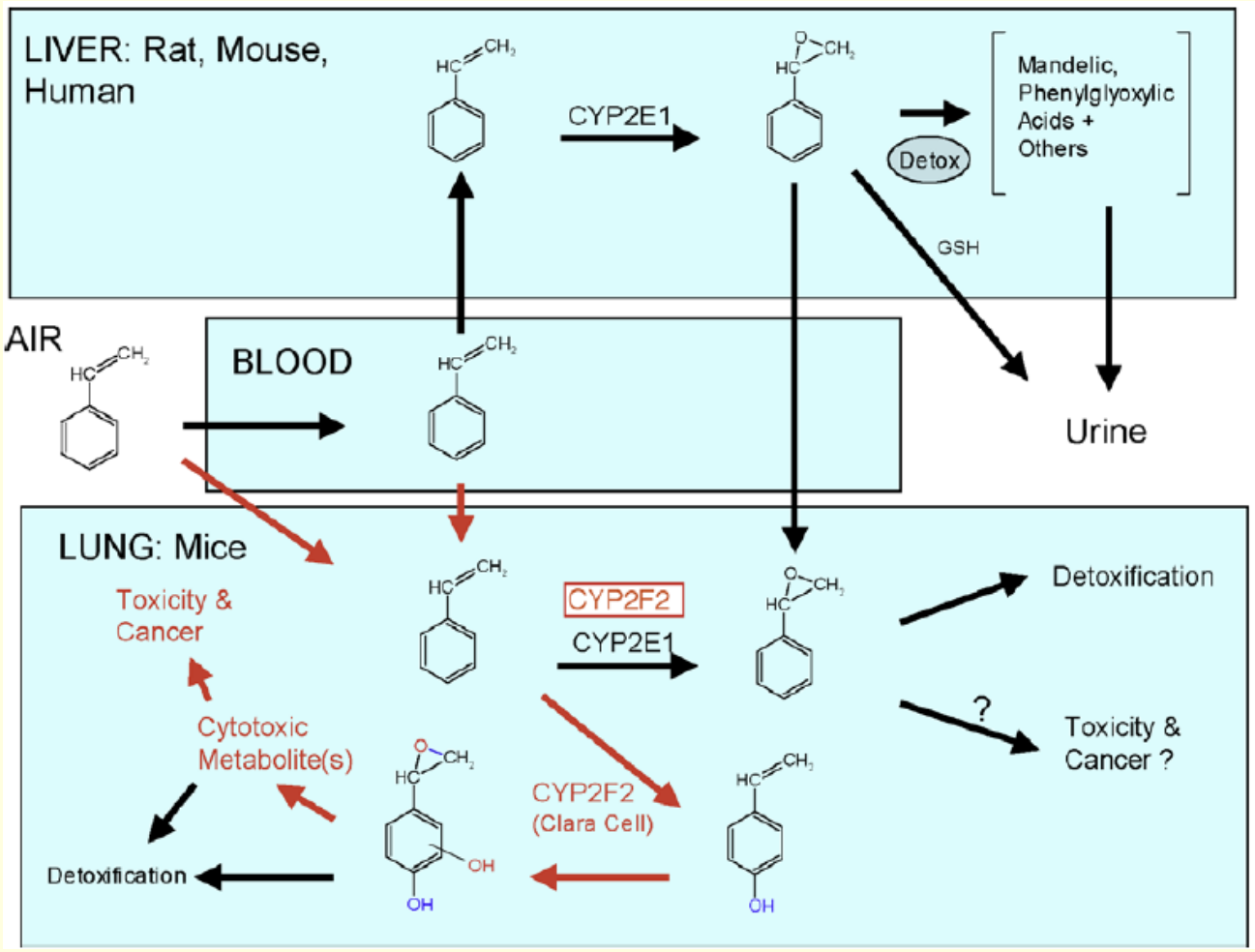
…On continued exposure, the increased cell replication leads to cellular crowding and then to hyperplasia in the terminal bronchioles. As the hyperplasia continues, it expands into the surrounding alveolar ducts. Some of these hyperplasias proceed to form adenomas in the mouse lung. Depending on the severity of the stress, a few of the adenomas may progress to carcinomas.

