Styrene Information and Research Center (SIRC)
1300 Wilson Boulevard, Suite 1200, Arlington, Virginia 22209
(703) 741-5010 Fax (703) 741-6010 Website www.styrene.org

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Dr. Ruth M. Lunn
NIEHS
P.O. Box 12233, MD EC-14
Research Triangle Park, NC 27709
lunn@niehs.nih.gov

THIS DOCUMENT SUBMITTED ELECTRONICALLY

RE: Comments on NTP Report on Carcinogens, Draft Background Document for Styrene

Dear Dr. Lunn:

The Styrene Information & Research Center[1] (SIRC) appreciates the opportunity to provide comments to the National Toxicology Program (NTP), in response to its *Federal Register* notice issuing a Draft Background Document for Styrene, for styrene's evaluation relative to potential inclusion in the 12th Report on Carcinogens (RoC). ***73 Fed. Reg. No.98, 29139 (May 20, 2008)***

On June 13, 2008, SIRC sent a letter to Dr. Samuel H. Wilson, NTP Acting Director, in response to the release of the Draft Background Document for Styrene (Document), expressing our organization's *serious concerns with the quality of the Document*; in particular noting that the Document appears to be written to focus primarily on data that might lead the Styrene Expert Panel to conclude that styrene could have human carcinogenic potential, but *omitting* critical assessments of key data that SIRC believes provide persuasive evidence that styrene should *not* be considered a human carcinogen concern.

[1] The Styrene Information and Research Center's (SIRC's) mission is to evaluate existing data on potential health effects of styrene, and develop additional data where it is needed. SIRC has gained recognition as a reliable source of information on styrene and helping ensure that regulatory decisions are based on sound science. For more information, visit http://www.styrene.org.

Because the primary goal of the July 21-22 Expert Panel meeting will be to agree upon, and recommend, a classification for styrene relative to potential listing in the RoC, SIRC feels that the current version of the Document does not begin to provide a complete, accurate, or balanced assessment of the styrene data on which the Expert Panel could base a thoughtful and scientifically sound conclusion as to styrene's human carcinogenic potential. In our letter, we requested that NTP postpone the Expert Panel meeting until such time as the Document could be revised to more evenly reflect the available data, and SIRC could provide the Panel with an assessment of the epidemiology data on styrene by an independent panel for its consideration.

In a June 26, 2008 letter, Dr. Wilson responded to SIRC, acknowledging our concerns but indicating that NTP feels the Expert Panel Meeting, as currently scheduled, would be the appropriate forum for SIRC to express its concerns with the limitations of the draft styrene Document.

Accordingly, we respectfully submit the attached detailed comments outlining our numerous issues with the limitations of the draft styrene Document. However, SIRC would like to note that – given the significant deficiencies we found in this document, and the limited amount of comment time to sufficiently address these issues – we do not consider the attached comments to be in any way a complete or definitive summary of the problems with the styrene Document.

As we noted in our June 13 letter to Dr. Wilson, the current styrene Document appears to rely heavily on the 2002 International Agency for Research on Cancer's last review of styrene, which is now seriously outdated due to the availability of significant new data. Indeed, very recently, both the European Union and Japan's Ministry of Economy, Trade and Industry (METI), after multi-year evaluations of the styrene data, each issued regulatory conclusions that styrene should <u>not</u> be classified as a carcinogen. Accordingly, the Expert Panel and NTP will need to provide very careful and scientifically sound justification for their own conclusions, should there be a recommendation to classify styrene, given that this would directly contradict the new determinations of other major international bodies – *and most especially given that the minimum threshold for inclusion in the RoC is that a substance is "reasonably anticipated to be a human carcinogen."*

SIRC strongly urges that the Styrene Expert Panel, as well as NTP, give careful consideration to the following comments on the deficiencies of the draft styrene Document in arriving at a conclusion as to styrene's human carcinogenic potential. We especially request that our written comments be given full consideration during the Expert Panel Meeting, given the extremely limited amount of time (7 minutes) during that meeting that SIRC will have to offer oral comments on the many concerns we have with this Document. SIRC continues to feel that the Draft Background Document on Styrene is sufficiently lacking in balance and scientific accuracy, to the point that the

Expert Panel should not form a recommendation on a classification for styrene until the Document has been appropriately updated.

This may include the need to postpone a Panel recommendation on styrene's classification until a future date, if the Panel concurs that the Document needs substantive revision by NTP. We do not believe that the simple inclusion of SIRC's comments in the collective styrene docket is an appropriate solution to this problem, as it will be the draft NTP styrene Document that will be cited in future, and not SIRC's comments. An unrevised styrene Background Document that is carried forward in the RoC process on styrene will reflect poorly on both the Expert Panel and NTP itself.

Listing of styrene in the RoC could have profound negative implications for the myriad of styrene-using industries, and for the many useful health- and life-enhancing products they manufacture. A carcinogen listing of styrene that is not grounded in a *thorough* review of the *best possible science* would be unfair and unwarranted – not only to the styrene industry, but also to the American public which the RoC is intended to inform.

SIRC thanks NTP for the opportunity to provide the following comments, and hopes that they will substantially contribute to the Expert Panel's ability to make an accurate conclusion on the available data on styrene.

Very truly yours,

Jack Snyder
Executive Director
Styrene Information & Research Center
1300 Wilson Boulevard – Suite 1200
Arlington, VA 22203
(703) 741-5010
Jack_Snyder@styrene.org

SIRC COMMENTS ON NTP DRAFT BACKGROUND DOCUMENT FOR STYRENE, FOR NTP REPORT ON CARCINOGENS

Introduction / Key Concerns

The NTP has developed a Report on Carcinogens Draft Background Document for Styrene (Document) for review by the Styrene Expert Panel, scheduled to meet July 21 & 22, 2008. SIRC finds that the Document is not balanced in its inclusion of studies, presentation of study findings, or assessment of evidence. Major changes in the Document are needed for it to be scientifically accurate. The Document often cites *partial* conclusions of other groups, but *ignores* conclusions from those groups that disagree with the apparent desired conclusions of the authors.

In the epidemiology section, the Document concludes that evidence of increased lymphohematopoietic (LH) cancers from styrene is strongest among styrene-butadiene rubber (SBR) workers; yet that analysis ignores the most recent study of SBR workers that reports *no* correlation of leukemia with styrene exposure. Furthermore, 10 to 100 fold higher exposures in reinforced plastics and composites (RPC) workers did not produce evidence of LH cancers. The assessment asserts that small non-significant differences from controls are evidence of cancer, without any criteria for the conclusions.

SIRC notes that Dr. Kolstad, lead author of one of the primary epidemiology studies referenced in the NTP Document, was also a contributor to the drafting of the epidemiology section of the styrene Document. SIRC submitted comprehensive comments to NTP (July 2004) when styrene was first proposed for review for the RoC, which addressed problems with the conclusions in the Kolstad study. However, SIRC's comments have not been reflected in the draft NTP document.

Overall, the epidemiology data provide neither "limited" nor "sufficient" evidence that styrene causes cancer in humans, upon which the Expert Panel or NTP could base a conclusion that styrene is "reasonably anticipated to be a human carcinogen" or "known to be a human carcinogen."

As agreed by the International Agency for Research on Cancer (IARC), the European Union, a Harvard Center for Risk Analysis Panel that assessed styrene, NTP's Center for the Evaluation of Risks to Human Reproduction, and the Agency for Toxic Substances and Disease Registry, an integrated assessment of 8 chronic studies in rats provides no evidence of increased tumors in rats. Based on 5 chronic studies in mice, only lung tumors are increased in mice. The draft NTP Document presents only the dogma that any effects from styrene are caused by its metabolite styrene-7,8-oxide. It presents two hypotheses for the mouse lung tumor mode of action based on styrene-7,8-oxide as the

causative agent. The assessment ignores several pieces of data that do not support these hypotheses.

