



National Toxicology Program  
U.S. Department of Health and Human Services

# REPORT ON CARCINOGENS

## MONOGRAPH ON

## *ORTHO-TOLUIDINE*

RoC MONOGRAPH 04

JULY 2014

# Report on Carcinogens Monograph on *ortho*-Toluidine

RoC Monograph 04

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National Toxicology Program  
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## Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Report on Carcinogens Monograph series began in 2012. Report on Carcinogens Monographs present the cancer hazard evaluations of environmental agents, substances, mixtures, or exposure circumstances (collectively referred to as “substances”) under review for the [Report on Carcinogens](#). The Report on Carcinogens is a congressionally mandated, science-based, public health document that provides a cumulative list of substances that pose a cancer hazard for people in the United States. Substances are reviewed for the Report on Carcinogens to (1) be a new listing, (2) reclassify the current listing status, or (3) be removed.

NTP evaluates cancer hazards by following a multistep process and using established criteria to review and integrate the scientific evidence from published human, experimental animal, and mechanistic studies. General instructions for the systematic review and evidence integration methods used in these evaluations are provided in the [Handbook for the Preparation of Report on Carcinogens Monographs](#). The handbook’s instructions are applied to a specific evaluation via a written protocol. The evaluation’s approach as outlined in the protocol is guided by the nature, extent, and complexity of the published scientific information and tailored to address the key scientific issues and questions for determining whether the substance is a potential cancer hazard and should be listed in the Report on Carcinogens. Draft monographs undergo external peer review before they are finalized and published.

The Report on Carcinogens Monographs are available free of charge on the [NTP website](#) and cataloged in [PubMed](#), a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these evaluations are included in the [Health Assessment and Workspace Collaborative](#). Information about the Report on Carcinogens is also available on the NTP website.

For questions about the monographs, please email [NTP](#) or call 984-287-3211.

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This report has been reformatted to meet new NTP publishing requirements; its content has not changed. The proposed substance profile is no longer part of the document because it is published in the 14th Report on Carcinogens.

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RoC Monograph on *ortho*-Toluidine

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## Peer Review

Peer review of the Draft RoC Monograph on *ortho*-toluidine was conducted by an ad hoc expert panel at a public meeting held December 12–13, 2013, in the Rodbell Auditorium at the National Institute of Environmental Health Sciences, David P. Rall Building, Research Triangle Park, NC (see <http://ntp.niehs.nih.gov/go/38854> for materials, minutes, and panel recommendations from the peer-review meeting). The selection of panel members and conduct of the peer review were performed in accordance with the Federal Advisory Committee Act and Federal policies and regulations. The panel members served as independent scientists, not as representatives of any institution, company, or governmental agency.

The charge to the Peer-Review Panel was as follows:

- (1) To comment on the draft cancer evaluation component, specifically, whether it is technically correct and clearly stated, whether NTP has objectively presented and assessed the scientific evidence, and whether the scientific evidence is adequate for applying the listing criteria.
- (2) To comment on the draft substance profile, specifically, whether the scientific evidence supports NTP's preliminary RoC listing decision for the substance (available in the RoC, first listed in the 13th edition, at <https://ntp.niehs.nih.gov/ntp/roc/content/profiles/toluidine.pdf>).

For each substance, the Panel was asked to vote on the following questions:

- (1) Whether the scientific evidence supports NTP's conclusion on the level of evidence for carcinogenicity from cancer studies in humans.
- (2) Whether the scientific evidence supports NTP's conclusion on the level of evidence for carcinogenicity from cancer studies in experimental animals.
- (3) Whether the scientific evidence supports NTP's preliminary policy decision on the RoC listing status of the substance.

This RoC monograph on *ortho*-toluidine has been revised based on NTP's review of the Panel's peer review comments. The Peer-Review Panel Report, which captures the Panel recommendations for listing status of *ortho*-toluidine in the RoC and their scientific comments, and the NTP Response to the Peer-Review Report are available on the Peer-Review Meeting webpage for *ortho*-toluidine (<http://ntp.niehs.nih.gov/go/38854>).

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## Abstract

**Introduction:** *ortho*-Toluidine is a high-production-volume synthetic chemical used to manufacture rubber chemicals, herbicide intermediates, dye intermediates, and the local anesthetic prilocaine. People are exposed to high levels of *ortho*-toluidine via inhalation or through the skin in workplaces that use it. *ortho*-Toluidine is found in urine and blood of individuals without known occupational exposure to the chemical, indicating more widespread exposure. Potential sources of exposure include smoking, medical and consumer products, and the environment.

**Methods:** The National Toxicology Program (NTP) conducted a cancer hazard evaluation of *ortho*-toluidine using systematic review methods to identify studies, evaluate study quality, integrate evidence across studies, and integrate evidence across data streams (human, animal, and mechanistic data). Using the Report on Carcinogen listing criteria, NTP reached conclusions on the strength of evidence for the carcinogenicity of *o*-toluidine from studies in experimental animals and humans, and the overall listing recommendation.

### Results and Discussion:

*Human cancer studies:* Epidemiological studies provide evidence of a causal relationship between exposure to *ortho*-toluidine and urinary bladder cancer based on consistent findings across diverse cohorts with different exposure conditions. Moreover, cancer risk increased with increasing level or duration of exposure, and the magnitude of the effect was large across studies.

*Cancer studies in experimental animals:* NTP concluded there was sufficient evidence of carcinogenicity of *ortho*-toluidine from studies in experimental animals. Dietary exposure to *ortho*-toluidine caused tumors (malignant or combined malignant and benign) of the urinary bladder and connective tissue (sarcoma) in rats of both sexes, subcutaneous tissue and mesothelium in male rats, blood vessels in male and female mice, and liver in female mice.

*Mechanistic data:* The mechanisms by which *ortho*-toluidine causes cancer are not fully understood. They likely include metabolic activation to reactive metabolites that bind DNA and proteins, mutagenicity, oxidative DNA damage, chromosomal damage, and cytotoxicity. Metabolic activation and genotoxic effects occur in both experimental animals and exposed humans.

**NTP Cancer Hazard Conclusion:** The conclusion of the cancer hazard evaluation was that *ortho*-toluidine should be listed as *known to be a human carcinogen* in the RoC. The Secretary of Health and Human Services approved the listing of *ortho*-toluidine in the 13th RoC. The rationale for the listing was sufficient evidence from studies in humans and supporting studies demonstrating the biological plausibility of mechanisms of its carcinogenicity in humans.

## Introduction and Methods

*ortho*-Toluidine (*ortho*-methylaniline, CASRN 95-53-4) is a synthetic chemical that is currently used in the United States in the manufacture of rubber chemicals, of a chemical intermediate for herbicides, of dye intermediates, and of the local anesthetic prilocaine. *ortho*-Toluidine has been listed in the Report on Carcinogens (RoC) as *reasonably anticipated to be a human carcinogen* since 1983 based on sufficient evidence of carcinogenicity from studies in experimental animals. Since that time, several cancer studies in humans have been published in the peer-reviewed literature, and the International Agency for Research on Cancer (2010, 2012) has concluded that *ortho*-toluidine is carcinogenic to humans (Group 1). *ortho*-Toluidine has been selected as a candidate substance for review for possible change in listing status in the RoC based on evidence of current U.S. exposure and an adequate database of cancer studies.

## Monograph Contents

This RoC monograph on *ortho*-toluidine reviewed the relevant scientific information and assesses its quality, applied the RoC listing criteria to the scientific information, and recommends an RoC listing status for *ortho*-toluidine. The monograph for *ortho*-toluidine provides information on the following topics: human exposure and chemical properties (Section 1), disposition and toxicokinetics (Section 2), cancer studies in humans (Section 3), cancer studies in experimental animals (Section 4), and mechanistic data and other related effects (Section 5), including studies of genetic toxicology and potential mechanisms of carcinogenicity, relevant toxicological effects. The information in Section 6 is a synthesis of Sections 2 through 5.

The scientific information reviewed in Sections 2 through 5 must come from publicly available, peer-reviewed sources. Information in Section 1, including chemical and physical properties, analytical methods, production, use, and occurrence, may come from publicly available, peer-reviewed or non-peer-reviewed sources.

## Process for Preparation of the Cancer Evaluation Component

The process for preparing the cancer evaluation component of the monograph included approaches for obtaining public and scientific input and using systematic methods (e.g., standardized methods for identifying the literature (see Appendix A), inclusion/exclusion criteria, extraction of data and evaluation of study quality using specific guidelines, and assessment of the level of evidence for carcinogenicity using established criteria).

The Office of the RoC (ORoC) followed the approaches outlined in the concept document, which discusses the scientific issues and questions relevant to the evaluation of *ortho*-toluidine carcinogenicity, the scope and focus of the monograph, and the approaches to obtain scientific and public input to address the key scientific questions and issues, for preparing the cancer evaluation component of the draft monograph. The ORoC presented the draft concept document for *ortho*-toluidine to the NTP Board of Scientific Counselors (BSC) at the June 21–22, 2012 meeting that provided opportunity for written and oral public comments and is available on the RoC website (<http://ntp.niehs.nih.gov/go/37898>), after which the concept was finalized and *ortho*-toluidine was approved by the NTP Director as a candidate substance for review.

## Key Scientific Questions and Issues Relevant for the Cancer Evaluation

The carcinogenicity of *ortho*-toluidine in experimental animals has been well established since the early 1980s, and no new data were identified to challenge these conclusions or to suggest that the findings in experimental animals are not relevant to humans. The monograph assesses the cancer studies in experimental animals that were not part of the original listing. The key questions and issues in the re-review of *ortho*-toluidine concern the evaluation of human cancer studies and mechanistic data.

### Questions Related to the Evaluation of Human Cancer Studies

- What is the level of evidence (sufficient, limited, or inadequate) for the carcinogenicity of *ortho*-toluidine from studies in humans? What are the tissue sites?
- What are the major potential confounders (i.e., co-exposures) for evaluating urinary bladder cancer risk in these studies?
- Can any association between urinary bladder cancer and exposure to *ortho*-toluidine be explained by exposure to these co-exposures or other risk factors for urinary bladder cancer?

### Questions Related to the Evaluation of Toxicological and Mechanistic Data

- What are the tumor sites that contribute to the sufficient evidence in experimental animals? Do the cancer studies in experimental animals published since the original listing provide additional information on the tumor sites?
- What are the potential mechanisms by which *ortho*-toluidine may cause cancer?
  - Is there evidence that these mechanisms occur in humans? Is there evidence to support biological plausibility for cancers of the liver in experimental animals and humans?

## Approach for Obtaining Scientific and Public Input

Additional scientific input from experts was obtained for exposure, human cancer studies, and disposition and toxicokinetics of *ortho*-toluidine. (Technical advisors are identified on the “CONTRIBUTORS” page.)

Public comments on scientific issues were requested at several times prior to the development of the draft RoC monograph, including the request for information on the nomination, and the request for comment on the draft concept document, which outlined the rationale and approach for conducting the scientific review. In addition, NTP posted its protocol for reviewing the human cancer studies for public input on the OROC webpage for *ortho*-toluidine (available at <http://ntp.niehs.nih.gov/go/37898>) several months prior to the release of the draft monograph. Public comment on *ortho*-toluidine is available at <http://ntp.niehs.nih.gov/go/37663>.

## Methods for Writing the Cancer Evaluation Component of the Monograph

The procedures by which relevant literature was identified, data were systematically extracted and summarized, and the draft monograph was written, together with the processes for scientific review, quality assurance, and assessment and synthesis of data, are described below.



The preparation of the RoC monograph for *ortho*-toluidine began with development of a literature search strategy to obtain information relevant to the topics listed above for Sections 1 through 5 using search terms developed in collaboration with a reference librarian (see Appendix A for a detailed description of the literature search strategy). The citations (N = 2,755) identified from these searches were uploaded to a web-based systematic review software for evaluation by two separate reviewers using inclusion/exclusion criteria, and 278 references were selected for final inclusion in the draft monograph using these criteria. Studies identified from the literature searches but excluded from the review include publications on chemicals other than *ortho*-toluidine (or relevant structurally related compounds such as *ortho*-toluidine metabolites and analogues), and studies involving exposure to *ortho*-toluidine that reported results for topics not covered in this monograph (see Monograph Contents).

Information for the exposure, relevant cancer, and mechanistic sections was systematically extracted in tabular format and/or summarized in the text, following specific procedures developed by ORoC, from studies selected for inclusion in the monograph. All sections of the monograph underwent scientific review and quality assurance (QA) (i.e., assuring that all the relevant data and factual information extracted from the publications have been reported accurately) by a separate reviewer. Any discrepancies between the writer and the reviewer were resolved by mutual discussion in reference to the original data source.

Strengths, weaknesses, and study quality of the cancer studies for *ortho*-toluidine in humans (see Appendix D) or experimental animals (see Appendix E) were assessed based on a series of a priori questions. For the human cancer studies, these questions and the guidelines for answering the questions were available in the protocol ([http://ntp.niehs.nih.gov/NTP/roc/thirteenth/Protocols/ortho-ToluidineProtocol\\_508.pdf](http://ntp.niehs.nih.gov/NTP/roc/thirteenth/Protocols/ortho-ToluidineProtocol_508.pdf)), which also outlines the approach for evaluating potential confounding from occupational co-exposures or other lifestyle factors. Relevant genotoxicity and mechanistic studies were also assessed for their strengths and weaknesses.

Human exposure information was assessed to determine whether the evidence indicates that a significant number of persons residing in the United States are exposed to *ortho*-toluidine (see Foreword for information regarding the congressional mandate for the RoC). However, for many substances, this information is not available, and typically, U.S. exposure can be inferred from data on use, production volume, occupational monitoring, environmental occurrence, estimated daily intake, and biomonitoring. Because cancer has a long latency period, past exposure is also considered in the assessment.

RoC listing criteria (see text box) were applied to the available database of carcinogenicity data to assess the level of evidence (sufficient, limited, or inadequate) for the carcinogenicity of *ortho*-toluidine from studies in humans and the level of evidence (sufficient, not sufficient) from studies in experimental animals. The approach for synthesizing the evidence across studies and reaching a level of evidence conclusion was outlined in the protocol. The initial conclusions do not integrate the human cancer, experimental cancer, and mechanistic data. The evaluation of the mechanistic data included a complete discussion and assessment of the strength of evidence for potential modes of action for *ortho*-toluidine-induced neoplasia, including metabolic activation, cytotoxicity, genetic-related effects, and epigenetic effects. The RoC listing criteria were then applied to the body of knowledge (cancer studies in humans and experimental animals and mechanistic data) for *ortho*-toluidine to reach a listing recommendation.

### RoC Listing Criteria

#### **Known To Be Human Carcinogen:**

There is sufficient evidence of carcinogenicity from studies in humans\*, which indicates a causal relationship between exposure to the agent, substance, or mixture, and human cancer.

#### **Reasonably Anticipated To Be Human Carcinogen:**

There is limited evidence of carcinogenicity from studies in humans\*, which indicates that causal interpretation is credible, but that alternative explanations, such as chance, bias, or confounding factors, could not adequately be excluded, OR

there is sufficient evidence of carcinogenicity from studies in experimental animals, which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site, or type of tumor, or age at onset, OR

there is less than sufficient evidence of carcinogenicity in humans or laboratory animals; however, the agent, substance, or mixture belongs to a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens as either known to be a human carcinogen or reasonably anticipated to be a human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to, dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub-populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals, but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

\*This evidence can include traditional cancer epidemiology studies, data from clinical studies, and/or data derived from the study of tissues or cells from humans exposed to the substance in question that can be useful for evaluating whether a relevant cancer mechanism is operating in people.

## Preparation of the RoC

As mentioned in “Monograph Contents,” the substance profile for *ortho*-toluidine contains the NTP’s listing recommendation proposed for the RoC, a summary of the scientific evidence considered key to reaching that decision, and data on properties, use, production, exposure, and Federal regulations and guidelines to reduce exposure to *ortho*-toluidine. NTP submits the substance profiles for *ortho*-toluidine and other newly reviewed candidate substances with recommended listing status to the NTP Executive Committee for consultation and then to the Secretary of HHS for review and approval. Upon their approval by the Secretary, the next Edition of the RoC is prepared in electronic format, transmitted to Congress, and published on the RoC website for the public (for more information on the Process for Preparation of the RoC, see <http://ntp.niehs.nih.gov/go/rocprocess>).

# 1. Properties and Human Exposure

*ortho*-Toluidine is a high-production-volume chemical (a classification based on imports exceeding 1 million pounds/year) used in the manufacture of rubber chemicals, of herbicide intermediates, of dye intermediates, and of the local anesthetic prilocaine. This section describes the chemical and physical properties of *ortho*-toluidine (Section 1.1); its uses and production (Section 1.2); exposure to *ortho*-toluidine and biomarkers for exposure (Section 1.3); exposure in the workplace (Section 1.4); potential exposure from other sources such as cigarette smoking, medical and consumer products, and food (Section 1.5); and potential for environmental exposure (Section 1.6). Section 1.7 synthesizes and summarizes the information in Sections 1.1 to 1.6. U.S. regulations and guidelines that potentially limit exposure to *ortho*-toluidine are in Appendix B. (Note: Links provided in the text connect directly to each table as it is discussed, and a link is provided at the end of each table to return to the text citing the table.)

## 1.1. Chemical Identification and Properties

*ortho*-Toluidine (Figure 1-1) is a synthetic chemical that exists at room temperature as a light-yellow liquid that darkens rapidly on exposure to air and light. It is described as having an aromatic odor (IPCS 1998). *ortho*-Toluidine may be classified as a monocyclic aromatic amine, arylamine, or alkyylaniline (IARC 2010e; Skipper et al. 2010; Yu et al. 2002). It is soluble in water and dilute acids; and miscible with ethanol, diethyl ether, and carbon tetrachloride (NLM 2011). Toxicological studies of *ortho*-toluidine using its hydrochloride salt (*ortho*-toluidine hydrochloride) are considered relevant for the cancer evaluation of *ortho*-toluidine because the use of the salt is unlikely to alter the observed health effects of the parent chemical (IPCS 1998). In addition, the reaction of *ortho*-toluidine with stomach acid will yield the hydrochloride salt as the test article (English et al. 2012). *ortho*-Toluidine hydrochloride is the solid salt form of *ortho*-toluidine that exists at room temperature as a green or white crystalline solid that is slightly soluble in water (University of Akron 2012a). Table 1-1 contains some chemical identification information for *ortho*-toluidine and *ortho*-toluidine hydrochloride.

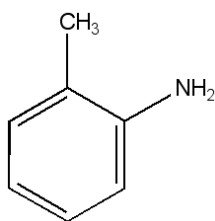


Figure 1-1. Chemical Structure of *ortho*-Toluidine

Table 1-1. Chemical Identification of *ortho*-Toluidine and *ortho*-Toluidine Hydrochloride

Characteristic	Information ( <i>ortho</i> -Toluidine)	Information ( <i>ortho</i> -Toluidine Hydrochloride)
Chemical Abstracts index name	<i>ortho</i> -toluidine	<i>ortho</i> -toluidine hydrochloride
CAS Registry number	95-53-4	636-21-5
Molecular formula	C <sub>7</sub> H <sub>9</sub> N	C <sub>7</sub> H <sub>9</sub> N•HCl

Characteristic	Information ( <i>ortho</i> -Toluidine)	Information ( <i>ortho</i> -Toluidine Hydrochloride)
Synonyms	1-amino-2-methylbenzene, 2-aminotoluene, <i>o</i> -methylaniline	2-toluidine hydrochloride, <i>o</i> -aminotoluene hydrochloride, methylaniline

Sources: IPCS (1998); NTP (1996).

Some physical and chemical properties for *ortho*-toluidine and *ortho*-toluidine hydrochloride are listed in Table 1-2.

**Table 1-2. Physical and Chemical Properties of *ortho*-Toluidine and *ortho*-Toluidine Hydrochloride**

Property	Information ( <i>ortho</i> -Toluidine)	Information ( <i>ortho</i> -Toluidine Hydrochloride)
Molecular weight	107.2 <sup>a</sup>	143.6 <sup>c</sup>
Melting point	< -15°C <sup>a</sup>	215°C <sup>d</sup>
Boiling point	198°C–201°C <sup>a</sup>	242.2°C <sup>d</sup>
Vapor pressure (mm Hg)	0.33 at 25°C <sup>a</sup>	0.293 at 25°C <sup>e</sup>
Equilibrium concentration (ppm)	434 <sup>h</sup>	385 <sup>h</sup>
Vapor density (air = 1)	3.69 <sup>a</sup>	–
Density	0.998 g/cm <sup>3</sup> at 20°C <sup>a</sup>	1.288 g/cm <sup>3</sup> at 20°C <sup>f</sup>
Solubility water (25°C) most organic solvents	16.6 g/L <sup>e</sup>	8.29 g/L <sup>e</sup>
Octanol/water partition coefficient (log <i>K</i> <sub>ow</sub> )	1.32 <sup>b</sup>	1.62 <sup>e</sup>
Critical temperature	717°K <sup>b</sup>	–
Henry's law constant	1.98 × 10 <sup>-6</sup> atm·m <sup>3</sup> /mol at 25°C <sup>b</sup>	2.10 × 10 <sup>-6</sup> atm·m <sup>3</sup> /mol at 25°C <sup>e</sup>
Conversion factors ( <i>ortho</i> -toluidine, <i>ortho</i> -toluidine hydrochloride in air) parts per million (ppm) to µg/m <sup>3</sup> µg/m <sup>3</sup> to parts per million (ppm)	µg/m <sup>3</sup> = 4,384.5 × (ppm) <sup>g</sup> ppm = 2.281 × 10 <sup>-4</sup> × (µg/m <sup>3</sup> ) <sup>g</sup>	µg/m <sup>3</sup> = 5,873.2 × (ppm) <sup>g</sup> ppm = 1.703 × 10 <sup>-4</sup> × (µg/m <sup>3</sup> ) <sup>g</sup>

<sup>a</sup>University of Akron (2012b).

<sup>b</sup>NLM (2011).

<sup>c</sup>NTP (1996).

<sup>d</sup>NLM (2005).

<sup>e</sup>NLM (2012a).

<sup>f</sup>University of Akron (2012a).

<sup>g</sup>Smarte.org (2008).

<sup>h</sup>Equilibrium concentration = (vapor pressure (mm Hg) × 1,000,000)/760.

## 1.2. Uses and Production

Although *o*-toluidine is no longer produced in the United States, it is still considered to be a high-production-volume chemical based on its importation into the United States in quantities of 1 million pounds or more per year (see table below). In the past it was produced in the United States in large amounts; in the early 1970s, production exceeded 454,000 kg (1 million pounds) (NLM 2005). In 2011, *o*-toluidine was reported to be manufactured by at least 15 companies

worldwide, including at least 3 in the United States (SRI Consulting 2012); however, in 2014, no U.S. producers of *ortho*-toluidine were identified. Reported recent and historical volumes of U.S. production, imports, and exports of *o*-toluidine are listed in the Table 1-3.

**Table 1-3. Production Data for *ortho*-Toluidine**

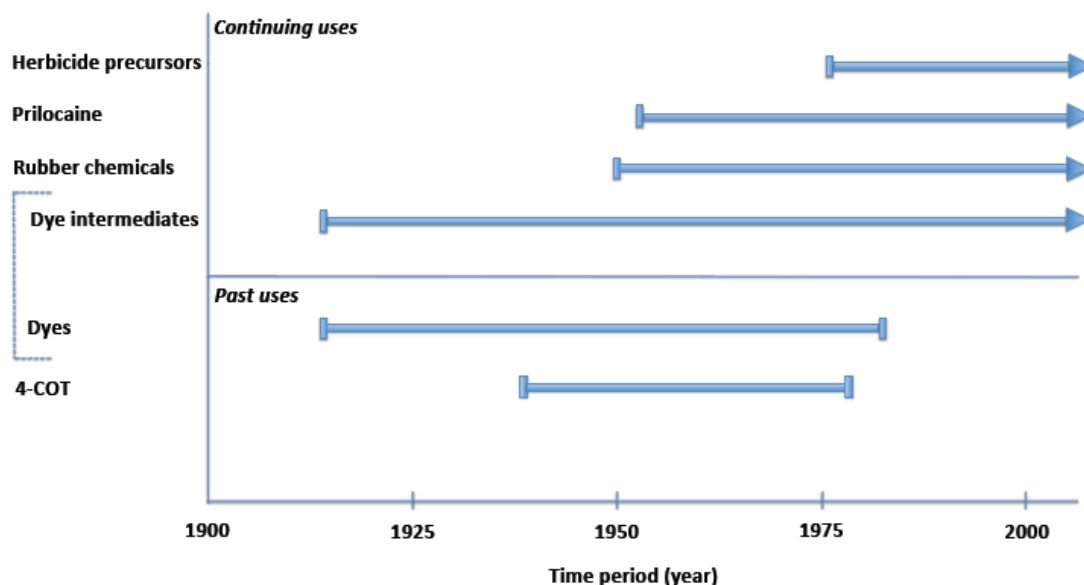
Category	Years Covered	Quantity in Pounds <sup>a</sup>
Chemical Data Reporting Rule <sup>b</sup>	2012	60.0 million
U.S. imports (recent)	2013	[52.7 million] (reported as 26.3 million kg)
U.S. imports (historical)	1989	[10.1 million] (reported as 4.6 million kg)
U.S. exports (recent)	2013	[3.1 million] (reported as 1.4 million kg)
U.S. exports (historical)	1989	[5.1 million] (reported as 2.3 million kg)

Sources: USEPA (2012a), SRI Consulting (2012), USITC (2012).

<sup>a</sup>From 1/2012, 3/2012, 7/2012 Internet searches; data subject to change.

<sup>b</sup>Formerly called the Inventory Update Rule.

*ortho*-Toluidine (either directly or as an intermediate) is currently used to make dyes, rubber chemicals, herbicides, and prilocaine; however, the major uses of *ortho*-toluidine in the United States have changed over time (see Figure 1-2). Major uses of *ortho*-toluidine in the past, i.e., in the early to mid-20th century, that have not continued to the present or have greatly diminished in the United States are uses in manufacture of large quantities of dyes (1910s to 1980s) and in manufacture of 4-chloro-*ortho*-toluidine (4-COT, an intermediate in the production of the acaricide, chlordimeform, and additionally as a dye intermediate) (1930s to 1970s) (Baptista 2012; IARC 2010c; Morris and Travis 1992). The dye industry in the United States began to decline in the 1970s when the 1974 oil crisis greatly increased costs of raw materials, and by 1977 to 1982 four of the largest dye makers had exited the business (Baptista 2012; Morris and Travis 1992). Production of *ortho*-toluidine-based dye and pigment intermediates is still reported by several companies in the United States, however.



**Figure 1-2. Timeline for Continuing (Current) and Past Uses of *ortho*-Toluidine in the United States**

Information obtained from the U.S. Environmental Protection Agency’s (EPA) Toxics Release Inventory (TRI) and Chemical Data Reporting (CDR) Rule indicates that *ortho*-toluidine continues to be used in the rubber chemicals, herbicide intermediates, and dye intermediates sectors as of 2010 (USEPA 2012b). Based on TRI data, two of the top five *ortho*-toluidine air emitters (see Section 1.6.1) reported production of rubber chemicals, and two other companies included herbicide intermediates among their products; the remaining company in the top five emitters reported production of toluidines. Inspection of data on *ortho*-toluidine manufacture or importation reported to EPA under the CDR Rule (formerly the Inventory Update Rule or IUR) identified one additional company that produced rubber chemicals and another that produced herbicide intermediates.

The largest current use of *ortho*-toluidine has been reported to be in production of 6-ethyl-*ortho*-toluidine as a herbicide intermediate, which is used to manufacture the widely used herbicides metolachlor and acetochlor (Bowers 2011). The potential for exposure to *ortho*-toluidine as part of this production would exist only during production of 6-ethyl-*ortho*-toluidine as the intermediate; that production is indicated in Figure 1-2 as “herbicide precursors.” Metolachlor was produced in the United States for more than 20 years from the late 1970s to the late 1990s (Bernard 2012), but no information on the source of the 6-ethyl-*ortho*-toluidine used during that period was identified. No export information on 6-ethyl-*ortho*-toluidine was identified. All current production of metolachlor has been reported to occur outside the United States (Bernard 2012), but no specific information on U.S. production of acetochlor was identified. No herbicides other than metolachlor and acetochlor whose intermediates are prepared from *ortho*-toluidine were identified.

Another important use for *ortho*-toluidine is as an intermediate for synthesis of rubber chemicals (e.g., the accelerant di-*ortho*-tolyl-guanidine or the antioxidant di-tolyl-phenyl-*para*-

phenylenediamine) and pharmaceuticals (e.g., prilocaine) (Hanley et al. 2012; IARC 2012; UNEP 2004). No uses of *ortho*-toluidine in the rubber chemicals manufacturing industry other than as intermediates to synthesize accelerants and antioxidants were identified. The largest release of *ortho*-toluidine to the air (68% of the total) reported to TRI in 2010 was by a company in the rubber industry (USEPA 2012b).

*ortho*-Toluidine can also be used in the manufacture of more than 90 dyes and pigments (e.g., acid-fast dyestuffs, azo pigment dyes, triarylmethane dyes, sulfur dyes, and indigo compounds) with acetoacet-*ortho*-toluidine, 3-hydroxy-2-naphthoyl-*ortho*-toluidine, 2-toluidine-5-sulfonic acid, and *ortho*-aminoazotoluene as four major intermediates (Bowers 2011). A search for producers of these intermediates identified three companies that reported use or storage of *ortho*-toluidine on site and included 2-toluidine-5-sulfonic acid or acetoacet-*ortho*-toluidine among their products (USEPA 2013a; 2013b). In the clinical laboratory, *ortho*-toluidine is also used as an ingredient in a reagent for glucose analysis, and for tissue staining (IARC 2012).

### 1.3. Exposure to *ortho*-Toluidine

Evidence for exposure to *ortho*-toluidine comes from biological monitoring data demonstrating worldwide exposure to *ortho*-toluidine in both occupationally and non-occupationally exposed individuals and in both smokers and non-smokers. Methods for biological indices for exposure to *ortho*-toluidine include measurement of *ortho*-toluidine and its *N*-acetyl-metabolites in urine and *ortho*-toluidine hemoglobin adducts in blood (Brown et al. 1995; Ward et al. 1996). Urinary levels provide an assessment of recent exposure, whereas assessment of hemoglobin adducts likely reflects cumulative dose to *ortho*-toluidine because of the 120-day half-life of human red blood cells. Table 1-4 reports studies measuring urine and hemoglobin adducts among occupationally and non-occupationally exposed people in the United States and other countries. (No analyses of *ortho*-toluidine in blood, serum, or urine specimens from the National Health and Nutrition Examination Survey (NHANES) have been identified.) The highest exposures were found for people exposed in the workplace (see Section 1.4), although low levels were reported among workers at a German chemical factory. In addition to the studies in Table 1-4, other studies detected *ortho*-toluidine in breast milk (DeBruin et al. (1999), as cited in IARC (2012)) and it was release from urinary bladder tissue or tumors after acid hydrolysis (Böhm et al. 2011). Potential for exposure from non-occupational sources includes cigarette smoking, medical and consumer products, food (see Section 1.5), and the environment (see Section 1.6).

**Table 1-4. *ortho*-Toluidine in Urine and Hb Adducts in Different Populations**

Population	Urine or Hb Adduct Levels	Urine or Hb Adduct	Reference
	(#): Mean (Range) Smokers	Levels (#): Mean (Range) Non-smokers	
<i>o</i> -Toluidine: Urine ( $\mu\text{g}/24$ hr unless otherwise specified)			
Volunteers, U.S.A.	6.3 $\pm$ 3.7 (16)	4.1 $\pm$ 3.2 (12)	el-Bayoumy et al. (1986)
Volunteers, Germany	0.204 $\pm$ 0.059 (10)	0.105 $\pm$ 0.026 (9)	Riedel et al. (2006)
Volunteers, Germany	0.13 (0.03–173) (145)	0.1 (0.03–34.25) (856)	Kütting et al. (2009)
Volunteers, Europe	0.179 $\pm$ 0.491 (1159)	0.064 $\pm$ 0.128 (399)	Lindner et al. (2011)

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Population	Urine or Hb Adduct Levels (#): Mean (Range) Smokers	Urine or Hb Adduct Levels (#): Mean (Range) Non-smokers	Reference
Chemical factory, Germany	0.6 ± 1.0 µg/L (22)	0.4 ± 1.1 µg/L (21)	Riffelmann et al. (1995)
Rubber factory, Germany	14.5 µg/L (mean) (36)	38.6 µg/L (mean) (15)	Korinth et al. (2007) <sup>a</sup>
NIOSH rubber chemical cohort, U.S.A. <sup>b</sup>	Exposed (post-shift) 132 ± 153 µg/L (15)	Exposed (post-shift) 80 ± 94 µg/L (27)	Ward et al. (1996) (see also Brown et al. (1995), Stettler et al. (1992))
	Nonexposed (post-shift) 2.8 ± 1.2 µg/L (9)	Nonexposed (post-shift) 2.8 ± 1.6 µg/L (16)	
Rubber chemical workers, U.S.A.	Exposed (post-shift) <sup>c</sup> 104 ± 111 µg/L (46) Unexposed (post-shift) <sup>c</sup> 2.7 ± 1.4 µg/L (31)		Teass et al. (1993)
<b><i>o</i>-Toluidine: Hb adducts<sup>d</sup> (pg/g Hb)</b>			
Volunteers, U.S.A.	100 ± 30 (12)	34 ± 10 (10)	Stillwell et al. (1987)
Volunteers, Italy	290–329 (means) (61)	188 (mean) (19)	Bryant et al. (1988)
Pregnant women, Germany	289 ± 25 pg/g Hb (27)	237 ± 65 (73)	Branner et al. (1998)
Children, Germany	–	487–632 (220)	Richter et al. (2001)
Patients, 24 hr post prilocaine administration, Germany	21,350 ± 15,000 (8)	22,330 ± 12,080 (17)	Gaber et al. (2007)
Rubber factory, Germany	1,900 <sup>d</sup> (mean) (36)	2,900 <sup>d</sup> (mean) (15)	Korinth et al. (2007) <sup>a</sup>
NIOSH rubber chemical cohort, U.S.A. <sup>b</sup>	Exposed (post-shift) <sup>e</sup> : 40,830 ± 32,518 (46)		Ward et al. (1996) (see also Stettler et al. (1992))
	Nonexposed (post-shift) <sup>e</sup> : 3,515 ± 6,036 (27)]		
Workers in a TNT factory, China		Exposed <sup>f</sup> : 675 Control <sup>f</sup> : 386	Jones et al. (2005)

<sup>a</sup>Air levels of *ortho*-toluidine higher among nonsmoking group, according to authors.

<sup>b</sup>See also Section 3.

<sup>c</sup>Smoking status not reported.

<sup>d</sup>Reported as 287 ng/L and 432 ng/L. Conversion factor for normal hemoglobin levels in blood: 15 g Hb/dL (12–16 g Hb/dL [for females], 14–18 g Hb/dL [for males]) (CDC 2010).

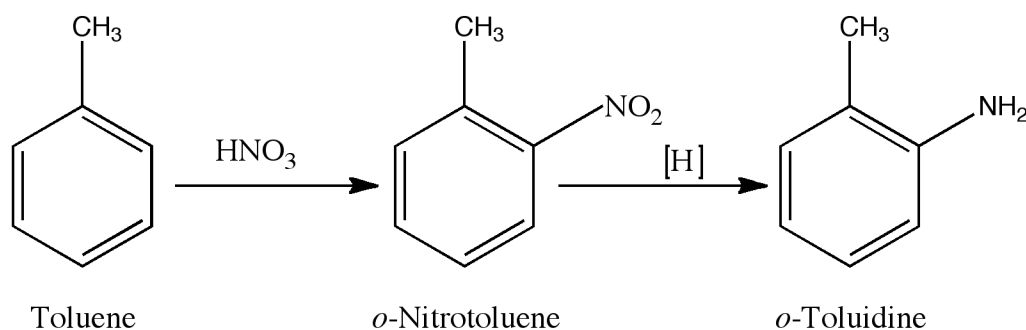
<sup>e</sup>Smokers and nonsmokers had similar values; combined group reported in table.

<sup>f</sup>Median values estimated from graph and converted from pmol adduct/g Hb (1 pmol *o*-toluidine = 107.2 pg). Smoking status not reported.

### 1.4. Characterization of Exposure in the Workplace

*ortho*-Toluidine is synthesized from toluene by a two-step process (Bowers 2011; IARC 2010e) (see Figure 1-3 and Appendix C). Mononitration of toluene produces three isomers (*ortho*-, *meta*-, and *para*-) of nitrotoluene, which can be separated by distillation prior to reduction of the nitro- group to an amine. Reduction of *ortho*-nitrotoluene to *ortho*-toluidine can be accomplished by several methods, but the most common method currently is a continuous vapor-phase hydrogenation process with a catalyst such as Raney nickel, copper, molybdenum, tungsten, vanadium, noble metals, or various supported metals.





**Figure 1-3. Chemical Synthesis of *ortho*-Toluidine from Toluene**

Occupational exposure to *ortho*-toluidine can occur by inhalation or skin contact (IARC 2010e) during its production, or during its use in production of chemical intermediates for pesticides, of rubber chemicals, or of intermediates for dyes and pigments. As small amounts of *ortho*-toluidine are also used in laboratory work, medical and laboratory personnel are exposed to low air concentrations of *ortho*-toluidine (USEPA (1984), Kauppinen et al. (2003), as cited in IARC (2012)).

Evidence that *ortho*-toluidine is absorbed following occupational exposure via inhalation or skin contact comes from studies measuring *ortho*-toluidine in urine or as hemoglobin adducts in rubber industry workers and other industries which use *ortho*-toluidine (Brown et al. 1995; Jones et al. 2005; Korinth et al. 2007; Stettler et al. 1992) (see Section 2.1.2). Note that the OSHA PEL for *ortho*-toluidine is 5 ppm and the ACGIH TLV-TWA is 2 ppm, and both occupational exposure limits include a skin notation, which indicates potential significant contribution to overall exposure via the cutaneous route, including mucous membranes and eyes, by contact with vapors, liquids, and solids. Consideration of the decrease in *ortho*-toluidine air levels relative to the ACGIH TLV-TWA of 2 ppm (with skin notation) highlights the importance of dermal exposure in occupational settings.

Limited information is available on exposure to *ortho*-toluidine in the various industries where *ortho*-toluidine is used or produced. Most of the exposure information found is from the human cancer studies, and details on the exposure conditions of those individual studies is presented in Appendix C, which is provided as background information to interpret the epidemiological studies. Exposure levels across industries are presented in Table 1-5 and biomonitoring data were presented in Table 1-4. No information was found on levels of exposure to *ortho*-toluidine during production of the pesticide intermediates (such as 6-ethyl-*ortho*-toluidine or 4-chloro-*ortho*-toluidine). The highest levels of exposures (air) were reported from a study of workers at a chemical plant in the former Soviet Union where *ortho*-toluidine was produced via reduction of *ortho*-nitrotoluene. Concentrations of *ortho*-toluidine in the air were two- to sevenfold higher than the maximum permissible concentration for the USSR of 3 mg/m<sup>3</sup> [0.7 ppm] (Khlebnikova et al. (1970), as cited in IARC (2012)). Dermal exposures also were reported (IARC 2012). Most of the air-monitoring data in the United States suggest that occupational exposure levels to *ortho*-toluidine are usually <1 ppm (e.g., up to 0.5 ppm in the dye and pigment industry) but may have been higher in the past.

**Table 1-5. Exposure to *ortho*-Toluidine in Various Industries**

Industry (Year)	TWA (Range), ppm <sup>a</sup>	Number of Samples	Reference
<i>ortho</i> -Toluidine production - distillation and extraction processes	[5.7–6.5] <sup>b</sup>	–	Khlebnikova et al. (1970) <sup>c</sup>
Rubber chemical industry	[0.1] <sup>d</sup>	7	Ward et al. (1996)
Dye and pigment industry - thioindigo production worker (1940)	<0.5	–	Ott and Langner (1983)
OSHA sampling data (1985–6, 1988, 1997, 2001) <sup>e</sup>	0.06–0.11	26 <sup>f</sup>	OSHA (2011)

<sup>a</sup>Values shown in brackets converted to ppm using conversion factor in Table 1-2.

<sup>b</sup>Reported as 25–28.6 mg/m<sup>3</sup>.

<sup>c</sup>As cited in IARC (2012).

<sup>d</sup>Reported as 516 µg/m<sup>3</sup>.

<sup>e</sup>OSHA Chemical Exposure Health Dataset.

<sup>f</sup>Only 6 samples from 1 facility in the rubber industry had detectable levels.

The most detailed exposure information was found for the rubber chemical industry. Air monitoring data were available from both a NIOSH assessment of a rubber chemical manufacturing plant carried out in 1990 (Figure 1-4) and company data from three intervals covering the period from 1976 to 2004 (Figure 1-5) (Hanley et al. 2012). NIOSH TWA breathing zone air sampling data or company-collected data for the rubber chemicals department (i.e., antioxidant, accelerant, and recycle process, and maintenance workers) indicate that the geometric mean breathing zone concentrations for *ortho*-toluidine for antioxidant production workers and maintenance personnel were similar and approximately twice those for accelerant production. Exposure levels among rubber chemical workers have decreased over time from a high of 0.1 ppm (time period from 1976 to 1979) to <0.02 ppm (time period from 1995 to 2004). Data reported for the same operations, e.g., “Maintenance,” are higher for the results from the NIOSH survey compared with the company-reported data, but the time periods covered differ, and the NIOSH data were based on fewer samples for each process.

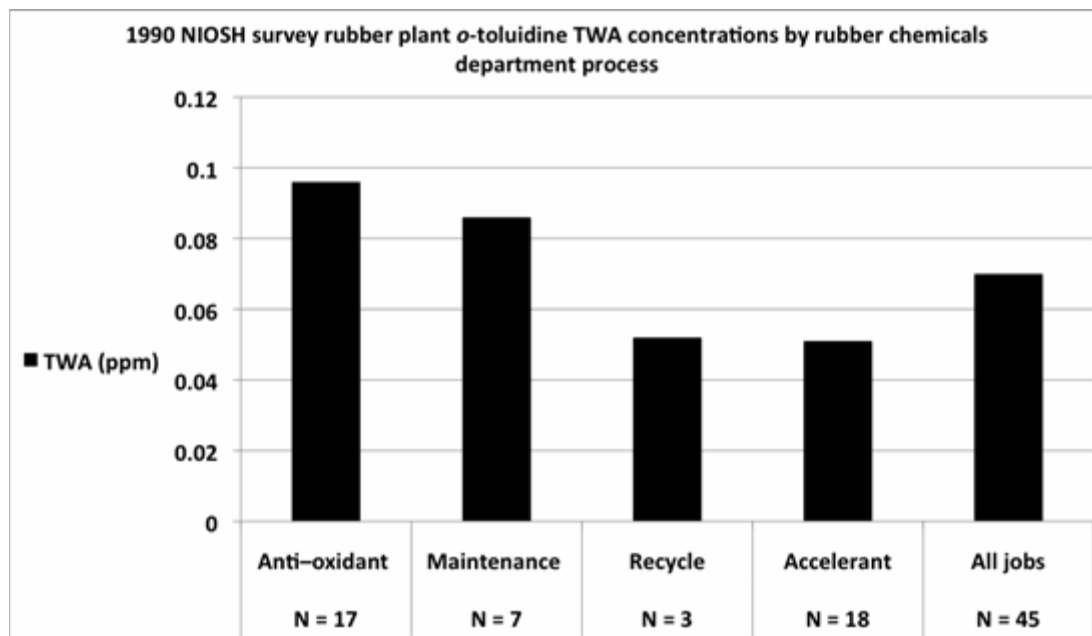


Figure 1-4. Air Monitoring Data (TWA Breathing Zone, Geometric Means) for *ortho*-Toluidine Collected by NIOSH in 1990 for Processes in the Rubber Chemicals Department

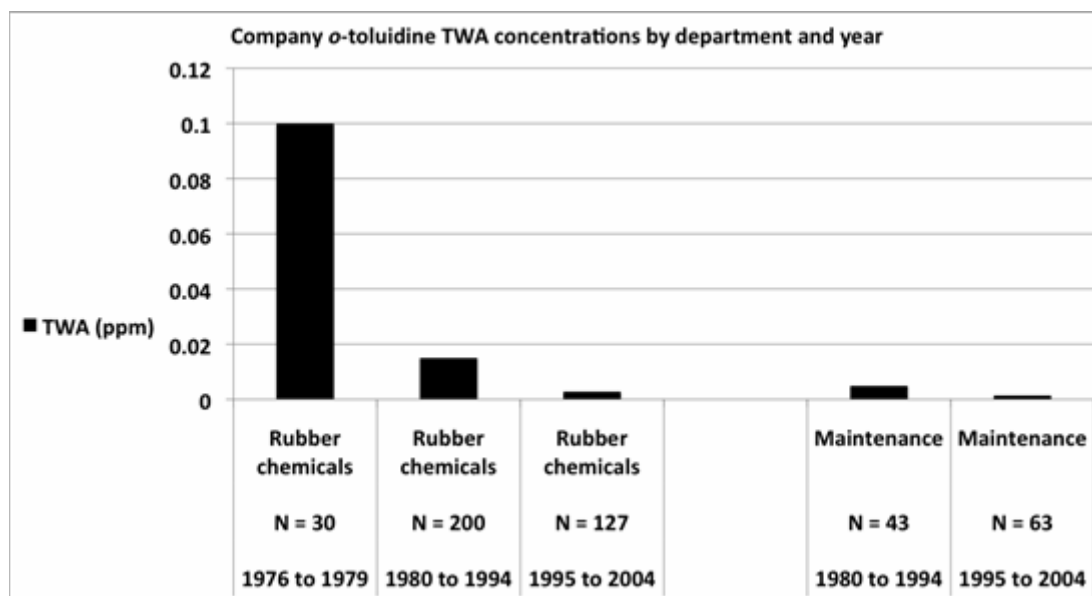


Figure 1-5. Air Monitoring Data (TWA Breathing Zone, Geometric Means) Collected by the Company by Department and Time Period

The continued use of *ortho*-toluidine in the rubber chemicals industry and for production of the herbicide intermediate and dye intermediates indicate that workers in the United States are currently exposed, although no current information is available on the number of workers in each industry. Data from the NIOSH National Occupational Exposure Survey (NOES), conducted between 1981 and 1983, estimated that about 30,000 workers, including approximately 15,500 women, were potentially exposed to *ortho*-toluidine during that time period (NIOSH 1990).

(Note: The NOES database has not been updated since July 1, 1990, and NIOSH has not conducted any national surveys of occupational exposure since that time.)

NIOSH recommends that worker exposure to *ortho*-toluidine be minimized through the use of engineering controls, work practices, and personal protective equipment (NIOSH 1991).

### **1.5. Potential for Exposure from Other Sources: Cigarette Smoking, Medical and Consumer Products, and Food**

*ortho*-Toluidine (and other arylamines) have been detected in the urine of non-occupationally exposed individuals in several studies (see Table 1-4); the sources proposed for the exposure underlying these levels include combustion products, passive smoking, dyestuffs in cosmetics and clothing (Riffelmann et al. 1995), and food (el-Bayoumy et al. 1986), but no data were identified to document contributions by any of these potential sources. *ortho*-Toluidine was released by acid hydrolysis from 11 of 12 urinary bladder tumor samples ( $8.72 \pm 4.49$  fmol/ $\mu$ g DNA) as well as from 13 of 46 urinary bladder epithelial tissue samples ( $0.24 \pm 0.63$ ) and from 10 of 46 urinary bladder submucosal tissue samples ( $0.27 \pm 0.70$ ) obtained from sudden death victims in Germany (Böhm et al. 2011) (see the footnote in Section 5.1.4 for further discussion of test methods used by Böhm et al. (2011)). The authors reported that the subjects had no known occupational exposure to aromatic amines and that smoking status had no significant effect on the adduct levels detected. *ortho*-Toluidine adducts to hemoglobin (Hb) were already detected in 25 patients before treatment with prilocaine as a local anesthetic (see below) (Gaber et al. 2007; IARC 2012). Several lines of evidence suggest that exposure to *ortho*-toluidine occurs for non-occupationally exposed individuals from sources such as cigarette smoking, medical products (e.g., the local anesthetic prilocaine), some consumer products (e.g., hair dyes), and possibly food.

*ortho*-Toluidine has been measured in mainstream cigarette smoke at 9 to 144 ng per cigarette (Stabbert et al. (2003), as cited in IARC (2012)). *ortho*-Toluidine concentrations in second-hand smoke have been reported to be approximately 20 times more than in first-hand smoke (Patrianakos and Hoffmann 1979). *ortho*-Toluidine urinary concentrations and hemoglobin adducts have generally been reported to be somewhat higher in non-occupationally exposed smokers than in non-smokers (el-Bayoumy et al. 1986; Riedel et al. 2006; Riffelmann et al. 1995), although in many studies the influence of smoking was weak (Kütting et al. 2009) (see Table 1-4). Among workers exposed to *ortho*-toluidine, there was little difference between exposure levels (urinary concentration or hemoglobin adducts) among workers that smoked compared with non-smoking workers (Korinth et al. 2007; Riffelmann et al. 1995; Ward et al. 1996).

Exposure to *ortho*-toluidine from medical products can occur from a product used for anesthesia. Prilocaine, a widely used local or topical anesthetic is required by U.S. Pharmacopeia standards to contain no more than 0.01% *ortho*-toluidine, but once in the body, prilocaine can be metabolized to release *ortho*-toluidine. Humans exposed to prilocaine excreted 0.75% of the dose as *ortho*-toluidine and 36.9% as hydroxylated *ortho*-toluidine (NTP 2000). The average amount of *ortho*-toluidine adducts to hemoglobin (Hb) increased 41-fold, from  $0.54 \pm 0.95$  ng/g Hb before treatment to  $22 \pm 13.2$  ng/g Hb at 24 hours after surgery in 25 patients who received prilocaine for local anesthesia (Gaber et al. 2007; IARC 2012). Information on sales of dental local anesthetics in the United States in the 11-year period from 1997 to 2008 indicate average

sales of approximately 18 million cartridges of prilocaine per year (Garisto et al. 2010), and recommended doses range from 1 to 8 72-mg cartridges per dental procedure (DrugsArea.com 2007). No specific information on the number of people exposed to prilocaine annually in the United States was identified, but based on the sales figures, a relatively small percentage of the population could be treated with prilocaine (assuming one cartridge per person, no more than 6% of the population could be exposed). Based on the current U.S. population of 314 million, potential exposure to this source of *ortho*-toluidine could still affect an estimated 18,000,000 people. The actual percentage of people could be lower, depending on the use of multiple doses for individual dental patients, but that would result in increased exposure levels for those patients. Prilocaine is also present with lidocaine in a topical local anesthetic cream used to numb the skin for needle insertion or minor surgical procedures; although these products for both prescription and over-the-counter sales are widely advertised on the Internet, no sales or use data for this preparation were found.

Exposure to *ortho*-toluidine can potentially result from exposure to dyes in consumer products, including some hair dyes, other dyes, and tattoos. Akyüz and Ata (2008, as cited in IARC 2012a) reported finding *ortho*-toluidine in 34 of 54 hair dyes tested in Turkey, at levels up to 1,547 µg/g. Hair dyes in Turkey were identified as available for sale on the Internet, but no information on the *ortho*-toluidine content of the dyes was identified (Alibaba.com 2013; Allbiz 2013). *ortho*-Toluidine was found in the fixative of a commercial hair-waving product at ng/g levels from a study in Sweden (Johansson et al. 2015). In the same study, hemoglobin adducts were reported to increase significantly with increasing numbers of hair waving treatments performed per week among non-smoking hairdressers, and a trend toward increasing concentrations with increased use of light-color permanent hair dye treatments was also found.

