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TOXICOLOGICAL REVIEW

OF

TRICHLOROACETIC ACID

(CAS No. 76-03-9)

In Support of Summary Information on the Integrated Risk Information System (IRIS)

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U.S. Environmental Protection Agency
Washington, DC

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LIST OF ABBREVIATIONS AND ACRONYMS

ACO	acyl-CoA oxidase
ACP	acid phosphatase
AHF	altered hepatic foci
AIC	Akaike's Information Criterion
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
BMD	benchmark dose
BMDL	95% lower confidence limit on the BMD
BMDS	benchmark dose software
BMR	benchmark response
BrdU	bromodeoxyuridine
CAR	constitutive activated/androstane receptor
CASRN	Chemical Abstracts Service Registry Number
CPK	creatine phosphokinase
CYP450	cytochrome P450
DCA	dichloroacetic acid
DEHP	di(2-ethylhexyl)phthalate
DEN	diethylnitrosamine
EC_{50}	median effective concentration
ENU	ethylnitrosourea
GD	gestation day
GGT	gamma-glutamyl transferase
GJIC	gap junctional intercellular communication
GSH	glutathione
GST	glutathione S-transferase
HPLC	high performance liquid chromatography
IAP	intracisternal A particle
IGF	insulin-like growth factor
IL	interleukin
i.p.	intraperitoneal(ly)
IRIS	Integrated Risk Information System
IUR	inhalation unit risk
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LINE	long interspersed nucleotide element
LOAEL	lowest-observed-adverse-effect level
LTR	long terminal repeat
MCA	monochloroacetic acid
MDA	malondialdehyde
5MeC	5-methylcytosine
MNU	N-methyl-N-nitrosourea
MOA	mode of action
MSW	multistage Weibull
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)

NCEA	National Center for Environmental Assessment
NHEERL	National Health and Environmental Effects Research Laboratory
NOAEL	no-observed-adverse-effect level
NRC	National Research Council
8-OHdG	8-hydroxy-2′-deoxyguanosine
ORD	Office of Research and Development
PB	phenobarbital
PBPK	physiologically based pharmacokinetic
PCO	palmitoyl-CoA oxidase
PFOA	perfluorooctanoic acid
POD	point of departure
PPAR	peroxisome proliferator-activated receptor
RfC	reference concentration
RfD	reference dose
SD	standard deviation
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
TCE	trichloroethylene
TNF-α	tumor necrosis factor-alpha
UF	uncertainty factor
U.S. EPA	U.S. Environmental Protection Agency

FOREWORD

The purpose of this Toxicological Review is to provide scientific support and rationale for the hazard and dose-response assessment in IRIS pertaining to chronic exposure to trichloroacetic acid (TCA). It is not intended to be a comprehensive treatise on the chemical or toxicological nature of TCA.

The intent of Section 6, *Major Conclusions in the Characterization of Hazard and Dose Response*, is to present the major conclusions reached in the derivation of the reference dose, reference concentration and cancer assessment, where applicable, and to characterize the overall confidence in the quantitative and qualitative aspects of hazard and dose response by addressing the quality of data and related uncertainties. The discussion is intended to convey the limitations of the assessment and to aid and guide the risk assessor in the ensuing steps of the risk assessment process.

For other general information about this assessment or other questions relating to IRIS, the reader is referred to EPA's IRIS Hotline at (202) 566-1676 (phone), (202) 566-1749 (fax), or hotline.iris@epa.gov (email address).

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1. INTRODUCTION

This document presents background information and justification for the Integrated Risk Information System (IRIS) Summary of the hazard and dose-response assessment of trichloroacetic acid (TCA). IRIS Summaries may include oral reference dose (RfD) and inhalation reference concentration (RfC) values for chronic and other exposure durations, and a carcinogenicity assessment.

The RfD and RfC, if derived, provide quantitative information for use in risk assessments for health effects known or assumed to be produced through a nonlinear (presumed threshold) mode of action. The RfD (expressed in units of mg/kg-day) is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. The inhalation RfC (expressed in units of mg/m^3) is analogous to the oral RfD, but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for both the respiratory system (portal-of-entry) and for effects peripheral to the respiratory system (extrarespiratory or systemic effects). Reference values are generally derived for chronic exposures (up to a lifetime), but may also be derived for acute (≤24 hours), short-term (>24 hours up to 30 days), and subchronic (>30 days up to 10% of lifetime) exposure durations, all of which are derived based on an assumption of continuous exposure throughout the duration specified. Unless specified otherwise, the RfD and RfC are derived for chronic exposure duration.

The carcinogenicity assessment provides information on the carcinogenic hazard potential of the substance in question and quantitative estimates of risk from oral and inhalation exposure may be derived. The information includes a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen and the conditions under which the carcinogenic effects may be expressed. Quantitative risk estimates may be derived from the application of a low-dose extrapolation procedure. If derived, the oral slope factor is a plausible upper bound on the estimate of risk per mg/kg-day of oral exposure. Similarly, an inhalation unit risk is a plausible upper bound on the estimate of risk per $\mu g/m^3$ air breathed.

Development of these hazard identification and dose-response assessments for TCA has followed the general guidelines for risk assessment as set forth by the National Research Council (NRC, 1983). U.S. Environmental Protection Agency (U.S. EPA) Guidelines and Risk Assessment Forum technical panel reports that may have been used in the development of this assessment include the following: *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986a), *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986b), *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991), *Interim*

Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity (U.S. EPA, 1994a), *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b), *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995), *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996), *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998), *Science Policy Council Handbook*: *Risk Characterization* (U.S. EPA, 2000c), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000a), *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000b), *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002), *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005c), *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b), *Science Policy Council Handbook: Peer Review* (U.S. EPA, 2006c), and *A Framework for Assessing Health Risks of Environmental Exposures to Children* (U.S. EPA, 2006a).

The literature search strategy employed for TCA was based on the chemical name, Chemical Abstracts Service Registry Number (CASRN), and multiple common synonyms. Any pertinent scientific information submitted by the public to the IRIS Submission Desk was also considered in the development of this document. Primary, peer-literature identified through June 2011 was included where that literature was determined to be critical to the assessment. The relevant literature included publications on TCA that were identified through Toxicology Literature Online (TOXLINE), PubMed, the Toxic Substance Control Act Test Submission Database (TSCATS), the Registry of Toxic Effects of Chemical Substances (RTECS), the Chemical Carcinogenesis Research Information System (CCRIS), the Developmental and Reproductive Toxicology/Environmental Teratology Information Center (DART/ETIC), the Environmental Mutagens Information Center (EMIC) and Environmental Mutagen Information Center Backfile (EMICBACK) databases, the Hazardous Substances Data Bank (HSDB), the Genetic Toxicology Data Bank (GENE-TOX), Chemical abstracts, and Current Contents. Other peer-reviewed information, including health assessments developed by other organizations, review articles, and independent analyses of the health effects data were retrieved and may be included in the assessment where appropriate. In particular, EPA notes that in 2004 the International Agency for Research on Cancer (IARC, 2004) evaluated TCA as "not classifiable as to its carcinogenicity to humans" (Group 3) based on "limited evidence" in experimental animals and "inadequate evidence" in humans. Newer studies on TCA have become available and are considered in this Toxicological Review. It should be noted that references have been added to the Toxicological Review after the external peer review in response to peer reviewers' comments and for the sake of completeness. These references have not changed the overall qualitative and quantitative conclusions. See Section 7 for a list of references added after peer review.

2. CHEMICAL AND PHYSICAL INFORMATION

TCA is a colorless to white crystalline solid with a sharp, pungent odor (NIOSH, 2003). The dissociation constant (pKa) for TCA at 25°C is 0.51. In aqueous solutions, TCA occurs almost exclusively in the ionized form as trichloroacetate anion. Common synonyms include trichloroethanoic acid and trichloro-methanecarboxylic acid. The structure of TCA is shown in Figure 2-1 and selected physical and chemical properties of TCA are provided in Table 2-1.

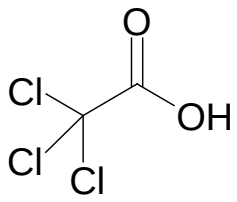


Figure 2-1. Trichloroacetic acid (TCA).

Table 2-1. Selected physical and chemical properties of TCA (CASRN 76-03-9)

Chemical formula	$C_2HCl_3O_2$	O'Neil (2001)
Molecular weight	163.39	O'Neil (2001)
Density	1.6126 g/mL at 64°C	Lide (2000)
Melting point	57.5°C	Lide (2000)
Boiling point	196.5°C	Lide (2000)
Vapor pressure	0.16 mmHg at 25°C	Liley et al. (1984)
Log pKa	0.51 at 25°C	Serjeant and Dempsey (1979)
Log K_{ow}	1.33	Hansch et al. (1995)
Water solubility	1,306 g/100 g at 25°C	Morris and Bost (2002)
Other solubilities	At 25°C, methanol, 2,143 g/100 g; ethyl ether, 617 g/100 g; acetone, 850 g/100 g; benzene, 201 g/100 g; *o*-xylene, 110 g/100 g	Morris and Bost (2002)
Henry's law constant	1.35×10^{-8} atm-m^3/mol at 25°C	Bowden et al. (1998)

TCA is used as a soil sterilizer and a laboratory intermediate or reagent in the synthesis of a variety of medicinal products and organic chemicals (HSDB, 2007). Medical applications of TCA include use as a reagent for the detection of albumin (Lewis, 1997), application as an antiseptic (Morris and Bost, 2002), and use as a skin peeling agent (Lee et al., 2002; Al-Waiz and Al-Sharqi, 2002; Coleman, 2001). TCA is also used industrially as an etching and pickling

agent for the surface treatment of metals and (in solution) as a solvent in the plastics industry (Koenig, 2005).

TCA can be formed as a combustion byproduct of organic compounds in the presence of chlorine (Juuti and Hoekstra, 1998). Stack gases of municipal waste incinerators have been reported to contain 0.37–3.7 µg/m^3 TCA (Mowrer and Nordin, 1987). TCA could be a photooxidation product of tetrachloroethylene and 1,1,1-trichloroethane in the atmosphere (Juuti and Hoekstra, 1998; Sidebottom and Franklin, 1996; Reimann et al., 1996b). Sidebottom and Franklin (1996) suggested that atmospheric degradation of chlorinated solvents could contribute only a minor amount of TCA to the atmosphere, based on the mechanistic and kinetic evidence, as well as the observed global distribution of TCA in precipitation. However, TCA has been detected in rainwater at a concentration range of 0.01–1 µg/L (Reimann et al., 1996a).

TCA is formed from organic material during water chlorination (IPCS, 2000; Coleman et al., 1980) and has been detected in groundwater, surface water distribution systems, and swimming pool water. TCA concentrations were measured in samples of disinfected drinking water collected under EPA's Information Collection Rule (U.S. EPA, 2000d). The mean concentrations of TCA (averaged across four distinct locations in the distribution system) were 3.28 and 13.25 µg/L in treated groundwater and surface water, respectively. TCA concentrations in drinking water are affected by water disinfection method, influent bromide concentration, influent total organic carbon (TOC) concentration, and temperature.

Human exposure to TCA occurs directly through the consumption and use of tap water disinfected with chlorine-releasing disinfectants (U.S. EPA, 2005a). TCA was detected in vegetables, fruits, and grains (Reimann et al., 1996a) and can be taken up into foodstuffs from the cooking water (U.S. EPA, 2005a). Therefore, human exposure to TCA can also occur via food consumption.

3. TOXICOKINETICS

3.1. ABSORPTION

Results from studies with rats and mice indicate that TCA is extensively absorbed by the gastrointestinal tract. In studies of excreta collected for up to 48 hours from male F344 rats and B6C3F$_1$ mice given single doses of [^{14}C]-labeled TCA in water ranging from 5 to 100 mg/kg, radioactivity detected in urine and in CO_2 in expired air represented about 57–72 and 4–8%, respectively, of the administered dose (Larson and Bull, 1992). Most of the urinary radioactivity was unmetabolized TCA, which accounted for 81–90% of the urinary radioactivity and 48–65% of the administered radioactivity. Urinary radioactivity in metabolites of TCA represented only minor amounts of the administered radioactivity: 1–3% for dichloroacetic acid (DCA) and 5–11% for a high performance liquid chromatography (HPLC) fraction coeluting with standards for glyoxylic acid, oxalic acid, and glycolic acid (which exist as glyoxylate, oxalate, and glycolate anions at physiological pH). Radioactivity detected in feces accounted for only about 2–4% of the administered radioactivity (Larson and Bull, 1992). In another study in which male B6C3F$_1$ mice were administered single 100 mg/kg doses of uniformly labeled [^{14}C]-TCA by gavage (constant volume of 10 mL/kg; vehicle not specified), the average distribution of radioactivity 24 hours after dose administration was about 55% in urine, about 5% in CO_2, and about 5% in feces, with the remainder in the carcasses (Xu et al., 1995). Radioactivity in urinary metabolites, expressed as percentage of the administered dose, showed the following distribution: 44.5% as trichloroacetate, 0.2% as dichloroacetate, 0.03% as monochloroacetate, 0.06% as glyoxylate, 0.11% as glycolate, 1.5% as oxalate, and 10.2% as unidentified compounds. Results from both of these studies are consistent with extensive absorption by the gastrointestinal tract, followed by rapid elimination in the urine, principally as the nonmetabolized parent compound.

Indicative of rapid absorption, TCA concentrations in the plasma or liver peaked in the first hour following oral dosing in other short-term studies with mongrel dogs (Hobara et al., 1988b) and male B6C3F$_1$ mice (administered either as aqueous free acid, neutral aqueous solution, or free acid in corn oil) (Styles et al., 1991). Likewise, peak blood concentrations of TCA were attained at a mean time of 1.55 hours after oral administration of single doses of 500 µmol/kg (82 mg/kg) TCA in water (adjusted to pH 7) to male F344 rats (Schultz et al., 1999). Comparison of the areas under the curve (AUCs) of plasma concentrations of TCA following oral administration and intravenous administration of TCA at the same dose level indicated that oral bioavailability of TCA was approximately equivalent to intravenous bioavailability (Schultz et al., 1999). The average ratio of oral:intravenous AUCs were 1.16. The 16% higher AUC value for oral exposure likely reflects measurement or statistical variability and/or differences in clearance rate by the two routes of administration. The mean absorption time, which was determined as the difference in the mean residence time in blood

following oral and intravenous dosing, was 6 hours for TCA. The mean absorption time is dependent on clearance from the blood as well as the absorption rate; therefore, the longer mean absorption time as compared to time-to-peak blood concentration of 1.55 hours may reflect slower clearance following oral dosing (Schultz et al., 1999).

Results from studies of urinary excretion of TCA by human subjects following 30-minute sessions in chlorinated swimming pool water indicate that TCA is rapidly absorbed by the skin (Kim and Weisel, 1998). TCA concentrations in pool water were measured before and after the subjects (two males and two females) either walked in the pool without submerging their heads (dermal exposure only) or swam (dermal exposure plus incidental oral exposure) in the pool for 30 minutes. TCA concentrations in the swimming pool water at various sessions varied from 57 to 871 µg/L, with a mean of 420 µg/L and a median of 278 µg/L. Entire urine voids were collected for at least 24 hours before exposure and 20–40 hours following exposure, at approximately 3-hour intervals. Additional urine samples were collected 5–10 minutes immediately before and after exposures. During the 24 hours prior to and following exposure, subjects avoided activities such as drinking chlorinated tap water or visiting the dry cleaner, which might have resulted in urinary TCA excretion. For each exposure session, the amount of urinary TCA associated with exposure was calculated for each subject from the amount of TCA excreted within 3 hours after exposure minus the amount excreted within 3 hours prior to exposure. Pre-exposure amounts of TCA in urine ranged from 155 to 1,183 ng, whereas postexposure amounts ranged from 294 to 1,590 ng. The amount of urinary TCA associated with the 30-minute exposure sessions ranged from 33 to 824 ng, depending on the subject and exposure session. Urinary excretion rates (ng/minute), calculated for various intervals before and after exposure, showed peaks at the postexposure 5–10-minute period that were about threefold higher than pre-exposure period rates. Excretion rates calculated for the first full 3-hour interval after exposure returned to values that were not discernable from pre-exposure rates. A scatter plot of the amount of urinary TCA per exposed body surface area (ng/m^2) in subjects under the dermal-exposure-alone scenario versus TCA exposure expressed as the TCA concentration in water multiplied by the exposure duration (µg/L × hour) indicated that urinary excretion (and, thus, presumably, dermal absorption) was higher with higher exposure. For exposures of about 20 and 420 µg/L × hour TCA, values for urinary TCA per surface area were about 10–50 ng/m^2 and 60–160 ng/m^2, respectively. The results from this study indicate that dermal absorption and subsequent urinary elimination of TCA are rapid, but are inadequate to provide more quantitative measures of dermal absorption for TCA, such as dermal permeability coefficients.

No studies were identified on the extent or rate of TCA absorption following inhalation exposure.

3.2. DISTRIBUTION

The tissue distribution of TCA following absorption has been most completely characterized in male F344 rats injected intravenously with radiolabeled [^{14}C]-TCA at doses of 0, 6.1, 61, or 306 µmol/kg (0, 1, 10, or 50 mg/kg) (Yu et al., 2000). TCA equivalent concentrations in plasma, red blood cells, and eight tissues (based on levels of detected radioactivity) were determined at various time points for up to 24 hours after injection (1, 3, 6, 9, and 24 hours). Peak concentrations in plasma and all tissues were observed at the postexposure first sampling. Levels of radioactivity in urine, feces, and expired air were also measured. Overall kinetic behavior was similar at all three doses (i.e., TCA equivalent concentrations declined with time in plasma and tissues, and first-order elimination rate constants were not consistently changed across tissues with increasing dose level). At early time points, the highest TCA equivalent concentrations were measured in plasma, followed by kidney, red blood cells, liver, skin, small intestine, large intestine, muscle, and fat; the relative order of these concentrations remained unchanged up to 3 hours following dosing. However, at 24 hours following dosing, the distribution pattern was changed, with the liver showing the highest TCA equivalent concentration. First-order rate constants for the disappearance of TCA equivalents from plasma and tissues were calculated and subsequently classified by the study authors into three groups: (1) fast elimination (rate constants between 0.081 and 0.156 h^{-1}) in plasma, red blood cells, muscle, and fat; (2) moderate elimination (rate constants between 0.064 and 0.077 h^{-1}) in kidney and skin; and (3) slow elimination (rate constants between 0.037 and 0.063 h^{-1}) in liver, small intestine, and large intestine.

To explore a possible explanation for the apparent differences in elimination kinetics of TCA in the plasma and liver of rats, Yu et al. (2000) compared the time courses of the distribution of nonextractable TCA equivalents (i.e., radioactivity from TCA metabolically incorporated into macromolecules) and extractable TCA equivalents in plasma and liver for up to 24 hours after injection. In both plasma and liver, nonextractable TCA equivalents increased to plateau levels within 6–10 hours after injection. Although the concentrations of nonextractable TCA equivalents in liver were higher than those in plasma, the total amount of TCA metabolized in these 24-hour studies (nonextractable TCA equivalents plus radioactivity in CO_2 in expired air) was estimated to be <20% of the administered dose.

