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Styrene Respiratory Tract Toxicity and Mouse Lung Tumors Are Mediated by CYP2F-Generated Metabolites

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Mice are particularly sensitive to respiratory tract toxicity following styrene exposure. Inhalation of styrene by mice results in cytotoxicity in terminal bronchioles, followed by increased incidence of bronchioloalveolar tumors, as well as degeneration and atrophy of nasal olfactory epithelium. In rats, no effects on terminal bronchioles are seen, but effects in the nasal olfactory epithelium do occur, although to a lesser degree and from higher exposure concentrations. In addition, cytotoxicity and tumor formation are not related to blood levels of styrene or styrene oxide (SO) as measured in chronic studies. Whole-body metabolism studies have indicated major differences in styrene metabolism between rats and mice. The major differences are 4- to 10-fold more ring-oxidation and phenylacetaldehyde pathways in mice compared to rats. The data indicate that local metabolism of styrene is responsible for cytotoxicity in the respiratory tract. Cytotoxicity is seen in tissues that are high in CYP2F P450 isoforms. These tissues have been demonstrated to produce a high ratio of *R*-SO compared to *S*-SO (at least 2.4 : 1). In other rat tissues the ratio is less than 1, while in mouse liver the ratio is about 1.1. Inhibition of CYP2F with 5-phenyl-1-pentyne prevents the styrene-induced cytotoxicity in mouse terminal bronchioles and nasal olfactory epithelium. *R*-SO has been shown to be more toxic to mouse terminal bronchioles than *S*-SO. In addition, 4-vinylphenol (ring oxidation of styrene) has been shown to be highly toxic to mouse terminal bronchioles and is also metabolized by CYP2F. In human nasal and lung tissues, styrene metabolism to SO is below the limit of detection in nearly all samples, and the most active sample of lung was approximately 100-fold less active than mouse lung tissue. We conclude that styrene respiratory tract toxicity in mice and rats, including mouse lung tumors, are mediated by CYP2F-generated metabolites. The PBPK model predicts that humans do not generate sufficient levels of these metabolites in the terminal bronchioles to reach a toxic level. Therefore, the postulated mode of action for these effects indicates that respiratory tract effects in rodents are not relevant for human risk assessment.

INTRODUCTION

Numerous studies have demonstrated marked differences in toxicity between rats and mice to styrene exposure, especially by inhalation. Mice are particularly sensitive to respiratory tract and hepatic toxicity from styrene. Both species have a qualitatively similar response of the nasal olfactory mucosa, with the mouse being much more sensitive. This paper lays out the toxicologic and metabolic data that explain the nasal and lung differences and their relevance for human risk assessment.

Inhalation of styrene at concentrations of 200 ppm or higher resulted in death of some mice after one to three exposures, with 30 to 50% lethality at about 250 ppm for 5 days (Morgan *et al*., 1993a,b,c, 1995; Cruzan *et al*., 1997; Sumner *et al*., 1997). At these concentrations, mice developed severe liver toxicity, which probably contributed to the cause of death. Mice that did not succumb to the initial styrene exposure survived continued exposure and had little evidence of liver toxicity after 13 weeks. Exposure of mice to 20 to 160 ppm styrene for 1 day to 2 years also resulted in toxicity to lung Clara cells and nasal olfactory cells (Cruzan *et al*., 1997, 2001). Evidence of increased lung tumors (mostly adenomas) was seen at concentrations of 40 to 160 ppm in an inhalation study and only equivocal evidence of lung tumors in two of four chronic toxicity/oncogenicity studies by gavage in mice (reviewed in Cruzan *et al*., 2001). No other tumor types were increased in these studies in mice. Based on the weight of the evidence, styrene induces lung tumors in mice, lung Clara cell toxicity, and nasal olfactory lesions.

Eight chronic toxicity/oncogenicity studies of styrene have been conducted in rats (see Cruzan *et al*., 1998, for review), in addition to numerous shorter term studies.

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In contrast to mice, rats can tolerate up to 1000 ppm via inhalation for 2 years with decreases in body weight, slight increases in liver weight, and nasal olfactory lesions. Nasal olfactory lesions were seen in a few rats exposed to styrene at concentrations as low as 50 ppm for 24 months; more severe lesions and a greater percentage of rats with olfactory lesions were seen at doses of 200 to 1000 ppm (Cruzan *et al*., 1998). By oral gavage, daily doses of 500 mg/kg/day were tolerated without effect. Daily doses of 1000 or 2000 mg/kg/day resulted in increased mortality and mild liver toxicity (NCI, 1979a). Nasal tissues were not examined, but olfactory lesions might be expected due to the high levels of blood styrene generated from these high doses. One study reported increased mammary tumors in females exposed by inhalation to concentrations of 25 to 300 ppm styrene 4 h/day 5 days/week for 1 year (followed by an additional 1 year of observation) without dose response (Conti *et al*., 1988), while the most recent, and only GLP-compliant, study found no difference from control at 50 or 200 ppm 6 h/day 5 days/week for 104 weeks and a dose-related decrease at 500 and 1000 ppm (Cruzan *et al*., 1998). No treatment-related effects on mammary tumors were reported in the other six chronic studies. No other tumor types were increased in rats in the chronic studies. Based on the weight of the evidence, styrene does not induce tumors in rats, but it does induce olfactory lesions.

Reinforced plastics fabrication workers may be exposed to 8-h time-weighted average styrene concentrations up to 50 ppm. A number of health surveys published in the 1960s and 1970s, when exposures were up to 100 ppm or higher, reported liver and lung function to be normal except for a few equivocal differences found inconsistently. A limited study of nasal histopathology found no abnormalities in reinforced plastics workers (Odkvist *et al*., 1985). In addition, no effect on olfactory function [based on standardized odor identification test and odor detection threshold for phenylethanol (rose)] was found in a recent study of reinforced plastics workers exposed to 30–50 ppm styrene for at least 5 years (Dalton *et al*., 2002). No styrene-related increases in lung tumors, or other tumor types, have been found in reinforced plastics workers (Wong *et al*., 1994; Kogevinas *et al.*, 1994). The lungs and olfactory tissue have not been found to be targets of styrene toxicity in humans.

In order to understand the differences in toxicity between rats and mice and the relevance of these findings for humans, a series of studies was conducted to understand the mode of action in rats and mice. The differential metabolism of styrene in rats, mice, and humans and the role of metabolism in the differential toxicity among species were studied. These studies demonstrate that tissues that are high in CYP2F and produce predominantly *R*-styrene oxide are most susceptible to styrene-induced toxicity.