An alternate mode of action was published in 2002 (Cruzan et al, 2002) and is ignored in the Document: Styrene is metabolized in mouse terminal bronchiolar cells by CYP2F2 to cytotoxic metabolites. The continued cytotoxicity leads to regenerative hyperplasia, and eventually tumors. Cytotoxicity from styrene occurs in tissues high in CYP2F (2F2 in mouse lung Clara cell and nasal olfactory epithelium cells, and 2F4 in rat nasal olfactory epithelium). Inhibition of CYP2F2 inhibits the cytotoxicity from styrene. Rats have less CYP2F4 in the lung Clara cells and do not produce sufficient metabolites to cause cytotoxicity or lung tumors. Even less human CYP2F1 is present in human tissues and 2F1 metabolizes even less styrene. Therefore, the mouse lung tumors from styrene exposure are not relevant to human risk.

Overall, there is no evidence of increased tumors in rats, and mouse lung tumors occur by a mode of action that is not relevant to humans.

SIRC believes that, based on a thorough evaluation of the effects of styrene in humans and experimental animals, it would not be scientifically valid for NTP to list styrene as either "reasonably anticipated to be a human carcinogen" or "known to be a human carcinogen" in the 12th Report on Carcinogens.

DETAILED COMMENTS

A. Epidemiology

The epidemiology section reviews studies from the reinforced plastics and composites (RPC), styrene-butadiene rubber (SBR) and monomer/polymer industry segments and properly notes there are differences in exposures among these groups. The differences include other chemical exposures, the degree of exposure to styrene, and lifestyle differences of the workers. However, these differences are not taken into account in assessing potential styrene effects.

1. Exposure Differences

The RPC industry has variable exposures among its workers. Being employed in the industry does not provide a good estimate of exposure. Some employees are defined as "laminators." Their job is to spray styrene-containing resin and chopped fiberglass onto an open mold and roll the mixture with paint rollers to imbed all the fiberglass into the resin. This results in very high exposures during application, but lower exposures when not laminating. Other workers in these facilities perform finishing work (sanding, assembling, etc.) on pieces of cured resin, where there is minimal styrene exposure.

Others are maintenance workers, supervisors, or office personnel. Past exposures for laminators have exceeded 100 ppm and often approached 200 ppm. During the periods that the available epidemiology studies estimated exposures (1940's to 1980), exposure to laminators was above 30 ppm. Potential confounding exposures include peroxides used to initiate the reaction between styrene and the resins, and solvents used for cleaning equipment. Many other applications of styrene are closed systems and have lower exposures. Without understanding individual job history, one cannot *estimate* exposure.

SBR production involves reaction of styrene and butadiene in essentially closed systems. Exposures are below 5 ppm. Recent re-evaluations have estimated styrene exposure in SBR manufacture to be less than 1 ppm. SBR workers were also exposed to butadiene and dimethyldithiocarbamate. Butadiene has been associated with increased leukemia in other studies.

Styrene exposure in monomer and polymer manufacturing has also been below 5 ppm. Styrene is manufactured by reacting benzene and ethylene (gas) to produce ethylbenzene, which is dehydrogenated. The system has to be closed to contain the ethylene. Exposures to styrene in this process come from fugitive emissions, and are very low. Workers in this industry segment are exposed to benzene and to a number of pigments used to color polystyrene.

Based on the principle of dose-response, workers in the RPC industries, especially laminators, have been consistently exposed to 10 to 100 fold higher styrene levels than workers in other styrene-related industries. There is also less potential for confounding exposures in the RPC industry. Therefore, if styrene exposure affects cancer, the effects should be most obvious among RPC workers. Effects in SBR or monomer/polymer workers that are not found in RPC workers are not likely to represent effects from styrene exposure.

2. Study Issues

The Kolstad cohort is treated in the draft Document as evaluating high exposure and low exposure, but there is no individual exposure assessment in the study. The authors reviewed the Danish industry registry for companies that might be involved in reinforced plastics. They then asked the company owners if they were involved in RPC. They also asked two suppliers of resin material to identify if each company listed was involved in RPC. The resin suppliers identified 386 companies with 36,525 employees as involved to some extent in RPC. The company owners identified 277 companies with 28, 518 employees as ever involved in RPC. The suppliers and owners agreed on 233 companies with 26,784 employees. The authors performed the rest of the analyses using the responses from the resin suppliers because they found a significant cancer increase using the suppliers' assessment and did not find one using the owners' assessment in a subset of the cohort. They further asked the suppliers if more or less than 50% of each company's workforce was involved in reinforced plastics. No attempt

was made to determine how many workers may have been laminators. Evaluation of high vs. low exposures is based on whether more or less than 50% of the workers may have been involved in RPC. RPC workers in the category where less than 50% were involved in RPC had the same exposure as workers in companies where more than 50% were involved in RPC. There were 32 leukemias among those who worked less than 1 year and were more than 10 years from first employment; this was reported as a significant increase. No attempt was made to determine if any of the 32 cases was actually exposed to styrene. Thus, this study's serious design limitations prohibit interpretation of a cancer association with styrene exposure.

Table 3-8, identifies Kolstad high exposed workers as "All workers employed in companies with 50% to 100% laminators." That is not true; this group was companies where 50 to 100% of employees were thought to be involved in some phase of RPC. In a typical RPC facility 10 to 20% of the workforce are actually laminators. Footnote "d" indicates that the Kogevinas analysis here excludes the Danish laminators. Kogevinas recognized that the Danish cohort was not all laminators and they are not included in the Kogevinas study as laminators; they are "workers with unspecified tasks." We note that the review of the Kolstad study in the draft Document was written by Dr. Kolstad himself. The table and text regarding Kolstad must be modified to correctly state the make-up and exposure of the cohort and correctly assess the conclusions that can scientifically be drawn from this study.

In the SBR industry, the Document concludes that "The evidence for lymphohematopoietic malignancies appears to be the strongest in the styrene-butadiene industry." (Summary, vii, line 2). Yet it excluded from quantitative assessment the most recent evaluation of the cohort (Graff et al., 2005) because it only examined lymphohematopoietic malignancies. Graff et al. (2005) found *no* consistent exposure-response trend with all leukemia, chronic myelogenous leukemia, or chronic lymphocytic leukemia, after adjusting for 1,3-butadiene. This inconsistency should have been explored, particularly because Graff et al. (2005) arguably describe exposure more completely. Thus, the study that provides evidence against the conclusion was *excluded*. Furthermore, since exposure in SBR industries is 10 to 100 fold lower than in RPC manufacturing, results in the RPC sector should carry much more weight than in the SBR segment.

Coyle et al. (2005) suggested that styrene might increase breast cancer because the rate of breast cancer in Texas counties correlated with Toxics Release Inventory (TRI) emissions from those counties. A review of the Coyle et al. study by Burns et al. (2006) noted that these results are likely to be an example of an ecological fallacy. Burns et al noted that the rate of breast cancer in Texas is *low* compared to the rest of the United States. Ambient styrene exposures in the Houston, TX area average 0.018 ppb. Industrial exposures are about 3 million times greater, but no excess risk of breast cancer has been found in these populations.

3. Causality Evaluations

The Document cites several small differences in SMR or RR between workers and controls or high and low exposure groups where the estimates are based on small numbers of cases and the 95% confidence limits encompass risk less than 1.00 as evidence of styrene-related increased cancer risk, calling them "statistically non-significant increases." *No criteria are provided for assessing non-significant risks.* Further, there are many more SMRs or RRs that are less than 1.00, but these are not regarded as evidence for a protective effect.

Table 3-8 is an attempt at evaluating cancer endpoints across RPC cohorts. Note that overall there is no increase for all LH, lymphoma, multiple myeloma, or leukemia, but a slight numerical increase for Hodgkin's disease, which was not statistically significant. Thus, there is no evidence of increase in any specific leukemia or lymphoma, or in a combination of these diseases, in RPC workers.

The NTP Document (Section 3.9) states "The risk of pancreatic cancer was increased across five studies of workers (three studies of the reinforced plastics industry, one study of the styrene monomer and polymer industry, and the cohort of bio-monitored workers) exposed to high levels of styrene. Moreover, among the highest styrene-exposure group in the reinforced plastics industry, there was an excess (1.77 fold) in the total number of observed cases across the four cohort studies compared to the total number of expected cases. There were also indications of an exposure-response relationship in the two of the four studies that assessed cumulative exposure or duration of exposure. However, no increased risk of pancreatic cancer was reported among styrene-butadiene workers." Only one of these was statistically significant (more than 50% workers in RPC of Kolstad et al., 1994). Further the studies in the monomer/polymer industry and the cohort of bio-monitored workers did not represent "high exposures to styrene." Thus the data do not support a conclusion of increased pancreatic cancer from styrene exposure.