…The analogous CYP2F4 in rats may be as capable of forming these cytotoxic metabolites as mouse CYP2F2; however, rats have much lower levels of CYP2F4 in terminal bronchioles and do not produce sufficient levels of these metabolites to cause cytotoxicity, hyperplasia, or lung tumors"

Key Events of MoA

- **Delivery of chemical to the respiratory system**
- **Metabolism *in* lung by CYP2F2**
- **Cytotoxicity mediated by reactive metabolites formed from CYP2F2 metabolism**
- **Sustained reparative hyperplasia**
- **Progression to late-developing tumors**

MoA as Illustrated by Styrene (Cruzan *et al.*, 2009)



Key Events: Dose & Temporal Relationships

Dose and temporal relationships of key events in mice.

Chemical	Metabolism by CYP2F2	Acute cytotoxicity	Sustained cytotoxicity	Hyperplasia	Tumors
Styrene	Yes	40 ppm*	20 ppm	160 ppm 3 months–20 ppm after 2 years	Only at 2 years: 40 ppm–males and 20 ppm–females
Ethylbenzene	Yes	750 ppm	750 ppm	750 ppm	750 ppm males only
Naphthalene	Yes	8 ppm	30 ppm	30 ppm	30 ppm females only
Cumene	Not tested	Not tested	250 ppm males 125 ppm females	250 ppm males 125 ppm females	250 ppm males 125 ppm females
α-Methylstyrene	Not tested	Not tested	300 ppm females	300 ppm females only	100 ppm females only (not significant)
Divinylbenzene	Not tested	Not tested	10 ppm males and females	10 ppm males and females	10 or 100, not 30 females only
Coumarin	Yes	150 mg/kg by gavage	None reported	None reported	200 mg/kg/day gavage males and females 275 mg/kg/day in diet–no increase
Benzofuran	Not tested	Not tested	120 mg/kg by gavage	120 mg/kg	120 mg/kg males and females

* Lowest concentration tested.

Cruzan *et al.*, *2009*

Key Event Weight of Evidence: Delivery to Respiratory System

- **Strong likelihood of delivery to lung resulting from inhalation exposures**
  - **Styrene, ethylbenzene, cumene, α-methylstyrene, napthalene, divinylbenzene**
- **Delivery to lung via systemic circulation (oral absorption)**
  - **Styrene, ethylbenzene, coumarin, napthalene, benzofuran: all are toxic to lung following oral dosing**
  - **1st pass metabolism attenuates amount of chemical delivered to systemic circulation**

Key Event Weight of Evidence: Metabolism Drives Toxicity

Event	Specificity	Biological plausibility	Qual. human relevance	Quant. human relevance
A. Toxicity results from metabolites generated in lung by CYP2F family				
A.1. Location of cytotoxicity consistent with location of CYP2F	High	CYP2F2 found in Clara cells; cytotoxicity occurs in Clara cells	Limited CYP2F1 in human lung	Cytotoxicity not likely
A.2. Cytotoxicity is consistent across chemicals	High	Cytotoxicity from styrene, ethylbenzene, α-methylstyrene, cumene, divinylbenzene, naphthalene, coumarin, benzofuran	No human lung cytotoxicity reported	Cytotoxicity not likely
A.3. Metabolism is necessary for effect	High	Inhibition of CYP2F2 inhibits toxicity	Limited if any metabolism in human lung	Metabolism not expected
A.4. Species differences in toxicity are consistent with CYP2F. Reactive metabolites are not formed by other CYPs	High	Mice have more 2F2 in lung than rats (2F4); humans have little 2F1; No decrease in lung toxicity in CYP2E1-knockout mice. No effect on lung toxicity by inhibitors of other CYPs.	Low metabolism indicates low likelihood of tumors	Tumors unlikely

Cruzan *et al., 2009*

- **Lung toxicity of styrene, SO, and napthalene blocked in CYP2F2 KO mice**
- **Lung toxicity of styrene and SO blocked in CYP2F1 humanized mice**