TARGET ORGANS IN LABORATORY ANIMALS

Lung

Inhalation or oral exposure to styrene has been reported to produce lung toxicity in mice, but not in rats. Effects in mice have been seen consistently in the terminal bronchioles, but no effects are reported in alveolar cells.

Gadberry *et al*. (1996) and Carlson (1997a) reported increased levels of the enzymes γ-glutamyltranspeptidase (GGT) and lactate dehydrogenase (LDH) in bronchoalveolar lavage fluid (BALF) obtained at necropsy 24 h after the ip injection of 600 mg/kg or greater styrene in mice. Histopathologic evaluation of the lungs did not demonstrate which cells were damaged. Ip injection of styrene oxide (SO, an intermediary metabolite of styrene) caused greater increases in GGT and LDH at lower doses (300 mg/kg) and after a shorter time period than did styrene. *R*-SO was more toxic than *S*-SO (Gadberry *et al*., 1996). In addition, 4-vinylphenol (4-VP), a ring-oxidized metabolite of styrene, is about 10-fold more potent than SO at inducing pneumotoxicity in mice as measured by increased lactate dehydrogenase and cells in bronchoalveolar lavage fluid, following ip administration (Carlson, 2001). Further, 4-VP caused extensive cellular damage in the terminal bronchioles as evidenced by exfoliation of epithelial cells into the lumen of the bronchioles leaving a very flattened bronchiolar surface (Carlson *et al.*, 2002), similar to that reported for naphthalene (Plopper *et al*., 1992). While the greater toxic response from 4-VP may be due to its inherent toxicity, there are other potential explanations for the difference between 4-VP and SO (rate of further metabolism, distribution) that require further investigation. Thus lung toxicity has been demonstrated in mice from styrene, SO, and 4-VP, with 4-VP being by far the most potent.

CD-1 mice examined immediately at the end of a 6-h inhalation exposure to 40 or 160 ppm styrene had multifocal necrosis and cell loss in bronchiolar epithelium, which was not evident 18 h later (Green *et al*., 2001a). Upon repeated exposures, there was decreased cytoplasmic staining of Clara cells and cell crowding in the terminal bronchioles (Cruzan *et al*., 1997). With longer duration of exposure, hyperplasia of the terminal bronchioles, sometimes extending into the alveolar ducts, occurred. With long-term (lifetime) exposure, lung tumors, primarily benign, were also reported for mice. Lung histopathologic effects have been reported for mice exposed to levels as low as 20 ppm styrene for 2 years (Cruzan *et al*., 2001). Effects in alveolar cells were not seen in any of these studies (Cruzan *et al*., 1997, 2001; Green *et al*., 2001a).

CD-1 and B6C3F1 mice have been exposed to styrene vapor in a series of studies at concentrations from 15 to 500 ppm, 6 h/day, from 1 to 14 days (Cruzan *et al*., 1997; Green *et al*., 2001a). These experiments

TABLE 1
Nonneoplastic Lung Pathology in Mice Exposed to Styrene Vapor for up to 24 Months

	Males					Females				
Concentration (ppm):	0	20	40	80	160	0	20	40	80	160
Number of **mice** examined										
12 months	10	10	9	10	10	10	10	9	10	10
18 months	8	10	10	8	6	8	9	8	9	10
24 (22.5) months	50	50	50	50	50	50	50	50	50	50
Finding										
Decreased eosinophilia of epithelial cells in terminal bronchioles										
12 months	0	6	2	10	10	0	5	9	10	10
18 months	0	9	10	8	6	0	8	8	9	10
24 (22.5) months	0	29	41	48	49	0	37	46	47	45
Bronchiolar epithelial hyperplasia										
12 months	0	0	2	9	10	0	0	4	7	10
18 months	0	7	9	8	6	0	7	8	9	10
24 (22.5) months	0	10	37	48	46	0	21	39	45	45
Bronchiolar epithelial hyperplasia extending into alveolar ducts										
12 months	0	0	2	9	10	0	0	0	0	7
18 months	0	1	3	5	5	0	0	3	3	7
24 (22.5) months	0	5	29	35	35	0	9	18	31	40
Bronchioloalveolar hyperplasia										
12 months	2	0	0	0	1	2	0	1	0	1
18 months	1	3	3	3	1	1	3	3	1	2
24 (22.5) months	18	23	30	40	38	6	15	25	18	21
Number of **rats** examined										
12 months	10	10	10	10	10	10	10	10	10	10
24 months	60	60	60	54	52	60	60	60	60	60
Finding										
Decreased eosinophilia of epithelial cells in terminal bronchioles:										
12 months	0	0	0	0	0	0	0	0	0	0
24 months	0	0	0	0	0	0	0	0	0	0
Bronchiolar epithelial hyperplasia										
12 months	0	0	0	0	0	0	0	0	0	0
24 months	0	0	0	0	0	0	0	0	0	0

consistently showed cell crowding, decreased staining, and increased cell replication in the Clara cells of the mouse bronchiolar epithelium. Increases in cell replication were seen at dose levels of 40 ppm and above after 3 days (Green *et al*., 2001a). In a 13-week subchronic study, CD-1 mice exposed to 0, 50, 100, 150, and 200 ppm styrene had changes of the bronchiolar epithelium characterized by decreased eosinophilia and focal crowding of nonciliated cells in bronchioles of mice exposed to 100 ppm and above. Increased labeling (by BrdU) of Clara, but not type II, cells was present after 2 weeks and to a limited extent after 5 weeks, but not at the end of 13 weeks (Cruzan *et al*., 1997). Although cell proliferation was not measured, similar histopathological findings were reported by Roycroft *et al.* (1992) in B6C3F1 mice exposed to up to 500 ppm styrene for 13 weeks.

In a 2-year inhalation chronic toxicity/oncogenicity study, groups of 70 CD-1 mice/sex were exposed to 0, 20, 40, 80, or 160 ppm styrene, 6 h/day, 5 days/week for up to 2 years (Cruzan *et al*., 2001). Based on observations from interim necropsies at 52 and 78 weeks and the terminal necropsy at 104 weeks, the lung effects progressed from decreased eosinophilia of the epithelium of the terminal bronchioles to hyperplasia of the terminal bronchiolar epithelium, and finally, to hyperplasia extending into alveolar ducts. With increasing duration, the exposure concentration at which effects were seen also decreased, such that at 104 weeks, mice in all dose levels were affected (Table 1).