Currently, 11 *ortho*-toluidine-based dyes are listed in the Colour Index as having commercial products, ranging from 2 to 193 depending on the dye (Freeman 2012). Most of the manufacturing involving these dyes occurs outside the United States and Europe, mainly in China and India. Many current commercial organic pigments, including Pigment Red 95, Pigment Red 148, and Pigment Red 253, are derived from *ortho*-toluidine. Pigment Red 95, Pigment Red 148, and Pigment Red 253 require the product from the reaction of *ortho*-toluidine with beta-oxynaphthoic acid for their production. The *ortho*-toluidine-based colorant Pigment Yellow 14 (193 commercial product listings in the Colour Index) requires production of the *ortho*-toluidine-based precursor acetoacet-*ortho*-toluidide. No information on specific use of these dyes in the United States was identified, but they are available from a number of suppliers, primarily in China and India, and no U.S. import restrictions were found.

Recent information from the European Union (EC 2013a; 2013b) identified two tattoo inks containing 89 mg/kg and 120 mg/kg of *ortho*-toluidine. Since most tattoo inks are purchased abroad via the Internet or during tattoo conventions (Shubhangi et al. 2013), the inks represent a potential source of exposure to *ortho*-toluidine for the 21% of the U.S. population who reported having at least one tattoo in a 2012 poll (Harris Interactive 2012).

In a German survey of primary and secondary amines in the human environment, a number of foods were examined; <0.1 ppm of *ortho*-toluidine was detected in a sample of broken beans; however, it was not found in 9 other samples of preserved vegetables, or in more than 16 other foods tested (Neurath et al. (1977), as cited in HSDB (2011)). Also, aminotoluene (toluidine), isomer unspecified, was found in shelled peas at a concentration of 0.4 ppm, in red cabbage at a

concentration of 0.2 ppm, in kale at a concentration of 1.1 ppm, in carrots at a concentration of 7.2 ppm, and in celery at a concentration of 1.1 ppm (NLM 2011). As cited in IARC (2012), Vitzthum et al. (1975) reported that *ortho*-toluidine has been detected as a volatile in black tea (IARC 2012). No information for *ortho*-toluidine was identified in the U.S. Food and Drug Administration's Total Diet Study (TDS) for market baskets 1991–3 through 2003–4 collected between September 1991 and October 2003 (FDA 2006).

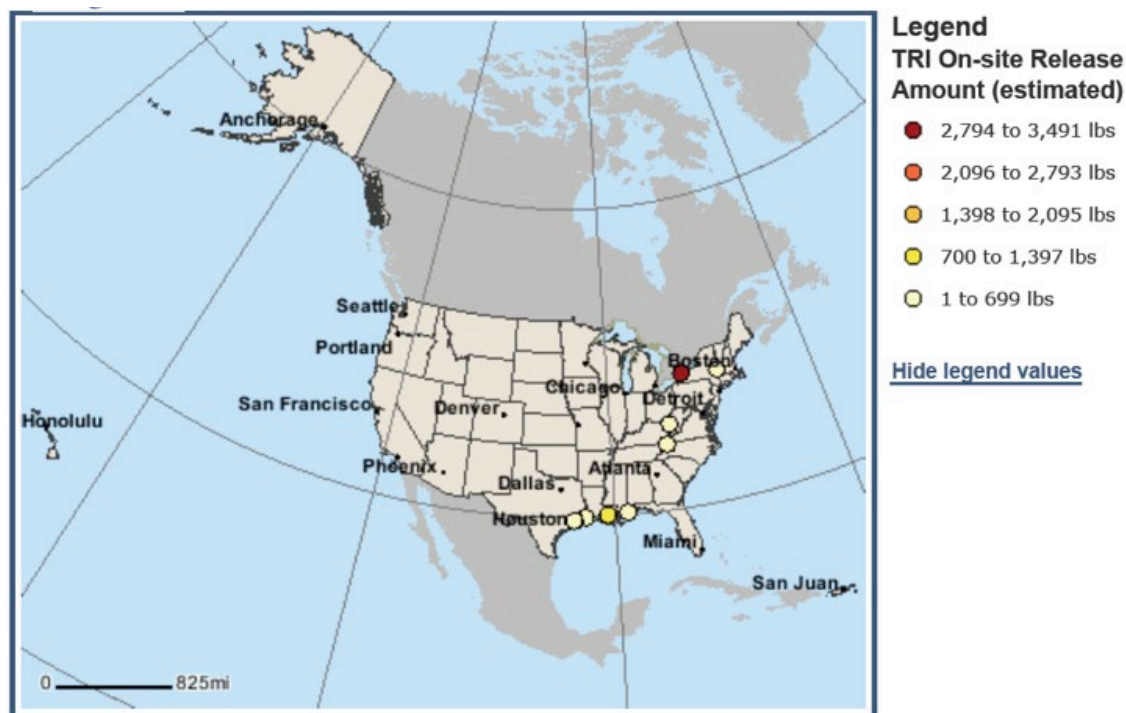
In summary, although exposure levels from all of these sources are lower relative to occupational exposures, almost everyone would be affected by exposure through food. In contrast, about 19% of U.S. adults smoked in 2010 (CDC 2012), and estimates for use of hair dye “indicate that more than one-third of women over age 18 and approximately 10% of men over age 40 use some type of hair dye” (Huncharek and Kupelnick 2005). Based on 2010 U.S. census data (Howden and Meyer 2011), approximately 43 million individuals or a little <15% of the U.S. population would be exposed to hair dyes of all types. As noted above, exposure to *ortho*-toluidine from the use of prilocaine as a dental anesthetic is estimated to occur to 6 percent or less of the U.S. population (estimated 18,000,000 people).

## 1.6. Potential for Environmental Exposure

People in the United States are potentially exposed to *ortho*-toluidine from the environment via releases into the atmosphere, as documented by TRI data (see Section 1.6.1), and data regarding people living close to emitting facilities (see Table 1-6). Occurrence data support these conclusions (see Section 1.6.2). In addition, *ortho*-toluidine hemoglobin adducts were found to be higher among children residing in cities compared with children residing in a largely rural environment in Germany suggesting the potential for environmental exposure because it is unlikely that food or personal products use differs in the three geographical areas. *ortho*-Toluidine hemoglobin adducts were lower in children from smoking homes compared with non-smoking homes.

### 1.6.1. Release of *ortho*-Toluidine to the Environment

*ortho*-Toluidine's production and use in the production of textile dyes or rubber vulcanization accelerators and antioxidants, and as an intermediate in organic synthesis results in its release to the environment through various waste streams (NLM 2011). According to the TRI, total reported on- and off-site release of *ortho*-toluidine was slightly over 6,600 pounds from 15 facilities in 2010 (USEPA 2012b). Releases to air accounted for 77.4% of total releases, disposal by underground injection for 19.6%, and releases to water for 3.0%. No releases were reported to either land or off-site disposal. Figure 1-6 shows a color-coded map of sites reporting on-site *ortho*-toluidine releases into the air, water, and ground to TRI in 2010 (NLM 2012b). The color of each circle indicates the amount of total on-site release for calendar year 2010. (See below for estimates of the numbers of individuals living near facilities reporting release of *ortho*-toluidine to the air.) Based on the visual depiction of *ortho*-toluidine releases in Figure 1-6, *ortho*-toluidine has been released at industrial facilities mostly in the northeast and southeast regions of the United States. The highest reported *ortho*-toluidine release is from a facility in Niagara Falls, NY, which is the site of the rubber chemical plant studied by NIOSH (see Section 1.4).



**Figure 1-6. Map of Reported TRI On-site *ortho*-Toluidine Releases for 2010**

The top 5 *ortho*-toluidine-emitting facilities released approximately 5,000 pounds of *ortho*-toluidine to the air, accounting for approximately 98% of total *ortho*-toluidine air emissions reported to TRI in 2010 (USEPA 2012b). Table 1-6 presents demographic data from EPA’s EJView website based on U.S. Census data for 2000 for areas within 0.5 miles and 1 mile of the top 10 *ortho*-toluidine-emitting facilities in 2010. Based on these data, approximately 1,400 people lived within 0.5 miles of these facilities, and 8,800 people within 1 mile of these facilities. These facilities are engaged in production of rubber chemicals, pesticide intermediates, dye intermediates, or *ortho*-toluidine. The numbers could be underestimates because census data are for residential populations and would not include workers who do not live within these boundaries. No data were identified for concentrations of *ortho*-toluidine in air near any of these sites.

**Table 1-6. Demographic Data for Areas within 0.5 Miles and 1 Mile of the Top 5 *ortho*-Toluidine-emitting Facilities in 2010**

City	State	<i>ortho</i> -Toluidine Air Emissions (Pounds)	Total Persons within 0.5 Miles	Total Persons within 1 Mile
Niagara Falls <sup>a</sup>	NY	3,491	763	4,428
Pasadena	TX	673	0	0
Geismar	LA	609	0	6
Pascagoula	MS	139	0	13
Kingsport	TN	111	137	2,942
<b>Total</b>		<b>5,023</b>	<b>900</b>	<b>7,389</b>

Sources: USEPA (2012a; 2012b).

<sup>a</sup>This plant is the same one assessed by Hanley et al. (2012) for exposure to *ortho*-toluidine and aniline.

Figure 1-7 shows a map of Superfund sites on the National Priorities List (NPL) where *ortho*-toluidine was listed as a site contaminant (NLM 2012b). Based on Figure 1-7, Superfund sites where *ortho*-toluidine was listed as a site contaminant also appear to be located primarily in the northeast and southeast United States.



Figure 1-7. Map of Superfund Sites at Which *ortho*-Toluidine Was Listed as a Site Contaminant

### 1.6.2. Fate and Occurrence

Environmental exposure to *ortho*-toluidine is possible based on its occurrence in water and sediment worldwide. However, no data for *ortho*-toluidine concentration levels in ambient air or soil have been identified. Table 1-7 presents concentration level data identified for *ortho*-toluidine in water and sediment. The highest levels occurred in sediment or water close to industrial areas.

Table 1-7. *ortho*-Toluidine Groundwater, Surface Water, and Sediment Concentration Levels

Location	Number of Samples/Frequency of Detection	Mean Conc. (Range), ppb	References <sup>a</sup>
<b>Groundwater</b>			
Hoe Creek coal gasification project, Northeastern WY	3	– (0.06–9.2) <sup>b</sup>	Stuermer et al. (1982)
<b>Surface Water</b>			
Rhine River, Netherlands	6% of samples	0.03 (1.8 max)	Wegman and De Korte (1981)
Rhine River tributaries, Netherlands	8% frequency of detection	– (0.07–0.2) <sup>c</sup>	Wegman and De Korte (1981)



Location	Number of Samples/Frequency of Detection	Mean Conc. (Range), ppb	References <sup>a</sup>
River Waal, Netherlands	Not reported	0.7 (–)	Meijers and Leer (1976)
Japan, industrial areas between 1974 and 1976	8 out of 68 samples	20 (–)	Kubota (1979)
<b>Sediment</b>			
Japan, industrial areas between 1974 and 1976	27 out of 68 samples	72 (–)	Kubota (1979)

– = no data available.

<sup>a</sup>As cited in NLM (2011).

<sup>b</sup>Combined concentration of 2- and 4-aminotoluene (*ortho*- and *para*-toluidine), sampled 15 months after completion of project.

<sup>c</sup>Range of means.

## 1.7. Synthesis and Summary

Several lines of evidence indicate that a significant number of people living in the United States are exposed to *ortho*-toluidine. This evidence includes widespread use and imports of large amounts of *ortho*-toluidine (53 million pounds imported in 2013), and biological monitoring data demonstrating exposure in both occupationally and nonoccupationally exposed individuals and in both smokers and nonsmokers.

The highest exposure to *ortho*-toluidine occurs in the workplace; urinary exposure levels were over 26-fold higher among workers exposed to *ortho*-toluidine than among unexposed workers (Ward et al. 1996). Exposure via inhalation and dermal contact also can occur in the workplace through the production and use of *ortho*-toluidine in the chemical industry.

Exposure occurs through these occupational uses, but the presence of *ortho*-toluidine in urine and as hemoglobin adducts in individuals without known occupational exposure supports more widespread exposure. One source of this exposure is primary and second-hand tobacco smoking, but exposure from consumer products (e.g., hair dyes, tattoos, *ortho*-toluidine based dyes), dental products (e.g., prilocaine), and food is also possible. Although nonoccupational exposure levels are lower than occupational exposure levels, these exposures are common, indicating that more people living in the United States are potentially exposed to *ortho*-toluidine from these sources than occupationally. No direct evidence for exposure of the general population to *ortho*-toluidine from environmental sources was identified, but TRI data indicate that production- and use-related releases of *ortho*-toluidine have occurred at numerous industrial facilities in the United States, and a biological monitoring study in Europe reported that exposure levels in children varied with geographical residence (lower in a largely rural environment) suggesting the importance of environmental sources.

## 2. Disposition and Toxicokinetics

The available data on the absorption, distribution, and excretion of *ortho*-toluidine in humans and experimental animals are summarized in Section 2.1, and metabolism is discussed in Section 2.2. Toxicokinetic data for *ortho*-toluidine were limited and are briefly reviewed in Section 2.1.1. Mechanistic implications of the processes of absorption, distribution, metabolism, and excretion are discussed further in Section 5.

### 2.1. Absorption, Distribution, and Excretion

#### 2.1.1. Experimental Animals

*ortho*-Toluidine is well absorbed following oral exposure based on studies in male rats (IARC 2000; 2010e). Absorption data for female rats, other species, or other routes of exposure were not available. Once absorbed, *ortho*-toluidine is rapidly distributed, metabolized, and excreted (Brock et al. 1990; Cheever et al. 1980; Son et al. 1980). Distribution data were available from rats exposed by subcutaneous (s.c.) injection or oral gavage. These studies show general agreement regarding tissues with the highest concentrations. In male F344 rats exposed to 50 or 400 mg/kg *ortho*-[methyl-<sup>14</sup>C]toluidine (2 animals/dose) by s.c. injection, radioactivity was detected in the liver, kidney, lung, spleen, colon, and urinary bladder 48 hours later (Son et al. 1980). The highest concentrations occurred in the liver; however, compared with the increase in dose, disproportionate increases of <sup>14</sup>C occurred in all tissues examined. These data imply saturation of some metabolic pathway(s) at the higher dose. Brock et al. (1990) reported more extensive tissue distribution data in male CrI:CD<sup>®</sup> BR rats 72 hours after receiving a single 500 mg/kg oral dose of radiolabeled *ortho*-[ring-U-<sup>14</sup>C]toluidine (Figure 2-1). The dose was selected as an approximate equivalent of the dietary level that produced tumors in the long-term carcinogenicity studies (see Section 4). The highest concentrations were found in whole blood, spleen, kidneys, and liver. Peak blood levels of undifferentiated radioactivity were reached at 24 hours, and the half-life for plasma elimination ranged from about 12 to 15 hours. The kinetic studies are of limited use in that it is unknown what percentage of the radioactivity in plasma is due to parent [<sup>14</sup>C]-*ortho*-toluidine, as opposed to metabolites. The major tumor sites in animals administered *ortho*-toluidine in feed (see Section 4) were the urinary bladder, spleen, and abdominal organs and connective tissue in rats and liver and blood vessels in mice. The urinary bladder also is the tumor site of interest in humans (see Section 3). Most of these tissues were among those with the highest accumulation of radioactivity in both distribution studies in rats.

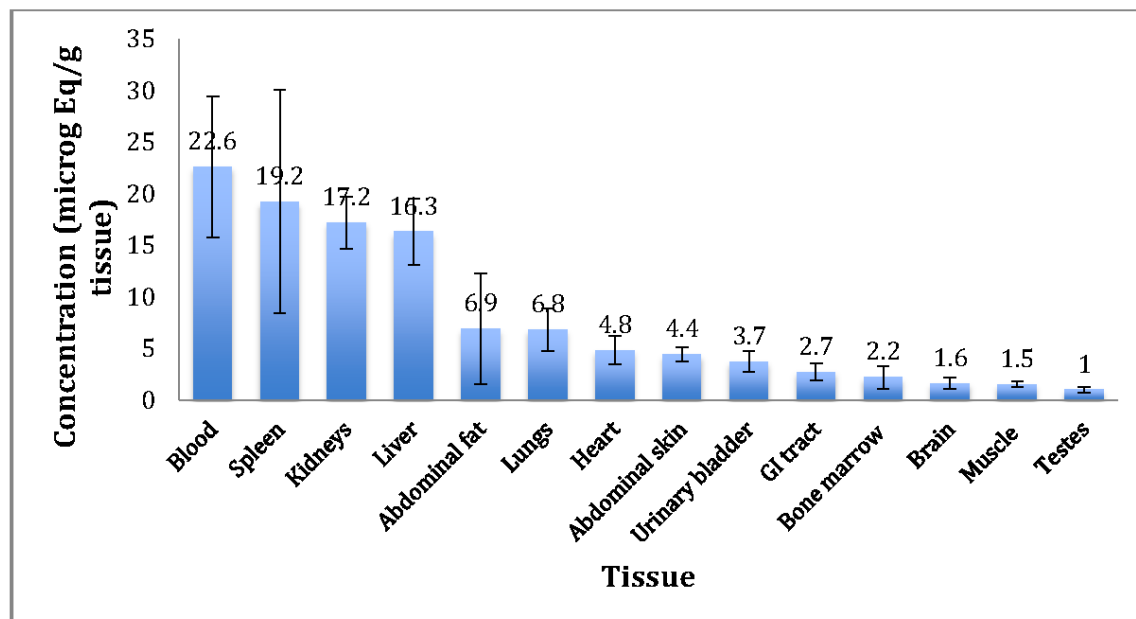


Figure 2-1. Tissue Distribution of Orally Administered *ortho*-Toluidine in Male Rats

Source: Brock et al. (1990) (mean  $\pm$  SD from four rats; samples collected at 72 h).

The primary route of excretion in rats was the urine regardless of exposure route. Following a single subcutaneous dose of either 50 or 400 mg/kg, 74% to 83% was excreted in the urine within 48 hours (Son et al. 1980) while more than 92% of administered radiation from an oral dose of 50 mg/kg was excreted in the urine within 24 hours (Cheever et al. 1980). Subcutaneous administration forces 100% absorption of the dose. The observation that urinary excretion is about the same for both routes implies near 100% absorption of the oral dose. The saturation of metabolic pathways is not as apparent in the urinary excretion data. Excretion via the lungs and feces were approximately 1% and 3%, respectively (Son et al. 1980). The amount of unmetabolized compound detected in the urine ranged from about 3.6% to 5.1% in male F344 rats administered 0.82 mmol/kg (about 88 mg/kg) (Kulkarni et al. 1983) or 400 mg/kg *ortho*-toluidine by s.c. injection (Son et al. 1980). The amount of unchanged toluidine excreted in rat urine also depended on the dose and isomer administered (Cheever et al. 1980). Male Sprague-Dawley rats administered a single oral dose (500 mg/kg) of *meta*- or *para*-toluidine excreted only 2.5% of the dose as the parent compound compared with 21% for *ortho*-toluidine, even though there were no major differences in the primary metabolic pathways (ring hydroxylation followed by sulfate or glucuronide conjugation) (see Section 2.2). Rats administered a lower oral dose of *ortho*-toluidine (50 mg/kg) excreted 36% of the dose unchanged.

### 2.1.2. Humans

The primary routes of exposure to *ortho*-toluidine in humans are the respiratory tract and skin (IARC 2000). Evidence that *ortho*-toluidine is absorbed following inhalation exposure comes from studies that measured *ortho*-toluidine in urine and/or as hemoglobin adducts in occupationally exposed workers (Jones et al. 2005; Korinth et al. 2007; Lüersen et al. 2006; Ward et al. 1996). Studies in different populations of rubber industry workers reported that *ortho*-toluidine concentrations in workplace air correlated significantly with concentrations in the

urine (Brown et al. 1995). A series of studies of *ortho*-toluidine exposed and non-exposed workers in the rubber chemicals industry reported that exposed workers had approximately 25 times higher post-shift urinary levels of *ortho*-toluidine compared with non-exposed workers and that the post-shift urinary exposure levels among exposed workers were sixfold higher than the pre-shift levels (Stettler et al. 1992). Pre-shift urinary levels of exposed workers were also higher than those of non-exposed workers suggesting that *ortho*-toluidine is not completely eliminated from the bodies of the exposed workers between work shifts (Brown et al. 1995; Stettler et al. 1992). *ortho*-Toluidine has been detected in the urine of non-smoking individuals with no known occupational exposure to *ortho*-toluidine, which implies exposure and absorption from contaminated food and water or from consumer or medical products (see Section 1).

Several *in vitro* studies reported that *ortho*-toluidine (either as neat compound or in a solution with phosphate buffer and 5% ethanol) was readily absorbed through excised human skin mounted on diffusion cells (Lüersen et al. 2006; Wellner et al. 2008). About 15% of the applied dose penetrated over 7 hours and 50% penetrated within 24 hours (Lüersen et al. 2006). Dermal absorption of *ortho*-toluidine was shown to be significant in studies of rubber industry workers (Korinth et al. 2008; Korinth et al. 2007). These studies monitored workplace air concentrations of *ortho*-toluidine, measured internal exposure based on either hemoglobin adducts or urinary excretion, and documented skin conditions of the workers. These studies demonstrated that the relative internal exposure depended more on the skin condition of the workers than on the concentrations of *ortho*-toluidine in the air. Internal exposure was significantly higher in workers with skin lesions (burns, erythema, or eczema) compared with workers with healthy skin. Furthermore, frequent use of skin barrier creams was shown to enhance dermal absorption of *ortho*-toluidine. Korinth et al. (2008) confirmed that skin barrier creams actually enhanced dermal absorption of *ortho*-toluidine *in vitro* using diffusion cells and excised human skin. The highest mean enhancement factors (based on maximum fluxes from the same skin donor in the same experimental series for untreated and treated skin) ranged from 6.2 to 12.3.

No tissue distribution studies were identified for humans.

## 2.2. Metabolism

Few studies have investigated *ortho*-toluidine metabolism in experimental animals or humans or have identified the specific cytochromes P450 involved. This section provides a brief review of the metabolism of aromatic amines and *ortho*-toluidine. Metabolism studies in experimental animals and humans are reviewed in Sections 2.2.1 and 2.2.2, respectively, and metabolic enzymes are discussed in Section 2.2.3.

### 2.2.1. Experimental Animals

Metabolism studies that were identified were in male rats exposed to *ortho*-toluidine by oral or subcutaneous injection (Kulkarni et al. 1983; Son et al. 1980); however, the studies by subcutaneous injection used higher doses and more sensitive methodologies. Son et al. (1980) used solvent extraction and chromatography on several absorbants to isolate metabolites. Nonextractable conjugated metabolites were hydrolyzed enzymatically and the aglycones were isolated by chromatography. GC/MS was used to identify the aglycones. The major metabolic pathways of *ortho*-toluidine in rats were *N*-acetylation and hydroxylation at the 4 position (*para* to the amine group), followed by glucuronide or sulfate conjugation. Glucuronidation,

acetylation, and oxidation are competing metabolic reactions in most species (Freudenthal et al. 1999). Sulfate and glucuronide-conjugated urinary metabolites comprised about half of the administered dose (Table 2-1). The ratio of sulfates to glucuronides was approximately 6:1. Minor metabolic pathways included hydroxylation at the 6 position (*ortho* to the amine group), oxidation of the methyl group, and oxidation of the amine group. A double acid conjugate of 4-amino-*meta*-cresol (thought to be the *N*-glucuronide of 4-amino-*meta*-cresyl sulfate) was identified but the amount was not reported. A subsequent study by Kulkarni et al. (1983) identified *N*-hydroxy-*ortho*-toluidine in rat urine using HPLC with an electrochemical detector. They noted that this metabolite might play a role in urinary bladder carcinogenicity (see Section 5.2.1). In the oral administration study (Cheever et al. 1980), unmetabolized *ortho*-toluidine and 4-amino-*meta*-cresol were detected, suggesting that ring hydroxylation was a major metabolic pathway in rats.

**Table 2-1. Metabolites of *ortho*-Toluidine Detected in Rat Urine**

Metabolite	Relative Amount (% of Dose) <sup>a</sup>
<i>Ether-extractable metabolites</i>	
<i>ortho</i> -Toluidine	3.6 <sup>b</sup> –5.1
Anthranilic acid	0.3
<i>N</i> -Acetyl-anthranilic acid	0.3
<i>N</i> -Acetyl- <i>ortho</i> -toluidine	0.2
<i>N</i> -Acetyl- <i>ortho</i> -aminobenzyl alcohol	0.3
<i>N</i> -Acetyl-4-amino- <i>meta</i> -cresol	0.3
4-Amino- <i>meta</i> -cresol	0.06
<i>ortho</i> -Azoxytoluene	0.2
<i>ortho</i> -Nitrosotoluene	≤0.1
<i>N</i> -Hydroxy- <i>ortho</i> -toluidine	0.1 <sup>b</sup>
<i>Acid-conjugated metabolites</i>	
4-Amino-cresyl sulfate	27.8
<i>N</i> -Acetyl-4-amino- <i>meta</i> -cresyl sulfate	8.5
2-Amino- <i>meta</i> -cresyl sulfate	2.1
4-Amino- <i>meta</i> -cresyl glucuronide	2.6
<i>N</i> -Acetyl-4-amino- <i>meta</i> -cresyl glucuronide	2.8
<i>N</i> -Acetyl- <i>ortho</i> -aminobenzyl glucuronide	1.3
Unidentified conjugated metabolites	5.9

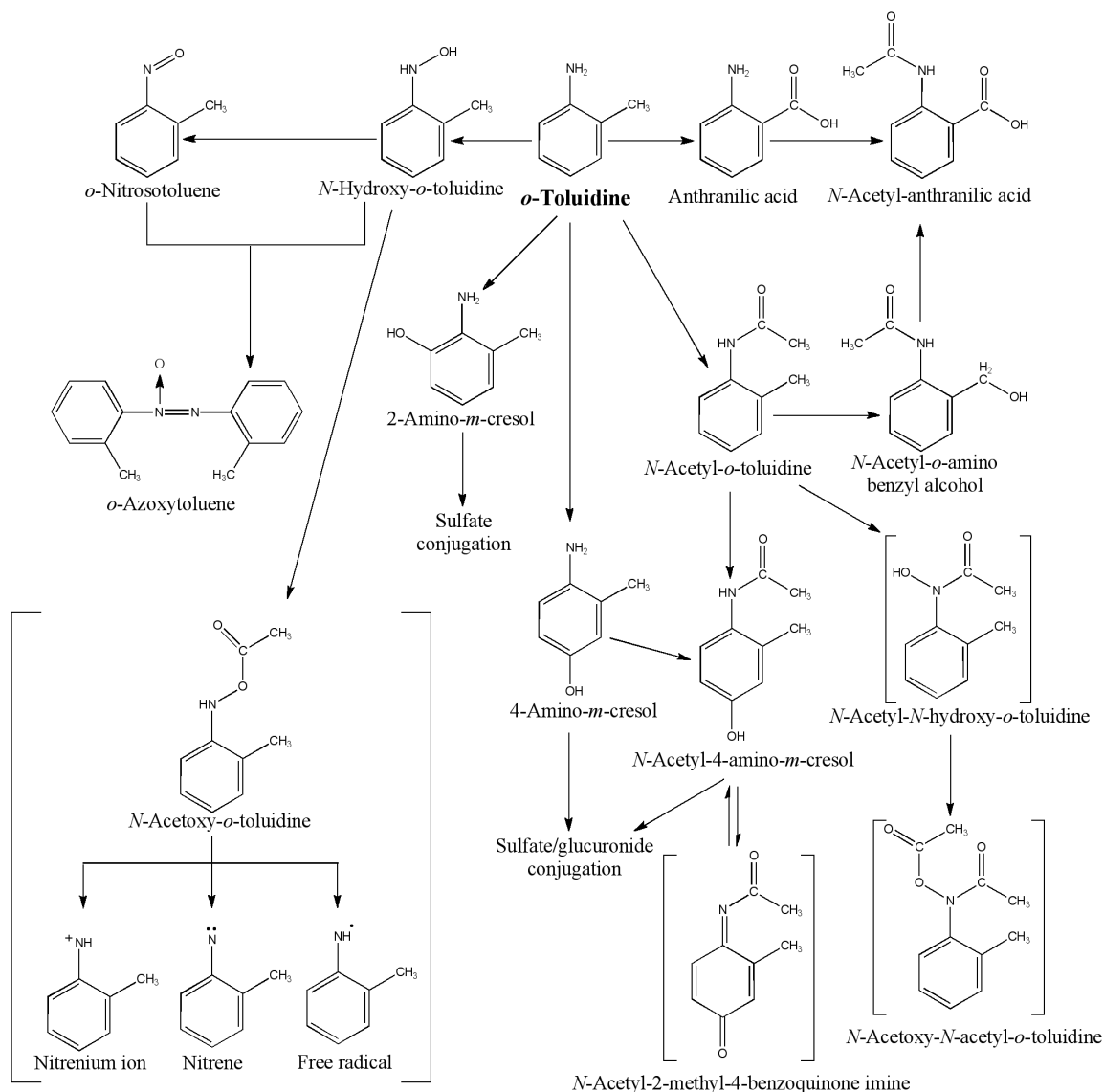
Sources: Kulkarni et al. (1983); Son et al. (1980).

<sup>a</sup>Measured 24 hours after a single s.c. dose of 400 mg/kg unless otherwise noted.

<sup>b</sup>Measured 6 hours after a single s.c. dose of 0.82 mmol/kg [~88 mg/kg].

In addition to the metabolites listed in Table 2-1, Gupta et al. (1987) predicted the existence of several metabolites based on data from other aromatic amines. These included *N*-acetoxy-*ortho*-toluidine (formed by acetylation of *N*-hydroxy-*ortho*-toluidine), *N*-acetyl-*N*-hydroxy-*ortho*-toluidine (formed by oxidation of *N*-acetyl-*ortho*-toluidine), *N*-acetoxy-*N*-acetyl-*ortho*-toluidine (formed by acetylation of *N*-acetyl-*N*-hydroxy-*ortho*-toluidine), 2-hydroxy-6-methyl-acetanilide (formed by acetylation of 2-amino-*meta*-cresol), and *ortho*-azotoluene (formed through a reaction of *ortho*-toluidine with *ortho*-nitrosotoluene). *N*-acetoxy-*ortho*-toluidine could undergo non-enzymatic breakdown to form several reactive electrophilic species (i.e., nitrenium ions, nitrene, and free radicals) that could bind to macromolecules. Another pathway involves oxidation of unconjugated phenolic metabolites to form reactive quinone imines (English et al. 2012; Skipper et al. 2010). Metabolic pathways of *ortho*-toluidine are shown in Figure 2-2.

## RoC Monograph on *ortho*-Toluidine



**Figure 2-2. Metabolism of *ortho*-Toluidine in Rats**

Sources: English et al. (2012); IARC (2000); Skipper et al. (2010); Son et al. (1980).

Brackets indicate postulated proximate or reactive metabolites based on data from other aromatic amines.

Toxicity studies of various aromatic amines in rats, mice, guinea pigs, rabbits, dogs, and monkeys show significant species differences in sensitivity to the carcinogenic effects of these chemicals (Freudenthal et al. 1999). The differences in species responses were related to differences in metabolism, particularly the ability of each species to *N*-acetylate aromatic amines, which is considered to be a detoxification step for urinary bladder cancer but an activating step for liver cancer. The mechanistic implications of these species differences in metabolism are discussed in Sections 5.2.1 and 5.2.2.

### 2.2.2. Humans

No studies of *ortho*-toluidine metabolism in humans were identified; however, metabolic pathways are expected to be similar to those reported in experimental animals based on studies of other aromatic amines and knowledge of the key metabolizing enzymes.

Ward et al. (1996) monitored aromatic amine exposure in workers at a chemical plant and reported that base hydrolysis was used to convert *N*-acetyl-*ortho*-toluidine to the parent compound prior to analysis, thus, indicating that *N*-acetylation of *ortho*-toluidine occurred in humans. Lewalter and Korallus (1985) noted that acetylation is a common metabolic pathway for aromatic amines but that only free, nonacetylated aromatic amines could lead to formation of hemoglobin (protein) adducts. Based on data for other aromatic amines, an oxidized metabolite (nitroso form) can come in contact and bind to hemoglobin in the blood (Skipper et al. 2010; Skipper and Tannenbaum 1994). Several studies have detected *ortho*-toluidine hemoglobin adducts in humans (Bryant et al. 1988; Korinth et al. 2007; Richter and Branner 2002; Richter et al. 2001; Seyler et al. 2010; Ward et al. 1996). The presence of hemoglobin adducts can be used as a biomarker of aromatic amine bioactivation to their ultimate carcinogenic form (Richter and Branner 2002; Skipper and Tannenbaum 1994). Methemoglobin is one of the principal acute effects observed in humans exposed to *ortho*-toluidine (English et al. 2012). The probable metabolic pathway leading to methemoglobinemia is metabolism of *ortho*-toluidine by hepatic cytochrome P450 enzymes to *N*-hydroxy-*ortho*-toluidine. Further oxidation of the *N*-hydroxy metabolite to nitrosotoluene occurs in the blood, thus generating methemoglobin.

*ortho*-Toluidine also is a metabolite of the commonly used local anesthetic prilocaine (Gaber et al. 2007). Gaber et al. (2007) also reported that *ortho*-toluidine-releasing hemoglobin adducts were increased up to 360-fold above background after prilocaine treatment. Prilocaine also is a well-known methemoglobin inducer (Gaber et al. 2007; Hjelm 1965; Lindenblatt et al. 2004). The amide bond in prilocaine is hydrolytically metabolized by human carboxylesterases (CES1A or CES2) *in vitro* to release *ortho*-toluidine (Higuchi et al. 2013).

### 2.2.3. Metabolic Enzymes

Cytochromes P450 (possibly CYP1A2 and CYP1A1) are responsible for *N*-hydroxylation of aromatic amines (Delclos and Kadlubar 1997; Windmill et al. 1997). Mammalian peroxidases also can catalyze oxidative metabolism of aromatic amines (Delclos and Kadlubar 1997; English et al. 2012). *o*-Toluidine increased cytochrome P450 content as well as the enzymatic activities, ethoxyresorufin-*O*-deethylase and ethoxycoumarin-*O*-deethylase, associated with CYP1A1, CYP1A2, and CYP2E1 (Leslie et al. 1988). In humans, CYP1A2 is expressed constitutively in the liver but not in other tissues, while CYP1A1 is predominantly expressed in extrahepatic tissues. Some studies in rats and humans suggest that CYP1A2 does not play a significant role in the metabolic activation of this compound (Gaber et al. 2007; IARC 2010e) but they were not definitive. Some data suggest that the ring-hydroxylation of *ortho*-toluidine is catalyzed by CYP2E1 (Diaz Gómez et al. 2006).

Jodynis-Liebert and Matuszewska (1999) studied the effects of toluidines and dinitrotoluenes on caffeine metabolism in rats and showed that *ortho*-, *meta*-, and *para*-toluidine induced CYP1A2 in the liver. CYP1A2-catalyzed *N*-hydroxylation is known to lead to bioactivation of many carcinogenic aromatic amines. Other studies have shown that recombinant human cytochromes P450 (CYP2A6, CYP2E1, CYP1A1, CYP2B6, CYP2D6, or CYP3A4) can metabolize the

aromatic amines 2,6-dimethylaniline and *ortho*-anisidine and might play a role in their bioactivation (Gan et al. 2001; Stiborová et al. 2005). Most of these P450s are expressed in the liver; however, CYP1A1 primarily is expressed in extrahepatic tissues, while 2E1, 2B6, 3A4, and 2D6 also have extensive extrahepatic activity. CYP1A1, 2D6 and 2E1 mRNA expression has been reported in human urinary bladder tissue and 1A1 also is expressed in human urinary bladder malignancies (Brauers et al. 2000).

In addition to P450-mediated *N*-hydroxylation, mammalian peroxidases (e.g., prostaglandin H synthase or myeloperoxidase) can metabolize ring-hydroxylated metabolites of aromatic amines to reactive quinone imines (Skipper et al. 2010). Phase II metabolic steps include *N*-acetylation, *O*-acetylation, *N,O*-transacetylation, *O*-sulfation, and glucuronidation (Woo and Lai 2004). Cytosolic arylamine *N*-acetyltransferases (NATs) catalyze *N*-acetylation, *O*-acetylation, and *N,O*-transacetylation reactions. Two mammalian cytosolic NATs have been identified in a variety of species, while three have been identified in mice (Delclos and Kadlubar 1997). Both of the NAT isozymes (NAT1 and NAT2) in humans are polymorphic and have different substrate specificities. NAT1 is expressed in most human tissues while NAT2 is predominantly expressed in liver, small intestine, and colon (Hein 2009). *O*-Sulfation reactions are catalyzed by cytosolic sulfotransferases (SULTs) (Windmill et al. 1997) and glucuronidation is catalyzed by UDP-glucuronosyltransferases in the endoplasmic reticulum (Woo and Lai 2004).

### 2.3. Synthesis

*ortho*-Toluidine can be absorbed after exposure in the air, on the skin, or through the gastrointestinal tract. Its distribution and excretion have been studied primarily in rodents where *ortho*-toluidine was detectable in blood, spleen, liver, kidneys, urinary bladder, and other tissues; excretion was mainly in the urine for all exposure routes with much smaller amounts eliminated through the lungs and in the feces.

*ortho*-Toluidine metabolism has only been investigated in male rats, thus, specific information on species and/or sex differences were not available. However, metabolism and toxicity data for various aromatic amines indicate that metabolic differences are important for species differences in toxicity. Cytochromes P450-mediated oxidation and acetylation, and glucuronide/sulfate conjugation reactions are the primary metabolic steps in all mammalian species studied. *N*-Hydroxylation has been identified as a key bioactivation step. The majority of the administered dose of *ortho*-toluidine is excreted as metabolites. More than half of a subcutaneous dose in rats was recovered in the urine as sulfate or glucuronide conjugates with smaller amounts of the parent molecule or acetylated metabolites.

Studies in the rat indicate that nitrosotoluene is formed from *N*-hydroxy-*ortho*-toluidine (Figure 2-2). Further metabolism of this molecule may lead to formation of reactive metabolites such as nitrenium ions, nitrene, and free radicals. Although no specific metabolism studies were available, there is indirect evidence based on formation of hemoglobin adducts and methemoglobin that nitrosotoluene is a metabolite of *ortho*-toluidine in humans. *N*-hydroxy-*ortho*-toluidine would be a likely intermediate in the formation of nitrosotoluene.



## 3. Human Cancer Studies

### Introduction

Human exposure to *ortho*-toluidine occurs mainly among industrial populations, where it is used primarily in the rubber chemical industry, as an intermediate in the manufacture of pesticides and of a variety of dyes and pigments, and in pharmaceuticals manufacturing (see Section 1, “Human Exposure”). Although exposure to *ortho*-toluidine can also occur via tobacco smoke, the environment, and consumer products, all of the available human studies have been conducted among occupationally exposed populations.

The human cancer studies section describes and evaluates the available epidemiological data on exposure to *ortho*-toluidine and cancer, reaching a level of evidence conclusion according to the approach outlined in the “Protocol: Evaluation of Human Cancer Studies on Exposure to *ortho*-Toluidine for the Report on Carcinogens” (hereafter referred to as “Protocol,” available at [http://ntp.niehs.nih.gov/NTP/roc/thirteenth/Protocols/ortho-ToluidineProtocol\\_508.pdf](http://ntp.niehs.nih.gov/NTP/roc/thirteenth/Protocols/ortho-ToluidineProtocol_508.pdf)). The steps in the cancer evaluation process, listed below, are captured in the following subsections or appendices.

- (1) Selection of the relevant literature included in the cancer evaluation (Section 3.1 and Appendix A, and in the Protocol).
- (2) Description of the study design, methodologies, and characteristics of the individual studies and identification of the tumor sites of interest (Section 3.2, Table 3-1 and Appendix D).
- (3) Assessment of study quality (Section 3.3, Table 3-2; Appendix D, Table D-3 and Table D-4).
- (4) Cancer assessment (Section 3.4): (a) evaluation of the cancer findings from the individual studies (Table 3-3) and (b) synthesis of the evidence for human carcinogenicity across studies.
- (5) Preliminary recommendation for the level of evidence of carcinogenicity (sufficient, limited, or inadequate) of *ortho*-toluidine from human studies (Section 3.5).

### 3.1. Selection of the Relevant Literature

Details of the procedures used to identify and select the primary studies and supporting literature for the human cancer evaluation are detailed in Appendix A and in the Protocol. The primary epidemiological studies considered for the cancer evaluation had to meet the following criteria:

(1) was a peer-reviewed, primary research study on potential exposure to *ortho*-toluidine, (2) provided exposure-specific analyses for *ortho*-toluidine or evidence that potential exposure to *ortho*-toluidine was highly likely based on author’s report or inferred using relevant knowledge of industrial processes, and (3) provided risk estimates for carcinogenesis endpoints or information needed to calculate risk estimates. Studies identified that met these criteria included six occupational historical cohort studies of cancer, and two population-based case-control studies (urinary bladder cancer and childhood acute lymphoblastic leukemia).

Studies excluded from the review were one case-control study of renal-cell cancer among paperboard printing workers (Sinks et al. 1992) that did not provide qualitative or quantitative

evidence of potential exposure (although pigments containing *ortho*-toluidine and other chemicals were reportedly used in the past) and several case-series of urinary bladder cancer, primarily among dyestuff manufacturers, that did not report or permit the calculation of risk estimates for potential exposure to *ortho*-toluidine (Conso and Pontal (1982); Genin et al. (1978); Khlebnikova et al. (1970); Oettel et al. (1968); Uebelin and Pletscher (1954); for a historical review of earlier case series, see e.g., Michaels (1988)). In general, no exposure data were reported in these studies, with the exception of the study of *ortho*-toluidine manufacturing workers in the former USSR by Khlebnikova et al. (1970), who reported ambient concentrations ranging from 0.5 to 28.6 mg/m<sup>3</sup> of *ortho*-toluidine and dermal exposure (see IARC 2010e).

### 3.2. Overview of the Selected Epidemiological Studies and Identification of Cancer Endpoints

This section provides an overview of the study population characteristics and methodologies of the individual studies included in the review (see Table 3-1) and identifies the primary cancer endpoint(s) of interest. For each of the reviewed studies, detailed data on study design, methods, and findings were systematically extracted from relevant publications, as described in the study Protocol, into Table D-1 and Table D-2 in Appendix D and in Section 3.4. Background exposure information for some of these epidemiological studies is available in Appendix C.

**Table 3-1. Human Cancer Studies of Potential Exposure to *ortho*-Toluidine**

Primary Reference	Name of Study	Exposure Assessment	Cancer Endpoints
<b>Cohort studies</b>			
Case and Pearson (1954), Case et al. (1954) <sup>a</sup>	U.K. dye workers	Exposure assigned based on company rolls of magenta manufacturing workers	Mortality (SMR): Urinary bladder cancer
Pira et al. (2010), Piolatto et al. (1991), Rubino et al. (1982)	Italian dye workers	Individual exposure assessment based on work history in departments producing <i>ortho</i> -toluidine, fuchsin, or safranine T dyes	Mortality (SMR): Urinary bladder cancer
Ott and Langner (1983)	U.S. dye workers	Individual exposure assigned by work history in one or both of departments producing bromindigo and thioindigo dyes	Mortality (SMR): All causes, all cancers, digestive, respiratory, urinary bladder cancers
Stasik (1988)	4-COT production workers	Employment in department producing or processing 4-chloro- <i>ortho</i> -toluidine	Mortality (SMR): All causes, stomach, urogenital, brain cancers Incidence (SIR): Urinary bladder cancer
Carreón et al. (2014; 2010), Hanley et al. (2012), Ward et al. (1991; 1996), Prince et al. (2000)	NIOSH (U.S.A.) rubber chemical workers	Individual exposure classified as definitely, probably (2 categories), and probably not <i>exposed to ortho</i> -toluidine, and by cumulative exposure based on work history and occupational hygiene data	Incidence (SIR, SRR, HR): Urinary Bladder cancer Mortality (SMR): All causes, all cancers, 8 specific sites (Prince et al. 2000)

Primary Reference	Name of Study	Exposure Assessment	Cancer Endpoints
Sorahan (2008), Sorahan et al. (2000), Sorahan and Pope (1993)	U.K. rubber chemical workers	Individual exposure assigned by history and 3 levels of duration of employment in department producing “Sopanox” rubber chemical agent	Incidence (SIR, SRR): Urinary bladder cancer Mortality (SMR): All causes, all cancers and >30 specific cancers (Sorahan et al. 2000) Combined incidence and mortality (RR): Urinary bladder cancer
<b>Population- or hospital-based case-control studies</b>			
Richardson et al. (2007)	Canadian case-control study	Job-exposure matrix based on self-reported occupational questionnaire and NIOSH NOES Survey data	Incidence (OR): Urinary bladder cancer
Castro-Jiménez and Orozco-Vargas (2011)	Colombian case-control study	Parental exposure to hydrocarbons including <i>ortho</i> -toluidine	Incidence (OR): Childhood acute lymphoblastic leukemia

4-COT = 4-chloro-*ortho*-toluidine; HR = hazard ratio; JEM = job-exposure matrix; NIOSH = National Institute for Occupational Safety and Health; NOES = National Occupational Exposure Survey (1983–1986); OR = odds ratio; RR = relative risk; SIR = standardized incidence ratio; SMR = standardized mortality ratio; SRR = standardized rate ratio.

<sup>a</sup>Although *ortho*-toluidine was not specifically mentioned in the study by Case and Pearson, exposure among magenta manufacturing workers is likely based on knowledge of the industrial process. In the U.K., the process for manufacturing magenta, resulting mainly in the production of magenta III (new fuchsin), involved condensation of *ortho*-toluidine and formaldehyde in the presence of *ortho*-nitrotoluene (Howe 1977).

Six occupational historical cohort studies of cancer among *ortho*-toluidine-exposed workers were identified: three cohort mortality studies were conducted among workers in the dyestuffs industry, two incidence/mortality studies in the rubber chemicals industry, and one incidence study in a plant manufacturing 4-chloro-*ortho*-toluidine (4-COT) (Table 3-1). Each of the studies conducted external or internal analyses on urinary bladder cancer risk, and four of the studies reported on some or all other cancer sites.

Two standardized cohort mortality studies of dyestuff workers were conducted among male workers manufacturing magenta: a study in the United Kingdom, hereinafter referred to as the U.K. dye workers study (Case and Pearson 1954) and a second study in Italy hereinafter referred to as the Italian dye workers study (Pira et al. 2010) (see Table 3-1). In the U.K. study, exposure to *ortho*-toluidine was inferred based on knowledge of the industrial process in the United Kingdom during that time period. Findings of the Italian dye workers study were reported in an initial mortality analysis to 1976 (Rubino et al. 1982) and in two follow-up studies to 1989 (Piolatto et al. 1991) and to 2003 (Pira et al. 2010). Exposure was assessed via individual work history. A third standardized mortality study was conducted among male workers engaged in the manufacture of thio- and bromindigo dyes in the United States (Ott and Langner 1983), hereinafter referred to as the U.S. dye workers study. Exposure to *ortho*-toluidine occurred only during the manufacture of thioindigo dyes and there was limited quantitative data documenting *ortho*-toluidine exposure.

A standardized urinary bladder cancer incidence analysis among male workers in Germany engaged in the manufacture of the azo dye and chlordimeform pesticide intermediate 4-COT, in

which *ortho*-toluidine is used as a precursor, has been reported by Stasik (1988) (hereafter referred to as the 4-COT production workers study).

Two standardized mortality and incidence studies in the rubber chemical industry, in the United States (conducted by NIOSH, hereinafter referred to as NIOSH rubber chemical workers) and United Kingdom (hereinafter referred to as the U.K. rubber chemical workers) were identified. Findings from the NIOSH rubber chemical workers were reported in a series of publications involving two different exposure assessments. An analysis of urinary bladder cases identified in the New York State Cancer Registry (Ward et al. 1991) and mortality (Prince et al. 2000) was conducted among male and female workers with a follow-up from 1973 through 1988, which assigned workers to categories of probability of exposure to *ortho*-toluidine based on employment in a department where *ortho*-toluidine was used. Air monitoring and biological monitoring of a subset of workers for *ortho*-toluidine and co-exposures were also conducted (Ward et al. 1991; 1996). An updated, detailed exposure survey of ambient levels of *ortho*-toluidine and co-exposures was subsequently conducted by Hanley et al. (2012). Urinary bladder cancer cases and deaths identified in the 1991 analysis were subsequently re-analyzed by Carreón et al. (2010), using the updated work histories and occupational hygiene data reported by Hanley et al. (2012). Carreón et al. (2014) reported on a reanalysis of this cohort, which (1) updated the follow-up until 2007, using incident urinary bladder cases identified in six State cancer registries, and (2) expanded the cohort to include workers hired from 1946 to 2006. Importantly, this study refined the exposure classification using department-job combinations and by approximately exposure ranks (Hanley et al. 2012). The (1991,2010, 2014) incidence analyses included internal analyses of duration of employment and time since first employment and the 2014 update incorporated internal analyses of cumulative exposure to *ortho*-toluidine for the first time. Incidence data from the most recent updated and expanded study are reported in this review (2014 incident cases and mortality data from the 2010 study) unless data from earlier reports were considered relevant to the assessment.

The United Kingdom study evaluated standardized cancer mortality and incidence among male rubber chemical manufacturing workers. Findings of an initial follow-up to 1986 were reported by Sorahan and Pope (1993), follow-ups to 1992 (incidence) and 1996 (mortality) by Sorahan et al. (2000), and follow-up to 2005 by Sorahan (2008). Analysis by duration of employment was conducted for this cohort but no quantitative data on *ortho*-toluidine exposure were reported.

Two case-control studies were identified: a population-based Canadian cancer registry study of male urinary bladder cancer in which job-exposure matrices of exposure to a range of occupational agents were applied (Richardson et al. 2007) (hereafter referred to as the Canadian case-control study) and a hospital-based case-control study in Colombia of childhood acute lymphoblastic leukemia (ALL) in association with parental exposure to a range of hydrocarbons, again using a job-exposure matrix (hereafter referred to as the Colombian case-control study) (Castro-Jiménez and Orozco-Vargas 2011).

The available database is only adequate to evaluate urinary bladder cancer. Urinary bladder cancer has been the primary focus of concern in human studies, driven in part by early case reports of increased numbers of these cancers among workers in the dyestuffs industry, in *ortho*-toluidine or 4-COT manufacturing, and in other amine manufacturing processes in which *ortho*-toluidine is used as an intermediate (see Section 3.1). Reporting is limited or absent for the majority of other tumor sites among the available cohort studies. The available case-control

studies on exposure to *ortho*-toluidine reported on only one other tumor site, childhood ALL, in the Colombian case-control study (Castro-Jiménez and Orozco-Vargas (2011); see Appendix D, Table D-2) in addition to the Canadian case-control study of urinary bladder cancer by Richardson et al. (2007). A statistically significant increase in ALL was observed among children whose parents were both estimated to have had potential occupational exposure to *ortho*-toluidine; however, potential confounding by one of a number of other hydrocarbons that were associated with an increase in ALL risk could not be ruled out. The available database on this cancer endpoint is inadequate to evaluate the relationship between exposure to *ortho*-toluidine and childhood ALL.

### **3.3. Assessment of the Quality of the Individual Studies**

Each primary study was assessed for its ability to inform hazard identification including the potential for biases and the adequacy of the “ability to detect an effect” and analytical methods, following the approach outlined in the Protocol for evaluating human cancer studies on exposure to *ortho*-toluidine. Details on the assessment of study quality are presented in Appendix D and in Table D-3 and details on the description of the study elements are in Appendix D, Table D-1 and Table D-2. Section 3.3.1 reports on the assessment of biases and other factors of study quality, Section 3.3.2 focuses on the assessment of potential confounding, and Section 3.3.3 integrates these assessments, reaching decisions on the utility of the individual studies to inform cancer identification.