4. Assessment of the Epidemiology Data

There is no consistent evidence of increased cancer among workers exposed to styrene.

a. There were more significant *decreases* in cancer incidence than *increases* in incidence.

b. Increases in LH cancers, including leukemia, were most consistent among SBR workers. After controlling for butadiene exposure, there were no increases related to styrene exposure (Graff et al., 2005). Increases in these cancers were not found among the RPC cohorts (Table 3-8).

c. Pancreatic cancer was not increased among highly exposed workers. Kolstad

reported increased pancreatic cancer among companies where more than 50% were involved in RPC, but relationship to being a laminator or to styrene exposure level was not determined.

Conclusion: Overall, there is not adequate evidence in the human data that styrene could be "reasonably anticipated to be a human carcinogen" or "known to be a human carcinogen."

B. Animal Cancer Studies

1. Rats – There are 8 chronic studies conducted in rats. The only question of increased tumors in rats centers on mammary tumors in females. Four gavage studies were negative. One drinking water study (although at low doses because of limited solubility of styrene in water) was reported as negative by the authors, but Huff (1984) reported a significant increased trend for combined benign and malignant mammary tumors. McConnell et al. (1986) reported that normally fibroadenomas (the vast majority of mammary tumors in rats) should not be combined with adenomas or adenocarcinomas. Thus, this study does not provide evidence of increased mammary tumors. Reference to the Huff analysis should be removed or the publication by NTP pathologists (McConnell et al.) should be added to the Document.

An inhalation study by Jersey et al. (1978) reported increased adenocarcinomas at the low dose (600 ppm), but not the high dose (1000 ppm) compared to the concurrent control group. They pointed out that control group incidence (0%) was low and the low dose incidence was within the historical control range. The authors concluded that styrene did not increase mammary tumors. Conti et al. (1988) reported increased mammary tumors at all exposure concentrations (25-300 ppm) in an inhalation study. It should be noted that the control incidence was below the control incidence in the Charles River database for S-D rats, and the incidences in the exposed groups were within the historical control range. Furthermore, there was no dose response among the treated groups. Cruzan et al. (1998) reported no increase at 50 or 200 ppm (within the range tested by Conti et al.) and significant dose-related decreases at 500 and 1000 ppm. Table 1 below from Cruzan et al. (1998) compares the results from all dose groups of all eight studies.

Conclusion: The Harvard Panel, IARC, NTP CERHR, ATSDR and European Union all concluded that styrene does not cause increased tumors in rats. SIRC agrees with these assessments that styrene does not cause increased tumors in rats.

Table 1. Mammary tumors results in rats exposed to styrene

Strain	Route of Exposure	Administered Daily Dose	Lifetime dose (g/kg)	Reported Response	Reference
SD	Inhalation	25 ppm	1.9	⇑	Conti et al., 1988
SD	Inhalation	50 ppm	3.9	⇑	Conti et al., 1988

SD	Inhalation	100 ppm	7.7	⇑	Conti et al., 1988
SD	Water	125 ppm	9.9	=	Beliles et al., 1985
SD	Inhalation	50 ppm	11.6	=	Cruzan et al., 1998
SD	Gavage	50 mg/kg/day	13.2	=	Conti et al., 1988
SD	Water	250 ppm	14.9	=	Beliles et al., 1985
SD	Inhalation	200 ppm	15.3	⇑	Conti et al., 1988
SD	Inhalation	300 ppm	23	⇑	Conti et al., 1988
F344	Gavage (m)	175 mg/kg/3x	42	=	NCI, 1979b
SD	Inhalation	200 ppm	45	=	Cruzan et al., 1998
BDIV	Gavage	500 mg/kg/wk	53	=	Ponomarkov, 1978
SD	Gavage	250 mg/kg/day	66	=	Conti et al., 1988
F344	Gavage	350 mg/kg/3x	84	=	NCI, 1978
SD	Inhalation	500 ppm	115	⇓	Cruzan et al., 1998
SD	Inhalation	600 ppm	115	?	Jersey et al., 1978
SD	Inhalation	1000 ppm	192	=	Jersey et al., 1978
SD	Inhalation	1000 ppm	230	⇓	Cruzan et al., 1998
F344	Gavage	500 mg/kg/day	264	=	NCI, 1979a
F344	Gavage	1000 mg/kg/day	396	=	NCI, 1979a
F344	Gavage	2000 mg/kg/day	792	=	NCI, 1979a

Conti studies dosed for 12 months; Gavage (m) was 30% b-nitrostyrene; 70% styrene – dose is styrene only; dosed 3 x/week.

2. Mice

Mice exposed to styrene develop lung tumors late in life. The draft Document proposes two hypotheses for the mode of action (MOA), but does not mention or evaluate the most supported hypothesis for mouse lung tumor MOA.

Both hypotheses put forward in the Document are based on proposed properties and effects of styrene-7,8-oxide. The toxic agent has not been identified, but there is good evidence that it is *not* styrene oxide (SO).

a. As pointed out in the NTP document, the Harvard Panel concluded that differences in lung concentrations of SO do not explain why mice get lung tumors from styrene exposure and rats do not.

b. Gavage administration of styrene oxide (550 mg/kg/day) to mice did not cause increased lung tumors, despite generating styrene oxide levels in the lungs at least equivalent to the level of SO from the metabolism of styrene in the mouse chronic inhalation study.

c. SO-DNA adducts from styrene exposure are very low (<1 adduct per 10^7 nucleotides) and are not greater in mouse lung than in rat lung.

d. Dr. Filser (Hofmann et al., 2005) concluded that SO is not the causative agent in mouse lung tumors from styrene inhalation exposure. Isolated perfused lungs of rats exposed to 1000 ppm (non-tumorigenic in chronic study) in inspired air produced 8 times as much SO as mouse lungs exposed to 40 ppm (lung tumors in chronic study).

Conclusion: IARC (2002) concluded that "the lung tumors were caused by lung metabolism of styrene and the process does not occur to a meaningful extent in humans." The European Union (2007) said "it is reasonable to conclude that the lung tumours seen in mice are unlikely to be of any relevance for human health." The current ATSDR (2008) draft says "Thus, mice appear to be very sensitive to the induction of lung tumors and the mechanism of inducing lung tumors is not likely to be relevant to humans." SIRC agrees with the conclusions of these organizations.

3. MOA for Mouse Lung Tumors

Another mode of action for styrene mouse lung tumors has been proposed but was not mentioned in the draft Document. Metabolism of styrene, and several related compounds, by CYP2F2 causes unique metabolites that cause cytotoxicity in the terminal bronchioles and lung tumors (Cruzan et al., 2002, 2005). The proposed MOA is supported by similar effects from at least 7 other structurally similar chemicals, some of which cannot form a vinyl epoxide.

SIRC is including this substantive assessment of the mouse lung tumor MOA data because we find this to be the *only* suggestion of tumor formation from styrene exposure, given the absence of cancer effects in rat and human data, and because the significant amount of available data addressing this MOA helps to distinguish the mouse effects as being *unrelated* to a human health concern.

The key characteristics are:

a. Target organs for cytotoxicity are consistent with location of CYP2F (mouse lung Clara cells and nasal olfactory epithelium, and rat nasal olfactory epithelium).

b. Inhibition of CYP2F2 (5P1P) inhibits cytotoxicity.

c. Inhibition of CYP2E1 (or CYP2E1-knockout mice) does not reduce cytotoxicity from styrene or 4VP (4-hydroxystyrene).

d. Ring-oxidized metabolites probably responsible; 4VP is toxic at 5 fold lower dose than SO.

e. Ring-oxidized metabolites similar in structure to toxic metabolite from coumarin.

f. Similar toxic and lung tumor response from ethylbenzene and cumene, which are not converted to vinyl epoxide.

g. Methyl group at 3 or 4 position of benzene ring (p-methylstyrene, 3-vinyltoluene) prohibit increased mouse lung tumors.

