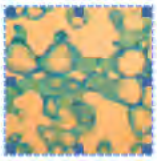

State-of-the-Science Workshop on Chemically-induced Mouse Lung Tumors: Application to Human Health Risk Assessment

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Session Abstracts

http://www.epa.gov/iris/irisworkshops/mltw/

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Disclaimer

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Contents

INTRODUCTION

The U.S. Environmental Protection Agency (EPA), National Center for Environmental Assessment (NCEA) is holding a two-day, state-of-the-science workshop on chemically-induced lung tumors in mice. The purpose of this workshop is to provide a forum for a scientific discussion of a broad range of evidence from human, animal, and *in vitro* studies with a focus on specific chemicals (ethylbenzene, naphthalene, and styrene) known to cause mouse lung tumors, in particular bronchiolar-alveolar adenomas[1] and carcinomas[2]. These types of tumors have been proposed to develop through a species-specific mode of action (MOA) based on metabolic and physiological susceptibility. In this workshop we will review information related to key events and human relevance considerations for all potential MOAs. In addition to the specific chemicals previously mentioned, which are in the process of assessment development for the Integrated Risk Information System (IRIS), other relevant chemicals, including cumene, coumarin, and fluensolfone, known to cause similar tumors will also be discussed. Discussion topics will cover biological similarities between mouse and human lung tumors, including comparative pathology, species concordance issues, and mechanistic evidence.

Workshop Goals – The goals of this workshop is to have an open discussion of evidence related to the formation of these types of mouse lung tumors and inform analysis and interpretation of this evidence in assessing the potential for human cancer risk. Follow-on meetings may occur after the workshop to continue discussions related to the goals of the workshop.

Meeting Scope and Meeting Plan – In order to adequately address all of the relevant issues, the workshop has been organized into four sessions. Each session will examine individual topic areas in detail, beginning with and continually referring back to the human relevance of data from animal and in vitro studies. The sessions are scheduled to reflect a progression of the discussion beginning with the evidence for lung tumors in humans – both in populations (epidemiology) and in individuals (human pathophysiology) – then moving into the use of animal models (esp. mice) to investigate tumor formation; consideration of the biological mechanisms leading to tumor formation; and wrapping up with consideration of key events at the cellular and subcellular level. The session titles are: (1) human cancer – epidemiology and pathophysiology; (2) comparative pathological evidence; (3) biological mechanisms; and (4) cellular, genetic, and molecular evidence. A summary discussion will conclude the workshop with a review the workshop discussions and by delineating any next steps.

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[1] Bronchiolar-Alveolar Adenoma – [synonyms: Alveolar Cell Adenoma; Alveolar/Bronchiolar (A/B) Adenoma; Bronchiolar Adenoma; Bronchiolo-alveolar Adenoma; Bronchoalveolar Adenoma] A benign epithelial neoplasm arising from either type II pneumocytes or club (Clara) cells in the area of the A/B junction in which the tumor cells form glands or gland-like structures; usually well circumscribed, tending to compress rather than infiltrate or invade adjacent tissue.

[2] Bronchiolar-Alveolar Carcinoma (or Adenocarcinoma) – [synonyms: Alveolar Cell Carcinoma; Bronchiolar Adenocarcinoma; Bronchiolo-alveolar Carcinoma; Bronchiolo-alveolar Adenocarcinoma; Bronchoalveolar Carcinoma] A malignant epithelial neoplasm arising from either type II pneumocytes or club (Clara) cells in the area of the A / B junction of the lung; microscopically, neoplastic cells are often cuboidal or columnar and form papillary structures; metastases may occur to regional lymph nodes or, less commonly, to more distant sites.

Sponsorship and Consultation – The workshop is being sponsored and organized by EPA with input from (1) a volunteer committee of outside experts (including representatives from academic institutions, State agencies, other Federal organizations, NGOs, and industry), and (2) an internal working group of experts from EPA and other Federal partners.

Purpose of the Session Abstracts

This document is being provided to assist workshop participants to be prepared for the session discussions with the intention to minimize the need for introductory presentations during the workshop. The following sections cover each session and are designed with two purposes in mind.

Topic Primer – The first purpose is to provide a brief introduction of the topic to be discussed for potential participants who may not be completely familiar with the topic. To assist in this introductory purpose, key references are provided to allow deeper investigation of the subject. The reference list provided with each session abstract is not comprehensive but should assist in becoming familiar with the topic. A more comprehensive list of references associated with the Mouse Lung Tumor Workshop is available from a "project page" designed for that purpose in the Health and Environmental Research On-line (HERO) database - http://epa.gov/iris/irisworkshops/mltw/. Footnotes are also provided at the bottom of the page when terms or acronyms are first introduced.

Discussion Guide – The second purpose is to provide a framework for the anticipated discussion with a list of more detailed discussion topics. Additional references relevant to those discussion topics are also provided, with the reference list organized by sub-topic to facilitate additional pre-workshop preparation. The intention is to define the key relevant issues which should be discussed in some detail in the workshop; additional topics/issues may be identified for later discussion (potentially in separate follow-on meetings after the workshop).

An appendix containing supplementary materials (more detailed background information, figures, tables, additional references, etc.) is also provided.

1. Session 1. Human Cancer – Epidemiology and Pathophysiology

Session Goals

- Provide a brief general overview of chemically-induced human lung cancer for context.
- Discuss human lung cancer hazard from the chemicals of interest to EPA (ethylbenzene, naphthalene, and styrene) and chemicals with similar types of lung tumors (cumene, coumarin, fluensolfone), beginning with effects on populations (epidemiology) and moving to effects on the individual (pathophysiology).
- Review of the evidence for upstream[3] events and mechanism(s) for observed toxic effects leading to tumor formation in humans (this topic will be discussed in more detail in Sessions 3 and 4).
- Review the strengths and weaknesses of the human studies on cancer in the lung and other tissues.

1.1. Background

Lung cancer is one of the leading causes of new cancer cases and the most common cause of cancer deaths in the United States, accounting for almost 30% of annual cancer mortality. Although smoking is an important risk factor for lung cancer, accounting for roughly 80% of all lung cancer deaths in both women and men, other risk factors include occupational and environmental exposures, particularly to second-hand smoke, asbestos, some organic chemicals, radiation, air pollution and many others (IARC, 2013; NCI, 2013a; American Cancer Society, 2012). Genetic susceptibility also contributes to lung cancer development (American Cancer Society, 2012).

Non-small cell lung cancer (NSCLC) constitutes the majority of human lung cancer cases (NCI, 2013b). The most common subtype of NSCLC is adenocarcinoma[4] (AC), regardless of smoking status. Current lung cancer terminology affects how tumors are classified; how this terminology is applied across epidemiologic and other human studies will also be considered. Many topics introduced in this session will be discussed in greater detail in the later sessions.

1.2. Discussion Topics

1. Classification of lung cancers found in humans.
2. Chemicals (excluding particles, metals, and tobacco smoking) which are causally or strongly associated with lung cancer in humans.
3. Epidemiological evidence regarding lung and other cancers for the three chemicals of interest.
4. Evidence in humans for the chemicals of interest to cause effects such as lung toxicity and oxidative damage associated with lung cancer development.
5. Genetic factors and polymorphisms which may modulate human lung cancer risk.

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[3] "Upstream" in this context refers to biological events (e.g., a genetic mutation) which take place in cells or tissues prior to an adverse outcome such as formation of a tumor. Upstream events are typically thought to be required before progression to the final outcome.

[4] Adenocarcinoma – A malignant neoplasm of epithelial cells with a glandular or gland-like pattern.

6. Key genetic susceptibility factors, and molecular alterations (including but not limited to genotoxicity, toxicity, and epigenetics) identified in human studies; how these events or markers elucidate signaling or disease pathways.

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2. Session 2. Comparative Pathological Evidence

Session Goals

- Discuss the utility of the standard mouse model for identifying potential human lung tumorigens.
- Investigate pathologic features of known mouse lung tumorigens and key pathologic changes related to the proposed CYP/cytotoxicity MOA and alternative MOAs.
- Review the known similarities and differences between the pathology of lung tumors (particularly chemically-induced bronchiolar-alveolar lung tumors) in mice and other species (especially humans).
- Review how mouse models (standard laboratory strains and genetically-engineered mouse models) have informed research on human lung cancer, particularly for chemically-induced bronchiolar-alveolar lung tumors.

2.1. Background

Lung tumors in mice share numerous morphological and molecular characteristics with human lung cancer. However, species differences also exist which may influence the human relevance assessment of mouse lung tumors. While lung tumors can arise spontaneously in mice, as in humans, mouse lung tumorigenesis can also be experimentally induced by chemical exposure, radiation, or direct genetic manipulation through molecular biology and selective breeding. For chemical exposures, lung is the second most frequent tumor site reported in pathology databases of the EPA and National Toxicology Program. In particular, mouse bronchiolar-alveolar tumors are proposed for some chemicals to originate in type II pneumocytes or club (Clara) cells[5] via pathways that might be species-specific. While rodent lung tumors are reported primarily in the mouse, they have also been observed in treatment-related response in the rat and other species. Genetically engineered mouse models (GEMMs) of lung cancer have also been developed which demonstrate a dramatic incidence and rapid progression of lung tumors in mice bred to contain specifically-mutated genes. These mice typically developed aggressive lung tumors within weeks to months, versus the months to years generally reported following exposure for chemically-induced mouse lung tumors. Molecular pathology analyses have revealed shared biological targets and pathways between mouse and human lung tumors; however, the human health relevance of lung tumors in mouse studies remains unclear. In this session we will review the comparative biology of mouse lung tumors, associated pathologic effects of known mouse lung tumorigens, and issues related to tissue and species concordance.