The binding of TCA in plasma and liver homogenate was also investigated (Yu et al., 2000). Data were fitted using a model that assumed binding and consisted of two components: low-specificity (nonsaturable, linear) anion binding and high-specificity (saturable, nonlinear) binding. Results from these in vitro binding studies indicated that reversible binding of TCA in rat plasma (presumably to serum albumin) was more extensive than binding in liver homogenates. Yu et al. (2000) hypothesized that TCA disappears from the liver more slowly than from the plasma because of a concentrating transport process in hepatocyte plasma membranes. In addition, theoretical calculations of cumulative urinary excretion of TCA,

assuming glomerular filtration of free, unbound plasma TCA (the only operable excretory process), indicated that actual urinary excretion rates of TCA were slower than the theoretical values (Yu et al., 2000). It was hypothesized that this difference may be due to the occurrence of reabsorption of TCA into renal tubules and/or from the bladder. Support for this hypothesis, which provides at least a partial explanation for the relatively high concentrations of TCA equivalents in the kidney, includes the observation of reabsorption of TCA into the systemic circulation following injection into the bladder of dogs (Hobara et al., 1988a, 1987).

Reversible binding of trichloroacetate anion to positively charged proteins in plasma has been hypothesized to play a role in determining the tissue distribution and elimination of TCA and has been demonstrated in both in vivo and in vitro studies (Lumpkin et al., 2003; Toxopeus and Frazier, 2002; Yu et al., 2000; Schultz et al., 1999; Toxopeus and Frazier, 1998; Templin et al., 1993).

Unbound TCA accounted for an average of 53 ± 4% (mean ± standard deviation [SD]) of the total TCA plasma concentration in blood samples collected at 0.25, 1, and 3 hours after intravenous injection of single doses of 500 µmol/kg (81.7 mg/kg) TCA to male F344 rats (Schultz et al., 1999). In this in vivo study, gas chromatography and electron capture detection were used to determine TCA concentrations in plasma samples and ultrafiltrates of plasma samples from which proteins with molecular weights >10,000–12,000 were removed. The blood/plasma concentration ratio for TCA was 0.76, indicating some propensity for TCA to partition to the plasma, and was consistent with the ability of TCA to bind plasma proteins.

Templin et al. (1993) estimated the degree of in vitro TCA binding to plasma proteins by incubating [^{14}C]-TCA (position of radiolabel not specified) at various concentrations with plasma obtained from unexposed male B6C3F$_1$ mice. The amounts of unbound and bound radioactivity were determined in samples removed after various incubation times, using ultrafiltration to remove proteins from the samples. At TCA concentrations <306 nmol/mL, approximately 50–57% of the TCA was bound to plasma constituents, while percentage binding decreased with increasing TCA concentrations. Approximately 41, 34, and 23% of TCA was bound to plasma constituents at TCA concentrations of 306, 612, and 1,224 nmol/mL, respectively.

Templin et al. (1995) measured the binding of TCA to plasma proteins in four different species: dog, rat, mouse, and human. Plasma samples were prepared from whole blood and incubated with 3–1,224 nmol/mL [^{14}C]-TCA at 37°C for 30 minutes. Binding of TCA to plasma constituents was analyzed by using a Scatchard plot and is summarized in Table 3-1. Binding of TCA to plasma proteins was higher in humans than in rats and mice.

Table 3-1. Binding of TCA to plasma proteins from different species[a]

Species	Binding[b]			K_d[c] (µM)
	6 nmol/mL	61 nmol/mL	612 nmol/mL	
Mouse	55%	52%	34%	46.1
Rat	53.5%	48.9%	38.3%	383.6
Dog	64.8%	58.5%	54.2%	No data
Human	84.3%	83.3%	74.8%	174.6

[a]Values are expressed as percent of [^{14}C]-TCA associated with protein fraction, expressed as mean value for two replications of pooled samples.
[b]Templin et al. (1995).
[c]Lumpkin et al. (2003).

K_d = Dissociation constant

Toxopeus and Frazier (1998) investigated the kinetics of TCA in isolated perfused rat liver from male F344 rats. The isolated perfused rat liver system was dosed with either 5 or 50 µmol of TCA, and TCA concentrations were monitored in perfusion medium supplemented with 4% bovine serum albumin and in bile for 2 hours. Liver viability was assessed by measuring lactate dehydrogenase (LDH) leakage into perfusion medium and by the rate of bile production. At the end of the exposure period, the concentration of TCA in liver was measured. In the study with 50 µmol TCA, the total TCA concentration (free and bound to bovine serum albumin) in perfusion medium decreased slightly during the first 30 minutes and then remained constant for the duration of the exposure period; the total TCA concentration in the perfusion medium was relatively constant in the study with 5 µmol TCA. At the high concentration, approximately 93% TCA was bound to bovine serum albumin, and the free TCA concentration averaged 15.4 µM at 5 minutes of exposure and 14.9 µM at 120 minutes of exposure. At the low concentration, 96% of the TCA was bound to protein and the free TCA concentration was approximately constant at 0.9–1 µM over the study period. The calculated free-TCA concentration in the liver intracellular space was higher than the free-TCA concentration in the perfusion medium. Enzyme leakage and bile flow were similar at both TCA exposure levels to those in the control liver, indicating the absence of hepatotoxicity. The authors concluded that the binding of TCA to bovine serum albumin in perfusion medium limits the uptake of TCA by the liver and that TCA is virtually unmetabolized by the liver. These findings are consistent with those from in vivo mouse studies (e.g., Templin et al., 1993) demonstrating TCA binding to plasma proteins and suggest that TCA kinetics may be influenced by plasma-protein binding. In a similar study conducted in the same laboratory, using concentrations of 50, 250, or 1,000 µM TCA (Toxopeus and Frazier, 2002), >90% of the TCA in the perfusion medium was bound to albumin, confirming the results for extent of binding obtained by Toxopeus and Frazier (1998).

Lumpkin et al. (2003) measured the in vitro binding of TCA at 13 concentrations ranging from 0.06 to 6,130 µM (0.01–1,000 µg/mL) to plasma proteins in samples of plasma from

humans, rats, and mice. Pooled plasma for each species was obtained from commercial sources. Neither donor strain (for rodents) nor donor sex were specified. Binding was determined by using an equilibrium dialysis technique. Plots of bound versus free TCA concentrations were compared with simulations from three binding models—a single saturable site model, a two saturable site model, and a saturable plus unsaturable site model—to explore the mechanistic basis for species differences. Plots of bound versus free TCA concentration indicated that the proportion of bound TCA is substantially higher for human plasma than for rodent plasmas. Decreases in the proportion of bound to free TCA at concentrations >307 µM were indicative of saturation of plasma binding. Human plasma showed the most pronounced binding over the tested range of concentrations, followed by rat, then mouse. Binding to human plasma was highest (86.8%) at the lowest quantifiable TCA concentration (0.12 µM). The bound fraction in human plasma remained relatively constant, with a mean value of 81.6% over a 3.7 order of magnitude increase in TCA concentration. In comparison, maximum and average quasi-steady-state bound fractions were 66.6 and 38.6% for the rat and 46.6 and 19.1% for the mouse, respectively.

Lumpkin et al. (2003) noted that the average value of TCA protein binding for the mouse was considerably lower than the range of 34–57% determined in vitro in male $B6C3F_1$ mice reported by Templin et al. (1993). The reason for the disparity is unclear, but Lumpkin et al. (2003) noted that Templin et al. (1993) used Scatchard analysis over a narrower range of TCA concentrations to estimate binding parameters. The best fits to the observed data were obtained by using the single saturable binding process model, but data limitations (inadequate number of data points at low TCA concentrations) precluded acceptable fits of the two-saturable-process model. Use of albumin rather than total plasma protein concentration also improved model fit. The calculated binding capacity (B_{max}) values for humans, rats, and mice were 709, 283, and 29 µM of TCA, respectively. The average number of binding sites per molecule of protein was 2.97, 1.49, and 0.17, respectively. The low number of binding sites observed for mice may indicate the existence of other ligands competing for TCA binding sites in mouse plasma. The dissociation constant values for humans, rats, and mice were 174.6, 383.6, and 46.1 µM, respectively. The higher binding capacity of human plasma was a product of a higher number of binding sites per molecule of protein and higher reported plasma concentrations of albumin (239 µM for humans versus 190–196 µM for rodents).

A possible toxicological significance of these findings for binding of TCA to plasma proteins is that the extent of plasma binding may influence the distribution of TCA from blood to target tissues to a degree that may influence species differences in susceptibility to TCA toxicity. Based on the results from these in vitro binding studies and published reports of peak plasma concentrations of total TCA in mice (580 µM) and rats (300 µM) following gavage exposure to 1,200 mg/kg trichloroethylene (TCE), Lumpkin et al. (2003) calculated that plasma levels of free TCA would be about four- to fivefold higher in mice than in rats at this dose level. Lumpkin et

al. (2003) speculated that this difference was consistent with the apparent relative susceptibility of mice to TCA-induced liver tumors. The relative susceptibility of rats and mice to TCA-induced liver tumors awaits confirmation from further research (as discussed in Section 4.7), as does the hypothesis that toxicokinetics of TCA in humans may be more like TCA toxicokinetics in rats than in mice.

Abbas and Fisher (1997) determined in vitro tissue:blood partition coefficients for TCA in B6C3F$_1$ mouse tissues by using a closed vial equilibration method. The tissue:blood partition coefficients were 1.18 for the liver, 0.88 for the muscle, 0.74 for the kidney, and 0.54 for the lung. Comparable empirical data for TCA tissue:blood partition coefficients in other species were not located.

No additional studies were identified that might confirm the nature and extent of species differences in TCA distribution. Indirect evidence, primarily from studies involving exposure to chlorinated solvents, suggests that TCA is available for systemic distribution in humans, as determined by appearance of TCA in the blood and urine. TCA is a metabolite of TCE and has been frequently measured in the urine or blood of humans exposed to TCE as a result of environmental contamination (Brüning et al., 1998; Skender et al., 1994; Vartiainen et al., 1993; Ziglio et al., 1983; Ziglio, 1981) and in volunteer studies (Fisher et al., 1998; Brashear et al., 1997; NIOSH, 1973). TCA is also found in the blood and urine of humans without known chlorinated solvent exposures (Hajimiragha et al., 1986) and in individuals exposed to low concentrations of TCA in swimming pool water (Kim and Weisel, 1998) and drinking water (Calafat et al., 2003; Froese et al., 2002; Kim and Weisel, 1998).

No studies investigating the toxicokinetics or degree of maternal-to-fetus or blood-to-breast milk transfer of TCA were located, although TCA has been detected in mouse fetuses and amniotic fluid following 1-hour inhalation exposures of pregnant C57BL mice (gestation day [GD] 17) to high concentrations of TCE or tetrachloroethylene (presumably 1,100–1,200 ppm) (Ghantous et al., 1986a). In these studies, peak TCA concentrations in fetuses and amniotic fluid were attained 4 hours after cessation of exposure.

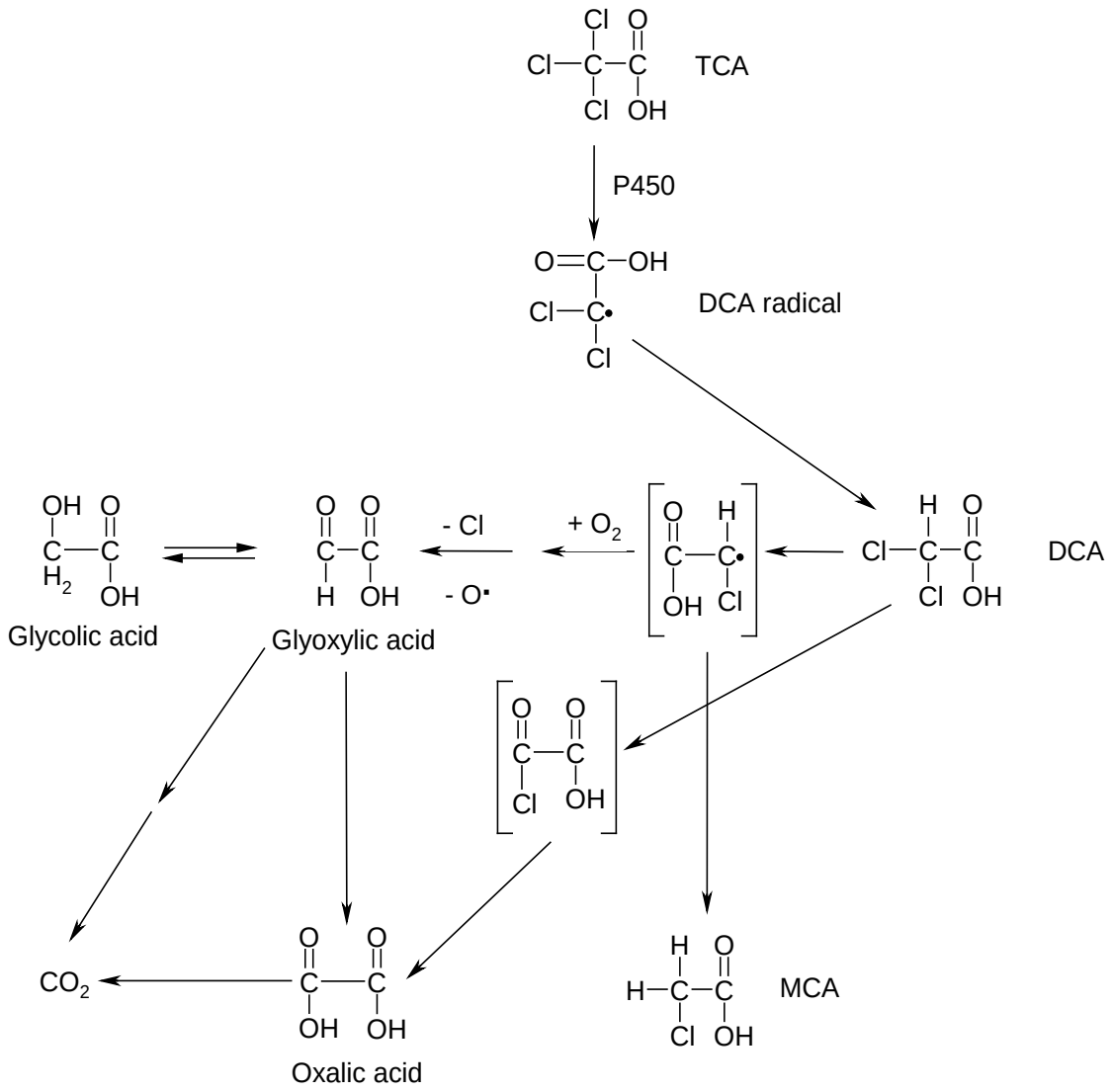
3.3. METABOLISM

As discussed in Sections 3.1 and 3.2, results from studies of rats and mice involving oral or intravenous administration of radiolabeled TCA indicate that TCA is only metabolized to a limited extent. Urinary excretion of nonmetabolized TCA accounted for about 48–55% of administered oral doses ranging from 5 to 100 mg/kg in rats and mice (Xu et al., 1995; Larson and Bull, 1992). Radioactivity in CO_2 collected in expired air accounted for 5–8% of administered doses in these studies, and amounts of radioactivity detected in individual metabolites in urine, such as DCA, monochloroacetic acid (MCA), glyoxylic acid, glycolic acid, and oxalic acid, were generally small, each accounting for less than 2 or 3% of administered doses (Xu et al., 1995; Larson and Bull, 1992). In contrast, orally administered radiolabeled

DCA is more extensively metabolized in rats and mice than is TCA (Larson and Bull, 1992). Based on measurement of radioactivity in expired CO_2 and in nonextractable radioactivity in plasma and tissues (i.e., radioactivity from metabolized TCA incorporated into macromolecules), Yu et al. (2000) estimated that <20% of an administered intravenous dose of 50 mg/kg TCA was metabolized in rats within 24 hours. Within 24 hours after injection of 1 or 50 mg/kg TCA, urinary excretion accounted for about 48 and 87%[1] and total exhaled CO_2 accounted for about 12 and 8% of the administered doses, respectively (Yu et al., 2000). These results are consistent with the idea that, at the higher dose level, metabolism of TCA may have been saturated, leading to an increased percentage of dose excreted as TCA in the urine and a decreased percentage of dose exhaled as metabolized CO_2. However, the distribution of radioactivity among TCA and potential metabolites in the urine was not quantified in this study (Yu et al., 2000), so confirmation of this idea awaits further research.

Figure 3-1 presents a proposed metabolic scheme for TCA, which is based on results from in vivo and in vitro studies in animals. The first proposed step is the reductive dehalogenation of TCA by cytochrome P450 (CYP450) enzymes, producing DCA via a free radical intermediate, the dichloroacetate radical. Early evidence in support of this step was restricted to the detection of radioactivity from TCA in urinary DCA (Xu et al., 1995; Larson and Bull, 1992) and the formation of lipid peroxidation byproducts following incubations of liver microsomes with TCA (Ni et al., 1996; Larson and Bull, 1992).

[1]These values were extracted from Figure 2 of the Yu et al. (2000) report.



Note: Molecules in brackets are intermediates proposed by Xu et al. (1995).

Sources: Adapted from Bull (2000); Lash et al. (2000); Merdink et al. (2000); Xu et al. (1995).

Figure 3-1. Proposed metabolic scheme for TCA.

Some uncertainty about the metabolic formation of DCA from TCA has been expressed, because DCA has been shown to form as an artifact during sample processing (Ketcha et al., 1996). Using analytical processes and methods to prevent the artifactual conversion of TCA to DCA, Merdink et al. (1998) reported that DCA was not detected in blood samples from male $B6C3F_1$ mice given single intravenous doses of 100 mg/kg TCA. Likewise, Yu et al. (2000) reported that radiolabeled DCA or other radiolabeled metabolites were not detected in plasma, urine, or other tissues collected from male F344 rats following intravenous injection of

[^{14}C]-labeled TCA, although metabolism of TCA was indicated in this study by the detection of radioactivity in exhaled CO_2 and in nonextractable materials (e.g., incorporated into cellular macromolecules) in plasma and tissue extracts. However, simulations with a pharmacokinetic model indicated that the rapid elimination of DCA from blood, relative to its formation, is consistent with the lack of accumulation of measurable amounts of DCA in the blood following injection of TCA (Merdink et al., 1998). Studies with a chemical Fenton reaction system and with suspensions of rat or mouse liver microsomes incubated with TCA detected the dichloroacetate radical by gas chromatography / mass spectrometry analysis following trapping of an adduct between the dichloroacetate radical and phenyl-tertiary-butyl nitroxide (Merdink et al., 2000), providing evidence for the occurrence of the metabolic conversion of TCA to DCA via reductive dehalogenation.

As shown in Figure 3-1, the reductive dechlorination of DCA to MCA has been proposed to proceed via a proposed monochloroacetate radical, which has also been proposed to be transformed to glyoxylic acid via oxidative dechlorination (Xu et al., 1995). Also shown in Figure 3-1 is a proposed oxidative dechlorination pathway that transforms DCA to oxalic acid via a proposed monochloroacetaldehyde intermediate (Xu et al., 1995). More direct evidence for these pathways is not available, and enzymes that may catalyze the reactions are not characterized. Glyoxylic acid can be metabolically transformed to glycolic acid and oxalic acid, as well as to CO_2, via mainstream carbon metabolic pathways (Figure 3-1).