#### **3.3.1. Assessment of Potential Bias, Analytical Methods, and Other Study Quality Characteristics**

##### **3.3.1.1. Selection and Attrition Bias**

Selection bias is not generally a major concern in cohort studies, with the exception of the potential for a healthy worker effect in studies using external (non-occupational) comparison groups (e.g., standardized mortality and incidence studies). Both the healthy worker hire effect and healthy worker survival effect tend to bias towards the null, so that positive associations are unlikely to be biased upward. The potential for a healthy worker hire effect can be indirectly assessed based on observed differences between all-cause and all-cancer mortality or incidence rates; no strong evidence of a healthy worker hire effect was identified in any of the cohort studies. No analyses were done in the studies to determine whether there was a healthy worker survival effect.

Overall, selection bias was not a major concern (i.e., potential for bias is not probable) in any of the studies with the exception of the 4-COT production worker study (Stasik 1988) (see Appendix D, Table D-3). The potential for a healthy worker effect was considered to be unlikely or minimal for analyses using internal controls in the two rubber chemical workers studies (Carreón et al. 2014; Sorahan 2008) and possible in the three dye worker cohort studies (Case and Pearson 1954; Ott and Langner 1983; Pira et al. 2010). The 4-COT production workers study (Stasik 1988) was initiated based on the findings of two urinary bladder cancer incident cases that were identified as a result of an earlier mortality study, and there is potential (probable) for a systematic bias, most likely towards finding a positive effect in this subcohort. The potential for selection bias in the Canadian case-control study (Richardson et al. 2007) was considered to be possible based on the use of cancer cases as controls and a low participation rate (60%). It is

difficult to predict whether using cancer cases as the referent group would bias the risk estimate towards the null because it is not known whether *ortho*-toluidine is a risk factor for other types of cancers.

Attrition bias was assessed via loss to follow-up in the cohort studies. Where reported, loss to follow-up was generally low and the potential for this type of bias was considered unlikely/minimal (Carreón et al. 2014; Ott and Langner 1983; Sorahan 2008) or possible (Pira et al. 2010). Information on follow-up was not available in two studies (U.K. dye workers and 4-COT production workers). Although loss to follow-up was higher (12%) in the Italian dye workers study (for the total cohort, but unknown for *ortho*-toluidine-exposed workers), there is no a priori reason to suspect that loss to follow-up would be related to exposure or disease status, and thus any potential bias would most likely be towards the null (Pira et al. 2010).

### 3.3.1.2. Information Bias: Exposure Endpoints

The adequacy of the characterization of exposure to *ortho*-toluidine and potential for misclassification was assessed based on quality of information provided to document exposure to *ortho*-toluidine at the individual level. Studies considered to have good or adequate exposure characterization were the two rubber chemical cohort studies and the Italian dye worker study. The most detailed and best-documented characterization of exposure was conducted in the NIOSH rubber chemical cohort (Carreón et al. 2014) because of the extensive ambient exposure measurements and biomonitoring data for a sample of workers, and the use of expert judgment, which included a review of records, plant walkthroughs, and interviews with company personnel (Hanley et al. 2012). The updated exposure assessment permitted the classification or reclassification of workers' exposure according to probability of exposure, duration, and cumulative exposure (as unit-days, using rank and continuous measures). (See Appendix C, Table C-1 and Section 1, Figure 1-4 and Figure 1-5 for more information on *ortho*-toluidine exposure levels among the different types of workers.)

Exposure assessment was based on individual work histories specific process information provided by the authors and expert judgment in the U.K. rubber chemical (Sorahan 2008) and Italian dye worker (Pira et al. 2010) studies (see Appendix B); although no quantitative exposure data were provided There is a higher potential for non-differential misclassification of exposure in these studies compared with the Carreón et al. (2014) study, but overall the exposure assessment was considered to be adequate to inform the evaluation of carcinogenicity.

The potential for misclassification of exposure was more of a concern in the U.K. and U.S. dye workers studies, 4-COT-production workers study, and the Canadian case-control study. In the U.K. dye worker study (Case and Pearson 1954), exposure to *ortho*-toluidine was inferred via knowledge of magenta manufacturing in the United Kingdom during the study time period. Although there was some limited ambient air and urine data in the 1940s indicating the presence of *ortho*-toluidine exposure in the U.S. dye worker study (Ott and Langner 1983), the potential for exposure misclassification was considered to be probable in this study because exposed workers (those producing thioindigo dyes) were grouped together with workers without potential exposure to *ortho*-toluidine (those producing bromindigo dyes). In addition, there were four alternative manufacturing processes, and *ortho*-toluidine might not have been used in all processes (see Appendix C). It also seems likely that not all 4-COT production and process workers were exposed to *ortho*-toluidine (Stasik 1988). In the Canadian case-control study

(Richardson et al. 2007), exposure to *ortho*-toluidine was assessed via self-reported occupational history and a job-exposure matrix (JEM). However, the quality of the JEM was limited because it was based on a U.S.-walk-through exposure survey (the NIOSH National Occupational Exposure Survey) in one calendar year and did not include information on specific jobs. In addition, it is not clear if there are differences in *ortho*-toluidine industries between Canada and the United States. Misclassification of exposure was considered to be probable in this study because of potential recall bias from using self-reported information and the unclear relevance of the JEM.

### 3.3.1.3. Information Bias: Disease Endpoints

The assessment of the potential for misclassification of urinary bladder cancer deaths or incident cases was based on the accuracy and completeness of ascertainment of vital status and diagnosis. Mortality data are typically less informative than incidence data for long survival cancers such as urinary bladder cancer, and the use of underlying cause of death from death certificates may underestimate cases of bladder cancer among deceased cohort members. In addition, cancer registries provide potentially greater diagnostic precision compared with death certificates; however, any cases that are not registered or occur outside of the state/region or time period covered may be missed. A strength of the updated NIOSH study (Carreón et al. 2014) was the expansion of the identification of cancer cases from the New York cancer registry (used by Ward et al. (1991)) to five additional cancer registries, together with increasing the follow-up time from 1988 through 2007.

Non-differential misclassification of cases, tending to bias the findings toward the null, was considered unlikely or minimal in the studies reporting cancer incidence (the two rubber chemical worker cohorts and the Canadian case-control study), but possible in the NIOSH and U.K. rubber chemical mortality analyses and the Italian and U.S. dye workers studies reporting mortality data. In the 4-COT production workers and U.K. dye workers studies, methods used to ascertain urinary bladder cancer cases (4-COT production workers) or deaths (U.K. dye workers) might have differed between the cohort and reference group, leading to the potential for a systematic (differential) misclassification of disease.

### 3.3.1.4. Ability to Detect an Effect and Adequacy of Analytical Methods

Factors influencing a study's ability to detect an effect include statistical power, levels, duration, level or range of exposure to *ortho*-toluidine, and length of follow-up in cohort studies. The statistical power (based on a one-sided test) to detect a twofold increased risk of urinary bladder cancer was limited in all the cohort studies except the NIOSH study (Carreón et al. 2014). In the Canadian case-control study, exposure to *ortho*-toluidine was rare (approximately 3.6% of cases; exposure prevalence not reported among controls) in the Canadian study, which limits its ability to detect an effect. All the cohort studies had adequate length of follow-up which, based on U.S. and European data on the latency of urinary bladder cancer, is considered to be 20 years. With respect to level, range, and duration of exposure, the ability to detect an effect was considered to be good for the NIOSH rubber chemical workers (Carreón et al. 2014), adequate for the cohorts of U.K. rubber chemical workers (Sorahan 2008), Italian dye workers (Pira et al. 2010), and U.K. dye workers (Case and Pearson 1954), and limited for the Canadian case-control study and cohort studies of U.S. dye workers (Ott and Langner 1983), and 4-COT production workers (Stasik 1988). In addition, no information on actual exposure levels was provided. The Canadian

case-control study is population- rather than occupation-based, and it is not clear how many of the participants who were assigned exposure to *ortho*-toluidine, particularly those in the “low” or “medium” exposure groups, would have had levels of exposure comparable to those typically experienced by the occupationally exposed workers in the cohort studies.

The adequacy of analytical methods was assessed based on whether the analyses included internal analyses, evaluated exposure-response relationships or time since first exposure, or adjusted for (or considered) potential confounding. Exposure-response relationships were evaluated in three studies: the two rubber chemical worker studies (Carreón et al. 2014; Sorahan 2008) and the Canadian case-control study (Richardson et al. 2007). Only two studies had formal statistical methods to evaluate confounding. The U.K. rubber chemical workers cohort study (Sorahan 2008) adjusted for occupational co-exposures but not for occupational risk factors for urinary bladder cancer, whereas the case-control study adjusted for some non-occupational risk factors but not occupational co-exposures. Although the NIOSH rubber chemical workers cohort study (Carreón et al. 2014) did not adjust for co-exposures in its analysis, it included extensive internal analyses, which help to mitigate potential confounding, and collected other information to evaluate potential confounding (see Sections 3.3.2 and 3.3.3). The U.K. rubber chemical workers study also conducted internal analyses. The three dye worker cohort studies excluded individuals with co-exposure to some known human bladder carcinogens (Case and Pearson 1954; Pira et al. 2010) or, in the U.S. study, to the known human carcinogens vinyl chloride and asbestos (Ott and Langner 1983). Although the Canadian case-control study conducted exposure-responses analyses, there is a high probability of misclassification to each of the exposure categories, and no information was provided in the number of cases and control for each exposure category. A strength of the study is that it controlled for cigarette smoking; however, no information on co-exposures was reported and no analyses controlling for any other occupational co-exposures were conducted. Analyses of the studies on dye workers (all three studies) and the 4-COT production workers were limited to external analyses with no consideration of exposure categories or accounting for potential confounders.

### **3.3.2. Assessment of Potential Confounding by Occupational Co-exposures to Known or Suspect Carcinogens**

Workers in both the dyestuffs industry and rubber chemical manufacturing industry may be exposed to a number of other chemicals, some of which may cause urinary bladder cancer or other cancers. In general, most studies did not assess exposure to potential confounders at the individual level or consider them in analytical models; however, in some cases the relative level of exposure to other chemicals compared with exposure to *ortho*-toluidine can be predicted from knowledge of the industrial process. The approach and conclusions of the evaluation of whether confounding from co-exposures is a concern in each study are captured in the table below and include the following steps:

- (1) A list of chemicals in each study of potential concern (e.g., reasonable potential of worker exposure) is compiled, together with the identification of co-exposures that may cause urinary bladder cancer (blue and orange font) or other cancers (green font) is co. (Table 3-3; see Appendix D, Table D-4 for background information regarding the potential carcinogenicity of the various co-exposures.)



- (2) Methods for assessing exposure to other chemicals or estimates of exposure compared to *ortho*-toluidine are evaluated (Table 3-2).
- (3) Analytical methods to consider or adjust for exposure to co-exposures are evaluated (Table 3-2).
- (4) An overall assessment of the potential for confounding is conducted.

The U.S., U.K., and Italian dye workers were potentially exposed to several suspected or known animal carcinogens, one of which, *ortho*-aminoazotoluene, causes urinary bladder cancer in animals. The U.K. and Italian dye workers were involved in magenta production, which is classified as carcinogenic to humans (IARC 2012); *ortho*-toluidine is one of the suspected co-exposures. Exposure data are lacking in each of the dye workers cohorts, however, and there is limited or no available evidence for the human carcinogenicity of the individual agents used in the dye production processes.

The German 4-COT production workers were exposed to 4-chloro-*ortho*-toluidine, for which there is some evidence of urinary bladder carcinogenicity in humans. The author noted that exposure to 4-COT was the predominant exposure, and it is not possible to separate potential effects of *ortho*-toluidine from those of 4-COT. It is also not clear whether workers in the U.S. dye workers study would also have been exposed to this substance as an intermediate in the thioindigo dye manufacturing processes.

With respect to potential confounding by co-exposures in the NIOSH cohort of rubber chemical workers, Hb adducts of 4-aminobiphenyl (the only known human bladder carcinogen with potential exposure in this population) were detected at very low levels and were similar in *ortho*-toluidine-exposed and unexposed workers (Ward et al. 1996), and ambient exposures were very low and intermittent (Hanley et al. 2012; Ward et al. 1991), which argues against 4-aminobiphenyl being a potential confounder. Workers were exposed to aniline, which is an animal carcinogen; however, exposure levels relative to *ortho*-toluidine were low. Nitrobenzene levels were mostly non-detectable (Hanley et al. 2012). The U.K. rubber chemical workers were likely to be exposed to several other chemicals if they worked in other departments, including aniline, phenyl- $\beta$ -naphthylamine (PBN), and 2-mercaptobenzothiazole (MBT), but evidence of carcinogenicity in humans of these substances is limited or inadequate for evaluation. In addition, internal analyses were conducted in which risk estimates for urinary bladder cancer incidence were adjusted for these co-exposures.

In the Canadian case-control study of bladder cancer, it is not clear whether individual observed cases were exposed to other chemicals; no adjustments were made in the analysis for multiple chemical exposures.

**Table 3-2. Co-exposures and Potential for Confounding**

Study	Co-exposures <sup>a</sup>	Exposure Assessment and Levels and Methods to Evaluate Potential for Confounding	Potential for Confounding
U.K. dye workers cohort Case et al. (1954); Case and Pearson (1954)	<i>High probability:</i> Magenta <i>Potential exposure</i> <sup>b</sup> [ <i>ortho-Nitrotoluene</i> ], [ <i>formaldehyde</i> ]	No exposure assessment at the individual level. Levels of potential co-exposures relative to <i>ortho</i> -toluidine cannot be evaluated. Excluded workers with exposure to auramine, benzidine, $\alpha$ -naphthylamine, or $\beta$ -naphthylamine.	Exposure to other known human urinary bladder carcinogens unlikely; exposure to animal or human carcinogens probable. Magenta production is a known human bladder carcinogen per IARC; however, any effects from magenta cannot be separated from its co-exposures (of which <i>ortho</i> -toluidine is a major suspect); studies of magenta in experimental animals are inadequate.
Italian dye workers cohort Piolatto et al. (1991); Pira et al. (2010); Rubino et al. (1982) <sup>c</sup>	<i>High probability:</i> <i>o</i> -Toluidine and 4,4'-methylene bis(2-methylaniline) production: [ <i>ortho-Nitrotoluene</i> ]; [ <i>4,4'-methylene-bis(2-methylaniline)</i> ] <i>Fuchsin and safranine T production:</i> Aniline, [ <i>ortho-aminoazotoluene</i> ], 2,5-diaminotoluene, fuchsin, safranine T	No exposure assessment at the individual level. Levels of <i>o</i> -nitrotoluene and 4,4'-methylene-bis(2-methylaniline) are predicted to be similar to that of <i>ortho</i> -toluidine. <sup>d</sup> Exposure to other chemicals probably lower. Excluded workers with exposure to benzidine, $\alpha$ -naphthylamine, or $\beta$ -naphthylamine.	Exposure to other known human urinary bladder carcinogens unlikely; exposure to animal urinary bladder carcinogens possible, exposure to animal carcinogens probable. See comment for Case and Pearson for magenta (e.g., fuchsin) production.
U.S. dye workers cohort Ott and Langner (1983)	<i>Potential exposure:</i> [ <i>Nitrobenzene</i> ]; [ <i>4-chloro-ortho-toluidine</i> ]; 4-chloro-acetyl- <i>o</i> -toluidine; thioindigo dyes; 1,2-dihydroacenaphthylene; 2-aminobenzoic acid	No exposure assessment at the individual level. Odor of nitrobenzene reported. Excluded individuals with high exposure to arsenicals, vinyl chloride, or asbestos.	Exposure to animal carcinogens or suspect human urinary bladder carcinogen (4-chloro- <i>ortho</i> -toluidine) probable for some workers in cohort.  (Note: This refers to the potential for confounding that remains after workers exposed to vinyl chloride or asbestos were excluded).

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Study	Co-exposures <sup>a</sup>	Exposure Assessment and Levels and Methods to Evaluate Potential for Confounding	Potential for Confounding
4-COT production workers cohort (Germany) Stasik (1988)	<i>High probability:</i> <b>[4-Chloro-ortho-toluidine]</b> <i>Potential exposure:</i> 6-Chloro- <i>ortho</i> -toluidine; <i>N</i> -acetyl- <i>ortho</i> -toluidine	No exposure assessment at the individual level. Authors state exposure to 4-chloro- <i>ortho</i> -toluidine was predominant.	High probability of potential confounding from exposure to a suspected human bladder carcinogen (4-chloro- <i>o</i> -toluidine). Study methods do not allow for attributing any observed effects to <i>ortho</i> -toluidine.
NIOSH (U.S.) rubber chemical workers cohort Carreón et al. (2010); Hanley et al. (2012); Ward et al. (1991); Ward et al. (1996)	<i>High probability:</i> <b>[Aniline]</b> <i>Potential exposure:</i> <b>[4-Aminobiphenyl]</b> (trace)	Air sampling of co-exposure available for <i>ortho</i> -toluidine exposure groups but not at the individual level; biomonitoring data (urine and <i>ortho</i> -toluidine-Hb adducts) available on subgroup of workers but not used in risk calculations.  <i>Definitely exposed jobs:</i>  Aniline: Breathing zone GM: slightly lower than <i>ortho</i> -toluidine; urine and aniline-Hb adducts lower than <i>ortho</i> -toluidine.  Nitrobenzene: Breathing zone GM: ~2.5 lower than <i>ortho</i> -toluidine; non-detectable in most samples.  4-Aminobiphenyl: observed in air at trace levels; 4-ABP-Hb adducts found at similar (low) levels in <i>ortho</i> -toluidine and unexposed workers.  Other co-exposures: levels NR, but evidence from process descriptions Hanley et al. (2012) suggest low or minimal potential exposure.  Internal analyses (probability, duration, and cumulative exposure to <i>ortho</i> -toluidine only).	No evidence that exposure to 4-aminobiphenyl (a known human bladder carcinogen) was a potential confounder in this study; exposure to other animal carcinogens possible.

RoC Monograph on *ortho*-Toluidine

Study	Co-exposures <sup>a</sup>	Exposure Assessment and Levels and Methods to Evaluate Potential for Confounding	Potential for Confounding
U.K. rubber chemicals workers cohort Sorahan (2008); Sorahan et al. (2000); Sorahan and Pope (1993)	<i>High probability:</i> “Sopanox” (1-tolylbiguanidine)  <i>Potential exposure</i> <sup>c</sup> : [Aniline]; MBT; PBN	Quantitative cumulative exposure assessment to MBT; duration of employment in aniline- and PBN-exposed departments.  Analysis of bladder cancers among <i>ortho</i> -toluidine exposed workers adjusted for exposure to MBT and PBN in internal analysis.	Exposure to other known human bladder carcinogens unlikely; methods to evaluate potential confounding adequate.
Canadian case-control study Richardson et al. (2007)	Other potential co-exposures among cases associated with <i>ortho</i> -toluidine exposure not reported.	No adjustment of case-control analysis for other occupational co-exposures was conducted.	Co-exposure to other carcinogens possible; inadequate data to evaluate potential confounding from co-exposures.

See also Table D-4.

GM = geometric mean; MBN = 2-mercaptobenzothiazole; ND = non-detectable; PBN = phenyl- $\beta$ -naphthylamine.

<sup>a</sup>Co-exposures include those where there is a reasonable potential for exposures, does not include co-exposure to very low or trace levels unless that chemical is a known human bladder carcinogen; blue font ([***bold italic type***] = known or potential human urinary bladder carcinogens, orange font [**bold type**] = animal urinary bladder carcinogen; green font [*italic type*] = animal or human carcinogen, not urinary bladder.

<sup>b</sup>Based on knowledge of manufacturing process (see Section 1). According to Howe (1977), U.K. magenta manufacture mainly resulted in Magenta III, produced by condensing *ortho*-toluidine with formaldehyde in the presence of *ortho*-nitrotoluene.

<sup>c</sup>According to the authors (Rubino et al. 1982), *ortho*-toluidine and 4,4'-methylene-bis (2-methylaniline) were made in one department and New Fuchsin (new magenta or Magenta III) was produced in a second by heating *ortho*-toluidine, 4,4'-methylene-bis (2-methylaniline) with *ortho*-nitrotoluene; workers in the second department also made safranin T by oxidizing *ortho*-toluidine and 2,5-diaminotoluene in the presence of aniline, with *ortho*-aminoazotoluene as an intermediate of the reaction (see Section 1). Workers in the *ortho*-toluidine-exposed subcohort worked in one or other of these departments.

<sup>d</sup>Based on knowledge of manufacturing process (see Section 1).

<sup>e</sup>Some workers may have been exposed to these chemicals via working in other departments.

### 3.3.3. Assessment of Potential Confounding by Other Risk Factors

In addition to the healthy worker effect and industrial co-exposures discussed above, exposed workers may differ from the comparison group with respect to demographic, lifestyle, or other risk factors. With the exception of smoking, there are few known non-occupational risk factors for urinary bladder cancer other than radiation and some chemotherapy drugs, and these would be unlikely to be related to *ortho*-toluidine exposure. There is strong evidence to suggest that smoking is an independent risk factor for urinary bladder cancer. In addition, *ortho*-toluidine has been detected in tobacco smoke (IARC 2010e). The NIOSH study (Carreón et al. 2014; Ward et al. 1991; Ward et al. 1996) was the only cohort study to examine the effect of smoking on urinary bladder cancer risk, first by assessing smoking status among an approximately 12% sample of workers and measuring *ortho*-toluidine, aniline, or 4-aminobiphenyl in urine and hemoglobin (Hb) adducts in a sample of smokers and nonsmokers (Ward et al. 1996). No differences were observed between smokers and non-smokers for *ortho*-toluidine in urine or *ortho*-toluidine Hb adducts. Secondly, the effect of the difference in smoking status among a subset of workers in comparison with the U.S. population on expected urinary bladder cancer risk was calculated, using an indirect adjustment method (using the method of Axelson and Steenland 1988) and incorporating a Monte Carlo sensitivity analysis to examine potential bias. The difference in smoking status between workers and the U.S. population was estimated to increase the risk of urinary bladder cancer incidence by 8%. (Carreón et al. 2014). The U.K. rubber chemical worker study reported a SMR for lung cancer for the overall cohort, which provides some indirect information on evaluating potential confounding from cigarette smoking. In the case-control study, analyses were adjusted for smoking, alcohol consumption, and demographic variables. In the Canadian case-control study, ethnicity, marital status, education, questionnaire respondent, alcohol, and smoking variables were considered by backwards stepwise conditional logistic regression.

### 3.3.4. Summary of the Utility of the Studies to Inform the Cancer Evaluation

Based on the methodological evaluation of the adequacy of study design, adequacy of exposure and disease assessment of cancer incidence or mortality, and ability to detect an effect of *ortho*-toluidine on urinary bladder cancer risk, the NIOSH and U.K. rubber chemical worker cancer incidence studies and the Italian dye workers cohort mortality study are considered to be of high or moderate utility to inform the cancer evaluation. The NIOSH cohort is considered to be the most informative study because it provides the best exposure assessment of *ortho*-toluidine and co-exposures. Exposure was not as well characterized (by e.g., ambient air monitoring or biomonitoring) in the U.K. rubber chemical and Italian dye workers studies; however, detailed job histories were available, and thus we can reasonably conclude that each worker was exposed to *ortho*-toluidine, although the levels of exposure are unknown. Strengths of the U.K. rubber chemical study are analysis by duration of employment and internal adjustment of potential confounding. The Italian dye workers study is of moderate but more limited utility due to the possibility of potential confounding, the smaller numbers of exposed workers, and higher overall number of workers lost to follow-up.

The cohort studies of U.K. and U.S. dye workers and the Canadian case-control study are of more limited utility because of the potential for misclassification of exposure and confounding by co-exposures. The U.S. dye workers study only has sufficient statistical power to detect a large effect. In addition, only 23% of the dye workers had more than 5 years of employment and

*ortho*-toluidine was used to make only one of the two types of dyes included in the potentially exposed group. Although the Canadian case-control study used a job-exposure matrix to assess exposure at the individual level, there is still potential for misclassification due to the limited quality of the exposure assessment and data are inadequate to evaluate potential confounding from occupational co-exposures in this study. In addition, exposure to *ortho*-toluidine (approximately 3.6% of cases, exposure prevalence not reported in controls) was rare among the study participants, which limits its ability to inform the cancer evaluation.

In the German 4-COT production worker study, it is not possible to reliably distinguish the potential effect of *ortho*-toluidine from that of 4-COT, a suspect human bladder carcinogen, and there is a risk of positive selection bias in this cohort; therefore, the study was considered to be methodologically inadequate for inclusion in the synthesis.

### **3.4. Cancer Assessment: Urinary Bladder Cancer**

This section summarizes and interprets the findings from the individual studies and synthesizes the evidence for urinary bladder cancer across the body of studies. Five cohort studies and one case-control study were considered adequate for inclusion in the evaluation of the level of evidence of carcinogenicity of *ortho*-toluidine from studies in humans. As noted in Section 3.3.4 (“Summary of the utility of the studies to inform the cancer evaluation”), based on the methodological strengths and limitations of these studies, the two (U.K. and NIOSH) rubber chemical cohorts of bladder cancer incidence and mortality and, to a lesser extent, the Italian dye workers cohort mortality study are considered the most informative for the evaluation of the overall findings, while the U.K. and U.S. dye workers cohort studies and the Canadian case-control study are of more limited utility. When both incidence and mortality data were available for the same study, incidence was considered to be more informative for evaluating urinary bladder cancer risk.

The key question for evaluating the level of evidence across the body of studies is whether any observed associations between *ortho*-toluidine exposure and urinary bladder cancer could be explained by chance, bias, or confounding by co-exposures or other risk factors. Several of the guidelines developed by Austin Bradford Hill (1965) are relevant to the evaluation of the level of evidence for human carcinogenicity, including the magnitude (strength) and consistency of any observed associations across studies; evidence for exposure-response relationships and associations with appropriate latency; and the degree to which chance, bias, and confounding could plausibly explain observed associations. Observed associations from methodologically limited studies or negative findings from such studies are given less weight in the overall evaluation than findings from methodologically strong studies.

#### **3.4.1. Individual Studies**

The findings for the six studies evaluating urinary bladder cancer and exposure to *ortho*-toluidine are summarized in Table 3-3 below. The interpretation of the studies, including the conclusions of the findings, whether they can be explained by confounding, and the overall quality of the evidence, is presented in the “Interpretation” column.

**Table 3-3. Urinary Bladder Cancer among *ortho*-Toluidine-exposed Workers**

Reference	Study Name Design/ Population Exposure Assessment	Exposure Group (N)	External Analysis: SMR or SIR (95% CI); # Obs Deaths or Cases	Internal Analysis: OR SRR, HR or RR (95%CI); # Obs Cases	Interpretation
Carreón et al. (2014) incidence analysis and Carreón et al. (2010) mortality analysis	U.S. (NIOSH) rubber chemical workers (1,786)	<u>Exposure probability</u> Probably not (562) Probably [1] <sup>b</sup> (155) Probably [2] <sup>c</sup> (195) Definitely <sup>d</sup> (900)	<u>Incidence (SIR)<sup>a</sup></u> 0.59 (0.07–2.12); <5 2.47 (0.67–6.33); <5 4.21 (1.15–10.8); <5 3.90 (2.57–5.68); 27	<u>Incidence (SRR)</u> 1.00 2.50 (0.42–14.8) 4.85 (0.81–29.0) 4.28 (1.02–18.0)	High risk estimates in incidence study Evidence of exposure-response relationship with duration and level of exposure.
United States	urinary bladder cancer	<u>Definitely exposed</u> <i>Employment duration (yr)</i> <5 5–9 ≥10 <i>p</i> <sub>trend</sub>	1.98 (0.80–4.08); <10 4.52 (0.93–13.2); <5 6.24 (3.63–9.99); 17	1.00 2.56 (0.60–10.9) 4.50 (1.74–11.6) <0.0001	Potential co-exposures unlikely to explain increase in risk
	Exposure assessment-based work history (jobs and dept.), industrial hygienist surveys, and expert assessment	<i>Time since first exposure (yr)</i> <10 10–19 20–30 >30 <i>p</i> <sub>trend</sub>	1.74 (0.04–9.68); <5 3.41 (0.93–8.72); <5 4.75 (2.17–9.02); 9 3.97 (2.11–6.79); 13	1.00 7.09 (0.76–66.2) 13.4 (1.59–11.3) 8.61 (1.11–67.0) 0.001	Potential confounding by smoking unlikely: estimated increase in SIR due to smoking = 1.08 based on 5% sample of smoking data, compared with national rates Overall quality of evidence: high
		<u>Cumulative rank (unit-days)</u> <i>Quartile model 10 yr. lag<sup>e</sup></i> <11,000 11,000 to <27,000 27,000 to <48,000 48,000+ <i>p</i> <sub>trend</sub>	1.32 (0.61–2.51); 9 3.37 (1.62–6.20); 10 5.44 (2.49–10.3); 9 6.13 (2.80–11.6); 9	1.00 3.05 (1.13–8.22) 6.37 (2.30–17.7) 7.34 (2.44–22.1) <0.001	
		<i>Continuous: Log-square<sup>f</sup></i> 3,652.5 18,262.5 36,525		<u>HR</u> 1.88 (1.48–2.42) 4.11 (2.39–7.23) 7.39 (3.42–16.4)	

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Reference	Study Name Design/ Population Exposure Assessment	Exposure Group (N)	External Analysis: SMR or SIR (95% CI); # Obs Deaths or Cases	Internal Analysis: OR SRR, HR or RR (95%CI); # Obs Cases	Interpretation
Sorahan (2008) United Kingdom	U.K. rubber chemical workers (2,160)  Historical cohort cancer mortality and incidence study of urinary bladder cancer  Exposure assessment based on detailed job history and expert assessment	<i>o</i> -Toluidine-exposed subcohort (53)  <u>Employment duration (yr)</u> Ref 0.1–4.9 ≥5 p <sub>trend</sub> (continuous)	SIR = 5.56 (1.51–14.22); 4 SMR = 11.16 (2.30–32.61); 3	Combined incidence and mortality  <u>Adjusted RR<sup>d</sup></u> 1.00 3.72 (1.21–11.4); NR 3.38 (0.67–17.0); NR p < 0.05	High risk estimates  Evidence of exposure response with duration of employment (exposure categories treated as continuous measure)  Significantly elevated risk after adjustment for co- exposures  Overall quality of evidence adequate to high
Pira et al. (2010) Italy	Italian dye workers (906)  Historical cohort mortality study  Exposure assessment based on work history and expert assessment	Fuchsin, safranine T, or <i>ortho</i> -toluidine mfr. (47)	SMR = 22.5 (8.3–49.0); 6	NR	Confounding by co- exposures unlikely but cannot be ruled out  High risk estimate but based on small numbers  Overall quality of evidence: adequate



RoC Monograph on *ortho*-Toluidine

Reference	Study Name Design/ Population Exposure Assessment	Exposure Group (N)	External Analysis: SMR or SIR (95% CI); # Obs Deaths or Cases	Internal Analysis: OR SRR, HR or RR (95%CI); # Obs Cases	Interpretation
Ott and Langner (1983) United States	U.S. dye workers (275)  Historical cohort mortality study  Exposure based on working in a production area associated with exposure to <i>o</i> -toluidine, but extent of exposure is unclear and misclassification likely	Bromindigo or thioindigo mfr. (117)	<u>Deaths</u> 0 observed deaths (Expected not reported; 1.2 expected in total cohort)	NR	No excess mortality  Limited power to detect effect due to potential exposure misclassification and low statistical power  Overall quality of evidence: limited
Case and Pearson (1954) United Kingdom	U.K. dye workers (4,622)  Historical cohort mortality study of urinary bladder cancer  Exposure inferred via manufacturing process	Magenta production (85)	SMR = [23.0 (5.87–62.8)]; 3	NR	High risk estimate but based on small numbers  Indirect but plausible evidence of exposure to <i>ortho</i> -toluidine  No clear evidence of carcinogenicity of co- exposures  Overall quality of evidence: limited

RoC Monograph on *ortho*-Toluidine

Reference	Study Name Design/ Population Exposure Assessment	Exposure Group (N)	External Analysis: SMR or SIR (95% CI); # Obs Deaths or Cases	Internal Analysis: OR SRR, HR or RR (95%CI); # Obs Cases	Interpretation
Richardson et al. (2007)	Canadian case-control study	Ever exposed (39)	—	<u>Adjusted OR<sup>b</sup></u> 1.01 (0.71–1.44); 39	No excess risk or exposure response relationship
Canada	Cancer registry-based study of urinary bladder cancer: 1,062 cases, 805 controls, 7 cancer controls  Exposure assessment based on JEM (NIOSH survey); concerns about quality of JEM)	<u>Probability of exposure</u> Low (NR) Medium (NR) High (NR)  P <sub>trend</sub>		1.49 (0.89–2.52); NR 0.46 (0.20–1.07); NR 1.10 (0.62–1.94); NR 0.79	Limited ability to detect an effect because of potential for exposure misclassification and rarity of exposure specific to <i>ortho</i> -toluidine in study.  Overall quality of evidence: limited

Numbers in [ ] calculated by RoC.

SMR = standardized mortality ratio; SIR = standardized incidence ratio; SRR = standardized rate ratio; OR = odds ratio; NR = Not reported; JEM = job exposure matrix.

<sup>a</sup>SIR based on rates for New York State excluding New York City.

<sup>b</sup>[1] Low and irregularly/occasionally.

<sup>c</sup>[2] Low and regularly.

<sup>d</sup>Moderate/high.

<sup>e</sup>Similar findings reported for unlagged and 20-yr lagged models and in Cox regression models using quartile or quintile categories of category rank exposure lagged for 10 yr.

<sup>f</sup>Cox regression model; cases matched to cases with risk sets on gender, race and attained age, birth yr. was not a confounder; HR reported for 1, 5, 10 yr at Rank 10 (corresponding unit-days reported in table); similar exposure-response found (albeit lower magnitudes) for log-linear model.

<sup>g</sup>Adjusted for exposure to MBT, PBN, aniline, age, and calendar period; trend calculated by treating 4 exposure categories as a continuous measure.

<sup>h</sup>Matched for age and additionally adjusted for ethnicity, years of smoking, alcohol consumption, and questionnaire respondent. (Other variables considered in the analysis were marital status, education, and other measures of smoking status. Reference group was 0 exposure group.

### 3.4.1.1. Studies of Rubber Chemical Workers

In the expanded and updated analysis of the NIOSH rubber chemical workers (Carreón et al. 2010), a statistically significant increased SIR was found among definitely exposed workers (SIR = 3.90; 95% CI = 2.57 to 5.68, 27 cases; SRR (vs. probably not exposed workers) = 4.28; 95% CI = 1.02 to 18.0). These data confirm the finding of a statistically significant risk of bladder cancer among exposed workers reported in both publications of the original cohort, which used two different exposure assessments (Carreón et al. 2010; Ward et al. 1991). The magnitude of the risk estimate among workers reclassified as having definitely (moderate to high) exposure in the 2014 study was somewhat lower than the previous reports, but the estimate was more precise.

There was strong evidence for a positive association between urinary bladder cancer risk and both cumulative rank exposure and exposure duration; risk estimates among the highest or longest exposed workers ranged between approximately five- and ninefold in most analyses. Among definitely exposed workers, the risk of urinary bladder cancer increased with longer duration of exposure in both external (SIR) or internal incidence analyses (using directly standardized rates, SRR, and <5 years duration as a referent group) and longer time since first exposure (SRR using <10 years as the referent group); statistically significant exposure response-relationships were observed in internal analyses ( $p_{\text{trend}} < 0.001$  for each analysis) (Carreón et al. 2010). Among all cohort members, urinary bladder cancer risk also increased with increasing quartiles of cumulative rank exposure in both external (SIR) and internal analyses (SRR, using the lowest exposure groups as the referent groups) and in unlagged, 10-year lagged, and 20-year lagged models for both types of analyses. Statistically significant, positive exposure-response relationships were observed in the unlagged ( $p_{\text{trend}} < 0.001$ ), 10-year lagged ( $p_{\text{trend}} < 0.001$ ) and 20-year lagged ( $p_{\text{trend}} = 0.037$ ) SRR analyses, albeit the 10-year lagged model was considered to have the best fit. In Cox regression analyses (10-year lagged, using the lowest exposure group as the referent group), cumulative rank exposure was significantly related to urinary bladder cancer risk in categorical models (quartile, quintile, decile, based on exposure distribution of cases) and continuous models (log-linear, log square). (Table 3-3 reports findings for several of these analyses.) Mortality analyses were not reported in the 2014 update, and analyses reported in the 2010 study were uninformative because of the small number of exposed cases; there was only one exposed case among definitely exposed workers (SMR = 1.96, 95% CI = 0.05 to 11.05, 1 exposed death).

It is unlikely that the increased risk of urinary bladder cancer in the NIOSH cohort could be explained by selection and information biases or confounding. There is no clear evidence of selection, ascertainment, or attrition bias (see Section 3.2, Table 3-2), and the high magnitude of the observed risk estimate mitigates against a risk of these types of biases. Based on detailed industrial hygiene data, biomonitoring data, individual work histories, and extensive use of records to reclassify exposure, there is documented evidence of substantial exposure to *ortho*-toluidine in the NIOSH cohort among workers in the “definitely exposed” category. It is unlikely that co-exposure to known or suspected carcinogenic substances or other risk factors could explain the observed associations between exposure to *ortho*-toluidine and urinary bladder cancer risk in the NIOSH cohort study. As noted, 4-aminobiphenyl (4-ABP) is the only co-exposure that has been clearly established as a urinary bladder carcinogen in humans; however, exposure to 4-ABP was very low (<1 ppm) (Hanley et al. 2012; Viet et al. 2009; Ward 1997; Ward et al. 1996), intermittent (1972 and 1985) (Ward et al. 1994; Ward 1997), and not

correlated with *ortho*-toluidine exposure (as assessed by hemoglobin adducts (Ward et al. 1996). Although workers were also exposed to aniline, it is unlikely that aniline can explain the association between *ortho*-toluidine and urinary bladder cancer. The available epidemiological studies have not found an association between aniline exposure and urinary bladder cancer (Case and Pearson 1954; IARC 1987; Sorahan 2008; USEPA 1994). Although aniline is an animal carcinogen (Group 3), it has not caused urinary bladder cancer in animals and it is considered a less potent animal carcinogen than *ortho*-toluidine (see Section 5, “Other Relevant Data”). Nitrobenzene, considered a Group 2B carcinogen by IARC is not associated with urinary bladder cancer in animals and, as noted, was mostly non-detectable in the plant (Hanley et al. 2012).

Smoking is also unlikely to explain the excess risk of urinary bladder cancer. As noted in Section 3.3.2, smokers and non-smokers had similar levels of *ortho*-toluidine that were not related to their occupational exposure status, and, when compared with national rates, smoking prevalence differences were estimated to potentially increase urinary bladder cancer risk in this cohort by only 8%, compared with the approximately three- and fourfold overall observed increases in association with *ortho*-toluidine exposure in external analysis and internal analyses respectively (Carreón et al. 2014). The indirect smoking adjusted SIR for the entire cohort was close to three-fold (SIR = 2.67, 95% Monte-Carlo limits for bias = 1.87 to 3.75).

Overall, the finding of an increased risk of urinary bladder cancer remained robust (1) using different methods to analyze exposure-response relationships, (2) adjusting for smoking, and (3) using sensitivity analyses, primarily related to different methods of case ascertainment in the different cancer registries.

Statistically significant increased risks of urinary bladder cancer incidence (SIR = 5.56; 95% CI = 1.51 to 14.22, N = 4) and mortality (SMR = 11.2, 95% CI = 2.30 to 32.61; 3 deaths) were observed in the U.K. rubber chemical cohort study. The major limitations of this study are the small number of exposed cases and the lack of quantitative information on exposure levels; however, neither of these limitations would create a false positive. Internal analyses showed a significant positive trend (continuous measure,  $p < 0.05$ ) for cumulative duration of employment in the *ortho*-toluidine department, after simultaneous adjustment for co-exposure to aniline, MBT and PBN (Table 3-3). Similar to the NIOSH study, there was no evidence of a potential for selection bias, and the potential for nondifferential exposure misclassification, is mitigated by the use of individual work histories to assign person-years at risk.

The U.K. rubber workers were also potentially exposed to 2-mercaptobenzothiazole (MBT), phenyl- $\beta$ -naphthylamine (PBN) and aniline, if they rotated to other departments of the plant where these chemicals were used, which approximately 74% of the *ortho*-toluidine-exposed workers did (Sorahan et al. 2000). Confounding by these exposures is considered to be unlikely. MBT and PBN have not been clearly associated with urinary bladder cancer in other epidemiological studies (Collins et al. 1999; Veys 1996), although they were associated with increased risks of urinary bladder cancer incidence in the U.K. rubber chemical cohort in an unadjusted (SRR) analysis. However, the relative risks were strongly attenuated and no longer statistically significant after simultaneous adjustment for co-exposure to *ortho*-toluidine, aniline, and either MBT or PBN in internal analyses by duration of employment. Although there was no information on cigarette smoking in this study, there was no excess risk of lung cancer among workers in the overall (not restricted to *ortho*-toluidine exposed workers) cohort (SMR = 0.91,

95% CI = 0.75 to 109; 120 deaths), suggesting that smoking habits among workers were not substantially different than the general population (reference group).

#### **3.4.1.2. Other Studies: Dye Workers and Case-control Study**

A large excess mortality was found in both studies of dye workers involved in magenta manufacturing: the Italian study (SMR = 22.5; 8.3 to 49.0, N = 6) and the U.K. study ([SMR = 23.0; 95% CI = 5.87 to 62.8], N = 3); however, exposure to *ortho*-toluidine in the U.K. study is inferred via industrial process and not definitely established. The use of individual work histories to assign person-years at risk helps mitigate the potential for misclassification of exposure in the Italian cohort. Workers in the Italian dye workers cohort who made *ortho*-toluidine were likely exposed at similar levels to *ortho*-nitrotoluene and 4,4'-methylene-bis (2-methylaniline), both of which are listed as reasonably anticipated to be human carcinogens (NTP 2011a; 2011b) based on sufficient evidence of carcinogenicity from studies in experimental animals; however, these chemicals did not cause cancer of the urinary bladder, although site concordance between animals and humans is not always observed. Workers who made fuchsin or safranin T were possibly exposed to the intermediate *ortho*-aminoazotoluene, for which there is some evidence of bladder tumors in animal studies. Three urinary bladder cancer deaths were reported among workers in the section making *ortho*-toluidine and two among workers in the dyemaking section (the location of the most recent death was not reported), with no overlap in exposure, according to the authors, suggesting that *ortho*-toluidine was the only common exposure. Effect modification cannot completely be ruled out, however, since co-exposures are mostly other aromatic amines that share common metabolic activation pathways.

In contrast to these studies, no association was found among U.S. dye workers who were involved in thioindigo manufacturing processes (Ott and Langner 1983); however, the study had low statistical power to detect an effect; only 8 cancer deaths (from all cancers) were expected in the subcohort, only 1.2 cases of urinary cancer were expected in the entire cohort of dye workers, and approximately 77% of workers had short-term employment (of 4 years or less). Thus, this study only had statistical power to detect large risk estimates. In addition, it is not known how many of the 117 workers were potentially exposed to *ortho*-toluidine because the subcohort also included workers who manufactured bromindigo dyes, who presumably were not exposed to *ortho*-toluidine. In summary, no firm conclusions can be drawn from the absence of observed urinary bladder cancer deaths in this cohort.

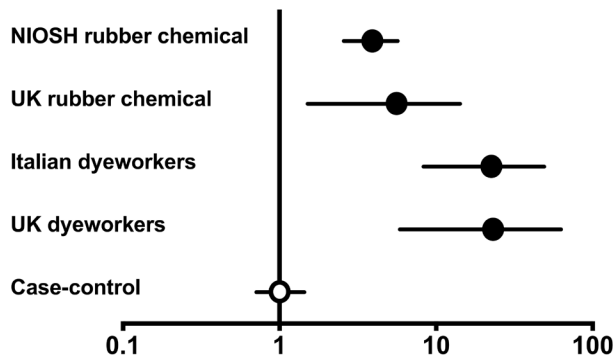
No association between *ortho*-toluidine and urinary bladder cancer was observed in the Canadian case-control study. However, the study had limited ability to detect an effect because of a limited exposure assessment and rarity of exposure to *ortho*-toluidine in this study population.

#### **3.4.2. Synthesis of Evidence across the Body of Studies**

Overall, there is credible evidence of an association between increased urinary bladder cancer risk and exposure to *ortho*-toluidine based on consistent findings across studies, the presence of exposure-response relationships (with cumulative rank exposure and employment duration) and large magnitudes of effect across studies.

An increased risk of urinary bladder cancer (incidence or mortality) was consistently observed in all four studies with adequate latency that used statistical or other methods capable of detecting an association (Carreón et al. 2014; Case and Pearson 1954; Pira et al. 2010; Sorahan 2008) (see

Figure 3-1). Both rubber chemical cohort studies (NIOSH and U.K.) of urinary bladder cancer incidence reported high and statistically significant increased SIRs of similar magnitude (approximately four- to fivefold) among exposed workers. Urinary bladder cancer mortality findings also point to an increase in urinary bladder cancer risk, although they vary across studies. In the rubber chemical cohorts, a 11-fold significantly elevated mortality risk was observed in the U.K. study; the NIOSH study was inadequate to evaluate mortality because only two deaths were observed in the entire cohort (Prince et al. 2000). These findings are supported by large and statistically significant increases (approximately 22-fold) in urinary bladder cancer mortality observed in two cohorts of magenta dye makers (U.K. and Italian) exposed to *ortho*-toluidine, both of which had a long follow-up period; both of these studies, as noted, have methodological limitations. Two studies did not find an excess of urinary bladder cancer among *ortho*-toluidine-exposed workers; however, the statistical power to detect an effect was low in the U.S. dye workers study (Ott and Langner 1983), and misclassification of exposure is a serious concern in both the U.S. dye workers cohort and the Canadian case-control study (Richardson et al. 2007).



**Figure 3-1. Risk Estimates for Urinary Bladder Cancer**

Risk estimates and 95% confidence intervals for urinary bladder cancer for five of the six studies that were considered in the assessment. SIR reported for the NIOSH and U.K. rubber-chemical cohorts, SMR for the Italian and U.K. dye workers cohort, and OR for the Canadian case-control study. No deaths were observed in the U.S. dye-worker study (Ott and Langner 1983). Solid circles = cohort studies, clear circle = case-control study.

Risks of urinary bladder cancer increased with increasing cumulative exposure level, duration, or time since first exposure to *ortho*-toluidine, which strengthens the arguments for causality. Among NIOSH rubber chemical workers definitely exposed to *ortho*-toluidine, statistically significant, positive exposure-response relationships ( $p_{\text{trends}}$  ranged from 0.037 to  $<0.001$ ) for cumulative exposure rank and urinary bladder incidence were observed in both lagged and unlagged analyses with risks ranging from approximately seven- to eightfold in the highest exposure category for each specific analysis (compared to the lowest exposure category) (Carreón et al. 2014). Risks also increased with exposure duration in both of the rubber chemical cohorts ( $p_{\text{trend}} < 0.001$  for Carreón et al. (2014), and  $p_{\text{trend}} < 0.005$  for Sorahan (2008)). and with time since first exposure in the NIOSH study ( $p_{\text{trend}} < 0.001$ ). (Employment duration was used as a surrogate of exposure in the UK study (Sorahan 2008).)

Limitations of the database are the limited number of methodologically adequate studies, and imprecise risk estimates in many studies because of the small number of urinary bladder cancer deaths or cases. In the updated NIOSH study, the number of exposed cases increased from 13 to

37, which resulted in more precise risk estimates. The findings of increased risks reported in the original report of this cohort (Ward et al. 1991), which have been reconfirmed and strengthened in both the 2010 reanalysis and the 2014 update (Carreón et al. 2014; Carreón et al. 2010), increases the confidence in the body of literature. Finally, the large magnitudes of the risk estimates across studies also reduce concerns that the strong associations between *ortho*-toluidine exposure and increased urinary bladder cancer risk are due to chance.

The next key question is whether the excess risk of urinary bladder cancer observed in these cohort studies can be attributed to exposure to *ortho*-toluidine or other factors. The NIOSH study provides strong evidence that the exposed cases/deaths were actually exposed to *ortho*-toluidine, and based on the authors' documentation and knowledge of the industrial processes, it is reasonable to assume that most, if not all, of the exposed cases or deaths in the other informative studies were exposed to *ortho*-toluidine (Pira et al. 2010; Sorahan 2008). In addition, the large magnitude of the risk estimates helps rule out bias due to exposure misclassification.

Another limitation is the lack of formal methods to evaluate confounding from exposure to smoking or other occupational carcinogens in many of the studies. Smoking can reasonably be ruled out in the NIOSH study, in which information was only available for a subset of workers. The large magnitude of risk estimates also provides high confidence for ruling out smoking across studies.

The most challenging issue is potential confounding from other occupational co-exposures. In the most informative study, the NIOSH cohort of rubber chemical workers, there is substantial evidence that *ortho*-toluidine is the most plausible agent causally related to the observed increase in urinary bladder cancer risk among workers considered to be definitely or probably exposed to *ortho*-toluidine (see above), and these conclusions are supported by the study of U.K. rubber chemical workers. Potential confounding by aniline, the principal common co-exposure in these two studies, can be reasonably ruled out because aniline has not been shown to cause urinary bladder cancer in experimental animals or humans in other epidemiologic studies (Case and Pearson 1954; IARC 1987; Sorahan 2008; USEPA 1994). Also, in the NIOSH cohort, workers were exposed to approximately three times higher levels of *ortho*-toluidine (which is a more potent animal carcinogen) than aniline, according to biomonitoring data reported by Ward et al. (1996). Potential confounding from occupational co-exposures is of greater concern for the two cohorts manufacturing magenta dyes (see above). Magenta manufacturing has been classified by IARC as a known to be carcinogenic to humans based largely on these two studies; however, IARC was unable to identify the specific chemical exposures, which include *ortho*-toluidine, responsible for the increased risk of urinary bladder cancer. In addition, urinary bladder cancer deaths were observed in both subgroups of workers in the Italian dye workers cohort study who were exposed to different process chemicals except for *ortho*-toluidine, strongly suggesting that *ortho*-toluidine is the common causal agent. Finally, the finding of urinary bladder cancer among rubber chemical workers who are not exposed to many of the chemicals found in the dye workers studies argues that *ortho*-toluidine contributes to the excess urinary bladder cancer among dye workers. In addition, there is no independent evidence that the other co-exposures cause urinary bladder cancer in humans, although some are animal carcinogens. The possibility of effect modification may exist and may help explain the observed large risk estimates, however.

Overall, the finding of increased urinary bladder cancer risk in separate cohorts with different exposure conditions and co-exposures lends strong support to the conclusion that *ortho*-toluidine is the common causal risk factor.

### **3.5. NTP's Level of Evidence Recommendation**

There is sufficient evidence of carcinogenicity from studies in humans based on epidemiological studies finding a causal association between increased risk of urinary bladder cancer and exposure to *ortho*-toluidine that is unlikely to be explained by chance, bias, or confounding. An increased risk of urinary bladder cancer was observed across studies of workers with different occupations and in different geographical locations. In addition, risks of urinary bladder cancer increased with increasing level and duration of exposure.



## 4. Studies of Cancer in Experimental Animals

*ortho*-Toluidine has been listed as *reasonably anticipated to be a human carcinogen* in the Report on Carcinogens (RoC) since 1983 based on sufficient evidence of carcinogenicity in experimental animals. This section reviews and assesses carcinogenicity studies in experimental animals exposed to *ortho*-toluidine. The steps in the cancer evaluation process are (1) identifying new studies published since, or not included in the original listing in 1983 (hereafter referred to as “new” studies) (Section 4.1), (2) assessing the quality of all studies (Section 4.2), (3) synthesizing the findings across studies (Section 4.3), and (4) reaching a preliminary conclusion on the level of evidence of carcinogenicity of *ortho*-toluidine from these studies (Section 4.4).

### 4.1. Identification and Overview of the Studies

The studies that formed the basis for the 1983 listing of *ortho*-toluidine in the RoC include (1) two feed studies in different strains of rats, one in male CD rats (Weisburger et al. 1978) and the other in male and female F344 rats (NCI 1979), and (2) two feed studies in different strains (Albino CD-1 [HaM/ICR] and B6C3F<sub>1</sub>) of male and female mice (NCI 1979; Weisburger et al. 1978) (see Table 4-1 for a brief overview of these studies). All of these feeding studies were two-year carcinogenicity studies that used two different exposure groups in addition to untreated controls. Rats and mice were exposed in the diet for two years in the NCI studies and for 18 months in the studies reported by Weisburger and colleagues (see Table 4-1).