Key Event Weight of Evidence: Cytotoxicity Leads to Hyperplasia

Event	Specificity	Biological Plausibility	Qual. Human Relevance	Quant. Human Rel.
B. Cytotoxicity leads to hyperplasia				
B.1. Increased cell replication only in Clara cells	High	Cell death leads to regenerative hyperplasia; cytotoxicity and cell replication both in Clara cells; does not occur in rats.	Hyperplasia not reported in human	Hyperplasia not likely
B.2. Increased cell replication sustained	Medium	Demonstrated through 5 weeks by BrdU; followed by cellular crowding in terminal bronchioles, then hyperplasia; does not occur in rat lung.	Not found	Not likely
B.3 Cytotoxic events (GSH depletion, oxidative stress)	Medium	GSH depletion demonstrated; oxidative stress may be present.	Not found in human tissue	Not likely

Cruzan *et al., 2009*

Key Event Weight of Evidence: Sustained Cell Proliferation Leads to Lung Tumors

Event	Specificity	Biological Plausibility	Qual. Human Relevance	Quant. Human Rel.
C. Sustained cell replication leads to lung tumors				
C1. Late occurring tumors	Medium	Consistent with epigenetic events	Difficult to find among human lung cancer incidence	Not likely
C2. Progression	Medium	Cell replication, leading to hyperplasia	If hyperplasia, then could expect tumors	Not likely
C3. Genotoxic events in lungs	High	Lack of genotoxic responses supports epigenetic MOA	No evidence	Not likely

Cruzan *et al., 2009*

Key Events Weight of Evidence: Ring-Oxidized Metabolites Drive Cytotoxicity and Lung Tumors

- Non-ring oxidized metabolites are non-cytotoxic and/or non-tumorigenic
  - 1-Phenylethanol, primary ethylbenzene metabolite, is not lung toxic or tumorigenic
  - SO, primary styrene metabolite, is not cytotoxic in CYP2F2 KO mice and is not a mouse lung carcinogen
  - 3,4-Dihydroxycoumarin is not a lung carcinogen
- Structural analogs that cannot form ring oxidized metabolites are not tumorigenic: 4-methylstyrene; vinyl toluene

Alternative MoA: Genotoxicity

Summary of *in vivo* genotoxicity studies in laboratory animals.