A detailed description of the data supporting the alternative MOA for styrene-induced mouse lung tumors, which is based on evidence from styrene and other structurally-related chemicals, is as follows:

A. **Postulated MOA for Mouse-Specific Lung Toxicity and Tumorigenicity**

CYP2F2 metabolism of several chemicals in terminal bronchiolar Clara cells in mice results in the generation of cytotoxic metabolites. Initial exposures lead to cytotoxicity in terminal bronchioles, followed by reparative cell replication. On continued exposure, the increased cell replication continues, leading to cellular crowding and then to hyperplasia in the terminal bronchioles. As the hyperplasia continues, it expands into the alveolar ducts. Some of this hyperplasia proceeds to form adenomas in the mouse lung, which has a high spontaneous incidence of adenomas in control mice. A few of the adenomas may progress to carcinomas. The analogous CYP2F4 in rats may be as capable of forming these cytotoxic metabolites; 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. Tissues that are high in CYP2F enzymes (CYP2F2 in mouse lung terminal bronchioles and nasal olfactory epithelium; CYP2F4 in rat nasal olfactory epithelium) develop cytotoxicity from these chemicals, which may or may not progress to tumors. Humans have very small amounts of the orthologous isozyme CYP2F1 in lungs or nasal turbinates. CYP2F1 appears to be much less active, if at all, in metabolizing these compounds. Therefore, no cytotoxicity or lung tumors are expected from human exposures to these chemicals. The key element of the hypothesis is that the lung-specific toxicity of this series of compounds converges on their metabolism to cytotoxic metabolites by mouse CYP2F2, which may differ in both specificity and rate of metabolism compared to rats and humans.

Examples of chemicals that are proposed to cause mouse lung tumors by this MOA: Coumarin, naphthalene, styrene, ethylbenzene, a-methylstyrene, cumene, divinylbenzene, benzofuran.

B. Key events

Key events in this MOA are: delivery of the chemical to the respiratory system, metabolism in lung, cytotoxicity in terminal bronchioles, cell replication, and tumors.

B.1. Delivery of Chemical to the Respiratory System

The respiratory system (nasal epithelium to alveoli) is the major interface between mammals and airborne chemicals in their environment. Inhalation of these chemicals delivers them directly to the cells lining the airways. Depending on the physico-chemical properties of the substance, at very low concentrations as much as 50% of the inhaled chemical in the airstream can be absorbed in the nose (Morris, 2000). In the presence of CYP metabolism inhibitors, about 10% of styrene is absorbed in the nasal region (Morris, 2000), but with metabolic activity up to 50% of the styrene is removed from the airstream in the upper respiratory tract of mice. These chemicals can also be absorbed directly into the cells of the terminal bronchioles (Clara cells) as well as the alveolar cells. When absorbed into alveolar cells, they pass into the blood capillaries and are distributed systemically in rats and mice, resulting in detectable blood concentrations of the parent compounds (Cruzan et al., 1998, 2001).

For coumarin, naphthalene, styrene, and ethylbenzene, there is good evidence of distribution of the chemical from all routes of exposure to all tissues, including respiratory epithelium. When exposure is by the oral route, first pass metabolism in the liver dramatically reduces the amount of chemical that is distributed to tissues through the blood stream (Sarangapani et al., 2002). However, metabolism and cytotoxicity in lung from oral exposure has been demonstrated for coumarin (NTP, 1993), naphthalene (Buckpitt et al, 2002), styrene (Green et al., 2001a), and ethylbenzene (Stott et al, 2003), indicating that these chemicals can be absorbed systemically and penetrate all organs after oral administration, and that the lung has a preferential capacity to metabolize systemically available concentrations of these compounds.

Qualitatively, delivery of these chemicals to lung cells does not appear to be species specific. This process is driven by the solubility of the chemicals in the various tissues, which should be approximately the same across species, including man, and by blood flow and minute volume, which may affect the quantity of these chemicals delivered to the lungs.

B.2. Metabolism in Lung

Many compounds are metabolized to cytotoxic metabolites by CYP2F2 in the Clara cells of the terminal bronchioles of mouse lung. The metabolite(s) responsible for cytotoxicity from most of these compounds in the terminal bronchioles have not been determined.

Coumarin: The major metabolite of coumarin in rats, mice, and humans is 7-hydroxycoumarin. However, coumarin is metabolized by CYP2F2 to coumarin-3,4-epoxide in mouse lung, which rearranges to 2-hydroxyphenylacetaldehyde (Born et al., 2002) and causes mouse lung cytotoxicity and lung tumors. Inhibition of CYP2F2 by 5-phenyl-1-pentyne (5P1P) eliminated the bronchiolar cytotoxicity from coumarin (Born et al., 2002). This metabolism occurs to a much lower extent in rats, which do not develop

lung cytotoxicity or lung tumors (Felter et al., 2006). Dihydrocoumarin is not capable of forming 3,4-epoxide and did not induce lung tumors in mice (NTP, 1993b).

Naphthalene: Pulmonary microsomes from mice metabolized naphthalene at approximately 8 times the rate of rat microsomes and produced mostly 1R,2S-naphthalene oxide, whereas rat microsomes produced mostly 1S, 2R- naphthalene oxide (Buckpitt et al., 2002). Inhibition of CYP2F2 by 5P1P eliminated the bronchiolar cytotoxicity from naphthalene (Buckpitt et al., 1995). Genter et al. (2006) demonstrated that CYP1A1 and CYP1A2 genes which are inducible by AHR in the mouse respiratory tract do not function to influence naphthalene toxicity, and confirm the results of Phimister et al. (2004) that CYP2F2 bioactivates naphthalene in lung and nasal tissues.

Styrene: For styrene, the first step in the major metabolic pathway is oxidation to S-styrene-7,8-oxide; this accounts for at least 80% of the metabolism of styrene in rats and mice (Sumner and Fennell, 1994; Cruzan et al., 2002). It should be noted that oral administration of styrene-7,8-oxide to mice at 275 mg/kg/day did not result in increased lung tumors, even though PBPK models indicate this dose of SO would result in a higher lung level of SO than from metabolism of styrene at 40 ppm by inhalation (Sarangapani et al., 2002). Further, Hofmann et al. (2006) demonstrated that *ex vivo* exposure to styrene in rat lungs at 1000 ppm (non-tumorigenic) produced 2.5 nmol styrene oxide/g lung vs. 0.25 from mouse lungs at styrene concentration of 40 ppm (tumorigenic). This led the authors to conclude that styrene oxide is not the agent responsible for mouse lung cytotoxicity from styrene exposure. In mouse lung, two alternate metabolic paths are prevalent; one involves formation of R-styrene-7,8-oxide and the other involves oxidation of the benzene ring (Cruzan et al., 2002; Bartels et al., 2005). Using selective inhibitors, Carlson determined that CYP1A, 2B, and 2E1 had little, if any, impact on Clara cell cytotoxicity of styrene, implying they are not involved in metabolic activation of styrene in the lung (Carlson 1997, Carlson et al., 1998). Inhibition of 2E1, or the use of 2E1 knockout mice demonstrated that 2E1 plays some role in the acute liver cytotoxicity of styrene, but has no impact on the lung toxicity (Carlson, 2004; Vogie et al., 2004). In studies of styrene, the inhibition of CYP2F2 by 5-phenyl-1-pentyne (5P1P) inhibited both the lung cytotoxicity and nasal cytotoxicity in CD-1 mice (Green et al., 2001a, b). 4-Vinylphenol (4VP, 4-hydroxystyrene) is a minor urinary metabolite of styrene and has been used as a substrate for further ring-oxidized metabolites of styrene. 4-VP is 10 times as toxic to mouse lung as styrene and 5 times as toxic as styrene-7,8-oxide (Carlson et al., 2002). Inhibition of CYP2F2 by 5P1P also inhibits the cytotoxicity of 4VP (Carlson, 2002), indicating that there is a subsequent metabolite of 4VP that is responsible for cytotoxicity. The metabolite(s) responsible for cytotoxicity from these compounds in the olfactory epithelium or terminal bronchioles have not been identified.