Cancer studies in experimental animals published since the review (or not included in the review) were identified by searching databases, comprehensive reviews, and citations from studies retrieved from the literature searches as described in Appendix A. Five publications published after the 1983 evaluation met the inclusion/exclusion criteria requiring that included studies evaluated exposure specifically to *ortho*-toluidine and either (1) report neoplastic lesions, or non-neoplastic or preneoplastic lesions relevant to carcinogenicity, or (2) are subchronic studies that provide information on dose selection (see Appendix A). These publications reported on seven studies including (1) three feed studies, two in male rats (Hecht et al. 1982; NCI 1979), and one in dogs (Pliss 2004), (2) three parenteral studies, all by subcutaneous injection, in rats (Pliss 2004), mice (Pliss 2004), and hamsters (Hecht et al. 1983), (3) and one tumor initiation study in genetically altered fish (Anders et al. 1991). An additional feed study (Takayama et al. 1989) in rats was identified in the literature search but was excluded from the review because exposure was to a mixture of 39 chemicals including *ortho*-toluidine hydrochloride.

All of the studies in rodents published since 1983 evaluated exposed animals to only one dose of *ortho*-toluidine. In the feeding study reported by Hecht et al. (1983) female rats were exposed to *ortho*-toluidine in the diet for 73 weeks and held for a total of 93 weeks. NTP (1996) reported on short-term exposure experiments using three different conditions: 13-week continuous exposure, 13-week exposure and held for a total of 26 weeks, and 26-week continuous exposure (NTP 1996). Five dogs (between 2 and 3 years) were exposed to *ortho*-toluidine, first in the food and later by gavage, for up to 9 years in the study reported by Pliss (2004); however, there were no untreated controls and two dogs died of non-cancer related deaths within three years of the study onset.

The subcutaneous studies in mice and rats were two-year carcinogenicity studies with animals receiving weekly subcutaneous injections for the entire study duration (Pliss 2004). The study in hamsters was shorter; the animals were injected weekly for 52 weeks and observed up to 87 weeks (Hecht et al. 1983).

The study by Anders et al. (1991) reported on melanomas (external evaluation) in genetically modified fish dosed for 4 months.

**Table 4-1. Overview of Studies of Exposure to *ortho*-Toluidine in Experimental Animals**

Route of Exposure	Species (Sex)	Exposure/Observation Duration	Reference
<b>Studies included in the 1983 evaluation<sup>a</sup></b>			
Feed	F344 rat (male and female)	101–104 wk	NCI (1979)
Feed	CD rat (male)	18 mo/24 mo	Weisburger et al. (1978)
Feed	B6C3F <sub>1</sub> mouse (male and female)	101–104 wk	NCI (1979)
Feed	Albino CD-1 (HaM/ICR) mouse (male and female)]	18 mo/24 mo	Weisburger et al. (1978)
<b>Studies published after (or not included) in the 1983 evaluation<sup>b</sup></b>			
Feed	F344 rat (male)	73 wk/93 wk	Hecht et al. (1982)
Feed	F344 rat (male)	13 wk/26 wk	NTP (1996)
Feed	Dog (female) <sup>c</sup>	9 yr	Pliss (2004)
Subcutaneous	Syrian golden hamster (male)	52 wk/87 wk	Hecht et al. (1983)
Subcutaneous	Rat (male and female) <sup>c</sup>	2 yr	Pliss (2004)
Subcutaneous	CC <sub>57</sub> BR mice (male and female)	2 yr	Pliss (2004)
<i>Per os</i>	Genetically altered fish	4 wk	Anders et al. (1991)

<sup>a</sup>All studies for the 1983 evaluation tested *ortho*-toluidine at two dose levels.

<sup>b</sup>All studies listed below tested *ortho*-toluidine at only one dose level.

<sup>c</sup>Strain or breed not specified.

## 4.2. Quality Assessment of Studies

The eleven studies, including the seven published after 1983 and four studies included in the previous evaluation, were systematically evaluated in a two-step process. The first step was to evaluate whether the level of detail reported for key elements of study design, experimental procedures, and carcinogenesis endpoints was adequate for evaluating its quality and interpreting its results. The reporting quality of key elements for 2 of the 11 studies was considered to be inadequate for cancer evaluation: the study in mice exposed to *ortho*-toluidine by subcutaneous injection (Pliss 2004) and the tumor initiation study in genetically altered fish (Anders et al. 1991).

The second step was to evaluate the nine remaining studies, which were adequately reported, for concerns for study quality, using a priori criteria, which might negatively impact the ability to assess carcinogenicity. Key factors considered in the quality assessment include characterization of the chemistry of the substance, dosing regimen, exposure and observation period, number of

animals per exposure group, monitoring of animal health, and assessment for neoplasm endpoints. Details of each study assessment and quality criteria are reported in Appendix E.

Eight of the remaining nine studies—the four feed studies in rats (Hecht et al. 1982; NCI 1979; NTP 1996; Weisburger et al. 1978), two feed studies in mice (NCI 1979; Weisburger et al. 1978), two subcutaneous studies, one in hamsters (Hecht et al. 1983) and one in rats (Pliss 2004)—were considered to have adequate quality to inform the carcinogenicity assessment. The feeding studies in rats and mice were considered to be more informative (e.g., had minimal concerns for most study quality elements) (Hecht et al. 1982; NTP 1996) than the two subcutaneous studies in rodents (Hecht et al. 1983; Pliss 2004). One of the feeding studies in rats, the NTP feed study in male rats, was a subchronic study, which by virtue of its design (e.g., short exposure and duration period) would usually not be considered able to detect tumors, due to the long latency period usually required for tumor development. Despite such limitations, preneoplastic and neoplastic findings were reported, and the study was included in the cancer assessment. One of the remaining nine studies, a feeding study in dogs, was considered to be inadequate to inform the cancer evaluation, primarily because no untreated control group of dogs was tested and there were also concerns about other study elements as described in Appendix E.

Overall, the studies in rodents provide an adequate database for evaluating the carcinogenicity of *ortho*-toluidine. Although some limitations in study design or reporting were observed in some studies, most of the limitations would bias towards not finding an effect (type-I error) and would not necessarily bias towards finding a “false” positive effect (type-II error).

### 4.3. Assessment of Neoplastic Findings

The studies in rats and mice previously evaluated for the 1983 listing of *ortho*-toluidine in the RoC provided sufficient evidence for its carcinogenicity based on tumors of the connective tissue (sarcoma) in the spleen and other organs in both sexes of rats, urinary bladder in female rats, mesothelium and subcutaneous tissue in male rats, blood vessels in both sexes of mice, and liver in female mice. This section integrates the findings across the new (two feeding studies in male F344 rats and two subcutaneous studies), and earlier studies (two feeding studies each in rats and mice). Findings from the four feeding studies in rats are reported in Table 4-2 (urinary bladder neoplasms), Table 4-3 (sarcoma), and Table 4-4 (other neoplasms); the two feeding studies in mice are reported in Table 4-5, and the two subcutaneous injection studies in rodents in Table 4-6. All studies are discussed below.

#### 4.3.1. Feed Studies: Rats

The new feeding studies also support the conclusions of the original listing that dietary exposure to *ortho*-toluidine causes neoplasms of the urinary bladder, connective tissue, subcutaneous tissue, mammary gland, and mesothelium in rats. These conclusions are based on studies that are considered to be of adequate quality to inform a cancer evaluation and thus increase the confidence in the conclusions. Strengths of the studies were lifetime exposure to doses approaching toxicity and performance of complete histopathological examination of all major organs. The Hecht study, which is a “new” study, was conducted in the same strain of male rats as one of the original studies (the NCI study) and, in general, confirmed the findings of the NCI study. The other “new” feeding study, the NTP subchronic study, was informative for the evaluation of neoplasms of the mesothelium. The new studies also provide some support that

*ortho*-toluidine causes urinary bladder neoplasms and mammary tumors in male rats in addition to females. Some types of neoplasms were observed in both male and female rats (urinary bladder, connective tissue, mammary gland), whereas other neoplasms may be sex specific (subcutaneous tissue and mesothelium). Some types of neoplasms may be strain specific (connective tissue, mesothelium, and mammary gland), although differences in study design (e.g., shorter exposure and observation period in the CD rat study compared with the F344 rat study) may also account for the differences in findings. Details on the findings of these tumor sites that support these conclusions are discussed below.

Urinary bladder cancer is a potential target site in human cancer studies. The collective data from the four feeding studies in experimental animals provide strong evidence that *ortho*-toluidine exposure causes urinary bladder neoplasms in female rats (a conclusion of the 1983 evaluation), with weaker evidence observed for male rats (Table 4-2) (conclusion supported by the new studies). In female F344 rats, dietary exposure to *ortho*-toluidine caused a dose-related statistically significant increased incidence of urinary bladder cancer (transitional-cell carcinoma) in both exposure groups with the incidence reaching 47% in the high-exposure group. This was considered to be a robust finding because spontaneous urinary bladder neoplasms in rats are rare; no carcinomas were observed in 989 female F344 rats from 20 feed studies (NIH-07 diet, which includes data up to 1999). In addition, the time to first observed tumor was shorter in high-dose females (65 weeks) compared with low-dose females (103 weeks). In male rats, dietary exposure to *ortho*-toluidine induced low incidences of urinary bladder neoplasms (statistically non-significant) in two strains of rats. Similar to females, spontaneous urinary bladder neoplasms are rare in both male F344 and CD rats: (1) 3 neoplasms (carcinoma or papilloma) and 1 carcinoma were observed in 991 rats from 20 studies using NIH-07 diet of F344 rats, and (2) 6 urothelial (transitional-cell) papilloma or carcinoma were observed in 30 studies of Charles River Crl:CD rats published between 1989 and 2002. The results in the chronic studies in rats are supported by the findings of a statistically significant increased incidence of transitional-cell hyperplasia of the urinary bladder in F344 male rats in the NTP subchronic study (13-week interim study and 26-week exposure study). NCI noted that there appeared to be a dose-related progression, especially in female rats, from transitional-cell hyperplasia to transitional-cell carcinoma of the urinary bladder.

**Table 4-2. Studies of Dietary Exposure to *ortho*-Toluidine in Rats: Urinary Bladder Hyperplasia and Neoplasm Incidence**

Reference Strain, Sex Study Duration	Exposure, ppm (# Rats)	Hyperplasia	Papilloma	Carcinoma	Carcinoma or Papilloma (Combined)	Comments
Weisburger et al. (1978)	0 (111) <sup>a</sup> 0 (25) <sup>b</sup>	–	–	5/111 (4.5) 0/16 <sup>d</sup>	–	<i>o</i> -Toluidine HCl (97%–99% pure). Survival similar between concurrent controls and exposed groups (16/25, 23/25, 24/25).
CD [Sprague-Dawley], Male 18 mo exposed (24 mo total)	8,000–4,000 (25) <sup>c</sup> 16,000–8,000 (25) <sup>c</sup>			3/23 (13) 4/24 (22)		Food consumption not reported for individual animals.
NCI (1979) F344, Male 101–104 wk	0 (20) 3,000 (50) 6,000 (50) Trend <sup>e</sup>	0/20 9/50 (18) 7/44 (16) NR	–	0/20 3/50 (6) 0/44 (0) p > 0.05	0/20 3/50 (6) 1/44 (2) p > 0.05	Technical grade (>99% pure, with one contaminant <1%). Body weights of both sexes of exposed rats lower than controls and dose-related.
NCI (1979) F344, Female 101–104 wk	0 (20) 3,000 (50) 6,000 (50) Trend <sup>e</sup>	0/20 21/45 (47) <sup>***</sup> 13/47 (28) <sup>**</sup> NR	–	0/20 9/45 (20)* 22/47 (47) <sup>***</sup> p < 0.001	0/20 10/45 (22)* 22/47 (47) <sup>***</sup> p < 0.001	Dose-related trends in mortality observed in both sexes; steep increases in mortality occurred after 70 wk in high-dose males, 75 wk in low-dose males, 80 wk in high-dose females and 90 wk in low-dose females. Food consumption not reported for individual animals.
Hecht et al. (1982) F344, Male 72-wk exposure (93 wk total)	0 (30) 4,000 <sup>f</sup> (30)	–	0/27 3/30	0/27 1/30	–	<i>o</i> -Toluidine HCl (purified, but purity not reported). Survival at week 22 was 18/30 for controls and 6/30 for exposed. Body weight of exposed reported as “somewhat lower than control group.”
NTP (1996) F344, Male						<i>o</i> -Toluidine HCl (100% pure). Survival 100% in all groups.

RoC Monograph on *ortho*-Toluidine

Reference Strain, Sex Study Duration	Exposure, ppm (# Rats)	Hyperplasia	Papilloma	Carcinoma	Carcinoma or Papilloma (Combined)	Comments
13-wk interim sacrifice	0 (10) 5,000 <sup>h</sup> (20)	0/10 10/20**	–	–	–	Mean body weights were 82%–89% lower in exposed groups than control group.
13-wk stop exposure <sup>g</sup>	0 (10), 5,000 <sup>h</sup> (20)	0/10 0/20	–	–	–	
26-wk continuous exposure	0 (10) 5,000 <sup>h</sup> (20)	0/10 17/18**	–	–	–	

\*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001 (compared with concurrent controls by Fisher's exact test).

<sup>a</sup>Pooled controls, controls from all compounds tested in study.

<sup>b</sup>Concurrent controls.

<sup>c</sup>Body weights in exposed groups <10% of concurrent control weights by 12 wk; doses reduced for remaining 60 wk. Low dose reduced from 8,000 to 4,000 mg/kg food, and high dose reduced from 16,000 to 8,000 mg/kg food.

<sup>d</sup>Six cases of subcutaneous fibroma or fibrosarcoma occurred along with transitional-cell carcinoma of the male rat urinary bladder.

<sup>e</sup>Trend calculated using Cochran-Armitage trend test.

<sup>f</sup>Mean Daily Dose: 62 mg/rat; Total dose of *o*-toluidine HCl: 31.3 g/rat; Average exposure of *o*-toluidine HCl per body weight per day of animals not reported.

<sup>g</sup>Rats exposed for 13 wk and observed for another 13 wk.

<sup>h</sup>Exposure (mg/kg/d) = 301 for 13-wk interim sacrifice, 304 for 13-wk stop exposure, and 285 for 26-wk continuous exposure.

Increased incidences of sarcoma in the spleen and other tissue sites were observed in rats of both sexes (NCI 1979) and in male rats in two different studies (Hecht et al. 1982; NCI 1979) (see Table 4-3). In the NCI study, *ortho*-toluidine exposure caused significant dose-related increased incidences of (1) multiple types of sarcoma (fibrosarcoma, angiosarcoma, osteosarcoma, and sarcoma NOS [not otherwise specified]) in the spleen and other abdominal organs in rats of both sexes, (2) specific sarcomas in multiple organs (fibrosarcoma in males, and osteosarcoma in females), and (3) multiple types of sarcoma and angiosarcoma in the spleen of females. The NCI (1979) noted that the histologic appearance of each type of sarcoma was similar regardless of the organs involved; they were of mesenchymal origin, closely related, and poorly differentiated. There was also a high incidence of invasion into adjacent organs and soft tissues and/or metastasis to other sites, suggesting that they were highly malignant. In the Hecht study, statistically significant increased incidences of sarcoma in the peritoneum and fibroma (benign neoplasm) in the spleen occurred in male F344 rats. Sarcoma in the spleen is a rare tumor in F344 rats; the historical control data for untreated rats from NTP oral studies using NIH-07 diet (which was also the diet used in the Hecht study) ranged from 0% to 0.4% (based on 1,003 tumors) for males and 0% to 0.1% in females depending on the specific type of sarcoma (<http://ntp.niehs.nih.gov/go/15482>).

Increased incidences of mesothelioma (tunica vaginalis or abdominal cavity and organs) were observed in males in all three studies of F344 rats but were only statistically significant in the NCI chronic study (see Table 4-4). In the NCI chronic study, the incidence of mesothelioma (multiple organs/tunica vaginalis) was higher (34%) in the low-dose exposure group than the high-dose exposure group (18%) and most of the mesotheliomas were located in the tunica vaginalis. The historical control rate for mesothelioma for the study laboratory in the NCI study was reported to be 0.4% (1/285). A small, but not statistically significant, excess of malignant mesothelioma (located in the peritoneal cavity) was observed in the exposed male F344 rats (16.6%) compared with the controls (7.4%) in the Hecht study, and thus the finding in this study is weaker than in the NCI study. The NTP subchronic study provides supporting evidence that dietary exposure to *ortho*-toluidine causes mesothelioma; malignant mesothelioma and mesothelial hyperplasia of the epididymides occurred in male rats exposed in the diet for only 13 weeks and held for an additional 13 weeks (stop-exposure). Although the findings were not statistically significant they are considered to be of biological significance because of the short exposure and observation duration (NTP 1996).

**Table 4-3. Studies of Dietary Exposure to *ortho*-Toluidine in Rats: Sarcomas**

Reference Strain, Sex Study Duration	Exposure, ppm (# Rats)	Multiple Tissues (Multiple Types)	Multiple Tissues (Specific Types)	Spleen (Multiple Types)	Spleen (Specific Types)	Comments <sup>a</sup>
Weisburger et al. (1978)	0 (111) <sup>b</sup> 0 (25) <sup>c</sup>	Not reported	Not reported	Not reported	Not reported	–
CD (Sprague-Dawley), male 18 mo exposed (24 mo total)	8,000–4,000 (25) <sup>d</sup> 16,000–8,000 (25) <sup>d</sup>					
NCI (1979) F344, male 101–104 wk	0 (20) 3,000 (50) 6,000 (50) Trend <sup>e</sup>	0/20 15/50 (30)** 37/49 (76)*** p < 0.001	<i>Fibrosarcoma</i> 0/20 8/50 20/49*** p < 0.001	0/20 8/49 (16) 4/42 (10) p > 0.05	<i>Fibroma</i> 0/20 10/49 (20)* 2/42 (5) p > 0.05	Multiple types of poorly differentiated sarcoma primarily include sarcoma, NOS (not otherwise specified), fibrosarcoma, angiosarcoma, or osteosarcoma in multiple organs or spleen
	0 (20) 3,000 (50) 6,000 (50) Trend <sup>e</sup>		<i>Sarcoma NOS</i> 0/20 3/50 (6) 11/49 (22)* p = 0.003			
NCI (1979) F344, female 101–104 wk	0 (20) 3,000 (50) 6,000 (50) Trend <sup>e</sup>	0/20 3/50 (6) 21/49 (43)*** p < 0.001	<i>Osteosarcoma</i> 0/20 0/50 18/49 (37)** p < 0.001	0/20 9/49 (18)* 2/49 (24)** p = 0.018	<i>Angiosarcoma</i> 0/20 7/49 (14) 9/49 (18)* p = 0.045	Multiple types: sarcoma, NOS, angiosarcoma or osteosarcoma
Hecht et al. (1982) F344, male 72 wk exp. (93 wk total)	0 (30) 4,000 (30) <sup>f</sup>	–	<i>Peritoneal cavity: sarcoma</i> 0/27 9/30 (30)**	–	<i>Fibroma</i> 0/27 10/30 (33)***	–



RoC Monograph on *ortho*-Toluidine

Reference Strain, Sex Study Duration	Exposure, ppm (# Rats)	Multiple Tissues (Multiple Types)	Multiple Tissues (Specific Types)	Spleen (Multiple Types)	Spleen (Specific Types)	Comments <sup>a</sup>
NTP (1996) F344, male 13-wk interim sacrifice; 13-wk stop exposure <sup>g</sup> ; 26-wk continuous exposure	0 (10) 5,000 (20)	None observed under any conditions	None observed under any conditions	None observed under any conditions	None observed under any conditions	–

\*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001 (compared with concurrent controls by Fisher's exact test).

<sup>a</sup>See Table 4-2 for information on purity, survival, body weight, or feed consumption.

<sup>b</sup>Pooled controls, controls from all compounds tested in study.

<sup>c</sup>Concurrent controls.

<sup>d</sup>Body weights in exposed groups <10% of concurrent control weights by 12 wk; doses reduced for remaining 60 wk. Low dose reduced from 8,000 to 4,000 mg/kg food, and the high dose reduced from 16,000 to 8,000 mg/kg food.

<sup>e</sup>Trend calculated using the Cochran-Armitage trend test.

<sup>f</sup>Mean daily dose: 62 mg/rat; Total dose of *o*-toluidine HCl: 31.3 g/rat; average exposure of *o*-toluidine HCl per body weight per day of animals not reported.

<sup>g</sup>Rats exposed for 13 weeks and observed for another 13 weeks.

Fibroma or fibrosarcoma of the subcutaneous tissues was observed in all three chronic feed studies (Hecht et al. 1982; NCI 1979; Weisburger et al. 1978) including two different strains of male rats (CD and F344). In male CD rats, statistically significant increased incidences of fibroma or fibrosarcoma were observed in both the low- and high-dose groups, but no further pathological information or stratification of incidence by type of neoplasm was provided (Weisburger et al. 1978). Increased incidences of fibroma were observed in male F344 rats in both the low- and high-dose groups in the NCI study; in addition to fibroma, a few osteosarcoma, myxosarcoma, fibrosarcoma, and unspecified sarcoma were also observed. The Hecht study reported a high incidence (83%) of fibromas occurring in the skin (presumably the subcutaneous tissue of the skin) in male F344 rats at the only dose tested.

Statistically significant increased incidences of fibroadenoma of the mammary gland were found in male F344 rats in the Hecht et al. (1982) study and in high-dose female F344 rats in the NCI (1979) study; a non-statistically significant excess of fibroadenoma was found in the low-exposure group males in the NCI (1979) chronic study. Fibroma and fibroadenoma of the mammary gland are benign, well-localized neoplasms with few mitotic figures. Some fibromas may actually be part of a fibroadenoma of mammary gland because the histological section reviewed contains only the fibrous components. In the Hecht study, two adenocarcinomas of the mammary gland were also observed. Occasionally, adenocarcinoma has been observed arising from within a fibroadenoma or there is a chemically induced increase suggesting that fibroadenoma sometimes progresses to adenocarcinoma (Boorman et al. 1990); however, no information on histogenesis was reported in the Hecht study. Mammary gland adenocarcinoma is rare in the male rat in F344 rats NTP studies (2/1,004 or 0.2% according to historical control data for NTP studies using the NIH-07 diet), which is the same strain used in the Hecht study.

**Table 4-4. Studies of Dietary Exposure to *ortho*-Toluidine in Rats: Mesothelium, Subcutaneous Tissue and Mammary Gland Neoplasm Incidence (%)**

Reference Strain, Sex Study Duration	Exposure, ppm (# Rats)	Mesothelium: Various Body Cavities <i>Mesothelioma</i>		Subcutaneous Tissue <i>Fibroma, Fibrosarcoma</i>	Mammary Gland <i>Fibroadenoma Adenocarcinoma</i>	Comments
Weisburger et al. (1978) CD [Sprague-Dawley], male 18-mo exposed (24 mo total)	0 (111) <sup>a</sup> 0 (25) <sup>b</sup> 8,000–4,000 (25) <sup>c</sup> 16,000–8,000 (25) <sup>c</sup>	–	–	<i>Fibroma and fibrosarcoma</i> 18/111 (16) 0/16 18/23 (78) [***] 21/24 (88) [***]	–	See Table 4-2 for information on purity, survival, and feed consumption
NCI (1979) F344, male 101–104 wk	0 (20) 3,000 (50) 6,000 (50) Trend <sup>d</sup>	<i>Tunica vaginalis</i> 0/20 10/50 (20) [*] 17/50 (34)*** 6/49 (12) NR	<i>Multiple organ</i> <sup>e</sup> 0/20 9/49 (18)* p > 0.05	<i>Fibroma</i> <sup>f</sup> 0/20 28/50(56)*** 27/49(55)*** p = 0.001	<i>Fibroadenoma</i> 0/20 7/50 (14) 1/49 (2) p > 0.05	See Table 4-2 for information on purity, survival, body weight, and feed consumption
NCI (1979) F344, female 101–104 wk	0 (20) 3,000 (50) 6,000 (50) Trend <sup>d</sup>	–	–	<i>Fibroma</i> 0/20 4/50 (8) 2/49 (4) p > 0.05	<i>Fibroadenoma</i> 6/20 20/50 (40) 35/49 (71)** p < 0.001	
Hecht et al. (1982) F344, male 72 wk exp. (93 wk total)	0 (30) 4,000 (30) <sup>g</sup>  0 (30) 4,000 <sup>g</sup>	<i>Peritoneal</i> <sup>h</sup> 2/27 (7) 5/30 (17)	–	<i>Fibroma</i> 1/27 (3) 25/30 (83)***	<i>Fibroadenoma</i> 0/27 11/30 (37)***  <i>Adenocarcinoma</i> <sup>i</sup> 0/27 2/30 (7)	See Table 4-2 for information on purity, survival, body weight, and feed consumption

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Reference Strain, Sex Study Duration	Exposure, ppm (# Rats)	Mesothelium: Various Body Cavities <i>Mesothelioma</i>	Subcutaneous Tissue <i>Fibroma, Fibrosarcoma</i>	Mammary Gland <i>Fibroadenoma Adenocarcinoma</i>	Comments
NTP (1996) F344, male 13-wk stop exposure <sup>j</sup>	0 (10) 5,000 (20) <sup>j</sup>	<i>Epididymis</i> – 0/10 2/20 (10) <sup>k</sup> No tumors in 13-wk interim sacrifice or 26-wk continuous exposure studies	–	–	See Table 4-2 for information on purity, survival, body weight, and feed consumption 2/20 mesothelial hyperplasia of the epididymis in stop-exposure study

\*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001 (compared with concurrent controls by Fisher's exact test).

[ ] = Statistical significance calculated by NTP using Fisher's exact test.

<sup>a</sup>Pooled controls, controls from all compounds tested in study.

<sup>b</sup>Concurrent controls.

<sup>c</sup>Body weights in exposed groups <10% of concurrent control weights by 12 wk; doses reduced for remaining 60 wk. Low dose reduced from 8,000 to 4,000 mg/kg food, and the high dose reduced from 16,000 to 8,000 mg/kg food.

<sup>d</sup>Trend calculated using the Cochran-Armitage trend test.

<sup>e</sup>Mesothelioma included malignant mesothelioma and mesothelioma NOS in tunica vaginalis and in other organs in the abdominal cavity.

<sup>f</sup>Malignant subcutaneous tumors including osteosarcoma, myxosarcoma, fibrosarcoma, sarcoma NOS were also observed in exposed males.

<sup>g</sup>Mean daily dose: 62 mg/rat; Total dose of *o*-toluidine HCl: 31.3 g/rat; average exposure of *o*-toluidine HCl per body weight per day of animals not reported.

<sup>h</sup>Located in muscular wall and organs of abdominal cavity.

<sup>i</sup>Fibroadenoma and adenocarcinoma combined – Control: 0/27, 4,000: 13/30 (43) [\*\*\*].

<sup>j</sup>Rats exposed for 13 weeks and held for another 13 weeks.

<sup>k</sup>Exposure = [304 mg/kg/d].

#### 4.3.2. Feed Studies: Mice

No new studies with adequate design and reporting were identified in mice, and the conclusions, based on the two feed studies in two different strains of mice (NCI (1979), B6C3F<sub>1</sub> strain; Weisburger et al. (1978), Albino CD-1 (HaM/ICR) strain) are the same as the 1983 evaluation. These studies were considered to be informative for cancer evaluation because they exposed animals to doses approaching toxicity for close to their lifetime and conducted complete histopathological examination on all major organs. Neoplasms of the blood vessels (hemangioma and hemangiosarcoma combined) were observed in both studies and liver cancer was observed in one study (Table 4-5). In the NCI study, a significant dose-related trend was observed in male but not female B6C3F<sub>1</sub> mice; most of the neoplasms observed in both the low and high doses were malignant neoplasms (hemangiosarcoma). Although the increases in incidences of the blood vessel neoplasms in male B6C3F<sub>1</sub> mice were not statistically significant, they were considered exposure related because they were outside the historical control range (hemangioma and hemangiosarcoma combined). The increased incidences of blood vessel neoplasms in male and female Albino CD-1 (HaM/ICR) mice were statistically significant in both the low- and high-dose groups; these doses were much higher than the dose used in the NCI study. Statistically significant dose-related increases in liver neoplasms (hepatocellular carcinoma and adenoma combined) were also observed in *ortho*-toluidine exposed B6C3F<sub>1</sub> females but not in Albino CD-1 (HaM/ICR) females; most of the neoplasms observed were carcinomas. This finding is consistent with other aromatic amines, as they often induce liver neoplasms in rodent studies.

#### 4.3.3. Subcutaneous Injection Studies in Rodents

No evidence of carcinogenicity was observed in studies of rats and hamsters administered *ortho*-toluidine by subcutaneous injection; however, the data are inadequate to conclude whether these negative findings are due to study design issues, species differences or route of exposure (see Table 4-6). No increases in specific neoplasm incidences were found in any of the studies. The incidence of all neoplasms in rats was reported to be statistically higher than in unexposed controls, but these findings have little biological meaning. As discussed in Section 4.1, these studies were considered to have limited ability to inform a cancer evaluation, and most of the limitations (e.g., less than lifetime exposure and observation duration, smaller numbers of animals, and uncertainty about whether necropsies were completed on all major organs) would decrease the sensitivity for detecting a neoplastic response.

**Table 4-5. Summary of Dietary *ortho*-Toluidine Hydrochloride Studies in Mice: Neoplasm Incidence (%)**

Reference Strain; Sex Study Duration	Doses: ppm, (# Animals)	Blood Vessels Hemangiosarcoma or Hemangioma	Liver Hepatocellular Adenoma or Carcinoma	Comments		
Weisburger et al. (1978)	0 (99) <sup>a</sup>	–	–	<i>ortho</i> -Toluidine hydrochloride (97%–99% pure)  Survival was similar between concurrent controls and exposed groups (Males: 14/25, 14/25, 11/25; Females: 15/25, 18/25, 21/25).		
Albino CD-1 (HaM/ICR; male)	0 (25) <sup>b</sup>	<i>Combined</i>	<i>Combined</i>			
	16,000–8,000 (25) <sup>c</sup>	5/99 (5)	0/14 (0)			
	32,000–16,000 (25) <sup>c</sup>	5/14 (36)*	5/14 (36)*			
18 mo exp/24 mo total		9/11 (82)*				
Weisburger et al. (1978)	0 (102) <sup>a</sup>	–	–	Body weights decreased by >10% of concurrent control weights at 12 wk.		
Albino CD-1 (HaM/ICR); female	0 (25) <sup>b</sup>	<i>Combined</i>	<i>Combined</i>			
	16,000–8,000 (25) <sup>c</sup>	9/102 (8.8)	0/15 (0)			
	32,000–16,000 (25) <sup>c</sup>	5/18 (28)*	5/18 (28)*			
18 mo exp/24 mo total		9/21 (43)*				
NCI (1979)		<i>Hemangio-sarcoma</i>	<i>Combined Neoplasms</i> <sup>e</sup>	<i>Carcinoma</i>	<i>Combined</i>	<i>o</i> -Toluidine hydrochloride (Technical grade, >99% pure, one contaminant <1%)  No significant dose-related trends were found for mortality.  Body weights were lower in exposed mice of both sexes and were dose related.
B6C3F <sub>1</sub> ; male	0 (20)	1/19 (5)	1/19 (5%) <sup>f</sup>	4/19 (21)	5/19 (26)	
2 yr (101–104 wk)	1,000 (50)	1/50 (2)	2/50 (4%)	16/50 (32)	19/50 (38)	
	3,000 (50)	10/50 (20)	12/50 (24%)	11/50 (22)	14/50 (28)	
	Trend <sup>d</sup>	p = 0.004	p = 0.002	p > 0.05	p > 0.05	
NCI (1979)		–	<i>Combined</i>	<i>Carcinoma</i>	<i>Combined</i>	Food consumption not reported for individual animals.
B6C3F <sub>1</sub> ; female	0 (20)		1/20 (5)	0/20	0/20 (0) <sup>g</sup>	
2 yr (101–104 wks)	1,000 (50)		1/49 (2)	2/49 (4)	4/49 (8)	
	3,000 (50)		3/50 (6)	7/15 (14)	13/50 (26)**	
	Trend <sup>d</sup>		p > 0.05	p = 0.015	p = 0.001	

\*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001 (compared with concurrent controls by Fisher's exact test).

<sup>a</sup>Pooled controls, controls from all compounds tested in study.

<sup>b</sup>Concurrent controls.

<sup>c</sup>Body weights in exposed groups decreased to <10% of concurrent control weights by 12 wk; doses reduced for remaining 60 wk. Low dose was reduced from 16,000 to 8,000 mg/kg food, and the high dose from 32,000 to 16,000 mg/kg food.

<sup>d</sup>Trend calculated using Cochran-Armitage trend test.

<sup>e</sup>Neoplasms of the blood vessels were predominantly located in either the periuterine or periepididymal fat in the abdominal viscera.

<sup>f</sup>Incidence in historical control male mice is 4% for hemangiosarcoma and 5% for hemangiosarcoma and hemangioma combined; the probability (in 50 animals) is <0.001 for obtaining 10 or more hemangiosarcoma and 12 or more combined neoplasms.

<sup>g</sup>Incidence of hepatocellular neoplasms in historical control female mice is 4.3% (range 0% to 11%).

**Table 4-6. Summary of Neoplasm Incidence in Subcutaneously Injected *ortho*-Toluidine Studies in Rats and Hamsters**

Reference Species (Strain; Sex Study Duration	Exposure Interval and Period Dose (# of Animals)	Neoplasm Incidence (%) or Findings	Comments
Pliss (2004) Rat (strain/source not reported); Male and female Up to 2 yr	1 d/wk, 18–24 mo <u>mg/injection</u> 0 (25 Male/25 Female) 30 (25 Male/25 Female ) Total maximum dose/animal: 3.25 g [estimated dose at study start: 300–375 mg/kg bw]	<u>Combined neoplasm<sup>a,b</sup></u> 4/38 (10.5) 12/30 (40)** Individual neoplasms not reported.	<i>o</i> -Toluidine (99.7% pure, with 0.2% <i>p</i> -toluidine) Survival of exposed rats (30/50) at time of first neoplasm (19.6 mo) in exposed rats was lower than controls (38/50) at time of first neoplasm (23 mo). Neoplasm incidences were not reported by sex or by tissue type.
Hecht et al. (1983) Hamster (Syrian Golden); Male 87 wk	1 d/wk, 52 wk <u>mmol [mg]/kg/wk</u> 0 (15) 1.9 [203] (15) Total maximum dose/animal: 99 mmol/kg [10.6 g/kg bw]	No statistically significant increase in neoplasm incidence in exposed hamsters compared with controls. Neoplasm incidence data not reported.	<i>o</i> -Toluidine pure as analyzed by TLC and gas chromatography; details not provided. Mean survival time (wk) in exposed hamsters (males 61.3) and females 57.8) were lower than controls (males 75.5, females 68.7).

\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  (compared with concurrent controls by Fisher's exact test).

bw = body weight.

<sup>a</sup>Neoplasms in control rats included 2 mammary fibroadenomas, 2 leucoses.

<sup>b</sup>Neoplasms in exposed rats included: 4 mammary fibroadenomas, 2 sarcomas and fibromas at the injection site, and 1 hepatic sarcoma, 1 renal plasmocytoma, 1 testicular lymphohemangioma, and 3 leucoses.

#### **4.4. NTP's Level of Evidence Recommendation**

There is significant evidence for the carcinogenicity of *ortho*-toluidine from studies in experimental animals based on increased incidences of malignant or combined benign and malignant tumors in rats and mice at multiple tissue sites. Dietary exposure to *o*-toluidine caused tumors of the urinary bladder and connective tissue (sarcoma) in rats of both sexes, subcutaneous tissue and mesothelium in male rats, blood vessels in male and female mice, and liver in female mice. Statistically significant increased incidences of benign tumors of the mammary gland (fibroadenoma) in male (Hecht et al. 1982) and female (NCI 1979) rats were also reported, which support the findings of sufficient evidence of the carcinogenicity of *ortho*-toluidine from studies in experimental animals.



## 5. Mechanistic Data and Other Relevant Effects

The purpose of this section is to review data that identify or elucidate potential mechanisms of action for the carcinogenic effects discussed in Sections 3 and 4. These data include but are not necessarily limited to: (1) genetic and related effects, (2) relevant toxicological effects (effects that may be associated with carcinogenicity or are a key feature of a proposed carcinogenic mechanism), (3) mechanistic considerations, and (4) carcinogenic effects of metabolites and analogues.

### 5.1. Genetic and Related Effects

The genetic and related effects of *ortho*-toluidine have been extensively investigated and reviewed (Danford 1991; IARC 2000; 2010e). Many of the data came from two international collaborative studies of short-term tests for carcinogens (Ashby et al. 1985; de Serres and Ashby 1981). The first of these studies included 42 coded chemicals and the second included 10 uncoded chemicals; *ortho*-toluidine was included in both studies. Some important features of these studies included the following: (1) more than 35 assays were used to represent all available types of short-term tests claimed to have potential as carcinogen prescreens, (2) multiple laboratories participated in the studies; (3) chemicals were prepared in pure form and dispatched to the participating laboratories from the same batch; (4) a common S9 preparation was provided but its use was optional; (5) all participating investigators were required to submit protocols prior to the beginning of the studies but no attempt was made to standardize protocols; (6) standard defined criteria were used to evaluate assay performance; and (7) data reporting was limited because of the number of chemicals involved.

Overall, the data show that *ortho*-toluidine has a wide range of genetic effects; however, there is substantial variation in results between different laboratories as well as minor variations in protocols. Data are presented for bacterial systems (Section 5.1.1), non-mammalian eukaryotes (Section 5.1.2), in vitro mammalian systems (Section 5.1.3), in vivo studies (Section 5.1.4), and genotoxicity studies of *ortho*-toluidine metabolites (Section 5.1.4.3). Results for genetic and related effects are synthesized in Section 5.1.5. All tables for this section, with the exception of the overall summary table, are provided in Appendix F.

#### 5.1.1. Bacterial Systems

*ortho*-Toluidine has a mutagenicity profile similar to other arylamines, with weakly positive results in bacteria and requiring specific assay conditions to demonstrate positive effects. *ortho*-Toluidine has been tested for reverse and forward mutations (using plate incorporation, preincubation, and fluctuation protocols) and prophage induction in *Salmonella typhimurium*; reverse mutations, differential toxicity, growth inhibition, and prophage induction in *Escherichia coli*; and DNA damage (*rec* assay) in *Bacillus subtilis*. Results from these studies are summarized in Appendix F, Table F-1.

*ortho*-Toluidine did not cause mutations in *S. typhimurium* or *E. coli* without metabolic activation. There were a few positive reverse mutation studies in *S. typhimurium* strains TA100, TA98, and TA1538 with metabolic activation only and, in most cases, with modifications to the standard protocol. These modifications included use of the fluctuation protocol (one study each with TA100 and TA1538), use of higher concentrations of S9 (30% rat or hamster, three studies

with TA100 and one with TA98), and/or specific preparations of S9 (phenobarbitol-induced rat S9, or phenobarbitone +  $\beta$ -naphthoflavone, one study each with TA98 and TA1538). Some negative findings were also found using the more sensitive fluctuation protocol in *Salmonella* strains TA98, 1535, and 1537. No clear differences were observed in studies using strains that detect base-pair mutations compared with strains detecting frameshift mutations. One forward mutation study in *S. typhimurium* strain BA13 was positive (arabinose resistance) with 30% rat S9 but negative with 10% rat S9. Two forward mutation studies (8-azaguanine resistance) in strain TM677 were negative. A host-mediated assay also reported that urine from *ortho*-toluidine-treated rats induced reverse mutations in TA98 but not in TA100.

The mutagenicity profile for *ortho*-toluidine in the Ames test is consistent with findings from studies on other arylamines. These studies suggest that the low mutagenic activity of arylamines in the Ames test might be explained by deficiencies in metabolizing enzymes (e.g., sulfotransferases and acetyltransferases) in *Salmonella*. One study found that the introduction of cDNA encoding hamster acetyltransferase in *S. typhimurium* TA1538 markedly enhanced the mutagenicity of arylamines in that test system (Kato and Yamazoe 1994). Similarly, Oda et al. (1999; 1995) reported increased sensitivity to aromatic amines in *Salmonella* tester strains over-expressing bacterial *O*-acetyltransferase (*O*-AT) or human *N*-acetyltransferases (NATs) compared with parent or *O*-AT deficient strains. This system is based on the induction of the *umuC* gene as part of the general SOS response to DNA damage. It is also possible that the type of damage induced by these carcinogens might include large-scale changes in genome structure (e.g., deletions, insertions, or translocations) that are not detected by the *Salmonella* tester strains. Other possibilities include a lack of sufficient quantities of the particular enzymes (with or without added S9 mix) required to activate certain carcinogens, or reactive intermediates might not be able to cross the cell wall. The specific conditions required in most of the positive *Salmonella* assays with *ortho*-toluidine, and results for other genetic toxicity assays support these possibilities.

Reverse mutations were reported with metabolic activation in only one of five studies with *E. coli*, and growth inhibition was reported with metabolic activation in two of six DNA-repair-deficient *E. coli* strains. Differential toxicity was reported without metabolic activation in one study with *E. coli* strains with different capacities for recombinational repair (*pol A*<sup>+</sup> and *pol A*<sup>-</sup>). The positive result was not confirmed in repeat studies in two different laboratories using the same protocol, or in another study that used the liquid suspension protocol. DNA damage was reported in one study with *rec* strains of *B. subtilis*, with and without metabolic activation.

Several studies investigated the co-mutagenic activity of norharman and *ortho*-toluidine (Appendix F, Table F-2). Norharman is found in cigarette smoke, coffee, alcoholic beverages, cooked meat and fish (formed as a pyrolysis product of tryptophan), and human urine (Gupta et al. 1989; Hada et al. 2001; Mori et al. 1996; Nishigaki et al. 2007; Oda et al. 2006). Norharman is non-mutagenic when administered alone but is co-mutagenic when administered with non-mutagenic or weakly mutagenic aromatic amines in the presence of S9. Norharman reacts with *ortho*-toluidine in the presence of S9 mix to form two mutagenic compounds (amino-3'-methylphenylnorharman and its hydroxyamino derivative). These compounds may be further activated by acetyltransferases (NATs). Oda et al. (2006) identified CYP1A1, CYP1A2, NAT1, and NAT2 as the activating enzymes.

### 5.1.2. Non-mammalian Eukaryote Systems

Studies in non-mammalian systems support the conclusion that *ortho*-toluidine is mutagenic under certain assay conditions (Appendix F, Table F-3). In contrast to studies in bacteria and fungi, 5 of 6 somatic mutation studies in *Drosophila* were positive. Exposure to *ortho*-toluidine did not cause mutations or genetic crossing-over (based on one study each in *Aspergillus*). Some positive studies in yeast (*Saccharomyces cerevisiae*) were reported for mutations, gene conversion, deletions, and aneuploidy with or without S9; however, most of the mutation and gene conversion studies were negative. Two studies of interchromosomal recombination in yeast were negative. Although many of the positive studies used an optimized yeast P448/P450 assay that eliminated the need for exogenous metabolic activation. Two studies that used the optimized yeast system reported a negative result (gene conversion). Conflicting findings were found for differential toxicity (based on only 2 studies).

### 5.1.3. Mammalian In Vitro Studies

Studies clearly show that *ortho*-toluidine induces a variety of genotoxic effects in human and rodent cells in vitro (Appendix F, Table F-4). Most of the in vitro genetic toxicology assays with *ortho*-toluidine were conducted as part of the second international collaborative study (Ashby et al. 1985) and were reviewed by Danford (1991) and IARC (2010e).

The available genotoxicity studies in human cells demonstrate that *ortho*-toluidine causes different types of genetic damage (including DNA and chromosomal damage) in various types of human cells. Genetic effects in human cells included DNA damage, unscheduled DNA synthesis (UDS), gene mutation, sister chromatid exchange (SCE), and micronucleus formation. Exogenous metabolic activation was required for UDS but not for SCE or micronucleus formation. All studies gave clear positive results with the exception of one inconclusive study for SCE. The inconclusive study showed a slight dose-related increase in SCE but did not reach a doubling of the control value and used a short incubation time (1 hour).

In general, similar types of genetic effects that were observed in human cells were also reported to occur in mammalian cells; however, for some types of damage, results were less consistent. The studies in non-human cells were conducted in a greater variety of cell types compared with the human cells. DNA damage was reported in rat hepatocytes, rat urinary bladder mucosa cells, and Chinese hamster ovary cells (CHO). DNA damage in both human and rat urinary bladder mucosal cells are of particular interest because the urinary bladder is a target site for carcinogenic effects in rats and humans (see Sections 3 and 4). The data also indicate that *ortho*-toluidine causes cytogenetic damage (SCE, chromosomal aberrations, micronuclei, or aneuploidy) in rodent cells; however, results were less consistent for SCE. There was no apparent requirement for S9 metabolic activation for cytogenetic effects, with the possible exception of chromosomal aberrations. In some studies, metabolic activation appeared to reduce SCE levels. The negative results for SCE, micronucleus, and chromosomal aberrations occurred when short treatment times were used (1 hour). The positive studies used treatment times of at least 2 hours.

Gene mutation studies in rodent cells were positive in over one half of the studies reviewed; however, the required conditions were unclear. Chinese hamster V79 cells and mouse L5178Y cells were the most frequently used but there were no apparent patterns related to locus, S9 presence or concentration, dose, or treatment time. UDS was positive in only one of eight studies

in rat or hamster hepatocytes. The one positive study used a relatively high dose compared with the negative studies. One study that investigated effects on the mitotic spindle in Chinese hamster lung cells was negative.

In addition, *ortho*-toluidine consistently induced cell transformation in rodent cells (Appendix F, Table F-5). Cell transformation assays measure morphological transformation as an early event in the in vitro neoplastic process and allow for a broader assessment of carcinogenic potential including both genotoxic and non-genotoxic events (Maire et al. 2012; Pant et al. 2012).

#### 5.1.4. In Vivo Studies

The genotoxic effects of *ortho*-toluidine have been investigated in more than a dozen studies. Endpoints evaluated include DNA adducts, DNA damage, single-strand breaks, clastogenic effects, altered sperm morphology, and DNA synthesis inhibition. The data indicate that *ortho*-toluidine forms DNA adducts and damages DNA and chromosomes, although certain types of genetic damage were only observed in some tissues.

##### 5.1.4.1. DNA Adducts

DNA adducts of *ortho*-toluidine can be synthesized by reacting the *N*-acetoxyarylamine derivative of *ortho*-toluidine with nucleosides (2'-deoxyguanosine or 2'-deoxyadenosine) or with calf thymus DNA (Jones and Sabbioni 2003; Marques et al. 1997; Marques et al. 1996). The dG-C8 adduct, which has been associated with point mutations and activation of the *c-H-ras* oncogene, was the most common; however, dC, dA, and dT adducts also were detected when incubated with calf thymus DNA.

Some studies indicate that *ortho*-toluidine forms DNA adducts in rats in vivo in liver and nasal mucosa (Appendix F, Table F-6). (Brock et al. 1990; Duan et al. 2008). DNA adducts were detected in rat liver, which is consistent with findings with studies of aromatic amines although they were not detected in the urinary bladder from rats. The mechanistic implications of DNA adducts and DNA damage are discussed in Sections 5.2.1 and 5.2.2.

Böhm et al. (2011) reported *ortho*-toluidine-releasing DNA adducts in 11 urinary bladder tumor samples (urothelial carcinomas) taken from 12 cancer patients and in 13 urinary bladder epithelial samples and 10 bladder submucosal tissue samples taken from 46 sudden death victims at autopsy (Appendix F, Table F-7), which were measured after acid hydrolysis. This method was not validated with synthesized DNA adducts to confirm that covalently bonded *ortho*-toluidine would be released. Although acid hydrolysis has not been validated for *ortho*-toluidine, it has been used for releasing DNA adducts of tobacco-specific nitrosamines and for all species of adducts, including phosphotriesters (Haglund et al. 2002; Lao et al. 2007a; 2007b). (The releasing DNA adducts for 1-(*N*-methyl-*N*-nitrosamino)-4-(3-pyridyl)-4-oxobutane [NNK] were later shown to be covalently bound (Haglund et al. 2002).) The major limitation of the study is that it does not provide definite evidence of covalent DNA binding. DNA-releasing adduct levels in bladder tumors were significantly higher than in non-cancerous bladder tissue but did not differ between smokers and non-smokers.

##### 5.1.4.2. Other Genotoxic Effects

Genotoxic effects were reported in two of the target organs where carcinogenic effects occurred (liver in mice and urinary bladder in rats and mice). In addition, *ortho*-toluidine induced DNA

strand breaks in the kidney, colon, and stomach in rats, and lung, stomach, and brain in mice (Appendix F, Table F-8). Micronucleus (MN) formation was detected in peripheral blood of rats and erythrocytes of *Pleurodeles waltl*, an amphibian, but not in mouse bone marrow or rat liver. No increase in chromosomal aberrations was observed in mice administered *ortho*-toluidine by intraperitoneal injection; this was the only study that evaluated chromosomal aberrations. One sperm morphology study was negative, and a second study was inconclusive. The latter study showed a statistically significant increase in abnormal sperm, but it did not meet all the criteria for a positive finding. DNA synthesis was inhibited in mouse testes but not mouse kidney.

#### 5.1.4.3. Genotoxic Effects of *ortho*-Toluidine Metabolites

*N*-Oxidized metabolites of *ortho*-toluidine were mutagenic in *Salmonella* while other metabolites were not mutagenic (Appendix F, Table F-9). Mutagenic activity of these metabolites is consistent with *N*-hydroxylation as a required step for metabolic activation (see Section 5.2.1). *S. typhimurium* tester strains TA98, TA100, TA1535, and TA1538 were used (Gupta et al. 1987; Hecht et al. 1979). No mutagenic activity was observed in TA1538. *N*-Hydroxy-*ortho*-toluidine and *ortho*-nitrosotoluene showed marked dose-related mutagenic activity toward TA100 and TA1535 with S9. When assayed without S9, these metabolites were non-mutagenic when tested to toxicity. *N*-Acetyl-*N*-hydroxy-*ortho*-toluidine showed slight mutagenic activity in TA1535 without S9 at 10  $\mu$ M/plate and a marginal mutagenic response in TA98 with S9 at 5 to 10  $\mu$ M/plate. Other studies showed that anthranilic acid was not mutagenic in *S. typhimurium* TA98, TA100, TA1535, TA1537, or TA1538 with or without metabolic activation when tested by plate incorporation or preincubation methods at maximum nontoxic concentrations (1,000 to 5,000  $\mu$ g/plate) (Chung et al. 1981) or in TA102 at concentrations up to 250  $\mu$ g/plate with or without rat S9 (Gadupudi and Chung 2011).

*N*-Hydroxy-*ortho*-toluidine and *ortho*-nitrosotoluene were potent frameshift mutagens in *S. typhimurium* TA98 in the presence of the co-mutagen norharman and S9 (Gupta et al. 1989). Other *ortho*-toluidine metabolites tested (see list from Appendix F, Table F-9) were not mutagenic even in the presence of norharman and S9. The *meta*- and *para*-toluidines and their nitroso or hydroxylamino metabolites were not mutagenic with or without norharman or S9. These data provide further evidence that *N*-oxidation is required for metabolic activation and that the *ortho*-toluidine is more mutagenic than the *meta* or *para* isomers.