Although the metabolism of TCA to DCA has been proposed, as shown in Figure 3-1, the mechanisms of dehalogenation of DCA have not been conclusively determined. The metabolism of both TCA and DCA to similar downstream metabolites, as shown in Figure 3-1, suggests that they may be sequential metabolites in the same pathway. For this reason, a brief summary of DCA metabolism is included in this review. For a more detailed analysis of data on DCA metabolism, the reader is referred to the IRIS *Toxicological Review of Dichloroacetic Acid* (U.S. EPA, 2003). DCA undergoes metabolic conversion via dechlorination and oxygenation to yield glyoxylate, oxalate, carbon dioxide, and several glycine conjugates, including hippuric acid (James et al., 1998; Lin et al., 1993; Evans, 1982; Crabb et al., 1981). In vitro experiments have demonstrated that conjugation with glutathione (GSH) also occurs and that this is the primary metabolic conversion pathway for DCA in the B6C3F$_1$ mouse, F344 rat, and human-liver cytosol (James et al., 1997; Lipscomb et al., 1995). The GSH-dependent oxygenation of DCA to form the initial major metabolite, glyoxylic acid, is catalyzed by glutathione S-transferase-zeta (GST-ζ) (Tong et al., 1998b, a).

Studies on enzyme pathways that might play a role in the metabolism of TCA are limited to one that evaluated the toxic effects of DCA and TCA on liver slices from male B6C3F$_1$ mice, as well as the metabolic capacity of the liver for these two compounds (Pravacek et al., 1996). To evaluate cytotoxicity (as evidenced by potassium content and liver enzyme leakage), the liver slices were exposed for up to 8 hours at concentrations of TCA ranging from 0 to 86 mM

(0–14 mg/mL) TCA. To determine if TCA treatments can alter phase I or phase II biotransformations, the liver slices were exposed to a low or high concentration of DCA or TCA, and the conversion of 7-ethoxycoumarin to 7-hydroxycoumarin (a measure of phase I metabolism) and formation of sulfate and glucuronide conjugates of hydroxycoumarin (a measure of phase II metabolism) were assessed. TCA treatment with 1,000 µg/mL increased phase I metabolism but had no effect on phase II metabolism at either 25 or 1,000 µg/mL. Metabolism of TCA was monitored by the rate of removal of the parent compound. The removal of TCA was not saturable at non-cytotoxic concentrations over the range of concentrations tested (0–5,000 µg/mL); thus, neither the K_m (the concentration at which half-maximal metabolic rate is reached) nor the V_{max} (maximum metabolic rate) was estimated. In contrast, DCA metabolism was saturable. Based on this difference in kinetics, Pravacek et al. (1996) suggested that TCA and DCA might be metabolized through distinct pathways, a finding consistent with other data demonstrating that the primary metabolic pathway for DCA is nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) and GSH dependent (e.g., Cornett et al., 1999; Cornett, 1997; Lipscomb et al., 1995), whereas the primary metabolic pathway for TCA appears to be mediated by CYP450 pathways. However, an alternative explanation for these data was noted, namely, that both TCA and DCA share a metabolic pathway that has a lower capacity for DCA.

TCA may be converted to DCA in situ in the gastrointestinal tract of mice, leading to the question of whether or not this process may influence levels of DCA in blood following exposure of mice to TCE (which is metabolically transformed to TCA) or TCA itself (Moghaddam et al., 1997; 1996). Under in vitro anaerobic conditions, microflora from the cecum of B6C3F_1 mice were clearly shown to convert TCA to DCA (Moghaddam et al., 1996). In contrast, gavage administration of 1,200 mg/kg TCE to control male B6C3F_1 mice and to mice whose gut was depleted of microflora by antibiotic treatment resulted in equivalent concentrations of DCA and other TCE metabolites (TCA, chloral hydrate, and trichloroethanol) in blood and liver (Moghaddam et al., 1997). These results suggest that metabolic formation of DCA by gut microflora does not influence circulating levels of DCA. In this study, antibiotic treatment resulted in large increases, compared with control values, in the total cecum content of TCA (4- and 9.5-fold at 4 and 8 hours after exposure), trichloroethanol (4.4- and 1.8-fold), and chloral hydrate (96- and 69-fold) but no significant change in total cecum content of DCA (93 and 74% of control values at 4 and 8 hours) (Moghaddam et al., 1997). The lack of a large effect of antibiotic treatment on DCA cecum content in situ, even when TCA levels were increased by this treatment, suggests that some other pathway may exist (other than conversion of TCA to DCA) for the appearance of DCA in the cecum of mice exposed to TCE.

In order to determine if TCA-induced lipid peroxidation is due to the formation of radical intermediates following dehalogenation of TCA by CYP450 enzymes, Austin et al. (1995) evaluated the effects of pretreating mice with TCA. Male B6C3F_1 mice were pretreated with 1,000 mg/L (estimated to be 228 mg/kg-day by the study authors) TCA in drinking water for

14 days and then administered 300 mg/kg TCA, DCA, or an equivalent volume of distilled water (control) by gavage as an acute challenge. Animals were sacrificed 9 hours following the acute challenge, and lipid peroxidation, peroxisome proliferation, and TCA-induced changes in phase I metabolism were measured. Measures of phase I metabolism included: (1) changes in 12-hydroxylation of lauric acid [an assay specific for CYP4A isoform activity, which is believed to be associated with induction of peroxisome proliferation in rats and mice (Gibson, 1989)]; (2) changes in *p*-nitrophenol hydroxylation (an assay specific for CYP2E1 activity); (3) immunoblot analysis for induction of CYP450 isoforms CYP2E1, CYP4A, CYP1A1/2, CYP2B1/2, and CYP3A1; and (4) total liver CYP450. Pretreatment with TCA increased 12-hydroxylation of lauric acid, demonstrating an increase in CYP4A activity (and apparently reflecting a peroxisome proliferation response), whereas *p*-nitrophenol hydroxylation was unchanged, indicating no effect on CYP2E1 activity. Immunoblot analysis, a measure of the amount of a protein, was consistent with the increase in CYP4A activity. Increased band intensities on the immunoblot appeared to occur at locations corresponding to those that have been identified as the CYP4A2 and CYP4A3 isoform bands. Similarly, immunoblot analysis was consistent with the absence of an effect on CYP2E1 activity and also showed no changes in CYP1A1/2, 2B1/2, or 3A1 protein levels. TCA pretreatment did not alter the overall amount of total liver microsomal P450. These data demonstrate that pretreatment of mice with TCA modifies the lipid peroxidation responses following acute challenge. The study authors suggested that this modification resulted from activities associated with peroxisome proliferation and might be related to a shift in the expression of P450 isoforms. The increased levels of CYP4A in TCA-pretreated mice are consistent with results observed in other studies with other peroxisome proliferators (Okita and Okita, 1992).

Results from another study with $B6C3F_1$ mice indicated that pretreatment with DCA or TCA in drinking water at concentrations of 2 g/L for 14 days had very little influence on the metabolism or kinetics of elimination of single 100 mg/kg gavage doses of [^{14}C]-labeled TCA (Gonzalez-Leon et al., 1999). Pretreated and control mice showed similar TCA blood concentration-time profiles. No significant differences in elimination kinetic parameters, such as volume of distribution, AUC, elimination half time, total body clearance, and renal clearance, were found between pretreated mice and control mice. The amount of radiolabel exhaled as CO_2, taken as an index of metabolism of TCA, was also not influenced by pretreatment. These results provide no evidence that pretreatment with TCA may induce levels of enzymes involved in the metabolism of TCA or inhibit metabolism of TCA or DCA (Gonzalez-Leon et al., 1999).

In summary, the available data on TCA metabolism in animal studies indicate that: (1) TCA is not as extensively metabolized as other chlorinated acids, such as DCA (Larson and Bull, 1992); (2) TCA is metabolically converted to DCA, but levels of DCA in blood, liver, and urine are low or not detectable, presumably due to rapid metabolic transformation of DCA into other metabolites (Yu et al., 2000; Merdink et al., 2000; Merdink et al., 1998; Xu et al., 1995;

Larson and Bull, 1992); (3) the metabolic conversion of TCA to DCA via reductive dehalogenation is likely catalyzed by CYP450 enzymes through the dichloroacetate radical intermediate (Merdink et al., 2000); (4) enzymes involved in TCA metabolism are poorly characterized; (5) microbial metabolism of TCA to DCA in the gut does not appear to influence circulating levels of DCA in the blood (Moghaddam et al., 1997; 1996); and (6) pretreatment of mice with TCA in drinking water does not markedly influence (e.g., enhance or inhibit) the metabolism or elimination kinetics of single challenge doses of TCA (Gonzalez-Leon et al., 1999; Austin et al., 1995).

3.4. EXCRETION

As described previously in Section 3.2, TCA in urine has been used as a biomarker for exposure to chlorinated solvents, which are metabolized to TCA, or exposure to disinfectant byproducts. This use is consistent with results from studies of rodents, clearly showing that, following oral or parenteral exposure to [^{14}C]-labeled TCA, TCA is principally eliminated from the body as the parent compound in the urine and that elimination of metabolites in the urine, elimination via the feces, and exhalation of completely metabolized TCA as CO_2 represent minor routes of elimination (Yu et al., 2000; Xu et al., 1995; Larson and Bull, 1992). For example, during a 48-hour period following administration of single doses of radiolabeled TCA ranging from 5 to 100 mg/kg to male F344 rats or male B6C3F$_1$ mice, radioactivity in urine, CO_2, and feces accounted for about 58–72, 4–8, and 2–4% of the administered dose, respectively (Larson and Bull, 1992). Non-metabolized TCA accounted for 81–90% of the radioactivity detected in the urine (Larson and Bull, 1992). Similarly, within 24 hours of intravenous injection of single doses of 1, 10, or 50 mg/kg radiolabeled TCA into male F344 rats, urinary excretion of radioactivity accounted for 48, 67, and 84% of the administered doses, respectively, whereas radioactivity in feces and CO_2 in expired air accounted for 4–8 and 8–12% of the administered doses, respectively (Yu et al., 2000).

Results from studies that monitored TCA concentration in bile from isolated rat livers perfused with TCA solution (Toxopeus and Frazier, 2002, 1998) or from dogs given intravenous doses of TCA (Hobara et al., 1986) indicate that rates of biliary excretion of TCA are low. For example, when isolated rat livers were perfused for 2 hours with medium containing initial TCA concentrations of 5 or 50 µM, excretion of TCA in bile was linear over time and cumulative excretion was 0.1% of the total dose by the end of the experiment (Toxopeus and Frazier, 1998). These results are consistent with the findings of low amounts of radioactivity in feces in the studies with radiolabeled TCA (Yu et al., 2000; Xu et al., 1995; Larson and Bull, 1992).

Studies comparing the relative importance of urinary, fecal, and exhalation routes of elimination in humans are not available.

Although elimination half-lives for TCA in urine were not reported in the available animal toxicokinetic studies involving direct exposure to TCA (e.g., Yu et al., 2000; Schultz et

al., 1999; Xu et al., 1995; Larson and Bull, 1992), the consistent finding of >50% of administered doses being excreted in the urine within 24-hours of dose administration is consistent with the hypothesis that significant portions of absorbed TCA can be rapidly eliminated from the body. However, the demonstrations of significant reversible binding of TCA to plasma proteins (e.g., Lumpkin et al., 2003; Toxopeus and Frazier, 2002, 1998; Templin et al., 1993) provide indirect evidence that bound TCA may contribute to TCA eliminated in the urine over periods of time longer than 24 hours after administration.

Limited support for a relatively slow elimination from the human body of at least some portion of absorbed TCA comes from a study of urinary TCA excretion in three human subjects during a 2-week period in which they ingested their normal tap water containing TCA, followed by a 2-week period in which tap water was replaced with bottled water containing no detectable TCA (Froese et al., 2002). TCA ingestion from tap water averaged 5.6 ± 3.1, 41 ± 27, and 73 ± 47 µg/day for the three subjects, reflecting substantial intrasubject and intersubject variability in daily intakes of TCA from tap water. TCA concentration was measured in first morning urine samples and normalized to creatinine concentration to adjust for differences in first morning urine volume. The logarithm of the creatinine-normalized TCA concentration was plotted against time during the bottled-water period and evaluated for a linear fit. The values for elimination half-life determined in this way ranged from 2.3 to 3.7 days.

In another study, three male volunteers ingested either 10 mg/kg trichloroethanol (in water), 3 mg/kg sodium trichloroacetate (in water), or 15 mg/kg chloral hydrate (in gelatin capsules) (Muller et al., 1974). The trichloroethanol and TCA concentrations in blood and urine were determined. The half-lives of TCA after ingestion of sodium trichloroacetate, trichloroethanol, and chloral hydrate were 50.6, 65.4, and 62.5 hours, respectively. Muller et al. (1974) demonstrated that the longer half-lives of TCA after ingestion of trichloroethanol and chloral hydrate were due to the prolonged formation of TCA from trichloroethanol, and the storage of trichloroethanol in the tissues, especially the fatty tissues.

When 15 mg/kg chloral hydrate was administered orally to a volunteer, there was a rapid increase in trichloroethanol and TCA concentrations, while no chloral hydrate could be measured. The half-life of trichloroethanol was about 7 hours, while the half-life of TCA was 4–5 days (Breimer et al., 1974).

Following inhalation exposure of five volunteers to 50 ppm TCE for 6 hours, the half-life of TCA was found to be 100 hours (Muller et al., 1972). Similarly, when five male volunteers were exposed to 100 ppm TCE for 2 weeks or 50 ppm TCE for 1 week, the half-lives of TCA were found to be 85.6 and 99 hours, respectively (Muller et al., 1974).

A study of urinary excretion of TCA following inhalation exposure to tetrachloroethylene (of which TCA is a metabolite) reported similar urinary elimination half-lives for TCA in humans. Volkel et al. (1998) exposed three male and three female human subjects and three male and three female Wistar rats to 10, 20, or 40 ppm tetrachloroethylene for 6 hours via

inhalation and measured metabolites in the urine. Urine was collected at intervals before exposure, during exposure, and up to 79 hours after beginning exposure. Urine was analyzed by gas chromatography / mass spectrometry for concentrations of DCA, TCA, and N-acetyl-S-(trichlorovinyl)-L-cysteine. TCA was the major metabolite recovered in the urine of both humans and rats. Half-lives of elimination of TCA from urine (estimated from the time course of TCA concentrations in urine following exposure) were 45.6 ± 2.5 hours in humans and 11.0 ± 1.2 hours in rats. It is uncertain if the apparent difference in elimination half-lives between humans and rats was due to species differences in rates of conversion of tetrachloroethylene to TCA, species differences in other processes more directly related to the appearance of TCA in the urine, or some other physiological difference between rats and humans.

In contrast to the relatively slow urinary excretion of TCA after cessation a 2-week exposure to tap water containing TCA (Froese et al., 2002) or cessation of a 6-hour inhalation exposure to perchloroethylene (Völkel et al., 1998), rapid urinary elimination kinetics of TCA were indicated in humans following exposure to TCA in swimming pool water (Kim and Weisel, 1998). In this study, four subjects (two/sex) walked in the pool for one 30-minute period (dermal exposure only) or swam (dermal exposure and presumed oral exposure from incidental ingestion of pool water during swimming) during a separate 30-minute period. TCA levels in the urine void collected 5–10 minutes after each 30-minute exposure period were elevated and generally returned to pre-exposure levels within 3 hours after exposure (i.e., were indistinguishable from pre-exposure levels). The relatively rapid return to pre-exposure levels within 3 hours after cessation of exposure is consistent with fast elimination kinetics in this study. However, as discussed in Section 3.1, there was large variability in the pre-exposure levels of TCA in urine[2], limiting the ability of this study to detect differences in pre- and postexposure levels of TCA in urine.

In summary, results from studies with animals indicate that urinary excretion of TCA is the principal route of elimination of TCA from the body (Yu et al., 2000; Xu et al., 1995; Larson and Bull, 1992). Other minor routes of elimination include urinary elimination of metabolites, exhalation of completely metabolized TCA as CO_2, and excretion of TCA in the bile or feces (Toxopeus and Frazier, 2002; Yu et al., 2000; Toxopeus and Frazier, 1998; Xu et al., 1995; Larson and Bull, 1992; Hobara et al., 1986). Although data on the kinetics of urinary elimination of TCA are limited, there are estimates that the half-life of TCA in urine from human subjects may be on the order of 2–3 days (Froese et al., 2002; Völkel et al., 1998). These findings are consistent with the idea that reversible binding of TCA to plasma proteins may influence the delivery of TCA to target tissues and prevent faster elimination of absorbed TCA in the urine.

[2]Pre-exposure amounts of TCA in urine ranged from 155 to 1,183 ng, whereas postexposure amounts ranged from 294 to 15,990 ng (Kim and Weisel, 1998).

3.5. PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS

Physiologically based pharmacokinetic (PBPK) models have not been developed for TCA. A PBPK model for TCE in humans (Fisher et al., 1998; Allen and Fisher, 1993) included a TCA compartment to account for metabolism of TCE. Fisher et al. (1998) concluded that further research is needed to explain the observed variability in urinary excretion of trichloroethanol glucuronide and TCA and the metabolic pathway resulting in the formation of DCA.

Chiu (2011) published a PBPK model for TCA in July 2011 after the current *Toxicological Review of Trichloroacetic Acid* was completed. Therefore, the availability of this model is not reflected in this assessment. The PBPK model by Chiu (2011) will be considered in future updates of the IRIS assessment for TCA.

Reference:

Chiu, WA. (2011) Trichloroacetic acid: updated estimates of its bioavailability and its contribution to trichloroethylene-induced mouse hepatomegaly. Toxicol 285(3):114-25.

4. HAZARD IDENTIFICATION

4.1. STUDIES IN HUMANS

4.1.1. Oral Exposure

No human epidemiology studies that evaluated TCA alone were located. Most of the human health data for chlorinated acetic acids concern components of complex mixtures of water disinfectant byproducts. These complex mixtures of disinfectant byproducts have been associated with increased potential for bladder, rectal, and colon cancer in humans [reviewed by Boorman et al. (1999) and Mills et al. (1998)] and adverse effects on reproduction [reviewed by Nieuwenhuijsen et al. (2000) and Mills et al. (1998)].

Most of the studies of human health effects following exposure to water disinfectant byproducts have used trihalomethanes and haloacetic acid concentrations as the exposure metric (Porter et al., 2005; King et al., 2005; Hinckley et al., 2005). These studies are not evaluated in this review as data on exposure to mixtures cannot be applied to the individual components of the mixture.

No clinical studies of the effects of oral or inhalation exposure of humans to TCA were located.