Ethylbenzene: *In vitro* studies examining comparative mouse, rat and human lung and liver microsomal metabolism of ethylbenzene have confirmed extensive metabolism in all three species to alkyl-oxidized metabolites, e.g., 1-phenylethanol (mouse > rat ~ human; Saghir et.al., 2006; 2007). 1-Phenylethanol was not pneumotoxic or tumorigenic

when tested in high-dose oral subchronic and chronic rat and mouse bioassays (NTP, 1990). No detectable lung toxicity was found from exposure to 1-phenylethanol, 2-phenylethanol, or phenylacetaldehyde in mice (Carlson et al., 2002). Use of GSH-trapping to detect putative cytotoxic catechol and hydroquinone metabolites confirmed the *in vitro* formation of these metabolites in mouse, rat and human liver microsomes, and in mouse and rat, but not human, lung microsomes. Similar to the generation of alkyl-oxidized metabolites, mouse lung microsomes exhibited substantially higher metabolic activity (mouse lung GSH-derived metabolites approximately 10X > rat lung; human lung not detectable; mouse lung GSH metabolites approximately 2X > mouse liver; mouse liver approximately 10X > rat and human liver). Although ring-oxidized metabolites accounted for a relatively small fraction of overall ethylbenzene metabolism, their selective elevation in mouse lung microsomes is nonetheless consistent with the hypothesized mode of action attributing preferential formation of lung-derived cytotoxic, ring-oxidized metabolites as driving the mouse lung specific toxicity of ethylbenzene. Interestingly, both mouse and rat lung microsomes exhibited decreasing amounts of ring-oxidized metabolite formation with increasing concentrations of ethylbenzene, suggesting the possibility of cytochrome P450 suicide inhibition by reactive ring-oxidized metabolite(s). This observation would also be consistent with the hypothesis of the formation of reactive cytotoxic metabolites in mouse lung. 5P1P inhibition studies are currently in progress. 4-Hydroxyethyl-benzene is the only metabolite of ethylbenzene that has been demonstrated to cause mouse lung cytotoxicity in 3-day studies (Kaufmann et al., 2005).

Cumene: In mice exposed to 14C-cumene, urinary metabolites included 4-(2-hydroxy-2-propyl) phenylsulfate, indicating ring oxidation (Ferguson et al., 2008).

Data on the metabolism of these compounds in human lung tissue are limited because of the difficulty obtaining adequate specimens for testing. However, limited data indicate that these metabolites are either not produced in human lung or are produced to a much lower degree (Vassallo et al., 2004; Buckpitt et al., 1986; Cruzan et al., 2002; Felter et al., 2006). Baldwin et al. (2004) found no detectable CYP2F in any lung subcompartments in rhesus macaque. Thus human lung and nasal cells would not be expected to develop cytotoxicity from these compounds.

B. 3. Cytotoxicity

Short term exposure to coumarin (Born et al., 1998), naphthalene (West et al., 2001), styrene (Cruzan et al. 2002), and ethylbenzene (Stott et al., 2003) all cause cytotoxicity in the terminal bronchioles of mouse lung, but not rat lung (Table 2). The target cells are the Clara cells lining the terminal bronchioles. Toxicity to alveolar cells does not occur. Single gavage doses of 150 and 200 mg/kg coumarin resulted in swelling and necrosis of Clara cells in the terminal bronchioles of male and female B6C3F1 mice (Born et al., 1998). Doses below 150 mg/kg did not cause toxicity. While coumarin caused mouse lung cytotoxicity and lung tumors (NTP, 1993a), dihydrocoumarin did not (NTP, 1993b).

Coumarin (NTP, 1993) causes cytotoxicity in the terminal bronchioles, but since it was administered orally the olfactory epithelium was not examined.

The cytotoxicity from naphthalene is summarized by Buckpitt and coworkers (2002). Briefly, parenteral administration of 50 mg/kg naphthalene results in swelling of the Clara cells (O'Brien et al., 1985); larger doses result in more severe effects, including a loss of apical blebs and decreased endoplasmic reticulum in Clara cells and denuding of Clara cells from the terminal bronchioles. For naphthalene, female mice are more susceptible than males. CYP2F2 bioactivates naphthalene in mouse lung terminal bronchiolar tissue to one or more reactive metabolites that induce cytotoxicity after depleting glutathione (Phimister et al., 2004; Genter et al., 2006). In rats, even at an ip dose of 1600 mg/kg, the Clara cells were apparently normal.

The cytotoxicity of styrene has been summarized by Cruzan et al. (2002, 2005). For styrene, cytotoxicity has been measured by increased cell replication following 3 inhalation (40 and 160 ppm) or ip (100 mg/kg) exposures (Green et al., 2001a; Kaufmann et al., 2005). Similarly, following 3 exposures, styrene metabolites styrene-7,8-oxide (100 mg/kg) and 4-hydroxystyrene (35 mg/kg), produced a greater increase in cell replication than the parent compound styrene (Kaufman et al., 2005). In the chronic mouse study (Cruzan et al., 2001), decreased staining of the Clara cells (an indicator of cytotoxicity) was reported in 50-70% of the mice exposed to 20 ppm for 12, 18 or 24 months and in more than 80% of those exposed to 40, 80, or 160 ppm. Increased cell proliferation has been reported at concentrations of 40 ppm or greater (20 ppm has not been examined). Bronchiolar hyperplasia was seen in a few mice exposed to 40 ppm for 12 months and in most mice exposed to 80 or 160 ppm; by 24 months bronchiolar hyperplasia was seen in up to 40% of the mice exposed to 20 ppm and in more than 75% of those exposed to 40, 80 or 160 ppm (Cruzan et al., 2001). Green and coworkers demonstrated that metabolism of styrene by CYP2F2 is necessary to cause the cytotoxicity (Green et al., 2001a).

Exposure of B6C3F1 mice to tumorigenic 750 ppm ethylbenzene exposures resulted in significantly increased S-phase DNA synthesis in the small airways after 1 week treatment (measured by BrdU incorporation); S-phase synthesis remained elevated after 4 weeks of exposures (non-significant approximate 2X increase; Stott, 2003). In addition, a re-evaluation of the mouse lung tissues from the ethylbenzene bioassay identified the presence of multifocal bronchiolar/parabronchiolar hyperplasia at the 750 ppm tumorigenic exposure level (Brown, 2000).

Administration of these chemicals results in GSH depletion. Phimister and coworkers (2004) demonstrated that administration of naphthalene resulted in GSH depletion. They further reported that lung GSH depletion precedes cellular injury, that lung GSH is depleted by levels of naphthalene that do not deplete liver GSH, and that liver GSH is not able to maintain lung GSH at normal levels following naphthalene administration. Carlson and coworkers have demonstrated glutathione (GSH) depletion in lung of mice

administered 200 mg/kg styrene ip, which lasted through 6 hours, but returned to normal levels by 12 hours (Turner et al., 2005).

B. 4. Cell replication

The mouse terminal bronchioles respond to the cytotoxic injury by generating replacement Clara cells. Increased cell labeling after short-term exposure has been demonstrated for styrene, naphthalene, ethylbenzene, and coumarin. Long-term exposure results in continued bouts of cytotoxicity and cell replication. Continually elevated cell replication leads to overproduction of Clara cells, leading to cellular crowding, followed by hyperplasia which can eventually extend into alveolar ducts (Cruzan et al., 2001). No increase in cell replication rates have been found in alveolar cells of mouse lungs from any of these compounds. No increase in cell replication rates was found in the lungs of rats exposed to styrene or ethylbenzene.

B. 5. Tumors

For coumarin (NTP, 1993), naphthalene (NTP, 1992), styrene (Cruzan et al., 2001), ethylbenzene (NTP, 1999), cumene (isopropylebenzene) (NTP, 2007a), alpha-methylstyrene (isopropenylbenzene) (NTP, 2007b), divinylbenzene (NTP, 2007c) and benzofuran (NTP, 1989), lung tumors were increased in mice, but not in rats. Tumors were found in the outer layer of the lung where the terminal bronchioles and alveoli intersect. Tumors generally encompass areas of alveoli and bronchioles and are termed "bronchioloalveolar adenomas" or "alveolarbronchiolar adenomas," depending on the pathologist. For all the chemicals in this class, tumors occurred late in life and were not life-shortening; i.e., increased tumors were found only at study termination. In general, the increases were in benign tumors. In the case of styrene, increased lung tumors were found only at the end of the 24-month study, but not at the 12 and 18 month interim sacrifices (Cruzan et al., 2001).