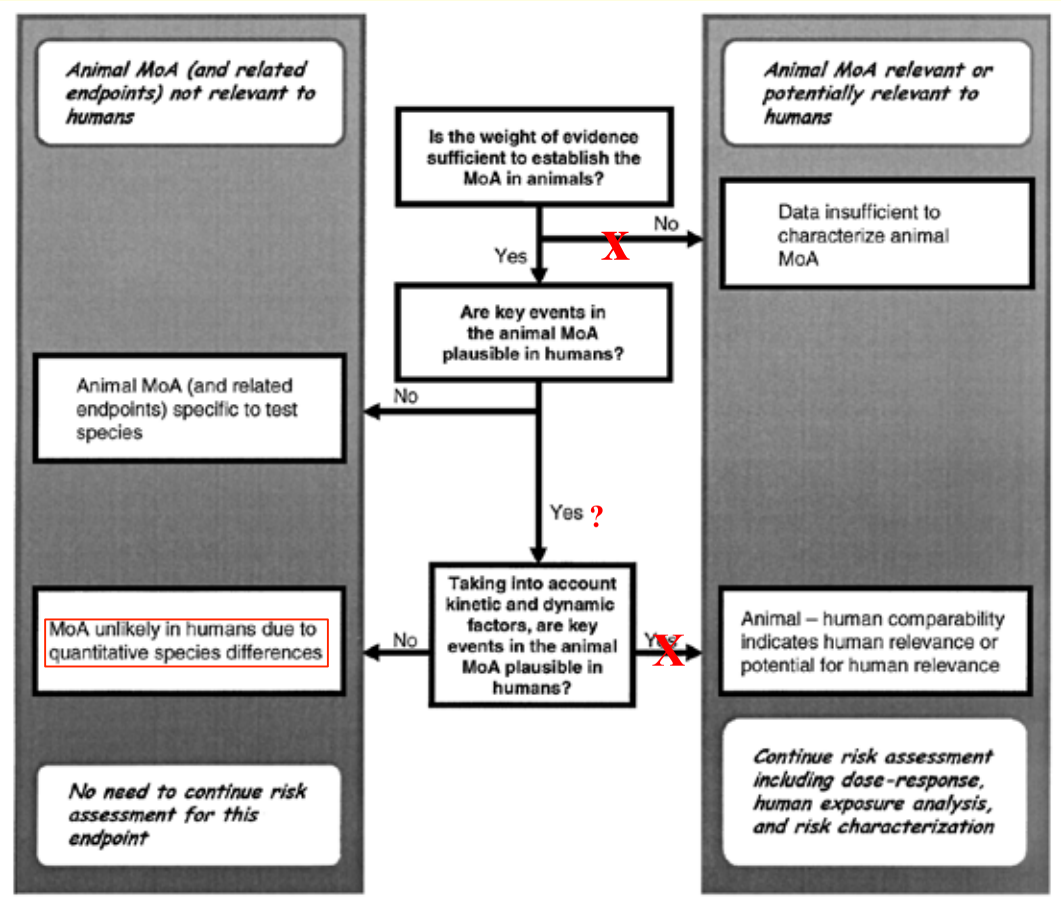
Micronucleus	Cumene	Negative 2 assays; equivocal 1 assay	Gulf Life Sciences Center (1985b), NTP (2007a)
	Divinylbenzene	Negative 2 assays	NTP (2007c)
	α-Methylstyrene	Negative 1 assay; equivocal 1 assay	NTP (2007b)
	Coumarin	Negative 2 assays	Api (2001), Edwards et al. (2000)
	Ethylbenzene	Negative 2 assays	NTP (1999), Mohtashamipur et al. (1985)
	Naphthalene	Negative 1 assay	Harper et al. (1984)
	Styrene	Positive 2 assays; negative 5 assays	See IARC (1994)
Chromosomal aberrations	Styrene	Positive 1 assay; negative 11 assays	See IARC (1994, 2002)
Sister chromatid exchange	Styrene	Positive 6 assays; negative 1 assay	See IARC (1994, 2002)
UDS	Ethylbenzene	Negative 1 assay	Clay (2001)

- **Styrene oxide (SO) hypothesized as putative genotoxic and cytotoxic agent, however, SO is not lung toxic in CYP2F2 KO or CYP2F1-humanized mice.**
- **Ethylbenzene cannot form alkyl-epoxide metabolite, yet still produces lung tumors.**

Uncertainties, Inconsistencies, Data Gaps

- **Human lung metabolism: difficult to obtain and study**
  - **Human lung metabolism preparations obtained from whole lung; low metabolism due to dilution of unidentified target cell metabolism? Humanized mice compensate?**
  - **However, human epidemiology not supportive of lung toxicity, even though for styrene, animal tumorigenicity dose overlaps human occupational exposures**
- **Most of chemicals in class cause tumors at multiple sites**
  - **Plausible alternative MoAs operating at other sites, e.g., CAR-receptor mouse liver tumors; accentuation of rat kidney nephropathy or α-2u-globulin male rat tumorigenicity**
- **Only styrene and napthalene studied in CYP2F2 KO and/or CYP2F1 mice; possible alternative metabolic activation MoAs**
  - **All chemicals in proposed class are non- or weakly genotoxic**

Human Relevance Framework: CYP2F2-Mediated Mouse Lung Tumors



From: Cohen *et al.*, Crit. Rev. Toxicol. 33: 581-589, 2003

Appendix C: Post Workshop Materials

IPCS/ILSI MOA/HR (WOE) Framework

"Key Events" established based on "Hill Criteria"

Comparison of "Key Events" & relevant biology between animals & humans

Q1. Is the weight of evidence sufficient to establish the MoA in animals?

Confidence?

Q2. Fundamental qualitative differences in key events?

Confidence?

Q3. Fundamental quantitative differences in key events?

Confidence?

