BEFORE THE
NATIONAL TOXICOLOGY PROGRAM

COMMENTS OF THE AMERICAN CHEMISTRY COUNCIL
Ethyl Benzene Panel
Plastics Division

ON

REPORT ON CARCINOGENS; AVAILABILITY OF THE DRAFT BACKGROUND DOCUMENT FOR STYRENE; REQUEST FOR COMMENTS ON THE DRAFT BACKGROUND DOCUMENT FOR STYRENE; ANNOUNCEMENT OF THE STYRENE EXPERT PANEL MEETING

Availability of Background Documents; Request for Comments; and Announcement of Meeting	)

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EXECUTIVE SUMMARY

The Ethylbenzene panel and the Plastics Division of American Chemistry Council (ACC) are submitting these comments in response to the notice dated May 20, 2008, announcing the availability of the National Toxicology Program's (NTP) Report on Carcinogens Draft Background Document for Styrene (Draft Document). This Draft Document will be peer-reviewed by an expert panel to be convened July 21-22, 2008. ACC offers these comments for consideration by the expert panel in its review of the scientific studies of styrene and their relevance to human health as part of the process for developing the 12th Report on Carcinogens (RoC). Since these comments emanate from two separate groups within the ACC we would like to request your consideration in having two witnesses at the hearing.

These comments submitted by ACC provide a compelling, scientifically supported and justified mode of action (MOA) for the carcinogenic effects of styrene in mouse lung which demonstrates that this carcinogenic response is species specific and therefore not relevant for extrapolating to humans. For all of the reasons discussed in these comments, ACC believes the Draft Document should be revised to consider fully this hypothesized alternative mode of action (MOA) for mice lung tumors, as well as the evidence supporting this MOA, and that the NTP Draft Document should be revised because these mouse-specific tumors provide insufficient evidence to support listing of styrene in the forthcoming RoC. Importantly, NTP's listing criteria anticipates substances, such as styrene, "for which there is evidence of carcinogenicity in laboratory animals, but there are compelling data indicating that the agent act through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans."

The scientific evidence that supports the alternative MOA for mouse lung tumors discussed in these comments includes the following:

- Tumors in animal studies are limited to one organ and species -- mouse lung (IARC, 2002).

- There are no positive genotoxicity studies in the target organ. Chromosomal aberrations following inhalation exposure at 125, 250 or 500 ppm for 2 weeks were negative (Kligerman *et al*., 1992). There was no initiation of lung tumors following 6 weeks of ip administration of styrene in A/J mice followed for 20 weeks (Brunneman *et al*., 1992).

- *In vivo* chromosomal aberrations (CA), micronuclei (MN) studies in rats and mice are negative (IARC, 2002; NTP, 2008).

- DNA adducts from styrene exposure occur at low levels ($<1 \times 10^7$ nucleotides) and are not specific or increased in mouse lung compared to other organs in mice or rats (Boogaard *et al*., 2000).

- Target organs for cytotoxicity from styrene are consistent with location of CYP2F (mouse lung Clara cells and nasal olfactory epithelium, and rat nasal olfactory epithelium) (Cruzan, 2001; 2002). There is no lung cytotoxicity in rats exposed to 1000 ppm styrene for up to 2 years; rats have lower levels of CYP2F4 (the rat ortholog to mouse 2F2) and cannot produce sufficient metabolites to cause toxicity.

- Inhibition of CYP2F2 with 5P1P inhibits mouse lung cytotoxicity (Green *et al.*, 2000a, b; Carlson *et al.*, 2002). There was no lung or nasal toxicity in mice inhaling styrene at 160 ppm for 2 weeks and co-treated with 5PIP, but increased cell replication was observed without 5P1P co-treatment.

- Inhibition of CYP2E1 (or CYP2E1-knockout mice) does not reduce cytotoxicity from styrene or 4VP (4-hydroxystyrene, a ring-oxidized metabolite likely generated by CYP2F2 metabolism) (Carlson, 2004; Vogie *et al*., 2004).

- Ring-oxidized metabolites probably responsible for lung cytotoxicity; 4VP is 5x more toxic than styrene -7, 8-oxide (SO) (Carlson *et al.*, 2002).

- Ring-oxidized metabolites from the structurally related compound coumarin likely drive mouse lung toxicity (Felter *et al*., 2006; Barels *et al.*, 2005).