[5] Club (Clara) Cells – [synonym: bronchiolar exocrine cell] A rounded, club-shaped, non-ciliated cell protruding between ciliated cells within bronchiolar epithelium; with secretory and metabolic functions.

2.2. Discussion Topics

2.2.1. Comparative Pathology

1. General anatomical and pathological features of mouse lung compared to other species.
2. Common mouse lung tumor histological types and their relation to histological types of human lung cancer.
3. Species, sex, and strain differences in historical control and treatment-related incidence of lung tumors.
4. Species comparison of associated pathologic events for mouse lung tumorigens.
5. Histologic or molecular features of spontaneous and chemically-induced mouse and human lung tumors.
6. Cell(s) of origin for chemically-induced mouse and human lung tumors.
7. Criteria for distinguishing bronchiolar-alveolar hyperplasia[6], adenoma, and carcinoma.
8. The relation of GEMMs to traditional guideline study models, particularly for CYP2F2-null mice.
   a. The ability of knock-out mouse models to effectively discriminate known rodent lung tumorigens versus non-tumorigens based on early key events.
   b. Pathologic features of GEMMs for lung cancer.
   c. The lung pathology phenotypes for common knock-out or transgenic mouse lung models.
   d. The translational limitations of these models.

2.2.2. Tissue/Species Concordance

1. General applicability of mouse lung tumorigenesis to understanding human lung tumor formation
   a. Similarities/differences in mouse and human lung tumorigenesis
   b. Areas of uncertainty in human lung tumor etiology which may affect the translatability of chemically-induced mouse lung tumorigenesis
   c. Morphological differences between mouse and human lungs that affect toxicokinetic/toxicodynamics parameters following inhalation or oral exposure.
2. The evidence for concordance between lung tumors in mice and potential tumors at other sites in humans.
   a. Other site-specific cancers observed in epidemiology studies for chemicals (excluding particles, metals, and tobacco smoking) with evidence of mouse lung tumors.
   b. The evidence for non-respiratory target sites for the three chemicals of interest.
3. Studies on upstream markers which may inform non-respiratory tumors.
4. The potential for observations of non-respiratory cancer which suggest involvement of other signaling or disease pathways common to the lung.

[6] Hyperplasia – An increase in the number of cells in a tissue or organ, excluding tumor formation, whereby the bulk of the part or organ may be increased. Hyperplasia is distinct from cancer but, depending upon the cause and tissue, may progress to a cancer.

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3. Session 3. Biological Mechanisms

Session Goals

- Discussion of biological mechanisms and relevance to potential MOAs.
- Discuss available ADME information for the three chemicals of interest (ethylbenzene, naphthalene, and styrene) as they relate to the formation of mouse lung tumors.
- Differences in ADME between mice and other species (especially rats and humans), and between the three chemicals.

3.1. Background

ADME (Absorption, Distribution, Metabolism, and Excretion) describes the set of processes which determine the concentration of a chemical, or its metabolites, in tissues of the body. Absorption refers to any process by which a chemical first enters the body, either through the respiratory tract (inhalation), gastrointestinal tract (oral ingestion), or skin (dermal absorption). Distribution is movement of a chemical within the body, which occurs primarily by diffusion within tissues and as convection as it is carried by the blood. Metabolism and other chemical reactions in the body alter chemical entities, typically as a means of eventually eliminating exogenous compounds, but intermediate metabolites are often the primary cause of a chemical's toxicity. Chemicals or their metabolites are often excreted in the feces and urine, but some are eliminated in the exhaled breath, sweat of sloughing of skin cells, or in expressed breast milk. Collectively these processes determine the concentration of the proximate toxicant at the site of toxicity. Further details and definitions of common ADME terms are available on line (http://pharmaxchange.info/press/2011/04/pharmacokinetics-basics-absorption-distribution-metabolism-and-excretion/).

The available information on ADME for each of the three chemicals (ethylbenzene, naphthalene, and styrene) will be reviewed in this session. Separate summaries and metabolism schemes for each chemical are provided in the Appendix.

Discussion will also focus on the mechanisms leading to development of mouse bronchiolar-alveolar tumors which are proposed to originate in type II pneumocytes or club (Clara) cells via a cytochrome P450 (CYP) /cytotoxicity-mediated mode of action (MOA) that might be species-specific for certain chemicals. In this session we will bring forward issues related to the pathologic features of mouse lung tumors as discussed in Session 2, and go into greater depth on key events (both pathologic and metabolic) in the proposed CYP/cytotoxicity and alternative MOAs.

3.2. Discussion Topics

1. Use of ADME to inform differences between effects in the lung and other tissues within the mouse, and differences between species/strains.
   - **a.** Contribution of species and tissue differences in metabolism (especially enzyme profiles and metabolites) to tumor formation.
   - **b.** Metabolites with cytotoxic, genotoxic, clastogenic, mitogenic, or epigenetic activity.

c. Metabolic pathways in mice and in the human lung: similarities, differences, and where comparative data do not exist.

d. Route of exposure differences (e.g., dietary versus inhalation) related to key effects leading to lung tumor formation.

e. Potential for metabolism in the liver to contribute to effects elsewhere in the body (e.g., nasal effects for naphthalene). First-pass effect considerations.

2. Mechanisms and resulting pathology in the context of potential to develop mouse lung tumors.

a. The time course of key pathologic effects for lung tumorigenesis in the mouse for known lung tumorigens.

b. Tumorigens which are known or suspected of inducing lung epithelial cell cytotoxicity and/or stress leading to regenerative proliferation and/or hyperplasia and/or for inducing genotoxic or other molecular events leading to tumor formation.

c. Morphologic criteria for cytotoxicity.

d. Evidence for mitogenicity[7] in lung epithelial cells and the potential to be distinguished from cytotoxicity

e. Downstream events that could distinguish a cytotoxic tumorigen versus a non-tumorigen versus a genotoxic tumorigen.

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[7] Mitogen – A substance that induces mitosis (cell division) and cell transformation, especially lymphocyte transformation.

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4. Session 4: Cellular, Genetic, and Molecular Evidence

Session Goals

- Review the types of cellular, genetic, epigenetic, and molecular effects evident from exposure to the chemicals of interest.
- Understand the role of cellular and molecular/genetic evidence in the development of lung tumors (both in mice and human).
- Discuss how biological mechanisms (ADME) on the three chemicals of interest (ethylbenzene, naphthalene, and styrene) lead to effects on the cellular and subcellular level, and the contribution of those effects on observable pathology.
- Review the evidence from the related chemicals (cumene, coumarin, fluensolfone) for any additional insights not available from studies on the chemicals of interest.

4.1. Background

Carcinogenesis involves a complex series of events that alter the cell signals from its extracellular environment, thereby promoting uncontrolled growth. These alterations could induce cell proliferation leading to tumor development. Knowledge of the biochemical and biological changes that precede tumor development may provide important insights for determining whether a cancer hazard exists. Thus, understanding the range of key steps in the carcinogenic process (whether it be mutagenesis, increased cell proliferation, cytotoxicity, or receptor activation) becomes essential for evaluating the mode of action of a particular agent. EPA has developed a framework for evaluating hypothesized carcinogenic mode of action (U.S. EPA, 2005).

4.1.1. Genotoxicity

Most simply, genetic toxicity or genotoxicity can be defined as adverse effects occurring on genetic material and their associated mechanisms within the cell. Genetic materials include the DNA and supporting structures (histones) which assist in packaging DNA into higher level organizational structures known as chromosomes. Various cellular machinery, used to translate, replicate, and repair the genetic code stored in DNA, can also be affected and can lead to genotoxic outcomes. In general, genotoxic chemicals may be mutagenic[8] or clastogenic[9]. In either case, cell transformation from a normally functioning cell may lead to formation of a cancerous cell if the altered cell does not go through a normal programmed death (apoptosis) to remove the threat.

It is well known that genotoxicity play a significant role in the development of tumor formation. Mutations in somatic cells can play a key role early in cancer initiation and might affect other stages of the carcinogenic process. All cancer cells acquire multiple mutations during carcinogenesis, therefore mutation induction or acquisition can be key events at some stage in all cancers.

[8] Mutagen – Any agent that promotes a mutation or causes an increase in the rate of mutational events, including radioactive substances, x-rays, or certain chemicals.
[9] Clastogen – An agent (certain chemicals, x-rays, ultraviolet light) that causes breaks in chromosomes.

DNA damage can occur as a direct DNA damage or indirect DNA damage. The results of these types of damage could be clastogenic or aneugenic. Examples of clastogenicity include chromosomal aberrations, sister chromatic exchanges (SCEs), and micronuclei. Mutations include, DNA strand breaks, DNA adducts (addition products), DNA cross-linking, and DNA covalent binding.

Determination of carcinogens that are operating through a genotoxic mode of action entails evaluation of the available data. One way of determining if a chemical is acting through a genotoxic mechanism is to assemble the relevant data (human, animal, in vivo, in vitro short-term testing results of individual genetic end points), evaluating the data against a current acceptance criteria (study quality, methodology used etc.), and determining the weight of evidence based on both the available data and evaluating against other existing information such as epidemiological data, ADME information etc. Such methodical evaluation will result in a better understanding of the role of an agent in the development of carcinogenesis.