The cells comprising the areas of hyperplasia in the terminal bronchioles stained immunohistochemically with high intensity for CC10, a protein found in normal Clara cells. Immunostaining for surfactant A, a protein more characteristic of normal alveolar Type II cells, was only rarely and faintly present (Cruzan *et al*., 2001). Electron microscopy of hyperplastic epithelium of the terminal bronchioles identified the predominant cell type as the Clara cell (Mullins, 1998). Decreased numbers of intracellular organelles, likely the secretory granules, were apparent in some of the Clara cells and may correlate with the decreased eosinophilia noted in the terminal bronchiolar cells.

Adverse effects in the lung were not reported in any of four gavage oncogenicity studies in various strains of mice (NCI, 1979a,b; Ponomarkov and Tomatis, 1978),

although two of the four studies reported equivocal increases in lung tumors. However, it is not clear how thoroughly the lungs were examined since inhalation was not the route of exposure. In a more recent study of lung effects, an increase in mouse Clara cell proliferation was seen when styrene was administered orally by gavage for 5 days at doses of 100 or 200 mg/kg/day, but not at 10 mg/kg/day (Green *et al.*, 2001a). However, no morphological changes were seen in this study at any of the dose levels.

No morphologic or cell proliferation effects were seen in the alveolar region in any of the mouse studies. Toxic effects in Clara cells have been reported following oral and inhalation exposure to styrene. Overall, these findings indicate that the Clara cell is the target cell for the toxic action of styrene in mouse lungs.

In contrast to the effects observed in mice, there were no styrene-related effects in the lungs of Sprague–Dawley rats (Cruzan *et al*., 1997) or F344 rats (Roycroft *et al*., 1992) at concentrations up to 1500 ppm for 3 months. Three long-term chronic toxicity/oncogenicity inhalation studies of styrene have been conducted in Sprague–Dawley rats without identifying the lung as a target organ. These include 0, 600, or 1000 ppm 6 h/day, 5 days/week for 18.3 (males) or 20.7 (females) months (Jersey *et al*., 1978); 0, 25, 50, 100, 200, or 300 ppm, 4 h/day, 5 days/week for 1 year (Conti *et al*., 1988); and 0, 50, 200, 500, or 1000 ppm 6 h/day 5 days/week for 24 months (Cruzan *et al*., 1998). In the most recent study, pale foci were seen the lungs of a few female rats exposed to 1000 ppm styrene for 2 years; histopathologic evaluation identified these as foamy alveolar macrophages and/or cholesterol cleft granulomas. These were judged not to be treatment-related lesions of lung tissue (Cruzan *et al*., 1998) and are not similar to lesions seen in the terminal bronchioles of mice (Cruzan *et al*., 2001).

Although rats have been given styrene orally in many studies, lung lesions have not been reported. Gavage studies were conducted at dose levels as high as 2000 mg styrene/kg (NCI, 1979a); 700 (males) or 350 (females) mg/kg styrene (NCI, 1979b); 5 days/week, for 78 or 79 weeks followed by 27–29 weeks without dosing prior to termination; and up to 250 mg/kg, 4–5 days/week, for 52 weeks followed by 52 weeks of observation (Conti *et al*., 1988). No lung effects were associated with 2 years of ingestion of styrene in the drinking water at 250 ppm (14 mg/kg/day for males and 21 mg/kg/day for females, dose limited by styrene solubility in water) in Sprague–Dawley rats (Beliles *et al*., 1985).

The terminal bronchiolar and type II alveolar cells from Sprague–Dawley rats exposed to levels up to 1500 ppm styrene for 2, 5, or 13 weeks (Cruzan *et al*., 1997) or 500 ppm for 1, 5, 6, or 10 exposures (Green *et al.*, 2001a) have also been examined for cell proliferation. There were no effects present on labeling indices for either bronchiolar cells or alveolar type II cells of rats at any exposure level or time point.

Although multiple studies discussed above found no styrene-related effects in the lungs of rats, Coccini *et al*. (1997) reported thickened alveolar septae in rats exposed to 300 ppm styrene, 6 h/day, 5 days/week, for 2 weeks. Electron microscopic evaluation detected a few alveolar type II cells or bronchiolar cells with dilated endoplasmic reticulum. Increased thickness of the alveolar septae was reported to be due to the presence of collagen fibrils. The ultrastructural effects were reported to be almost completely reversed 3 weeks after cessation of exposure with only mild alterations in the cytoplasm of some bronchiolar or type II cells still present. Similar effects were reported from intraperitoneal injection of 40 or 400 mg styrene/kg for 3 days, but not at 4 mg/kg/day (Coccini *et al*., 1997). The reason for these findings by Coccini and co-workers is unknown, but the numerous studies with much greater exposure concentrations and longer durations suggest that these effects, if present, did not progress, but regressed.

Thus in contrast to mice, lung toxicity in rats from styrene exposure is either nonexistent, or very subtle, compared to the overt toxicity in mice. In addition, in rats styrene does not induce progressive lesions based on histopathologic evaluation of eight long-term studies. Furthermore, lung toxicity has not been reported in humans.

Nasal Olfactory Epithelium

A single exposure of CD-1 mice to 80 ppm, but not 40 ppm, styrene resulted in early atrophy/degeneration of olfactory epithelial cells with dilatation of Bowman's glands. Continued exposure resulted in replacement of olfactory cells by ciliated columnar cells (respiratory-like), Bowman's gland hyperplasia, and debris (Cruzan *et al*., 2001). After 13 weeks of exposure at 100–200 ppm nearly all CD-1 mice had atrophy of olfactory epithelium and dilatation of Bowman's glands (Cruzan *et al*., 1997). At 50 ppm, approximately half of the mice were similarly affected. Fewer mice at each dose level also had atrophy of olfactory nerve fiber and replacement of olfactory cells with respiratory cells. After 2 years of exposure at 20, 40, 80, or 160 ppm, treatment-related changes were also present in the nasal passages in male and female mice (Cruzan *et al*., 2001). The major findings were respiratory metaplasia of the olfactory epithelium and changes of the underlying Bowman's glands, including dilatation, respiratory metaplasia, epithelial hyperplasia, eosinophilic material/debris, cholesterol clefts, atrophy of nerve fibers, and turbinate bone changes. The lesions showed progression with time.