The incidence of lung tumors was not increased in mice exposed to dihydrocoumarin (not able to form 3,4-epoxide), 4-methylstyrene (not able to form 4-hydroxystyrene), mixture of 3- and 4-methylstyrene (vinyltoluene, not able to form 3- or 4-hydroxystyrene), styrene-7,8-oxide, or 1 phenylethanol (side-chain oxidation product of ethylbenzene).

C. Adequacy of Evidence of MOA in animals

C.1. Strength of Association

Chronic inhalation exposure of ethylbenzene, styrene, naphthalene, cumene, alpha-methylstyrene, divinylbenzene, and coumarin have all been shown to increase the incidence of lung tumors among mice, but not rats. Cytotoxicity and increased cell replication have been studied in coumarin, naphthalene, styrene, and ethylbenzene; in

mice, all four cause terminal bronchiolar cytotoxicity and increased cell replication at exposure levels comparable to the tumorigenic levels (Table 2). For coumarin, naphthalene, and styrene, it has been demonstrated that inhibition of CYP2F2 inhibits the cytotoxicity and cell replication. Structurally similar chemicals (dihydrocoumarin, 2-, 3-, or 4-methylstyrene) that cannot be oxidized by CYP2F2 to active intermediates did not cause cytotoxicity or mouse lung tumors. Other chemicals have not been tested.

C.2. Consistency of Association

Cytotoxicity from these chemicals occurs in organs with high levels of CYP2F family. CYP2F2 (mouse) is expressed largely in Clara cells in the lung airways (most notably in the terminal bronchioles) and in the nasal olfactory epithelium, with little or none present in the liver. Extensive research has shown that there is a strong association between CYP2F expression levels and tissue susceptibility to naphthalene cytotoxicity (Buckpitt et. al., 2002). Styrene (Cruzan et al., 1997, 2001), naphthalene (NTP, 1992), cumene (NTP, 2007a), and alpha-methylstyrene (NTP, 2007b) cause cytotoxicity in the terminal bronchioles and nasal olfactory epithelium in mice. Ethylbenzene causes cytotoxicity in the terminal bronchioles, but not in the nasal olfactory epithelium at the concentrations tested (NTP, 1999). In rats, CYP2F4 is expressed mainly in the nasal olfactory epithelium, with lesser amounts in the lung. Styrene (Cruzan et al., 1997, 1998), naphthalene (NTP, 2000), cumene (NTP, 2007a), and alpha-methylstyrene (NTP, 2007b) cause cytotoxicity in the nasal olfactory epithelium of rats, but not in the lung terminal bronchioles. Ethylbenzene does not cause cytotoxicity in either lung or olfactory epithelium in rats (NTP, 1999). Coumarin does not cause cytotoxicity in rat lung or nasal olfactory epithelium. In humans, CYP2F1 is expressed at very low levels in the lung, much lower than CYP2F4 in the rat. Therefore, it is not surprising that these chemicals have not been reported to cause cytotoxicity in human lung cells.

C.3. Specificity of Association

Mice have a much greater number of Clara cells than do rats, which have a much greater number than humans. In addition, mouse Clara cells have much more CYP2F2 than the amount of CYP2F4 found in rat Clara cells. Human lung Clara cells have barely detectable levels of CYP2F1. Thus mice have the greatest number of target cells for toxicity, and those target cells have the greatest capacity to produce toxic metabolites.

Toxicity in mice occurs in 2 organs which contain high levels of CYP2F2: nasal olfactory mucosa (chronic cytotoxicity, limited cellular replacement, cells replaced with respiratory-like cells), and lung (chronic cytotoxicity, rapid cellular replacement in kind, hyperplasia). Toxicity in both olfactory mucosa and Clara cells is prevented if CYP2F2 is inhibited by 5P1P. In rat lung and liver, with very little CYP2F4, these chemicals are metabolized primarily via CYP2E1. Rat nasal olfactory tissue contains a large amount of CYP2F4, in addition to CYP2E1 (Green, 2001b). In rat nasal olfactory tissue, large

amounts of the toxic metabolites from these compounds are formed and cytotoxicity is seen from many of them.

D. Qualitative Relevance of the Animal MOA for Humans

The key events for this mouse lung tumor MOA are presented in Table 2.

Lung tumors are quite prevalent in humans, mostly related to cigarette smoking. These are thought to arise from bronchiolar cells and may involve cytotoxicity, as well as genotoxicity. This suggests that cytotoxicity in bronchioles of humans from chemicals could contribute to the formation of lung tumors.

The MOA proposes that the toxic effects in mice are due to metabolism by CYP2F2. Rats have lower levels of CYP2F4 in terminal bronchioles and do not produce sufficient metabolites to cause cytotoxicity or lung tumors. Humans have much lower amounts of CYP2F1 and one would expect they would produce much lower levels of cytotoxic metabolites than in mice or even rats. If human CYP2F1 could produce sufficient metabolites from a chemical to produce bronchiolar cell cytotoxicity, it could conceivably lead to lung tumors

Table 2. 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 to 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
Alpha-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

E. Quantitative Relevance of the Animal MOA for Humans

Given that the qualitative impacts of the proposed MOA on tumor outcomes are not fully defined, quantitative differences between mice and humans must also be considered and include: (1) Rodent exposures in the bioassays are orders of magnitude higher than expected human exposure; (2) Mouse lung has a larger fraction than the

human lung with respect to Clara cells (Plopper et al., 1980a, b); (3) Rates of metabolism for these chemicals in lung microsomes exhibit clear species differences, with rates in mice being greater than the corresponding rates in humans (Green et a., 2001; Vassallo *et al.*, 2004; Saghir *et al.*, 2006) and (4) Background rates for lung tumors are higher in male mice (~14%) than in humans (~7%, SEER, 2006). Given these species differences, the MOA is assumed to be plausible in humans, but humans are expected to be much less sensitive than mice to the pulmonary effects of these chemicals. Because rat lungs contain more CYP2F4 than human lungs contain CYP2F1 and rats do not develop cytotoxicity or lung tumors from these chemicals, it is *very unlikely* that any chemical that causes mouse lung tumors by this MOA and does not cause rat lung tumors will cause human lung tumors.

Conclusion on Mode of Action: The mouse lung tumors are generated following CYP2F2 metabolism in terminal bronchioles; the resulting unique metabolites cause cytotoxicity, leading to regenerative hyperplasia and eventually tumors.

Detailed Recommended Text Changes to the Draft Background Document on Styrene:

p. x, line 11: Cruzan et al., 1998 reported dose-related decrease in mammary tumors, not included.

p. xiv, line 13: One reviewer considered there to be increased CAs in highly exposed workers, another reviewer disagreed. Need to acknowledge this is not agreed on.

p. xiv, line 17-25 The summary of the mechanistic data relied only on the conclusions of the Harvard Panel (Cohen et al., 2002). IARC had different conclusions; they concluded MOA not likely in humans.

p. 172, line 24: Reported Huff analysis of combined mammary tumors: not appropriate because fibroadenomas, not related to adenocarcinomas. McConnell et al. (1986) indicate that mammary fibroadenomas should not be combined with malignant mammary tumors unless a continuum has been demonstrated within a given study. No such continuum was demonstrated in the Beliles drinking water study. Therefore, combining them, as Huff did (1984) is not appropriate and should be removed from the Document.

McConnell EE, Solleveld HA, Swenberg JA, Boorman GA. (1986). Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. JNCI 76: 283-289.

p. 183, line 12: Huff interpretation. This should be removed; see above comment.

p. 183, line 21: Conti et al., 1988, concludes that styrene by inhalation caused increased mammary tumors. The incidence of tumors in the treated groups were within the historical control range reported by Charles River for Sprague-Dawley rats. Based on 28 studies: range of adenocarcinomas: 8.6-58%, mean 22%; fibroadenomas: 13-62%, mean 38%.

