Table 4-12. Summary of studies in rats.

Studies	Design: dose, duration and initial group size	Comments on study	Results male	Results female
Oral				
F344 rats (NCI, 1979a)	500, 1000 or 2000 mg/kg, 5 days/wk, 78 weeks (mid- & high dose) or 103 weeks (low dose) 20/control group; 50/treatment group	Poor survival of high dose groups, small control group	No significant increase in tumors	No significant increase in tumors
BD IV rats (Ponomarkov andTomatis, 1978)	1,350 mg/kg prenatally and 500 mg/kg weekly postweaning until death Controls: 36–39, Dosed: 71–73	Limited dosage regimen, once/wk dosing, limited reporting	No significant increase in tumors	No significant increase in tumors
Sprague Dawley rats (Conti *et al.* 1988)	50 or 250 mg/kg, 4–5 days/wk for 52 weeks and held until death 40/dose group	Mortality in high dose females, short treatment duration, low doses, limited reporting	No significant increase in tumors	No significant increase in tumors
Sprague Dawley rats (Beliles *et al.* 1985)	125 or 250 ppm in drinking water (7.7 – 14mg/kg/day in males and 12 – 20.5 mg/kg/day in females) for 2 years Controls: 76 (m), 106 (f) Dosed: 50 (m), 70 (f)	Low doses, limited reporting;	No significant increase in tumors	Small increase in mammary fibroadenoma
Inhalation				
Sprague Dawley rats (Conti *et al.* 1988)	25, 50, 100, 200 or 300 ppm, 4 hrs/day, 5 days/wk for 52 weeks and held until death	Limited dosing regimen, limited reporting	No significant increase in tumors	malignant mammary tumors increased in multiple groups, with significant trend
Sprague Dawley rats (Jersey *et al.* 1978)	600 or 1,200/1,000 ppm, 6 hrs/day, 5 days/wk, for 18.3 months (males) or 20.7 months (females)	Original report and data not available in published literature; limited reporting in reviews, high incidence of pneumonia	No significant increase in tumors	Small increase in leukemia/lymphosarcoma, with a significant trend
Sprague Dawley rats (Cruzan *et al.* 1998)	50, 200, 500 or 1000 ppm, 6 hrs/day, 5 days/wk for 104 weeks	No major limitations	Positive dose-related trend in interstitial testicular tumors	Pituitary and malignant mammary tumors decreased in all dose groups.

occurred primarily 1 and 3 bases following adduct bypass, when the lesion is positioned in the major groove of the template-primer stem (Latham *et al.* 2000).

Similar replication assays have been performed using *E. coli* Klenow fragment, Sequenase 2.0, T4 polymerase holoenzyme, polymerase α, and polymerase β, *in vitro*. In all instances, lesion bypass is sensitive to both the local sequence context and the chirality of the α-N^6-dA styrene-7,8-oxide adducts. For example, in the 5'-AXG-3' sequence, adducts having (*R*) stereochemistry are bypassed, whereas stereochemically-identical lesions in other sequence contexts are often poor substrates. Similarly, (*R*) vs. (*S*) α-N^6-dA adducts introduced within identical sequences are often bypassed nonequivalently. The degree of adduct-directed termination and translesion synthesis during replication is also dependent on the choice of polymerase. Templates that are poor substrates for bypass synthesis with one enzyme often read through much more efficiently when a different polymerase is used (Latham *et al.* 1995). Similar studies have been conducted using reconstituted *E. coli* Pol III. Replication is poorly processive and strongly terminated by styrene-7,8-oxide lesions in 33-mer templates, although the same enzyme shows efficient bypass of the same adducts in M13 DNA (Latham *et al.* 1996). No data are available regarding replication by Y-family polymerases, *in vitro*.