4.1.2. Epigenetics

In addition to genetic alterations, the study of epigenetics has been providing additional insight into mechanisms of carcinogenesis. Epigenetics includes methylation/demethylation processes of DNA, and micro RNA activation/inactivation. Evidence is recently emerging on the potential role of epigenetics in the MOA of mouse lung tumors. Although limited data base is available on epigenetic mechanism, any such data can be evaluated in the realm of mode of action and weight of evidence for evaluating carcinogenesis or lung tumors. Discussion on this topic, however, will focus on associated studies which may provide clues to the role of epigenetics in chemical-induced carcinogenesis.

4.1.3. Molecular Toxicology

Molecular and high throughput data are being generated that may be useful to better understand the adverse outcome pathways leading to formation of mouse lung tumors, and perhaps make comparisons to similar outcomes in humans. Discussion in this topic area will focus on the existing evidence or near-term potential for relevant data to be generated from these advancing areas of research.

4.2. Discussion Topics

1. Evidence for a role of genotoxicity/mutagenicity in the development of the specific mouse lung tumors being discussed in this workshop (e.g., mutational events, clastogenicity, other genotoxic mechanisms). Consider the role of metabolites and ROS to cause DNA damage and repair, as well as damage to other cellular organelles (cytotoxicity).
2. Epigenetics
   a. Potential for epigenetic mechanisms to play a role in the development of mouse lung tumors.
   b. Evidence of epigenetic alterations due to chemical exposure leading to the formation of mouse lung tumors.
3. Relevance of more recent methods (e.g., Tox21 and Next Generation) to generate data on gene expression, toxicity pathways, and epigenetics that could inform the MOA for these mouse lung tumors.
4. Most in vivo genotoxicity data are for tissues other than the lung and in vitro data are difficult to relate to specific tissues. How do we consider these other (in vivo and in vitro) data?

5. Potential for linkages of data seen in humans (e.g., cellular markers, methylation changes, genomic sequencing or GWAS studies) to the mouse lung tumor data.
6. Morphologic effects, molecular features, or biomarkers that contribute to lung tumorigenesis in the mouse.
   a. Key somatic and inherited mutations which need to be considered.
   b. Comparative transcriptional profiles and pathway analyses.

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APPENDIX A. SUPPLEMENTARY MATERIALS

Appendix A Contents

A.1. Session 1

A.1.1. Human Lung Cancer Overview

Lung cancer is one of the leading causes of new cancer cases and the most common cause of cancer deaths in the United States, accounting for almost 30% of annual cancer mortality. Lung cancer mortality rates have declined beginning in the 1990s in men and more recently in women, reflecting patterns in decreased smoking prevalence (Siegel et al., 2013; American Cancer Society, 2012). A different pattern emerges for lung cancer incidence, however, with rates declining in men since the 1990s while remaining steady in women (Siegel et al., 2013). While smoking is an important risk factor for lung cancer, accounting for roughly 80% of all lung cancer deaths in both women and men, other risk factors include some occupational and environmental exposures, particularly to second-hand smoke, asbestos, some organic chemicals, radiation, air pollution and paint (occupational) (IARC, 2013; NCI, 2013c; American Cancer Society, 2012). Genetic susceptibility also contributes to lung cancer development (American Cancer Society, 2012)..

Non-small cell lung cancer (NSCLC) constitutes the majority of human lung cancer cases. The most common subtype of NSCLC is adenocarcinoma (AC), regardless of smoking status. Inconsistencies in the classification of AC led to a 2006 recommendation to use clinical, radiological, molecular, and imaging features and tumor progression, from pre-invasive to invasive, to classify this tumor type (Travis et al., 2011b; Travis et al., 2011a). Another major recommendation by Travis et al. was to use terms that better described tumor growth progression instead of bronchial-alveolar carcinomas (BAC).

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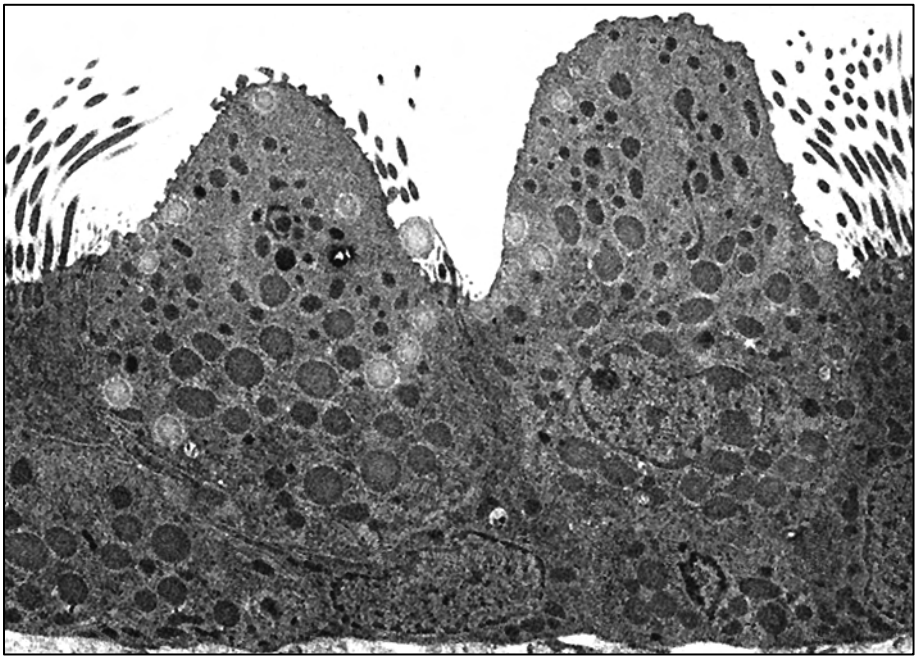
A.2. Session 2

A.2.1. Genetically-engineered Mouse Models (GEMMs)

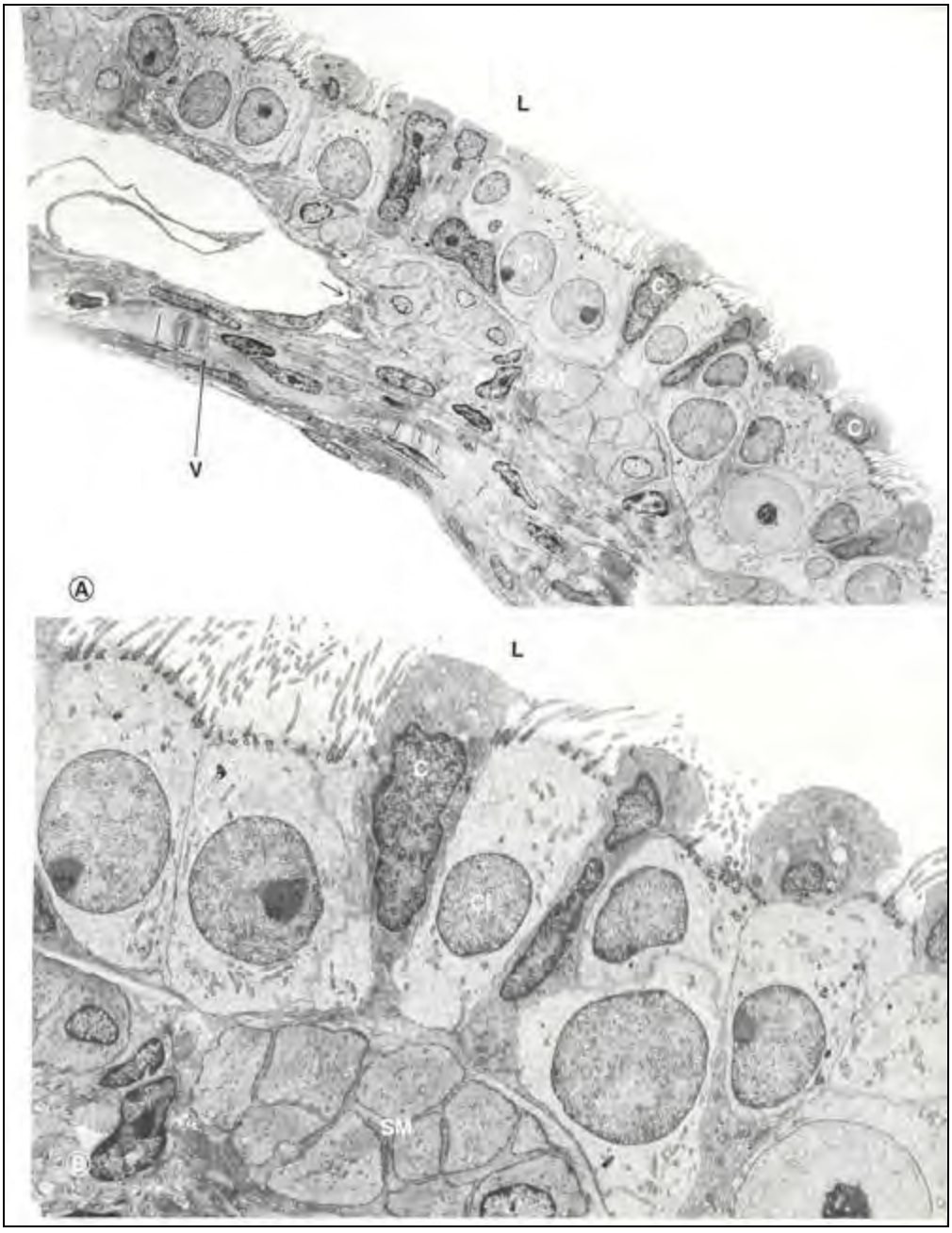
Lung tumors in mice share numerous morphological, histological, and molecular characteristics with human lung cancer, and typically develop in 1–2 years, or roughly 1/10 the time required for human lung carcinogenesis. While lung tumors can arise spontaneously in mice, as in humans, mouse lung tumorigenesis can also be experimentally induced by chemical exposure, radiation, or direct genetic manipulation through molecular biology and selective breeding.