In addition to the reverse mutation assays in *Salmonella* discussed above, several other genotoxicity studies with the *ortho*-toluidine metabolite anthranilic acid also were negative. McFee et al. (1989) reported that anthranilic acid did not induce chromosomal aberrations or micronuclei in male B6C3F<sub>1</sub> mice but did have a positive effect on SCE in two of three trials. It did not induce DNA strand breaks in the pSP-72 plasmid at concentrations up to 400  $\mu$ M (Gadupudi and Chung 2011), and it did not induce micronuclei in rodent V79, CHL, or CHO cells or in human peripheral blood lymphocytes, liver, or TK6 cells at doses up to 10 mM (Fowler et al. 2012). DNA strand breaks were not increased in mouse lymphoma cells (L5178Y/TK) with or without metabolic activation, but mutations at the TK locus were increased without metabolic activation (Garberg et al. 1988). Umeda et al. (1989) reported that anthranilic acid did not increase transformation frequency in X-ray-irradiated BALB/3T3 cells, but 3-hydroxyanthranilic acid (a metabolite of anthranilic acid) had a weak, though statistically significant, activity.

### 5.1.5. Synthesis of Results

Overall, the large database of genetic and related effects indicates that *ortho*-toluidine can bind to DNA, cause DNA and chromosomal damage, and induce mutations. For some genetic endpoints, positive findings were only observed when certain protocols were used or were limited to specific cell or tissue types. Genetic damage was observed in some tissues or cells that are target sites for carcinogenicity in experimental animals or humans.

Most of the data were reported in two international collaborative studies published in the early to mid-1980s. Only a few genetic toxicology studies were published since that time. A limitation of the database is there are no studies in humans with known exposure to *ortho*-toluidine; however, there are studies in human cells and human tissues. Studies in bacteria, mammalian cells, exposed animals, or cells from humans were considered to be the most informative. The overall assessment of the genetic effects of *ortho*-toluidine is summarized in Table 5-1. Findings were considered to be positive in the table if a consistent pattern was observed across studies, i.e., any conflicting findings across a specific endpoint could be explained by conditions of the assays, such as protocol, tissue or cell type, species, or metabolic activation.

**Table 5-1. Genetic and Related Effects of *ortho*-Toluidine**

Effect	Bacteria	In Vitro		In Vivo
		Rodents	Humans	Rodents
DNA adducts <sup>a</sup>	NT	NT	NT	+ <sup>a</sup>
Mutation	(+) <sup>b</sup>	(-)	+	NT
DNA damage/strand breaks	NT	+	+	+ <sup>c</sup>
SCE	NT	+	±	+
Chromosomal aberrations	NT	+	NT	- <sup>d</sup>
Micronucleus	NT	±	+	+ <sup>e</sup>
Aneuploidy	NT	+ <sup>f</sup>	NT	NT
Inhibition of cellular communication	NT	+	NT	NT
Differential toxicity	-	NT	NT	NT
Cell transformation	NT	+	NT	NT
Inhibition of DNA synthesis	NT	NT	NT	± <sup>g</sup>
Unscheduled DNA synthesis	NT	-	+	NT

- = negative; + = positive; (+) = weakly positive; ± = inconclusive; (-) = mostly negative; NT = not tested.

<sup>a</sup>Adducts detected in calf thymus DNA.

<sup>b</sup>Positive in some tissues: male rat liver and nasal mucosa; negative in male rat urinary bladder and white blood cells and female rat liver. A few positive studies with modified protocols primarily using different metabolic activation systems, comutagenic activity with norharman, and some *N*-oxidized metabolites were mutagenic; overall assessment weakly mutagenic.

<sup>c</sup>Positive in several tissues: rat: urinary bladder, kidney, colon, and stomach; mouse: liver, lung, urinary bladder, stomach, and brain.

<sup>d</sup>Only one study reviewed.

<sup>e</sup>Positive in some tissues of some species: rat reticulocytes and newt erythrocytes; negative in mouse bone marrow and rat liver.

<sup>f</sup>Positive in some tissues/species: hamster lung V79 cells and liver fibroblasts, negative in rat liver epithelial cells.

<sup>g</sup>Positive in mouse testes, negative in mouse kidney.

Evidence that *ortho*-toluidine can bind DNA comes from findings of adducts in liver and nasal mucosa of rats exposed to *ortho*-toluidine. *ortho*-Toluidine is considered to be weakly

mutagenic. Although *ortho*-toluidine was not mutagenic in most bacteria studies, a few reverse mutation studies in *Salmonella* were positive under certain conditions (fluctuation protocol, high concentrations of rat or hamster S9, and/or certain preparations of induced S9), and one forward mutation study was positive with 30% hamster S9. This is consistent with findings observed for other aromatic amines. *ortho*-Toluidine was co-mutagenic with norharman and some oxidative metabolites of *ortho*-toluidine also were mutagenic in bacteria. It was also mutagenic in human lymphoblast cell lines (TK6 and AHH-1); however, findings were mixed in mammalian cells, which did not seem to be explained by exogenous metabolic activation. These conclusions are supported by the studies in non-mammalian eukaryotes; *ortho*-toluidine was clearly mutagenic in *Drosophila* and weakly mutagenic in yeast (see Appendix F, Table F-3).

DNA damage was reported in both in vitro and in vivo mammalian systems. Other cytogenetic effects (SCE, chromosomal aberrations, or micronuclei) were consistently induced in mammalian cells when incubation times of 2 hours or more were used. Clastogenic effects also were observed in vivo but not in all tissues examined. There is evidence that *ortho*-toluidine causes large-scale chromosome damage in yeast and mammalian cells (e.g., deletions, insertions, translocations, and/or aneuploidy) and oxidative damage to DNA. The only study that examined chromosomal aberrations (mouse bone marrow) was negative. Micronucleus formation was observed in rat reticulocytes but not in rat liver or mouse bone marrow. Aneuploidy also occurred in mammalian cells. Most cell transformation studies in several mammalian cell types were positive.

Genetic damage was observed in cells or tissue of some of the target sites (liver in mice and urinary bladder in rats) for carcinogenicity in experimental animals and humans (urinary bladder). DNA adducts were observed in liver but not urinary bladder tissue of rats exposed to *ortho*-toluidine. DNA damage was observed in both human and rat urinary mucosal cells, and in urinary bladder tissues of rats and liver tissue of mice exposed to *ortho*-toluidine.

## 5.2. Mechanistic Considerations

Aromatic amines include many chemicals that are carcinogenic, mutagenic, and/or are hemotoxicants (Benigni and Passerini 2002). In general, the same metabolic schemes, same kind of genotoxic lesions, and similar tissue specificity have been described for many aromatic amines and support a common mode of action for this chemical class (IARC 2010d). Recent investigations indicate that the biochemical mechanisms of action for the monocyclic compounds may be more complex than for the more extensively studied multi-ring aromatic amines (Skipper et al. 2010); however, data specific for *ortho*-toluidine are limited. Several aromatic amines are known human bladder carcinogens (benzidine, 4-aminobiphenyl, and 2-naphthylamine) or are listed as *reasonably anticipated to be a human carcinogen* based in part on increased incidences of bladder tumors in animals (2-acetylaminofluorene and 4-chloro-*ortho*-toluidine). IARC has classified *ortho*-toluidine as a Group 1 carcinogen based on sufficient evidence of carcinogenicity in humans (IARC 2010e) IARC (2010b) classified another bicyclic aromatic amine, 4,4'-methylenebis(2-chloroaniline) (MOCA or MBOCA), as a Group 1 carcinogen based on inadequate evidence in humans and sufficient evidence in experimental animals for its carcinogenicity supported by a common mode of action for monocyclic aromatic amines (which include *ortho*-toluidine) and evidence of genotoxicity and adducts in urothelial cells or lymphocytes from workers with known occupational exposure to MOCA.

No criteria have yet been identified that can separate genotoxic from non-genotoxic, or carcinogenic from non-carcinogenic monocyclic aromatic amines (IARC 2010d). However, quantitative structure-activity relationship (QSAR) models suggest that carcinogenic potency and carcinogenic activity of aromatic amines are described by different molecular properties (Benigni et al. 2000; Benigni and Passerini 2002; Franke et al. 2010; Franke et al. 2001). The QSARs for carcinogenicity were similar to those for mutagenicity in *Salmonella* and suggested that similar mechanisms were responsible for the mutagenic and carcinogenic activity of aromatic amines. Thus, results from the QSAR models are consistent with the importance of metabolic activation in aromatic amine carcinogenicity and mutagenicity. Electronic properties (measures of chemical reactivity or the ability to undergo metabolic transformation) and steric characteristics were the most important factors related to carcinogenic activity, while hydrophobicity (log P) was the most important factor for carcinogenic potency. Carcinogenic potency increased with increasing hydrophobicity (particularly for monocyclic amines) and increasing energy of the highest occupied molecular orbital (EHOMO). EHOMO is a parameter for oxidation reactions and indicates the propensity of the toxic amines to form *N*-hydroxylamines. Hydrophobicity is a primary determinant of absorption, distribution, and the interaction of xenobiotics with metabolizing enzymes.

The epidemiology data (see Section 3) identified the urinary bladder as a target for *ortho*-toluidine-induced cancer in humans. Carcinogenicity studies in experimental animals indicate that the urinary bladder, liver, mesenchymal tissue in the spleen and other visceral organs, the mesothelial lining of the testis and epididymis (tunica vaginalis), subcutaneous tissue, mammary gland, and blood vessels are target sites for *ortho*-toluidine (see Section 4). The data suggest the following: (1) multiple mechanisms are involved, (2) mechanisms may vary with target tissue, and (3) aromatic amines generally must be mutagenic and cytotoxic to induce a carcinogenic effect. Although the mechanisms of carcinogenicity in the various target tissues are not completely understood, the following sections discuss possible mechanisms of carcinogenicity for the target tissues.

### 5.2.1. Urinary Bladder Tumors

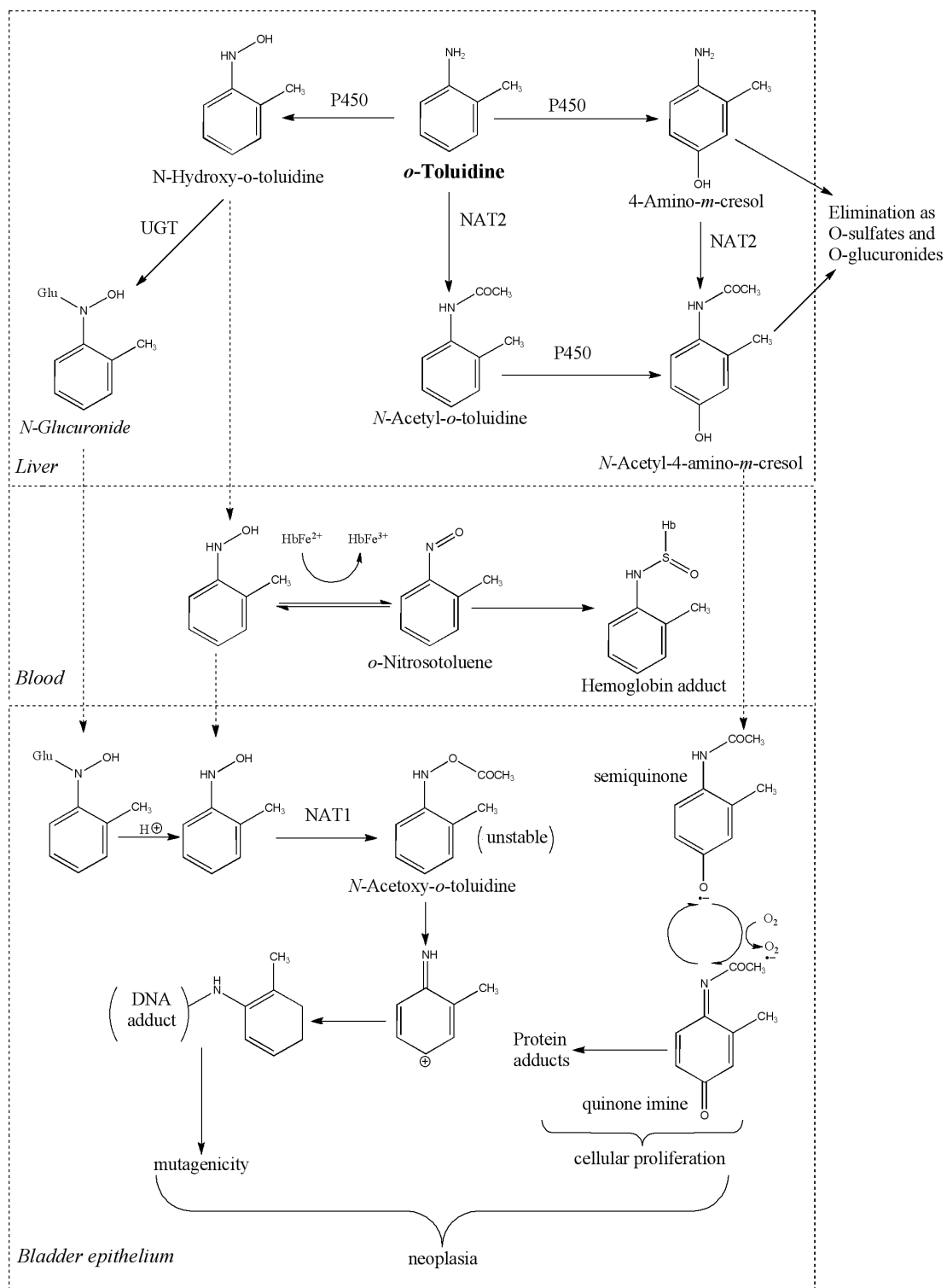
Proposed mechanisms associated with urinary bladder carcinogenesis by aromatic amines include metabolic activation, direct and indirect genotoxic effects, and non-genotoxic mechanisms (e.g., cytotoxicity and sustained cell proliferation) (Skipper et al. 2010). Polymorphic expression of some of the metabolizing enzymes has been linked to aromatic amine exposure and urinary bladder cancer risk in humans. Genotoxic effects have been reported for *ortho*-toluidine and some of its metabolites (reviewed in Section 5.1) and cytotoxicity and cell proliferation are known to be important promoting factors in aromatic amine-induced carcinogenicity (Bitsch et al. 1999; IARC 2010d; Neumann 1986; 2007; Poirier et al. 1995; Woo and Lai 2004).

A comprehensive model of the possible mode of carcinogenic action of *ortho*-toluidine in the human and rodent urinary bladder was recently developed by (English et al. 2012). The proposed key events based on data from *ortho*-toluidine and other aromatic amines are illustrated in Figure 5-1 and include the following: (1) initial bioactivation by hepatic P450 enzymes to *N*-hydroxy-*ortho*-toluidine, (2) further bioactivation in the urinary bladder transitional epithelium via NAT1 yielding *N*-acetoxy-*ortho*-toluidine that hydrolyzes to nitrenium ions that can bind to DNA, (3) semiquinone/quinone redox cycling of unconjugated phenolic metabolites in the



transitional epithelium producing reactive oxygen species (ROS), oxidative cellular damage and compensatory cell proliferation, (4) mutations from direct or indirect DNA damage to urinary bladder epithelial cells, and (5) development of urinary bladder tumors. *N*-hydroxy-arylamines also can be further metabolized to reactive *N*-sulfonyloxy-arylamines or *N*-hydroxyarylamine *N*-glucuronides in the liver and these metabolites also may contribute to the genotoxicity and cytotoxicity of *ortho*-toluidine (English et al. 2012; Sabbioni and Sepai 1995). These key events, supporting data, and data weaknesses for the proposed mechanisms are discussed in the following sections.

## RoC Monograph on *ortho*-Toluidine



**Figure 5-1. Proposed Key Events in the Mode of Action of *ortho*-Toluidine-induced Urinary Bladder Cancer**

Source: English et al. (2012); Kadlubar and Badawi (1995).

### 5.2.1.1. Metabolic Activation

It is known that aromatic amines require metabolic activation to exert toxic effects (IARC 2010a). Although not completely understood, activation steps include both phase-I oxidation and phase-II conjugation (primarily acetylation or sulfation) reactions (Skipper et al. 2010; Woo and Lai 2004). The first step involves *N*-hydroxylation (*N*-oxidation) or *N*-acetylation in the liver to form an aryl-*N*-hydroxylamine or an *N*-acetylated (arylamide) derivative. *N*-Acetylation is usually considered a detoxification pathway; however, arylamides can undergo further metabolic activation in the liver to form reactive and toxic metabolites (Delclos and Kadlubar 1997; Woo and Lai 2004). Studies in experimental animals indicate that *N*-acetylation of aromatic amines is a detoxification step for urinary bladder carcinogenesis while *N*-hydroxylation is an obligatory step for carcinogenic activity (Freudenthal et al. 1999; Windmill et al. 1997).

Following *N*-oxidation or *N*-acetylation in the liver, further activation can occur through *N,O*-transacetylation, *O*-acetylation, or *O*-sulfation to form acyloxy-arylamines or sulfonyloxy-arylamines (English et al. 2012; Hein 1988a; 1988b; Windmill et al. 1997; Woo and Lai 2004). These reactions are catalyzed by cytosolic *N*-acetyltransferases (NAT1 and NAT2 in humans) and cytosolic sulfotransferases. Acyloxy-arylamines and sulfonyloxy-arylamines are highly reactive esters that can form electrophilic arylamidonium or arylnitrenium ions via heterolysis of the ester bond. Aryl-*N*-hydroxylamines may be further oxidized to nitroso derivatives in the blood. Nitroso metabolites form hemoglobin adducts and generate methemoglobin (see Appendix F, Table F-10) (English et al. 2012; Eyer 1983). However, the *N*-acetoxy derivative of *ortho*-toluidine so far has been identified only as a postulated metabolite based on studies with other aromatic amines. In addition, Hein et al. (1993) showed that the *N*-acetylation of *ortho*-toluidine by human NAT1 and NAT2 was consistently lower than for most other arylamines, including *para*-toluidine, 4-aminobiphenyl, 2-aminofluorene, 2-naphthylamine, and *para*-aminobenzoic acid. Zhang et al. (2006) also reported that *ortho*-toluidine binding to NAT1 was lower than that of 4-aminobiphenyl and *para*-aminobenzoic acid, which is because of steric hindrance by the methyl group. Both studies showed better activity for NAT2 than NAT1.

The phase-II activation steps are in competition with glucuronide or glutathione conjugation (Woo and Lai 2004). Glucuronide formation usually is a detoxification pathway, but these metabolites may be cleaved nonenzymatically in the mildly acidic pH of the urinary bladder (typically found in humans and dogs) or enzymatically by  $\beta$ -glucuronidase and further activated to arylnitrenium ions that can covalently bind to urothelial DNA (English et al. 2012; Freudenthal et al. 1999; Woo and Lai 2004; Yu et al. 2002). The *N*-hydroxy metabolites of aromatic amines also may enter the urinary- bladder lumen in the unconjugated form and, thus, would immediately be available for reabsorption into the bladder mucosa and binding to urothelial DNA (Kadlubar et al. 1991). There also is some evidence in dogs and humans that the *N*-glucuronide of the parent amine may reach the bladder. In this case, hydrolysis in the urine would release the free parent compound where it may be *N*-hydroxylated (activated) directly by the bladder epithelium (Freudenthal et al. 1999). Thus, *N*-hydroxy-*ortho*-toluidine conjugates are formed in the liver, and based on hypothesis developed from metabolism of other aromatic amines, are proposed to enter the blood, and to be transported to the bladder where they form reactive nitrenium ions that can react with DNA in the transitional epithelium (English et al. 2012; Kadlubar and Badawi 1995; Riedel et al. 2006).

In general, glutathione conjugation is a detoxification reaction. Although glutathione conjugates have been described for other aromatic amines (Delclos and Kadlubar 1997; Skipper et al. 2010; Woo and Lai 2004), no glutathione conjugates were identified in metabolism studies with *ortho*-toluidine (see Section 2).

Further evidence of the importance of *N*-oxidation of *ortho*-toluidine as a metabolic activation step for bladder carcinogenicity was shown in a comparative carcinogenicity study of *ortho*-toluidine hydrochloride and *ortho*-nitrosotoluene in male F344 rats (Hecht et al. 1982). Test compounds were administered in feed for 72 weeks and all animals were killed at 93 weeks. Rats treated with the *N*-oxidized metabolite (*ortho*-nitrosotoluene) developed significantly more urinary bladder tumors (16/30) than rats treated with *ortho*-toluidine (4/30).

#### 5.2.1.2. Hemoglobin Adducts

The finding of hemoglobin adducts in humans provides indirect evidence for the relevance of the *N*-oxidation metabolic pathway in humans. *ortho*-Toluidine-releasing hemoglobin or albumin adducts have been reported in in vitro studies (Stettler et al. (1992), see Appendix F, Table F-8), in vivo studies of exposed rats and mice (Birner and Neumann (1988); Cheever et al. (1992); DeBord et al. (1992); Stettler et al. (1992), see Appendix F, Table F-8), and in several studies in humans, including workers with a known excess of urinary bladder cancer, non-occupationally exposed populations from various countries (see Table 1-4) and in patients treated with the local anesthetic prilocaine (which is metabolized to *ortho*-toluidine) (Gaber et al. 2007). (See Section 1 for additional information on *ortho*-toluidine hemoglobin adducts in human studies.) Hemoglobin adducts are formed from the reaction of hydroxy-*ortho*-toluidine metabolites with hemoglobin and most of the studies measured adducts released from the hemoglobin via base hydrolysis. Although the possibility that hydroxyl metabolites formed from ring oxidation cannot be ruled out, the hemoglobin adducts are believed to be formed from the *N*-hydroxy metabolites. An in vitro study of a related aromatic amine (3,4-dichloroaniline) reported that its *N*-hydroxy metabolite with hemoglobin was an order of magnitude greater than its ring-hydroxylated metabolite (Cheever et al. 1992). Studies of other aromatic amines that cause urinary bladder cancer have shown that *N*-hydroxy-amines may be further oxidized to nitroso derivatives in the blood, generating methemoglobin and forming hemoglobin adducts (Skipper and Tannenbaum 1994). Birner and Neumann (1988) reported a positive correlation between hemoglobin-binding index and methemoglobin formation for six monocyclic amines that were studied (*ortho*-toluidine was not tested). Hemoglobin adducts are considered as biological markers of aromatic amine bioactivation to their ultimate carcinogenic metabolites and have been associated with bladder cancer risk in a population-based study (Gan et al. 2004). One study also reported a correlation between hemoglobin adducts and DNA adducts of 4-aminobiphenyl in exfoliated urothelial cells (Talaska et al. 1991).

#### 5.2.1.3. Polymorphisms in Metabolizing Enzymes

Polymorphic expression of some of the metabolizing enzymes has been linked to aromatic amine exposure and urinary bladder cancer risk in humans. Polymorphisms have been described for human NAT1 and NAT2 (Kadlubar and Badawi 1995; Talaska 2003; Yu et al. 2002). The human population can be divided into slow, intermediate, and fast acetylator phenotypes based on polymorphic expression of NAT2. Since *N*-acetylation in the liver is a detoxifying pathway for the urinary bladder, slow acetylators would be expected to detoxify aromatic amines less

efficiently. Epidemiological data indicate that slow acetylators are more susceptible to aromatic amine-induced bladder cancer. In addition, the human urinary bladder has appreciable NAT1 activity and can catalyze *O*-acetylation of *N*-hydroxylamines to electrophilic *N*-acetoxy derivatives. There is some evidence that cigarette smokers that carry the high-activity NAT1 allele, especially when combined with the NAT2 slow acetylation genotype, are more likely to develop bladder cancer.

#### 5.2.1.4. Sex and Species Considerations

Urinary bladder neoplasms were significantly increased in rats but not in mice (see Section 4); however, mice are less sensitive for developing urinary bladder cancer than rats (Frith et al. 1994). Although both male and female rats developed urinary bladder tumors, the lower incidence in male rats (which was not statistically significant but considered to be treatment related because of their rarity) compared with female rats might be related to sex-specific patterns of metabolic activation and detoxification (English et al. 2012). No metabolic studies have been conducted in female rats (see Section 2.2); however, English et al. (2012) reported that GSTM1 (a phase-II enzyme involved in arylamine detoxification) is more abundant in male mouse liver compared with females. If a similar pattern is true for rats, this would be consistent with a lower tumor incidence in male rats. There also is some evidence that women have a higher capacity for arylamine activation than men (Castelao et al. 2001). In addition, Richter et al. (2001) reported that levels of hemoglobin adducts of toluidine isomers tended to be lower in boys than in girls.

#### 5.2.1.5. Genetic Damage

Genotoxic effects of *ortho*-toluidine that are potentially relevant to bladder cancer have been reported; they include demonstration that *ortho*-toluidine can bind DNA and damage DNA and chromosomes (see Section 5.1 and Appendix F).

Some *N*-oxidized metabolites of *ortho*-toluidine are mutagenic with exogenous metabolic activation (see Section 5.1.5) and are carcinogenic (see Section 5.2.1.4). Thus, the *N*-oxidized derivatives of arylamines or arylamides are considered the proximate carcinogenic forms (Hein 1988a; Kato and Yamazoe 1994). Studies using calf thymus DNA or in exposed rats have demonstrated that *ortho*-Toluidine can form DNA adducts (Duan et al. 2008; Marques et al. 1996) and *ortho*-toluidine releasing DNA were detected in the urinary bladder tumor and tissue from humans (Böhm et al. 2011); however, because of methodological limitations, covalent binding was not confirmed. No DNA adducts were detected in the urinary bladder of exposed rats (Duan et al. 2008). Thus, evidence for DNA adduct formation in the urinary bladder tissue is less clear. Evidence that *ortho*-toluidine causes DNA damage in the urinary bladder includes DNA strands breaks detected in human cells in vitro and in rats and mice in vivo.

Evidence for the relationships between DNA adduct formation, mutagenicity, and carcinogenicity comes from studies of other aromatic amines. A study in dogs reported that the extent and relative persistence of total DNA binding of several bicyclic and polycyclic aromatic amines (4-aminobiphenyl, 2-acetylaminofluorene, 1-naphthylamine, 2-naphthylamine, 4-nitrobiphenyl, benzidine, and *N*-acetylbenzidine) correlated with the compounds' carcinogenic potency in the urinary bladder (Beland et al. 1983). The adduct profiles were very similar to those obtained when the *N*-hydroxy metabolites of these compounds were reacted with DNA in

vitro. Further studies in *S. typhimurium* showed that the C8-deoxyguanosine adduct was correlated with frameshift reversions and could be important for tumor initiation.

Although several studies have suggested that aromatic amines cause urinary bladder cancer by forming DNA adducts and inducing mutations, there is evidence that gross chromosome alterations may also be involved. A correlation between formation of DNA adducts and induction of chromosomal aberrations has been demonstrated in the liver of mice exposed to the aromatic amine benzidine (Talaska et al. 1987). Cytotoxic levels of 4-aminobiphenyl caused chromosomal instability (CIN, i.e., selective loss of chromosomes 4 and 6) in urinary bladder and liver cells; the specific chromosome lost varied according to cell type (Saletta et al. 2007). CIN not only results in aneuploidy but also is associated with loss of heterozygosity, an important mechanism for inactivation of tumor suppressor genes. Thus, aromatic amines may affect tumorigenesis by inducing CIN. These data are consistent with previous studies of bladder cancer in humans that show complex patterns of aneuploidy (Höglund et al. 2001; Phillips and Richardson 2006; Sandberg 2002) and provide further evidence that exposure to specific carcinogens can select for tumor cells with distinct forms of genetic instability (Bardelli et al. 2001; Brevik and Gaudernack 1999).

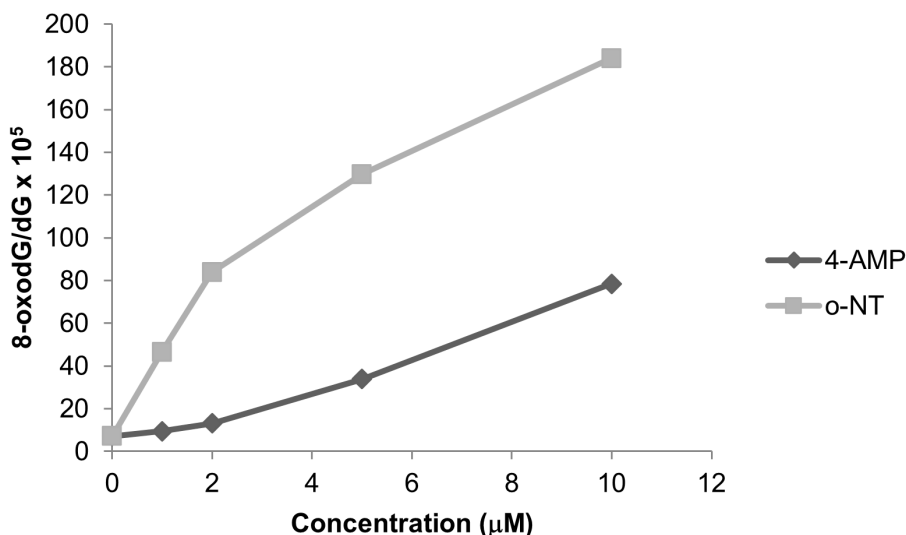
The genetic toxicology data for *ortho*-toluidine (see Section 5.1) are consistent with this hypothesis. Although *ortho*-toluidine is a weak mutagen, it induced aneuploidy, DNA strand breaks, chromosomal damage, and cell transformation in mammalian cells. It also caused DNA damage or strand breaks in the liver and kidney of exposed mice, SCE in bone marrow of exposed hamsters and mice, and micronuclei in peripheral blood cells of exposed rats.

#### 5.2.1.6. Oxidative DNA Damage

Oxidative DNA damage may be more important for the mutagenic and carcinogenic effects of monocyclic compounds compared with the bicyclic and polycyclic aromatic amines (Kawanishi et al. 2002; Ohkuma et al. 1999). DNA adducts can result in tumor initiation while oxidative products of DNA bases can be highly mutagenic and are involved in tumor initiation and promotion (Kawanishi et al. 2002). Many organic carcinogens, including aromatic nitro and amino compounds, induce oxidative DNA damage through generation of copper or iron-catalyzed reactive oxygen species (ROS) (Murata and Kawanishi 2011).

Two metabolites of *ortho*-toluidine (4-amino-3-methylphenol (also called 4-amino-*meta*-cresol) and *ortho*-nitrosotoluene) caused oxidative DNA damage in DNA fragments from human *c-Ha-ras* and *p53* genes and induced 8-oxodG lesions in calf thymus DNA (Ohkuma et al. 1999). The presence of both NADH and Cu(II) was required for *ortho*-nitrosotoluene, but Cu(II) alone was sufficient for 4-amino-3-methylphenol. Oxidative lesions (8-oxodG) in calf thymus DNA increased with dose for both metabolites (Figure 5-2). *ortho*-Nitrosotoluene also caused oxidative damage in human leukemia (HL-60) cells (Watanabe et al. 2010). Two distinct mechanisms of Cu(II)-mediated DNA damage were proposed. 4-Amino-3-methylphenol is autooxidized to form the aminomethylphenoxyl radical while NADH reduces *ortho*-nitrosotoluene to the *ortho*-toluolhydronitroxide radical in the presence or absence of Cu(II). These radicals react with oxygen to form superoxide ion and hydrogen peroxide. Cu(II) is reduced by superoxide ion to Cu(I), and the interaction of Cu(I) and hydrogen peroxide generates ROS that are capable of damaging DNA. This is the same mechanism proposed by. Superoxide dismutase (SOD), catalase, and the Cu(I) chelator, bathocuproine, reduced the amount of DNA

damage. 8-OxidG is known to cause GC to TA transversions (Ohkuma et al. 1999; Shibutani et al. 1991). The mutation spectrum for the *p53* gene in human urinary bladder carcinomas and bladder tumor cell lines has shown that GC to TA transversions are relatively common (Williamson et al. 1994).



**Figure 5-2. Formation of 8-oxodG Lesions in Calf Thymus DNA Treated with Metabolites of *ortho*-Toluidine in the Presence of Cu(II)**

Source: Ohkuma et al. (1999).

4-AMP = 4-amino-3-methylphenol; *o*-NT = *o*-nitrosotoluene.

#### 5.2.1.7. Semiquinone/Quinone Redox Cycling, Cytotoxicity, and Cellular Proliferation

The cytochrome P450-mediated *N*-hydroxylation pathway is not the only phase-I oxidative metabolic pathway for aromatic amines. Direct ring oxidation of aromatic amines produces aminophenols that generally are regarded as detoxification products because they are usually conjugated with sulfate or glucuronic acid and excreted (English et al. 2012; Skipper et al. 2010). If the aminophenols are not conjugated, they can undergo peroxidase-catalyzed oxidation to form quinone imines (Figure 5-1). Although peroxidase activity in the liver is low compared with monooxygenase activity, other tissues, including the urinary bladder, contain high levels of peroxidase.

Concentrations of one of the phenolic metabolites of *ortho*-toluidine (*N*-acetyl-4-amino-*meta*-cresol) in rat urine were correlated with urinary bladder-cell proliferation (UNEP 2004). These data support the assertion that toxicity to the urinary bladder epithelium may be associated with reactive oxygen species generated by the redox cycling of *N*-acetyl-4-amino-*meta*-cresol and its quinone imine counterpart, or with direct damage mediated by the electrophilic quinone imine itself. The induction of reactive oxygen species can lead to oxidative damage to DNA.

Several studies have reported cytotoxic and proliferative effects in the urinary bladder following subacute, subchronic, and chronic exposure of rats and mice to *ortho*-toluidine. Ekman and Strömbeck (1947; 1949) reported that the tumorigenic action of azo compounds was likely due to metabolism to toluidines because albino rats fed a diet containing *ortho*-toluidine or *meta*-

toluidine developed epithelial changes in the bladder mucosa that were similar to those observed in rats fed tumorigenic azo compounds. These changes included keratosis, metaplasia, early epithelial proliferation, and a tendency to incipient papillomatosis. Both *meta*- and *ortho*-toluidine are metabolites of 2,3'-azotoluene and urinary bladder tumors occurred only when the metabolic products of 2,3'-azotoluene were in direct contact with the bladder mucosa (Strömbeck 1946).

The incidence of proliferative nonneoplastic lesions in the urinary bladder was significantly increased in rats or occurred only in dosed animals in two NTP carcinogenicity studies of *ortho*-toluidine (NCI 1979; NTP 1996). Neoplastic effects and preneoplastic effects (hyperplasia of transitional-cell epithelium) observed in the urinary bladder were reviewed in Section 4. A dose-related progression from hyperplasia of transitional-cell epithelium to transitional-cell carcinoma in the urinary bladder was observed, particularly in female rats. This lesion was reversible because complete regression occurred in the stop-exposure group (NTP 1996).

### 5.2.2. Other Tumors

Very few mechanistic data for other tumor sites were identified. Reactive *N*-oxidized metabolites or *ortho*-toluidine reach the blood as evidenced by hemoglobin adducts detected in exposed humans and experimental animals, and at least one study reported micronucleus formation in peripheral blood of rats (see Sections 5.1.4, 5.2.1, 5.2.2). Limited information is available on mechanisms for liver tumors and splenic tumors (see below). In addition to *ortho*-toluidine, other aromatic amines often induce liver neoplasms in rodents.

Liver tumors were significantly increased in female mice exposed to *ortho*-toluidine but not in male or female rats (see Section 4); however, placental glutathione *S*-transferase (PGST)-positive foci (a preneoplastic lesion) were significantly increased in male rats in a subchronic study (NTP 1996). Metabolic activation to reactive metabolites is a key step in aromatic amine-induced liver neoplasms, differences in metabolism may contribute to interspecies differences in susceptibility; *N*-acetylation is an activating reaction in the liver. Liver tumors in rodents exposed to aromatic amines likely result from *N*-hydroxy-*N*-acetylarylamine metabolites, which are formed from *N*-acetylation of *N*-hydroxy metabolites in the liver. The relative capability of NAT enzyme systems from liver cytosol of various mammalian species to acetylate the amine function of three carcinogenic aromatic amines (2-aminofluorene, 4-aminobiphenyl, and 2-aminonaphthalene) was highest in hamster followed by guinea pig, but enzyme activity in mouse liver cytosol was about an order of magnitude higher than rat liver cytosol (Lower and Bryan 1973). The comparatively greater acetylating activity in mouse liver could explain the greater susceptibility of mice to liver tumors with exposure to *ortho*-toluidine.

Exposure to *ortho*-toluidine caused DNA strand breaks in liver tissue from exposed mice, suggesting a role for genotoxicity. However, data on interactions of *ortho*-toluidine with DNA are limited. DNA binding alone does not necessarily explain the differences in carcinogenic potency observed among various aromatic amines or tissue-specific responses because biological events (e.g., promotion, DNA repair, DNA adduct persistence, cytotoxicity) in addition to macromolecular binding influence the expression of carcinogenic potential (Brock et al. 1990).

The spleen is a specific tumor target for *ortho*-toluidine based on evidence from studies in rats (see Section 4) (NCI 1979). Splenic fibrosarcomas are rare in rats, occurring in about 0.4%



(4/1,003) of males and 0% (0/1,000) of females in historical controls in dietary studies ([http://www.ntp.niehs.nih.gov/ntp/research/database\\_searches/historical\\_controls/path/r\\_orlfd.txt](http://www.ntp.niehs.nih.gov/ntp/research/database_searches/historical_controls/path/r_orlfd.txt)). The morphologic similarity of splenic fibrosis and capsule hyperplasia to the sarcomas suggested that these lesions were preneoplastic and that the treatment-related splenic lesions appeared to be precursors to the neoplastic process (NCI 1979; Short et al. 1983). There is limited mechanistic data that is specific for *ortho*-toluidine-induced splenic tumors; however, data on aromatic amines suggest that the carcinogenic effects of *ortho*-toluidine in the spleen are associated with oxidative stress and erythrocyte toxicity (Ciccoli et al. 1999; Khan et al. 1997). Two metabolites of *ortho*-toluidine, 4-amino-3-methylphenol and *ortho*-nitrosotoluene, have been shown to cause oxidative damage in DNA (see Section 5.2.1), although no direct evidence of oxidative damage to the spleen was identified. Exposure to *ortho*-toluidine results in an increased incidence of several nonneoplastic effects in the spleen of rats that are consistent with a proposed mode-of-action involving toxic effects resulting from damaged red blood cells reaching the spleen. Histopathologic effects (fibrosis and mesothelial capsule hyperplasia) consistent with enhanced erythrocyte toxicity were reported by Short et al. (1983), and both NTP carcinogenicity studies (NCI 1979; NTP 1996) also reported nonneoplastic effects in the spleen of rats (congestion, hematopoietic cell proliferation, hemosiderin pigmentation, capsule fibrosis, capsule-lymphatic angiectasis).

### 5.3. Structural Comparison Studies

Several lines of evidence point to a role for the methyl group in the *ortho*-position in the carcinogenicity of *ortho*-toluidine. Rats administered single oral doses of *ortho*-, *meta*-, or *para*-toluidine excreted higher concentrations of unchanged *ortho*-toluidine (21% to 36%) compared with 2.5% for the *meta*- or *para*- isomers (Cheever et al. 1980). Thus, the higher concentration of unmetabolized *ortho*-toluidine in urine might partially explain why only the *ortho*-isomer caused urinary bladder tumors in rats.

Hecht et al. (1979) investigated the structure-mutagenicity relationships among several monocyclic (including aniline and *ortho*-toluidine) and bicyclic aromatic amines and their *N*-oxidized derivatives and reported that the derivatives having a methyl group *ortho* to the amine group were generally more mutagenic in the Ames test. QSAR models suggest that substitution at the *ortho*-position also was an important factor that affected carcinogenic potency. Steric bulk at the *ortho*-position decreased potency by preventing enzymatic access to the nitrogen and formation of the reactive intermediate; however, substitution of chloro-, methyl- (e.g., *ortho*-toluidine), or methoxy- groups at this position enhanced potency (Benigni et al. 2000; Benigni and Passerini 2002; Franke et al. 2010; Franke et al. 2001).

The genotoxic effects of many aromatic amines are likely initiated by DNA adducts (Beyerbach et al. 1996). *N*-Hydroxy-arylamines (*N*-oxidation products of aromatic amines form hemoglobin, albumin, and DNA adducts (Cheever et al. 1992; Sabbioni 1992; Sabbioni and Sepai 1995). The dG-C8 adduct, which has been associated with point mutations and activation of the *c-H-ras* proto-oncogene, is the most common (Beland and Kadlubar 1985; Beland et al. 1997; Marques et al. 1997; Marques et al. 1996). Other sites of DNA adducts include the N<sup>2</sup> and O<sup>6</sup> atoms of guanine and the C8 and N<sup>6</sup> atoms of adenine (Branco et al. 1999; Delclos and Kadlubar 1997; Jones and Sabbioni 2003). Several studies reported that *ortho*-toluidine formed adducts with nucleosides, nucleotides, and DNA in vitro and that the three-dimensional conformation of various single-ring arylamine (methyl-, ethyl-, and dimethylaniline) adducts affected their ability

to induce mutations (Beland et al. 1997; Beyersbach et al. 1996; Jones and Sabbioni 2003; Marques et al. 1997; Marques et al. 1996). Adducts that contained alkyl groups *ortho*- to the amine function (e.g., *ortho*-toluidine and 2,6-dimethylaniline) tended to be more mutagenic than their *meta*- or *para*-substituted analogues. Spectroscopic and theoretical data indicated that conformational differences among these adducts depended on the location of the alkyl group. The increased biological activity was attributed in part to the greater propensity of *ortho*-alkylated adducts to adopt a higher percentage of low-energy *syn* conformers that increased with the substitution pattern in the order *para* < *meta* < *ortho* < *ortho, para* < *ortho, meta* (Marques et al. 1996). The *syn* conformation enables an arylamine adduct to be inserted into the DNA helix.

## 6. Overall Cancer Evaluation – Synthesis of Human, Animal, and Mechanistic Data

This section synthesizes the information from toxicological, mechanistic, and human epidemiological studies, and applies the RoC listing criteria to that body of knowledge to reach a preliminary listing recommendation. The evidence for urinary bladder cancer, which is the only tumor site that has been adequately studied in humans, is discussed in Section 6.1 and the available data for other tumor sites associated with exposure to *ortho*-toluidine are briefly discussed in Section 6.2.

### 6.1. Urinary Bladder Cancer

Evidence that *ortho*-toluidine is a known human bladder carcinogen comes from human cancer studies finding increased risks of urinary bladder cancer in humans, in concert with studies showing that it causes cancer in experimental animals, including site concordance at the same tissue site in rats and studies demonstrating the biological plausibility of mechanisms of its carcinogenicity in humans.

#### 6.1.1. Human Cancer Studies

There is credible evidence of an association between increased urinary bladder cancer risk and exposure to *ortho*-toluidine based on consistent findings across studies, the presence of statistically significant, positive exposure-response relationships with cumulative rank exposure and duration, and large magnitudes of effect across studies. Potential confounding by occupational co-exposures can reasonably be ruled out in the studies of rubber chemical workers. Although there is limited information on cigarette smoking, potential confounding from smoking can reasonably be ruled out in the NIOSH cohort, and it is unlikely to explain the large risk estimates found in the smaller studies. Finally, the finding of increased urinary bladder cancer risk in different cohorts with different exposure conditions and different co-exposures lends strong support to the conclusion that *ortho*-toluidine is the common causal risk factor (see Section 3).

#### 6.1.2. Studies of Cancer in Experimental Animals

There is site concordance for urinary bladder cancer; *ortho*-toluidine causes urinary bladder neoplasms in both female and male rats although the evidence is stronger in females. The urinary bladder cancer findings in female rats are considered to be robust because of the relatively high tumor incidences, the rarity of the tumor in this species, and the shorter time to first observed tumor in the high-dose females than in the low-dose females (NCI 1979). Although the incidences of urinary bladder cancer are lower in male rats and not statistically significantly increased, they are considered to be exposure related because they are very rare, and were observed in all three carcinogenicity studies. The observation of a significantly increased incidence of transitional-cell hyperplasia of the urinary bladder in male rats after 13 or 26 weeks of exposure in subchronic exposure study (NTP 1996) (see Section 4) supports this conclusion.

### 6.1.3. Mechanistic Evidence

Although the mechanisms of carcinogenicity of *ortho*-toluidine are not completely understood, the available evidence suggests that they are complex and involve several key modes of action, including metabolic activation that results in binding of reactive metabolites to DNA and proteins, mutagenicity, oxidative DNA damage, chromosomal damage, and cytotoxicity. The key metabolic activation steps and genotoxic effects occur in both experimental animals and humans. *ortho*-Toluidine is an aromatic amine, and some chemicals in this class are known human bladder carcinogens: 4-aminobiphenyl, benzidine, and 2-naphthylamine. In addition, *ortho*-toluidine has a similar toxicological profile as another aromatic amine, 4,4'-methylenebis(2-chloroaniline), which is classified as carcinogenic to humans based on strong mechanistic data involving metabolic activation similar to that proposed for *ortho*-toluidine.

#### 6.1.3.1. Metabolic Activation Leading to Formation of Hemoglobin and DNA Adducts

Metabolism of monocyclic aromatic amines, including *ortho*-toluidine, involves many competing activating and deactivating pathways including *N*-acetylation, *N*-oxidation and hydroxylation, and ring oxidation (C-hydroxylation). Cytochrome P450-mediated *N*-hydroxylation to the *N*-hydroxy derivative (*N*-hydroxy-*ortho*-toluidine), a proximate carcinogenic metabolite, occurs in the liver. *N*-hydroxy-*ortho*-toluidine is transported to the urinary bladder and can be bioactivated via conjugation by sulfation and/or acetylation by cytosolic NATs or SULTs. The postulated (based on comparison with other aromatic amines) conjugated form, *N*-acetoxy-*ortho*-toluidine, is a reactive ester that forms electrophilic arylnitrenium ions that can bind DNA.

Studies of other aromatic amines that cause urinary bladder cancer have shown that during the transport of the *N*-hydroxy-arylamines to the urinary bladder, bioreactive metabolites (nitroso form) can form and bind to hemoglobin in the blood (Skipper and Tannenbaum 1994). Thus, hemoglobin adducts may reflect the amount of amine that an individual takes up from the environment, converts to its proximate carcinogenic form (i.e., *N*-hydroxy-toluene for *ortho*-toluidine), and exports into blood.

Evidence suggesting that this pathway is relevant to humans comes from numerous studies that detected hemoglobin adducts in *ortho*-toluidine-exposed humans (following both occupational and nonoccupational exposures), consistent with studies in experimental animals. Hemoglobin adducts are thought to be formed from the *ortho*-toluidine metabolite, *ortho*-nitrosotoluene, which also causes urinary bladder cancer in rats (Hecht et al. 1982).

#### 6.1.3.2. Metabolic Activation Leading to Oxidative DNA Damage

Other activation pathways for aromatic amines include peroxidase-catalyzed reactions that form quinone imines from non-conjugated phenolic metabolites. The urinary bladder, a primary target for carcinogenic aromatic amines, contains high peroxidase activity. Semiquinone/quinone redox cycling of unconjugated phenolic metabolites in the transitional epithelium can produce reactive oxygen species (ROS), oxidative cellular damage and compensatory cell proliferation. Support for this mechanism comes from studies of (1) *ortho*-toluidine (via its metabolites) induced oxidative DNA damage in cultured cells (HL-60), DNA or DNA fragments from key genes (*c-Ha-ras* oncogene and the *p53* tumor-suppressor gene) thought to be involved in carcinogenesis (Ohkuma et al. 1999; Watanabe et al. 2010) and (2) *ortho*-toluidine induced DNA damage

(strand breaks) in cultured human urinary bladder cells and urinary bladder cells from rats and mice exposed to *ortho*-toluidine.

### 6.1.3.3. Genetic and Related Effects

Overall, the extensive data on genetic and related effects of *ortho*-toluidine indicate that it can cause DNA and chromosomal damage and induce mutations. Although *ortho*-toluidine is a weak mutagen in bacteria, it causes mutations in human lymphocytes and cell transformation in rodent cells in vitro, and two *ortho*-toluidine metabolites (*N*-hydroxy-*ortho*-toluidine and *ortho*-nitrosotoluene) are potent mutagens in bacteria with mammalian metabolic activation. DNA adducts have been detected in liver (similar to other aromatic amines) and nasal (but not urinary bladder) tissue from exposed rats. *ortho*-Toluidine-releasing DNA adducts have been reported in human urinary bladder tumors and in normal human urinary bladder tissue (presumably as a result of background environmental exposure albeit covalent binding was not demonstrated (see Section 5.)). Exposure to *o*-toluidine also caused DNA damage in other tissues in addition to the urinary bladder including bladder, kidney, colon, and stomach in exposed rats, and liver, lung, stomach, and brain in exposed mice.

There is evidence that *ortho*-toluidine also causes large-scale chromosome damage in yeast and mammalian cells (e.g., deletions, insertions, translocations, and/or aneuploidy). Exposure to aromatic amines has been shown to induce chromosomal instability in genetically stable urinary bladder cancer cells. Chromosomal instability is associated with aneuploidy and loss of heterozygosity. Aneuploidy is a common feature of bladder cancer in humans and loss of heterozygosity is an important mechanism for inactivation of tumor-suppressor genes.

## 6.2. Other Tumor Sites

In addition to urinary bladder neoplasms, exposure to *ortho*-toluidine also caused increases in the incidence of (malignant or combined malignant and benign) tumors in the blood vessels of male and female mice, liver of female mice, subcutaneous tissue in male and female rats, and connective tissue and mesothelium in male rats (see Section 4). It also caused benign tumors of the mammary gland in rats of both sexes. Few mechanistic data are available for most of these tumor sites; and no compelling information was found to disregard the relevance to humans. In the liver, metabolic activation to reactive metabolites is a key step in aromatic amine-induced liver neoplasms; *N*-acetylation is an activating reaction in the liver. NAT enzyme activity for other aromatic amines in rat liver cytosol was about an order of magnitude lower than in mouse liver cytosol and may explain the greater susceptibility to liver cancer in mice (Lower and Bryan 1973). *ortho*-Toluidine caused single-strand breaks in exposed mice, suggesting that DNA damage might play a role in *ortho*-toluidine carcinogenicity. In addition, hemoglobin adducts have been reported in exposed mice, suggesting the role of reactive (hydroxylated) metabolites.

Several studies also examined the unique splenotoxic effects of *ortho*-toluidine and related compounds, and the evidence suggests that splenic tumors in rats result from a secondary response at cytotoxic doses associated with erythrocyte damage, vascular congestion, hemosiderin deposition, iron overload, hemorrhage, hyperplasia, and fibrosis. No mechanistic data were found for tumors at other sites.

### **6.3. NTP's Listing Recommendation**

*ortho*-Toluidine is known to be a human carcinogen based on sufficient evidence from studies in humans in concert with (1) sufficient evidence from cancer studies in animals, including site concordance for cancer in female and male rats and (2) mechanistic data demonstrating biological plausibility in humans.