4.1.2. Dermal Exposure

Identified case reports demonstrate the corrosive potential of TCA to human skin. Depending on concentration and duration of contact, TCA can denature and precipitate protein. This characteristic has been used clinically in chemical skin peeling treatments for many years. TCA at concentrations ranging from 15 to 35% has been used in skin peeling treatments to treat conditions such as actinic damage, scars, wrinkles, and dyspigmentation (Cotellessa et al., 2003; Lee et al., 2002; Coleman, 2001; Kang et al., 1998; Witheiler et al., 1997; Tse et al., 1996; Moy et al., 1996; Chiarello et al., 1996; Rubin, 1995). Concentrations of ≥45% have an increased risk of causing scarring. Histological studies (Tse et al., 1996; Moy et al., 1996) indicate that the TCA-induced skin damage is characterized by epidermal loss, early inflammatory response, and collagen degeneration. Adverse side effects or complications resulting from these treatments are uncommon (Fung et al., 2002; Coleman, 2001) and are usually mild in severity (Fung et al., 2002). Reported side effects in patients receiving the skin peel procedure have included infection (Coleman, 2001), persistent (>1 month) erythema (Al-Waiz and Al-Sharqi, 2002; Coleman, 2001), transient hyperpigmentation (Fung et al., 2002; Lee et al., 2002; Coleman, 2001), acne or cyst formation (Lee et al., 2002; Coleman, 2001), keratoacanthomas[3] (Cox, 2003), and fine crusting (Kim et al., 2002). One case reported conjunctivitis and abrasions that involved

[3]Keratoacanthomas are round, firm, usually flesh-colored growths with a central crater that is scaly or crusted.

25% of the cornea when 35% TCA inadvertently entered the eye (Fung et al., 2002); complete corneal healing occurred, suggesting that the response to TCA was reversible under the reported exposure conditions. Nunns and Mandal (1996) reported two cases of inflammation of the vulva caused by the use of TCA in topical treatments of genital warts. Wilson et al. (2001) did not report any adverse side effects in patients (n = 95) treated for genital warts with either TCA, cryotherapy, or electrocautery.

4.2. SUBCHRONIC AND CHRONIC STUDIES AND CANCER BIOASSAYS IN ANIMALS—ORAL AND INHALATION

4.2.1. Short-term and Subchronic Studies

4.2.1.1. *Oral*

4.2.1.1.1. *Rats.* Short-term and subchronic (≤90 days) oral exposure studies are summarized in Table 4-1. Mather et al. (1990) evaluated toxicological effects in male Sprague-Dawley rats (10/dose group) dosed with neutralized TCA in drinking water at concentrations of 0, 50, 500, or 5,000 ppm (approximately 0, 4.1, 36.5, or 355 mg/kg-day) for 90 days. Animals were weighed at the beginning of the study and at the time of necropsy. Blood was collected at the time of sacrifice for clinical chemistry analysis (blood urea nitrogen, creatinine, glucose, alanine aminotransferase [ALT], alkaline phosphatase [ALP], cholesterol, total protein, albumin, calcium, phosphorus, creatine phosphokinase [CPK], and gamma-glutamyl transferase [GGT]). In addition, the following immune function parameters were evaluated: antibody production, delayed hypersensitivity, natural killer cell cytotoxicity, and production of prostaglandin E2 and interleukin (IL)-2. Hepatic peroxisomal and microsomal enzyme induction was also assessed. At sacrifice, a complete necropsy was performed, and the liver, kidneys, and spleen were weighed.

Table 4-1. Summary of acute, short-term, and subchronic studies evaluating effects of TCA after oral administration in rats and mice

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects[a]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Rats								
Goldsworthy and Popp (1987)	F344 rats (males, 5–6/group)	Oral, gavage	10 d	0 or 500 mg/kg-d in corn oil	Hepatic and renal peroxisome proliferation, increased relative liver weight	Not determined	500	The cyanide-insensitive PCO activity assay was used to measure the peroxisome proliferative response. Liver:body weight ratio was significantly increased.
DeAngelo et al. (1989)	Sprague-Dawley, F344, and Osborne-Mendel rats (males, 6/group/ strain)	Oral, drinking water	14 d	0, 212, 327, or 719 mg/kg-d	Hepatic peroxisome proliferation induction (Osborne-Mendel and F344 rats)	327	719	Peroxisome proliferation was observed only in Osborne-Mendel and F344 rats. These results suggest that Sprague-Dawley rats were the least sensitive of the three strains evaluated to peroxisome proliferation.
Davis (1990)	Sprague-Dawley rats (6/sex/ dose)	(A) Oral, drinking water	(A) 14 d	(A) 0, 5.2, 20.8, 81.9, or 309 mg/kg-d	(A) Limited endpoints were monitored. No effects were observed on weight gain, urine volume, and osmolarity, plasma glucose, and liver lactate levels	(A) Not determined	(A) Not determined	(B) At 0.15 mg/kg, plasma glucose levels were also decreased in females. These results are consistent with effects on intermediary carbohydrate metabolism. Similar effects were not observed in the 14-d study (A).
		(B) Oral, gavage	(B) Three doses over 24 hrs	(B) 0, 0.15, or 0.4 mg/kg in water, neutralized with sodium hydroxide	(B) Decreased plasma (45%) and liver lactate (48%) levels in females; decreased plasma lactate (30%) level in males at high dose; decreased plasma glucose level (25%) in females at high dose.	(B) Not determined	(B) 0.15	
Mather et al. (1990)	Sprague-Dawley rats (males, 10/dose)	Oral, drinking water	90 d	0, 4.1, 36.5, or 355 mg/kg-d	Increased absolute spleen weight; increased relative liver and kidney weights; increased liver, kidney, and spleen sizes; peroxisome proliferation	36.5	355	

Table 4-1. Summary of acute, short-term, and subchronic studies evaluating effects of TCA after oral administration in rats and mice

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects[a]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Bhat et al. (1990)	Sprague-Dawley rats (males, 5/group)	Oral, drinking water	90 d	0 or 825 mg/kg-d	Decreased body weight gain, minor changes in liver morphology, collagen deposition, perivascular inflammation of the lungs	Not determined	825	¼ of the LD_{50} (3,300 mg/kg) was administered daily.
Acharya et al. (1997; 1995)	Wistar rats (males, 5–6/dose)	Oral, drinking water	10 wks	0 or 3.8 mg/kg-d	Decreased terminal body weight, liver and kidney histopathologic changes, increased glycogen, changes in liver lipid and carbohydrate homeostasis, decreased kidney GSH	Not determined	3.8	Doses were estimated based on default drinking water intake values for rats. 3.8 mg/kg-d is judged as an equivocal LOAEL because the observed severity of the observed liver changes was considered minimal.
Celik (2007)	Sprague-Dawley rats (female)	Oral, drinking water	50 d	0 or 300 mg/kg-d	Increase in serum AST, ALT, CPK, and ACP activities; increase in SOD and catalase activities in brain, liver, and kidney tissues	Not determined	300	
Mice								
Goldsworthy and Popp (1987)	$B6C3F_1$ mice (males, 7–8/group)	Oral, gavage	10 d	0 or 500 mg/kg-d in corn oil	Induction of hepatic and renal peroxisome proliferation; increased relative liver weight	Not determined	500	Cyanide-insensitive PCO activity assay was used to measure the proliferative response. Liver:body weight ratio significantly increased.
DeAngelo et al. (1989)	$B6C3F_1$, C3H, Swiss-Webster, C57BL/6 mice (males, 6/group)	Oral, drinking water	14 d	0, 261, or 442 mg/kg-d	Increased relative liver weight, peroxisome proliferation (PCO activity)	Not determined	261	C57BL/6 mice were more sensitive than the other strains to peroxisome proliferation.

Table 4-1. Summary of acute, short-term, and subchronic studies evaluating effects of TCA after oral administration in rats and mice

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects[a]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Sanchez and Bull (1990)	$B6C3F_1$ mice (males, 12/group)	Oral, drinking water	14 d	0, 75, 250, or 500 mg/kg-d	Increased liver weight; hepatocyte proliferation (DNA labeling)	75	250	Doses were estimated based on default drinking water intake values for male $B6C3F_1$ mice. At 500 mg/kg-d, there was a slightly increased hepatocyte diameter because of increased glycogen deposition.
Dees and Travis (1994)	$B6C3F_1$ mice (5/sex/dose)	Oral, gavage	11 d	0, 100, 250, 500, or 1,000 mg/kg-d in corn oil	Increased absolute and relative liver weight; increased hepatocyte labeling	Not determined	100	
Austin et al. (1995)	$B6C3F_1$ mice (males, 6/group)	(A) Oral, drinking water (B) Oral, gavage	(A) 14 d (B) Single dose	(A) 0 or 250 mg/kg-d (B) 0 or 300 mg/kg in distilled water, pH adjusted to 7.0 with 5 N NaOH	(A) Increased relative liver weight (B) Decreased TBARS[b]; increased PCO, catalase, and CYP4A activities	Not determined	250	(A) Doses were estimated based on default drinking water intake values for male $B6C3F_1$ mice. (B) Acute administration occurred after a 14-d pretreatment period.
Austin et al. (1996)	$B6C3F_1$ mice (males, 6/group)	Oral, gavage	Single dose	0, 30, 100, or 300 mg/kg in water, pH adjusted to 7 using 5 N NaOH	Oxidative stress (increased 8-OHdG[c] levels)	Not reported	Not reported	Doses were estimated based on default drinking water intake values for male $B6C3F_1$ mice; 8-OHdG[c] levels at 30 or 100 mg/kg were not reported.

Table 4-1. Summary of acute, short-term, and subchronic studies evaluating effects of TCA after oral administration in rats and mice

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects[a]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Parrish et al. (1996)	$B6C3F_1$ mice (males, 6/group)	Oral, drinking water	3 or 10 wks	0, 25, 125, or 500 mg/kg-d	Increased absolute and relative liver weights; peroxisome proliferation (increased PCO activity and increased 12-hydroxylation of lauric acid)	25	125	Doses were estimated based on default drinking water intake values for male $B6C3F_1$ mice; results were similar for the 3- and 10-wk evaluations; 8-OHdG[c] levels were not affected by TCA.
Kato-Weinstein et al. (2001)	$B6C3F_1$ mice (males, 5/dose)	Oral, drinking water	(A) 4 or 8 wks (B) 12 wks	(A) 750 mg/kg-d (B) 0, 75, 250, or 750 mg/kg-d	Increased absolute and relative liver weights; decreased liver glycogen content	Not determined	75	Doses were estimated based on default drinking water intake values for male $B6C3F_1$ mice.
Laughter et al. (2004)	SV129 wild-type mice; PPAR[d] α-null mice (males, 3–5/group)	Oral, drinking water	7 d	0, 62.5, 125, 250, or 500 mg/kg-d	Induction of markers of peroxisome proliferation in wild-type but not PPAR[d] α-null mice at 2.0 g/L; induction of CYP4A at 1.0 g/L. Wild-type mice receiving the high dose exhibited centrilobular hepatocyte hypertrophy	125	250	Doses were estimated based on default drinking water intake values for male $B6C3F_1$ mice.

[a]The effects listed in this table may have occurred either at the LOAEL or at higher doses.
[b]TBARS = thiobarbituric acid-reactive substances.
[c]8-OHdG = 8-hydroxy-2′-deoxyguanosine.
[d]PPAR = Peroxisome proliferator-activated receptor.

ACP = acid phosphatase; AST = aspartate aminotransferase; LD_{50} = median lethal dose; LOAEL = lowest-observed-adverse-effect level; NOAEL = no-observed-adverse-effect level

Histopathologic examination was conducted on the brain, heart, lungs, kidneys, spleen, thymus, pancreas, adrenals, testes, lymph nodes, gastrointestinal tract, urinary bladder, muscle, and skin. TCA administration did not affect body weight at any dose. At 355 mg/kg-day, relative liver and kidney weights were significantly ($p \leq 0.05$) increased (7 and 11%, respectively) compared with controls. At the high dose, hepatic peroxisomal enzyme activity was significantly (15%, $p \leq 0.05$) increased (as measured by palmitoyl-CoA oxidase [PCO] activity). The liver, spleen, and kidney of high-dose animals were enlarged; however, no microscopic lesions were observed at any dose. No consistent treatment-related effects were seen on clinical chemistry or immune function parameters. EPA determined that the no-observed-adverse-effect level (NOAEL) for this study was 36.5 mg/kg-day and the lowest-observed-adverse-effect level (LOAEL) was 355 mg/kg-day, based on increased liver size and weight and peroxisome proliferation as well as statistically significantly increased kidney weight and size and increased spleen size.

In a subchronic study, Bhat et al. (1990) administered ¼ of a median lethal dose (LD_{50}) of TCA, DCA, or MCA in drinking water to male Sprague-Dawley rats (five/dose) for 90 days. Based on the reported LD_{50} of 3,300 mg/kg for TCA, ¼ of this value would correspond to an administered dose of approximately 825 mg/kg-day. Body weights were monitored throughout the study. The animals were sacrificed after 90 days of exposure, and the liver, lung, heart, spleen, thymus, kidney, testes, and pancreas were removed and weighed. These organs and the brain were microscopically examined. Liver sections were also stained for collagen deposition. No other toxicity parameters were evaluated. TCA exposure resulted in a significant depression (17%, $p < 0.0001$) of body weight gain throughout the exposure period. Toxicologically significant changes in liver weight were not observed. Exposure to TCA induced minimal to moderate collagen deposition (an indication of liver injury) in portal triads and large central veins in 4/5 animals (minimal collagen deposition was observed in 1/5 controls). Morphologic changes in the liver included portal vein dilation/extension of minimal to moderate severity in 5/5 TCA-treated animals. Perivascular inflammation of the lungs occurred at unspecified incidences. EPA determined that the only dose tested in this study, 825 mg/kg-day, was a LOAEL based on significantly reduced body weight gain.

In a 50-day drinking water study (Celik, 2007), 4-month-old female Sprague-Dawley rats were administered 2,000 ppm (300 mg/kg-day, assuming a default water intake of 0.15 L/kg-day) TCA (numbers unknown), while the control group received natural spring water. At the end of the study, blood samples were collected. Animals were sacrificed, and brain, liver, and kidney samples were obtained. Serum marker enzymes (aspartate aminotransferase [AST], ALT, CPK, acid phosphatase [ACP], ALP, and LDH), erythrocytes and tissue antioxidant defense systems (GSH, GSH reductase, superoxide dismutase [SOD], GST catalase), and malondialdehyde (MDA) (a product of lipid peroxidation) were measured.

TCA significantly increased serum AST, ALT, CPK, and ACP activity ($p \leq 0.05$) in treated rats. A slight but insignificant increase in MDA was found in the erythrocytes and liver. The antioxidant enzymes, SOD and catalase, were significantly increased in the brain, liver, and kidney. However, no changes in GSH, GSH reductase, or GST activities were found in any tissue. Celik (2007) concluded that elevated serum marker enzymes probably resulted from damage to liver cells by TCA and subsequent leakage of the enzymes into plasma and that the increases in SOD and catalase activities in the tissues after TCA treatment were probably due to increased generation of reactive oxygen species.

Acharya et al. (1995) evaluated liver and kidney toxicity of TCA as part of a study on the interactive toxicity of tertiary butyl alcohol and TCA. Young male Wistar rats (50 days old) (five to six/dose) were exposed to water containing 0 or 25 ppm or approximately 0 or 3.8 mg/kg-day, assuming a default water intake of 0.15 L/kg-day TCA (U.S. EPA, 1988) for 10 weeks. Animals were weighed weekly during treatment, and food and water consumption were recorded daily. Blood was taken from animals after the 10-week exposure, and the following parameters were evaluated: succinate dehydrogenase, ALP, ACP, AST, ALT, and serum triglyceride, cholesterol, and glucose levels. In addition, glycogen, triglyceride, cholesterol, GSH, lipid peroxidation, and diene conjugation were determined in liver homogenates. Microscopic examination of tissues was not performed.

In animals treated only with TCA, terminal body weight was decreased by approximately 17% in the absence of changes in food consumption (data not shown). Little, if any, TCA-induced liver toxicity was observed. Relative liver weight did not differ significantly in TCA-treated animals. No significant changes were detected in AST, ALT, ALP, or ACP. In contrast to the serum markers of liver necrosis, indicators of lipid and carbohydrate homeostasis were affected by TCA. Succinate dehydrogenase activity was increased by roughly 30% compared with controls. Liver triglyceride and cholesterol levels were significantly decreased, while liver-glycogen levels were increased approximately eightfold. Serum cholesterol levels were also increased approximately twofold. The study authors suggested that this profile of carbohydrate and lipid changes was consistent with the onset of hepatomegaly, which would increase the energy demands of the liver and activate succinate dehydrogenase, leading to increased oxidative phosphorylation and mobilization of lipids (decreased liver triglyceride and cholesterol). There was little evidence for induction of oxidative stress in the liver. Kidney, but not liver, GSH levels were decreased to approximately 66% of control values and no increase in lipid peroxidation was observed in the liver.

In a follow-up study using the same exposure protocol (Acharya et al., 1997), histopathologic changes in the liver and kidney were evaluated. The study authors noted that minimal hepatic alterations were observed in the TCA treatment group, indicating that the 3.8 mg/kg-day dose was marginally toxic. Liver histopathologic changes that were noted included centrilobular necrosis, hepatocyte vacuolation, loss of hepatic architecture, and

hypertrophy of the periportal region. Incidence and severity data were not reported for these lesions. Hypertrophy of the periportal region observed in the latter study may have accounted for the observed marginal increase in liver weight in the former study. The magnitude of the severity of these changes was reportedly small (the magnitude of the response could not be accurately quantified from the reported figures) and is consistent with the absence of effects on serum-liver enzymes in the earlier study (Acharya et al., 1995).

Histopathologic changes were also noted in the kidneys of TCA-treated animals and included degeneration of renal tubules with syncytial arrangement of the nucleus in the epithelial cells, degeneration of the basement membrane of Bowman's capsule, diffused glomeruli, vacuolation of glomeruli, and renal tubular proliferation in certain areas (incidence and severity not reported). Based on the liver and kidney histopathologic changes at the single dose tested, the study authors (Acharya et al., 1997) indicated that TCA is a liver and kidney toxicant.

Taken together, the two studies (Acharya et al., 1997; Acharya et al., 1995) suggest that the single dose tested, 3.8 mg/kg-day, is an apparent LOAEL. However, a number of questions regarding these studies preclude a definitive determination of the LOAEL. First, Acharya et al. (1995) noted a lack of increase in liver enzyme activity. Although liver histopathologic changes were observed, they were described as "only marginal" by the authors. The authors did not discuss the severity of the histopathologic changes in relation to untreated controls, and no incidence data were provided. Therefore, it is not clear whether the effects observed at the single TCA-only dose that was evaluated were adverse. Due to this uncertainty, EPA determined that 3.8 mg/kg-day could be best described as an equivocal LOAEL. It should be noted that Wistar rats were actually more sensitive than mice to increases in cyanide insensitive acyl-CoA oxidase (ACO) activity by TCA (Elcombe, 1985).

The ability of TCA to induce peroxisome proliferation and oxidative stress has been evaluated in a number of studies. Goldsworthy and Popp (1987) investigated the ability of TCA to induce hepatic and renal peroxisome proliferation (as assessed by the cyanide-insensitive PCO activity assay) in adult male F344 rats (five to six/dose) given 0 or 500 mg/kg-day TCA in corn oil via gavage for 10 consecutive days. Toxicological parameters other than liver and kidney weights were not evaluated. Hepatic peroxisomal enzyme activity increased significantly ($p < 0.05$) in rats receiving TCA, resulting in levels of enzyme activity approximately 2.8-fold greater than in controls. Liver-to-body-weight ratios were also significantly (41%, $p < 0.05$) increased relative to those in controls. Body weight gain was not changed. Renal peroxisomal enzyme activity was significantly ($p < 0.05$) increased by approximately 1.8-fold over that in controls in rats. Kidney weights were not affected by treatment. This study demonstrated that TCA treatment induced peroxisome proliferation in the livers and kidneys of male F344 rats.