Members of the RAS gene family harbor the most frequent initial mutations in a variety of human cancers. Mouse lung cancer models have been developed which demonstrate a dramatic incidence and rapid progression of lung tumors in mice bred to contain specifically-mutated K-ras genes. These mice typically developed aggressive lung tumors within weeks to months, versus the months to years generally reported following exposure for chemically-induced mouse lung tumors. While experimentally useful, these GEMMs do not present the mutational diversity commonly observed following chemically-induced tumorigenesis, because the mutations are pre-determined by specific breeding. Although accelerated onset of lung tumors could be beneficial by reducing time and costs, some lung tumors in GEMMs grow so rapidly that they do not acquire the subsequent enabling mutations common to both human lung cancer and chemically-induced mouse lung tumor models.



Note the short surface microvilli, abundant secretory vesicles in the cytoplasm, and basal nuclei within the club cells. (Image provided courtesy of Connie Cummings and the NIH/National Institute of Environmental Health Sciences.)

Figure A-1. Terminal bronchiole showing two dome-shaped club (Clara) cells between ciliated cuboidal epithelial cells.



Legend: C, Clara Cell; Ci, ciliated epithelial cell; L, lumen of bronchiole; SM, Smooth Muscle; V, vein (Fig. A); arrow, point of separation of venous and bronchiolar walls (Panel A). Panel A, 2,500 X; Panel B, 5,130 X
[Courtesy of Visual Histology (VisualHistology.com), with the permission of David T. Moran Ph.D.
http://www.visualhistology.com/products/atlas/VHA_Chpt11_The_Respiratory_System.html, accessed July 30, 2013]

Figure A-2. Cross sections through the terminal bronchiole of the squirrel monkey.

A.3. Session 3

A.3.1. Ethylbenzene

Ethylbenzene appears to show kinetics typical of an inhaled gas eliminated by saturable metabolism in mice (Charest-Tardif et al., 2006) and rats (where competitive clearance was shown) (Haddad et al., 2000). However, it has also been shown to induce CYPs 2E1, 2B1, and 2B2, and reduce 2C11 expression in rats, with all of these effects being time-dependent with on-going exposure (e.g., after an initial spike of 4-5-fold increase in CYP 2E1 activity after a single day of exposure, the increase declines to around 2-fold with subsequent days of treatment) (Bergeron et al., 1999).

In the mouse liver and lung CYP 2F2 appears to mediate a significant portion of ethylbenzene oxidation to reactive metabolites, as quantified by binding to microsomal protein (Saghir et al., 2010; Saghir et al., 2009). The link from protein binding to cytotoxicity has not been demonstrated for ethylbenzene. A 13-week inhalation bioassay found no histopathological changes in mouse (or rat) lungs for exposure up to 1,000 ppm ethylbenzene (NTP, 1992a).. Further, it is not clear that the ethylbenzene metabolites involved are specific to CYP2F2, as inhibition of CYP2E1 also significantly reduced the observed binding.

Figures

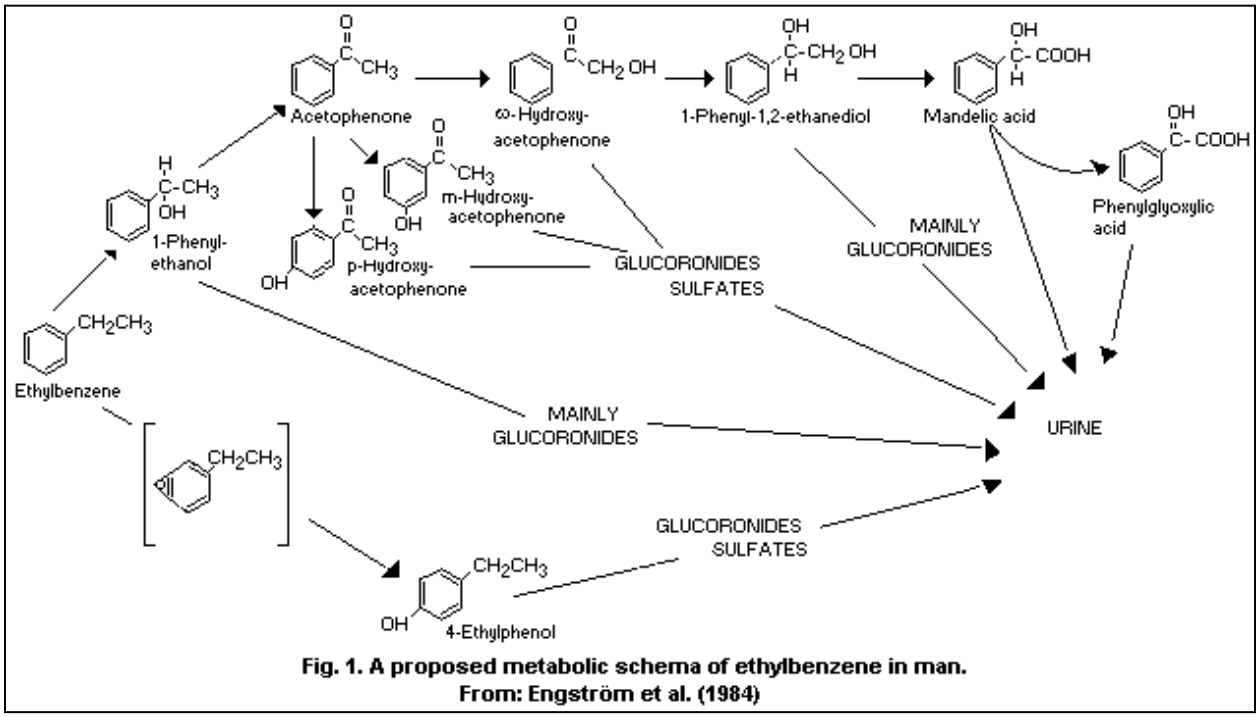


Fig. 1. A proposed metabolic schema of ethylbenzene in man.
From: Engström et al. (1984)

From IPCS, 1996; http://www.inchem.org/documents/ehc/ehc/ehc186.htm.

Figure A-3. Metabolic scheme for ethylbenzene in humans.

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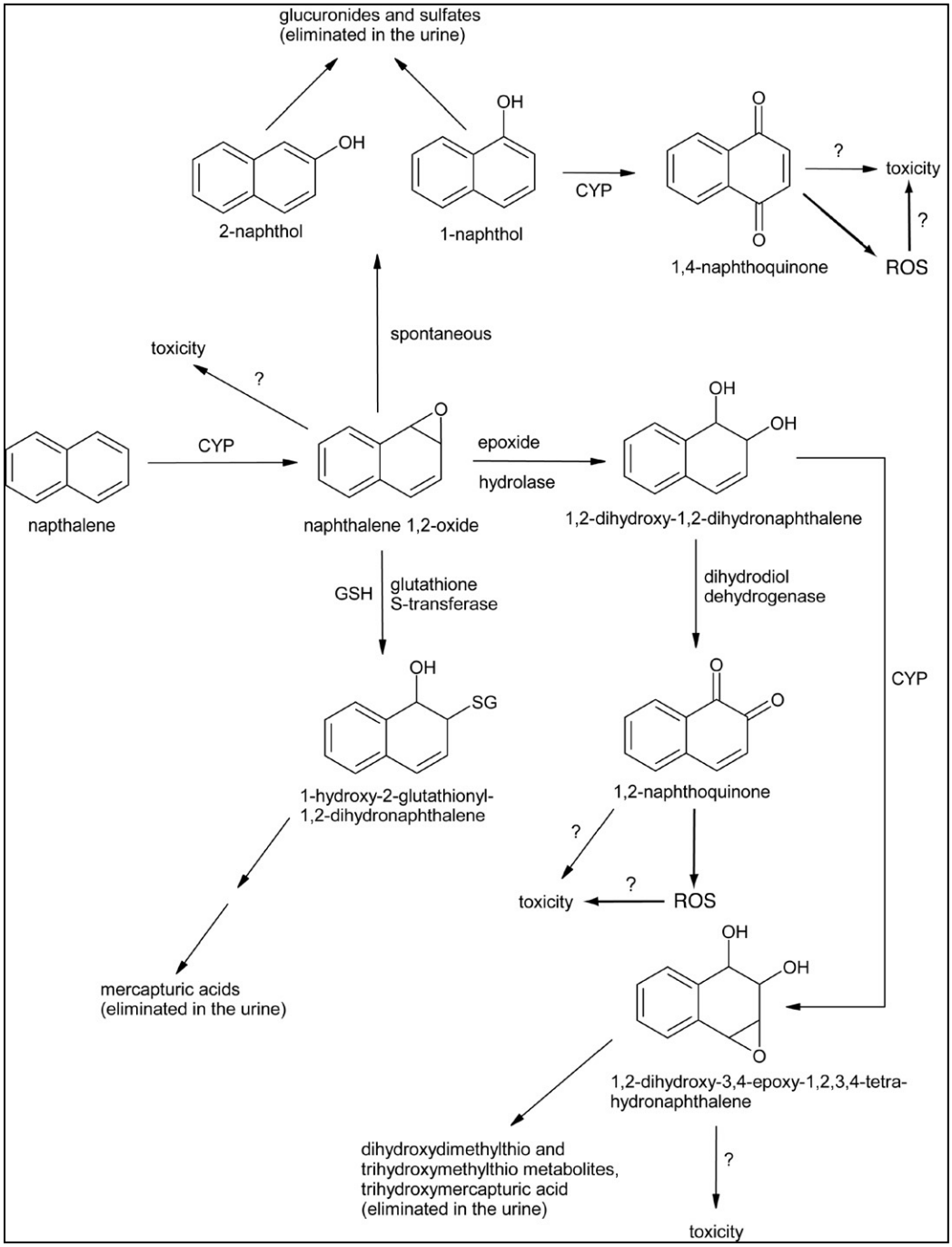
A.3.2. Naphthalene

The role of metabolism in tissue-specific naphthalene-induced cytotoxicity and tumorigenicity was reviewed at a state-of-the-science meeting in 2006, as reported by Bogen et al. (2008). The abstract of that paper makes a number of statements on the then state-of-the-science and identifies critical areas of uncertainty, which is presented here as a starting point for workshop discussions; quoting of this text does not constitute endorsement of these statements.