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## Abbreviations

<sup>1</sup> H NMR:	proton nuclear magnetic resonance
4-AMP:	4-amino-3-methylphenol
4-COT:	4-chloro- <i>ortho</i> -toluidine
8-OH-dG:	8-hydroxydeoxyguanosine
AAF:	2-acetylaminofluorene
ACGIH:	American Conference of Governmental Industrial Hygienists
ADME:	absorption, distribution, metabolism, and excretion
ALL:	acute lymphoblastic leukemia
CA:	chromosomal aberration
CASRN:	Chemical Abstracts Service registry number
CDR:	Chemical Data Reporting Rule
CHO:	Chinese hamster ovary
CIN:	chromosomal instability
dA:	deoxyadenosine
DEN:	diethylnitrosamine
dG:	deoxyguanosine
DNA:	deoxyribonucleic acid
EG:	ethylguanine
EHOMO:	energy of the highest occupied molecular orbital
Endo III:	endonuclease III
EPA:	Environmental Protection Agency
FDA:	Food and Drug Administration
FU:	follow-up
G:	guanine
GC/MS:	gas chromatography/mass spectroscopy
GI:	gastrointestinal
GIS:	Geographic Information System
GM:	geometric mean
Hb:	hemoglobin
HDAC:	histone deacetylase
HEG:	(2-hydroxyethyl) guanine

HGPRT:	hypoxanthine-guanine phosphoribosyl transferase
HHE:	Health Hazard Evaluation
HIC:	highest ineffective concentration
HID:	highest ineffective dose
HPLC:	high-performance liquid chromatography
hr:	hour
HWE:	healthy worker (hire or survival) effect
I:	inconclusive
i.p.:	intraperitoneal
i.v.:	intravenous
IARC:	International Agency for Research on Cancer
ICD-9:	International Classification of Diseases, Ninth Revision
IDLH:	immediately dangerous to life and health
IUR:	Inventory Update Rule
JEM:	job-exposure matrix
kg:	kilogram
L:	liter
LEC:	lowest effective concentration
LED:	lowest effective dose
Log $K_{ow}$ :	logarithm of octanol/water partition coefficient
LOH:	loss of heterozygosity
m <sup>3</sup> :	cubic meter
MBT:	2-mercaptobenzothiazole
MG:	methylguanine
mg:	milligram
mL:	milliliter
MN:	micronucleus
MS:	mass spectrometry
N.D.:	not detected; not determined
NA:	not applicable
NAT1:	<i>N</i> -acetyltransferase 1
NAT2:	<i>N</i> -acetyltransferase 2
NCE:	normochromatic erythrocyte

NDMA:	<i>N</i> -nitrosodimethylamine
NHANES:	National Health and Nutrition Examination Survey
NIOSH:	National Institute for Occupational Safety and Health
NLM:	National Library of Medicine
NOES:	National Occupational Exposure Survey
NPL:	National Priorities List
NR:	not reported; none reported
NS:	not significant
nt:	nucleotides
NT:	not tested
<i>o</i> -NT:	<i>o</i> -nitrosotoluene
OR:	odds ratio
OSHA:	Occupational Safety and Health Administration
OTM:	olive tail moment
PBN:	phenyl- $\beta$ -naphthylamine
PCE:	polychromatic erythrocyte
PCNA:	proliferating cell nuclear antigen
PEL:	permissible exposure limit
PGST:	placental glutathione <i>S</i> -transferase
p.o.:	per os (oral administration)
ppm:	parts per million
QSAR:	quantitative structure-activity relationship
RBC:	red blood cell
RLV:	Rauscher-leukemia virus
ROS:	reactive oxygen species
RQ:	reportable quantity
RR:	relative risk
SA7:	Simian Agent 7 adenovirus
SAFE:	significance analysis of function and expression
SCE:	sister-chromatid exchange
SIC:	Standard Industrial Classification
SIR:	standardized incidence ratio
SMR:	standardized mortality ratio

SOCMI:	synthetic organic chemical manufacturing industry
SOD:	superoxide dismutase
SPA:	solid phosphoric acid
SRR:	standardized rate ratio
SSB:	single strand break
TDS:	Total Diet Study
TL:	tail length
TLC:	thin-layer chromatography
TLV-TWA:	threshold limit value – time-weighted average
TRI:	Toxics Release Inventory
TSCA:	Toxic Substances and Recovery Act
TSFE:	time since first employment
UDS:	unscheduled DNA synthesis
UK:	United Kingdom
WBC:	white blood cell
wt%:	weight percent
µg:	microgram

## Glossary

**Accelerant:** A substance that increases the speed of curing of rubber.

**Ames assay:** The Ames *Salmonella*/microsome mutagenicity assay is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations.

**Aneuploidy:** An abnormality involving a chromosome number that is not an exact multiple of the haploid number (one chromosome set is incomplete).

**Arabinose resistance:** The L-arabinose resistance test with *Salmonella typhimurium* (Ara test) is a forward mutation assay that selects a single phenotypic change (from L-arabinose sensitivity to L-arabinose resistance) in a unique tester strain (an *araD* mutant).

**Ascertainment bias:** Systematic failure to represent equally all classes of cases or persons supposed to be represented in a sample.

**Attrition bias:** Systematic differences between **comparison groups** in withdrawals or exclusions of **participants** from the results of a study.

**Biexponential process:** A process of drug (or xenobiotic) clearance with two phases with different rates. The first phase often involves rapid distribution of a drug to peripheral tissues, while the second phase represents clearance mechanisms that eliminate the drug from the body. (See “Two-compartment pharmacokinetic model.”)

**Biodegradation:** Biotransformation; the conversion within an organism of molecules from one form to another. A change often associated with change in pharmacologic activity.

**Boiling point:** The boiling point of the anhydrous substance at atmospheric pressure (101.3 kPa) unless a different pressure is stated. If the substance decomposes below or at the boiling point, this is noted (dec). The temperature is rounded off to the nearest °C.

**Colour index:** A compendium of dyes and pigments prepared in the United Kingdom by the Society of Dyers and Colourists and in the United States by the American Association of Textile Chemists and Colorists. The index serves as a common reference database of information on generic names, chemical structures, companies offering dyes and pigments, and technical application details of many colorants.

**Comet assay:** single cell gel electrophoresis for assessment of DNA damage in presumptive target tissues.

**Critical temperature:** The temperature at and above which a gas cannot be liquefied, no matter how much pressure is applied.

**Differential selection:** Selective pressure for self-renewal. Gene mutations that confer a growth or survival advantage on the cells that express them will be selectively enriched in the genome of tumors.

**Disposition:** The description of absorption, distribution, metabolism, and excretion of a chemical in the body.



**Double acid conjugate:** a compound formed by the joining of two acids.

**Epigenetic mechanisms:** Changes in gene function that do not involve a change in DNA sequence but are nevertheless mitotically and/or meiotically heritable. Examples include DNA methylation, alternative splicing of gene transcripts, and assembly of immunoglobulin genes in cells of the immune system.

**Genomic instability:** An increased propensity for genomic alterations that often occurs in cancer cells. During the process of cell division (mitosis) the inaccurate duplication of the genome in parent cells or the improper distribution of genomic material between daughter cells can result from genomic instability.

**Healthy worker hire effect:** Initial selection of healthy individuals at time of hire so that their disease risks differ from the disease risks in the source (general) population.

**Healthy worker survival effect:** A continuing selection process such that those who remain employed tend to be healthier than those who leave employment.

**Henry's Law constant:** The ratio of the aqueous-phase concentration of a chemical to its equilibrium partial pressure in the gas phase. The larger the Henry's law constant the less soluble it is (i.e., greater tendency for vapor phase). The relationship is defined for a constant temperature, e.g., 25°C.

**Host-mediated assay:** This assay evaluates the genotoxicity of a substance to microbial cells introduced (e.g., by intravenous injection) into a host animal. The host animal receives the test compound orally and therefore acts as a source of chemical metabolism, distribution, and excretion of the test compound.

**Keratosis:** A localized horny overgrowth of the skin, such as a wart or callus.

**Loss of heterozygosity:** If there is one normal and one abnormal allele at a particular locus, as might be seen in an inherited autosomal dominant cancer susceptibility disorder, loss of the normal allele produces a locus with no normal function. When the loss of heterozygosity involves the normal allele, it creates a cell that is more likely to show malignant growth if the altered gene is a tumor suppressor gene.

**Melting point:** The melting point of the substance at atmospheric pressure (101.3 kPa). When there is a significant difference between the melting point and the freezing point, a range is given. In case of hydrated substances (i.e., those with crystal water), the apparent melting point is given. If the substance decomposes at or below its melting point, this is noted (dec). The temperature is rounded off to the nearest °C.

**Metabolic activation:** The chemical alteration of an exogenous substance by or in a biological system. The alteration may inactivate the compound, or it may result in the production of an active metabolite of an inactive parent compound.

**Metaplasia:** A change of cells to a form that does not normally occur in the tissue in which it is found.

**Methemoglobin:** A form of hemoglobin found in the blood in small amounts. Unlike normal hemoglobin, methemoglobin cannot carry oxygen. Injury or certain drugs, chemicals, or foods

may cause a higher-than-normal amount of methemoglobin to be made. This causes a condition called methemoglobinemia.

**Micronuclei:** Small nuclei separate from, and additional to, the main nucleus of a cell, produced during the telophase of mitosis or meiosis by lagging chromosomes or chromosome fragments derived from spontaneous or experimentally induced chromosomal structural changes.

**Miscible:** A physical characteristic of a liquid that forms one liquid phase with another liquid (e.g., water) when they are mixed in any proportion.

**Molecular weight:** The molecular weight of a substance is the weight in atomic mass units of all the atoms in a given formula. The value is rounded to the nearest tenth.

**Mutations:** A change in the structure of a gene, resulting from the alteration of single base units in DNA, or the deletion, insertion, or rearrangement of larger sections of genes or chromosomes. The genetic variant can be transmitted to subsequent generations.

**National Priorities List:** The list of national priorities among the known releases or threatened releases of hazardous substances, pollutants, or contaminants throughout the United States and its territories. The NPL is intended primarily to guide the EPA in determining which sites warrant further investigation.

**Non-differential misclassification:** The probability of erroneous classification of an individual, a value, or an attribute into a category other than that to which it should be assigned is the same in all study groups.

**Norharman:** A beta-carboline widely distributed in the environment.

**Normochromatic erythrocyte:** A mature erythrocyte that lacks ribosomes and can be distinguished from immature, polychromatic erythrocytes by stains selective for RNA.

**Osmotic mini pump:** A miniature implantable infusion pump that is used to continuously infuse laboratory animals with a drug or other material. Absorption of water from surrounding tissues by osmosis through an outer rigid shell provides the means by which the material is forced out of a collapsible internal chamber at a constant rate.

**Personal protective equipment:** Specialized clothing or equipment, worn by an employee to minimize exposure to a variety of hazards. Examples of PPE include such items as gloves, foot and eye protection, protective hearing devices (earplugs, muffs) hard hats, respirators, and full body suits.

**Plate incorporation:** A commonly used procedure for performing a bacterial reverse mutation test. Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate-incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. After two or three days of incubation, revertant colonies are counted and compared with the number of spontaneous revertant colonies on solvent control plates.

**Poly-3 trend test:** A survival-adjusted statistical test that takes survival differences into account by modifying the denominator in the numerical (quantal) estimate of lesion incidence to reflect more closely the total number of animal years at risk.

**Polychromatic erythrocyte:** A newly formed erythrocyte (reticulocyte) containing RNA.

**Proto-oncogene:** A gene involved in normal cell growth. Mutations (changes) in a proto-oncogene may cause it to become an oncogene, which can cause the growth of cancer cells.

**p<sub>trend</sub>:** Level of statistical significance of a change over time in a group selected to represent a larger population.

**S<sub>9</sub> metabolic activation:** The chemical alteration of an exogenous substance by or in a biological system. The alteration may inactivate the compound, or it may result in the production of an active metabolite of an inactive parent compound.

**Selection bias:** An error in choosing the individuals or groups to take part in a study. Ideally, the subjects in a study should be very similar to one another and to the larger population from which they are drawn (for example, all individuals with the same disease or condition). If there are important differences, the results of the study may not be valid.

**Sister-chromatid exchange:** The exchange during mitosis of homologous genetic material between sister chromatids; increased as a result of inordinate chromosomal fragility due to genetic or environmental factors.

**Solubility:** The ability of a substance to dissolve in another substance and form a solution. The Report on Carcinogens uses the following definitions (and concentration ranges) for degrees of solubility: (1) *miscible* (see definition), (2) *freely soluble*- capable of being dissolved in a specified solvent to a high degree (>1,000 g/L), (3) *soluble*- capable of being dissolved in a specified solvent (10–1,000 g/L), (4) *slightly soluble*- capable of being dissolved in a specified solvent to a limited degree (1–10 g/L), and (5) *practically insoluble*- incapable of dissolving to any significant extent in a specified solvent (<1 g/L).

**Specific gravity:** The ratio of the density of a material to the density of a standard material, such as water at a specific temperature; when two temperatures are specified, the first is the temperature of the material and the second is the temperature of water.

**Spot test:** Qualitative assay in which a small amount of test chemical is added directly to a selective agar medium plate seeded with the test organism, e.g., *Salmonella*. As the chemical diffuses into the agar, a concentration gradient is formed. A mutagenic chemical will give rise to a ring of revertant colonies surrounding the area where the chemical was applied; if the chemical is toxic, a zone of growth inhibition will also be observed.

**Steric bulk:** An indicator of the stability of the spatial arrangement of atoms in a molecule.

**Toxicokinetics:** The mathematical description (toxicokinetic models) of the time course of disposition of a chemical in the body.

**TOXMAP:** A Geographic Information System from the National Library of Medicine that uses maps of the United States to help users visually explore data from EPA's TRI and Superfund programs.

**Transitions:** DNA nucleotide substitution mutation in which a purine base is substituted for another purine base (adenine → guanine or guanine → adenine) or a pyrimidine base for another pyrimidine base (cytosine → thymine or thymine → cytosine).

**Transversions:** DNA nucleotide substitution mutation in which a purine base (adenine or guanine) is substituted for a pyrimidine base (cytosine or thymine) or vice versa.

**Tunica vaginalis:** The serous membranous covering of the testis.

**Two-compartment pharmacokinetic model:** A two-compartment pharmacokinetic model resolves the body into a central compartment and a peripheral compartment. The central compartment generally comprises tissues that are highly perfused such as heart, lungs, kidneys, liver, and brain. The peripheral compartment comprises less well-perfused tissues such as muscle, fat, and skin. A two-compartment model assumes that, following drug administration into the central compartment, the drug distributes between that compartment and the peripheral compartment. However, the drug does not achieve instantaneous distribution (i.e., equilibrium), between the two compartments. After a time interval ( $t$ ), distribution equilibrium is achieved between the central and peripheral compartments, and elimination of the drug is assumed to occur from the central compartment.

**Type-I error:** The error of rejecting a true null hypothesis, i.e., declaring that a difference exists when it does not.

**Type-II error:** The error of failing to reject a false null hypothesis, i.e., declaring that a difference does not exist when in fact it does.

**Vapor density, relative:** A value that indicates how many times a gas (or vapor) is heavier than air at the same temperature. If the substance is a liquid or solid, the value applies only to the vapor formed from the boiling liquid.

**Vapor pressure:** The pressure of the vapor over a liquid (and some solids) at equilibrium, usually expressed as mm Hg at a specific temperature ( $^{\circ}\text{C}$ ).

## Appendix A. Literature Search Strategy

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This document identifies the data sources, search terms, and search strategies that were used to identify literature for the draft monograph on *ortho*-toluidine (CASRN 95-53-4). The literature search strategy used for *ortho*-toluidine involved several approaches designed to identify potentially useful information for the broad range of topics covered by a Report on Carcinogens (RoC) monograph, as listed below.

- Properties and Human Exposure (focusing on the U.S. population)
- Disposition (ADME) and Toxicokinetics
- Human Cancer Studies
- Studies of Cancer in Experimental Animals
- Mechanistic Data and Other Relevant Effects
- Genetic and Related Effects
- Mechanistic Considerations

The methods for identifying the relevant literature for the draft *ortho*-toluidine monograph including (1) the search strategy, (2) updating the literature search, and (3) review of citations using web-based systematic review software are illustrated in Figure A-1 and discussed below. The detailed literature search strategy, including all database sources, and exclusion/inclusion criteria, are available at <http://ntp.niehs.nih.gov/go/37898>.

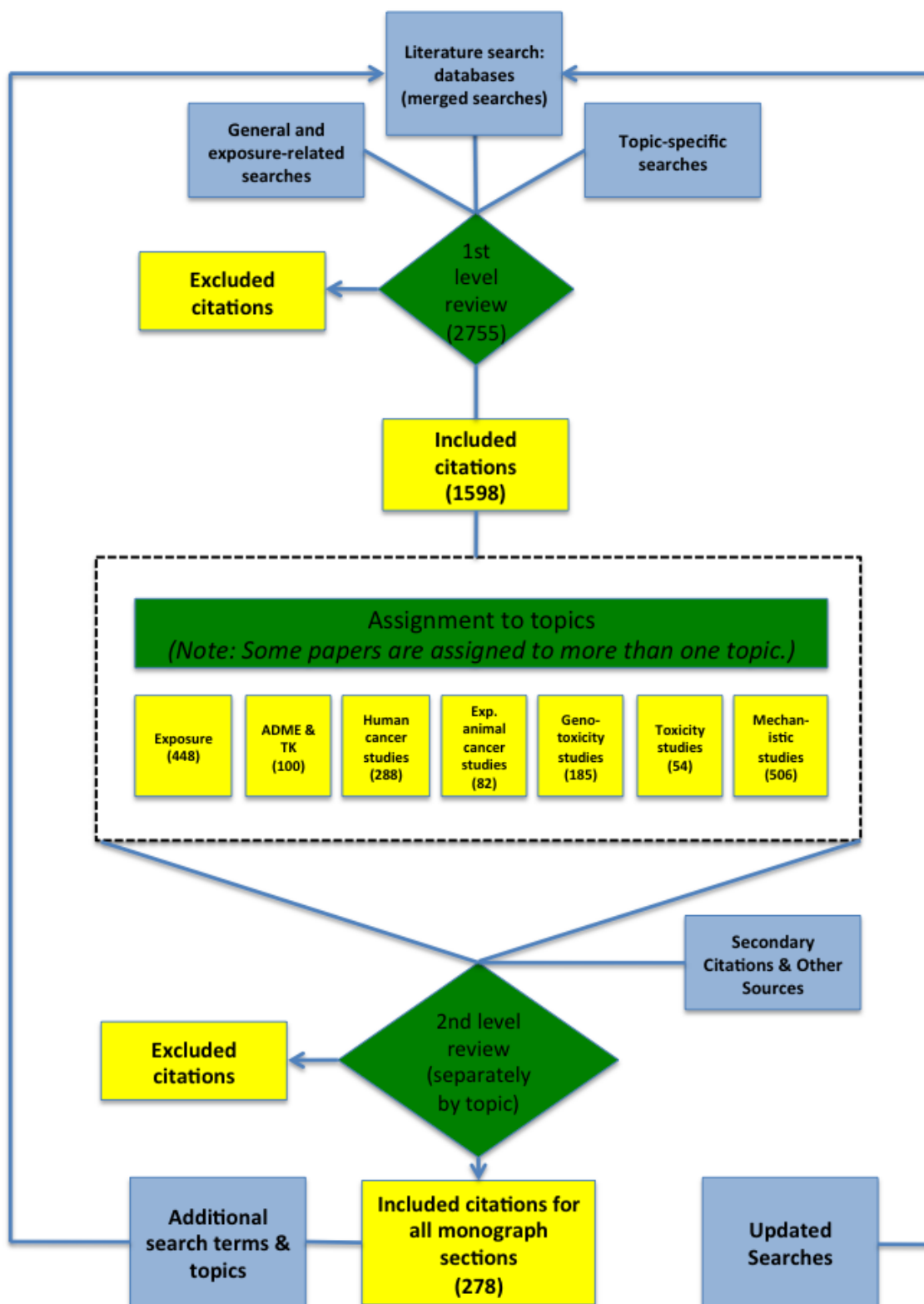


Figure A-1. Literature Search Strategy and Review

## A.1. Search Strategies

Relevant literature is identified using search terms, data sources, and strategies as discussed below.

- (1) **General data search:** This search covers a broad range of general data sources (see *ortho*-Toluidine Literature Search Strategy, available at <http://ntp.niehs.nih.gov/go/37898>) for information relevant to many or all of the wide range of monograph topics pertaining to *ortho*-toluidine.
- (2) **Exposure-related data search:** This search covers a broad range of potential sources (see *ortho*-Toluidine Literature Search Strategy, available at <http://ntp.niehs.nih.gov/go/37898>) for exposure-related information and physical-chemical properties.
- (3) **Database searches in PubMed, Scopus, and Web of Science:** The majority of the primary literature used to draft the *ortho*-toluidine monograph was identified from searches of these three extensive databases available through the NIEHS Library. Synonyms, metabolites, and the chemical class for *ortho*-toluidine were identified from the sources listed in Table A-1 and the search terms are listed in Table A-2. The substance search terms were combined with the search terms for each of the monograph topics listed above to create the specific literature searches. See Table A-2 for details on this approach and Table A-3 for topic-specific search terms. Searches for human cancer studies are somewhat unique because they involve the identification of search terms for exposure scenarios that might result in exposure of people to *ortho*-toluidine. For *ortho*-toluidine, these exposure-related search terms were based on its use in the manufacture of dyes, rubber chemicals, and an herbicide intermediate, and those search terms were combined with search terms specific for human cancer (see Table A-2 and Table A-3).
- (4) **QUOSA library of occupational case-control studies** search of the QUOSA-based library of approximately 6,000 occupational case-control studies, approximately 60% of which are currently available as searchable full-text pdfs, was conducted using the synonyms “o-toluidine,” “ortho-toluidine,” “95-53-4,” “2-toluidine,” “1-amino-2-methylbenzene,” “2-amino-1-methylbenzene,” “2-aminotoluene,” “o-aminotoluene,” “ortho-aminotoluene,” “2-methyl-1-aminobenzene,” “2-methylaniline,” “o-methylaniline,” “ortho-methylaniline,” “o-methylbenzenamine,” “ortho-methylbenzenamine,” “2-methylphenylamine,” “2-tolylamine,” “o-tolylamine,” “ortho-tolylamine.”
- (5) **Special topic-focused searches:** No special topic-focused searches were conducted for *ortho*-toluidine, but searches for general information on potential confounders were conducted.
- (6) **Secondary sources:** Citations identified from authoritative reviews or from primary references located by literature search, together with publications citing key papers identified using the Web of Science “Cited Reference Search,” were also added.

Because *ortho*-toluidine has been listed in the Report on Carcinogens as *reasonably anticipated to be a human carcinogen* since 1983, searches for studies of cancer in experimental animals



were limited to studies published since 1982. The monograph relies primarily on authoritative reviews for information on exposure, toxicokinetics, and genotoxicity, but literature searches were conducted to update that information from the date of the most recent comprehensive review by IARC (Working group met in 2008, literature searches were conducted from 2007).

## A.2. Updating the Literature Search

The literature searches will be updated prior to submitting the draft monograph for peer review and prior to finalizing the monograph. Monthly search alerts for *ortho*-toluidine synonyms, metabolites, chemical class, exposure scenarios (human cancer), and topic-focused searches were created in PubMed, Scopus, and Web of Science, and the results of these searches from the closing date of the initial search will be downloaded for review.

## A.3. Review of Citations Using Web-based Systematic Review Software

Citations retrieved from literature searches were uploaded to web-based systematic review software and screened using inclusion and exclusion criteria. Multi-level reviews of the literature were conducted, with initial reviews (Level 1) based on titles and abstracts only to identify citations that could be excluded and to assign the included literature to one or more monograph topics; subsequent reviews (Level 2) for literature assigned to the various monograph topics (Exposure, ADME & TK, Human cancer studies, etc.) were based on full-text (i.e., PDFs) of the papers and were carried out by the writer and scientific reviewer for each monograph section. Two reviewers, at least one of whom is a member of the ORoC at NIEHS, participated at each level of review. The inclusion/exclusion criteria are available in the *ortho*-Toluidine Literature Search Strategy document, available at <http://ntp.niehs.nih.gov/go/37898>.

**Table A-1. Data Sources for *ortho*-Toluidine Searches**

Information Type	Data Sources
Synonyms	National Library of Medicine databases (e.g., ChemIDplus, Hazardous Substances Data Bank)
Metabolites	Son et al. (1980)

**Table A-2. Literature Search Approach for *ortho*-Toluidine**

Substance	Search Terms	Topics (Combined with) <sup>a</sup>
<i>o</i> -Toluidine synonyms	<i>o</i> -toluidine, <i>o</i> -toluidine, 95-53-4, 2-toluidine, 1-amino-2-methylbenzene, 2-amino-1-methylbenzene, 2-aminotoluene, <i>o</i> -aminotoluene, <i>o</i> -aminotoluene, 2-methyl-1-aminobenzene, 2-methylaniline, <i>o</i> -methylaniline, <i>o</i> -methylaniline, <i>o</i> -methylbenzenamine, <i>o</i> -methylbenzenamine, 2-methylphenylamine, 2-tolylamine, <i>o</i> -tolylamine, <i>ortho</i> -tolylamine	Human exposure Toxicokinetics Human cancer studies Cancer studies in experimental animals Genotoxicity Toxicity Mechanism

RoC Monograph on *ortho*-Toluidine

Substance	Search Terms	Topics (Combined with) <sup>a</sup>
<i>o</i> -Toluidine metabolites and their synonyms	Anthranilic acid, <i>N</i> -acetyl-anthranilic acid, <i>N</i> -acetyl- <i>o</i> -toluidine, <i>N</i> -acetyl- <i>o</i> -aminobenzyl alcohol, <i>o</i> -azoxytoluene, <i>o</i> -nitrosotoluene, <i>N</i> -hydroxy- <i>o</i> -toluidine, 4-amino-cresyl sulfate, <i>N</i> -acetyl-4-amino- <i>m</i> -cresyl sulfate, 2-amino- <i>m</i> -cresyl sulfate, 4-amino- <i>m</i> -cresyl glucuronide, 4-acetyl-4-amino- <i>m</i> -cresyl-glucuronide, <i>N</i> -acetyl- <i>o</i> -aminobenzyl glucuronide	Human cancer studies Cancer studies in experimental animals (for the mechanistic section) Genotoxicity Toxicity Mechanism
Chemical class synonyms	Monocyclic aromatic amines, arylamines, alkylanilines	Cancer studies in experimental animals (for the mechanistic section) Genotoxicity Toxicity Mechanism
Exposure scenarios (Dye industry, rubber chemical manufacturing, and herbicide manufacturing)	(dyestuff OR (dye AND (manufacturing OR manufacture))) OR rubber chemicals OR <i>o</i> toluidine OR <i>o</i> -toluidine OR chloro- <i>o</i> -toluidine OR chloro- <i>o</i> -toluidine OR aniline OR ((manufacture OR manufacturing OR production) AND magenta) OR metolachlor OR acetochlor	Human cancer studies

<sup>a</sup>Search terms for each of these topics were developed in consultation with an informational specialist.

**Table A-3. Search Terms for Monograph Topics for *ortho*-Toluidine**

Monograph Topic	Search Terms Used in PubMed, Scopus, and Web of Science	MeSH Terms Used in PubMed
Exposure	exposure OR occurrence OR oral OR dermal OR air OR water OR food OR soil OR environmental pollut* OR environmental exposure* OR occupational exposure*	(“Environmental Pollutants” [MeSH] OR “Environmental Pollution” [MeSH])
ADME/ Toxicokinetics	<i>Toxicokinetic search terms</i> - administration OR absorption OR distribution OR tissue distribution OR bioavailab* OR biological availability OR metaboli* OR biotransform* OR activat* OR bioactivat* OR detoxif* OR excret* OR clearance OR eliminat* OR kinetic* OR pharmacokinetic* OR toxicokinetic* OR cytochrome P450  <i>Combine with AND</i>  <i>Animal study search terms</i> - in vivo OR animal* OR mouse OR mice OR rat OR hamster OR guinea pig OR rabbit OR monkey OR dog	<i>Toxicokinetic search terms</i> - "Pharmacokinetics"[Mesh] OR "Metabolism"[Mesh] OR "Cytochrome P450 Enzyme System"[Mesh]
Human Cancer	<i>Cancer search terms</i> - cancer OR mortality OR follow-up OR incidence)  <i>Combine with AND</i>  <i>Epidemiology search terms</i> - epidemiologic* OR workers OR case-control OR cohort OR case-report OR case-series	None

RoC Monograph on *ortho*-Toluidine

Monograph Topic	Search Terms Used in PubMed, Scopus, and Web of Science	MeSH Terms Used in PubMed
Animal Tumors	<p><i>Cancer search terms-</i> cancer OR neoplasm* OR carcinogen* OR malignan* OR oncogene* OR tumor* OR tumour*</p> <p><i>Combine with AND</i></p> <p><i>Animal study search terms-</i> animal* OR mouse OR mice OR rat OR hamster OR "guinea pig" OR rabbit OR monkey OR dog</p>	<p><i>Cancer search terms-</i> "Neoplasms"[Mesh]) OR "Carcinogens"[Mesh]</p>
Genotoxicity	<p>genetic toxicology" OR clastogen* OR "DNA strand break*" OR "unscheduled DNA synthesis" OR "UDS" OR aneuploid OR aneuploid* OR polyploid OR polyploid* OR "neoplastic cell transformation" OR "chromosom* aberration*" OR cytogenetic OR cytogenetic* OR "DNA adduct*" OR "DNA damage" OR "DNA repair" OR crosslink* OR "germ-line mutation" OR micronucle* OR mutagen OR mutagen* OR mutation OR mutation* OR oncogen* OR "sister chromatid exchange" OR "SCE" OR "SOS response*" OR "Ames test" OR "gene expression" OR "cell proliferation" OR cytotoxic OR cytotoxic* OR "comet assay"</p>	<p>"DNA Damage"[Mesh] OR "DNA Repair"[Mesh] OR "Mutagens"[Mesh] OR "Mutation"[Mesh] OR "Cytogenetic Analysis"[Mesh] OR "Oncogenes"[Mesh] OR "Mutagenicity Tests"[Mesh]</p>
Toxicity	<p>toxic* OR toxin*OR cytotoxic* OR (nephrotoxic* OR hepatotoxic* OR pneumotoxic* OR thyrotoxic*</p>	<p>"Toxic Actions"[Mesh]) OR "Toxicity Tests"[Mesh]) OR "adverse effects" [Subheading]</p>
Mechanisms of Carcinogenicity	<p>((mode OR mechanism*) AND action) OR (carcinogen OR genetic OR epigenetic OR inhibit* OR promot* OR interact* OR activate* OR detoxific* OR "oxidative damage" OR alkylat* OR adduct)) AND ((animal OR animals OR mouse OR mice OR rat OR hamster OR "guinea pig" OR rabbit OR monkey OR dog OR pig) OR (person* OR people OR individual* OR subject* OR participant*))</p>	-

## Appendix B. Regulations and Guidelines

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## **B.1. Regulations**

### **B.1.1. U.S. Environmental Protection Agency (EPA)**

#### ***B.1.1.1. Clean Air Act***

*National Emission Standards for Hazardous Air Pollutants*: Listed as a hazardous air pollutant.

#### ***B.1.1.2. Comprehensive Environmental Response, Compensation, and Liability Act***

Reportable quantity (RQ) = 100 lb.

#### ***B.1.1.3. Resource Conservation and Recovery Act***

*Listed Hazardous Waste*: Waste codes for which the listing is based wholly or partly on the presence of *o*-toluidine or *o*-toluidine hydrochloride = U222, U328, K112, K113, K114.

Listed as a hazardous constituent of waste.

### **B.1.2. Occupational Safety and Health Administration (OSHA)**

This legally enforceable PEL was adopted from the 1968 ACGIH TLV-TWA shortly after OSHA was established. The PEL may not reflect the most recent scientific evidence and may not adequately protect worker health.

Permissible exposure limit (PEL) = 5 ppm.

Potential for dermal absorption.

## **B.2. Guidelines**

### **B.2.1. American Conference of Governmental Industrial Hygienists (ACGIH)**

Threshold limit value – time-weighted average (TLV-TWA) = 2 ppm.

Potential for dermal absorption.

### **B.2.2. National Institute for Occupational Safety and Health (NIOSH)**

Immediately dangerous to life and health (IDLH) limit = 50 ppm.

Listed as a potential occupational carcinogen.

Potential for dermal absorption.

## Appendix C. Background Exposure Information on Epidemiological Studies

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The available epidemiological studies evaluating potential exposure to *ortho*-toluidine and cancer include workers in industries manufacturing either (1) dyestuffs, (2) synthesis of 4-chloro-*ortho*-toluidine, or (3) certain rubber chemicals (see Section 1.4). These occupational settings also involve potential exposure to other substances. Many of the studies do not have detailed information on exposure to *ortho*-toluidine or other co-exposures; however, some of this information can be inferred from knowledge of the manufacturing process, or dye chemistry. In order to provide background information for interpreting these epidemiological studies, the nature of the potential for exposure to *ortho*-toluidine and to other intermediates or end products in these manufacturing processes is discussed below.

## **C.1. Manufacture of Dyestuffs**

The review of epidemiological studies of dyestuffs manufacture (see Section 3) with potential exposure to *ortho*-toluidine includes four different cohorts ([1] Case et al. (1954) and Case and Pearson (1954), [2] Rubino et al. (1982), [3] Stasik (1988), and [4] Ott and Langner (1983)) that examined the incidence of urinary bladder cancer in relation to manufacturing processes that potentially involved exposure to *ortho*-toluidine. Occupational exposure to *ortho*-toluidine can be inferred from knowledge of dye production processes in dye worker cohort studies where very few or no quantitative data are available on exposure to *ortho*-toluidine (Freeman 2012). In general, exposure to *ortho*-toluidine was more likely to have occurred in manufacturing facilities prior to the adoption of formal regulations regarding building ventilation and personal protective equipment (e.g., adoption of OSHA regulations in the mid-1970s).

### **C.1.1. Aniline Production in Great Britain: Case et al. (1954) and Case and Pearson (1954)**

Case et al. (1954) and Case and Pearson (1954) reported findings of urinary bladder cancer among dyestuff workers, employed from 1910 to 1952 at 21 firms in Great Britain. Although the authors do not mention exposure to *ortho*-toluidine in their study, potential exposure might have occurred among both aniline and magenta manufacturing workers (see below) based on the production processes of the first half of the 20th century. The benzene preparations used in the early part of the 20th century varied in purity and often contained toluene. Crude benzene was marketed in preparations identified based on the percentage that distills before 100°C is reached as 90s (75% benzene, 24% toluene, 1% xylene), 50s (50% benzene, 40% toluene, 10% xylene), and 30s (10% benzene, 60% toluene, 30% xylene) (Cain and Thorpe 1905). When the crude benzene preparations were subjected to the processes of nitration and reduction, a mixture of aniline and toluidines (*ortho*-, *meta*-, and *para*- in the approximate proportions 15:9:1) resulted. The probability of exposure to *ortho*-toluidine among aniline manufacturing workers must be considered as low because of the uncertainty about the degree of purity of the benzene, especially as it might have changed over the 42 years of exposure covered by these studies.

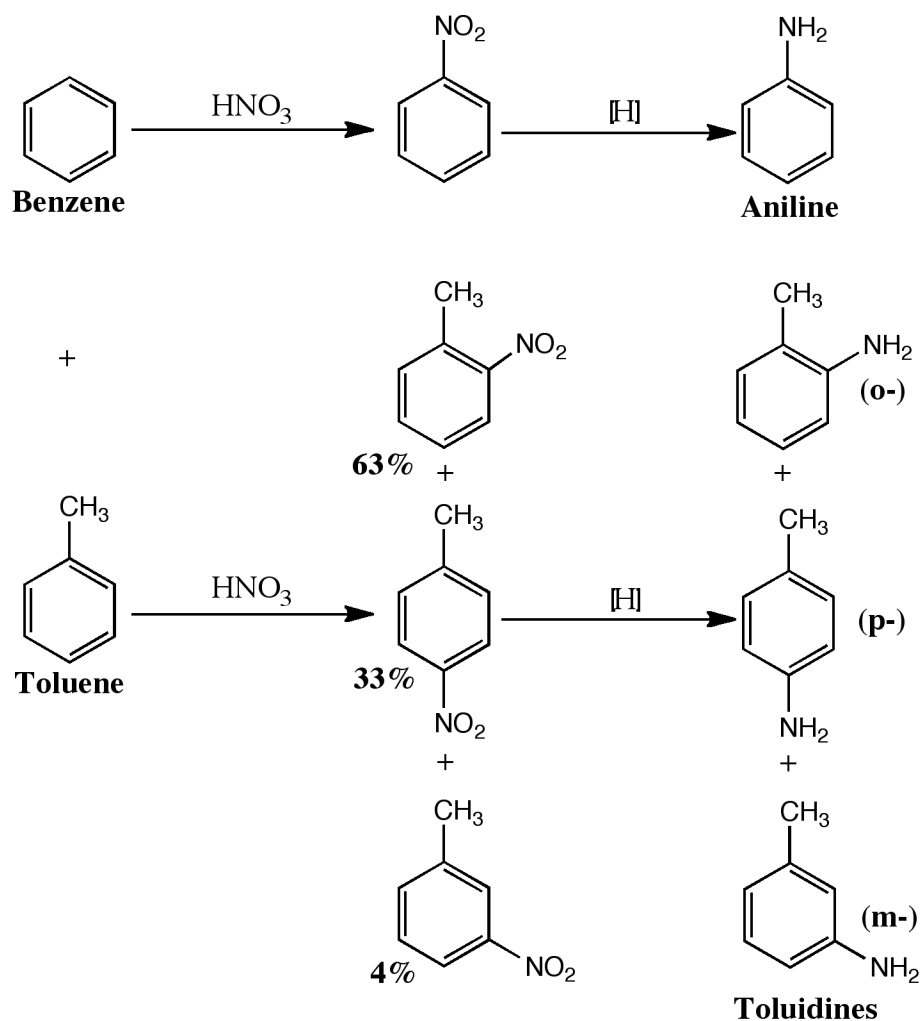
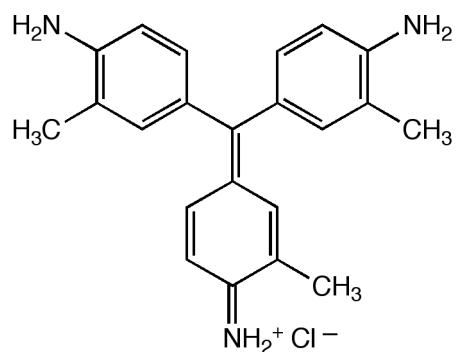


Figure C-1. Synthesis of Aniline from Benzene and Mixed Isomers of Toluidine from Toluene by Successive Nitration and Reduction Reactions

### C.1.2. Magenta Manufacturing in Great Britain: Case et al. (1954), Pira et al. (2010)

The studies of dyestuff manufacture and use workers by Case et al. (1954) and Case and Pearson (1954) covered the period of 1910 to 1952 and included manufacture of magenta and other dyes. (Magenta III, also called “New Fuchsin” or new magenta, III is the product generally produced in the United Kingdom (Figure C-2).





### Magenta III

**Figure C-2. Structure of Magenta III**

In the United Kingdom, the process for manufacturing magenta, resulting mainly in the production of magenta III, involved condensation of *ortho*-toluidine and formaldehyde in the presence of *ortho*-nitrotoluene (Howe 1977). One can reasonably infer that workers in the study by Case et al. (1954) and Case and Pearson (1954) were exposed to *ortho*-toluidine (see Section 3).

A sub-cohort of dyestuff workers engaged in manufacture of fuchsin or *ortho*-toluidine was included in the bladder cancer mortality study reported by Pira et al. (2010) (see Section 3). Earlier studies of dyestuff workers from the same plant were published by Rubino et al. (1982) and Piolatto et al. (1991); the latter publication reported findings for magenta and Safranin T manufacturing workers combined. Safranin T workers had potential co-exposures to 2,5-diaminotoluene and aminoazotoluene; Pira et al. (2010) reported estimates that appear to be specific for *ortho*-toluidine and magenta manufacturing workers. The process for making *ortho*-toluidine and 4,4'-methylene bis(2-methylaniline) as precursors for fuchsin (identified as New Fuchsin by Rubino et al. 1982) are shown in Figure C-3. *ortho*-Toluidine was used as a precursor for the production of New Fuchsin (Figure C-4). Rubino et al. (1982) reported that the *ortho*-toluidine production department was housed in a different building within the factory area from the building in which New Fuchsin was produced. The process for producing the fuchsin dye was described as involving heating a mixture of *ortho*-toluidine, 4,4'-methylene bis(2-methylaniline), and aniline with a step at the end of the process for the recovery of excess *ortho*-toluidine.

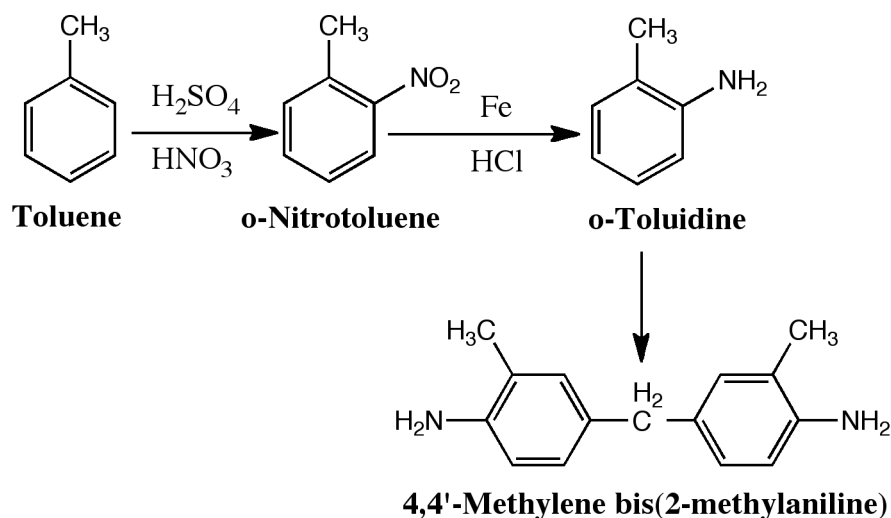


Figure C-3. Synthesis of *ortho*-Toluidine and 4,4'-Methylene bis(2-methylaniline) for Production of Fuchsin (New Fuchsin)

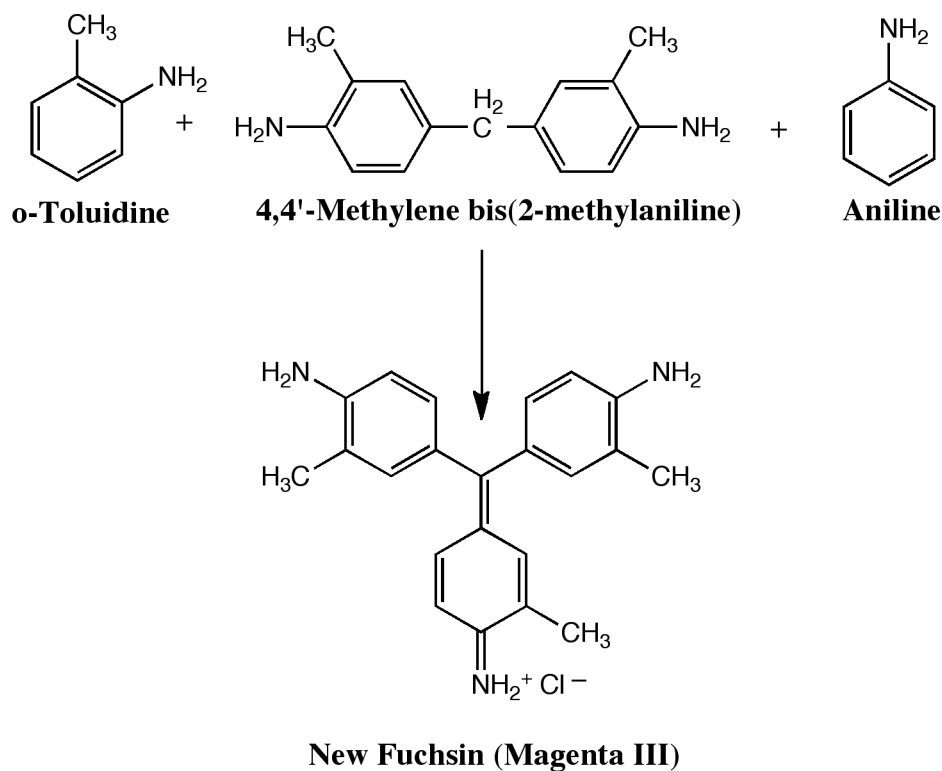


Figure C-4. Synthesis of New Fuchsin from *ortho*-Toluidine, 4,4'-Methylene bis(2-methylaniline), and Aniline

### C.1.3. Manufacture of Dyes and Dye Intermediates in the United States: Ott and Langner (1983)

Ott and Langner reported that U.S. dye production workers employed in the bromindigo and thioindigo production area of a manufacturing facility between 1914 and 1958 were potentially exposed to *ortho*-toluidine. Industrial hygiene measurements taken at the plant during the mid-

1940s for *ortho*-toluidine in breathing zone or area samples were consistently <0.5 ppm, and results of medical surveillance that included measurement of *ortho*-toluidine in urine ranged from less than the limit of detection of 0.3 ppm up to 1.7 ppm. *ortho*-Toluidine was included in a list of raw materials and intermediate products for production of thioindigo, which consisted of four alternately run batch processes involving manufacture of the following dyes: thioindigo (pink B), scarlet G, red 3B, and red 2B; however, no other details on the chemistry of these processes were reported that would indicate which process(es) involved exposure to *ortho*-toluidine. *ortho*-Toluidine might have been used to make intermediates, e.g., anthranilic acid or *ortho*-nitrobenzoic acid, that can be used in the manufacture of thioindigoid dyes (Freeman 2012). In addition to *ortho*-toluidine, raw materials and intermediate products for the four thioindigo processes included 1,2 dihydroacenaphthylamine, sodium nitrite, chloroacetic acid, 4-chloro-*ortho*-toluidine, 4-chloro-acetyl-*ortho*-toluidine, thioindoxyl, chlorosulfonic acid, ethanol, and 2-aminobenzoic acid.

## C.2. Manufacture of Other Intermediates

The chemical intermediate 4-chloro-*ortho*-toluidine (4-COT) can be used in the production of dyes and pigments and of the pesticide chlordimeform. Stasik (1988) studied a facility in Germany that produced and processed 4-COT, but the author did not report the end use for that product. Possible exposure to *ortho*-toluidine existed for a sub-cohort of 4-chloro-*ortho*-toluidine production and processing workers from that cohort (see Section 3). 4-Chloro-*ortho*-toluidine is produced by acetylation of *ortho*-toluidine to yield *N*-acetyl-*ortho*-toluidine, followed by chlorination to yield chloro-*N*-acetyl-*ortho*-toluidines, which are hydrolyzed to yield 4-chloro-*ortho*-toluidine (see Figure C-5). 6-chloro-*ortho*-toluidine originates from chlorination in the 6-position of *N*-acetyl-*ortho*-toluidine (simultaneous with chlorination in the 4 position) and carries over to the hydrolysis step, creating 6-chloro-*ortho*-toluidine as a by-product (Freeman 2012).

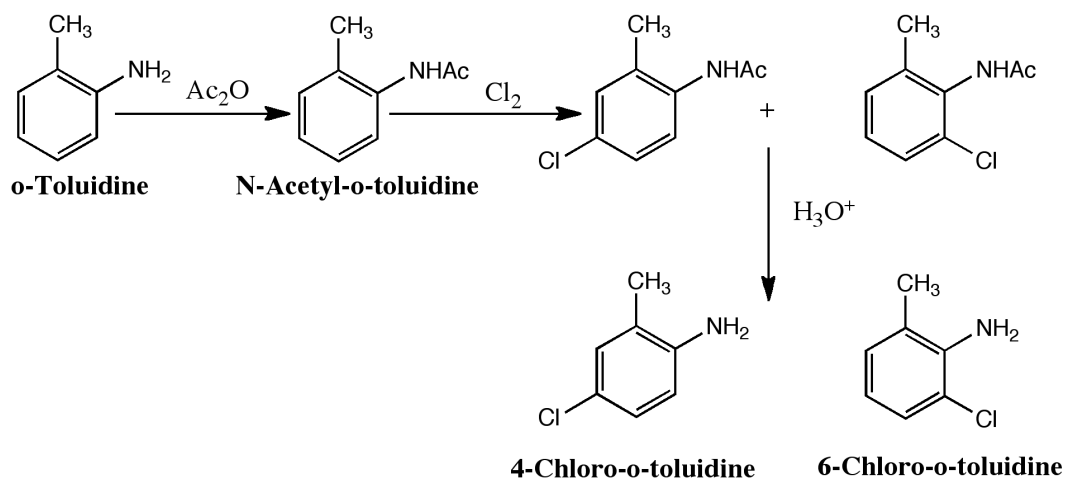


Figure C-5. Chemistry Associated with Synthesis of 4-Chloro-*ortho*-toluidine

## C.3. The Rubber Chemical Industry and Exposure to *ortho*-Toluidine

The major source of information on exposure to *ortho*-toluidine in the rubber chemical production industry is from the cohort studied by Carreón et al. (2014) and earlier by Carreón et

al. (2010) and Ward et al. (1991) (see Section 3). Exposure data for the workers in this cohort have been reported by Ward et al. (1996), Ruder et al. (1992), Teass et al. (1993) and Hanley et al. (2012). The exposure data reported by Hanley et al. (2012) are discussed below. Additional information was obtained from the studies by Sorahan and Pope (1993), Sorahan et al. (2000), and Sorahan (2008) of the rubber industry in the United Kingdom.

### **C.3.1. Manufacture of a Rubber Antioxidant in the United States: Hanley et al. (2012)**

The subcohort studied by Carreón et al. (2014; 2010) (Section 3) was also the subject of a retrospective exposure assessment, which was described in the publication by Hanley et al. (2012). Workers were engaged (1) from 1957 onwards in the production of rubber antioxidants from *ortho*-toluidine, aniline, hydroquinone, toluene or xylene, and a proprietary catalyst; phenol and diphenylamine were the reaction byproducts, and (2) from the mid-1950s to either 1970 or 1994, depending on the product, the production of accelerants from nitrobenzene, aniline, and benzothiazole. 4-aminobiphenyl was a suspected contaminant in some of the process chemicals and was found in trace amounts (<1 ppm) in 3 of 9 bulk samples of process chemicals (Ward et al. 1991). Both antioxidants and accelerators were produced and packaged in parallel operations in the same rooms of the plant's rubber chemicals department (Hanley et al. 2012). The highest breathing zone exposures to *ortho*-toluidine were measured for the antioxidant process and for maintenance (see Figure 1-4), with measurements for all departments ranging from 0.020 to 0.37 ppm (N = 45). The geometric mean for company-collected TWA breathing zone samples from the rubber chemicals department (see Figure 1-5) were highest in 1976 to 1979 (0.1 ppm, N = 30), and declined to 0.015 ppm (N = 200) in 1980 to 1994 and 0.0028 ppm (N = 127) in 1995 to 2004. Ambient air levels of aniline in the rubber chemicals were approximately half of those for *ortho*-toluidine, and nitrobenzene levels were almost all non-detectable. Biomonitoring data for *ortho*-toluidine, aniline, and 4-aminobiphenyl in urine and Hb adducts collected in 1990 (Ward et al. 1996), which would integrate ambient and dermal exposure, indicated that exposure to *ortho*-toluidine was approximately 3–4 times that of aniline. Hanley et al. (2012) identified a number of changes or procedures from the beginning of rubber chemical manufacture in 1954 through 1988 that had the potential to impact exposure to *ortho*-toluidine and other chemicals. The major change that potentially increased exposure was a steady increase in chemical use, including *ortho*-toluidine, for production of rubber chemicals from 1960 to 1970. Changes that could potentially reduce exposure included (1) initiation of an air monitoring program in 1975–1976, (2) replacement of a manual charging operation for adding chemicals with an automated premix reactor feed system in 1978, (3) establishment of a company policy for personal protective equipment in or around 1980, and (4) various changes in shower and laundry facilities, material recycling, leak protection, and automated bagging during the 1990s.

Although quantitative exposure data were not available for individual workers, Hanley et al. (2012) were able to assign them to one of three categories (i.e., “definitely exposed,” “probably exposed,” or “probably not exposed”) based on the departments in which the individuals had worked using updated information from “a plant walkthrough, interviews with employees, management and union representatives, and review of records.” The same categories had been used to categorize exposure for the earlier study by Ward et al. (1991), but the assessment by Hanley et al. (2012) involved several changes in assignments by departments. In the expanded and updated analysis conducted by Carreón et al. (2014), exposure rank estimates based on department, job and year, multiplied by duration of exposure, enabled NIOSH industrial

hygienists, blinded to case status, to assign cumulative exposure ranks to 225 department-job classifications (Carreón et al. 2014).