Elcombe (1985) demonstrated species differences in peroxisome proliferation after TCA treatment in vivo and in vitro. Male Wistar rats and male Swiss mice were administered 10–200 mg/kg-day TCA in corn oil by gavage for 10 consecutive days. Control animals received

10 mL/kg corn oil vehicle alone. The animals were sacrificed 24 hours following the final dose, and the livers were excised and homogenized. Liver catalase activity and cyanide insensitive palmitoyl CoA oxidation (a peroxisomal β-oxidation marker) were determined spectrophotometrically.

In a separate study, Elcombe (1985) isolated hepatocytes from rats and mice. The isolated cells were seeded in a tissue culture flask and incubated at 37°C. Human hepatocytes were prepared from liver obtained from brain-dead renal transplant donors. TCA (up to a noncytotoxic concentration of 5 mM), dissolved in N,N-dimethylformamide, was added to the monolayer cultures at each 24-hour medium change. Ninety-six hours after seeding, the hepatocytes were harvested. Protein content and cyanide insensitive palmitoyl CoA oxidation in the cell homogenate were determined.

Dose-related increases in cyanide insensitive palmitoyl CoA oxidation were observed in rats and mice after TCA treatment. At doses of 200 mg/kg-day TCA for 10 days, 6.5-fold (Wistar rat) and 4.8-fold (Swiss mouse) increases in peroxisomal β-oxidation were observed. Peroxisome volume densities were increased concomitantly with β-oxidation activity. On the other hand, TCA had no effect on hepatic catalase activity.

Dose-related increases in cyanide insensitive palmitoyl CoA oxidation were also observed in cultured rat and mouse hepatocytes exposed to TCA (Elcombe, 1985). No stimulation of peroxisomal β-oxidation was observed, however, in cultured human hepatocytes prepared from two human liver samples and treated with TCA.

DeAngelo et al. (1989) conducted a series of experiments in three strains of rats and four strains of mice to determine relative species and strain sensitivities to the induction of hepatic peroxisome proliferation by chloroacetic acids (results of the mouse studies are described later in this section). Male Sprague-Dawley, F344, and Osborne-Mendel rats (six/dose/strain) received drinking water supplemented with 0, 6, 12, or 31 mM (approximately 0, 212, 327, or 719 mg/kg-day as calculated by the study authors) for 14 days. Hepatic PCO activity was used to assess peroxisome proliferation in all three strains. Carnitine acetyl-CoA transferase activity (another peroxisomal enzyme marker) was determined only in Sprague-Dawley rats, and induction of the peroxisome proliferation-associated protein was evaluated in high-dose Sprague-Dawley rats. Morphometric analysis of peroxisome proliferation was conducted by electron microscopy on liver sections from two high-dose Sprague-Dawley rats. No other toxicological parameters were evaluated.

TCA treatment did not significantly affect body weights or liver-to-body-weight ratios in either Osborne-Mendel or F344 rats. The final mean body weight of Sprague-Dawley rats was significantly reduced at 719 mg/kg-day when compared with controls (16% reduction). No effects were seen on liver-to-body-weight ratios in any of the strains. PCO activity was elevated in Osborne-Mendel rats by 2.4-fold and in F344 rats by 1.6-fold over control values at the high dose. In contrast, PCO activity was not affected in treated Sprague-Dawley rats at any dose.

Carnitine acetyl-CoA transferase activity, however, was increased by 321% above the controls in Sprague-Dawley rats at the high dose (significant increases were not observed at lower doses), but the volume fraction of cytoplasm from hepatic tissue occupied by peroxisomes was decreased to less than half that seen in controls in this strain. The reason for this paradoxical effect was not addressed. Taken together, these observations suggest that Sprague-Dawley rats are not sensitive to peroxisome proliferation in response to TCA exposure under the experimental conditions tested. EPA determined that the NOAEL and LOAEL values for peroxisome proliferation were 327 and 719 mg/kg-day, respectively, in both Osborne-Mendel and F344 rats.

Collectively, the data in rats suggest that short-term exposure to TCA primarily affects the liver, although effects on the kidneys and lungs have also been observed. Liver effects have included increased size and weight, collagen deposition, indications of altered lipid and carbohydrate metabolism, and peroxisome proliferation. Strain differences were also evident. An equivocal LOAEL of 3.8 mg/kg-day (liver and kidney pathology) was identified in 10-week studies in Wistar rats (Acharya et al., 1997; Acharya et al., 1995). In a 90-day study (Mather et al., 1990), a higher LOAEL of 355 mg/kg-day (increase in liver and kidney weight and peroxisome proliferation) was identified in Sprague-Dawley rats.

4.2.1.1.2. ***Mice.*** Short-term and subchronic studies in mice are summarized in Table 4-1. The available studies in mice have primarily been conducted to evaluate TCA-induced effects on the liver and the mode of action (MOA) underlying hepatic effects. No toxicity studies that evaluated a complete suite of toxicological parameters (e.g., body weight, clinical pathology, gross pathology, and microscopic pathology of a comprehensive set of tissues) in mice were located.

Goldsworthy and Popp (1987) investigated the ability of TCA to induce hepatic and renal peroxisome proliferation as assessed by the cyanide-insensitive PCO activity assay in adult male B6C3F$_1$ mice (seven to eight/dose) given 0 or 500 mg/kg-day in corn oil for 10 days via gavage. Relative liver and kidney weight were the only other toxicological parameters evaluated. Hepatic peroxisomal enzyme activity increased significantly ($p < 0.05$) in mice receiving TCA, resulting in levels of enzyme activity that were 280% those of the controls. Renal peroxisomal enzyme activity was significantly ($p < 0.05$) increased to 305% of control levels in mice. Liver-to-body weight ratios were also significantly increased (40%; $p < 0.05$) relative to controls.

DeAngelo et al. (1989) investigated the effects of TCA exposure on hepatic peroxisome proliferation by using four strains of male mice (B6C3F$_1$, C3H, Swiss-Webster, and C57BL/6). Groups of six mice per strain and dose were exposed to TCA in drinking water that contained 0, 12, or 31 mM (approximately 0, 261, or 442 mg/kg-day) TCA for 14 days. No effects were seen on body weight, but liver-to-body-weight ratios were significantly increased at both dosages in all four strains. The activity of PCO was elevated in all four strains for all TCA dose groups.

PCO levels were 276, 325, and 456% above controls at 12 mM and 648, 644, and 678% above controls at 31 mM for Swiss-Webster, C3H, and $B6C3F_1$ mice, respectively. PCO activity in C57BL/6 mice was increased by 2,100 and 2,500% above control levels at the high and low doses for TCA, respectively, indicating that this is a particularly sensitive strain of mouse.

In another phase of this study, catalase activity was increased by 461% above controls in $B6C3F_1$ mice at the high dose, with accompanying increases in the level of peroxisome proliferation-associated protein and number and size of peroxisomes in liver cytoplasm. The results indicated that mice, in general, are more sensitive than rats to the effects of TCA on peroxisome proliferation, as indicated by PCO activity. As described previously, levels of PCO activity in F344 and Osborne-Mendel rats were increased only by approximately 63 and 138%, respectively, at an approximate TCA dose of 719 mg/kg-day, and no significant effects on PCO activity occurred at 327 mg/kg-day in any strain. No effects were seen on this parameter in Sprague-Dawley rats at any dose (DeAngelo et al., 1989).

Several studies have evaluated the ability of TCA to induce oxidative stress in the liver of treated mice. These studies range from single-dose studies to 10-week studies. In an acute study by Austin et al. (1996), male $B6C3F_1$ mice (six/group) were treated with a single oral dose of TCA (0, 30, 100, or 300 mg/kg) in water, adjusted to pH 7 using 5 N NaOH. Mice were deprived of food for 3 hours prior to dosing. Liver nuclear DNA was extracted to assess increases in 8-hydroxy-2′-deoxyguanosine (8-OHdG) adducts, a measure of oxidative damage to DNA resulting from oxidative stress. TCA has been shown to induce lipid peroxidation in rodents (Larson and Bull, 1992), and compounds that produce oxidative stress also increase 8-OHdG, which is capable of inducing DNA base transversions that might be involved in the carcinogenic process (Chang et al., 1992). A significant increase in 8-OHdG in nuclear DNA in the liver was observed in the 300 mg/kg group at 8–10 hours post-dosing. The maximum 8-OHdG level was observed at 8 hours and was an increase of approximately 33% [estimated from Chang et al. (1992), Figure 3] over controls. The 8-OHdG levels in groups dosed with 30 or 100 mg/kg were not reported.

Austin et al. (1996) reported that the maximum concentration of TCA-induced thiobarbituric acid-reactive substances (TBARS) (an indicator of lipid peroxidation) occurred in the liver of mice 9 hours after dosing. In an earlier study, Larson and Bull (1992) also reported that the maximum concentration of TBARS occurred at 9 hours post-dosing in the livers of mice given 2,000 mg/kg TCA. The Larson and Bull (1992) study reported that a single oral dose of TCA 9 hours after dosing induced TBARS levels 1.15-, 1.7-, 2-, and 2.7-fold over controls at 100, 300, 1,000, and 2,000 mg/kg, respectively. Austin et al. (1996) suggested that the ability of haloacetates to increase both TBARS and 8-OHdG levels indicates that oxidative stress may be related to their hepatocarcinogenicity. The concordance between TBARS and 8-OHdG levels also suggested a common mechanism of induction of these two markers. Neither a NOAEL nor

a LOAEL were identified for Austin et al. (1996) because no other measures of liver or systemic toxicity were reported.

Parrish et al. (1996) evaluated the ability of haloacetic acids to induce oxidative DNA damage in the livers of mice. Male B6C3F_1 mice (six/group) were exposed to 0, 100, 500, or 2,000 mg/L TCA in drinking water for either 3 or 10 weeks. The study authors did not estimate the average daily doses resulting from exposure to these concentrations. Based on default water-intake values of 0.25 L/kg-day for male B6C3F_1 mice (U.S. EPA, 1988), the corresponding doses were approximately 0, 25, 125, or 500 mg/kg-day. Body weight and liver weight were evaluated. Several indicators for peroxisome proliferation were measured, including cyanide-insensitive PCO activity and increased 12-hydroxylation of lauric acid, which have been identified in other studies as "classical" responses resulting from exposure to compounds that are known peroxisome proliferators (Parrish et al., 1996). The level of 8-OHdG in liver nuclear DNA was also evaluated as an indicator of oxidative DNA damage. No histopathologic examination or standard clinical chemistry measurements were performed.

No differences in body weight were observed for any of the treatments (Parrish et al., 1996). The absolute liver weight was increased at the high dose, and relative liver weight was increased at the mid and high dose (by 13 and 33%, respectively) following exposure for 3 weeks ($p < 0.05$). After 10 weeks of exposure, the absolute liver weights were significantly increased at the mid dose and higher, and there were statistically significant increases in relative liver weight beginning at the mid dose (increases of 12 and 35%, respectively). Significant dose-related increments in cyanide-insensitive PCO activity were observed in mice treated at all TCA doses for 3 weeks (indicating peroxisome proliferative changes before liver weight changes); these increases persisted when treatment was extended to 10 weeks. Significantly increased 12-hydroxylation of lauric acid was also observed after 3 and 10 weeks of TCA exposure (the response was statistically significant at the high dose), whereas 8-OHdG levels were unchanged at both time periods. Thus, oxidative damage to genomic DNA as measured by 8-OHdG adducts did not occur with prolonged TCA treatment, even though peroxisome proliferation was induced, as indicated by increased PCO activity and 12-hydroxylation of lauric acid. The authors concluded that the lack of an increase in 8-OHdG indicated that this type of DNA base damage was not likely to be associated with the initiation of cancer by TCA; either the formation of these adducts was inhibited or their repair was enhanced with continued TCA treatment. The increased relative liver weight of approximately 10% at the mid dose (125 mg/kg-day) was accompanied by a significant increase in PCO activity but not 12-hydroxylation of lauric acid. The magnitude of these changes at the high dose was greater, with relative liver weight increasing approximately 35% over controls and significant increases in both indicators of peroxisome proliferation. Microscopic examination of the liver was not conducted in these experiments. However, based on significant increases in relative liver weight ($p < 0.05$)

accompanied by markers of peroxisome proliferation, EPA considered the mid dose of 125 mg/kg-day a LOAEL. The low dose of 25 mg/kg-day is considered a NOAEL.

Austin et al. (1995) tested whether TCA pretreatment would alter the lipid-peroxidation response of a subsequent acute dose of TCA. They also explored the relationship between TCA-induced lipid peroxidation and the ability of TCA to induce markers of peroxisome proliferation or CYP450s following short-term treatments. Male $B6C3F_1$ mice (18/group) were treated with 0 or 1,000 mg/L TCA for 14 days, which corresponds to estimated average doses of approximately 0 or 250 mg/kg-day based on the default water intake of 0.25 L/kg-day for male $B6C3F_1$ mice (U.S. EPA, 1988). For the lipid peroxidation experiments, the water or TCA pretreated mice were divided into six/group and administered 300 mg/kg of TCA, DCA, or an equivalent volume of distilled water by gavage (control) as an acute challenge. Animals were sacrificed 9 hours after the acute challenge. The livers were removed and homogenized, and the following endpoints were evaluated: (1) lipid peroxidation response, as measured by the production of TBARS; (2) indicators of peroxisome proliferation, as measured by increased PCO activity, increased catalase activity, and changes in microsomal 12-(ω) hydroxylation of lauric acid (an indicator for the activity of CYP4A); (3) hydroxylation of *p*-nitrophenol (as an index of CYP2E1 activity); and (4) protein levels for a panel of CYP450s, as described in Section 3.3. In addition to measurements following 14 days of treatment, TBARS levels were also measured for the acute-challenge experiments.

No changes in water consumption or body weight were observed, although relative liver weight was increased by 29% after 14 days of TCA treatment. TCA-treated mice had a lower mean TBARS level as compared with controls, but the difference was not statistically significant. In the acute challenge experiment, TCA-pretreated mice exhibited a significant decrement in TBARS in liver homogenates, following acute dosing with either TCA or DCA, as compared with animals that received the same acute challenge but had not been pretreated. In contrast to the decrease in TBARS induced by TCA pretreatment, PCO, catalase, and CYP4A activities were increased by 4.5-, 1.7-, and 2-fold, respectively, with TCA pretreatment. The TCA-pretreated group showed no increase in CYP2E1 activity and no changes in the overall amount of total liver microsomal P450. These data demonstrate that treatment of mice with TCA reduced lipid peroxidation responses but increased other markers that have been associated with peroxisome proliferation. The study authors suggested that the reduction in the TBARS response observed in TCA-pretreated animals resulted from activities associated with peroxisome proliferation, although it was not clear if the modifications were due to altered haloacetate metabolism or induction of systems that would quench reactions subsequent to lipid peroxidation initiation. The increased levels of CYP4A in TCA-pretreated mice are consistent with results observed in other studies with other peroxisome proliferators (Gibson, 1989). Peroxisomes were not measured directly. However, based on significant increases in relative liver weight and

several indirect markers of peroxisome proliferation (PCO, catalase, and CYP4A activities), the single dose tested, 250 mg/kg-day, is considered a LOAEL for this study.

In summary, the ability of TCA to induce oxidative stress responses, such as lipid peroxidation and oxidative DNA damage, and the relationship between these responses and indicators of peroxisome proliferation or altered CYP450 activities has been tested in a series of studies following acute or short-term TCA dosing in mice (Parrish et al., 1996; Austin et al., 1996; Austin et al., 1995; Larson and Bull, 1992). TCA induces both lipid peroxidation (TBARS) and oxidative DNA damage (8-OHdG) following administration of single oral doses. These increases appear transient, however, since neither lipid peroxidation (Austin et al., 1995) nor 8-OHdG formation (Parrish et al., 1996) were increased in multiple-dose studies. In contrast, responses associated with peroxisome proliferation were induced following TCA dosing for up to 10 weeks (Parrish et al., 1996; Austin et al., 1995). These results suggest that peroxisome proliferation is more likely than oxidative stress responses to be associated with liver toxicity observed in subchronic studies.

Sanchez and Bull (1990) investigated the effects of trichloroacetate on reparative hyperplasia in the livers of male B6C3F_1 mice (12 animals/dose group). TCA was administered in the drinking water for 14 days at concentrations of 0, 300, 1,000, or 2,000 mg/L, which correspond to estimated average doses of approximately 0, 75, 250, or 500 mg/kg-day based on the default water intake of 0.25 L/kg-day for male B6C3F_1 mice (U.S. EPA, 1988). Food and water consumption were recorded during the exposure period. After 14 days of exposure, animals were sacrificed, their livers and kidneys were removed and weighed, hepatocyte diameter was determined, and cell proliferation in the liver was assessed using [^{3}H]thymidine labeling after 2-day (n = 4), 5-day (n = 4), or 14-day (n = 12) treatments. Liver weight was significantly ($p < 0.05$) increased compared with controls at 250 (23%) and 500 mg/kg-day (38%). Hepatocyte diameter was significantly increased (13%; $p < 0.05$) at 500 mg/kg-day. Periodic acid-Schiff's reagent-positive material (glycogen) was confined to periportal areas. Necrosis was evident in 2 of 20 sections examined from high-dose animals, but it was not possible to determine whether this low frequency was treatment related. A significant ($p < 0.05$) increase in incorporation of [^{3}H]thymidine into hepatic DNA was seen at 5 and 14 days at the highest dose. However, this effect was not correlated with replicative synthesis of DNA as measured by autoradiography. These data suggest that other processes must account for the increased incorporation of radiolabel. The study authors suggested increased DNA repair synthesis or alterations in thymidine pool size as possible explanations for the observed results but noted that the mechanism for [^{3}H]thymidine uptake could not be determined based on the available data. EPA determined the LOAEL for this study to be 250 mg/kg-day for increased liver weight, and the NOAEL to be 75 mg/kg-day.

Dees and Travis (1994) evaluated the ability of TCA to induce DNA synthesis in the livers of male and female B6C3F_1 mice. Mice (five/sex/dose) were given gavage doses of 0,

100, 250, 500, or 1,000 mg/kg-day TCA in corn oil for 11 days. Twenty-four hours after the last dose, [^{3}H]thymidine was administered intraperitoneally (i.p.). Six hours later, the mice were sacrificed and their livers were removed. Liver samples were subsequently fixed for histopathologic examination and evaluation of DNA synthesis (based on incorporation of the radiolabeled thymidine). Final mean body weight and liver weight were also determined. There were no clinical signs of toxicity at the time of sacrifice, and no significant effects on body weight or body weight gain were observed. Absolute and relative liver weights were statistically significantly increased in all male and female treatment groups when compared with controls. In males, the relative liver weight was increased by 15% (at 500 mg/kg-day) to 28% (at 250 mg/kg-day), and the increases were not dose related. In contrast, the relative liver weight in females was increased by ≤9% at all doses, indicating that males may be more sensitive than females.