- Similar toxic and lung tumor responses are seen with both ethylbenzene and cumene, which are not converted to vinyl epoxide (NTP, 1999; NTP, 2007a).

- Methyl group insertion at 3 or 4 position of the aromatic ring (p-methylstyrene, 3-vinyltoluene) does not result in increased mouse lung tumors, although there is still some positive genotoxicity studies *in vitro* (IARC, 1994).

Apart from the compelling scientific merit of including meaningful consideration of the alternative MOA for mice lung tumors, ACC believes that the Draft Document must include this information to be consistent with Section 515(a) of the Treasury and General Government Appropriations Act for Fiscal Year 2001 (commonly referred to as the Information Quality Act (IQA)) and the U.S. Department of Health and Human Services (HHS), National Institutes of Health (NIH) Guidelines implementing the Office of Management and Budget's (OMB) IQA Guidelines. These authorities require the use and reliance upon "the best available science and supporting studies conducted in accordance with sound and objective scientific practices, including peer reviewed studies when available." Moreover, the IQA requires that NTP use "data collected by accepted methods or best available methods" and "be comprehensive, informative, and understandable."

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INTRODUCTION

The American Chemistry Council is submitting these comments in response to the notice dated May 20, 2008, announcing the availability of the National Toxicology Program's (NTP) Report on Carcinogens Draft Background Document for Styrene (Draft Document).[1] This Draft Document will be peer-reviewed by an expert panel to be convened July 21-22, 2008. ACC offers these comments for consideration by the expert panel in its review of the scientific studies of styrene and their relevance to human health as part of the development of the 12th Report on Carcinogens (RoC).

The Draft Document includes evaluation of some, but not all, of the relevant scientific literature on cancer studies of workers exposed to styrene, cancer studies of experimental animals exposed to styrene, and mode of action (MOA) analyses. The Draft Document appears to conclude that styrene is carcinogenic in humans and cites both human and animal data to support that conclusion. Unfortunately, the Draft Document has not considered nor integrated other clearly relevant and important analyses of the epidemiology and animal cancer MOA information that, when examined in a weight-of-evidence approach, clearly do not support a conclusion that styrene is "reasonably anticipated to be human carcinogen."

In the epidemiology studies, slightly higher but not statistically significant standardized mortality ratios (SMR) or relative risks (RR) from a few published studies are cited as evidence of carcinogenicity. However, statistically significant decreases in cancer risk are

[1] 73 Fed. Reg. 29139 (May 20, 2008).

apparently and inappropriately not included in the analysis. Importantly, the Draft Document neglects to note that increases in cancer incidence reported in the cited studies were attributed by study authors to other chemicals, and not to styrene specifically

In the cited animal cancer bioassays, recent and comprehensive reviews by the International Agency for Research on Cancer (IARC), the European Union (EU), the Harvard Center of Risk Analysis, and the Agency for Toxic Substances and Disease Registry (ATSDR) have all concluded styrene does not increase cancer in rats, yet the Draft Document asserts increased mammary tumors and leukemia/lymphoma in rats. An integration and meaningful consideration of the entire rat database does not support the conclusion of increased cancer in rats. In addition, with regard to the increase of styrene-induced mouse lung tumors, the NTP analysis of MOA relies on selected datasets and neglects to include important scientifically relevant studies that offer important insight into the lack of relevance of styrene rodent carcinogenicity to human health. ACC's comments focus on an alternative MOA that has been thoroughly published in the scientific literature, and yet was ignored in the Draft Document.

The Draft Document hypothesizes that styrene is carcinogenic through its metabolite styrene-7,8-oxide (SO). It cites several studies that support this hypothesis, but ignores important and relevant data that do not support the hypothesis. In the metabolism and toxicity sections of the Draft Document, in particular, many of the studies cited appear to support a different hypothesis, *i.e.,* that styrene produces mouse lung toxicity and tumors through metabolism by a cytochrome P450 2F2 isozyme (CYP2F2) that is unique to this species. Importantly, there is no mention or consideration of this alternative MOA in the Draft Document.