**Table C-1. Exposure Classifications Used in NIOSH Rubber Chemical Cohort Studies (Carreón et al. 2014; Carreón et al. 2010; Hanley et al. 2012; Ward et al. 1991)**

Exposure Category	Ward et al. (1991)		Carreón et al. (2010)		Carreón et al. (2014)	
	Job Title	Total (N)	Job Titles	Total (N)	Job Titles	Total (N)
Definitely exposed	Ever employed: 1946–1988 Rubber chemical dept.	708	Ever employed: 1946–1988 Rubber chemical dept., maintenance, laboratory (inc. quality control, R&D)	962	Ever employed: 1946–2006 Dept.-jobs with moderate/high exposure e.g., rubber chemical, maintenance	900
Possibly exposed	Ever employed: 1946–1988: Maintenance, shipping/packaging/warehouse, janitorial, yard work Never in rubber chemicals dept.	288	Ever employed: 1946–1988 Shipping/packaging/warehouse, janitorial, yard work	187	Ever-employed: 1946–2006 Probably exposed low/regular e.g., shipping, warehouse, lab	195
			Temporary assignment from headquarters, none assigned depts. Never in rubber chemicals, maintenance, or laboratory depts.		Probably exposed low/irregular e.g., nurse, supervisor, janitor	155
Probably not exposed	Never employed in above depts. All work prior to Jan 1, 1957	753	Never in above depts. All work prior to Jan 1, 1957	600	Never employed 1946–2006 in depts. with direct exposure to <i>ortho</i> -toluidine, aniline, or nitrobenzene, e.g., secretary, accountant All work prior to Jan 1, 1957	562

### **C.3.2. Manufacture of a Rubber Antioxidant in the United Kingdom: Sorahan and Coworkers**

Sorahan and Pope (1993), Sorahan et al. (2000), and Sorahan (2008) studied a cohort of workers at a chemical production factory that manufactured and used 2-mercaptobenzothiazole (MBT) and phenyl- $\beta$ -naphthylamine (PBN) along with vulcanization inhibitors and accelerators, antioxidants, and other proprietary products for the rubber industry in the United Kingdom (see Section 3). Exposure to *ortho*-toluidine occurred from 1936 to 1979, in the department manufacturing *ortho*-tolybiguanide (also called Soprox), which was produced by reacting *ortho*-toluidine with dicyanamide in the presence of hydrochloric acid. No information was provided on exposure levels. Exposure to aniline, MBT, and PBN occurred in other departments; however, some workers switched departments.

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This appendix contains background information related to the cancer assessment on *ortho*-toluidine in humans including (1) detailed data information on study design, methods, and findings for the cohort studies (Table D-1) and case-control studies (Table D-2), (2) detailed information on the quality assessment of the individual studies (Table D-3) and (3) background carcinogenicity information on co-exposures in the cohort studies (Table D-4).

## D.1. Methodologies and Study Characteristics of the Selected Epidemiological Studies and Identification of Cancer Endpoints

The data from the eight cohort studies and two nested case-control studies (as delineated in Table 3-1) were systematically extracted from relevant publications, as described in the study protocol, and are summarized in the tables below.

**Table D-1. Cohort Studies of *ortho*-Toluidine Exposure: Study Characteristics and Overall Findings**

Study Characteristics and Overall Findings	
<b>Case and Pearson (1954): U.K. Dyeworkers</b>	
Related Reference	Case et al. (1954)
Geographic Location	United Kingdom
Population Characteristics	
Exposed cohort and ascertainment	<p><u>Eligibility criteria for exposed cohort:</u> Workers without contact with auramine, benzidine, or <math>\alpha</math>- or <math>\beta</math>-naphthylamine and who worked at least 6 months between 1910 and 1952 (referred to as nominal roll)</p> <p><u>Exposed cohort:</u> All male workers who had contact (manufacture or use) with magenta (N = 85) at 21 British chemical factories</p> <p><u>Total cohort:</u> Male workers (4,622) at 21 firms who had contact with aniline, benzidine, <math>\alpha</math>- or <math>\beta</math>- naphthylamine from 1910 to 1952</p> <p><u>Follow-up:</u> 1921–1952</p> <p><i>Loss to follow-up:</i> Not reported</p>
Reference population	General population (men): 15,875 deaths due to urinary bladder cancer in Wales and England (1921–1952)
All-cause and all-cancer mortality	Not reported
Study Design and Analytical Methods/Control for Confounding	<p>Historical cohort study on urinary bladder cancer mortality</p> <p>Expected number of cases based on mean rate of male deaths from bladder cancer in Wales and England (up to 1952). Comparative composite cohort analysis, which accounts for age</p> <p>Excluded workers with exposure to benzidine, <math>\alpha</math>-naphthylamine, or <math>\beta</math>-naphthylamine</p>
Exposure Data and Information Assessment	

	<b>Study Characteristics and Overall Findings</b>
Exposure: levels and co-exposures	Magenta manufacturing: levels and types of magenta NR Possible (depending on process): <i>ortho</i> -nitrotoluene, formaldehyde, aniline: levels NR
Exposure assessment	Exposure inferred via manufacturing process of magenta
Assessment: other exposure	Not reported
Disease assessment	Firm, hospital and death certificates, and coroner's records and confirmed by other sources <u>Missing data:</u> Authors note that cases from earlier time points might have been missed
<b>Pira et al. (2010): Italian Dyeworkers</b>	
Related Reference	Decarli et al. (1985); Piolatto et al. (1991); Rubino et al. (1982)
Geographic Location	Italy
Population Characteristics	
Exposed cohort and ascertainment	<u>Eligibility criteria:</u> Worked at least 1 year from 1922–1972 and were alive in 1946 <u>Exposed cohort:</u> 47 male workers involved only in the manufacture of fuchsin or <i>ortho</i> -toluidine (last update) <u>Total cohort:</u> 906 male dyestuff workers exposed to aromatic amines from 1922 to 1972; 2003 update on 590 <u>Follow-up:</u> 1951–2003; censored at age 85 <u>Loss to follow-up:</u> total cohort: 112 (12.4%) lost to follow-up before 1989, <4% after 1989
Reference population	1951–1980: national mortality rates 1981–2003: regional mortality rates
All-cause and all-cancer mortality	Total cohort only All causes SMR = 1.50 (1.36–1.66); 394 All cancers SMR = 2.11 (1.80–2.46); 163
Study Design and Analytical Methods/ Control for Confounding	Historical cohort mortality study SMR and CI calculated using Poisson distribution for observed deaths and adjusted for age (in 5-year age groups) and for 5-year calendar period Workers exposed to benzidine and/or $\alpha$ - or $\beta$ -naphthylamine were excluded from cohort.
Exposure Data and Information Assessment	

## RoC Monograph on *ortho*-Toluidine

	<b>Study Characteristics and Overall Findings</b>
Exposure: levels and co-exposures	<p><i>ortho</i>-Toluidine: levels not reported</p> <p><i>ortho</i>-Toluidine production section: potentially exposed to <i>ortho</i>-nitrotoluene, 4,4'-methylene bis(2-methylaniline): levels NR</p> <p>Fuchsin and safranin T production section: potentially exposed to 2,5-diaminotoluene, aniline, and aminoazotoluene as well as dye products: levels NR</p>
Exposure assessment	Exposure for each person was assessed by linking individual records with knowledge of chemical processes
Assessment: other exposure	Personnel and factory records
Disease assessment	Death certificates from regional registration offices
<b>Ott and Langner (1983): U.S. Dyeworkers</b>	
Related Reference	None
Geographic Location	United States
Population Characteristics	
Exposed cohort and ascertainment	<p><u>Eligibility criteria</u> Employed as of 1940 or hired subsequently, without high exposure to arsenicals, vinyl chloride, or asbestos</p> <p><u>Exposed cohort</u>: Bromindigo and thioindigo subcohort; 117 male workers</p> <p><u>Total cohort</u>: 342 male workers assigned to 3 aromatic amine-based dye production areas; 275 did not have exposure to arsenicals, vinyl chloride, or asbestos</p> <p><u>Follow-up</u>: 1940–1975</p> <p><u>Loss to follow-up</u>: NR; 124 left company, but followed via Social Security</p>
Reference population	U.S. mortality rates for whites
All-cause and all-cancer mortality	<p>Bromindigo and thioindigo subcohort</p> <p>All causes SMR = [0.79 (0.56–1.09)]; 36</p> <p>All cancers SMR = [1.5 (0.81–2.56)]; 12</p>
Study Design and Analytical Methods/ Control for Confounding	<p>Historical cohort mortality study</p> <p>SMR based on Poisson distribution assumptions</p> <p>Expected numbers: mortality rates for whites (5-yr interval)</p> <p>Person-years accumulated on a month-by-month basis from date of eligibility</p> <p>Excluded individuals with high exposure to arsenicals, vinyl chloride, or asbestos</p>
Exposure Data and Information Assessment	

RoC Monograph on *ortho*-Toluidine

	<b>Study Characteristics and Overall Findings</b>
Exposure: levels and co-exposures	<p><i>ortho</i>-Toluidine: Breathing zone and area measurements in the 1940's</p> <p>Air &lt;0.5 ppm</p> <p>Urine – undetected to 1.7 ppm</p> <p>Nitrobenzene, 4-chloro-<i>ortho</i>-toluidine, 4-chloro-acetyl-<i>ortho</i>-toluidine, 1, 2-dihydroacenaphthylene, 2-aminobenzoic acid, thioindigo dyes: levels NR</p> <p>Odor of nitrobenzene reported (threshold levels for odor is 5 ppb/volume)</p>
Exposure assessment	Exposure assessed via work history; all jobs held in production areas of interest and dates of employment used to calculate person-years of employment in each area
Assessment: other exposure	Work history
Disease assessment	<p>Vital status determined by record search performed by Social Security Administration</p> <p>Underlying causes of death were coded according to the Seventh or Eighth Revision of the International Classification of Diseases (ICD), and were grouped for statistical purposes by categories used in the Eighth Revision</p>
<b>Stasik (1988): 4-COT Production Workers</b>	
Related Reference	Stasik et al. (1985)
Geographic Location	Germany
Population Characteristics	
Exposed cohort and ascertainment	<p><u>Eligibility criteria:</u> Workers with higher exposure employed before 1970</p> <p><u>Exposed subcohort:</u> 116 higher exposed 4-chloro-<i>ortho</i>-toluidine production and processing workers</p> <p><u>Total cohort:</u> 335 male 4-chloro-<i>ortho</i>-toluidine production and processing workers employed between 1929 and 1982</p> <p><u>Follow-up:</u> 1967–1985</p> <p><u>Loss to follow-up:</u> Not reported</p>
Reference population	<p>Incidence (sex and age-specific) using non-contiguous regional rates</p> <p>Earlier publication: mortality study: national and regional rates</p>
All-cause and all-cancer mortality	<p>Subcohort</p> <p>All-causes SMR = 1.12 (0.68–1.73); 19</p> <p>All-cancers SMR = 1.45 (0.47-3.38); 5 (regional mortality rates)</p>

## RoC Monograph on *ortho*-Toluidine

<b>Study Characteristics and Overall Findings</b>	
Study Design and Analytical Methods/ Control for Confounding	<p>Historical cohort study of bladder cancer incidence; post-hoc analysis initiated by findings of cluster of bladder cancer (2 cases) after completion of mortality study</p> <p>Earlier study: historical cohort mortality study</p> <p>SIR: expected rates were calculated by multiplying person years at risk by age-specific bladder cancer incidence (regional)</p>
Exposure Data and Information Assessment	
Exposure: levels and co-exposures	<p>Duration of higher exposure to 4-chloro-<i>ortho</i>-toluidine (before 1970); 14.0 years (median); total exposure duration (before and after 1970); 25.5 years (median)</p> <p>4-chloro-<i>ortho</i>-toluidine, <i>N</i>-acetyl-<i>ortho</i>-toluidine</p> <p>6-chloro-<i>ortho</i>-toluidine</p> <p>Benzidine and <math>\beta</math>-naphthylamine were not used in the plant</p>
Exposure assessment	<p>Exposure classification based on employment status, i.e., production or processing worker</p> <p>Information on duration of employment of cases, latency, age at beginning of employment</p>
Assessment: other exposure	Smoking status known for bladder cases
Disease assessment	<p>Bladder cancer incidence cases: hospital and clinical records</p> <p>Histopathological diagnosis made (when possible) according to WHO classification</p> <p>Mortality: source not reported, ICD-9 codes</p> <p>Information on age at diagnosis of bladder cancer</p>
<b>Carreón et al. (2014): U.S. Rubber Chemical Workers</b>	
Related Reference	Carreón et al. (2010); Hanley et al. (2012); Markowitz and Levin (2004); Prince et al. (2000); Ward et al. (1991); Ward et al. (1996)
Geographic Location	United States
Population Characteristics	

	<b>Study Characteristics and Overall Findings</b>
Exposed cohort and ascertainment	<p><u>Eligibility criteria:</u> Employed for 1 day or more at the plant between 1946 and 2006</p> <p><u>Exposed cohort:</u> Groups of workers definitely, probably, or probably not exposed to <i>o</i>-toluidine, classification varies by publication</p> <p><u>Total cohort:</u> 1,875 (majority male) chemical plant workers</p> <p><u>Follow-up:</u> Incidence: 1976–2007</p> <p><u>Mortality:</u> 1946–1988 (Carreón et al. 2010)</p> <p><u>Loss to follow-up:</u> 33 workers died and 30 were otherwise lost to follow-up prior to earliest start of cancer registry (1976)</p>
Reference population	<p><u>Incidence rates:</u> New York State excluding NYC (local), Pennsylvania, California, Ohio, Texas, Florida State cancer registries</p> <p><u>Mortality:</u> United States mortality (national)</p> <p><u>Internal:</u> Lowest exposure group</p>
All cause and all-cancer mortality	<p>Definitely exposed workers</p> <p>All-causes SMR = 0.96 (0.7–1.2); 65</p> <p>All-cancers SMR = 1.1 (0.66–1.8); 17</p>
Study Design, Analytical Methods/Control for Confounding	<p>Historical cohort study on bladder cancer: incidence and mortality</p> <p>Life table methods: SIR and SRR, stratified by age and calendar year, for probability of exposure, exposure duration, cumulative rank, and time since first exposure categories. SMR (2010) – probability of exposure</p> <p>Cox proportional hazards regression for cumulative rank (continuous, categorical) adjusted for sex, race, age at diagnosis and birth year</p> <p>Smoking: indirect methods to adjust and Monte Carlo sensitivity analysis using data on subset of smokers</p>

<b>Study Characteristics and Overall Findings</b>																									
<b>Exposure Data and Information Assessment</b>																									
Exposure: levels and co-exposures	<p>TWA Breathing zone concentrations: Rubber Chemical dept 1976–2004 (Hanley et al. 2012)</p> <p><u>Range, ppm (GM range, ppm)</u></p> <p><i>o</i>-Toluidine: ND–1.8 (0.003–0.10)</p> <p>Aniline: 0.026–1.7 (0.0009–0.081)</p> <p>Nitrobenzene: ND–0.16 (0.004–0.038)</p> <p>4-aminobiphenyl (4-ABP): trace amounts detected in bulk samples</p> <p><i>Biomonitoring data Ward et al. (1991; 1996)</i></p> <table style="margin-left: auto; margin-right: auto;"> <thead> <tr> <th></th> <th style="text-align: center;"><u>Unexposed</u></th> <th style="text-align: center;"><u>Exposed</u></th> </tr> </thead> <tbody> <tr> <td colspan="3"><i>Urine (µg/L)</i></td> </tr> <tr> <td><i>o</i>-Toluidine:</td> <td style="text-align: center;">2.8</td> <td style="text-align: center;">98.7<sup>d</sup></td> </tr> <tr> <td>Aniline:</td> <td style="text-align: center;">3.9</td> <td style="text-align: center;">29.8<sup>d</sup></td> </tr> <tr> <td colspan="3"><i>Adducts (pg/g Hb)</i></td> </tr> <tr> <td><i>o</i>-Toluidine:</td> <td style="text-align: center;">3,515</td> <td style="text-align: center;">40,830<sup>d</sup></td> </tr> <tr> <td>Aniline:</td> <td style="text-align: center;">3,163</td> <td style="text-align: center;">17,441<sup>d</sup></td> </tr> <tr> <td>4-ABP:</td> <td style="text-align: center;">74.5</td> <td style="text-align: center;">81.7</td> </tr> </tbody> </table>		<u>Unexposed</u>	<u>Exposed</u>	<i>Urine (µg/L)</i>			<i>o</i> -Toluidine:	2.8	98.7 <sup>d</sup>	Aniline:	3.9	29.8 <sup>d</sup>	<i>Adducts (pg/g Hb)</i>			<i>o</i> -Toluidine:	3,515	40,830 <sup>d</sup>	Aniline:	3,163	17,441 <sup>d</sup>	4-ABP:	74.5	81.7
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Aniline:	3,163	17,441 <sup>d</sup>																							
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Exposure assessment	<p>Workers classified into exposure probability categories and relative exposure ranks based on dept.-job-year combinations. Each dept.-job category was assigned a relative exposure rank for various calendar years. Cumulative rank = (time in each dept. job X exposure rank) summed over all jobs worked</p> <p>Occupational hygiene data used to develop exposure assessment.</p> <p>Missing data: none (records available for all workers 1946–2006)</p>																								
Assessment: other exposure	Smoking histories collected on 18% non-random sample of current and former employees.																								
Disease assessment	<p>Cases: 6 state cancer registries; company and union medical records</p> <p>Deaths: death certificates in company records or Social Security Administration</p>																								
<b>Sorahan (2008): U.K. Rubber Chemical Workers</b>																									
Related Reference	Sorahan and Pope (1993); Sorahan et al. (2000)																								
Geographic Location	Wales (UK)																								
Population Characteristics																									

	<b>Study Characteristics and Overall Findings</b>
Exposed cohort and ascertainment	<p><u>Eligibility criteria</u>: At least 6 months' employment between 1955 to 1984</p> <p><u>Exposed cohort</u>: 53 male workers with potential exposure to <i>o</i>-toluidine who worked in the "Sopanox" department of a rubber chemical factory.</p> <p><u>Total cohort</u>: 2,160 male rubber production workers (hourly employees); subcohorts also established for workers exposed to 2-mercaptobenzothiazole (MBT), aniline, and phenyl-<math>\beta</math>-naphthylamine (PBN)</p> <p><u>Follow-up</u>: Censored at age 85</p> <p style="padding-left: 20px;">Mortality: 1955 to 2005</p> <p style="padding-left: 20px;">Incidence: 1971 to 2005</p> <p><u>Loss to follow-up</u>: 14 subjects emigrated, 32 untraced</p>
Reference population	<p><u>Mortality</u>: Serial mortality rates for England and Wales</p> <p><u>Incidence</u>: expected numbers from national cancer registration</p> <p><u>Internal analysis</u>: Cohort members not exposed to <i>ortho</i>-toluidine</p>
All-cause and all-cancer mortality	<p><i>ortho</i>-Toluidine subcohort</p> <p>All-causes SMR = 1.00 [0.60–1.58]; 17</p> <p>All-cancers SMR = 1.37 [0.60–2.71]; 7</p>
Study Design and Analytical Methods	<p>Historical mortality and morbidity study of bladder cancer</p> <p>External analysis: SMR and SRR using person years at risk</p> <p>Internal analysis: Poisson regression – employment duration was categorized into three levels; combined incidence and mortality</p> <p>Adjusted internal analysis: Controlled for exposure to aniline, MBT and PBN, age and calendar period</p>
Exposure Data and Information Assessment	
Exposure: levels and co-exposures	<p>"<i>ortho</i>-Toluidine was used in the Sopanox department from 1936 to 1979; aniline, MBT and PBN not used in this department</p> <p>Overlap of membership in 4 subcohorts (<i>ortho</i>-toluidine, aniline, MBT, and PBN)</p> <p>Levels of co-exposures not reported except for MBT</p>
Exposure assessment	<p>Job History (unique job-department titles) for 1930 to 1988</p> <p>Cumulative duration of employment in <i>ortho</i>-toluidine-exposed department calculated using 3 time-dependent variables</p>



<b>Study Characteristics and Overall Findings</b>	
Assessment: other exposure	JEM created for MBT - cumulative exposure, cumulative duration of exposure for PBN and niline
Disease assessment	Mortality - death certificate from U.K. Office of National Statistics Morbidity - registration of malignant tumors <u>External analysis</u> : SMR limited to underlying cause on death certificate; SIR limited to malignant urinary bladder tumors [ICD-9: 188] <u>Internal analysis</u> : Primary cancer of the urinary bladder mentioned on death certificate or diagnosis of malignant or benign bladder tumor in cancer registries cancer registry data

[ ] = calculated based on observed vs. expected cases, not reported by authors.

NR = not reported; SMR = standardized mortality ratio; CI = confidence interval; ppm = parts per million; ppb = parts per billion; ICD = International Classification of Diseases; SIR = standardized incidence ratio; WHO = World Health Organization; NYC = New York City; TWA = time-weighted average; GM = geometric mean; ND = not detectable; SRR = standardized rate ratio; MBT = 2-mercaptobenzothiazole; PBN = phenyl- $\beta$ -naphthylamine; JEM = job-exposure matrix.

**Table D-2. Case-control Studies of *ortho*-Toluidine Exposure: Study Characteristics and Findings**

<b>Study Characteristics and Overall Findings</b>	
<b>Richardson et al. (2007): Canadian Case-Control Study</b>	
Related References	None
Geographic Location	Canada
Population Characteristics	
Cases: selection and ascertainment	<u>Cases</u> : 1,062 males with urinary bladder cancer <u>Case eligibility criteria</u> : Diagnosed with urinary bladder cancer at 20+ yr old, between 1983 and 1990 and returned a lifetime occupational questionnaire; identified from the British Columbia Cancer Registry <u>Participation rate</u> : 60.1% (15,463 of the 25,726 eligible cancer registrants returned a questionnaire). Final number of cases and controls based on those that provided completed occupational histories
Controls: selection and ascertainment	<u>Referents</u> : 8,057 males with other cancer <u>Referent eligibility criteria</u> : All cancers excluding urinary bladder, lung, and unknown sites, diagnosed between 1983 and 1990, and completed lifetime occupational questionnaire; identified from the British Columbia Cancer Registry <u>Matching criteria</u> : Matched on year of birth and year of diagnosis of cases; median matching ratio of controls to cases 7:1
Length of follow-back	Lifetime
Loss to follow-back	5.6% of cases and 4.3% controls excluded due to incomplete occupational histories

	<b>Study Characteristics and Overall Findings</b>
Study Design and Analytical Methods	<p>Population cancer registry-based case-control design</p> <p>Analysis by cumulative exposure and test for linear trend</p> <p>Corrected for multiple comparisons; chemicals selected for further analysis if (a) associated with at least 3 of 9 cases or (b) IARC suspected or known bladder carcinogens</p> <p><u>Confounding</u>: Ethnicity, marital status, education, questionnaire respondent, alcohol, smoking variables analyzed by backwards stepwise conditional logistic regression</p>
Exposure Data and Information Assessment	
Exposure: levels and co-exposures	Not reported
Exposure assessment	<p>Lifetime JEM constructed based on self-reported occupational history and NIOSH NOES (1983–1986) occupational exposure survey using U.S. occupational and industry codes</p> <p>Estimated ever-exposed plus estimated cumulative exposure converted to ordinal scale (0 = no exposure, 1 = low, 2 = medium, 3 = high)</p> <p>Exposure truncated 5 yr before diagnosis</p>
Assessment of co-exposures and other potential confounders	<p>No assessment of occupational co-exposures</p> <p>Assessment of non-occupational co-exposures assessed via questionnaire</p>
Disease assessment	Histologically confirmed urinary bladder cancer (ICD-9) identified from cancer registry
<b>Castro-Jiménez and Orozco-Vargas (2011)</b>	
Related References	None
Geographic Location	Colombia
Population Characteristics	
Cases: selection and ascertainment	<p><u>Cases</u>: 85 cases of acute lymphoblastic leukemia</p> <p><u>Case eligibility criteria</u>: All children &lt;15 years old diagnosed Jan 2000 to Mar 2005 not adopted, living with biological parents, no previous cancer, both parents agree to participate. Identified from hospital registries of 6 tertiary care centers, Colombia</p>
Controls: selection and ascertainment	<p><u>Referents</u>: 85 matched controls</p> <p><u>Referent eligibility criteria</u>: Healthy, never diagnosed with cancer, not adopted, living with biological parents. Residents of neighborhoods in which cases lived</p> <p><u>Matching criteria</u>: Matched on sex and age at diagnosis of cases <math>\pm 2</math>–3 years; ratio of controls to cases = 1:1</p>

	Study Characteristics and Overall Findings
Length of follow-back	(a) 2 years prior to conception; (b) pregnancy to diagnosis of case
Study Design and Analytical Methods	Hospital-based cases; population-based controls <u>Confounding</u> : crude (using age and sex matched cases and controls) and multivariate analysis considering maternal age, education, breastfeeding, periconceptual disease, maternal disease in pregnancy, urban/rural residency, prenatal parental smoking, parental alcohol or drug use, birth weight, gestational age
Exposure Data and Information Assessment	
Exposure: levels and co-exposures	List of 13 hydrocarbons or hydrocarbon groups based on IARC classification of known or suspected human carcinogens (Group 2A), including <i>ortho</i> -toluidine; levels not reported
Exposure assessment	JEM based on self-reported parental occupation (industry) and home/environment exposure (questionnaire) <i>ortho</i> -Toluidine exposure assigned by occupational physician based on SIC code for parental occupation and agents observed in NIOSH 1983–6 NOES Survey by SIC code
Assessment of co-exposures and confounders	JEM used to assign exposure by occupational physician based on parental questionnaire data using SIC-coded parental occupation and agents observed in NIOSH 1983–6 NOES Survey
Disease assessment	Cases of acute lymphoblastic leukemia (ICD NR) identified from institutional registers and histologically verified

IARC = International Agency for Research on Cancer; JEM = job-exposure matrix; NIOSH = National Institute for Occupational Safety and Health; NOES = National Occupational Exposure Survey; ICD = International Classification of Diseases; SIC = Standard Industrial Classification; NR = not reported.

## D.2. Assessment of Potential Bias, Analytical Methods, and Other Study Quality Characteristics

Biases in observational studies are often classified into three major categories: (1) selection bias, (2) information bias, and (3) confounding (discussed in Section 3.3.2). Studies with lower potential for bias are generally considered to be the most informative for cancer evaluation. However, the presence of a potential bias in a study does not necessarily mean that the findings of the study should be disregarded. Therefore, an important step in the process of evaluating biases is to determine the probable impact of the described biases on study results—that is, the magnitude of distortion and the direction in which each bias is likely to affect the outcome of interest (if known). The impact of the potential bias or confounding on the study findings is discussed in the cancer assessment (see Section 3.4).

For this review, overall conclusions on the concern for the potential (unlikely/minimal, possible, or probable) of selection and information bias and the adequacy of other quality factors (good,

adequate, or limited) for each study were made using the questions and guidelines outlined in the protocol. In some cases, there is inadequate information to evaluate the level of concern. The guidelines describe the ideal methods and design for each study element. The terms used for defining the potential for bias are as follows:

- Unlikely/minimal: Information from study designs and methodologies indicate that the potential for bias is unlikely or minimal and are close to the ideal study characteristics.
- Possible: Study designs or methodologies are close to but less than ideal, recognizing that in observational studies, there is almost always some methodological or informational limitation and thus some potential for certain types of bias.
- Probable: Study designs or methodologies suggest that the potential for a specific type of bias is likely.
- Unknown: Insufficient information is provided to enable an evaluation to be made.

If adequate information is available, each type of bias is also characterized as to whether it is differential or non-differential. Differential (systematic) biases in the selection of study participants or information assessment are related to both exposure and disease status and have the potential to bias findings in one direction or another, whereas non-differential (random) biases, which are not related to both exposure and disease, tend to reduce the precision of the risk estimates and often bias the findings toward the null. For example, occupational cohort studies may have limited exposure data across exposure groups, increasing the potential for nondifferential exposure misclassification, and may also have the potential for a healthy worker (hire or survival) effect, a type of selection bias that tends to bias findings away from finding an effect (if present) in studies where the comparison group comes from the general population.

An overview of the approach and conclusions is discussed in Section 3.3 and details of the quality assessment are provided below in Table D-3.

**Table D-3. Summary of Study Quality**

Study	Selection Bias Attrition Bias (Loss to Follow-up)	<i>ortho</i> -Toluidine Exposure Assessment: Adequacy	Urinary Bladder Cancer Assessment: Adequacy	Ability to Detect an Effect for Urinary Bladder Cancer and Analytical Methods: Adequacy
Case and Pearson (1954) U.K. dye workers	<p><i>Selection bias</i></p> <p><u>Possible</u>: Healthy worker effect, which would bias positive findings towards the null, is always possible in occupational cohort studies, however, authors did not report all-cause mortality.</p> <p><i>Loss to follow-up</i></p> <p><u>Unknown</u></p>	<p><i>Exposure characterization</i></p> <p><u>Limited</u>: Exposure inferred from process<sup>a</sup></p> <p><i>Misclassification of workers' exposure</i></p> <p><u>Possible (non-differential)</u>: Exposure not assigned at the individual level; exposure category assigned according to production process.</p>	<p><i>Misclassification of deaths</i></p> <p><u>Probable (differential)</u>: Urinary bladder cancers identified and cross-verified via multiple sources for cohort, which may differ from assessment of deaths in the general population. Deaths diagnosed outside region may be missed.</p> <p><i>Misclassification of cases</i></p> <p><u>Possible (non-differential)</u>: Mortality studies will miss cases that do not result in death, a concern for urinary bladder cancer, which has a higher survival rate.</p>	<p><i>Ability to detect an effect</i></p> <p><u>Overall limited</u>:</p> <p>Statistical power: 8.9% power to detect twofold increase in risk. <u>Adequate</u> probability of moderate or higher exposure, exposure ND but likely high since workers were employed in the early 1900's. <u>Adequate</u> length of follow-up for total cohort.</p> <p><i>Analysis</i></p> <p><u>Limited</u>: Observed/expected only, using national comparison data.</p>

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Study	Selection Bias Attrition Bias (Loss to Follow-up)	<i>ortho</i> -Toluidine Exposure Assessment: Adequacy	Urinary Bladder Cancer Assessment: Adequacy	Ability to Detect an Effect for Urinary Bladder Cancer and Analytical Methods: Adequacy
Pira et al. (2010) Italian dye workers	<p><i>Selection bias</i></p> <p><u>Possible</u>: All-cause mortality not decreased among total cohort but not reported for <i>ortho</i>-toluidine subcohort.</p> <p><i>Loss to follow-up</i></p> <p><u>Possible</u>: 12% of total cohort lost to follow-up before 1989, &lt;4% lost to follow-up after 1989 but no information on loss to follow-up in <i>o</i>-toluidine subcohort or whether loss to follow-up is related to exposure and disease.</p>	<p><i>Exposure characterization</i></p> <p><u>Adequate</u>: Specified as part of process by authors; no quantitative exposure data.</p> <p><i>Misclassification of workers' exposure</i></p> <p><u>Possible (non-differential)</u>: Individual person-years of exposure assigned based on detailed work history; workers did not change departments or jobs.</p>	<p><i>Misclassification of deaths</i></p> <p><u>Possible (non-differential)</u>: Diagnosis by death certificates subject to potential misclassification; ICD codes (if used) not stated.</p> <p><i>Misclassification of cases</i></p> <p><u>Possible (non-differential)</u>: Mortality data only.</p>	<p><i>Ability to detect an effect</i></p> <p><u>Overall limited</u>:</p> <p>Statistical power; 11.7% power to detect wofold increase in risk. <u>Adequate</u> probability of moderate or higher exposure. Level of exposure ND but likely high since workers were employed in the early 1900s. <u>Adequate</u> length of follow-up for total cohort.</p> <p><i>Analysis</i></p> <p><u>Limited</u>: External mortality analysis by department only.</p>

RoC Monograph on *ortho*-Toluidine

Study	Selection Bias Attrition Bias (Loss to Follow-up)	<i>ortho</i> -Toluidine Exposure Assessment: Adequacy	Urinary Bladder Cancer Assessment: Adequacy	Ability to Detect an Effect for Urinary Bladder Cancer and Analytical Methods: Adequacy
Ott and Langner (1983) U.S. dye workers	<p><i>Selection bias</i></p> <p><u>Possible</u>: Statistically non-significant decrease in all-cause SMR &lt;1.0 in subcohort suggests a potential healthy worker effect.</p> <p><i>Loss to follow-up bias</i></p> <p><u>Unlikely/minimal</u>: Follow-up was conducted using company and social security records; no evidence to suggest the potential for bias.</p>	<p><i>Exposure characterization</i></p> <p><u>Limited</u>: Limited quantitative exposure assessment of air and urine at one time point but estimates likely relevant for only some of the workers.<sup>b</sup></p> <p><i>Misclassification of workers' exposure</i></p> <p><u>Probable (non-differential)</u>: No information on exposure at the individual subcohort level. Subcohort combined workers making thioindigo dyes with those making bromindigo dyes. <i>o</i>-Toluidine appears to have been used as an intermediate in thioindigo dye production, but its use in four different processes is unclear. Bromindigo dyes were produced in a different section of the building (separated by a firewall) and did not involve <i>o</i>-toluidine.</p>	<p><i>Misclassification of deaths</i></p> <p><u>Possible (non-differential)</u>: Diagnosis by death certificates.</p> <p><i>Misclassification of cases</i></p> <p><u>Possible (non-differential)</u>: Mortality study only.</p>	<p><i>Ability to detect an effect</i></p> <p><u>Overall limited</u></p> <p>Statistical power: ~15% power to detect twofold increase in risk. <u>Limited</u> probability of moderate or higher exposure. Only 77% workers were employed &lt;5 years; level of exposure known only for one time period and number/extent of workers exposed to <i>o</i>-toluidine unknown. <u>Adequate</u> length of follow-up for subcohort.</p> <p><i>Analysis</i></p> <p><u>Limited</u>: External mortality analysis by department and duration of employment. Reporting also limited. Urinary bladder cancer (observed/expected) only reported for the entire cohort (since there were 0 cases).</p>

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Study	Selection Bias Attrition Bias (Loss to Follow-up)	<i>ortho</i> -Toluidine Exposure Assessment: Adequacy	Urinary Bladder Cancer Assessment: Adequacy	Ability to Detect an Effect for Urinary Bladder Cancer and Analytical Methods: Adequacy
Stasik (1988) 4-COT production workers	<p><i>Selection bias</i></p> <p><b>Probable:</b> Differential bias towards finding an effect.</p> <p>Incidence study was initiated based on findings of 2 urinary bladder cancer cases while conducting a mortality study of these workers.</p> <p><i>Loss to follow-up bias</i></p> <p>Unknown</p>	<p><i>Exposure characterization</i></p> <p><b>Limited<sup>c</sup>:</b> No quantitative exposure assessment; not clear if processing workers were exposed to <i>o</i>-toluidine.</p> <p><i>Misclassification of workers' exposure</i></p> <p><b>Probable (non-differential):</b> no exposure assessment at the individual level.</p>	<p><i>Misclassification of cases</i></p> <p><b>Probable (differential):</b> Poor documentation; cases were identified using unspecified hospitals and other institutes, which may differ from methods used to identify expected cases in regional cancer registry.</p>	<p><i>Ability to detect an effect</i></p> <p><b>Overall: Limited</b></p> <p>Statistical power: 8.5% power to detect twofold increase in risk. <b>Limited</b> probability of moderate or higher exposure: level/duration exposure ND. <b>Limited</b> follow-up</p> <p><i>Analysis</i></p> <p><b>Limited:</b> External incidence analysis of workers in 4-COT production department included in analysis only if employed prior to 1970. Non-contiguous regional comparison; limited data on cancer endpoints.</p>



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Study	Selection Bias Attrition Bias (Loss to Follow-up)	<i>ortho</i> -Toluidine Exposure Assessment: Adequacy	Urinary Bladder Cancer Assessment: Adequacy	Ability to Detect an Effect for Urinary Bladder Cancer and Analytical Methods: Adequacy
Carreón et al. (2014); Carreón et al. (2010) (mortality) NIOSH (U.S.) rubber chemical workers	<p><i>Selection bias</i></p> <p><u>Unlikely/minimal</u> in analyses using internal controls, possible in external analysis. Statistically non-significant decrease in all-cause mortality among definitely exposed workers suggests a potential healthy worker effect, which would bias towards the null.</p> <p><i>Loss to follow-up bias</i></p> <p><u>Unlikely/minimal</u>: Multiple sources of data used to ascertain vital status at end of follow-up period.</p>	<p><i>Exposure characterization</i></p> <p><u>Good</u>: Extensive quantitative measurement of <i>ortho</i>-toluidine in air. <i>o</i>-Toluidine and <i>o</i>-toluidine Hb adducts measured in sample of workers.</p> <p><i>Misclassification of workers' exposure</i></p> <p><u>Unlikely/minimal (non-differential)</u>: Workers grouped according to definitely (high/moderate), probably (low/regular), probably (low/irregular), and probably not exposed based on updated detailed occupational histories (including jobs and dept.), exposure characterization of work areas/processes by investigators and company plus biological monitoring (described above).</p>	<p><i>Misclassification of cases</i></p> <p><u>Unlikely/minimal (non-differential)</u>: Incident cases identified and cross-verified via multiple sources including cases who moved from the NY to Pennsylvania, California, Ohio, Texas, and Florida state cancer registries. Any cases who moved elsewhere would be missed.</p> <p><i>Misclassification of deaths</i></p> <p><u>Possible (non-differential)</u>: Death certificates, company records or SSA used to identify deaths.</p>	<p><i>Ability to detect an effect</i></p> <p><u>Overall good</u>: Statistical power approx. 93% for incidence and approx. 11% for mortality to detect twofold increased risk among definitely exposed workers. <u>Good</u> probability of moderate or higher exposure: based on extensive industrial hygiene and biomonitoring data. <u>Good</u> length of follow-up</p> <p><i>Analysis</i></p> <p><u>Good</u>: SIR and SRR analyzed by exposure probability, duration of employment, time since first employment and cumulative exposure (lagged and unlagged) ranked categories. HR for cumulative exposure also analyzed by Cox regression for ranked and continuous measures of exposure. SMR analyzed only by 2010 exposure probability categories. No formal methods for controlling for confounding from co-exposures or smoking but biomonitoring data provide adequate data to evaluate (see Table D-2) and smoking information on subset of workers.</p>

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Study	Selection Bias Attrition Bias (Loss to Follow-up)	<i>ortho</i> -Toluidine Exposure Assessment: Adequacy	Urinary Bladder Cancer Assessment: Adequacy	Ability to Detect an Effect for Urinary Bladder Cancer and Analytical Methods: Adequacy
Sorahan (2008) U.K. rubber chemical workers	<p><i>Selection bias</i></p> <p><u>Unlikely/minimal</u> in analyses using internal controls, possible in external analysis.</p> <p>All-cause mortality not reported for <i>o</i>-toluidine subcohort.</p> <p><i>Loss to follow-up bias</i></p> <p><u>Unlikely/minimal</u>: 2% (emigration + not traced) for mortality; 3% for incidence.</p>	<p><i>Exposure characterization</i></p> <p><u>Adequate</u>:<sup>d</sup> no quantitative exposure data.</p> <p><i>Misclassification of workers' exposure</i></p> <p><u>Possible (non-differential)</u>:</p> <p>Person-years at risk in <i>o</i>-toluidine department based on individual work histories.</p>	<p><i>Misclassification of cases</i></p> <p><u>Unlikely/minimal (non-differential)</u>:</p> <p>Cases identified via national regional cancer registry using ICD-8, 9, and 10 coding).</p> <p><i>Misclassification of deaths</i></p> <p><u>Possible (non-differential)</u>:</p> <p>National death registry used.</p>	<p><i>Ability to detect an effect</i></p> <p><u>Overall adequate</u>:</p> <p>Statistical power: 17.3% power to detect twofold increase in risk.</p> <p><u>Adequate</u> probability of moderate or higher exposure: level/duration exposure ND but levels likely to be high. <u>Adequate</u> length of follow-up for total cohort.</p> <p><i>Analysis</i></p> <p><u>Good</u>: Included external analysis and internal RR analysis by duration of exposure, adjusted for 3 other exposures.</p>

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Study	Selection Bias Attrition Bias (Loss to Follow-up)	<i>ortho</i> -Toluidine Exposure Assessment: Adequacy	Urinary Bladder Cancer Assessment: Adequacy	Ability to Detect an Effect for Urinary Bladder Cancer and Analytical Methods: Adequacy
Richardson et al. (2007) Canadian Case-control study	<i>Selection bias</i> <u>Possible</u> : Use of cancer cases as controls may bias towards the null if <i>o</i> -toluidine is a risk factor for those cancers, but unknown if <i>o</i> -toluidine causes cancers at other sites. Participation rate 60% which may increase potential for bias.	<i>Exposure characterization</i> <u>Limited</u> : Study is population-based; JEM is limited because exposure classification by industry and occupation based on a (separate) qualitative walk-through exposure survey in one calendar year. No information on specific jobs. <i>Exposure misclassification</i> <u>Probable (non-differential)</u> : Cumulative exposure is the product of the probability of exposure, based on the limited JEM, and duration of employment based on self-reported occupational history, which may have been subject to recall error.	<i>Misclassification of cases</i> <u>Unlikely/minimal (non-differential)</u> : Cases and controls identified via cancer registry.	<i>Ability to detect an effect</i> <u>Overall limited</u> Statistical power to detect twofold increase in risk could not be calculated (larger number of exposed cases [39] than most cohort studies). Exposure to <i>o</i> -toluidine rare among study participants (~3.6% cases, exposure prevalence not reported among controls). <u>Unknown</u> probability of moderate or higher levels of exposure: levels/duration exposure ND but likely to be imprecise over lifetime <i>Analysis</i> <u>Limited</u> : ORs adjusted for demographic/lifestyle factors but no control for potential confounding by co-exposures; analysis by 3 categories of exposure level but categories likely to be imprecise.

HWE = healthy worker (hire or survival) effect; JEM = job-exposure matrix; ICD = International Classification of Diseases; ND = not determined; OR = odds ratio; RR = relative risk; SIR = standardized incidence ratio; SMR = standardized mortality ratio; SRR = standardized rate ratio.

<sup>a</sup>*o*-Toluidine exposure can be inferred among the magenta production group via knowledge of the processes used in the UK Howe (1977) (also see Appendix C).

<sup>b</sup>Inadequate data on the thioindigo and bromindigo dye manufacturing process were provided to determine the probability or levels of potential exposure to *ortho*-toluidine, and it is not used in conventional methods of making these specific dyes (see Section 2); however, ambient and urine levels of *ortho*-toluidine were reported in only one (1940s) survey.

<sup>c</sup>*ortho*-Toluidine is a precursor in the synthesis of 4-chloro-*ortho*-toluidine (by acetylation of *ortho*-toluidine to producing *N*-acetyl-*o*-toluidine and further chlorination and hydrolysis to produce 4-chloro-*ortho*-toluidine) and would be expected to occur in comparable amounts relative to the product, based on the amount of feedstock (see Section 2); however, no detailed data on the process, or qualitative or quantitative data on levels of *ortho*-toluidine were provided.

<sup>d</sup>*ortho*-Toluidine is the principal precursor in the synthesis of Sapanox (*ortho*-tolylbiguanide) where it is reacted with dicyanamide and hydrochloric acid; workers in the Sapanox department reportedly had “appreciable” exposure to *ortho*-toluidine Sorahan and Pope (1993).

<sup>e</sup>Number of exposed controls were not provided but were estimated from odds ratios and number of exposed cases.

### D.3. Background Carcinogenicity Information on Co-exposure of Interest

**Table D-4. Carcinogenicity Information on Co-exposures of Interest<sup>a</sup>**

Co-exposure	Studies	Comments	Cancer Evaluation	Urinary Bladder Cancer Evaluation
N-acetyl- <i>o</i> -toluidine	Stasik (1988) <sup>b</sup>	–	No evaluation	–
<i>o</i> -Aminoazotoluene	Pira et al. (2010) <sup>b</sup>	No human data	IARC- Group 2B	Limited evidence in animals
2-Aminobenzoic acid	Ott and Langner (1983) <sup>b</sup>	Insufficient data in experimental animals for evaluation	IARC- Group 3	–
4-Aminobiphenyl	Carreón et al. (2014) <sup>b</sup>	–	IARC- Group 1 RoC- RAHC	IARC/RoC Sufficient evidence in humans
Aniline	Pira et al. (2010) <sup>c</sup> Sorahan (2008) <sup>c</sup> Carreón et al. (2014) <sup>c</sup>	–	IARC- Group 3	Not a target site in experimental animals; no evidence from human studies
4-Chloro-acetyl- <i>ortho</i> -toluidine	Ott and Langner (1983) <sup>b</sup>	–	No evaluation	–
4-Chloro- <i>ortho</i> -toluidine	Ott and Langner (1983) <sup>b</sup> Stasik (1988) <sup>c</sup>	–	RoC- RAHC IARC- Group 2A	IARC/RoC Limited evidence in humans
6-Chloro- <i>ortho</i> -toluidine	Stasik (1988) <sup>c</sup>	–	No evaluation	–
2,5-Diaminotoluene	Pira et al. (2010) <sup>c</sup>	No human data Insufficient data in experimental animals for evaluation	IARC- Group 3	–
1,2-Dihydroacenaphthylene	Ott and Langner (1983) <sup>b</sup>	–	No evaluation	–
Formaldehyde	Case and Pearson (1954) <sup>b,c</sup>	–	IARC- Group 1 RoC- Known to be a human carcinogen	Not a target site in humans or experimental animals

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Co-exposure	Studies	Comments	Cancer Evaluation	Urinary Bladder Cancer Evaluation
Fuchsin (New Magenta)/Magenta III <sup>c</sup>	Case and Pearson (1954) <sup>c,e</sup> Pira et al. (2010) <sup>c</sup>	IARC: Magenta production is a known human bladder carcinogen (Group 1); IARC could not separate effects of magenta from its co-exposures (which include <i>ortho</i> -toluidine) Magenta I inadequate studies in experimental animals CI Basis Red 9 (IARC/RoC) Sufficient evidence of carcinogenicity in experimental animals (not urinary bladder)	Magenta (not defined) IARC- Group 2B CI basic red 9 (Magenta 0/para-magenta) RoC- RAHC	–
4,4-Methylene-bis (2-methylaniline)	Pira et al. (2010) <sup>c</sup>	No human data	RoC- RAHC IARC- Group 2B	Not a target site in experimental animals
2-Mercaptobenzothiazole	Sorahan (2008) <sup>c</sup>	–	Not evaluated	Increased risk in Sorahan study, which is diminished after controlling for exposure to <i>ortho</i> -toluidine ; no independent evidence in other epidemiological studies (Collins et al. 1999; Veys 1996)
Nitrobenzene	Ott and Langner (1983) <sup>b</sup> Carreón et al. (2014) <sup>c</sup>	No human data	RoC- RAHC IARC- Group 2B	Not a target site in experimental animals
<i>ortho</i> -Nitrotoluene	Case and Pearson (1954) <sup>b,d</sup> Pira et al. (2010) <sup>c</sup>	No human data	RoC- RAHC IARC- Group 2A	Not a target site in experimental animals
Phenyl-β-naphthylamine	Sorahan (2008) <sup>c</sup>	–	IARC- Group 3	Increased risk in Sorahan study, which is diminished after controlling for exposure to <i>ortho</i> -toluidine; no independent evidence
Safranine T	Pira et al. (2010) <sup>c</sup>	–	No evaluation	–
Thioindigo dyes	Ott and Langner (1983) <sup>c</sup>	–	No evaluation	–
1-Tolylbiguanidine (“Sopanox”)	Sorahan (2008) <sup>c</sup>	–	No evaluation	Product in <i>ortho</i> -toluidine dept; no independent evidence

IARC = International Agency for Research on Cancer; Group 1 = carcinogenic to humans; Group 2A = probably carcinogenic to humans; Group 2B = possibly carcinogenic to humans; Group 3 = not classifiable as to its carcinogenicity in humans; RoC = Report on Carcinogens; RAHC = reasonably anticipated to be a human carcinogen.

<sup>a</sup>Carcinogenicity information is reported for co-exposures where there were more than trace amounts of exposure to a chemical (unless it was a known human bladder carcinogen); carcinogenicity information was obtained from IARC and RoC evaluations or from literature searches (in the absence of an IARC or RoC evaluation).

<sup>b</sup>Little evidence of exposure.

<sup>c</sup>Probably exposed.

<sup>d</sup>According to Howe (1977), U.K. magenta was prepared by condensing *ortho*-toluidine with formaldehyde in the presence of *ortho*-nitrotoluene as an oxidizing agent.

# Appendix E. Assessment of the Quality of the Individual Animal Cancer Studies on Exposure to *ortho*-Toluidine

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Seven publications, reporting on eleven studies, were identified that were either long-term carcinogenicity studies or reported neoplastic lesions in experimental animals exposed to *ortho*-toluidine (See Section 4, Cancer studies in Experimental Animals). Each of these primary studies was systematically evaluated in a two-step process by first evaluating whether the level of detail reported for key elements of study design, experimental procedures, and carcinogenesis endpoints was adequate for evaluating its quality and interpreting its results. Studies that were adequately reported were further evaluated for concerns for study quality that might negatively impact the ability to assess carcinogenicity. Those studies with adequate reporting and that did not have major concerns of study quality are included in the cancer assessment described in Section 4, Cancer Studies in Experimental Animals.

### **E.1. Adequacy of Reporting for *ortho*-Toluidine Studies**

The reporting quality of key elements for two of the eleven studies was considered to be inadequate for cancer evaluation: a study in mice exposed to *ortho*-toluidine by subcutaneous injection (Pliss 2004) and a tumor initiation study in genetically altered fish (Anders et al. 1991). In the study in mice, the total number of animals exposed, and the duration period were not clearly defined; the authors noted that some deaths occurred in the exposed animals, and more animals were added later, but did not provide any details on the cause of death, the exposure duration of the animals that died, and the timing of the addition of new mice to the study.

There is no reference to validation or standardization of this fish model for cancer detection. The almost complete lack of description of study design, study validation, and data analysis makes this study inadequate for evaluation of carcinogenicity. The reporting is inadequate for details such as the tumor incidence of untreated fish, necropsy procedures, the sex used, dose selection rationale or toxicities, duration of observation, and purity of chemical.

### **E.2. Study Quality Assessment**

Table E-1 (rats) and Table E-2 (other animals) report the assessment of nine studies that were considered to have adequate reporting quality, including feeding studies in rats (Hecht et al. 1982; NCI 1979; NTP 1996; Weisburger et al. 1978), mice (NCI 1979; Weisburger et al. 1978) dogs (Pliss 2004) and subcutaneous injection studies in hamsters (Hecht et al. 1983) and rats (Pliss 2004). Study quality was assessed using questions related to the following study performance elements: substance characterization, animal husbandry, study design, endpoint assessment, and data interpretation. In most cases, each question inquires whether there are concerns (minimal, some, major, unknown, i.e., no information reported) that the quality of a specific study element is adequate for attributing any neoplastic endpoints to exposure to *ortho*-toluidine. In general, the ranking of the concerns for the study elements is based on how far each study element deviates from the ideal (see below).

The assessment of the overall quality of a study is based on consideration of the individual elements and how they impact the usefulness of that study in assessing carcinogenicity. Studies that were given the most weight in the evaluation (e.g., those with no or minimal concerns in key elements) are those with the characteristics listed below.

- Use a chemical that is representative of the candidate substance (in terms of purity and stability) so that any observed effects can be attributed to the candidate substance.
- No evidence of poor animal husbandry conditions (such as high mortality due to infection). Often information on animal husbandry conditions is not known and while this information is desirable, it is not a requirement.
- Exposure of animals to high enough doses (resulting in tolerable toxicities) for a sufficiently long duration (approaching the lifetime of the animal), but not to a dose that limits survival over the exposure period. The use of more than one dose level is ideal but is not a requirement.
- Have an appropriate comparison group (e.g., ideally unexposed, sham treated concurrent controls). The absence of an appropriate control group, by itself, is sufficient for judging a study to be inadequate for cancer evaluation.
- Have adequate statistical power to detect an effect, which is based on the number of animals used in a study, the incidence of tumors in control vs. treated group, and the rarity of the tumor.
- Perform full necropsies and histopathological examinations on all tissues. Ideally, animals are exposed to multiple doses that allow for statistical comparisons to the control group and dose-response analysis.