Histopathologic changes were observed for both males and females at 1,000 mg/kg-day. Histopathologic changes included a slight increase in the eosinophilic cytoplasmic staining of hepatocytes near the central veins (incidence not reported). The increase in eosinophilic staining was accompanied by a loss of cytoplasmic vacuoles. In the intermediate zone, subtle changes in cellular architecture were noted, including that the normally parallel pattern of hepatic cords was in disarray. Dee and Travis (1994) indicated that the appearance resembled areas of nodular cellular proliferation but did not discuss their criteria for evaluation of this lesion. In TCA-treated mice, [^{3}H]thymidine incorporation (observed in autoradiographs) was mostly localized in the intermediate zone in cells that resembled mature hepatocytes, while labeling in controls occurred primarily in the peri-sinusoidal cells. Similar patterns of labeling were observed in male and female mice. In addition, mitotic figures (indicative of dividing cells) were observed in the livers of TCA-treated mice but not in controls, and these dividing cells had often incorporated the radiolabel into the DNA. The observed mitotic figures and active labeling of dividing cells suggest the labeling of newly replicated DNA rather than labeling of damaged DNA as proposed by Sanchez and Bull (1990). The number of mature hepatocytes labeled with [^{3}H]thymidine appeared to increase with increasing TCA dose, reaching a maximum of approximately 2.5-fold increase at 1,000 mg/kg-day (no statistical analysis was reported). In contrast, the proportions of radiolabel incorporated into other cells (principally small peri-sinusoidal cells) remained relatively constant at all TCA doses.

Incorporation of [^{3}H]thymidine in extracted liver DNA also increased as TCA dose increased. In female mice, labeling was 1.1-, 2.0-, 2.9-, and 3.3-fold the control value at 100, 250, 500, and 1,000 mg/kg-day, respectively. In male mice, labeling was 1.3-, 1.4-, 1.8-, and 2.0-fold the control value at 100, 250, 500, and 1,000 mg/kg-day, respectively. The increase in DNA synthesis ([^{3}H]thymidine/µg DNA) became statistically significant at ≥250 mg/kg-day for female mice and ≥100 mg/kg-day for males. No difference in total liver DNA content (mg DNA/g liver) was observed. Peroxisome proliferation was not quantified. Dee and Travis (1994) concluded that their results are consistent with an increase in DNA synthesis and cell

division/proliferation in response to TCA treatment. The authors further suggested that, since only slight histopathologic effects were observed at the highest dose, it was unlikely that the increased DNA synthesis and cell division were secondary to tissue repair. Based on the increased relative liver weight (16%) at 100 mg/kg-day, accompanied by an increase in the [^{3}H]thymidine incorporation (1.3-fold) in male mice and supported by the histopathologic evidence of cell proliferation, EPA determined that 100 mg/kg-day was the LOAEL for this study. A NOAEL was not observed.

Kato-Weinstein et al. (2001) evaluated the ability of several haloacetic acids to affect liver glycogen content, serum insulin levels, and serum glucose levels in mice. Groups of five male B6C3F$_1$ mice were exposed daily to neutralized TCA (>98% pure) in the drinking water at 3 g/L for 4 or 8 weeks and at 0.3, 1, or 3 g/L for 12 weeks. The concentrations provided correspond to estimated average doses of approximately 0, 75, 250, or 750 mg/kg-day, respectively, based on a reference water intake value of 0.25 L/kg-day for male B6C3F$_1$ mice (U.S. EPA, 1988). Body and liver weights were recorded, and liver glycogen content and serum glucose and insulin levels were determined after 4, 8, or 12 weeks of exposure. Localization of glycogen in the liver was evaluated by periodic acid-Schiff's reagent staining.

TCA treatment did not affect body weight at any tested concentration. Relative liver weights were significantly ($p \leq 0.05$) greater than controls at all exposure groups, and absolute liver weights were significantly ($p \leq 0.05$) greater than controls at all exposure groups except in mice exposed at 0.3 g/L for 12 weeks. The magnitude of these increases was 20–50% greater than controls. The time course for liver glycogen content was significantly lower [approximately 25–33% as estimated from Figure 1A in Kato-Weinstein et al. (2001); $p \leq 0.05$)] than in controls after 8 and 12 weeks of treatment at 3 g/L. After 12 weeks of treatment, liver glycogen concentration was significantly decreased at all tested concentrations. No consistent or dose-related effects on insulin or glucose levels were observed at any concentration of TCA in this study. Histopathologic examination of livers from control animals revealed that glycogen-rich (strong periodic acid-Schiff's reagent staining) and glycogen-poor (low periodic acid-Schiff's reagent staining) cells were mixed in each hepatic zone, with slightly higher numbers of glycogen-rich cells in the portal area. In comparison, periodic acid-Schiff's reagent staining was confined to the periportal region in animals exposed to 0.3 and 1.0 g/L of TCA. This observation suggests that glycogen depletion occurred in the central lobular area as a result of depletion of glycogen from cells that appear to concentrate it in the liver of control mice. This result can be compared with observations made by Bull et al. (1990) and Sanchez and Bull (1990), who reported that TCA-treated animals displayed less evidence for glycogen accumulation and noted that when staining occurred, it was more prominent in the periportal portions than in centrilobular portions of the liver acinus.

Laughter et al. (2004) exposed wild-type SV129 mice (males, 3-5/group) and a mouse strain lacking a functional form of peroxisome proliferator-activated receptor (PPAR)α (PPARα-

null mice, males, 3-5/group) to TCA at 0, 0.25, 0.5, 1, or 2 g/L in the drinking water (neutralized) for 7 days. These concentrations correspond to estimated doses of approximately 0, 62.5, 125, 250, or 500 mg/kg-day, respectively, based on a reference water intake value of 0.25 L/kg-day for male $B6C3F_1$ mice (U.S. EPA, 1988). Wy-14,693 at 50 mg/kg was given as the positive control. Following exposure, the mice were sacrificed, and livers were removed and weighed. Subsamples of liver were processed for histopathologic examination, analysis of CYP4A and ACO protein expression, and measurement of PCO activity. Exposure to TCA increased liver-to-body-weight ratios in wild-type mice, but the response was not statistically significant. Exposure to TCA induced markers of peroxisome proliferation in wild-type mice but not PPARα-null mice. Exposure to 1 or 2 g/L TCA significantly increased the level of CYP4A protein, and exposure to 2 g/L significantly increased PCO and ACO activity in liver homogenates from wild-type mice only, indicating that PPARα is necessary for TCA to induce lipid metabolism enzymes associated with peroxisome proliferation. Centrilobular hepatocyte hypertrophy was observed in wild-type mice exposed to 2 g/L TCA, but not in PPARα-null mice exposed to the same concentration. The results of this study indicate that TCA induces peroxisome proliferation through activation of PPARα.

4.2.1.2. *Subchronic Inhalation Studie*s

No short-term or subchronic toxicity studies for TCA were identified for exposure by the inhalation route.

4.2.2. Chronic Studies and Cancer Assays

Long-term oral toxicity studies for TCA are available for rats and mice. The available data are summarized in Table 4-2 (noncancer data) and Table 4-3 (cancer and tumor promotion data).

Table 4-2. Summary of chronic studies evaluating noncancer effects of TCA after oral administration in rats and mice

Reference[a]	Species	Exposure route	Exposure duration	Doses evaluated	Noncancer effects evaluated	Effects[b]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Rats									
DeAngelo et al. (1997)	F344 rats (males, 50/group)	Oral, drinking water	104 wks	0, 3.6, 32.5, or 364 mg/kg-d	Body weight, ALT and AST activity, histopathology (liver, kidneys, spleen, testes, excised lesions at interim and terminal sacrifice; comprehensive histopathologic exam in high-dose group at terminal sacrifice), peroxisome proliferation	Decreased body weight, increased serum ALT activity; mild hepatocellular necrosis; increased peroxisome proliferation	32.5	364	Time-weighted average daily doses were calculated by the study authors; a comprehensive set of tissues was examined microscopically.
Mice									
DeAngelo et al. (2008)	$B6C3F_1$ mice (males, Study 1: 50/group; Study 2: 58/group; Study 3: 72/group; 27–30/dose at terminal sacrifice; 5/dose at interim sacrifices)	Oral, drinking water	Study 1: 60 wks Studies 2 and 3: 104 wks	Study 1: 0, 8, 68, or 602 mg/kg-d; Study 2: 0 or 572 mg/kg-d; Study 3: 0, 6, or 58 mg/kg-d (doses based on nominal drinking water concentrations; see text)	Body weight, liver weight, serum LDH activity, liver PCO activity, hepatocyte proliferation, histopathologic examination for gross lesions, liver, kidney, spleen, and testis at interim and terminal necropsies; complete histopathologic examination on five mice from the high-dose and control groups	LOAEL based on increased liver weight, hepatic necrosis, LDH activity (30 wks), and testicular degeneration. Other effects at higher doses included decreased body weight and hepatic inflammation. Increased liver PCO activity and labeling index for nuclei outside of hepatic proliferative lesions were observed at mid and high doses.	8	68	Time-weighted average daily doses were calculated by the study authors; a comprehensive set of tissues was examined microscopically.

Table 4-2. Summary of chronic studies evaluating noncancer effects of TCA after oral administration in rats and mice

Reference[a]	Species	Exposure route	Exposure duration	Doses evaluated	Noncancer effects evaluated	Effects[b]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Pereira (1996)	$B6C3F_1$ mice (females, 38–134/group)	Oral, drinking water	51 or 82 wks	0, 78, 262, or 784 mg/kg-d	Body and liver weight, liver histopathology	Increased relative liver weight	78	262	Increased liver weight was observed after 82 wks at 262 mg/kg-d; 262 mg/kg-d was judged to be an equivocal LOAEL in the absence of other measures of liver toxicity.
Bull et al. (1990)	$B6C3F_1$ mice (5–35 mice/ dose/time point, see text) (B) (11 males/ dose)	Oral, drinking water	(A) 52 wks (with interim sacrifices at 15, 24, and 37 wks) (B) 37 wks + 15-wk recovery	(A) 0, 164, or 329 mg/kg-d (B) 0 or 309 mg/kg-d	Liver and kidney weight and histopathology	Increased absolute and relative liver weight, cytomegaly, modest glycogen accumulation, accumulation of lipofuscin in liver	Not determined	164	Only the liver and kidneys were evaluated; dose was estimated by the authors.
Herren-Freund et al. (1987)	$B6C3F_1$ mice (males, 22–33/group)	Oral, drinking water	61 wks	0, 500, or 1,250 mg/kg-d	Liver weight and histopathology	Increased absolute and relative liver weight	Not determined	500	Only the liver was examined microscopically.

[a]Cancer studies that evaluated noncancer endpoints are included in this table; data from von Tungeln et al. (2002) were not included in this table because animals were not dosed by the oral route (i.p. injection).
[b]The effects listed in this table may have occurred either at the LOAEL or at higher doses.

Source: adapted from U.S. EPA (2005a).

Table 4-3. Summary of cancer bioassays and tumor promotion studies of TCA in rats and mice

Reference	Species	Study type	Exposure route	Exposure duration	Doses evaluated	Results	Comments
Cancer bioassays							
Rats							
DeAngelo et al. (1997)	F344 rats (males, 50/group)	Cancer assay, multiple organs	Oral, drinking water	104 wks	0, 3.6, 32.5, or 364 mg/kg-d	Negative for tumor induction	A comprehensive set of tissues was microscopically examined; only about 30 animals/ concentration were exposed for >60 wks.
Mice							
DeAngelo et al. (2008)	$B6C3F_1$ mice (males, 25–42/dose at terminal sacrifice; five/dose at interim sacrifices)	Cancer bioassay	Oral, drinking water	Study 1: 60 wks; interim sacrifices at 4, 15, 30, and 45 wks Studies 2 and 3: 104 wks (doses based on nominal drinking water concentrations; see text)	Study 1: 0, 8, 68, or 602 mg/kg-d; Study 2: 0 or 572 mg/kg-d; Study 3: 0, 6, or 58 mg/kg-d	Positive for liver tumors starting at 45 wks	Liver, kidneys, spleen, and testes were evaluated microscopically for tumors; complete histopathologic evaluation was conducted on other organs for five mice from the control and high-dose groups.
Pereira (1996)	$B6C3F_1$ mice (females, 38–134/ group)	Cancer bioassay	Oral, drinking water	51 or 82 wks	0, 78, 262, and 784 mg/kg-d	Positive for liver tumors at 51 and 82 wks	Only the liver was evaluated for tumors.
Bull et al. (2002)	$B6C3F_1$ mice (males, 20 or 40/group)	Cancer bioassay	Oral, drinking water	52 wks	0, 120, or 480 mg/kg-d	Increased incidence of liver tumors	Only the liver was microscopically examined; doses were estimated based on a default water intake of 0.24 L/kg-d.
Bull et al. (1990)	$B6C3F_1$ mice (5–35 mice/ dose/time point, see text) (B) 11 males/ dose	Chronic toxicity study with microscopic examination of the liver	Oral, drinking water	(A) 52 wks (interim sacrifices at 15, 24, and 37 wks) (B) 37 wks + 15-wk recovery (males only)	(A) 0, 164, or 329 mg/kg-d (females only treated with 329 mg/kg-d for 52 wks) (B) 0 or 309 mg/kg-d	Males: dose-related increase in liver tumors at 52 wks Females: no liver tumors found	Hepatoproliferative lesions were only observed in males, but noncancer effects were reportedly similar in incidence and severity in males and females; only the liver and kidneys were evaluated.

Table 4-3. Summary of cancer bioassays and tumor promotion studies of TCA in rats and mice

Reference	Species	Study type	Exposure route	Exposure duration	Doses evaluated	Results	Comments
Von Tungeln et al. (2002)	$B6C3F_1$ mice (23–24/sex/ dose, males and females)	Neonatal cancer assay	i.p. injection	Doses administered at 8 and 15 ds of age; tumors evaluated at 12 or 20 mo of age	Total dose of 16 or 33 mg/kg over a 2-d period (at 8 and 15 d of age)	Negative for tumor induction	TCA induced oxidative stress, but there was no significant increase in tumors in the neonatal mouse.
Tumor promotion studies							
Rats							
Parnell et al. (1988)	Sprague-Dawley rats (males, 6–12/dose and sampling time)	Promotion, multiple organs, partially hepat-ectomized rats	Oral, drinking water	Up to 12 mo	0, 6, 60, or 600 mg/kg-d	GGT-positive foci in liver	TCA promoted GGT-positive foci in DEN-initiated rats at all doses evaluated, but only one rat showed a liver carcinoma. TCA showed no activity as an initiator.
Mice							
Herren-Freund et al. (1987)	$B6C3F_1$ mice (males, 22–33/group)	Cancer assay and tumor promotion, liver	Oral, drinking water	61 wks	0, 500, or 1,250 mg/kg-d	Positive for tumor production and for tumor promotion	ENU was used as an initiator. Only the liver was microscopically examined; liver tumors were observed either with or without ENU pretreatment.
Pereira and Phelps (1996)	$B6C3F_1$ mice (females, 8–40/ group)	Cancer assay and tumor promotion	Oral, drinking water	Up to 52 wks	0, 78, 262, or 784 mg/kg-d	Positive with or without MNU initiation	MNU was used as an initiator. Only the liver was examined for tumors.
Pereira et al. (2001)	$B6C3F_1$ mice (males and females, 14–16/sex)	Tumor promotion	Oral, drinking water	31 wks	0 or 960 (females) or 1,000 (males) mg/kg-d	Males: significant increase in liver and kidney tumors in TCA-treated mice initiated with MNU Females: insignificant increase in liver and kidney tumors in mice initiated with MNU and promoted by TCA	Only the liver and kidneys were examined for tumors; MNU was used as an initiator; statistically significant increases in tumor yield were only observed in males.

Table 4-3. Summary of cancer bioassays and tumor promotion studies of TCA in rats and mice

Reference	Species	Study type	Exposure route	Exposure duration	Doses evaluated	Results	Comments
Pereira et al. (1997)	$B6C3F_1$ mice (females, 20–45/ dose)	Tumor promotion	Oral, drinking water	44 wks	0, 235, or 980 mg/kg-d	Positive for liver tumors	MNU was used as an initiator; only the liver was microscopically examined.
Bull et al., (2004)	$B6C3F_1$ mice (males, 10/group)	Tumor promotion	Oral, drinking water	36 wks	0, 0.1, 0.5 or 2.0 g/l	Positive for tumor promotion	VC was used as an initiator. Only the liver was microscopically examined.

DEN = diethylnitrosamine; MNU = N-methyl-N-nitrosourea; VC = vinyl carbamate

4.2.2.1. *Oral Studies*

4.2.2.1.1. *Rats*

4.2.2.1.1.1. *Chronic studies*. DeAngelo et al. (1997) evaluated the tumorigenicity of TCA in male F344 rats exposed for 104 weeks via drinking water. Groups of 50 rats received TCA in drinking water (adjusted to physiological pH) at 0, 50, 500, or 5,000 mg/L, resulting in time-weighted mean doses of 0, 3.6, 32.5, or 364 mg/kg-day as calculated by the study authors. Dosing was initiated at 28–30 days of age. Interim sacrifices (18–21 rats/group) were conducted at 15, 30, 45, and 60 weeks, and gross lesions in the body and internal organs were examined. The organs examined histologically at the interim and terminal sacrifices were liver, kidney, spleen, and testes. The survivors were sacrificed at 104 weeks. At study termination, blood from all treatment groups was analyzed for serum AST and ALT activity, and livers were analyzed for cyanide-insensitive PCO activity and extent of hepatocyte proliferation ([^{3}H]thymidine incorporation). At sacrifice, all animals were subjected to a complete necropsy. A comprehensive set of tissues, including all major organs, was examined microscopically in high-dose rats. The liver, kidney, spleen, and testes were examined in the remaining dose groups.

Survival in dosed animals was similar to that in controls (79, 75, 59, and 76% in the control, low-, mid-, and high-dose groups, respectively), and there were no significant differences in water consumption between exposed and control groups. A maximum tolerated dose was reached, as indicated by a 10.7% decrease in the final mean body weight of the high-dose animals relative to controls. Absolute liver weight was decreased by 11% at the high dose. No significant differences from the control values were observed in the absolute and relative weights of the kidney, spleen, or testes. AST activity was significantly decreased in the mid-dose group, but the data did not show a dose-related trend. ALT activity increased in a dose-related manner, and the response was statistically significant at the high dose. Peroxisome proliferation in the livers of animals exposed to the high dose (364 mg/kg-day) of TCA was significantly increased, based on a twofold increase in cyanide-insensitive PCO activity throughout the exposure period. There was no evidence of a dose-related increase in hepatocyte proliferation. Most nonneoplastic hepatic lesions were spontaneous and age related. A minimal-to-mild, treatment-related increase in hepatic cytoplasmic vacuolization was evident at the low and mid doses, but not at the high dose (data not shown). A mild increase in the severity of hepatocellular necrosis was observed in high-dose animals (data not shown). No treatment-related histopathologic changes were noted for the kidney, spleen, or testes. No dose-related increases in the incidences of neoplasms or hyperplasia were observed in the liver or other tissues. Animals for interim sacrifices were from the same exposed groups. The number of animals at final sacrifice ranged from 19 to 24/dose group. Hence, the power of detection of this bioassay was limited by the relatively small group sizes. DeAngelo et al. (1997) determined the study NOAEL and LOAEL values to be 32.5 and 364 mg/kg-day, respectively, based on

decreased body weight, increased serum ALT activity, mild hepatocellular necrosis, and increased peroxisome proliferation.