Through this alternative MOA, CYP2F2 metabolizes styrene, and several structurally related compounds, to form unique metabolites that cause cytotoxicity in the terminal bronchioles of mice but not of humans. The resulting ongoing regenerative cell proliferation in mice leads to prolonged hyperplasia and eventually lung tumors that are specific to mice (Cruzan *et al*., 2002). 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." Moreover, the EU cancer animal bioassay stated that "it is reasonable to conclude that the lung tumours seen in mice are unlikely to be of any relevance for human health" (EU, 2007). The current ATSDR (2008) draft states, "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."

The scientific evidence that supports this MOA includes the following:

- Tumors in animal studies are limited to one organ and species -- mouse lung (IARC, 2002).
- There are no positive genotoxicity studies in the target organ. Chromosomal aberrations following inhalation exposure at 125, 250 or 500 ppm for 2 weeks were negative (Kligerman *et al*., 1992). There was no initiation of lung tumors following 6 weeks of ip administration of styrene in A/J mice followed for 20 weeks (Brunneman *et al*., 1992).
- *In vivo* chromosomal aberrations (CA), micronuclei (MN) studies in rats and mice are negative (IARC, 2002; NTP, 2008).
- DNA adducts from styrene exposure occur at low levels ($<1 \times 10^7$ nucleotides) and are not specific or increased in mouse lung compared to other organs in mice or rats (Boogaard *et al*., 2000).
- Target organs for cytotoxicity from styrene are consistent with location of CYP2F (mouse lung Clara cells and nasal olfactory epithelium, and rat

nasal olfactory epithelium) (Cruzan, 2001; 2002). There is no lung cytotoxicity in rats exposed to 1000 ppm styrene for up to 2 years; rats have lower levels of CYP2F4 (the rat ortholog to mouse 2F2) and cannot produce sufficient metabolites to cause toxicity.

- Inhibition of CYP2F2 with 5P1P inhibits mouse lung cytotoxicity (Green *et al.*, 2000a, b; Carlson *et al.*, 2002). There was no lung or nasal toxicity in mice inhaling styrene at 160 ppm for 2 weeks and co-treated with 5PIP, but increased cell replication was observed without 5P1P co-treatment.

- Inhibition of CYP2E1 (or CYP2E1-knockout mice) does not reduce cytotoxicity from styrene or 4VP (4-hydroxystyrene, a ring-oxidized metabolite likely generated by CYP2F2 metabolism) (Carlson, 2004; Vogie *et al*., 2004).

- Ring-oxidized metabolites probably responsible for lung cytotoxicity; 4VP is 5x more toxic than SO (Carlson *et al.*, 2002).

- Ring-oxidized metabolites from the structurally related compound coumarin likely drive mouse lung toxicity (Felter *et al*., 2006; Barels *et al.*, 2005).

- Similar toxic and lung tumor responses are seen with both ethylbenzene and cumene, which are not converted to vinyl epoxide (NTP, 1999; NTP, 2007a).

- Methyl group insertion at 3 or 4 position of the aromatic ring (p-methylstyrene, 3-vinyltoluene) does not result in increased mouse lung tumors, although there is still some positive genotoxicity studies *in vitro* (IARC, 1994).

Based on the above observations, ACC believes the Draft Document should be revised to consider fully the hypothesized alternative MOA for mouse lung tumors, and to review and evaluate the significant evidence supporting the alternative MOA for mice lung tumors, and to revise the Draft Document to conclude that these mouse-specific tumors provide insufficient evidence to support listing of styrene in the forthcoming RoC. Importantly, NTP's listing criteria anticipates substances, such as styrene, "for which there is evidence of carcinogenicity in laboratory animals, but there are compelling data indicating that the agent act

through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans." (*See* listing criteria at http://ntp.niehs.nih.gov/index.cfm?objectid=03C9CE38-E5CD-EE56-D21B94351DBC8FC3.)

A detailed description of the data supporting this alternative MOA for styrene-induced mouse lung tumors, based on evidence from styrene and other structurally-related chemicals, is set forth below. Following this scientific description is a brief discussion of the reasons why the ACC believes that the Information Quality Act (IQA) and implementing Department of Health and Human Services (HHS) Guidelines require meaningful and explicit consideration of the alternative MOA for mice lung tumors in the Draft Document.