An ideal study would have the following characteristics, which are related to interpreting the study. In general, with the exception of route of exposure, these do not contribute as much weight to the overall evaluation of the study as the characteristics related to the validity of the study discussed above.

- The use of an exposure route comparable to human exposure.
- The use of animal model that is sensitive for detecting tumors and does not have high background rates for the observed tumors. Studies in both sexes are more informative than those testing only one sex. Often this information is not available.
- Availability of historical control data, which can be helpful in assessing the significance of a finding, especially in the case of rare tumors, lower powered studies, or assessment of background tumor incidences. Rare tumors will be considered in the assessment even if their incidences do not reach significance.
- Appropriate reporting of incidence data and statistical methods. If statistical tests are not reported, the study should at a minimum present incidence data for specific tumors so that statistical tests can be run.

Studies having elements that are judged to have some or major concerns may still be considered in the evaluation or can be considered to provide support to the more informative studies. It should also be noted that some concerns about a study element (such as inadequate observation and exposure period and statistical power) would decrease the sensitivity of a study to detect an effect; however, if despite these limitations positive findings were described, these studies would inform a cancer assessment. In contrast, other concerns (poor purity of the chemical) may lead to false positive or false negative results, and thus in general, studies having major concerns are considered to be inadequate for cancer evaluation.



**Table E-1. Assessment of the Quality of Cancer Studies in Rats**

	<b>Weisburger et al. (1978)</b>	<b>NCI (1979)</b>	<b>Hecht et al. (1982)</b>	<b>NTP (1996)</b>	<b>Pliss (2004)</b>
<b>Substance characterization</b>					
Are there concerns that the purity solubility and stability of the chemical are not adequate for attributing any neoplastic effects to the substance?	<b>Concerns: minimal</b> Tested for purity, but only a non-specific purity was reported as 97% to 99% for most chemicals. Stability was only tested for 3 days and results were not reported.	<b>Concerns: minimal</b> Purity was tested and found to be >99%. Stability testing was not reported.	<b>Concerns: minimal</b> Reported as purified by recrystallization and melting point determination. No stability tests were reported.	<b>Concerns: minimal</b> Purity (100%) and stability in feed tested.	<b>Concerns: minimal</b> Purity reported as >99.7% with 0.2% <i>p</i> -toluidine as a contaminant. No stability tests were reported.
<b>Animal husbandry</b>					
Are there concerns that the quality of the animal husbandry (e.g., care, diet, maintenance, and disease surveillance) is not adequate for attributing any neoplastic effects to the substance?	Few details were reported.	<b>Concerns: minimal</b>	<b>Concerns: some</b> No information on disease surveillance. Housing, diet described.	<b>Concerns: minimal</b> Studies done under U.S. FDA Good Laboratory Practice regulations; subclinical disease surveillance performed.	Minimal information on housing reported. No other information reported.
<b>Study design</b>					
Are there concerns that the study design did not include randomization of animals to dose groups and blinding of dose groups?	No information reported	No information reported	No information reported	<b>Concerns: minimal</b> Animals were distributed randomly into groups of approximately equal initial mean body wts.	No information reported

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	<b>Weisburger et al. (1978)</b>	<b>NCI (1979)</b>	<b>Hecht et al. (1982)</b>	<b>NTP (1996)</b>	<b>Pliss (2004)</b>
Are there concerns that the dosing regimen (dose selection and dose groups, or other factors) is either not adequate for detecting a neoplastic effect (if present) or for attributing any tumor effects to the substance?	<b>Concerns: minimal</b> Two dose levels were tested and were based on the “maximum tolerated dose” of a 30-day study. Results of the 30-day study were not reported. Initial doses caused >10% body weight loss and were decreased after 3 months.	<b>Concerns: minimal</b> Two dose levels were tested and were based on the “MTD” of a 7-week study. These dose levels did not significantly decrease survival.	<b>Concerns: minimal</b> Only one exposed dose level tested; dose was toxic based on decreased survival after 88 wk (20% survival) compared to untreated controls (60% survival). Survival in control and treated groups comparable through week 68 of study.	<b>Concerns: minimal</b> One dose level tested, dose was toxic based on decreased of body weight gain (up to 27%) in the exposed group.	<b>Concerns: some</b> Only one dose level (30 mg, s.c.) tested, reported as “maximally tolerated dose;” however, no other information was provided
Are there concerns that the study duration (exposure and observation) is not adequate to detect a neoplastic effect, if present?	<b>Concerns: minimal</b> Exposure occurred for 18 months and observation was for 2 years.	<b>Concerns: minimal</b> Exposure and observation occurred for two years.	<b>Concerns: minimal</b> The exposure (73 wk) and observation duration (93 wk) were adequate although do not quite meet the ideal conditions of close to lifetime exposure.	<b>Concerns: major</b> Exposure (13 wk) and observation (26 wk) durations were short, only one dose level.	<b>Concerns: minimum</b> Study duration 2 years
Are there concerns that the concurrent control group was not adequate for evaluating the study?	<b>Concerns: minimal</b> A single group control and pooled control were used.	<b>Concerns: minimal</b> A concurrent control group was used that had less, but still sufficient numbers of, mice (20/sex) than the exposed groups (50/sex).	<b>Concerns: minimal</b>	<b>Concerns: minimal</b>	<b>Concerns: minimal</b>
Are there concerns that the study does not have adequate statistical power (number of animals per exposure and control group) to detect a neoplastic effect, if present?	<b>Concerns: some</b> 25 animals/exposed group and untreated control group, though a pooled control group had 111 animals.	<b>Concerns: minimal</b>	<b>Concerns: minimal</b> 30 males/exposed group. Some concern for rare tumors.	<b>Concerns: some</b> 20 animals/exposed group in the exposed groups; 10 animals in untreated control group.	<b>Concern: some</b> 25 animals/exposed group and untreated controls.

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	Weisburger et al. (1978)	NCI (1979)	Hecht et al. (1982)	NTP (1996)	Pliss (2004)
<b>Endpoint assessment</b>					
Are there concerns that the assessment of study outcome (gross and microscopic tissue analysis) was not done blind?	No information reported	No information reported	No information reported	No information reported	No information reported
Are there concerns that the methods to access tumor outcome and the pathology procedures (necropsy, histology, or diagnosis) are not adequate for attributing the effects?	<b>Concerns: minimal</b> Complete necropsies and histological examinations were done on all major organs and gross lesions of animals that died after the first 6 months.	<b>Concerns: minimal</b> Complete necropsies and histological examinations were done on all major organs and gross lesions.	<b>Concerns: minimal</b> Complete necropsies and histological examinations were done on all major organs and gross lesions.	<b>Concerns: minimal</b> Complete necropsies and histological examinations were done on all organs and confirmed by an independent pathology laboratory.	<b>Concerns: some</b> Necropsies and histology were performed; however, it is unclear whether all major organ systems were examined.
<b>Data interpretation</b>					
Are there concerns that survival-related effects could affect attributing the study findings to exposure?	<b>Concerns: minimal</b> No treatment related survival effects.	<b>Concerns: some</b> Decrease in survival at the high-dose group in both sexes. The study of males was stopped three weeks early because of low survival.	<b>Concerns: some</b> Survival was decreased to 20% in the exposed group compared with 60% in the untreated controls; however, survival did not sharply decrease until 18 months exposure.	<b>Concerns: minimal</b> No treatment related survival effects	<b>Concerns: some</b> Authors state that 30 animals (presumably of the 50 males and females combined) were available after 19.5 months and that 38 of 50 controls survived until 23 months
Are there concerns that the route of exposure is not adequate for evaluating the potential for human carcinogenicity?	<b>Concerns: minimal</b> Feeding	<b>Concerns: minimal</b> Feeding	<b>Concerns: minimal</b> Feeding	<b>Concerns: minimal</b> Feeding	<b>Concerns: some</b> Subcutaneous exposure

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	<b>Weisburger et al. (1978)</b>	<b>NCI (1979)</b>	<b>Hecht et al. (1982)</b>	<b>NTP (1996)</b>	<b>Pliss (2004)</b>
Are there concerns about the animal model (source, species, strain, or sex) that could affect study interpretation?	<b>Concerns: some</b> Only males were tested.	<b>Concerns: minimal</b>	<b>Concerns: minimal</b> Only male Fischer 344 rats were tested; concerns would be if no effects were observed.	<b>Concerns: minimal</b> Only male Fischer 344/N rats were tested; concern would be if no effects were observed.	<b>Concerns: some</b> An unspecified strain of male and female rats from an unreported source were tested.
Are historical control data reported? If not, this would be a concern for rare tumors, or tumors with high background.	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>No</b>	<b>No</b>
Are there concerns that reporting of the data and statistical analyses are inadequate for evaluating the results?	<b>Concerns: minimal</b>	<b>Concerns: minimal</b>	<b>Concerns: some</b> Authors used chi-squared method to test for statistical significance, but no evidence that analyses were on o-tol exposed vs. control animals. Incidence data was available to perform pairwise comparisons using Fisher exact test.	<b>Concerns: minimal</b>	<b>Concerns: major</b> Data were not presented in a format that would allow for statistical analyses. All tumors combined for statistical analyses; no comparison of tumor burden to controls.
<b>Overall assessment of study quality and utility for cancer assessment</b>					
Does this study have utility for cancer assessment? What is the overall level of concern for the quality of the study, and how would any concerns affect its interpretation?	<b>Yes</b> , minimal concerns in most key elements	<b>Yes</b> , minimal concerns in most key elements;	<b>Yes</b> , minimal concerns in most key elements	Positive findings (such as neoplasia, pre-neoplastic lesions), <b>yes</b> , minimal concerns Negative findings, <b>no</b> , major concerns Insufficient exposure and observation duration period.	<b>Yes</b> , some concerns: Most of the concerns would affect negative findings: one dose level, limited statistical power, uncertainty of completeness of necropsies

**Table E-2. Assessment of the Quality of Cancer Studies in Other Experimental Animals: Mice, Hamsters, and Dogs**

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	Weisburger et al. (1978)	NCI (1979)	Hecht et al. (1983) (Hamsters)	Pliss (2004) (Dogs)
<b>Substance characterization</b>				
Are there concerns that the purity solubility and stability of the chemical are not adequate for attributing any neoplastic effects to the substance?	<b>Concerns: minimal</b> Tested for purity, but only a non-specific purity was reported as 97% to 99% for most chemicals. Stability was only tested for 3 days and results were not reported.	<b>Concerns: minimal</b> Purity was tested and found to be >99%. Stability testing was not reported.	<b>Concerns: minimal</b> Reported as “pure” as determined by gas and thin layer chromatography; no stability tests were reported.	<b>Concerns: minimal</b> Purity reported as >99.7% with 0.2% <i>p</i> -toluidine as a contaminant. No stability tests were reported.
<b>Animal husbandry</b>				
Are there concerns that the quality of the animal husbandry (e.g., care, diet, maintenance, and disease surveillance) is not adequate for attributing any neoplastic effects to the substance?	Few details were reported.	<b>Concerns: minimal</b>	<b>Concerns: some</b> No information on disease surveillance. Housing, diet described.	Minimal information on housing reported. No other information reported.
<b>Study design</b>				
Are there concerns that the study design did not include randomization of animals to dose groups and blinding of dose groups?	No information reported	No information reported	No information reported	No information reported
Are there concerns that the dosing regimen (dose selection and dose groups, or other factors) is either not adequate for detecting a neoplastic effect (if present) or for attributing any tumor effects to the substance?	<b>Concerns: minimal</b> Two dose levels were tested and were based on the “maximum tolerated dose” of a 30-day study. Results of the 30-day study were not reported. Initial doses caused >10% body weight loss and were decreased after 3 months.	<b>Concerns: minimal</b> Two dose levels were tested and were based on the “MTD” of a 7-week study. These dose levels did not significantly decrease survival.	<b>Concerns: minimal</b> Only one dose level tested, dose was toxic based on decreased survival in the exposed group compared to control group.	<b>Concerns: some</b> Maximum tolerated dose exceeded (125 mg/kg, s.c.) in the first month; route of exposure changed to gelatin capsules by mouth for first month followed by gavage. No further lethal toxicities. Only one dose group (125 mg/kg) was tested.

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	<b>Weisburger et al. (1978)</b>	<b>NCI (1979)</b>	<b>Hecht et al. (1983) (Hamsters)</b>	<b>Pliss (2004) (Dogs)</b>
Are there concerns that the study duration (exposure and observation) is not adequate to detect a neoplastic effect, if present?	<b>Concerns: minimal</b> Exposure occurred for 18 months and observation was for 21 months.	<b>Concerns: minimal</b> Exposure and observation occurred for two years.	<b>Concerns: some</b> The duration of exposure (52 wk) was short at 52 wk, observation duration (87 wk) was adequate although does not quite meet ideal condition (lifetime 2.5–3 yr).	<b>Concerns: minimal</b> Exposed close to lifespan (9 years)
Are there concerns that the concurrent control group was not adequate for evaluating the study?	<b>Concerns: minimal</b> A single group control and pooled control were used.	<b>Concerns: minimal</b> A concurrent control group was used that had less, but still sufficient numbers of, mice (20/sex) than the exposed groups (50/sex).	<b>Concerns: minimal</b>	<b>Concerns: major</b> No untreated control group was tested
Are there concerns that the study does not have adequate statistical power (number of animals per exposure and control group) to detect a neoplastic effect, if present?	<b>Concerns: some</b> 25 animals/sex/exposed group and untreated control group, though a pooled control group had 99 males and 102 females.	<b>Concerns: minimal</b>	<b>Concerns: some</b> 15 animals/group/sex	<b>Concerns: major</b> Only 3 dogs were exposed for more than 3 years
<b>Endpoint assessment</b>				
Are there concerns that the assessment of study outcome (gross and microscopic tissue analysis) was not done blind?	No information reported	No information reported	No information reported	No information reported
Are there concerns that the methods to assess tumor outcome and the pathology procedures (necropsy, histology, or diagnosis) are not adequate for attributing the effects?	<b>Concerns: minimal</b> Complete necropsies and histological examinations were done on all major organs and gross lesions of animals that died after the first 6 months.	<b>Concerns: minimal</b> Complete necropsies and histological examinations were done on all major organs and gross lesions.	<b>Concerns: minimal</b> Complete necropsies and histological examinations were done on all major organs and gross lesions.	<b>Concerns: some</b> Necropsies and histology were performed; however, it is unclear whether all major organ systems were examined.

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	<b>Weisburger et al. (1978)</b>	<b>NCI (1979)</b>	<b>Hecht et al. (1983) (Hamsters)</b>	<b>Pliss (2004) (Dogs)</b>
<b>Data interpretation</b>				
Are there concerns that survival-related effects could affect attributing the study findings to exposure?	<b>Concerns: minimal</b> No treatment related survival effects.	<b>Concerns: minimal</b> No treatment related survival effects.	<b>Concerns: some</b> Mean survival time (wk) was decreased in the exposed groups compared to the untreated controls (males 61.3 vs. 75.5; and females 57.8 vs. 68.7); may have decreased sensitivity for late occurring neoplasms.	<b>Concerns: some</b> Authors state that 2 of the 5 dogs died within a month after exposure, a 3rd dog died after ~3 yr.
Are there concerns that the route of exposure is not adequate for evaluating the potential for human carcinogenicity?	<b>Concerns: minimal</b> Feeding	<b>Concerns: minimal</b> Feeding	<b>Concerns: some</b> Subcutaneous exposure	<b>Concerns: minimal</b> Feeding/gavage
Are there concerns about the animal model (source, species, strain, or sex) that could affect study interpretation?	<b>Concerns: minimal</b>	<b>Concerns: minimal</b>	<b>Concerns: minimal</b> Both sexes of Syrian Golden hamsters were tested.	<b>Concerns: minimal</b> Dog considered to be a sensitive model human urinary bladder cancer.
Are historical control data reported? If not, this would be a concern for rare tumors, or tumors with high background.	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>No</b>
Are there concerns that reporting of the data and statistical analyses are inadequate for evaluating the results?	<b>Concerns: minimal</b>	<b>Concerns: minimal</b>	<b>Concerns: major</b> Incidence data and statistical analyses were not reported. Stated that the incidence was not significantly different from control group.	<b>Concerns: major</b> Insufficient data to evaluate results

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	<b>Weisburger et al. (1978)</b>	<b>NCI (1979)</b>	<b>Hecht et al. (1983) (Hamsters)</b>	<b>Pliss (2004) (Dogs)</b>
<b>Overall assessment of study quality and utility for cancer assessment</b>				
Does this study have utility for cancer assessment? What is the overall level of concern for the quality of the study, and how would any concerns affect its interpretation?	<b>Yes</b> , minimal concerns in most key elements	<b>Yes</b> , minimal concerns in most key elements	<b>Yes</b> , some concerns in key elements.  Most of the concerns would affect negative findings: short duration of exposure and observation; only one dose, limited statistical power	<b>No</b> , major concerns with key elements  The lack of concurrent controls prevents attributing any tumor findings to exposure.



## Appendix F. Data Tables for Genotoxicity and Other Relevant Data

### Tables

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The 14 tables on the following pages contain data discussed in the “Mechanisms and Other Relevant Effects” section.

Genotoxicity data are reported for in vitro studies of *ortho*-toluidine mutagenicity in bacteria and yeast (Table F-1), co-mutagenic effects of *ortho*-toluidine and norharman in bacteria (Table F-2), genetic effects of *ortho*-toluidine in non-mammalian eukaryotes (Table F-3), in vitro genotoxicity studies of *ortho*-toluidine in mammalian cells (Table F-4), cell transformation studies of *ortho*-toluidine in rodent cells (Table F-5), DNA adducts from *ortho*-toluidine exposure in vivo (Table F-6), *ortho*-toluidine-releasing DNA adducts in bladder tissue from cancer patients and sudden death victims (Table F-7), genotoxicity effects of *ortho*-toluidine in vivo (Table F-8), mutagenicity of *ortho*-toluidine metabolites (Table F-9), hemoglobin and albumin adducts formed in vitro and in vivo (Table F-10).

**Table F-1. Genetic Effects of *ortho*-Toluidine in Bacteria**

Bacteria Strains	Endpoint	Res. <sup>a</sup> – S9	Res. <sup>a</sup> + S9	HID (µg/Plate) <sup>b</sup>	LED (µg/Plate) <sup>b</sup>	Comments
<i>S. typhimurium</i>	Reverse mutation					
TA92	(base pair: plate	0/1	0/1	2,000		TA100 positive in one study with 30% hamster S9 but negative with rat S9.
TA100	incorporation, pre-	0/26	5/26	10,000	100 µg/mL	Two positive studies with rat S9 (30%) and one positive study with the fluctuation test with rat S9.
TA102	incubation, or fluctuation	0/2	0/2	10,000		
TA1535	method)	0/17	0/18	10,000		Most recent study (An et al. 2010) reported weak positive response in TA100 with rat S9 (concentration not specified) at 2,000 to 4,000 µg/plate
G46		0/1	0/1	1,000 µg/mL		
<i>S. typhimurium</i>	Reverse mutation					
TA97	(frameshift: plate	0/3	0/3	10,000		One positive study with TA98 required 30% rat S9. The other positive study with TA98 required phenobarbitol-induced rat S9.
TA98	incorporation, pre-	0/25	2/26	10,000	1.3 µg/mL	
TA1537	incubation, or fluctuation	0/16	0/16	10,000		One positive study with TA1538 used the fluctuation test and a mixed-induced S9 (phenobarbitone + β-naphthoflavone) and the other positive study used the standard plate incorporation method and Aroclor-induced rat S9.
TA1538	method)	0/15	2/16	10,000	10 µg/mL	
C3076		0/1	0/1	1,000 µg/mL		
D3052		0/1	0/1	1,000 µg/mL		
<i>S. typhimurium</i>	Reverse mutation					
TA98	(host-mediated assay)	0/1	1/1	1,000	500	Urine extracts from rats administered 300 mg/kg <i>o</i> -toluidine was mutagenic in TA98 with 30% hamster S9.
TA100		0/1	0/1	1,000		
<i>S. typhimurium</i>	Forward mutation					
BA13	arabinose resistance	NT	1/1	965	480	BA13 positive only with 30% rat S9, negative with 10% rat S9 at 965 µg/plate. TM677 studies used standard protocols with Aroclor or phenobarbitol induced rat liver S9.
TM677	8-azaguanine resistance	0/1	0/2	500		
<i>S. typhimurium</i>	Prophage induction, SOS	0/1	0/1	1,670	–	–
TA1535/pSK102	repair, DNA strand breaks or cross-links					
<i>E. coli</i>	Reverse mutation	0/5	1/5	1,000	50 µg/mL	Positive in one study with 10% rat S9 but negative in four other studies.
WP2 <i>uvrA</i>						

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Bacteria Strains	Endpoint	Res. <sup>a</sup> – S9	Res. <sup>a</sup> + S9	HID (µg/Plate) <sup>b</sup>	LED (µg/Plate) <sup>b</sup>	Comments
<i>E. coli</i> EMT-1,-2,-3,-4	Zorotest lambda prophage, <i>gal</i> gene expression	0/1	0/1	5,000	–	–
<i>E. coli</i> JC2921 JC5519	rec assay (growth inhibition)	0/1 0/1	1/1 1/1	NR	NR	Negative in four other DNA repair deficient strains (JC7623, 7689, 8471, and 9238).
<i>E. coli</i> <i>rec</i> strains	Differential toxicity	1/3	0/2	2,500	20 µL/disc	Positive result was not confirmed in repeat studies in two different labs.
<i>E. coli</i> W3110 (pol A <sup>+</sup> ) P3478 (pol A <sup>-</sup> )	Differential toxicity (liquid suspension)	0/1	0/1	250	–	No difference in survival in the polymerase deficient strain compared to the wild-type strain.
<i>E. coli</i> 58-161 envA C600	Prophage lambda induction, SOS repair	NT	1/1	–	2,500	Tested with metabolic activation only. Chemical-induced induction was 25-fold greater than spontaneous induction.
<i>B. subtilis</i> H17 <i>rec</i> <sup>+</sup> M45 <i>rec</i> <sup>-</sup>	rec assay (DNA damage)	1/1	1/1	–	NR	Only one study conducted. Used the more sensitive spore method rather than vegetative cells. Only reported the maximum dose tested (20 µL/disc). Rat and yellowtail fish S9 used.

Sources: An et al. (2010); Danford (1991); IARC (2010e).

HID = highest ineffective dose; LED = lowest effective dose; NR = not reported; NT = not tested; Res. = Results.

<sup>a</sup>Number of positive studies/number of studies reviewed.

<sup>b</sup>HIDs are the same value with or without S9. Since no studies were positive without S9, the LEDs values apply only to the few positive studies with S9.

**Table F-2. Co-mutagenic Effects of *ortho*-Toluidine and Norharman in Bacteria**

Bacteria Strains	Endpoint	Results <sup>a</sup>		Reference
		– Norharman (HID/LED µg/mL)	+ Norharman (HID/LED µg/mL)	
<i>S. typhimurium</i> TA98 TA100	Reverse mutation (plate incorporation)	– (100) – (100)	+ (20) + (20)	Nagao et al. (1978) (cited in Danford (1991))
<i>S. typhimurium</i> TA98 TA100 TA1537	Reverse mutation (plate incorporation)	– (500) – (500) – (500)	+ (25) + (25) + (25)	Nagao and Takahashi (1981) (cited in Danford (1991))
<i>S. typhimurium</i> TA98	Reverse mutation (pre-incubation)	– (25)	+ (25)	Nagao et al. (1978) (cited in Danford (1991))
<i>S. typhimurium</i> TA98 TA100 TA1535 TA1538	Reverse mutation (pre-incubation)	– (100) – (100) – (100) – (100)	+ (20) – (100) – (100) + (20)	Sugimura et al. (1982) (cited in Danford (1991))
<i>S. typhimurium</i> TA98	Reverse mutation (pre-incubation)	– (100)	+ (100)	Mori et al. (1996)
<i>S. typhimurium</i> TA98	DNA adducts	– (4 mg)	+ (4 mg)	
<i>S. typhimurium</i> YG1024	DNA adducts	– (4 mg)	+ (4 mg)	Hada et al. (2001)

– = negative; + = positive; HID = highest ineffective dose; LED = lowest effective dose.

<sup>a</sup>All positive responses occurred with metabolic activation.

**Table F-3. Genetic Effects of *ortho*-Toluidine in Non-mammalian Eukaryotes**

Test System	Endpoint	Results <sup>a</sup>		HID (µg/mL)	LED (µg/mL)
		– S9	+ S9		
<i>Aspergillus nidulans</i>	Forward mutation	0/1	NT	504	–
	Genetic crossing-over	0/1	NT	2,520	–
<i>Saccharomyces cerevisiae</i>	Differential toxicity	1/2	1/2	1,000	300
	Gene conversion	1/8	2/8	2,000	50
	Mutations	2/8	1/7	2,500	378
	Deletion assay	2/2	1/1	–	1,000
	Interchromosomal recombination	0/2	0/2	8,000	–
<i>Schizosaccharomyces pombe</i>	Aneuploidy	2/3	2/2	1.5 µL/mL	50
	Forward mutation	0/1	0/1	1,920	–
<i>Drosophila melanogaster</i>	Somatic mutation and recombination		5/6	2 mM (feed)	0.94 mM (feed)

Sources: Danford (1991); IARC (2010e), Brennan and Schiestl (1999).

HID = highest ineffective dose; LED = lowest effective dose; NT = not tested.

<sup>a</sup>Number of positive studies/number of studies reviewed.

Table F-4. In Vitro Genetic Toxicology Studies in Mammalian Cells

Endpoint	Cell Type (Assay)	Results <sup>a</sup> -S9 No. Studies	LED (µg/mL)	HID (µg/mL)	Results <sup>a</sup> +S9 No. Studies	LED (µg/mL)	HID (µg/mL)
<b>Human cells</b>							
DNA damage/strand breaks	MCL-5 <sup>b</sup> (comet assay)	1/1	454	–	–	–	–
	Exfoliated milk cells <sup>b</sup> (comet assay)	1/1	[91]	–	–	–	–
	Urinary bladder mucosa <sup>b</sup> (comet assay)	1/1	[1,714]	–	–	–	–
Unscheduled DNA synthesis	HeLa cells	0/3	–	1,000	3/3	25	–
Gene mutations	TK6 lymphoblasts (TK)	0/1	–	750	1/1	450	–
	AHH-1 lymphoblasts <sup>b</sup> (HGPRT)	1/1	300	–	–	–	–
Sister chromatid exchange	Lymphocytes	1/2 <sup>c</sup>	[21.4]	–	0/1	–	600
Micronucleus	Lymphocytes	1/1	[214]	–	0/1	–	[960]
<b>Rodent cells</b>							
DNA damage/strand breaks	Rat hepatocytes <sup>b</sup> (alkaline elution)	1/1	319	–	–	–	–
	Rat urinary bladder mucosa <sup>b</sup> (comet assay)	1/1	[857]	–	–	–	–
	Chinese hamster ovary (CHO) (alkaline sucrose gradient)	1/2	4,280	5,360	2/2	2,140	–
Unscheduled DNA synthesis	Rat hepatocytes <sup>b</sup>	1/6	107	53.5	–	–	–
	Syrian golden hamster hepatocytes <sup>b</sup>	0/2	–	10.7	–	–	–
Gene mutations	CHO ( <i>Hprt</i> , ouabain resistance)	0/1	–	500	?/1	–	–
	Chinese hamster lung (CHL) V79 ( <i>Hprt</i> , ouabain resistance)	1/4	500	2,000	0/4	–	1,000
	Mouse lymphoma L5178Y ( <i>Tk</i> )	2/6	10	1,004	0/5	–	1,004
	Mouse lymphoma L5178Y ( <i>Hprt</i> )	1/2	200	1,300	1/2	200	1,300
	L5178Y (ouabain resistance)	0/2	–	1,004	0/2	–	1,004
	Mouse BALB/c 3T3 (ouabain resistance)	–	–	–	1/1	250	–
Sister chromatid exchange	CHO	3/5	300	[2,140]	1/5	?	[2,140]

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Endpoint	Cell Type (Assay)	Results <sup>a</sup> –S9 No. Studies	LED (µg/mL)	HID (µg/mL)	Results <sup>a</sup> +S9 No. Studies	LED (µg/mL)	HID (µg/mL)
Chromosomal aberrations	CHL	1/1	268	–	1/1	268	–
	Rat liver epithelial cells (RL <sub>4</sub> ) <sup>b</sup>	1/1	21.8	–	–	–	–
	CHO (plasma from exposed rats)	1/1	400 <sup>d</sup>	–	–	–	–
	Chinese hamster liver fibroblasts (CH1-L) <sup>b</sup>	1/1	12	–	–	–	–
	CHO	1/3	500	[2,140]	2/3	250	[2,140]
	CHL	0/1	–	1,500	1/1	1,000	–
Micronucleus	RL <sub>4</sub> <sup>b</sup>	1/1	700	–	–	–	–
	CHO	0/1	–	[4,286]	0/1	–	[4,286]
Aneuploidy/polyploidy <sup>c</sup>	Syrian hamster embryo (SHE)	1/1	NR	–	–	–	–
	CH1-L <sup>b</sup>	1/1	60	–	–	–	–
	CHL	1/1	1,000	–	0/1	–	1,500
Mitotic spindle	RL <sub>4</sub> <sup>b</sup>	0/1	–	700	–	–	–
	CH1-L <sup>b</sup>	0/1	–	120	–	–	–
Inhibition cellular communication	CHL V79 <sup>b</sup>	2/3	5	535	–	–	–

Sources: Danford (1991); IARC (2010e); Martin (2000a; 2000b) (DNA damage in human exfoliated milk cells), Robbiano et al. (2002) (DNA damage in human bladder mucosa and rat bladder mucosa cells).

[ ] Indicates data provided by original author (e.g., as molarity) was converted to µg/ml for ease of comparison.

HID = highest ineffective dose; LED = lowest effective dose; NR = not reported; ? = inconclusive.

<sup>a</sup>Number of positive studies/number of studies reviewed.

<sup>b</sup>Cells with an intrinsic metabolizing system; no testing +S9.

<sup>c</sup>One test was positive and the other was inconclusive.

<sup>d</sup>In vivo metabolism: CHO cells exposed to plasma from rats treated with 400 mg/kg *ortho*-toluidine.

<sup>e</sup>All studies examined 100 metaphases with 2 to 3 replicates/dose and compared results to negative and positive controls.

**Table F-5. Cell Transformation Studies of *ortho*-Toluidine in Rodent Cells**

Cell Type	Results -S9	Results +S9	HID (µg/mL)	LED (µg/mL)	Reference
Mouse BALB/c 3T3	-	NT	2		Maeshima et al. (2010)
	+ <sup>a</sup>	NT		800	Tanaka et al. (2012)
	-	+	330	150	Matthews et al. (1985)
Mouse Bhas 42 <sup>b</sup> initiation assay	-	NT	800		Sakai et al. (2010)
	+	NT		300	
Syrian hamster embryo (SHE) cells <sup>c</sup>	+	NT		50	Ahmadzai et al. (2012)
	+ <sup>d</sup>	NT		40	Pant et al. (2012)
	+	NT		20–600 <sup>e</sup>	Maire et al. (2012)
	+	NT		1	Barrett and Lamb (1985)
	+	NT		100	Sanner and Rivedal (1985)
	+	NT		750	Kerckaert et al. (1998)
	+	NT		965	Hatch and Anderson (1985)
SHE-SA7 <sup>c</sup>	(+)	NT			
Mouse C3H/10T1/2	-	(+)		600	Lawrence and McGregor (1985)
	+	NT		500	Nesnow et al. (1985)
Chinese hamster ovary cells	-	-	500		Zdzienicka et al. (1985)
Baby hamster kidney (BHK-21) cells	+	+		362	Daniel and Dehnel (1981)
	NT	+		250	Styles (1981)
RLV/F344 rat embryo cells <sup>c</sup>	(+)	NT		10	Suk and Humphreys (1985)

- = negative; + = positive; (+) = weak positive; HID = highest ineffective dose; LED = lowest effective dose; NT = not tested; RLV = Rauscher-leukemia virus; SA7 = Simian Agent 7 adenovirus.

<sup>a</sup>Tested in three labs: initial results were positive in 2 labs and negative in another although the number of foci was increased at the highest dose. Retest was clearly positive.

<sup>b</sup>BALB/c 3T3 clone transfected with an oncogenic murine *ras* gene (v-Ha-*ras*).

<sup>c</sup>Cells with an intrinsic metabolizing system.

<sup>d</sup>Tested in three labs: initial result in one lab was inconclusive due to cytotoxicity. Retest at lower concentration was positive.

<sup>e</sup>Tested positive in four labs but different LEDs in each lab.



**Table F-6. DNA Adducts from *ortho*-Toluidine Exposure In Vivo**

Reference	Sex:Species (No./Group)	Tissue	Exp. Route <sup>a</sup>	Dose or HID/LED (mg/kg)	Results	Evaluation: Comments
Brock et al. (1990)	M:CrI:CD rats (4–5)	Liver	gavage	500	<1 pmol/μg	Positive: binding peaked at 24 hr. Levels were lower than measured with <i>para</i> -toluidine (see Section 5.2.2), specific adduct was not identified
Jones and Sabbioni (2003)	F:Wistar rats (2)	Liver	Gavage	~54 <sup>b</sup>	Possible dG adduct	Negative: tentatively identified dG adduct determined to be false positive. Detection limit: <3.1/10 <sup>8</sup> bases
Duan et al. (2008)	M:F344 rats (3)	liver nose bladder WBCs	gavage (1 or 7 daily doses)	54 54 322 322	1.2/10 <sup>7</sup> nt 0.55/10 <sup>7</sup> nt ND ND	Positive: liver and nasal mucosa, single and multiple doses, specific adducts were not identified Negative: urinary bladder and WBCs Detection limit: ~1/10 <sup>8</sup> bases

Exp. = Exposure; HID = highest ineffective dose; LED = lowest effective dose; ND = not detected; nt = nucleotides; WBCs = white blood cells.

<sup>a</sup>Single dose unless otherwise noted.

<sup>b</sup>Administered 0.5 mM/kg = ~54 mg/kg.

**Table F-7. *ortho*-Toluidine-releasing DNA Adducts (fmol/ $\mu$ g DNA  $\pm$  S.D.) in Bladder Tissue from Cancer Patients (CP) and Sudden Death Victims (SDV)<sup>a</sup>**

Group	CP			SDV	
	(N)	Epithelial	Submucosal	(N)	Bladder Tumor
Non-smokers	27	0.136 $\pm$ 0.280	0.292 $\pm$ 0.784	7	10.580 $\pm$ 3.055***
Smokers	19	0.378 $\pm$ 0.910	0.232 $\pm$ 0.571	5	6.120 $\pm$ 5.193*
All	46	0.236 $\pm$ 0.625	0.267 $\pm$ 0.697	12	8.722 $\pm$ 4.491***

\* $p < 0.05$ ; \*\*\* $p < 0.001$  (Mann-Whitney test; compared to bladder tissue from sudden death victims).

Source: Böhm et al. (2011).

<sup>a</sup>Direct evidence for covalent binding of *ortho*-toluidine with DNA was not provided by the authors.

**Table F-8. Genotoxic Effects of *ortho*-Toluidine In Vivo**

Reference	Test System (Time after Dosing hr)	Sex:Species (No./Group)	Tissue	Exp. Route <sup>a</sup>	Dose or HID/LED (mg/kg)	Results	Evaluation:Comments			
Cesarone et al. (1982)	DNA SSB (4)	M: Swiss CD1 mice (8)	liver	i.p.	0	$21 \times 10^{-3}/\text{mL}^b$	Positive			
			kidney		100	$63 \times 10^{-3}$	Results reported as mean elution rate (see footnote).			
					0	$22 \times 10^{-3}$				
					100	$51 \times 10^{-3}$				
Robbiano et al. (2002)	DNA damage (20)	M: Sprague- Dawley rats (3)	bladder	gavage	$\frac{1}{4}$ LD <sub>50</sub> <sup>c</sup>	~7.4*	Positive (bladder)			
			kidney		$\frac{1}{2}$ LD <sub>50</sub>	9.7*	Negative (kidney and liver)			
			liver		$\frac{1}{2}$ LD <sub>50</sub>	~1	Comet assay. Results reported as the ratio of treated/control tail moment.			
					$\frac{1}{2}$ LD <sub>50</sub>	~1				
Sekihashi et al. (2002)	DNA damage (3, 8, 24)	M: ddY mice (4)		gavage	600 ( $\frac{1}{2}$ LD <sub>50</sub> )	<u>Sampling time (h)</u>				Positive (Bold) stomach (3** & 8*** h), liver (8* h), bladder (3** & 8*** h), lung (3*** & ***8 h), brain (8* h) Untreated animals as negative controls DNA migration (μm) = length of whole comet – diameter of comet head 50 nuclei measured/organ/animal
			stomach			0	3	8	24	
			colon			5.8	12.0	12.6	5.0	
			liver			2.8	6.2	4.3	4.8	
			kidney			2.4	2.2	8.5	3.5	
			bladder			2.0	5.0	6.6	2.2	
			lung			5.1	16.7	21.5	9.9	
			brain			2.2	17.1	12.3	2.8	
			bone marrow			0.9	7.3	9.2	1.2	
						0.8	1.9	3.8	1.8	
Sekihashi et al. (2002)	DNA damage (3, 8, 24)	M: Wistar rats (4)		gavage	600 ( $\frac{1}{2}$ LD <sub>50</sub> )	<u>Sampling time (h)</u>				Positive (bold) stomach (8* & 24* h), colon (24* h), kidney (3** h), bladder (8* h) Untreated animals as negative controls DNA migration (μm) = length of whole comet – diameter of comet head 50 nuclei measured/organ/animal
			stomach			0	3	8	24	
			colon			13.0	15.0	30.6	27.4	
			liver			10.7	15.1	20.0	22.7	
			kidney			1.8	2.3	5.4	2.4	
			bladder			2.8	23.7	15.4	7.9	
			lung			8.0	13.1	17.7	16.9	
			brain			2.4	6.7	4.4	3.4	
			bone marrow			1.8	2.7	1.0	1.8	
						1.6	1.2	0.5	1.0	

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Reference	Test System (Time after Dosing hr)	Sex:Species (No./Group)	Tissue	Exp. Route <sup>a</sup>	Dose or HID/LED (mg/kg)	Results	Evaluation:Comments
Neal and Probst (1983)	SCE (18)	F: Chinese hamsters (3)	bone marrow	i.p. or gavage <sup>d</sup>	0	4.1 ± 1.3	Weak positive 25 metaphases/animal were scored (SCE/metaphase).
					100	4.5 ± 1.6	
					200	5.6 ± 2.0*	
					300	5.6 ± 2.1*	
McFee et al. (1989)	SCE (23)	M: B6C3F <sub>1</sub> mice (4)	bone marrow	i.p.	0	3.3 ± 0.21	Positive 2nd trial conducted at 450 and 600 mg/kg. Significant at 450 but not at 600. 25 metaphases/animal scored. Pronounced sedation occurred at doses >300 mg/kg.
					150	4.5 ± 0.28	
					300	6.1 ± 0.69	
					600	6.5 ± 0.35*	
					Trend	p = 0.0001	
	CA (17)	M: B6C3F <sub>1</sub> mice (7-8)	bone marrow	i.p.	0	1.5 ± 0.69	Negative 50 metaphases/animal scored. Results represent % aberrant cells.
					150	0.75 ± 0.37	
					300	1.1 ± 0.60	
					600	0.57 ± 0.37	
	MN (24, 48, 72)	M: B6C3F <sub>1</sub> mice (8)	bone marrow	i.p.	0	2.16-2.65	Negative Data are the mean MN/1000 PCEs scored. Considerable fluctuation in percentage of PCEs observed but no apparent pattern. Values shown indicate range from 24 to 72 hrs.
					75	0.56-2.39	
					150	1.57-2.28	
					300	2.00-3.02	
				Trend	p = 0.15-0.31		

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Reference	Test System (Time after Dosing hr)	Sex:Species (No./Group)	Tissue	Exp. Route <sup>a</sup>	Dose or HID/LED (mg/kg)	Results	Evaluation:Comments
Salamone et al. (1981)	MN (30, 48, 72, 96)	M: B6C3F <sub>1</sub> mice (5)	bone marrow	i.p.	270 × 2 169 337	1 (72 hr) 5 (30 hr) 2 (72 hr)	Negative Data are the sum of MN/500 PCEs/mouse for 4 to 5 mice at peak sampling time. Multiple sampling times used to increase sensitivity. Phase 1 mice received 2 injections (24 hr apart) at 80% LD <sub>50</sub> and sampled at 48, 72, and 96 hr. Phase 2 mice received single injection at 50% or 100% LD <sub>50</sub> and sampled at 30, 48, and 72 hr). LD <sub>50</sub> = 0.338 mL/kg or about 337.3 mg/kg.
Tsuchimoto and Matter (1981)	MN (6)	M/F: CD-1 mice (4)	bone marrow	i.p.	0 40 × 2 80 × 2 160 × 2	0.08 (0.07–0.13) 0.08 (0.0–0.13) 0.05 (0.0–0.13) 0.15 (0.0–0.27)	Negative Two doses given 24 hr apart. Mice were killed 6 hr after the second dose. Data are the mean % micronucleated PCEs and range (1,500 PCEs scored/animal).
Suzuki et al. (2005)	MN (3–5 days)	M: F344 or SD rats <sup>d</sup> (4)	liver	i.p. or gavage <sup>d</sup>	<u>Lab 1</u> 0 300 600 <u>Lab 2</u> 0 300 600	0.05 0.1–0.11 0.01–0.06 0.04 0.04–0.08 0.01–0.04	Negative Data are the mean % micronucleated hepatocytes (2,000 hepatocytes scored/animal) and range over sampling times (3 to 5 days) Experiments conducted independently in two laboratories.

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Reference	Test System (Time after Dosing hr)	Sex:Species (No./Group)	Tissue	Exp. Route <sup>a</sup>	Dose or HID/LED (mg/kg)	Results	Evaluation:Comments
	MN (2 days)	M: F344 or SD rats <sup>d</sup> (4)	Blood	i.p. or gavage <sup>d</sup>	<u>Lab 1</u> 0 300 600 <u>Lab 2</u> 0 300 600	0.08 ± 0.06 0.25 ± 0.11* 0.36 ± 0.09** 0.21 ± 0.06 0.19 ± 0.03 0.46 ± 0.11**	Positive Data are mean % micronucleated reticulocytes (2,000 scored/animal on day 2) ± S.D.
Fernandez et al. (1989)	MN (12 days)	Newt larvae (15)	RBCs	Tank water	0 ppm 4 ppm 20 ppm 40 ppm	5.3 (87) 9.3 (140) 13.1 (197)* 27.2 (408)*	Positive Results are mean frequencies of micronucleated RBCs/1,000 cells (total no. cells with MN).
Topham (1980)	Transmissible genetic damage: abnormal sperm morphology (5 wk)	M: (CBA × BALB/c)F <sub>1</sub> mice (5)	Sperm	i.p.	400 × 5	Small increase at high doses	Negative Dose range 50 to 500 mg/kg, 500 mg/kg dose was toxic, repeat studies did not confirm original observation. All tests included positive controls.
Topham (1981)					100 × 5 250 × 5	1.4% increase 1.7% increase	Inconclusive: Increase was statistically significant compared to controls but did not fulfill all criteria for a positive response (i.e., response was < double the response in negative controls). Dose range 50 to 500 mg/kg, 500 mg/kg dose was lethal. At least 250 sperm examined. All tests included positive controls.

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Reference	Test System (Time after Dosing hr)	Sex:Species (No./Group)	Tissue	Exp. Route <sup>a</sup>	Dose or HID/LED (mg/kg)	Results	Evaluation:Comments
Amlacher and Ziebarth (1979) (cited in Danford (1991))	DNA synthesis inhibition.	?: suckling mouse	Kidney	i.p. or intra-gastric	15%–30% of LD <sub>50</sub>	No effect	Negative Measured as mean decrease of silver grains on autoradiograph per 150 renal cell nuclei compared to nuclei of untreated controls. Few details provided in original reference or in Danford (1991).
Seiler (1977)	DNA synthesis inhibition	M: Mouse (3–4)	Testes	gavage	200	75%*	Positive Results reported as % of tritiated thymidine incorporation compared to concurrent controls. Only one dose tested.

\*p < 0.05 compared with controls; \*\*p < 0.01 compared with controls.

CA = chromosomal aberration; Exp. = Exposure; HID = highest ineffective dose; i.p. = intraperitoneal; LED = lowest effective dose; MN = micronucleus; PCEs = polychromatic erythrocytes; RBCs = red blood cells; SCE = sister chromatid exchange; SSB = single strand breaks.

<sup>a</sup>Single dose unless otherwise noted.

<sup>b</sup>Values represent the elution rate (K) where  $K = \ln(\text{DNA\% retained on filter})/\text{eluted volume}$ .

<sup>c</sup>LD<sub>50</sub> value was not reported, HSDB (2011) reported rat oral LD<sub>50</sub> of 750 mg/kg, NTP (1996) reported values of 900 to 2,951 mg/kg.

<sup>d</sup>Authors tested multiple chemicals but did not provide further detail.

**Table F-9. Mutagenicity of *ortho*-Toluidine Metabolites in *Salmonella* Strains**

Metabolite	TA100 (-S9)	TA100 (+S9)	TA1535 (-S9)	TA1535 (+S9)	TA98 (-S9)	TA98 (+S9)	HID/LED ( $\mu$ M/plate)
<i>N</i> -Hydroxy- <i>o</i> -toluidine	-	+	-	+	-	-	0.5/1
<i>o</i> -Nitrosotoluene	-	+	-	+	-	-	0.5/1
<i>N</i> -Acetyl- <i>o</i> -toluidine	-	-	NT	NT	-	-	10
<i>N</i> -Acetyl- <i>N</i> -hydroxy- <i>o</i> -toluidine	-	-	+	-	-	+	5/10
<i>N</i> -Acetoxy- <i>N</i> -acetyl- <i>o</i> -toluidine	-	-	NT	NT	-	-	10
2-Hydroxy-6-methyl-acetanilide <sup>a</sup>	-	-	NT	NT	-	-	10 <sup>c</sup>
<i>o</i> -Azoxytoluene	-	-	NT	NT	-	-	10 <sup>c</sup>
<i>o</i> -Azotoluene	-	-	NT	NT	-	-	10 <sup>c</sup>
Anthranilic acid <sup>b</sup>	-	-	-	-	-	-	5,000 $\mu$ g/ plate

Sources: Chung et al. (1981); Gadupudi and Chung (2011); Gupta et al. (1987); Hecht et al. (1979).

HID/LED = highest ineffective dose/lowest effective dose; - = negative; + = positive; NT = not tested.

<sup>a</sup>*N*-acetyl-2-amino-*meta*-cresol.

<sup>b</sup>Also tested negative in *S. typhimurium* strains TA1537, TA1538, and TA102.

<sup>c</sup>Tested doses not explicitly stated; HID inferred from highest doses tested with other negative compounds.



**Table F-10. Hemoglobin and Albumin Adducts Formed In Vitro and In Vivo**

Reference	Exposure Conditions Route of Exposure <i>o</i> -Toluidine Concentration Time of Sacrifice	Protein	Adduct Detection	Results	Comments
<b>In vitro (protein)</b>					
Stettler et al. (1992)	130 nmol rat liver microsomes and cofactors 10 to 60 minutes	Albumin (rat or human)	Radioactivity	No appreciable binding	Authors state that conditions may not have been optimal
Stettler et al. (1992)	130 nmol rat liver microsomes and cofactors 10 to 60 minutes	Hb	Radioactivity	8–9 ng/mg Hb	Microsomes prepared from phenobarbital-induced animals increased <i>o</i> -toluidine binding 1.5-fold (27.2 ng/mg) compared to control microsomes (16.2 ng)
<b>In vivo studies</b>					
Birner and Neumann (1988)	B6C3F <sub>1</sub> mice (female) Gavage 0.47 to 0.6 mmol/kg 24 hr sacrifice	Hb	Base hydrolysis GC	HBI = 2.1 ± 0.4	–
Birner and Neumann (1988)	Wistar rats (female) Gavage 0.47 to 0.6 mmol/kg 24 hr sacrifice	Hb	Base hydrolysis GC	HBI = 4.0 ± 0.65	–

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Reference	Exposure Conditions Route of Exposure <i>o</i> -Toluidine Concentration Time of Sacrifice	Protein	Adduct Detection	Results	Comments
DeBord et al. (1992)	Sprague-Dawley rats (male) i.p.: <i>Dose effect study</i> : 10, 20, 40, 50, or 100 mg/kg bw 4 hr sacrifice <i>Time course study</i> : 50 mg/kg, 2–72 hr sacrifice; 100 mg/kg, 4 hr–28 d sacrifice p.o. 100 mg/kg 24 hr sacrifice	Hb	Radioactivity	<i>Dose effect study</i> : Highest level (23.0 ± 5.1 ng/mg Hb) at highest dose (100 mg/kg) HBI = 51.8 <i>Time course study (50 mg/kg)</i> : Highest level (14.2 ± 2.6 ng/mg Hb) at 24 hr p.o. 10.0 ± 0.8 ng/mg Hb	Hb peak at 24 hr in both time course studies Radioactivity still available at 28 days Half-life 12.3 days Pretreatment with phenobarbital (PB) or [3-naphthoflavone Increased binding in PB-induced animals considered not statistically significant; large rat to rat variability in the treatment group Due to high variability of findings, study considered inadequate to determine role of metabolic activation.
DeBord et al. (1992)	Sprague-Dawley rats (male) i.p. Same design as Hb study for dose effect and time course (albumin not analyzed in p.o. animals)	Albumin	Radioactivity	Dose effect: Highest level (32 ng/mg albumin) at highest dose (100 mg/kg) <i>Time course study (50 mg/kg)</i> : Highest level (15.6 ng/mg Hb) at 4 hr	Albumin peak at 4 hr in both time course studies No radioactivity at 72 hr (50 mg/kg) or 14 d (100 mg/kg) Half-life = 2.6 d
Stettler et al. (1992)	Sprague-Dawley rats (male) <sup>a</sup> i.p. 110, 20, 40, 50 100 mg/kg 4 hr	Hb	Base hydrolysis Radioactivity/ HPLC or GC	Dose-related response	Higher doses: decreased in the percent of bound <i>o</i> -toluidine that could be hydrolyzed suggesting other adducts are formed that are not susceptible to base-hydrolysis

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Reference	Exposure Conditions Route of Exposure <i>o</i> -Toluidine Concentration Time of Sacrifice	Protein	Adduct Detection	Results	Comments
Cheever et al. (1992)	Sprague-Dawley rats (male) <i>Route of exposure study:</i> 50 mg/kg/wt i.p. and po 4 hr sacrifice Time course studies: 50 mg/kg i.p. up to 72 hr; 100 mg/kg i.p. up to 28 d <i>Dose effect study:</i> 10 to 100 mg/kg bw i.p. 4 hr	Hb	Base hydrolysis Thin layer chromatography/ HPLC or GC-MS	<i>Route of exposure study:</i> i.p. $3.5 \pm 1.6$ ng/mg Hb p.o. $0.4 \pm 0.6$ ng/mg Hb <i>Time course studies</i> 50 mg/kg: Highest level ( $3.7 \pm 0.5$ ng/mg Hb) at 24 hr; 100 mg/kg: adducts detected up to 28 d <i>Dose Effect study</i> Highest level ( $7.5 \pm 0.9$ ng/mg Hb) at 100 mg/kg	Half-life = 11 day (following single i.p. dose of 100 mg/kg)
Cheever et al. (1992)	Sprague-Dawley rats (male) Same design as Hb study	Albumin	Base hydrolysis Thin layer chromatography/ HPLC or GC-MS	<i>Time course study:</i> (50 mg/kg): Highest level (2.5 ng/mg albumin) at 4 hr	Not detected at 24 hr

HBI = hemoglobin binding index = (mmol bound/mmol Hb)/mmol dose/kg body wt; Hb = hemoglobin.

\*Species and sex not clear, but male Sprague-Dawley specified as being used for the preparation of the phenobarbital-induced microsomes.



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