> "Major conclusions reached concerning scientific claims of high confidence were that: (1) rat nasal tumor occurrence was greatly enhanced, if not enabled, by adjacent, histologically related focal cellular proliferation; (2) elevated incidence of mouse lung tumors occurred at a concentration (30 ppm) cytotoxic to the same lung region at which tumors occurred, but not at a lower and less cytotoxic concentration (tumorigenesis NOAEL = 10 ppm); (3) naphthalene cytotoxicity requires metabolic activation (un-metabolized naphthalene is not a proximate cause of observed toxicity or tumors); (4) there are clear regional and species differences in naphthalene bioactivation; and (5) target tissue anatomy and physiology is sufficiently well understood for rodents, non-human primates and humans to parameterize species-specific physiologically based pharmacokinetic (PBPK) models for nasal and lung effects. Critical areas of uncertainty requiring resolution to enable improved human cancer risk assessment were considered to be that: (1) cytotoxic naphthalene metabolites, their modes of cytotoxic action, and detailed low-dose dose-response need to be clarified, including in primate and human tissues, and neonatal tissues; (2) mouse, rat, and monkey inhalation studies are needed to better define in vivo naphthalene uptake and metabolism in the upper respiratory tract; (3) in vivo validation studies are needed for a PBPK model for monkeys exposed to naphthalene by inhalation, coupled to cytotoxicity studies referred to above; and (4) in vivo studies are needed to validate a human PBPK model for naphthalene."

Buckpitt et al. (2002) specifically reviewed the metabolic mechanisms of naphthalene-induced respiratory tract toxicity and concluded that CYP2F2 plays a significant role in the mouse lung. Cruzan et al. (2009) indicate in their review that the naphthalene-induced toxicity is secondary to glutathione depletion induced by the compound (in the mouse lung).

Figures



Reprinted with permission of Elsevier Inc.; Bogen et al. (2008): Adapted from Buckpitt et al. (2002); Waidyanatha et al. (2002); ATSDR, (2005);
CYP, cytochrome P450 enzymes(s) including (but perhaps not limited to) CYP2F;
SG, glutathionyl moiety; GSH, reduced glutathione; ROS, reactive oxygen species.

Figure A-4. Bogen et al.'s (2008) "Scheme for naphthalene metabolism and formation of multiple reactive metabolites that may be involved in naphthalene toxicity."

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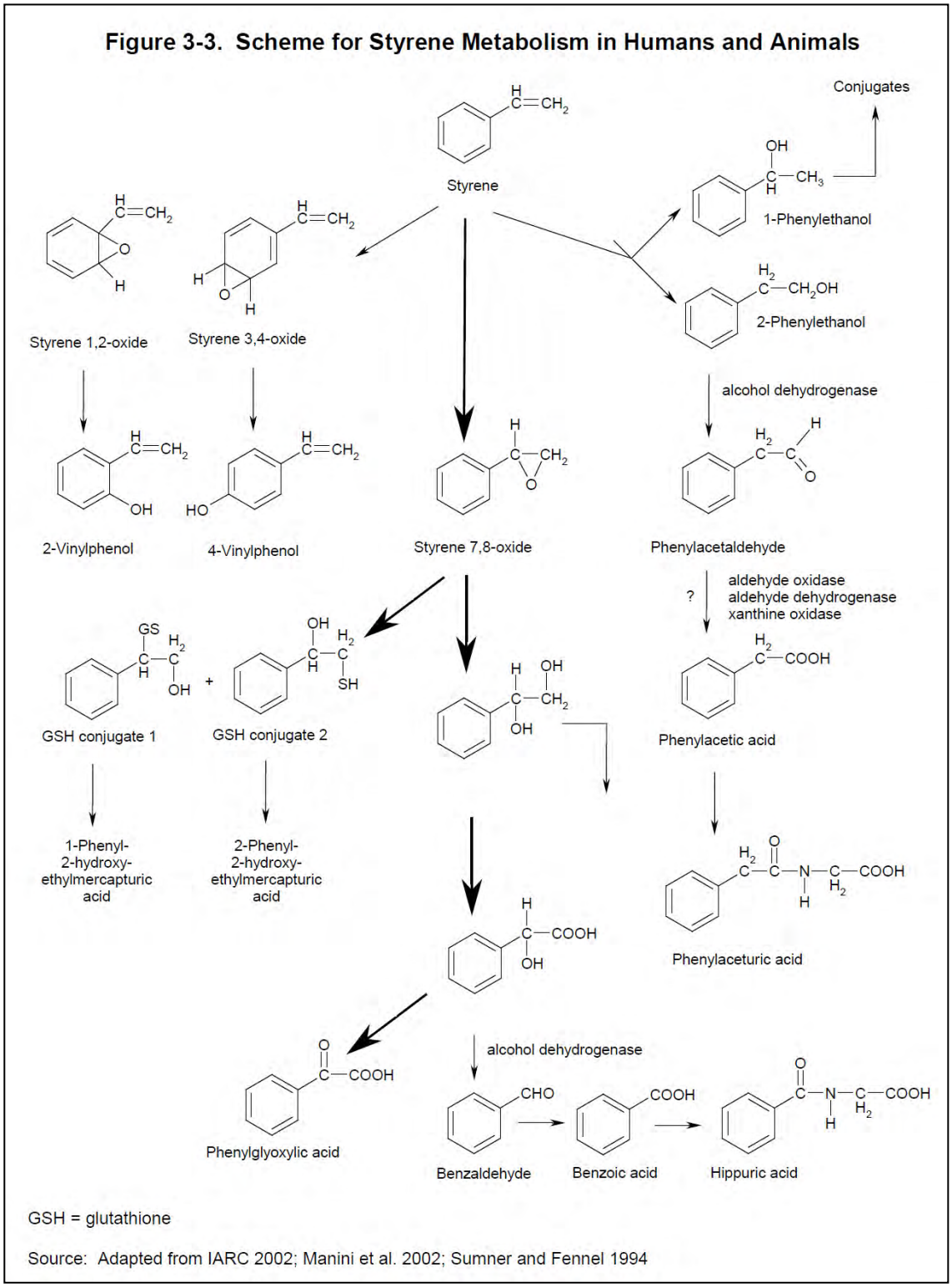
A.3.3. Styrene

Sumner and Fennell (1994) provides a general review on styrene's metabolism in laboratory animals and humans, which has been subsequently updated in papers by Manini et al. (2002) and Linhart et al. (2010). Autoradiographic studies were described for both species with clearly differing distributions: "nonvolatile material" was found in the lung, kidney, and intestinal and gastric mucosa of CD1 mice after IV exposure while most of the material found in Sprague Dawley rats was in fat or kidneys at lower concentrations. The lung was *not* mentioned as a site of accumulation in NMRI mice, however. Toxic endpoints mentioned in the review of Sumner and Fennell (1994) included hepatotoxicity, reproductive and developmental toxicity, irritation of the respiratory tract and central nervous system, and alterations of the nasal mucosa (accumulation in the olfactory bulb observed by autoradiography is also mentioned). Data on stereoselective metabolism are described and the higher mutagenicity of R-styrene oxide in the *Salmonella* assay is mentioned. The specific activities and stereo-selectivity of a number of CYPs are described, but not 2F2. The role of species differences in metabolism in inducing the higher sensitivity of mice to styrene-induced hepatotoxicity is discussed, but the differences described were essentially quantitative (e.g., the Vmax used in PBPK models for styrene oxidation in mice was 2-3 fold higher than in rats) rather than a qualitative difference that would indicate zero risk or toxicity in species other than the mouse.

Carlson and colleagues have published a number of research studies (Carlson, 2012; Carlson et al., 2006; Carlson, 2004; Carlson et al., 2000; Carlson, 1997a, b) and review articles (Cruzan et al., 2009; Carlson, 2008) reporting and analyzing data on styrene metabolism as it occurs in lung vs. liver, predominantly in mice but with supporting data for humans and rats. These data make it fairly clear that CYP 2F2 has a predominant role in mouse lung metabolism of styrene to cytotoxic metabolites and a significant but not exclusive role in mouse liver. For example, styrene-induced hepatotoxicity is reduced in CYP2E1 knockout mice (KO) while pneumotoxicity was not (Carlson, 2004). Microsomal incubation of styrene using tissues from CYP2F2 KO vs. wild type mice found that hepatic metabolism of styrene to R-styrene oxide was reduced by 26%, but hepatic metabolism to the S enantiomer was *increased* by 16% in the KO

mice; lung metabolism to the R enantiomer was reduced 70% and to the S enantiomer reduced by 41% (Carlson, 2012). Lung cytotoxicity as measured by BALF cell counts and protein levels was completely eliminated in the KO mice while hepatotoxicity was only somewhat reduced. However these data also indicate that over half of the metabolism in mouse lung to the most mutagenic styrene oxide enantiomer (the S form) occurs via other CYPs.