Postulated MOAs
D-R/Temporal Relationships
Consistency, Specificity
Biological Plausibility

Implications of Kinetic & Dynamic Data for Dose– Response

From: Meek & Klaunig, 2010

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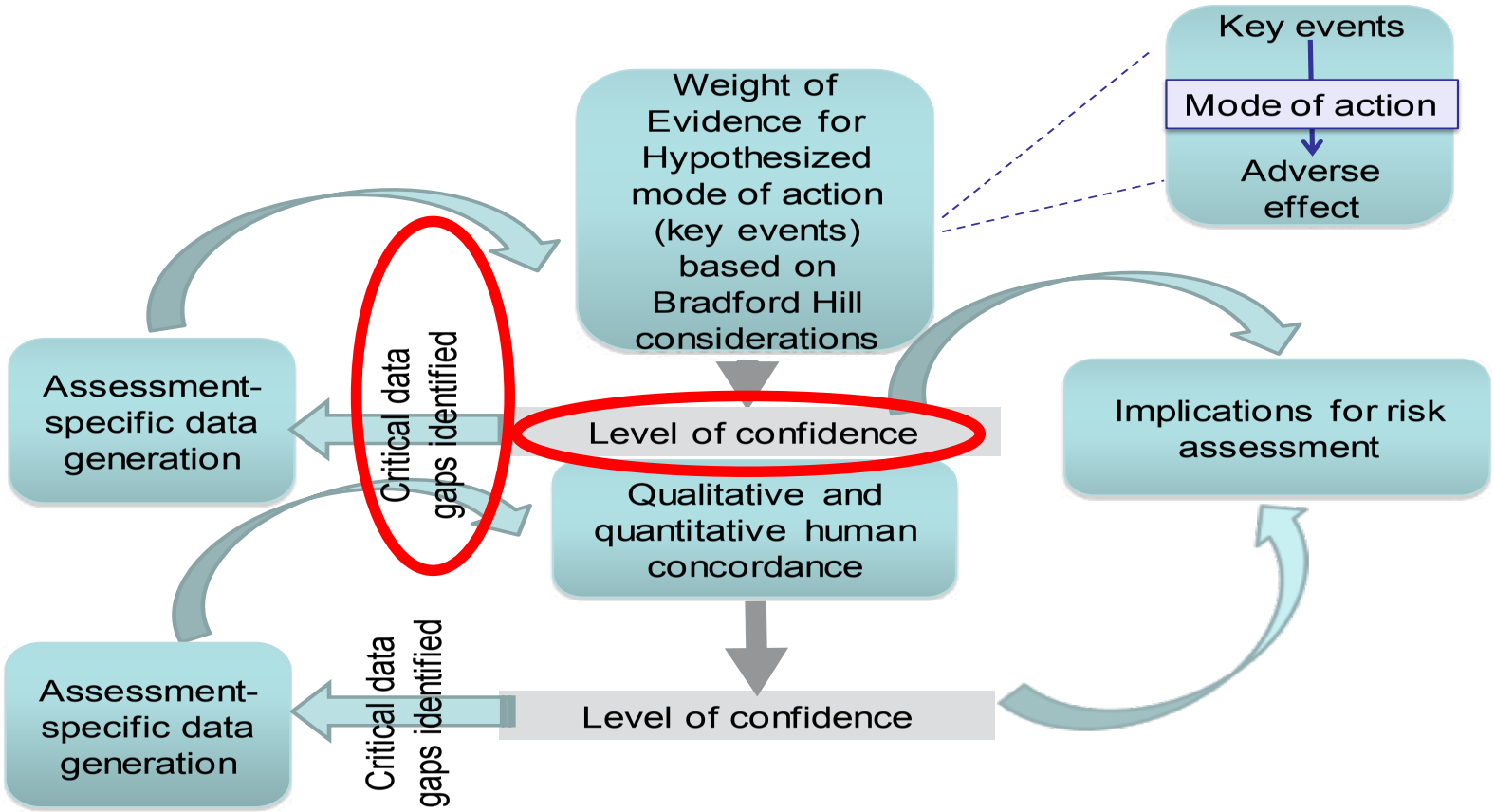
Modified MOA Framework



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First Step
Possible MOAs

From: Meek & Klaunig, 2010

Possible MOA	Nature of Evidence *(Chemical Specific or Related Compounds)*	Initial Conclusion
Direct Acting Mutagen?	Pattern of Genotoxicity Results	Likely or not?
Reductive Metabolic Pathway?	Pattern inconsistent	Likely or not?
Oxidative metabolism/sustained cytotoxicity/prolifera-tive regeneration	Multiple studies on key events	Likely or not?