Styrene-related histopathological changes were seen in the nasal olfactory epithelium of male and female CD (Sprague–Dawley) rats exposed to 1000 or 1500 ppm

for 13 weeks (Cruzan *et al*., 1997). At 500 ppm two of 20 rats were affected; no differences from control were seen at 200 ppm. After 2 years of exposure, olfactory lesions (atrophy/degeneration in epithelium, prominent Bowman's glands, atrophy/dilatation/hypertrophy/hyperplasia of Bowman's glands) were present at exposure concentrations of 50 to 1000 ppm in CD rats (Cruzan *et al*., 1998).

Based on these findings, the nasal olfactory effects in mice occurred at lower concentration levels and were more severe than in rats. In humans, a limited study found no increase in nasal symptoms or histology scores in 11 reinforced plastics workers exposed to 47–59 ppm styrene for 1 to 16 years (Odkvist *et al*., 1985). In addition, olfactory function was not decreased in a group of 52 reinforced plastics workers exposed to 30–60 ppm styrene for at least 4 years (Dalton *et al*., 2002).

TABLE 2
Species Differences in Styrene Metabolism as Indicated by Urinary Metabolites Metabolic Pathway

Species	SO-EH (%)	SO-GSH (%)	PAA (%)	Ring (%)
B6C3FI mouse	49–52	33–35	12–17	4–8
CD-1 mouse	51–59	20–27	21–22	4–8
F344 rat	68–72	23–26	3–5	<1
Human (2–4 h)	95	ND[a]	5	ND
Human (4–9 h)	100	ND[a]	ND	ND

Note. SO-EH, styrene oxide and epoxide hydrolase to mandelic acid, etc.; SO-GSH, glutathione conjugates; PAA, products derived from phenylacetaldehyde, such as phenylacetic and phenylaceturic acids, Ring, ring-opened compounds with or without GSH conjugation. ND, not detected.

[a] Other authors have reported trace amount of GSH conjugates in human urine, which were below the limits of detection in this study; see Manini *et al.* (2000), Maestri *et al.* (1997), and Ghittori *et al.* (1997).

METABOLISM AND BIOKINETICS: DIFFERENCES AMONG MOUSE, RAT, AND HUMAN

Urinary Metabolites

The major metabolic pathways of styrene in mouse, rat, and human are shown in Fig. 1. In mice there are four metabolic pathways for styrene:

1. Oxidation of the side chain of styrene by P450s to SO, which is further converted by an epoxide hydrolase (EH) to the styrene glycol, with subsequent conversion to acidic metabolites, such as mandelic, phenylglyoxylic, and hippuric acids.
2. Oxidation of the side chain of styrene by P450s to SO which is further converted by glutathione transferase (GST) to GSH adducts which are eventually excreted as mercapturic acids in the urine.
3. Formation of phenylacetaldehyde either by rearrangement of SO or P450 conversion of styrene to phenylethanol, which is followed by conversion to phenylacetic and phenylaceturic acids.
4. Oxidation of the benzene ring of styrene (possibly leading to the formation of 4-vinylphenol and possibly additional oxidation of the side chain). These intermediates may result in ring opening which can lead to further oxidation to acids or conjugation with GSH.

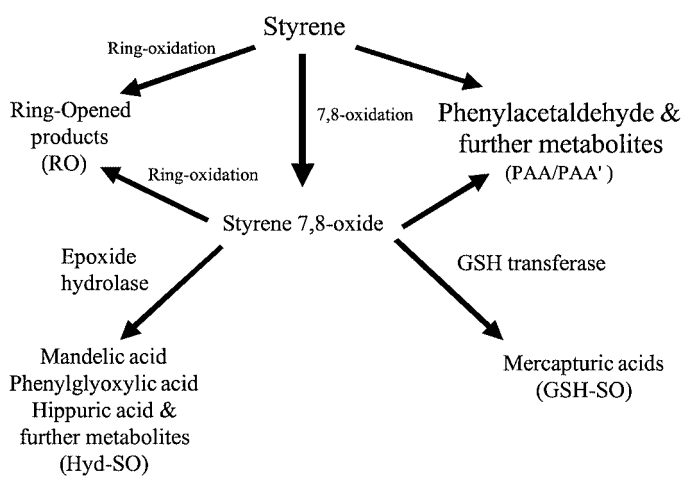

FIG. 1. Metabolic scheme for styrene (taken from Sumner *et al.*, 2001, which is generalized from Sumner and Fennell, 1994).

Differences in styrene metabolism among humans, rats, and mice have been studied by examining differences in patterns of urinary metabolites. These differences are summarized in Table 2 and reflect data from rats and mice exposed to 125, 250, or 500 ppm styrene for 6 h (Sumner *et al.*, 1995) and humans exposed to 50 ppm styrene for 2 h (Johanson *et al*., 2000).

The urinary metabolite data indicate important qualitative differences in metabolism among the species. The epoxide hydrolase pathway accounts for the greatest percentage removal of SO in rats and mice, but the use of GSH conjugation is also an important metabolic pathway for removal of styrene oxide for them. The major differences between rats and mice are in the proportion of styrene metabolism through the PAA (~4 vs ~20%) and ring-opened (<1 vs ~6%) pathways. Based on urinary metabolites, humans metabolize styrene almost exclusively via the epoxide hydrolase pathway, with less metabolized via the PAA pathway than in either rats or mice. No more than trace amounts of GSH conjugates or ring-opened metabolites occur in humans exposed to styrene. Thus the ring oxidation and PAA pathways are used much more in the metabolism of styrene in mice than in rats; these pathways play very small roles in styrene metabolism in humans. These data demonstrate that there are major species differences, both qualitative and quantitative, in the overall metabolism of styrene, which leads us to ask if there are target organ differences in styrene metabolism that might explain differences in toxic response.

Hepatic Metabolism of Styrene

Based on physiologically based pharmacokinetic (PBPK) models (Csanady *et al*, 1994; Sarangapani *et al*., 2002), metabolism of styrene by liver is probably the most important for removing styrene from an exposed animal and is largely responsible for the body burden/blood level of SO. However, blood levels of SO were much higher in rats at nontumorigenic concentrations (1000 ppm) than in mice at levels (40–160 ppm) that caused increased lung tumors (Cruzan *et al*., 2001). Since blood levels of SO do not correlate with increased incidence of lung tumors, it would appear that toxic effects of styrene in mouse lung tissue are likely the result of localized tissue metabolism of styrene and/or that SO is not the proximate toxicant.