I. THE ALTERNATIVE MOA FOR MOUSE-SPECIFIC LUNG TOXICITY AND TUMORIGENICITY DOES NOT SUPPORT LISTING STYRENE IN THE 12TH RoC

In mice, CYP2F2 metabolism of several chemicals, including styrene, in terminal bronchiolar Clara cells 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 these areas of hyperplasia proceed to form adenomas in the mouse lung, which have a high spontaneous incidence of adenomas in control mice (*see* Table 1. In these mice, a few of the adenomas may progress to carcinomas.

The analogous CYP2F4 in rats may be as capable of forming these cytotoxic metabolites. Rats, however, 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. In humans, 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 (*see* Table 1). The key element of the MOA hypothesis is that the lung-specific toxicity of these chemicals is predicated on their metabolism to cytotoxic metabolites by mouse CYP2F2, which differs in both specificity and rate of metabolism compared to rats and humans.

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Table 1. Examples of Chemicals Shown by Studies to Cause Mouse, but Not Rat, Lung Tumors

Chemical	**Exposure Route and Dose (Strain)**	**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) Inhalation: 0, 20, 40, 80, 160 ppm (CD-1)	0/20; 6/50; 9/50 17/50; 24/50; 36/50; 30/50; 36/50	0/20; 1/50; 3/50 6/50; 16/50; 17/50; 11/50; 27/50
Ethylbenzene	Inhalation: 0, 75, 250, 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	13/50; 6/50; /50; 9/50	2/50; 7/50; 5/50; 6/50

Chemical	Exposure Route and Dose (Strain)	Lung Tumors in Male Mice	Lung Tumors in Female Mice
	(B6C3F1)		
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

A. The MOA's Key Events Have Been Elucidated

As described below, the key events in the MOA are: delivery of the chemical to the respiratory system, metabolism in lung, cytotoxicity in terminal bronchioles, cell replication, and tumors.

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 physio-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). Metabolism and cytotoxicity in lung from oral exposure, however, 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 humans and by blood flow and minute volume, which may affect the quantity of these chemicals delivered to the lungs.

2. Metabolism in Lung

In the mouse, many compounds, including styrene, 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. Data on the metabolism of these compounds in human lung tissue are somewhat limited because of the difficulty in obtaining adequate specimens for testing. Available data, however, 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). Moreover, 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.

a. Styrene

For styrene, the first step in the major metabolic pathway is oxidation to S-styrene-7,8-oxide which accounts for at least 80% of the metabolism of styrene in rats and mice (Sumner and Fennell, 1994; Cruzan *et al*., 2002). Oral administration of styrene-7,8-oxide (SO) 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). These data led the authors to conclude that SO 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 SO (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.

b. Coumarin

The major metabolite of coumarin in rats, mice, and humans is 7-hydroxycoumarin. Coumarin, however, is metabolized by CYP2F2 to coumarin-3,4-epoxide in mouse lung, which rearranges to 2-hydroxyphenylacetaldehyde (Born *et al*., 2002) and has been shown to cause mouse lung cytotoxicity and lung tumors. Inhibition of CYP2F2 by 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).

c. 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.

d. 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 glutathione (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 MOA 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).

e. Cumene

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

3. Cytotoxicity

a. Styrene

The cytotoxicity of styrene in mice has been summarized by Cruzan *et al*. (2002, 2005). For styrene, cytotoxicity has been measured by increased cell replication following three inhalation (40 and 160 ppm) or ip (100 mg/kg) exposures (Green *et al*., 2001a; Kaufmann *et al*., 2005). Similarly, following three exposures, styrene metabolites SO (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 in mice (Green *et al*., 2001a).

b. Coumarin

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) were shown to cause cytotoxicity in the terminal bronchioles of mouse lung, but not rat lung (*see* Table 2 below). 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.

c. Naphthalene

The cytotoxicity from naphthalene is summarized by Buckpitt *et al.* (2002). In sum, 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. Female mice were shown to be more susceptible to naphthalene 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.

d. Ethylbenzene

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 *et al.*, 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 styrene, coumarin, naphthalene, and ethylbenzene results in glutathione (GSH) depletion. Phimister *et al.* (2004) demonstrated that administration of naphthalene resulted in GSH depletion, and 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 GSH depletion in the lungs 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).

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 has 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.

5. Tumors

For coumarin (NTP, 1993), naphthalene (NTP, 1992), styrene (Cruzan *et al.*, 2001), ethylbenzene (NTP, 1999), cumene (isopropylebenzene) (NTP, 2007a), α-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), SO, or 1 phenylethanol (side-chain oxidation product of ethylbenzene).