Much of the lung cytotoxicity appears to be related to the ring-oxidation of styrene to styrene to 4-vinylphenol (4-VP, 4-hydroxystyrene) as inhibition of this pathway was shown to reduce lung (and nasal) toxicity (Green et al., 2001b; Green et al., 2001a) and (Carlson, 2004) found 4-VP to be a very potent pneumotoxicant. However the toxicity of 4-VP appears to be mediated by further metabolism via *both* CYP2E1 and CYP2F2, as specific inhibitors for each enzyme were found to significantly reduce 4-VP-induced pneumotoxicity in mice (Carlson, 2002).



From ATSDR (2010) Toxicological Profile for Syrene, 2010.

Figure A-5. Styrene metabolism.

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A.4. Session 4

Genetic Activity Profile (GAP) is the term for a bar graph containing test results from multiple toxicological effects. The graphs display effective (or ineffective) doses across an array of short-term tests measuring genetic and related damage. GAPs are presented here for ethylbenzene, naphthalene, styrene, and styrene oxide.

The GAP database was assembled for the U.S. Environmental Protection Agency (EPA) in the 1990s (Waters et al., 1991) using data from the International Agency for Research on Cancer (IARC) or from the EPA.

Phylogenetic Order of Short-term Tests

Tests are organized in the profiles and data listings according to the test organism based on the phylogeny of the test species as follows:

Nonmammalian

- Prokaryotes (primarily bacteria)
- Lower eukaryotes (primarily yeast and fungi)
- Plants
- Insects

Mammalian

- Animals (primarily rodents)
- Humans

Within the mammalian group, tests are divided into in vitro or in vivo subgroups.

Endpoints of Short-term Tests

Short-term tests for genetic and related effects measure an array of DNA, chromosomal and mutagenic effects, including the following endpoints:

- DNA damage
- Recombination (including gene conversion)
- Gene mutation
- Sister chromatid exchange
- Micronuclei
- Chromosomal aberrations
- Aneuploidy
- Morphological cell transformation

Some tests in the GAP database do not have a single endpoint, and GAP includes such a category for assays using "body fluids or host-mediated activation."

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Figures

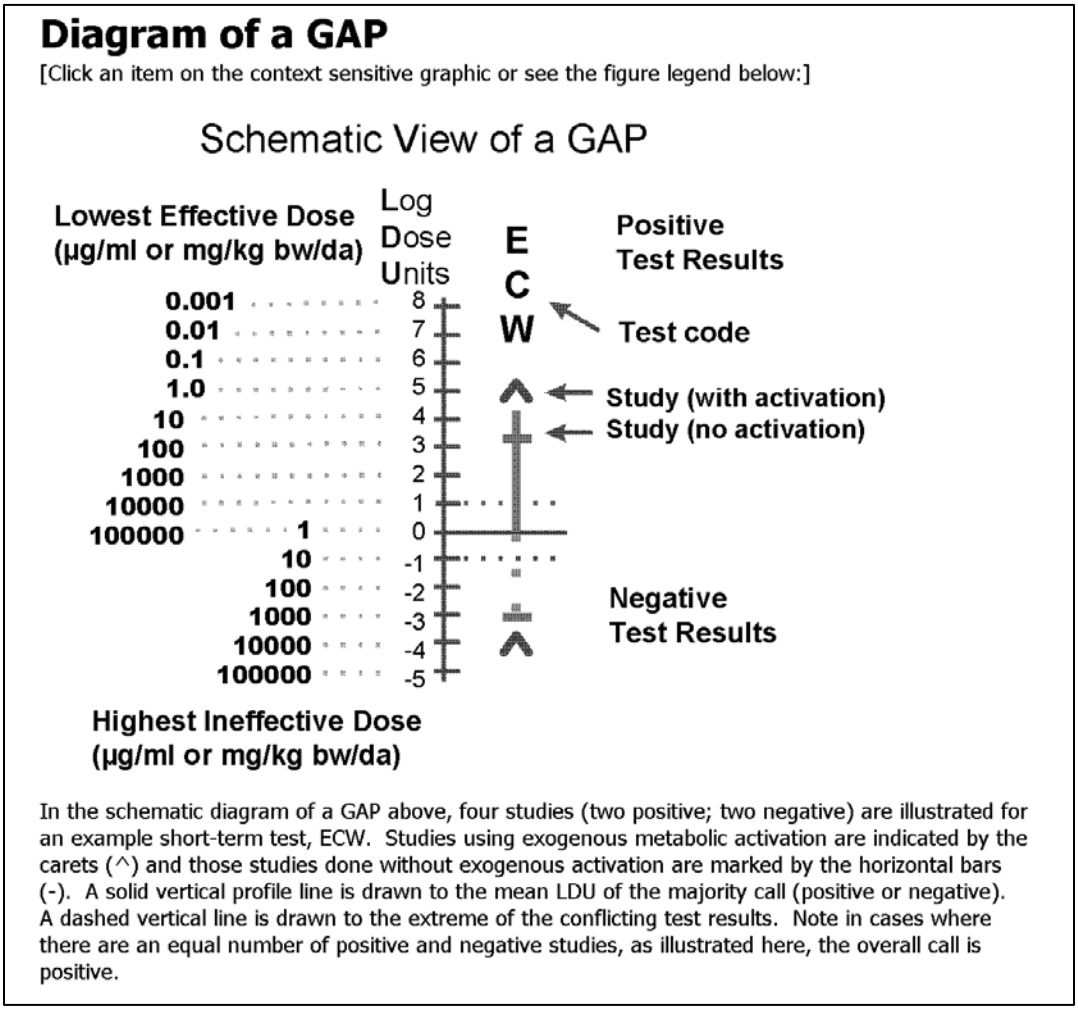

Figure A-6. Diagram of a Genetic Activity Profile (GAP).

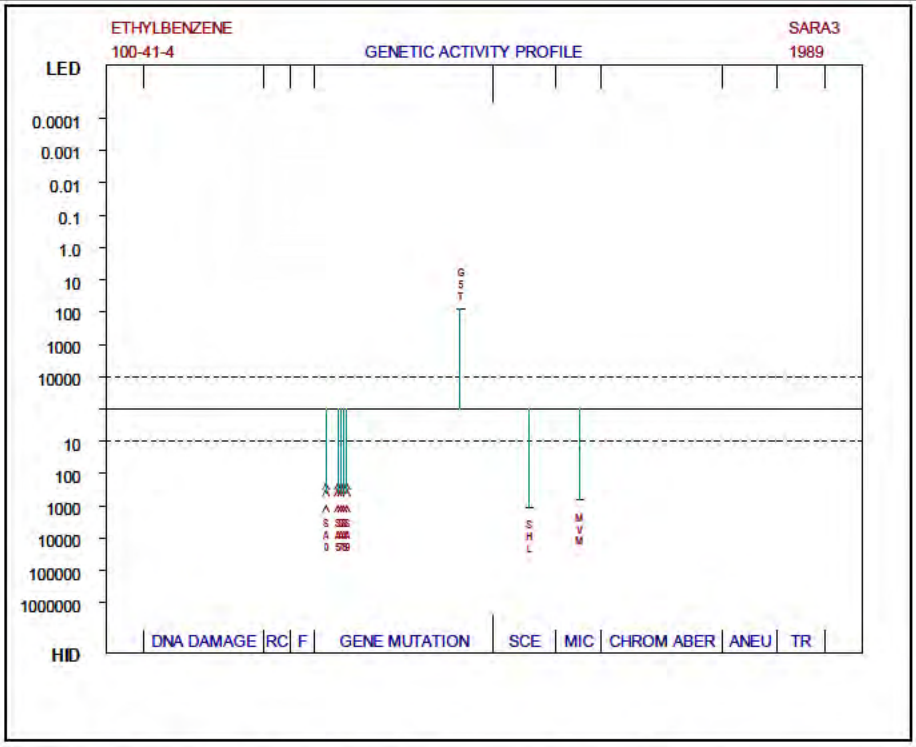

Figure A-7. Genetic Activity Profile for ethylbenzene by endpoint.