Pulmonary Metabolism of Styrene

Various studies have shown that microsomal preparations from mouse lung can metabolize styrene to styrene oxide (Carlson 1997a,b; Carlson *et al.*, 2000; Green *et al*., 2001a). When isolated rat and mouse Clara-cell-enriched fractions were compared for styrene-metabolizing activity, the total amount of SO (pmol/10^6 cells/min) formed was about fivefold higher in Clara cells from mice than from rats (Hynes *et al.*, 1999). Furthermore a comparison of the ratio of the formation of *R*-SO to *S*-SO shows that the mouse Clara cells produces about 3 times more of the *R*-enantiomer than the *S*-enantiomer, while rat produces more of the *S*-enantiomer (Hynes *et al*., 1999). This species difference in production of the styrene oxide enantiomers means that mouse Clara cells produce about 15 times more *R*-SO enantiomer than rat Clara cells. This is important because Gadberry *et al*. (1996) demonstrated that the *R*-enantiomer is a more potent pneumotoxicant and hepatotoxicant than the *S*-enantiomer.

Of the various cytochrome P450 isozymes present in mouse lung tissue, CYP2F2 and CYP2E1 have been identified as being the most important in the pulmonary metabolism of styrene to SO (Carlson, 1997b; Green *et al*., 2001a). Other cytochromes, notably CYP1A and CYP2B, appear to play little or no role in the biotransformation of styrene in mouse lung (Carlson *et al*., 1998; Hynes *et al*., 1999).

Incubation of hepatic microsomes from CD-1 mice with styrene resulted in the formation of SO, but the formation of 4-VP was not detected (Carlson *et al*., 2001). The authors concluded that this could be because it was not formed or because it was rapidly metabolized to further metabolic products. They reported that 4-VP readily disappeared when incubated with microsomes from rat and mouse liver and lung in the presence of NADPH, indicating oxidative metabolism. Mouse liver was about three times as active as rat liver and mouse lung about eight times as active as rat lung. Further studies indicated that CYP2E1 and CYP2F2 were important in the metabolism of 4-VP in mouse liver and that CYP2F2 was more important in the metabolism of 4-VP in mouse lung than was CYP2E1, similar to the metabolism of styrene to SO. They were not able to identify the metabolite(s) formed, but lack of UV absorbance suggests the metabolites are not aromatic; i.e., they may be ring opened.

The relationship between P450-mediated styrene metabolism and pulmonary toxicity in the mouse has been demonstrated by Green *et al*. (2001a). Using bronchiolar cell replication as a marker of toxicity, mice treated with 5-phenyl-1-pentyne, an inhibitor primarily of CYP2F2 and to a lesser extent of CYP2E1, showed no evidence of lung cytotoxicity or increased cell replication when exposed to styrene at 160 ppm 6 h/day for 4 days. In comparison, mice not treated with 5-phenyl-1-pentyne showed evidence of cell necrosis and loss of cells, believed to be Clara cells, with subsequent increases in cell replication. The results demonstrate that Clara cell toxicity and cell replication seen in mice following treatment with styrene are associated with the metabolism of styrene by CYP2F2 and/or CYP2E1. It has been postulated (Green *et al*., 2001b) that styrene is oxidized primarily by CYP2F2 in tissues that produce high *R*:*S*-SO ratios because: (1) inhibition by 5-phenyl-1-pentyne (more potent inhibitor of 2F2 than 2E1) prevents a toxic response and (2) purified human CYP2E1 (expressed by *Escherichia coli*) generated an *R*:*S*-SO ratio of 0.48. The most recent data also indicate that styrene toxicity could be mediated through 4-VP (or a further metabolite), which is also metabolized primarily by CYP2F2 in mouse lung.

Although there are only limited published data, human pulmonary tissue does not appear to be particularly effective in metabolizing styrene to SO. Nakajima *et al*. (1994) estimated styrene metabolism in human lung microsomes of 0.0065 nmol/min/mg protein, which is about 100-fold lower than the activity of rat microsomes. Carlson *et al*. (2000), using microsomes from eight human samples, found only one sample expressing any styrene-metabolizing activity (0.088 nmol/min/mg protein) while the other seven samples were devoid of styrene-metabolizing activity. All eight human lung samples did however possess P450-metabolizing activity as indicated by their ability to metabolize benzene, which is metabolized in mouse lung by CYP2E1 and CYP2F2. In a similar investigation, Filser *et al*. (2002) failed to find any styrene metabolism in human lung homogenates even though P450 activity was detected using ethoxycoumarin as a substrate. Using human lymphoblastoid cell cultures that express CYP2F1, no conversion of styrene to SO could be measured, although these cells do metabolize naphthalene (Yost and Carlson, personal communication).

We note that the CYP2F family contains similar enzymes from different species, but the studies outlined above suggests they may have somewhat different

abilities to metabolize styrene. CYP2F1 is found in human tissues, but it does not appear to metabolize styrene. CYP2F2 is found in mice; it readily metabolizes styrene. CYP2F4 is found in rats (particularly nasal tissue); it readily metabolizes styrene.

To determine which pulmonary cells are responsible for the metabolism of styrene, Hynes *et al.* (1999) and Carlson *et al.* (2000) used enriched fractions of Clara cells and type II pneumocytes. Data from these studies showed that essentially all of the metabolic activity was associated with Clara cells with little or none being found in the type II cells in either rats or mice (Hynes *et al.*, 1999). The data showing that styrene was metabolized by Clara cells but not type II pneumocytes is consistent with the cytochrome P450 isozyme pattern in the cells; i.e., Clara cells contain CYP2E1 and CYP2F2 while type II cells do not (Forkert, 1995; Buckpitt *et al.*, 1995). A comparison of the isozyme pattern in mouse and rat lung indicates the levels of both isozymes CYP2E1 and CYP2F2 are higher in the mouse Clara cell compared to the rat. These metabolic differences are consistent with toxicity differences in that styrene exposure results in toxicity to mouse Clara cells but not type II cells or rat lung cells.