The Report on Carcinogens Draft Background Document for Styrene contains an evaluation of the cancer studies of workers exposed to styrene, cancer studies of experimental animals exposed to styrene and mode of action analyses. The studies in rats are summarized on pages ix and x, and discussed in detail in Section 4.2, pages 170-179.

The studies are reported on individually, but there is no assessment of the consistency and dose response of this group of studies. In some cases the report of the outcomes of individual studies is misleading or incomplete. The document accurately states that none of the rat studies found increased lung tumors and that none of the gavage studies found any increases in any tumor. However, the document asserts that the drinking water and inhalation studies demonstrate increased mammary tumors in female rats. This assertion is not supported by the data.

The authors of the drinking-water study reported no increase in mammary (or any other) tumors. (Beliles et al., 1985). In an analysis of this and other studies, Huff (1984) asserted that there was a statistically significant trend for increased mammary tumors if one combined the malignant and benign tumors (fibroadenoma, adenoma, adenocarcinoma) in females. According to the NTP publication on combining benign and malignant tumors for statistical analysis, 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 report. This reference should also be removed from the summary table on page 173.

The Conti et al., 1988 report (p. 183, line 21) concludes that styrene by inhalation caused increased mammary tumors. The incidence of total mammary tumors (which should not be combined) and the incidence of malignant mammary tumors in all treated groups was greater than in the controls. The section concludes with the statement that IARC considered the study to

be inconclusive. The report should further add that 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%.

The Cruzan et al., 1998 study is reported in square brackets to suggest it was flawed by incomplete histopathology. However, the histopathologic evaluation of the study follows standard practice, including EPA and OECD study guidelines for carcinogenicity studies, in that all tissues from control and high-dose animals were examined microscopically and organs identified as affected in the high dose were examined in the three lower doses. This reference to incomplete histopathology should be removed.

The document asserts that the incidence of mammary tumors in the controls was abnormally high, based on a 1992 historical control database. The studies in this control database were conducted at least 8 years before the subject study and may not reflect historical control incidences at the time of the study. The rats were obtained from Charles River and the control incidence in the Cruzan et al study was within the historical control range of studies in the Charles River database.

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. The table below (table 2) demonstrates the response of mammary tissue in all the 8 rat studies. The only

increases shown were at the lowest doses tested. Similar doses in other studies did not confirm the finding of increased mammary tumors.

The findings of these studies need to be evaluated in the context of dose-response. Table 2 below was taken from Cruzan et al., 1998 evaluating all the individual dose groups from all the rat studies of styrene. It is clear from this table that the only dose groups suggesting increased mammary tumors were at the low end of the dose range and other studies did not find increased mammary tumors at similar doses.

Table 2 Evaluating all the individual dose groups from all the rat studies of styrene (Cruzan et al., 1998)

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.

The results under inhalation in Table 4-11 (p. 185) do not reflect the results of the rat inhalation studies of styrene accurately. One study (Conti et al., 1988) reported increased malignant mammary tumors, but another (Cruzan et al., 1998) found no increase at similar doses and decreased malignant mammary tumors at higher doses. From a scientific standpoint these data should not be ignored..

The rat studies are presented individually, but not integrated by dose. The summary notes increased mammary tumors by Conti (concentrations 25-300 ppm; control incidence lower than historical controls) and in the low dose of the study by Jersey et al, 1978 (but within the historical control range). The report does not mention that higher exposure concentrations resulted in decreased mammary tumors. Cruzan et al., 1998, demonstrated no increase in mammary at 50 or 200 ppm and significantly decreased mammary tumors at 500 and 1000 ppm. All three studies were conducted by inhalation in Sprague-Dawley rats. The table above shows that only the very lowest doses were reported to increase mammary tumors and all incidences were within the Charles River historical control range based on 24 studies in Sprague-Dawley rats. The Harvard Panel, IARC, NTP CERHR, ATSDR and EU all concluded that styrene does not cause increased tumors in rats.

II. THE EVIDENCE FOR THE ALTERNATIVE MOA IN ANIMALS IS ROBUST AND COMPELLING AND SHOULD BE FULLY CONSIDERED AND INTEGRATED INTO THE BACKGROUND DOCUMENT

A. Strength of Association

Chronic inhalation exposure of ethylbenzene, styrene, naphthalene, cumene, α-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 (*see* Table 3 below). 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.