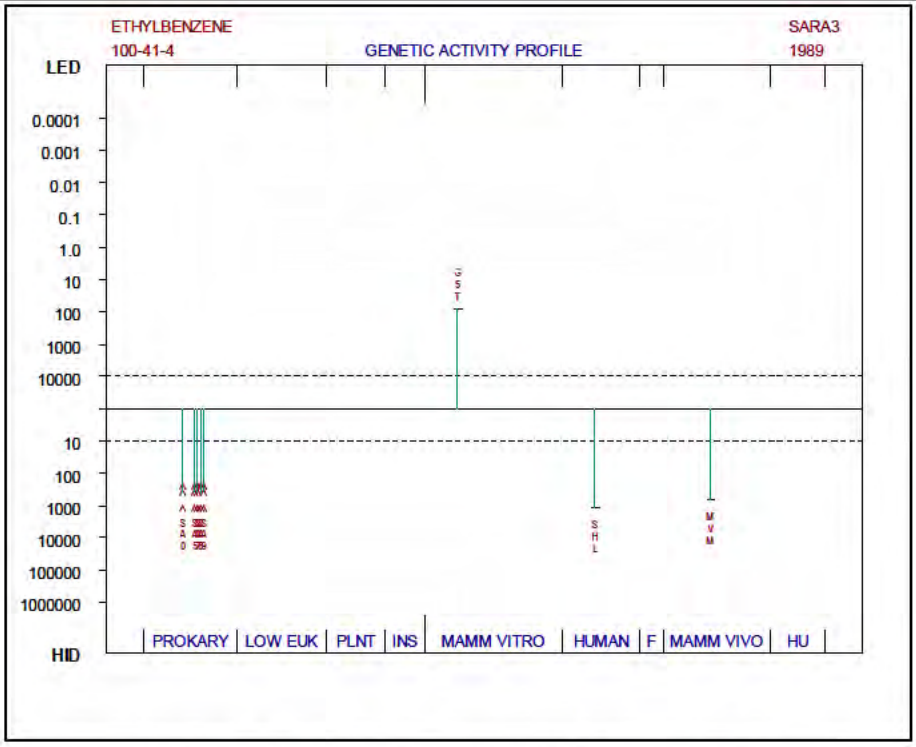

Figure A-8. Genetic Activity Profile for ethylbenzene phylogentically.

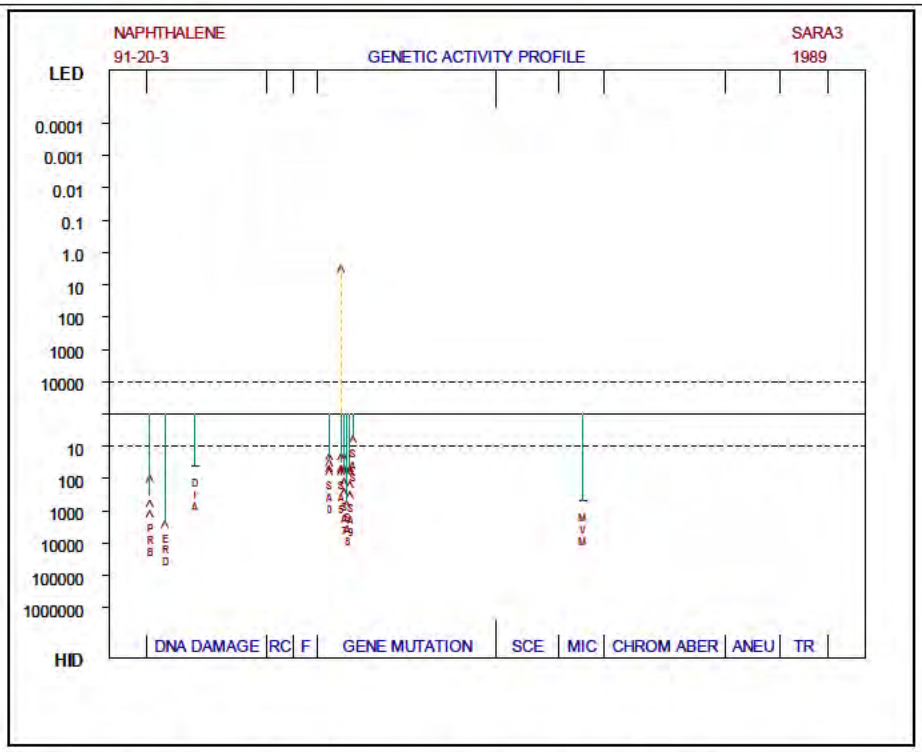

Figure A-9. Genetic Activity Profile for naphthalene by endpoint.

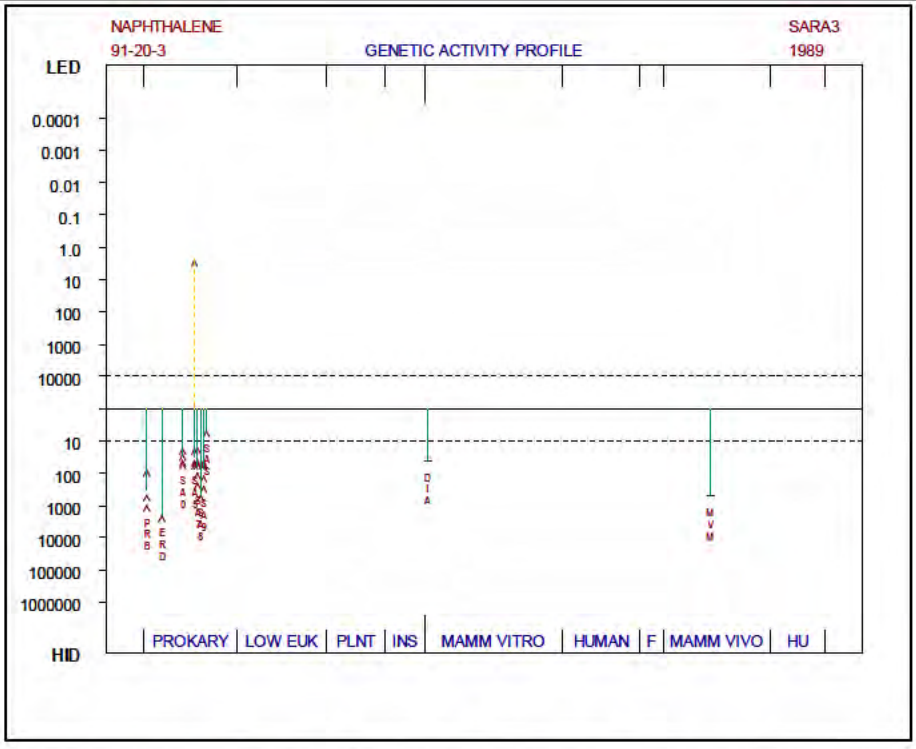

Figure A-10. Genetic Activity Profile for naphthalene phylogenetically.

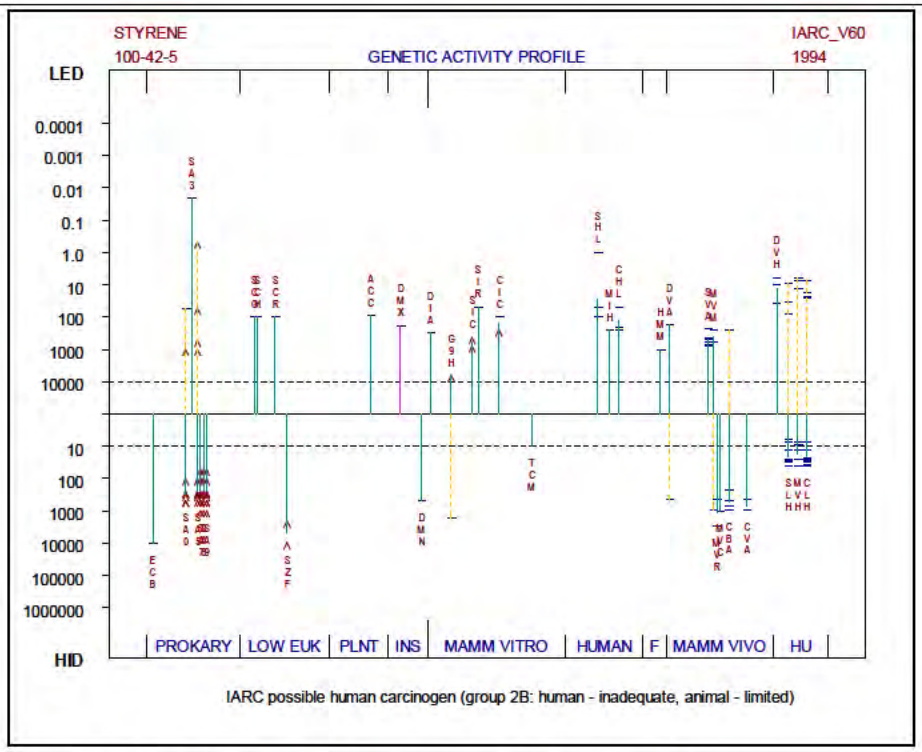

Figure A-11. Genetic Activity Profile for styrene phylogenetically.

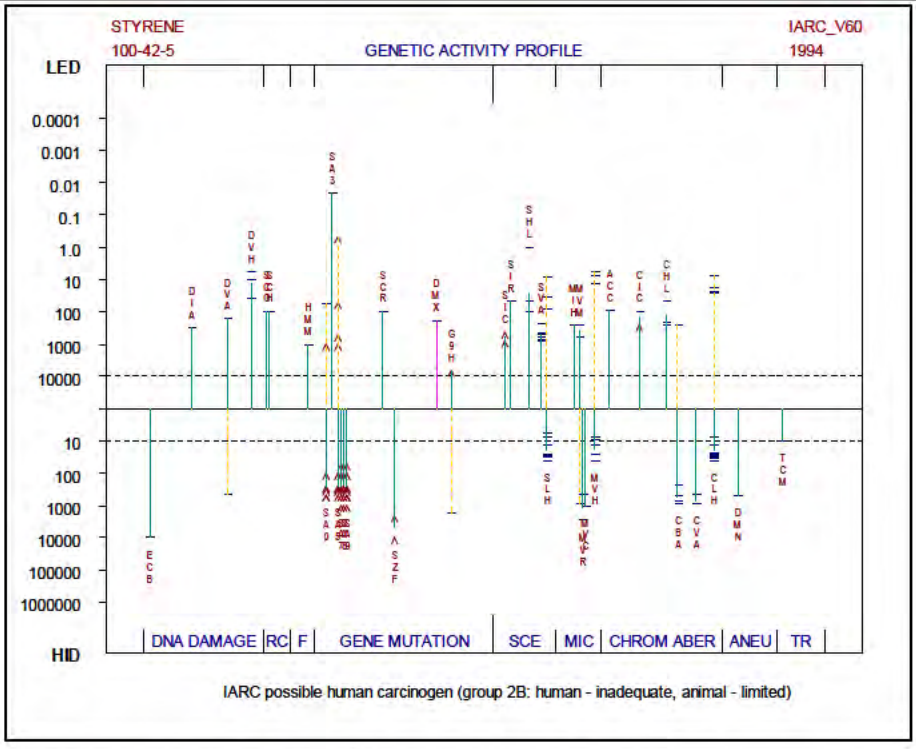

Figure A-12. Genetic Activity Profile for styrene by endpoint.