Using PBPK modeling, Sarangapani *et al.* (2002) concluded that the target tissue concentration of SO is primarily due to local metabolism of styrene (Fig. 2). Based on metabolic data from isolated Clara cells (Hynes *et al.*, 1999) the PBPK model predicts that the concentration of *R*-SO predominates in the terminal bronchioles of mice, while *S*-SO predominates in rats (Table 3). The model predicts a maximum level of 2 μM *R*-SO in the terminal bronchioles of rats due to saturation of styrene metabolism at 500–600 ppm. This is only one-half the level of *R*-SO achieved in the terminal bronchioles of mice exposed to 20 ppm styrene (Table 3). The fact that 20–40 ppm styrene caused an increase in lung tumors in mice, while 500 and 1000 ppm did not cause increased lung tumors in rats may be explained by the lack of sufficient *R*-SO in rat terminal bronchioles. Other factors, such as greater susceptibility in mice, or effects from a different metabolite, such as 4-VP, may also be involved. The Sarangapani *et al.* model predicts that at a given airborne concentration, the level of total SO in the terminal bronchioles of mice is approximately 10-fold higher than in the terminal bronchioles of rats and 100-fold higher than in humans (Fig. 3). In humans, the maximum concentration of SO in the terminal bronchioles ($\sim$0.09 μM) is reached at an airborne concentration of 200 ppm; this is the concentration found in the lungs of mice exposed to 0.1 ppm.

TABLE 3

Steady-State Concentrations of *R*-SO and *S*-SO in the Terminal Bronchioles in Mice and Rates for Exposure Concentrations Used in the Respective Rodent Chronic Bioassays

	R-SO (nmol/ml)	*S*-SO (nmol/ml)	*R*/*S* Ratio
Mouse exposure (ppm)			
20	4.3	1.5	2.87
40	5.3	2.0	2.65
80	6.2	2.5	2.48
160	7.2	3.3	2.2
Rat exposure (ppm)			
50	0.65	0.93	0.7
200	1.6	2.2	0.73
500	1.98	2.7	0.73
1000	2.0	2.75	0.73

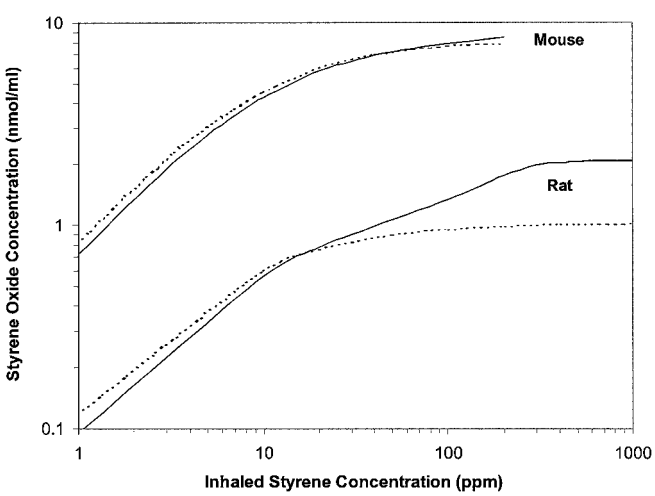

FIG. 2. Model simulations comparing steady state SO concentration in the terminal bronchioles with (solid line) and without (dotted line) accounting for systemic metabolism (i.e., P450 activity in the liver) in both mice and rats. Target tissue concentration of SO is primarily due to local metabolism, rather than systemic delivery (from Sarangapani *et al.*, 2002).

The model predictions demonstrate that SO concentrations in terminal bronchioles of rats and mice are largely determined by local tissue metabolism of styrene. They predict that *R*-SO levels in the terminal bronchioles of rats do not exceed the minimally tumorigenic concentration in mice. Their model also predicts that humans cannot be exposed to a level of styrene that produces this level of SO in the terminal bronchioles.

The pulmonary metabolism studies and PBPK model of styrene led to the following conclusions:

1. Styrene metabolism is highest in mouse lung tissue, at a lower level in rat lung tissue, and barely detectable in human lung tissue.

2. *R*-Styrene oxide, the more toxic enantiomer, is preferentially formed in mouse lung, while *S*-SO is preferentially formed in rat lung.

3. Metabolism of styrene to SO is carried out primarily, if not exclusively, by Clara cells; no

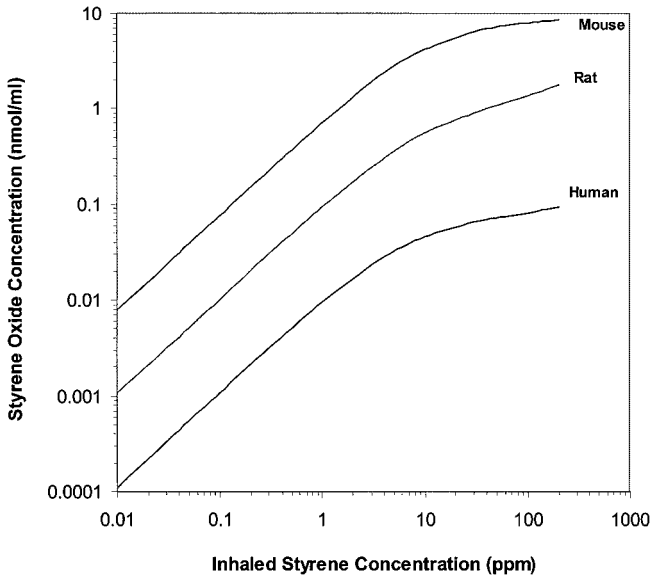

FIG. 3. Model simulation of steady-state SO concentration in the terminal bronchioles in mouse (solid line), rat (dashed line) and human (dotted line) for a wide range of inhaled ST concentration. Tissue SO concentration is approximately 10-fold lower in the rat terminal bronchioles compared to the mouse and a 100-fold lower in the human target tissue compared to the mouse. The tissue concentrations reflect P450 activity in the Clara cell rich terminal/transitional bronchioles in all three species (from Sarangapani *et al*., 2002).

styrene metabolism has been detected in type II pneumocytes.

4. Pulmonary toxicity seen in the mouse is caused by metabolite(s) of styrene formed primarily by CYP2F2.

5. PBPK modeling indicates that the maximal concentration of SO in human terminal bronchioles at any exposure level is less than that present in the terminal bronchioles of mice exposed to 0.1 ppm styrene.

Nasal Metabolism of Styrene

In rats and mice, the uptake of styrene in the upper respiratory tract is partly dependent on metabolism of styrene (Morris, 2000). When present in the air at 5 ppm, up to 40% of styrene was taken up in the isolated upper respiratory tract of normal rats and mice. The percentage absorbed decreased with increasing concentration, demonstrating saturation of metabolism. Saturation of uptake occurred at a lower airborne concentration of styrene in rats than in mice, indicating a greater metabolic capacity in mice than in rats. In rats and mice pretreated with metyrapone (a P450 inhibitor), uptake of styrene was about 10% of the airborne amount, regardless of concentration, between 5 and 200 ppm.