From: Meek et al. submitted

Modified BH Considerations

Dose Response & Temporal Concordance	**Dose response** – Are the key events observed at doses below or similar to those associated with the apical effect? **Temporality** – Are the key events observed in hypothesized order?
Consistency, specificity	Is the incidence of the toxic effect consistent less than that for the key events? Is the sequence of events reversible if dosing is stopped or a key event prevented?
Biological plausibility	Is the pattern of effects across species/strains consistent with the hypothesized mode of action? Does the hypothesized mode of action make sense based on broader knowledge (e.g., biology, established mode of action)?

From: Meek & Klaunig, 2010

Dose – Response and Temporality

Temporal

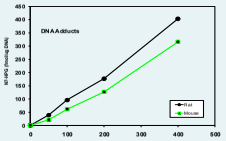
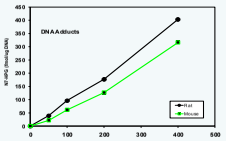
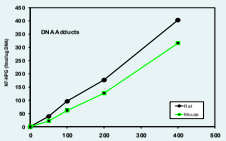
Dose (mg/kg bw/day)	***Key event 1***	***Key event 2***	***Key event 3***
0.2 (2 ppm)	+ 4 weeks	+ 52 weeks	
1 (10 ppm)	++ 4 weeks	++ 52 weeks	+ 107 weeks
4 (40 ppm)	+++ 4 weeks	+++ 13 weeks	++ 52 weeks

Dose-Response

+ = severity

From: Meek et al. submitted

Concordance Table with Dose-Response

Key Event	**Animal**	**Human**	**Strength**	**Quantitative Concordance**	**Quant. Dose-Resp.**
Metabolism by CYP2E1	Correlation with binding of metabolites	Relevant enzyme in kidney and liver	Considerable In animals; limited but relevant to humans	PBPK model incorporating metabolic rates, enzyme affinities and distribution based on *in vitro* human data supported by *in vivo* data	
Sustained cell damage and repair (cytotoxicity; proliferation)	In all cases at doses that induce tumours	Liver and kidney target organs in humans	Considerable in animals, possible in humans but limited data	No data	
Liver & kidney tumours	Mice & rats	Possible	Considerable in animals,; highly plausible in humans	No data	

From: Meek et al. submitted

Comparative Weight of Evidence

Cytotoxic Mode of Action

Bradford Hill criterion/factor	Supporting Weight of Evidence	Potentially Inconsistent Evidence
Dose Response Temporal Concordance	Metabolism, cytotoxicity, proliferation precede tumours; tumors observed only at cytotoxic (BMD Analysis) *(qualify based on nature & number of studies)*	
Strength, consistency, specificity	Consistency in repeated studies & different labs & across species, sexes routes & levels of biological organization *(#s)* correlating with extent of metabolism . No adverse effects without relevant enzyme in null mice. Incidence for tumors less than that for key events & tissue recovery in reversibility studies	
Biological Plausibility	Consistency with state of knowledge on cancer	

Mutagenic Mode of Action

Bradford Hill criterion/factor	Supporting Weight of Evidence	Potentially Inconsistent Evidence
Dose Response Temporal Concordance		Parent compound negative for mutation in a range of in vitro and in vivo bioassays *(qualify based on nature and number of studies)*
Strength, consistency, specificity		The pattern of genotoxicity results consistent with what would be expected for the hypothesized mode of action (e.g., not mutagenic in a range of assays; metabolite induces mutation at cytotoxic doses)
Biological Plausibility		Pattern of results for genotoxicity inconsistent with that observed for chemicals known to act via a mutagenic mode of action

References Cited

Meek, M.E., Klaunig, J., 2010. Interpreting available data and identifying critical data gaps for benzene risk assessment. Chem-Biol Interact. 184, 279-285.

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