4.2.2.1.1.2. ***Tumor initiation and promotion studies.*** Parnell et al. (1988) investigated the initiating and promoting effects of TCA by using two short-term tests: the rat hepatic enzyme-altered foci assay and stimulation of peroxisomal-dependent PCO activity in the liver. In the initiation protocol, male Sprague-Dawley rats (6–12/treatment/time point) underwent a two-thirds partial hepatectomy or sham operation as control, followed 24 hours later by a single gavage dose of 10 mg/kg diethylnitrosamine (DEN) (a known initiator) or 1,500 mg/kg of TCA. Additional groups of hepatectomized rats began a regimen of exposure to 5,000 mg/L of TCA in drinking water (about 600 mg/kg-day) for 10, 20, or 30 days to assess the effects of an extended initiation period. Two weeks following the initiation period, all groups were promoted for the remainder of the study (up to 12 months after beginning the promotion phase) with 500 mg/L phenobarbital (PB) in the drinking water. Animals were randomly sampled 24 hours after the end of the initiation period, 24 hours prior to the start of the promotion phase, and 3, 6, and 12 months after beginning promotion. In the initiation study, the positive control is the group with partial hepatectomy, treated with DEN as the initiator and PB for promotion.

In the promotion protocol, rats (6–12/treatment/time point) underwent the two-thirds hepatectomy or sham operation followed 24 hours later by administration of a single 10 mg/kg oral dose of DEN (the initiator) or distilled water (control). Promotion was begun 2 weeks later by the addition of 500 mg/L PB (the positive control) or 0, 50, 500, or 5,000 mg/L TCA [equivalent to doses of about 0, 6, 60, or 600 mg/kg-day as calculated by using the chronic water intake factor of 0.12 L/kg-day for Sprague-Dawley rats (U.S. EPA, 1988)] to the drinking water. The test animals were randomly sampled at 2 weeks and 1, 3, 6, and 12 months after beginning promotion. In the initiation bioassay, only the positive control group showed a statistically significant induction of GGT-positive foci at the 3-, 6-, and 12-month evaluation intervals. (The development of GGT-positive foci has been closely linked to the subsequent development of both neoplastic nodules and hepatomas.) None of the groups that received initiation doses of TCA or the associated controls exhibited significant induction of GGT-positive foci. Since TCA did not induce GGT-positive foci (as did the tumor initiator DEN), TCA did not appear to be an initiator based on the results of this assay.

In the promotion bioassay, GGT-positive foci were induced in the positive control (partial hepatectomy/DEN/PB) at all evaluation intervals. Exposure of rats to 50, 500, or 5,000 mg/L TCA as a promoter for 6 or 12 months produced a significant increase in the number and size (mean area) of GGT-positive foci over the negative control groups (partial hepatectomy alone, partial hepatectomy/DEN, or TCA alone). At 3 months, rats in the 50 and 5,000 mg/L TCA promotion groups also had significantly greater numbers of GGT-positive foci compared with the negative controls (data on size of foci were not reported for this time point). The

promotion protocol also resulted in a statistically significant, but weak (10–20% greater than controls), increase in peroxisomal-specific PCO activity at the 5,000 mg/L drinking water level. No significant gross or histopathologic lesions, hepatomegaly, or changes in organ-to-body-weight ratios could be attributed to TCA exposure and only one hepatocellular carcinoma in an animal from the partial hepatectomy/DEN/5,000 mg/L TCA group was found in this study. The study authors concluded that TCA has significant, but relatively weak, tumor-promoting activity in the tested bioassay model. It should be noted that the observed promotion effect was from both partial hepatectomy and TCA. There was no study group that treated sham-operated rats with DEN, followed by TCA. Partial hepatectomy can function as a promoter by itself.

4.2.2.1.2. *Mice*

4.2.2.1.2.1. *Chronic studies*. DeAngelo et al. (2008) evaluated the induction of hepatocellular neoplasia in male B6C3F$_1$ mice exposed to TCA in drinking water in three studies. Male B6C3F$_1$ mice were exposed to nominal concentrations of 0.05, 0.5, or 5 g/L TCA in the drinking water (50/dose at study initiation) for 60 weeks (Study 1); 0 or 4.5 g/L TCA (58 animals/group) for 104 weeks (Study 2); or 0, 0.05, or 0.5 g/L TCA (72/group) for 104 weeks (Study 3). The pH of the dosing solutions was adjusted to 6.0–7.1 by the addition of 10 N sodium hydroxide. Mice in the control group in Study 1 received 2 g/L sodium chloride in the drinking water, while those in Study 2 received 1.5 g/L neutralized acetic acid to account for any taste aversion of TCA in dosing solutions. In Study 3, deionized water served as the control. Body weights and water consumption were measured twice monthly for the first 2 months and then monthly afterwards. In Study 1, groups of five animals from each dose group were examined at necropsy at 4, 15, 31, and 45 weeks. In Study 2, serial necropsies were conducted at 15, 30, 45, and 60 weeks. In Study 3, serial necropsies were conducted at 26, 52, and 78 weeks.

At interim and terminal necropsies, gross lesions, livers, kidneys, spleens, and testes were examined by a board-certified veterinary pathologist. For all other tissues, a complete pathological examination was performed on five mice from the high-dose and control groups. If the number of any histopathologic lesions in a tissue was significantly increased above that in the control animals, then that tissue was examined in all TCA dose groups. To determine long-term hepatocellular damage during TCA treatment, arterial blood was collected at 30 and 60 weeks (Study 1) and 4, 30, and 104 weeks (Study 2), and serum LDH activity was measured. Portions of liver tissue from the interim-sacrifice animals (5/group/duration) were frozen and analyzed for PCO activity, a marker of peroxisome proliferation. Five days prior to each scheduled necropsy, osmotic pumps containing 200 µL [^{3}H]thymidine (62–64 Ci/mmol) or 20 mg/mL bromo-deoxyuridine (BrdU) (Study 3) were implanted subcutaneously. Autoradiography using paraffin-embedded sections of liver was performed to evaluate hepatocyte proliferation, as measured by the incorporation of [^{3}H]-labeled thymidine or BrdU into nuclear DNA. The

labeling index was calculated by dividing the number of labeled hepatocyte nuclei (S-phase) by the total number of hepatocyte nuclei scored.

For Study 1, time-weighted mean doses of 8, 68, and 602 mg/kg-day were calculated by the study authors from nominal TCA concentrations (0.05, 0.5, and 5 g/L, respectively) and drinking water consumption data for the low-, mid-, and high-dose groups.[4] Animals in the mid- and high-dose groups consumed significantly less water than the controls. The study authors estimated the mean doses to be 572 mg/kg-day for a nominal drinking water concentration of 4.5 g/L TCA (Study 2), and 6 and 58 mg/kg-day for nominal concentrations of 0.05 and 0.5 mg/kg-day (Study 3).

No decrease in animal survival was found at any TCA dose in any of the three studies. Exposure to TCA in drinking water decreased body weight by 15% in the high-dose group relative to the control. Significant, dose-related increases in absolute and relative liver weights were observed in the 0.5 and 5 g/L treatment groups at all scheduled sacrifices, with the exception of the 0.5 g/L dose group at 30 days.

Nonneoplastic alterations in the liver and testes were seen at study termination at 60 weeks and appeared to be dose related (Tables 4-4 and 4-5). The nonneoplastic alterations observed in the liver included hepatocellular cytoplasmic alteration, necrosis, and inflammation. Cytoplasmic alterations were observed in all treatment groups; however, the incidence did not increase monotonically with dose. These lesions were most prominent in the 5 g/L TCA group throughout the study and were most severe after 60 weeks of treatment. The alterations were characterized by an intense eosinophilic cytoplasm with deep basophilic granularity and slight cytomegaly. The distribution ranged from centrilobular to diffuse. Hepatic necrosis was observed in the middle- and high-dose group at all time points and was reported to be most severe at 30–45 weeks; the study report provided only combined data for the 30- and 45-week interim sacrifices (Table 4-5). A significant increase in the severity of inflammation was seen in the high-dose group at 60 weeks. A dose-related increase in serum LDH activity (a measure of liver damage) was observed at 30 weeks, and significant increases were measured in the 0.5 and 5.0 g/L dose groups. No change in LDH activity was found in any treatment groups at 60 weeks. No other hepatic changes showed statistically significant increases in incidence or severity level. An increased incidence of testicular tubular degeneration was seen in the 0.5 and 5 g/L treatment groups (Table 4-4). No treatment-related changes were observed in the spleen or kidney.

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[4]DeAngelo et al. (2008) also reported measured TCA concentrations in drinking water. Doses calculated by EPA based on those concentrations and reported drinking water consumption are as follows:
Study 1: time-weighted mean doses were calculated as 7.7, 68.2, and 602.1 mg/kg-day for measured TCA concentrations of 0.05, 0.48, and 5.06 g/L, respectively.
Study 2: time-weighted mean dose was calculated as 571.5 mg/kg-day for a measured TCA concentration of 4.43 g/L.
Study 3: time-weighted mean doses were calculated as 6.7 and 81.2 mg/kg-day for measured TCA concentrations of 0.06 and 0.70 g/L, respectively.

Table 4-4. Incidence and severity of nonneoplastic lesions in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks

Lesion	Treatment	Control	0.05 g/L TCA	0.5 g/L TCA	5 g/L TCA
	Dose[a]	**0**	**8**	**68**	**602**
	Number[b]	30	27	29	29
Hepatocellular cytoplasmic alteration	Incidence[c]	7%	48%[e]	20.6%[e]	93%[e]
	Severity[d]	0.10 ± 0.40	0.70 ± 0.82	0.34 ± 0.72	1.60 ± 0.62[e]
Hepatocellular inflammation	Incidence[c]	10%	0	7%	24%[e]
	Severity[d]	0.13 ± 0.40	0	0.07 ± 0.03	0.24 ± 0.44
Testicular tubular degeneration	Incidence[c]	7%	0	14%[e]	21%[e]
	Severity[d]	0.10 ± 0.40	0	0.17 ± 0.47	0.21 ± 0.41

[a]Time-weighted mean daily dose in mg/kg-day based on nominal TCA concentration in drinking water as reported in DeAngelo et al. (2008). Doses calculated by EPA using measured concentrations are 7.7, 68.2, and 602.1 mg/kg-day.
[b]Number of animals examined.
[c]Percentage of animals with alteration.
[d]Severity: 0 = no lesion, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe (reported as the average severity of all animals in the dose group).
[e]Statistically significant from the control group, $p \leq 0.05$.

Source: DeAngelo et al. (2008).

Table 4-5. Incidence and severity of hepatocellular necrosis at 30–45 weeks in male B6C3F$_1$ mice exposed to TCA in drinking water

Treatment	Control	0.05 g/L TCA	0.5 g/L TCA	5 g/L TCA
Dose[a] (mg/kg-d)	**0**	**8**	**68**	**602**
Number[b]	10	10	10	10
Incidence[c]	0	0	30.0%	50.0%
Severity[d]	0	0	0.50 ± 0.97	1.30 ± 1.49[e]

[a]Time-weighted mean daily dose in mg/kg-day based on nominal TCA concentration in drinking water as reported in DeAngelo et al. (2008). Doses calculated by EPA using measured concentrations are 7.7, 68.2, and 602.1 mg/kg-day.
[b]Number of animals examined.
[c]Percentage of animals with alteration.
[d]Severity: 0 = no lesion, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe (reported as the average severity of all animals in the dose group).
[e]Statistically significant from the control group, $p \leq 0.05$.

Source: DeAngelo et al. (2008).

Areas of inflammation (at high dose only) and necrosis (at mid- and high-dose) were present during the early course of TCA administration, but abated after week 60 in all studies. Similarly, LDH activity was elevated in the mid- and high-dose groups at week 30 but not at week 60. Cytoplasmic alterations occurred as early as week 4 and persisted throughout the three

studies at all doses, indicating that this effect did not correlate with other nonneoplastic changes in the liver. For the 60-week study, EPA determined the LOAEL for effects on the liver (increased liver weight, hepatic necrosis, and serum LDH activity at 30 weeks) and testes (testicular tubular degeneration) to be 0.5 g/L (68 mg/kg-day) and the NOAEL to be 0.05 g/L (8 mg/kg-day).

Exposure to TCA induced tumors in the liver at 60 weeks (Table 4-6). There were significant dose-related trends for increased prevalence and multiplicity of adenomas and carcinomas. The prevalence and numbers of hepatocellular carcinomas and hepatocellular adenomas were significantly increased in the high-dose group. The number of animals with either lesion was significantly increased in the 0.5 g/L treatment group. Neoplasia was first seen in all dose groups after 45 weeks of treatment. The prevalence and number of tumors in the 5 g/L group were 60% (3/5 animals with a lesion) and 0.80 lesions/animal. One hepatocellular carcinoma was found in the 0.5 g/L group and one hepatocellular adenoma was found in the 0.05 g/L group. No induction of tumors was reported in other organs.

Table 4-6. Prevalence and multiplicity of hepatocellular tumors in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks

Neoplasia type[c]	Treatment	Control	0.05 g/L TCA	0.5 g/L TCA	5 g/L TCA
	Dose[a]	**0**	**8**	**68**	**602**
	Number[b]	30 (30)	27 (30)	29 (30)	29 (30)
HA	Prevalence[d]	7%	15%	22%	38%[f]
	Multiplicity[e]	0.07 ± 0.05[e]	0.15 ± 0.07	0.24 ± 0.10	0.55 ± 0.15[f]
HC	Prevalence[d]	7%	4%	21%	38%[f]
	Multiplicity[e]	0.07 ± 0.05	0.04 ± 0.04	0.28 ± 0.22	0.41 ± 0.11[f]
HA or HC	Prevalence[d]	14%	15%	38%[f]	55.%[f]
	Multiplicity[e]	0.13 ± 0.06	0.19 ± 0.09	0.52 ± 0.14[f]	1.00 ± 0.19[f]

[a]Time-weighted mean daily dose in mg/kg-day based on nominal TCA concentration in drinking water as reported in DeAngelo et al. (2008). Doses calculated by EPA using measured concentrations are 7.7, 68.2, and 602.1 mg/kg-day.
[b]Number of animals examined at terminal sacrifice. Parentheses = number of animals/group scheduled for terminal necropsy. Data for interim-sacrifice animals not included.
[c]HA = hepatocellular adenoma, HC = hepatocellular carcinoma, HA or HC = either hepatocellular adenoma or hepatocellular carcinoma.
[d]Percentage of animals with a lesion as reported in the study report.
[e]Number of lesions/animal, mean ± standard error of the mean.
[f]Statistically significant from the control group, $p \leq 0.05$.

Source: DeAngelo et al. (2008).

Significant increases above the control values were also observed for the prevalence and multiplicity of adenomas, carcinomas, and either adenomas or carcinomas for mice exposed to 4.5 g/L TCA for 104 weeks (Study 2) or 0.5 g/L TCA for 104 weeks (Study 3) (Table 4-7).

Neoplastic lesions observed at organ sites other than the liver were considered spontaneous for the male mouse and did not exceed the tumor incidences when compared to a historical control database.

Table 4-7. Incidence of hepatocellular tumors in male B6C3F$_1$ mice exposed to TCA in drinking water for 104 weeks

	Treatment	**Control**			**4.5 g/L TCA**
	Dose[a]	**0**			**572**
Neoplasia type[c]	Number[b]	25 (32)			36 (43)
HA	Prevalence[d]	0			59%[f]
	Multiplicity[e]	0			0.61 ± 0.16[f]
HC	Prevalence[d]	12%			78%[g]
	Multiplicity[e]	0.20 ± 0.12			1.50 ± 0.22[f]
HA or HC	Prevalence[d]	12%			89%[f]
	Multiplicity[e]	0.20 ± 0.12			2.11 ± 0.25[f]
	Treatment	**Control**	**0.05 g/L TCA**	**0.5 g/L TCA**	
	Dose[a]	**0**	**6**	**58**	
	Number[b]	42 (50)	35 (50)	37 (50)	
HA	Prevalence[d]	21%	23%	51%[f]	
	Multiplicity[e]	0.21± 0.06	0.34 ± 0.12	0.78 ± 0.15[f]	
HC	Prevalence[d]	55%	40%	78%[f]	
	Multiplicity[e]	0.74 ± 0.12	0.71 ± 0.19	1.46 ± 0.21[f]	
HA or HC	Prevalence[d]	64%	57%	87%[f]	
	Multiplicity[e]	0.93 ± 0.12	1.11 ± 0.21	2.14 ± 0.26[f]	

[a]Time-weighted mean daily dose in mg/kg-day calculated over 104 weeks based on nominal TCA concentration in drinking water as reported in DeAngelo et al. (2008). Doses calculated by EPA using measured concentrations are 6.7, 81.2, and 571.5 mg/kg-day, respectively.
[b]Animals surviving ≥78 weeks, parentheses = number of animals/group scheduled for terminal necropsy. Data for interim-sacrifice mice not included.
[c]HA = hepatocellular adenoma; HC = hepatocellular carcinoma; HA or HC = either hepatocellular adenoma or hepatocellular carcinoma.
[d]Number of animals with a lesion/number of animals examined × 100%.
[e]Mean number of lesions ± standard error of the mean.
[f]Statistically significant from the control group, $p \leq 0.03$.

Source: DeAngelo et al. (2008).

Liver PCO activity was significantly increased at the mid and high doses at intervals of 4, 15, 30, 45, and 60 weeks when compared with control values. The range of PCO activity for mice exposed to 0.5 and 5 g/L was 129–260 and 326–575%, respectively, above the control value. The mean PCO activity (averaged over the five intervals) is summarized in Table 4-8. Autoradiographs of the livers from animals exposed to 5 g/L TCA showed significantly increased labeling of hepatocyte nuclei at 30 weeks (about 3-fold) and 40 weeks (about 2.5-fold). Increased nuclear labeling was observed in the mid-dose treatment group at 60 weeks (about

threefold). These data indicate that TCA induced treatment-related tumors in male mice at doses that also induced peroxisome proliferation and hepatocyte proliferation.

Table 4-8. Mean PCO activity in male B6C3F$_1$ mice exposed to TCA in drinking water for up to 60 weeks

	Control	0.05 g/L TCA (8 mg/kg-d)[b]	0.5 g/L TCA (68 mg/kg-d)[b]	5 g/L TCA (602 mg/kg-d)[b]
Mean PCO activity (nmol NAD reduced/min/mg protein)[a] (% control)	2.59 ± 1.04	2.85 ± 0.86 (117%)	4.75 ± 1.16 (200%)	11.99 ± 3.04 (475%)

[a]Mean PCO activity ± SD was calculated as an arithmetic mean of the PCO activity for weeks 4, 15, 30, 45, and 60. PCO activity for each time point was based on 5 mice/group/time point. The total number of mice for each concentration was 25 (with the exception of 24 mice for the 5 g/L TCA group).
[b]Time-weighted mean daily dose in mg/kg-day based on nominal TCA concentration in drinking water as reported in DeAngelo et al. (2008). Doses calculated by EPA using measured concentrations are 7.7, 68.2, and 602.1 mg/kg-day.

Source: DeAngelo et al. (2008) and email dated March 12, 2010, from Anthony DeAngelo, National Health and Environmental Effects Research Laboratory (NHEERL), Office of Research and Development (ORD), U.S. EPA, to Diana Wong, National Center for Environmental Assessment (NCEA), ORD, U.S. EPA.