Nasal olfactory epithelium in both rats and mice produce SO from styrene at about the same rate with an *R*:*S* ratio of about 3, indicating the primary role of CYP2F2 in mice and 2F4 in rats. Nasal respiratory epithelium from rats and mice produced SO at about half the rate of olfactory epithelium. In contrast, production of SO was not detected in nine human nasal samples (Green *et al*., 2001b). Olfactory epithelium from rat was more efficient at removing SO via epoxide hydrolase than from mice (V_{max} ratio about 10×; Green *et al*., 2001b). In both rats and mice, the K_m for epoxide hydrolase was much lower in respiratory epithelium than in olfactory epithelium, especially for *R*-SO. Human nasal tissues possessed epoxide hydrolase and GSH transferase activity for both *R*- and *S*-SO (Green *et al*., 2001b). The authors concluded that the differential nasal toxicity between rats and mice could be explained by extensive metabolism of styrene in both species, but a more effective removal of SO in rats than in mice. They further concluded that styrene is unlikely to be toxic to human nasal epithelium.

Prior administration of 5-phenyl-1-pentyne, a cytochrome P450 inhibitor, to mice exposed by inhalation to 40 and 160 ppm styrene, 6 h/day, for 3 days protected against cellular damage in the nasal olfactory epithelium, demonstrating that the toxic entity is derived from the cytochrome P450 metabolism of styrene (Green *et al.*, 2001b).

Evaluation of Metabolic and Biokinetic Data

The metabolic and biokinetic data led to the following conclusions:

1. There are significant species differences in the activities of styrene- and SO-metabolizing enzymes. In those tissues with high levels of CYP2F2 (mouse lung Clara cells, rat and mouse nasal olfactory epithelium), *R*-SO is the main first metabolite from styrene and tissue damage is found.

2. The toxicity of styrene in lung and nasal tissues is caused by one or more metabolites derived from oxidation of styrene by CYP2F2 which include *R*-SO and 4-VP or derivatives from 4-VP.

3. The concentration of SO in lung and nasal tissue is mainly due to the local metabolism of styrene. Exposure due to the presence of SO in the blood is of little importance.

4. Differences in nasal toxicity from styrene in rats and mice are consistent with a greater ability of rat nasal tissue to remove SO, especially *R*-SO, by epoxide hydrolase.

5. Little if any SO is formed in human lung or nasal tissue, but epoxide hydrolase is very active in both human lung and nasal tissue to remove any small amounts formed or that migrates into the cell from the blood. Human lung is incapable of producing sufficient SO to achieve the bronchiolar concentration produced in mice at 0.1 ppm. Thus no lung or nasal toxicity is expected in humans from styrene exposure.

DNA ADDUCT FORMATION *IN VIVO* AFTER STYRENE INHALATION EXPOSURE IN THE MOUSE AND RAT

Styrene is metabolized to SO, a biologically reactive metabolite. A number of *in vitro* and *in vivo* studies have demonstrated SO adducts to proteins and DNA (IARC, 1994a,b). Therefore, it is important to determine if styrene exposure results in an increased target organ burden of DNA adducts. Low levels of N^7- and O^6-guanine DNA adducts were reported in mice exposed to styrene by ip injections of 29 to 450 mg/kg (Pauwels *et al*., 1996). The authors reported adduct levels corresponding to approximately 15 per 10^8 nucleotides in liver and lung using a ^{32}P-postlabeling technique. Otteneder *et al*. (1999) commented that this method was not as sensitive as claimed by the authors and results less than 30 adducts per 10^8 nucleotides by this method should be viewed with caution. Otteneder *et al*. (1999) reported that no adducts of O^6-hydroxyethylphenyl guanine were detected (limit of detection 30 per 10^8 nucleotides) using this same ^{32}P-postlabeling assay in mice which had received 1, 5, 6, or 10 six-hour inhalation exposures to 160 ppm styrene (5 days/week). In addition, Otteneder *et al*. (1999) reported 70 O^6-guanine adducts per 10^8 nucleotides in livers of female Sprague–Dawley rats exposed to 1000 ppm styrene for 2 years.

In the most recent experiment using [*ring*-U-^{14}C]styrene, mice were exposed by inhalation to 160 ppm and rats to 500 ppm for 6 h (Boogaard *et al*., 2000). DNA adducts were determined both at the end of the exposure or 42 h later. A high-specific-activity styrene (52 mCi/mmol) was used so that chromatographic analysis would reveal the presence of any adducts present at levels greater than 0.1 adducts per 10^8 nucleotides. The N^7-guanine adduct was the most prevalent in rat liver; it was present at 3.2 adducts per 10^8 nucleotides at the end of the 6-h exposure and at 2.1 adducts per 10^8 nucleotides 42 h later. Other adducts had likewise diminished and some were not detectable at 42 h. In rat lung, adduct levels were lower than in rat liver; the N^7-guanine adduct was present at 1 adduct per 10^8 nucleotides at the end of the 6-h exposure and at 0.5 adducts per 10^8 nucleotides 42 h later. In mouse liver, the N^7-guanine adduct was present at $<$1 adduct per 10^8 nucleotides at the end of the 6-h exposure and at 3 adducts per 10^8 nucleotides 42 h later. In mouse lung, the N^7-guanine adduct was present at 1 adduct per 10^8 nucleotides at the end of the 6-h exposure and at 0.5 adducts per 10^8 nucleotides 42 h later, the same as in rats. Three unidentified adducts were present in mouse liver at levels up to 4.7 adducts per 10^8 nucleotides at the end of the exposure; the one present in greatest concentration increased to 12 adducts per 10^8 nucleotides 42 h later. No increase in adducts was found in mouse lung compared to rat lung. These data indicate that increased lung tumors in mice are not accompanied by an increase in DNA adducts in mouse lung cells. In addition, the authors reported that DNA adducts were not higher in mouse lymphocytes than in lung cells, implying that SO migrating from blood was not the major cause of DNA adducts in lung cells.

The covalent binding index (CBI) as defined by Lutz (1979) was found to be similar for both species and organs: approximately 0.3 in liver and lung of rats and mice; at 42 h CBI values in liver were 0.14 and 0.44 for rats and mice, respectively. These values confirm the earlier estimates made by Cantoreggi and Lutz (1993) that styrene (and its metabolites) reactions with DNA *in vivo* are very limited.