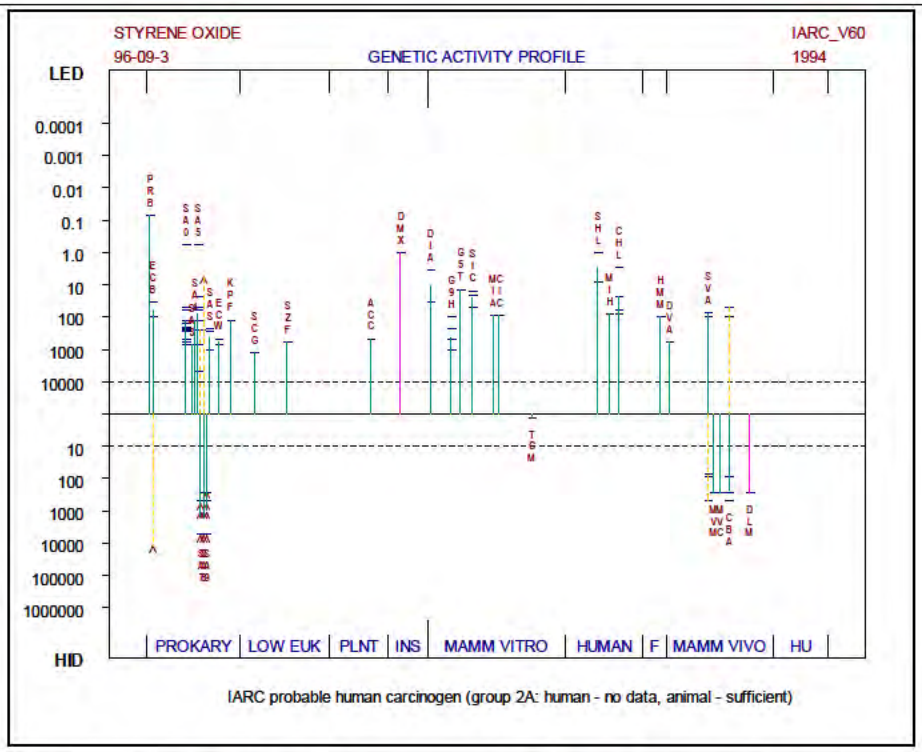

Figure A-13. Genetic Activity Profile for styrene oxide phylogenetically.

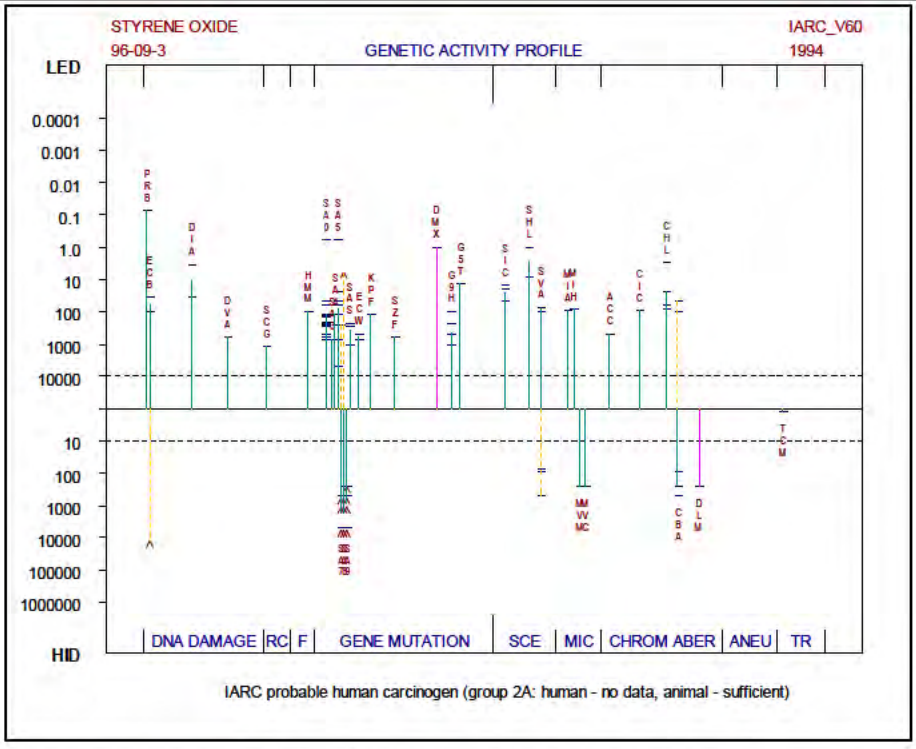

Figure A-14. Genetic Activity Profile for styrene oxide by endpoint.

Table A-1. List of short-term tests used in GAP figures for ethylbenzene, naphthalene, styrene, and styrene oxide

Code[a]	Definition[a]
ACC	Allium cepa, chromosomal aberrations
BHP	Binding (covalent) to RNA or protein, human cells in vivo
BVD	Binding (covalent) to DNA, animal cells in vivo
BVP	Binding (covalent) to RNA or protein, animal cells in vivo
CBA	Chromosomal aberrations, animal bone-marrow cells in vivo
CHL	Chromosomal aberrations, human lymphocyte in vitro
CIC	Chromosomal aberrations, Chinese hamster cells in vitro
CIH	Chromosomal aberrations, other human cells in vitro
CIR	Chromosomal aberrations, rat cells in vitro
CLH	Chromosomal aberrations, human lymphocytes in vivo
DIA	DNA strand breaks, cross-links or related damage, animal cells in vitro
DLM	Dominant lethal test, mice
DMN	Drosophila melanogaster, aneuploidy
DMX	Drosophila melanogaster, sex-linked recessive lethal test
DVA	DNA strand breaks, cross-links or related damage, animal cells in vivo
DVH	DNA strand breaks, cross-links or related damage, human cells in vivo
ECB	E. coli (or E. coli DNA), strand breaks, cross-links or related damage
ECW	Escherichia coli WP2 uvrA, reverse mutation
EC2	Escherichia coli WP2, reverse mutation
ERD	Escherichia coli rec strains, differential toxicity
G5T	Gene mutation, mouse lymphoma L5178Y cells, TK locus
G9H	Gene mutation, Chinese hamster lung V-79 cells, hprt locus
HMM	Host mediated assay, microbial cells in animal hosts
KPF	Klebsiella pneumonia, forward mutation
MIA	Micronucleus test, animal cells in vitro
MIH	Micronucleus test, human cells in vitro
MVC	Micronucleus test, hamsters in vivo
MVH	Micronucleus test, human cells in vivo
MVM	Micronucleus test, mice in vivo
MVR	Micronucleus test, rats in vivo
PRB	Prophage, induction, SOS repair, DNA strand breaks, or cross-links
SAS	Salmonella typhimurium (other miscellaneous strains), reverse mutation
SA0	Salmonella typhimurium TA100, reverse mutation
SA2	Salmonella typhimurium TA102, reverse mutation

Table A-1 (Continued): List of short-term tests used in GAP figures for ethylbenzene, naphthalene, styrene, and styrene oxide

Code[a]	Definition[a]
SA3	Salmonella typhimurium TA1530, reverse mutation
SA4	Salmonella typhimurium TA104, reverse mutation
SA5	Salmonella typhimurium TA1535, reverse mutation
SA7	Salmonella typhimurium TA1537, reverse mutation
SA8	Salmonella typhimurium TA1538, reverse mutation
SA9	Salmonella typhimurium TA98, reverse mutation
SCG	Saccharomyces cerevisiae, gene conversion
SCH	Saccharomyces cerevisiae, homozygosis by recombination or gene conversion
SIC	Sister chromatid exchange, Chinese hamster cells in vitro
SIR	Sister chromatid exchange, rat cells in vitro
SLH	Sister chromatid exchange, human lymphocytes in vivo
SPM	Sperm morphology, mouse
SPR	Sperm morphology, rat
SVA	Sister chromatid exchange, animal cells in vivo
SZF	Schizosaccharomyces pombe, forward mutation
TCM	Cell transformation, C3H 10T1/2 mouse cells

[a]As defined in Waters et al. (1988) as three-letter test codes after the test codes used by the GENE-TOX Program (Waters and Auletta, 1981).

A.5. Additional References

A more comprehensive list of references associated with the Mouse Lung Tumor Workshop is available from a "project page" designed for that purpose in the Health and Environmental Research On-line (HERO) database – http://hero.epa.gov/mltw/.

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