> Charles River Laboratories. (2004) .Compilation of Spontaneous Neoplastic Lesions and Survival in Crl:CD (SD) Rats from Control Groups(Information Prepared by *Mary L.A. Giknis, Ph.D., Charles B. Clifford, D.V.M., Ph.D.),* Charles River Laboratories, Wilmington, MA.

The important finding of the Cruzan et al., 1998 rat study was a dose-related *decrease* in mammary adenocarcinomas, which did not verify the reported increased mammary tumors by Conti et al. at much lower exposure concentrations in the same strain of rat. Table 1, found earlier in these comments, demonstrated the response of mammary tissue in all the 8 rat studies. The only increases were at the lowest doses tested. Similar doses in other studies did not confirm the finding of increased mammary tumors.

p. 185, Table 4-11. The results under inhalation do not reflect the results of the rat inhalation studies of styrene accurately. One study reported increased malignant mammary tumors, but another reported no increase at similar doses and decreased malignant mammary tumors at higher doses. From a scientific standpoint, the decrease cannot be ignored.

p. 187, line 6: This is, in general, an accurate presentation. However, the Kraeling and Bronaugh study reported on two chemicals. However, the study *tables* should be referenced, and not the *abstract*, to determine that 1.2% (line 22) of the styrene penetrated the skin, 91% of that which penetrated was in the receptor fluid (line 23), and only 0.1% (line 24) remained in the skin.

p. 188, line 2: The Riihimaki and Pfaffli study is a bit confusing. The text suggests that there were 3 subjects tested with styrene, not 10 as indicated in the NTP document, and the comparison on estimated percent absorbed is really based on large differences in the exposure levels (600 ppm for percutaneous and 10 ppm for inhalation), thus it actually has little meaning.

p. 188, line 15: The Luderer et al. study is really a *review*, not a research paper. SIRC could not locate the recalculation on dermal uptake which is cited in the NTP document.

p. 189, line 13: Hofmann et al. demonstrated that rat lungs exposed to 1000 ppm styrene produced 8 times as much SO as mouse lungs exposed to 40 ppm. The

conclusion from the Hofmann paper was that SO is not responsible for producing mouse lung tumors.

p. 192: Figure 5-1 has one questionable metabolite. The phenylglycine in the figure is not mentioned by anyone other than Manini (to whom the figure is attributed). In Manini's paper it is noted that this is a *hypothesized* metabolite that has never been identified in either animals or humans, therefore it would be more accurate to omit it.

p. 193, lines 4 and 5: It should be noted that only one of the human lung samples had any measureable styrene-metabolizing activity. The way the document current reads, one would assume all the human lung samples did. This is a *very* important distinction when attempting to determine species differences in bioactivation.

p. 195, line 7 to 11: The section in the document which describes the Boogaard et al. study is somewhat misleading. Boogaard really is an inhalation study followed by isolation of the cells for analysis. The last sentence of this section (lines 12 and 13) does not make a great deal of sense --type II cells and Clara cells are found in different parts of the lung (alveolae versus bronchioles), so this is misleading.

p. 197, line 27: The figure is really about 73%, not one-half.

p. 198, line 14: SIRC could not find the information in the paper cited for total CYP450 as mentioned in the NTP document.

p. 199, line 29: Greater toxicity in CYP2E1 knockout mice exposed to 4VP does *not* indicate that 4VP is the toxic agent. 4VP is not toxic when administered in animals pretreated with 5P1P. These studies indicate that CYP2E1 is *not* important in lung toxicity from styrene and 4VP, but metabolism from CYP2F2 *is*.

p. 200, line 4: The studies reported by Arand et al. really deal with mutant forms of the enzyme. Relevance to the importance to styrene metabolism in an intact organism is unclear.

p. 204, line 11: The assessment of neurobehavioral effects from workplace styrene exposure remains unclear, with conflicting results reported ranging from slight effects as low as 22 ppm to no effects as high as 100 ppm. Insufficient details were provided in the Benignus *et al*., 2005 paper to reconstruct and, therefore, fully assess the validity of their meta analysis of neurobehavioral effects of styrene. However, their extrapolation of urinary styrene metabolites into air styrene exposures did not use typical extrapolation relationships, and did not account for reductions in workplace styrene exposures during the course of the studies included. Two of the four studies included in evaluation of styrene effects on CRT do not meet the accepted criteria for CRT endpoints. Their use of a linear model precludes estimation of a no-effect level, and is not justified by the data. The assumption of a cumulative (duration) effect is counter to

the results of two of the four CRT studies, which explicitly looked for, but found *no* effect of duration of exposure on CRT. Further, any change in color discrimination from styrene exposure has no clinical impact, contrary to their assertion, and no impact of styrene exposure on driving ability or automobile accidents has been demonstrated.

p. 207, line 13: Delzell determined the effect was not present in the updated cohort.

> Delzell E; Sathiakumar N; Graff J; Matthews R. 2005.Styrene and ischemic heart disease mortality among synthetic rubber industry workers. J Occup Environ Med. 47:1235-1243.

p. 208, line 4: The prolactin levels of the workers were within the normal human range. Thus, the significance of this observation is unclear.

p. 214, line 27: Gamer et al. reported that 4VP produced nearly double cell proliferation (19x) than SO (10x) and at 1/3rd dose.

p. 219, lines 22 to 27: The last part of the first paragraph should cite Filser et al. (1999) rather than Cohen et al.

p. 219, line 20: For rats, should be 2.5 not 2.6.

p. 219, last line: Statement regarding Trevor Green's published work is incorrect. The rate for microsomal epoxide hydrolase activity was faster in the rat.

p. 220, line 24: The work by Linhart is a review of the work of Carlson et al. and not original data.

p. 222, lines 25 to end of section: The material on regarding the toxicokinetic analysis of Cohen et al. is a problem. In a paper by Csanady, G.A, Kessler, W., Hoffmann, H.D., and Filser, J.G. entitled "A toxicokinetic model for styrene and its metabolite styrene-7,8-oxide in mouse, rat and human with special emphasis on the lung" which appeared in Toxicology Letters, vol. 138, pages 75-102, on page 77 in a description of the model development, it is stated that "Model compartments representing pulmonary blood, liver, muscle, fat, and the richly perfused tissue group are connected by the arterial and the venous blood, which are also described as distinct compartments. Tissue uptake of ST and SO from the arterial blood entering the compartments is described as a perfusion-limited process. Metabolism of ST and SO is assumed to occur in liver and lung solely. Metabolism of SO in the liver is modeled as previously described (Eq. (5); Csanady et al., 1994). Unfortunately, in this equation there was a typographic error, which was overlooked by some modelers who therefore tried unsuccessfully to copy our previous model (Cohen et al., 2002). In order to avoid possible future errors, all equations describing the new model are presented in Appendix A." Therefore the validity of the conclusions from the study of Cohen et al. is unknown.

p. 222, line 31: The conclusion of Cohen was based on their PBPK model. Other models give different results. It is important to understand the construction of the 3 models because they largely explain the different outputs. The Cohen model assumes that all styrene metabolism takes place in the liver and that lung SO level is determined by the blood SO level. The Filser model assumes that styrene is metabolized in both the liver and the lung, but averages styrene lung metabolism over the whole lung, although only the Clara cell are capable of metabolizing styrene; this model assumes a uniform distribution of styrene in all lung cells. The Sarangapani model is based on metabolism of styrene in both the liver and specifically in the terminal bronchioles of the lung and predicts styrene oxide levels in the terminal bronchioles.

p. 226, line 4: The Document states that there is some evidence that O^6 adducts build up over time. Very low levels were found after 95 weeks of exposure to 1000 ppm in rats and were not detected in mice exposed to 160 ppm for 2 weeks.

p.237, line 16-25: The significance of DNA adducts in NMRI mice exposed to 175 or 350 ppm is not clear, since exposure to these levels is lethal to some CD-1 and B6C3F1 mice (up to 50% at 250 ppm). Morgan et al., 1993, Cruzan et al., 1997.

p. 267, line 17: the Document lists 17 human CA studies as positive, IARC lists 3 of those as negative. The Document indicates that positive results were observed in studies with higher levels of exposure. In the accompanying table, the human CA studies are arranged by exposure. Note that the proportion of positive and negative studies does not change with exposure concentration and the study with the highest exposure (Fleig 1978) was considered as negative by IARC. In the RoC Document there were 8 studies with reported exposures above 50 ppm; 5 were + and 3 – (IARC did not report one of these studies, which was considered negative by the RoC Document). If one considers the highest 15 exposure studies (above 25 ppm), there were 10+, 5-; in the lower half there were 9+ and 6-. Note that 3 of 4 studies with the lowest exposures were reported as +. A more appropriate statement is that there are mixed results for CA studies in humans.