In summary, all DNA-binding studies with styrene in rats and mice have shown no increase in adducts in mice compared to rats or in mouse lung (tumor response) compared to liver (no tumor response). Thus, the DNA adduct data suggest that lung tumors in mice are not due to increased DNA adduct formation in the target cells. Moreover, the very low CBI values calculated in these studies are consistent with the hypothesis that tumor formation in mice exposed to styrene is unlikely to be associated with a primary genotoxic event.

EVALUATION OF POSSIBLE MODES OF ACTION RESPONSIBLE FOR THE LUNG TUMOR FORMATION IN THE MOUSE

This section evaluates the available scientific evidence for genotoxic and nongenotoxic modes of action for the development of lung tumors in mice.

Genotoxic Mode of Action

The genotoxicity data on styrene are equivocal. Most studies of styrene do not report increased mutations. Chromosomal aberrations and micronuclei were not seen in most animal studies, but several studies indicated a weak induction of sister chromatid exchange. The frequency of alkali labile sites/DNA strand breaks is increased in some studies. A minority of reinforced plastics worker studies indicate increased chromosomal aberrations, micronuclei, or sister chromatid exchange (IARC, 1994a).

Styrene is metabolized, at least in part, to SO, which is mutagenic in several *in vitro* systems (IARC, 1994b). Exposure of humans or animals to styrene results in increased levels of hemoglobin and DNA adducts derived from SO. The *R*-enantiomer of SO has been shown to be slightly more reactive than the *S*-enantiomer in the Ames assay (Pagano *et al*., 1992; Seiler, 1990; Sinsheimer *et al*., 1993), although Watabe *et al*. (1978) did not find a difference. Chromosome aberrations were induced in mouse bone marrow cells in one study by the

(*S*)-enantiomer of SO, but not with the (*R*)-enantiomer (Sinsheimer *et al*., 1993). These data indicate that styrene is capable of interacting with DNA in animals and humans.

Two studies suggest a lack of styrene-related genotoxic response in mouse lung cells. Inhalation of styrene at 125, 250, or 500 ppm for 14 days by B6C3F1 mice did not result in increased chromosomal aberrations in lung (Kligerman *et al*., 1992). No increase in lung tumors occurred in a lung tumor initiation assay in A/J mice (Brunnemann *et al*., 1992).

Nongenotoxic Mode of Action

The styrene tumor profile suggests a nongenotoxic mode of action by EPA evaluation criteria (EPA, 1996). An increased tumor incidence has been reported in only one species at only one site, and the tumor type is a common one. The increase in tumor incidence was seen only at study termination (24 months) and not at interim sacrifices. The tumors did not result in early mortality in the styrene-exposed mice. Furthermore, the tumor response was accompanied by organ toxicity and persistent cell turnover. In addition, a screening assay for genotoxic carcinogens in A/J mice was negative for styrene (Brunnemann *et al*., 1992). All these aspects of the styrene database support a nongenotoxic mode of action.

The metabolic profile of styrene helps explain species differences in response and supports a nongenotoxic mode of action. The metabolism of styrene in mouse lung Clara cells produces high levels of styrene metabolites which cause Clara cell toxicity. The cellular damage results in reparative responses including increased Clara cell proliferation and hyperplasia of Clara cells. No increased frequency of DNA adducts was found in lung Clara cells of mice exposed to styrene. Rats and humans have a lower metabolism of styrene in lung and more rapid removal of metabolites; thus they do not develop toxicity, increased cell proliferation, hyperplasia, or lung tumors.

Styrene is metabolized to SO, which is genotoxic in *in vitro* experiments and is classified as a probable human carcinogen by IARC (1994b). It should be noted, however, that increased tumors were produced in both rats and mice only at the site of contact with SO (forestomach following gavage administration) and that considerable tissue damage preceded tumor formation. Skin application of SO did not result in increased incidence of tumors in the skin or any other site. The lack of systemic tumors from administration of SO supports a nongenotoxic mode of action for SO even though it has been found to be genotoxic. This premise is consistent with the styrene database, which indicates a nongenotoxic mode of action at the site of bioactivation.

CONCLUSIONS

In mouse lung Clara cells and nasal olfactory epithelium, styrene is metabolized primarily by CYP2F2 to produce *R*-SO. These tissues are also highly sensitive to cytotoxicity following styrene exposure. Inhibition of CYP2F2 eliminates the styrene-induced cytotoxicity. In mouse lung, a regenerative hyperplasia results from the cytotoxicity, and increased incidences of lung tumors were found only after more than 18 months of repeated exposures.

In rats CYP2F4 is much less prevalent in lung tissue; rat lung produces primarily *S*-SO and cytotoxicity is not seen. No increases in lung tumors have been reported in any of the eight chronic rat studies of styrene. In rat nasal tissue, CYP2F4 is present to a large extent and a high level of *R*-SO is produced. Epoxide hydrolase in rat tissue is more active than in mouse nasal tissue, resulting in a more efficient removal of SO and less nasal toxicity in rats than in mice.

In humans very limited metabolism of styrene occurs in the lung or nasal tissue. Both lung and nasal tissue possess epoxide hydrolase activity and, thus, should be able to rapidly remove any SO either formed in the tissue or which might have migrated from the blood.

Lung tumors in mice most likely result from a nongenotoxic mode of action as a result of cytotoxicity leading to hyperplasia. No lung tumors are seen in rats exposed to styrene, where there is no evidence of cytotoxicity. No styrene-induced lung tumors would be expected in humans, which possess even less ability to metabolize styrene in lungs than rats. Indeed, no styrene-related increase in lung tumors have been reported in human cohort mortality studies.

Nasal olfactory lesions in rats and mice are the result of local metabolism of styrene. Differences in toxicity among species are explained by metabolic differences. Olfactory lesions are not expected in humans exposed to styrene because no styrene metabolism was detected in human nasal tissue. Loss of olfactory function was not found in a study of workers exposed to styrene for at least 5 years.

We conclude that styrene respiratory tract toxicity in mice and rats, including mouse lung tumors, is mediated by CYP2F-generated metabolites. The PBPK model predicts that humans do not generate sufficient levels of these metabolites in the terminal bronchioles to reach a toxic level. Therefore, the postulated mode of action for these effects indicates that respiratory tract effects in rodents are not relevant for human risk assessment.

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