- Some DNA adducts are xenobiotic specific (see page 326, line 2) but many are not (e.g., methylated adducts). Furthermore adducts formed by oxidative damage can be formed by many agents. In the same paragraph (line 6), 8-hydroxy-2'-deoxyguanosine is listed among the adducts in styrene-exposed individuals, and this most definitely is not styrene-specific. Delete first sentence (about adducts in general, lines 3-4), note and group the styrene-specific adducts together, and move 8-OH-dG to the end of the list of adducts.
- Page 326, lines 11 and 12 [“N7-guanine adducts are the predominant type, but are efficiently repaired…. O^6-guanine adducts…. but are persistent…”]: Delete “efficiently” and add “more” before persistent.
- “adducts may result in depurination, may cause single-strand breaks, or may be repaired directly” (page 326, lines 13-14). Delete “or may be repaired directly.”
- Page 326, line 19 should be GC to TA instead of GC to AT.
- Replace “adducted in” on page 326, line 29 with “bonded at” or “bound at” and “adducted” on page 326, line 28 and page 327, line 2 with “bonded” or “bound.”
- Delete lines 3-7 on page 327 (This text mentions the geometry of the N^6 adenine adducts and the GC to AT transition is not a major mutagenic lesion).

16. Section 5.5 Mechanistic studies and considerations (General)
    - Add information in Section 5.5.3 on cytotoxicity/epigenetic mechanisms. Additional discussion could be included that relates the cytotoxicity of styrene in susceptible Clara cells or in human bronchial epithelial cells to cellular proliferation and mechanisms of clonal expansion of styrene-induced, or spontaneous mutants. Bogen *et al.* (2008) publication on naphthalene is a good example of this mechanism. Suggested text follows:

      In a report of deliberations of a scientific panel at the Naphthalene State-of-the-Science Symposium on the pathogenesis of respiratory tumor formation in rodents (Bogen *et al.* 2008), the following hypothesis for naphthalene-induced neoplasia was presented. Rats chronically exposed to naphthalene by inhalation (NTP 2000) had chronic nasal tissue damage associated with regenerative hyperplasia, atypical hyperplasia, and in a few cases, adenoma formation. This association supports the hypothesis that an increase in tumor incidence after naphthalene exposure is occurring through a cytotoxic mechanism. Chronic cytotoxicity can result in a clonal expansion of cells and increased susceptibility to malignant transformation. Further, this panel reported that elevated incidences of mouse lung tumors occurred only with exposure to

cytotoxic concentrations of naphthalene. Metabolic activation was required and mouse Clara cells had the greatest capacity to metabolize naphthalene. Clara cells were also highly susceptible to naphthalene-induced cytotoxicity. Therefore, this section discusses cytotoxicity of styrene metabolites as a possible mechanism of styrene-induced carcinogenesis.

- Add discussion of styrene metabolism to 4-vinylphenols that are further oxidized to quinone reactive intermediates. Quinones participate in reactive oxygen mediated damage and cytotoxicity. Similar to naphthalene, this cytotoxicity could be a mechanism of clonal expansion of initiated cells.
- NTP to research relevance of cytotoxicity and tumorigenicity of ethylbenzene in mice and rats (Chan *et al.* 1998; Stott *et al.* 2003; Seghir *et al.* (submitted for publication) in regard to mechanistic considerations for styrene.
- Discuss Roder-Stolinski *et al.* 2008. Styrene induces an inflammatory response in human lung epithelial cells via oxidative stress and NF-kB activation. This paper characterized the inflammatory response of human alveolar epithelial cells to styrene. They reported that human lung epithelial cells (A549 cell line) exposed to styrene (10^{-1} to 10^{6} μg/m^3) via the gas phase for 20 h had significantly increased (~ 1.4 fold; $P < 0.05$) release of the inflammatory mediator protein, monocyte chemoattractant protein (MCP-1) at 10^{2} μg/m^3 and higher, which could be inhibited by antioxidants. Styrene exposure (at the same concentrations that caused an increased MCP-1 release) also increased GSTP1 mRNA expression, which was inhibited by pre-treatment with antioxidants and significantly ($P < 0.05$) increased phosphorylation of NF-κB (10^{-1} to 10^{5} μg/m^3) and iκB (10^{0} to 10^{6} μg/m^3). The authors suggested that the activation of NF-κB signaling by styrene was mediated via a redox-sensitive mechanism.