Table 3: Human CA Studies by Exposure

Author	date	E/C	ppm	urinary	Results	IARC
Fleig	1978	14/20	50-300		+	-
Camurri	1983/4	2-7/2-7	<90		+	+
Anderson	1980	36/37	(75)	1204	+	+
Maki-Paakanen	1991	17/17	70		+	+
Jablonica	1988	11/11	58		-	-
Theiss	1980	24/31	58		-	-
Forni	1988	40/40	<57		+	+
Vodicka	2004a	86/42	(50)	798	-	NR
Somaroska	1999	44/19	46		+	?
Sorsa	1991	109/54	43		-	-
Hogstedt	1979	6/6	11-92		+	+

Meretoga	1977	10/5	(45)	721	+	NR
Watanabe	1981	16/13	(41)	650	-	-
Meretoga	1978	26/6	(35)	570	+gaps	+
Tomanin	1992	50/54	26		+	+
Dolmerski	1983	30/2	<23		+gaps	NR
Oberheitman	2001	14/7	<23		-	NR
Maki-Paakanen	1987	21/21	23 (8-60)		-	-
Watanabe	1983	18/16	(22)	350	-	-
Tates	1994	46/23	20		+	NR
Artuso	1995	46/51	(20)	319	+high group	NR
Anwar	1995	18/18	(20)	328	+	NR
Pohlova	1985	2 plants	(14)	<226	+ at one	-
Hansteen	1984	18/9	13		+gaps	-
Hagmar	1989	11/14	13		-	-
Migliore	2006	72/89	8.5 (1-123)		-	NR
Vodicka	2004c	84/16	<3		+	NR
Van Sittert	1985	200/135	<1.5		+	NR
Lazutka	1999	97/90	<1.4		+	NR
Biro	2002	10/25	NR		-	NR

p. 270, line 21: SIRC questions why "(styrene-industry sponsored)" is added after the Henderson and Speit reference? A study should be evaluated on its scientific merits, not its sponsorship. Other studies cited do not reference sponsorship. Such a notation would seem to carry an implied message that the data should be considered suspect purely because the research was industry–sponsored. If NTP believes the study is flawed due to industry sponsorship, this should be so stated from a scientific perspective. Regardless of the intent, *the parenthetical notation should be deleted*. Further, the Document provides no conclusion on the Henderson review. Henderson and Speit point out deficiencies in a number of studies, both positive and negative, and recommend those studies *not* be included in an evaluation. Since the Document lists the Henderson review, it should make some comment on the conclusions of that review. Should deficient studies be included in an evaluation, or omitted?

p. 305, line 4. It is confusing that most of the early studies of SCE in styrene-exposed workers were negative when exposures in the industry were much higher, and most of those conducted since 1994 were reported as increased SCE when exposures were at least 4 fold lower than in the earlier studies.

p. 324, Table 5-18. Table does not match description above. Text says mutation studies in humans are "inconclusive to weakly positive," while the table says "weakly positive." Text says "results of clastogenic effects are inconclusive"; Table indicates CAs "weakly positive", while SCE and MN had "equally positive and negative results." On page 290, the Document says there is no compelling evidence of effect on micronucleus in humans. This is not the same as "equal numbers of positive and negative studies."

p. 325, line 5: Statement by Huff is inappropriate for SO: SO is not highly reactive, it has a half-life in blood *in vitro* of ~30 minutes. There is no indication of increases in "liver, harderian gland, and circulatory system neoplasms in mice," "Zymbal's gland and brain tumors in rats" or "mammary gland tumors in both rats and mice" from exposure to SO. There were only forestomach tumors in rats and mice, and liver tumors in the low dose of mice, as stated in the next sentence. There is no reason to put this sentence in and it indicates that SO is NOT like other epoxides described by Huff.

p. 325, line 1. This section makes an assumption that the tumorigenic activity of styrene is caused by styrene oxide. This is not supported by the scientific data. Cohen et al. conclude that differences in SO levels do not explain mouse lung tumors, Hofmann et al., conclude that mouse lung tumors are not caused by SO, Cruzan et al. (2002, 2005) indicate that CYP2F2 generated metabolites are responsible and that ring-oxidized metabolites are likely the cause.

p. 325, line 7: Lijinsky reported increased liver tumors in the low dose only in *male* mice exposed to styrene oxide. There was no increase in female or in high-dose males.

p. 325, line 23: IARC concluded that lung metabolism of styrene was the likely MOA and that is not a likely MOA in humans.

p. 325, line 24, 25: Overall, the data do not support increased mammary tumors or lymphatic cancers in rats from styrene exposure. See comments above.

p. 325, line 26, 27: See comments on human studies; this needs to reflect an overall assessment of the studies, not just "have been reported in some studies."

p. 329, line 18: The studies of Chung et al. 2006 are basically irrelevant to the lung cytotoxicity. They were conducted in a liver transgenic cell-line that overexpresses CYP2E1. Studies by Carlson's lab have shown that inhibition of CYP2E1 or genetic removal (CYP2E1 knockout mice) does not reduce the lung cytotoxicity of styrene. Green et al. (2001) demonstrated that inhibition of CYP2F2 by 5P1P prevented the cytotoxicity of styrene in mouse lung and nasal epithelium.

p. 330, line 7: The studies by Gadberry do not necessarily indicate that SO is responsible for the lung cytotoxicity of styrene. 4-vinylphenol was toxic at 5 times lower concentration than was SO. It is likely that a further metabolite of both of them is responsible for the cytotoxicity. Studies by Bartels et al., 2005 indicated that 3,4-dihydroxystyrene and 4-hydroxystyrene-7,8-oxide could be trapped with excess GSH from incubation of styrene or 4-VP using lung microsomes.

Bartels M, Rick D, Zhang F, Leibold E, Gelbke H, Cruzan G. (2005). In vitro metabolism of 4-vinylphenol and styrene in mouse, rat and human microsomes. The Toxicologist 84: abstract 1563.

p. 330, line 15: Although Cohen et al. (2002) identified CYP2E1 as important in the cytotoxicity of styrene, later studies in Carlson's lab have demonstrated that CYP2E1 does not play an important role lung cytotoxicity.

p. 330, line 27: The Cohen model assumes that all metabolism of styrene occurs in the liver and does not include lung metabolism. Thus it cannot explain mouse and rat differences.

p. 331, first paragraph: The conclusions of Cohen et al. about styrene oxide lead logically to the conclusion that SO is not responsible for the cytotoxicity from styrene in mouse lung terminal bronchioles.

p. 331, end of paragraph 1: Cruzan et al., 2002 proposed a mode of action based on the available metabolic, pathologic, and tumor data. This proposed MOA was that CYP2F2 in the terminal bronchioles of mice generates metabolites that cause cytotoxicity, leading to regenerative cell proliferation, hyperplasia and eventually tumors. Additional studies since then in Carlson's lab, in Cruzan et al., 2005 and Bartels et al., 2005 have further supported this MOA. *This NTP document needs to address this proposed MOA in the same manner that it addressed the discussions of the MOA by Cohen et al. The hypothesis of the MOA needs to be stated, along with a discussion of the supporting data, with a conclusion of the strengths and weaknesses.*

p. 331, section 5.5.4: The genotoxicity and cytotoxicity data cited in this section do *not* support any conclusion on the MOA. The role of CYP2F2 metabolism needs to be addressed.

REFERENCES

Following are references relating to the discussion of the mouse lung tumor mode of action (section 3: MOA for Mouse Lung Tumor, beginning page 11), which have not already been cited in the NTP Background Document.

In the interest of brevity, citations of studies referenced in the NTP Document and noted in these comments may be found in the References section of the NTP Draft Background Document for Styrene, beginning at page 377: http://ntp.niehs.nih.gov/files/Styrene_Whole_Document.pdf

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