B. Consistency of Association

Cytotoxicity from ethylbenzene, styrene, naphthalene, cumene, α-methylstyrene, divinylbenzene, and coumarin 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 α-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 α-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. 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. Moreover, 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 two organs that 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.

III. THE ALTERNATIVE MOA IS NOT OPERATIVE IN HUMANS

Lung tumors prevalent in humans are 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.

Through this alternative MOA, 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 therefore would be expected to produce much lower levels of cytotoxic metabolites than in mice or even rats. The key events for this mouse lung tumor MOA are presented in Table 3.

Table 3. 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	Only at 2 years: 40 ppm -- males and 20

Chemical	Metabolism by CYP2F2	Acute Cytotoxicity	Sustained Cytotoxicity	Hyperplasia	Tumors
				years	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

Quantitative differences between mice and humans underscore the MOA's specificity to mice, but not humans. These differences 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 styrene and other chemicals in lung microsomes exhibit clear species differences, with rates in mice being greater than the corresponding rates in humans (Green *et al*., 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). Although the MOA is assumed to be plausible in humans, given these species differences, humans are expected to be much less sensitive than mice to the pulmonary effects of these chemicals. Moreover, rat lungs contain more CYP2F4 than human lungs contain CYP2F1, yet rats have not been shown to develop cytotoxicity or lung tumors from these chemicals. It is therefore highly unlikely that styrene or any other chemical that causes mouse, but not rat, lung tumors by this alternative MOA will cause human lung tumors.

IV. THE IQA COMPELS THE INCLUSION AND MEANINGFUL CONSIDERATION OF THE ALTERNATIVE MOA FOR MICE LUNG TUMORS

In its current form, the Draft Document fails to meet the clear requirements of the IQA.[2] The Act directed the Office of Management and Budget (OMB) to issue government wide guidelines that provide policy and procedural guidance for ensuring and maximizing the quality, objectivity, utility, and integrity of information that federal agencies, including NTP, disseminate to the public. Moreover, the IQA imposes a higher standard of care with respect to influential documents, which the Draft Document plainly qualifies.

To ensure that the requirements of the IQA and the U.S. Department of Health and Human Services' (HHS) implementing guidelines[3] are satisfied, NTP must ensure that the Draft Document does not suffer from a lack of quality, objectivity, utility, or integrity. Because the Draft Document is "highly influential" as this term is defined under the IQA, the standard set forth under the Safe Drinking Water Act (SDWA) applies. This standard requires that NTP use the "best available science and supporting studies conducted in accordance with sound and objective practices, including peer reviewed studies when available." Moreover, the IQA requires that NTP use "data collected by accepted methods or best available methods" and be comprehensive, informative, and understandable.

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[2] 44 U.S.C. 3516 note.

[3] Guidelines for Ensuring the Quality of Information Dissemination to the Public, available at http://aspe.hhs.gov/infoquality/Guidelines/index.shtml.

ACC believes that it has set forth above a compelling, scientifically-supported alternative MOA that is based on published, credible science that plainly falls within the scope of the SDWA standard applicable to highly influential science information such as the Draft Document. NTP must now include and meaningfully consider this information because rigorous scientific discourse requires such consideration; Congress expected no less in passing the IQA, and HHS demands as much under the HHS Guidelines to which NTP information disseminated to the public is subject. Accordingly, ACC respectfully requests that NTP include and meaningfully consider the alternative MOA for mice lung tumors in a manner consistent with its legal obligations pursuant to the IQA.

CONCLUSION

For all the reasons noted above, ACC believes the Draft Document should be revised to consider fully the studies published in the scientific literature which underpin the alternative MOA for mice lung tumors, and revise the Draft Document by integrating these data and this MOA into the assessment, using a weight of evidence framework to arrive at a conclusion with respect to the application of this MOA to human health. ACC believes that there is a compelling, scientifically supported and justified mode of action (MOA) for the carcinogenic effects of styrene in mouse lung which demonstrates that this carcinogenic response is species specific and therefore not relevant for extrapolating to humans, thereby supporting the conclusion that these mouse-specific tumors provide insufficient evidence to support listing of styrene in the forthcoming RoC.

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