17. Section 5.5.3
    - Page 331, line 17: Add discussion of proposed mechanism that discusses a possible role of styrene-induced elevation in prolactin levels as a potential mechanism for the induction of breast cancer.
    - Add a brief discussion of epoxides in mammary tissue (role of CYP1B1) in Section 5.5 on page 325. Several chemicals associated with induction of mammary tumors are epoxides or are metabolized to epoxides (includes glycidol, benzene, 1,3-butadiene, and ethylene oxide; see Dunnick *et al.* 1995), and it has been hypothesized that the mammary gland may efficiently metabolize chemicals to their epoxides (Bennett and Davis, 2002), and therefore, may be a target tissue for the induction of epoxide-induced tumors.
    - Page 329: change subheading 5.5.3 to “Cytotoxic effects of styrene on mouse lung” (rather than styrene oxide)
    - Section 5.5.3: Add additional text after line 17 on page 331 and to summary in Section 5.5.4. Suggested text as follows:

      Similar to the first of the three factors put forward by Cohen *et al.* (2002) as possible mechanisms for the development of hyperplasia and lung tumors in mice (see above), Cruzan *et al.* (2002) proposed that interspecies differences in styrene toxicity are most likely explained through CYP2F-generated metabolites (2F2 in mice, 2F4 in rats, and 2F1 in humans). They noted that almost all of the effects of cytotoxicity and tumor formation were seen in tissues that are high in CYP2F isoforms and that CYP2F inhibitors prevented cytotoxicity (see Section 5.1.3.4). Ring-oxidation metabolites, including 4-vinylphenol, are ~6-fold higher in mice compared with rats, and 4-vinylphenol is more potent than styrene-7,8-oxide as a pneumotoxicant (see Section 5.1.3.4). Also, styrene metabolism occurs primarily in Clara cells (see Sections 5.1.3.3 and 5.1.3.4), and mice produce higher levels of toxic metabolites (*R*-styrene-7,8-oxide, 4-vinylphenol, and oxidized reactive intermediates of 4-vinylphenol), and

have a lower level of epoxide hydrolase activity than rats or humans (see Sections 5.1.3.1 and 5.1.3.2). They stated that PBPK models predict that human do not generate sufficient levels of these metabolites in the terminal bronchioles to reach toxic levels. Cruzan stated that the tumor profile of styrene suggests a non-genotoxic mode of action since he felt that the tumors in animals, were common, reported in only one species and one site, did not occur at the 12 month sacrifice, and were associated with organ toxicity and cell turnover. Studies published after Cruzan's 2002 proposal that evaluated the role of CYP2F2, ring-oxidized metabolites, and cytotoxicity in the lung are discussed in Sections 5.1.3.4 and 5.2.2.2).

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David Phillips, Ph.D., D.Sc. (Chair)

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Attachment 1 to Section 4 Subgroup Report. Excerpts on Comparisons with Historical Controls from the IARC Preamble and a Recent NTP 2008 Report

Preamble to the *IARC Monographs*
(amended January 2006)

"It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls, particularly when historical controls show high between-study variability and are, thus, of little relevance to the current experiment. In analysing results for uncommon tumours, however, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, gender and strain, as well as other factors such as basal diet and general laboratory environment, which may affect tumour-response rates in control animals (Haseman *et al.* 1984; Fung *et al.* 1996; Greim *et al.* 2003)."

This may be found at the following link or obtained from WHO/IARC:
http://monographs.iarc.fr/ENG/Preamble/currentb3studiesanimals0706.php

From the May 2008 NTP Technical Report on the Toxicology and Carcinogenesis Studies of Methylene Blue Trihydrate (CAS No. 7220-79-3) in F344/N Rats and B6C3F1 Mice (Gavage)

"The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential treatment-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical database must be generally similar."

Attachment 2 to Subgroup Section 4 Report: Laboratory historical control incidence of leukemia-lymphosarcoma for Jersey *et al.* 1978

Study number	Males	Females
1	3/80 (3.75%)	0/79 (0%)
2	0/25 (0%)	0/25 (0%)
3	None	2/159 (1.26%)
4	0/50 (0%)	None
5	2/90 (2.22%)	0/90 (0%)
6	5/85 (5.88%)	2/86 (2.32%)
7	4/112 (3.57%)	None
8	5/189 (2.64%)	5/189 (2.64%)
9	2/80 (2.5%)	2/80 (2.50%)
10	0/100 (0%)	0/100 (0%)
Total	21/811 (2.59%)	11/808 (1.36%)