Bull et al. (1990) examined the induction of tumors in the liver of male and female B6C3F$_1$ mice given TCA in drinking water (neutralized to pH 6.8–7.2). Groups of mice (males: 24/high dose, 11/low dose, 35 controls; females: 10/group) were exposed to neutralized TCA (males: 0, 1, or 2 g/L; females: 0 or 2 g/L) for 52 weeks. Interim sacrifices were performed at 15, 24, and 37 weeks on separate groups of male mice (five/group). An additional group of 11 males received 2 g/L TCA for 37 weeks, followed by a 15-week recovery period. The 0, 1, and 2 g/L concentrations used in this study corresponded to estimated average doses of 0, 164, and 329 mg/kg-day as calculated from data for total dose provided in the study report. The approximate average dose for the 37-week exposure with recovery was 309 mg/kg-day.

No effects of treatment on survival or body weight were observed. Body weight and food and water consumption data were recorded but not reported. A significant increase in the relative liver weight was seen in the 1 g/L males (30% increase from control), 2 g/L males (63% increase), and 2 g/L females (25% increase) at 52 weeks when compared with controls. No changes in kidney weights were observed. Mild intracellular swelling and some indication of glycogen accumulation in the periportal region were observed in the livers of treated male and female mice at 52 weeks. Male mice in the 2 g/L group had a dose-related accumulation of lipofuscin near proliferative lesions (no incidence reported) and hyperplastic liver nodules (9/24).

The incidences of hepatocellular adenomas in male mice were 0/35 (0%), 2/11 (18%), and 1/24 (4%), and the incidences of hepatocellular carcinomas were 0/35 (0%), 2/11 (18%), and 4/24 (17%) in the 0, 1, and 2 g/L exposure groups, respectively. Female mice did not develop

any tumors in response to TCA treatment and might be less sensitive to TCA treatment than males. However, fewer female mice (52 weeks: 2 g/L, 10 females) were evaluated in this study than were male mice (37 weeks: 2 g/L, 11 males; 52 weeks: 1 g/L, 11 males; 2 g/L, 24 males), which may have limited the ability of the study to detect tumors in female mice. Fifteen weeks into the 37-week study, exposure to 2 g/L resulted in hepatocellular carcinomas in 3/11 (30%) male mice, but hepatic adenomas had not occurred by that time. Since the maximum exposure duration in this study was only 52 weeks, this study may not have evaluated mice for an adequate length of time to observe the full carcinogenic potential of TCA. In addition, the numbers of animals tested were less than adequate. EPA determined that the LOAEL for noncancer effects was 164 mg/kg-day based on increase in liver weight, cytomegaly, and modest glycogen accumulation.

Pereira (1996) administered 0, 2.0, 6.67, or 20.0 mmol/L TCA (0, 327, 1,090, or 3,268 mg/L) (neutralized with sodium hydroxide to pH 6.5–7.5) in drinking water to female $B6C3F_1$ mice from 7 to 8 weeks of age until sacrifice after 360 days (51 weeks) or 576 days (82 weeks) of exposure. A control group of 134 mice was administered 20 mmol sodium chloride. There were 93, 46, and 38 mice in the low-, mid-, and high-dose groups, respectively. Estimates of daily doses resulting from exposure to treated drinking water were not reported. Based on the default water intake for female $B6C3F_1$ mice of 0.24 L/kg-day, calculated from the default body weight in an allometric equation (U.S. EPA, 1988), the estimated doses were 0, 78, 262, and 784 mg/kg-day. Drinking water consumption was monitored during the first 4 weeks of exposure. Body weights were monitored throughout the study. At sacrifice, livers were collected, weighed, and processed for histopathologic examination.

Drinking water consumption was decreased only for the first week for the high-dose group. Body weight was decreased beginning after 51 weeks of treatment with 20 mmol/L TCA. Body weights were significantly decreased ($p < 0.05$) by approximately 10% on sporadic occasions beginning at week 51 until study termination. Relative liver weight increased with dose (linear regression coefficient, r = 0.991). The relative liver weights of the high-dose group increased by approximately 40% over controls at 51 weeks, and liver weights for the mid- and high-dose groups increased by approximately 25 and 60% over controls, respectively, after 82 weeks. EPA determined the NOAEL to be 2.0 mmol/L (78 mg/kg-day) and the LOAEL to be 6.67 mmol/L (262 mg/kg-day) based on increased in liver weight. This study was not designed, however, to evaluate noncancer effects of TCA. The identification of a LOAEL at 6.67 mmol/L based on liver weight is supported by short-term studies in $B6C3F_1$ mice that have reported some evidence for glycogen accumulation (Sanchez and Bull, 1990), increased DNA synthesis in hepatocytes (Dees and Travis, 1994), and peroxisome proliferation (Parrish et al., 1996) at TCA doses that increased liver weights.

The incidence of hepatocellular carcinoma was significantly increased ($p < 0.05$) at 20 mmol/L (784 mg/kg-day) after 51 weeks (control: 0/40, 0%; 2.0 mmol/L [78 mg/kg-day]:

0/40, 0%; 6.67 mmol/L [262 mg/kg-day]: 0/19, 0%; 20.0 mmol/L [784 mg/kg-day]: 5/20, 25%). At 82 weeks, the incidence of foci of altered hepatocytes was significantly increased at 6.67 and 20.0 mmol/L (10/90, 11.1%; 10/53, 18.9%; 9/27, 33.3%; 11/18, 61.1%, respectively). The incidence of hepatocellular adenomas was significantly increased at 20.0 mmol/L (2/90, 2.2%; 4/53, 7.6%; 3/27, 11.1%; 7/18, 38.9%, respectively), and the incidence of hepatocellular carcinomas was significantly increased at 6.67 and 20.0 mmol TCA (2/90, 2.2%; 0/53, 0%; 5/27, 18.5%; 5/18, 27.8%, respectively).

As part of experiments designed to evaluate if TCA alone was responsible for TCE-induced liver tumors, Bull et al. (2002) exposed 40 male B6C3F_1 mice to neutralized TCA in drinking water at 2 g/L for 52 weeks (Experiment 1) and 20 male mice at 0.5 or 2 g/L for 52 weeks (Experiment 2). Controls (12 in Experiment 1 and 20 in Experiment 2) were given untreated drinking water. After exposure, animals were sacrificed and livers were removed, weighed, grossly examined, and processed for histopathologic examination. No other tissues were examined histologically. The estimated doses resulting from exposure to these concentrations were not reported. However, based on reference water intake of 0.24 L/kg-day for male B6C3F_1 mice (U.S. EPA, 1988), the estimated doses used in this study were 0, 120, and 480 mg/kg-day. Groups of animals were also exposed to TCE, DCA, and various concentrations of a mixture of DCA and TCA. Those results are not fully discussed in the context of this toxicological review.

Random tumor samples were stained with an anti *c-jun* antibody; all tumors were analyzed for mutation frequency and spectra of the H-*ras* codon 61; and these results were compared with those from DCA- and TCE-induced tumors. Proteins involved in the mitogen-activated protein kinase-signaling cascade (Ras, MeK, active Erk1/2, and *c-fos*) were examined by western blotting in order to determine if the three common codon 61 mutations of *ras* had different effects on downstream effectors. Tumor incidence and multiplicity were significantly ($p < 0.05$) greater than controls at all TCA exposure concentrations. Tumor incidence in animals exposed to TCA at 2 g/L for 52 weeks (Experiment 1) was 33/40 compared with 4/12 in controls; tumor incidences in mice exposed to TCA at 0.5 or 2 g/L for 52 weeks (Experiment 2) were 11/20 and 9/20, respectively, compared with an incidence of 1/20 in controls. All tumor cells from TCA-treated mice were nonreactive with the *c-jun* antibody (*c-jun*$^-$), which is consistent with previous reports (Stauber and Bull, 1997). The mutation frequency at H-*ras* codon 61 in TCA-induced tumors (44%) was lower than the frequency of codon 61 mutations (56%) in spontaneous liver tumors in B6C3F_1 mice but higher than that in TCE-induced tumors (21%). The H-*ras* mutation spectrum of TCA-induced tumors did not differ significantly from that of historical controls. TCA had no effect on activation of the mitogen-activated protein kinase cascade.

4.2.2.1.2.2. *Tumor promotion studies*. Herren-Freund et al. (1987) investigated the initiation/promotion potential of TCA in male B6C3F$_1$ mice (22–33/group). At 15 days of age, mice were pretreated with a single i.p. dose of ethylnitrosourea (ENU) as a tumor initiator at doses of 0 mg/kg (uninitiated control, treated with 2 μL/g sodium acetate and 5 g/L TCA), 2.5 mg/kg (2 and 5 g/L TCA groups), or 10 mg/kg (5 g/L TCA group only). Following pretreatment, TCA was administered in the drinking water at concentrations of 2 or 5 g/L (500 or 1,250 mg/kg-day) as calculated by using a subchronic water intake factor of 0.25 L/kg-day (U.S. EPA, 1988) from 4 to 65 weeks of age. The negative control groups for tumor promotion (22–23 animals/group) received 2 g/L sodium chloride in drinking water and 0, 2.5, or 10 mg/kg ENU. The mice were sacrificed after 61 weeks of exposure. Survival data were not reported.

Significant decreases of 9–12% in final mean body weight were observed in the 5 g/L TCA groups relative to the corresponding sodium chloride control. Absolute and relative liver weights were significantly increased (by 41–73%) in all TCA treatment groups relative to the corresponding sodium chloride control group. The incidences of hepatocellular adenomas and hepatocellular carcinomas were significantly increased in the uninitiated group receiving 5 g/L TCA when compared with the uninitiated sodium chloride control group (see Table 4-9). The incidences of hepatocellular adenomas and hepatocellular carcinomas were significantly increased in the TCA groups initiated with 2.5 mg/kg ENU. Mice initiated with 10 mg/kg ENU and then administered 5 g/L TCA also showed an increase in the incidence of hepatocellular carcinomas, although the increase was not statistically significant. Thus, TCA enhanced the incidence of hepatocellular adenomas and carcinomas above control levels, with or without prior initiation. The study authors concluded that TCA acted as a complete carcinogen in B6C3F$_1$ mice.

Table 4-9. Incidence of adenomas and hepatocellular carcinomas in B6C3F$_1$ mice treated with ENU and TCA

ENU(µg/g body wt)	TCA (as promoter) (g/L)	Incidence of adenomas (%)	No. adenomas per animal[a]	Incidence of carcinomas (%)	No. carcinomas per animal[a]
0	Sodium chloride	2/22 (9)	0.09 ± 0.06	0/22 (0)	0
2.5	Sodium chloride	1/22 (5)	0.05 ± 0.05	1/22 (5)	0.05 ± 0.05
0	5	8/22 (3)[b]	0.50 ± 0.16[b]	7/22 (32)[b]	0.50 ± 0.17[b]
2.5	2	11/33 (33)[b]	0.42 ± 0.12[b]	16/33 (48)[b]	0.64 ± 0.14[b]
2.5	5	6/23 (26)[b]	0.30 ± 0.12[b]	11/23 (48)[b]	0.57 ± 0.21[b]
10.0	5	11/28 (39)	0.61 ± 0.16	15/28 (54)	0.93 ± 0.22

[a]The number of adenomas per animal and the number of carcinomas per animal are expressed as the mean ± standard error of the mean.
[b]Significantly different from the corresponding sodium chloride control group not treated with TCE, DCA, or PB as determined by the Fisher's exact test; $p < 0.01$.

Source: Herren-Freund et al. (1987).

Pereira and Phelps (1996) assessed liver tumor promotion activity by TCA in female B6C3F$_1$ mice. Test animals were treated with 25 mg/kg of the tumor initiator N-methyl-N-nitrosourea (MNU) at 15 days of age or given 4 mL/kg sterile saline (vehicle control). Starting at 7 weeks of age, animals were administered neutralized TCA in drinking water at concentrations of 0, 2.0, 6.67, or 20.0 mmol/L (0, 327, 1,090, or 3,268 mg/L) for either 31 weeks (n = 8–15/group) or 52 weeks (n = 39 for MNU controls, 40 for the low-dose TCA-only group, 19 for the mid- and high-dose TCA-only groups, and 6–23 for TCA + MNU groups). Dose estimates were not reported by the study authors. The drinking water concentrations used resulted in doses of approximately 0, 78, 262, or 784 mg/kg-day based on the default drinking water value of 0.24 L/kg-day for female B6C3F$_1$ mice (U.S. EPA, 1988). A recovery group (n = 11) was removed from treatment after 31 weeks and retained for an additional 21 weeks.

At 31 weeks, treated animals exhibited a slight, dose-related linear increase in relative liver weights. At 31 and 52 weeks, no significant increase in foci of altered hepatocytes, adenomas, or carcinomas was observed in mice that received MNU only. In mice administered TCA but not initiated with MNU, the only tumorigenic response was a slight increase in the yield of hepatocellular carcinomas/animal (0.50 tumors/mouse) in the high-dose group (784 mg/kg-day) after 52 weeks of treatment. Animals initiated with MNU and treated with TCA exhibited an increase in liver tumors following both 31 and 52 weeks of exposure in the 784 mg/kg-day group and following 52 weeks of exposure in the 262 mg/kg-day group. Both the numbers of adenomas/mouse and carcinomas/mouse were statistically elevated as compared with controls, and the tumor yield generally increased with exposure duration from 31 to 52 weeks. However, there was no significant increase in the yield of altered hepatocyte foci at either time point in any dose group. The concentration-response relationships for total

lesions/mouse (foci plus tumors) after both 31 and 52 weeks of treatment were best described by a linear regression line.

When exposure to 784 mg/kg-day TCA was terminated after 31 weeks and the animals were held for an additional 21 weeks, the yield of tumors/mouse remained stable (1.50 and 1.64 at 31 and 52 weeks, respectively). However, the yield of hepatocellular carcinomas increased from 0.20/mouse in mice exposed for 31 weeks to 0.73/mouse in mice held to 52 weeks. When treatment continued between weeks 31 and 52, the yield of tumors/mouse rose from 1.50 at 31 weeks to 4.21 at study termination. These findings indicate that, although the occurrence of additional TCA-promoted tumors was dependent on continuous treatment, the stability and progression to carcinoma appeared to be independent of further treatment. Histochemical staining indicated that >71% of tumors promoted with either 262 or 784 mg/kg-day TCA were basophilic and did not contain GST-π, a phase II conjugation enzyme highly expressed in some tumor types, except for very small areas comprising <5% of the tumor. The predominantly basophilic nature of the tumors promoted by TCA is consistent with the character of lesions induced by tumorigenic compounds that are rodent peroxisomal proliferators, but "spontaneous" liver tumors in mice have also been reported to be predominantly basophilic and lacking GST-π (Pereira and Phelps, 1996).

Pereira et al. (2001) administered MNU to B6C3F$_1$ mice (16 males and 14 females) via i.p. injection at 30 mg/kg, then exposed the MNU-initiated mice to TCA at 4 g/L in the drinking water for 31 weeks. Based on reference drinking water intake values for B6C3F$_1$ mice (0.25 and 0.24 L/kg-day for males and females, respectively), male and female mice received approximately 1,000 and 960 mg/kg-day, respectively. After the treatment period, the liver and kidneys were removed, weighed, and microscopically examined. The study was designed to evaluate the effects of chloroform on TCA-induced tumor promotion, and only the TCA-only treated groups are discussed in this review. Relative liver weight was significantly ($p < 0.001$, 75% in males and 35% in females) increased compared with controls. A significant ($p < 0.05$) increase in the number of mice with liver tumors (adenomas + adenocarcinomas) was observed in TCA-treated males initiated with MNU (incidence of 13/16 compared with 2/8 MNU-treated controls). These tumors were >97% basophilic. Although an increase was also observed in females (incidence of 6/14 compared with 2/29 controls), the increase was not statistically significant ($p < 0.05$). Similarly, an increase in kidney tumors was also observed in male mice initiated with MNU and promoted by TCA (incidence of 0/8 in MNU-only treated controls compared with an incidence of 14/16 in MNU + TCA treated mice). Incidences of kidney tumors in female mice were not significantly increased compared with MNU-treated controls (incidence not reported). The study authors also investigated hypomethylation of the *c-myc* gene in liver and kidney tumors from TCA-treated mice. These results are discussed in Section 4.5.1.

In a study designed to compare the promotion of liver tumors in TCA- and DCA-treated mice initiated with MNU, Pereira et al. (1997) exposed female B6C3F$_1$ mice (20–45/dose) to

TCA at 6 or 25 mmol/L in drinking water with or without addition of various concentrations of DCA for 44 weeks. Based on reference water intake for female B6C3F$_1$ mice of 0.24 L/kg-day (U.S. EPA, 1988), the estimated doses were 0, 235, and 980 mg/kg-day. Body weight was monitored throughout the study. Livers were removed, weighed, and microscopically examined for presence of tumors. Liver sections were also stained immunohistochemically for GST-π. A significant increase in adenomas was observed in TCA-only treated mice at 25 mmol/L (0.52 tumors/mouse compared with 0.07 tumors/control mouse) but not at 6 mmol/L (0.15 tumors/mouse). The tumors from TCA-treated mice were exclusively basophilic and were generally without GST-π (with the exception of four carcinomas at 25 mmol/L TCA), which is consistent with the results reported by Pereira and Phelps (1996). In contrast, tumors from DCA-treated mice were primarily eosinophilic and were positive for GST-π. When TCA and DCA were administered together (25 mmol/L TCA + 15.6 mmol/L DCA), the tumor yield increased synergistically. At the lower concentration, the relationship was at least additive. The tumors in the livers from mice treated with DCA + TCA were more consistent with the characteristics of DCA-induced livers (eosinophilic and containing GST-π). These data suggest that TCA and DCA both promote tumor formation; however, the different tumor characteristics are consistent with the conclusion that the mechanisms for the tumor-promoting activity of each compound are different.

Bull et al. (2004) examined interactions in the tumor-promoting activity of TCA, dichloroacetate, and carbon tetrachloride administered in drinking water to male B6C3F$_1$ mice that were initiated with vinyl carbamate. In trials involving TCA only, TCA showed liver tumor promoting activity that increased with duration of exposure and dose.

4.2.2.2. *Inhalation Studies*

No chronic toxicity studies or cancer studies in animals exposed by inhalation to TCA are available.

4.2.2.3. *Studies Using Other Routes of Exposure*

Von Tungeln et al. (2002) evaluated the neonatal tumorigenicity of TCA in B6C3F$_1$ mice (23–24 animals/sex/dose) in two bioassays. For each assay, TCA was dissolved in dimethyl sulfoxide and administered via i.p. injections at 8 and 15 days of age. In Assay A, neonatal mice were given a total dose of 2,000 nmol (approximately 33 mg/kg based on a reference body weight of 0.01 kg for B6C3F$_1$ mice at weaning) (U.S. EPA, 1988) and were sacrificed at 12 months of age. In Assay B, neonatal mice were given a total dose of 1,000 nmol (approximately 16 mg/kg) and were sacrificed at 20 months of age. 4-Aminobiphenyl was used as the concurrent positive control. Dimethyl sulfoxide solvent control groups (23–24 mice/sex) were included in each assay. At sacrifice, all test animals were necropsied for gross tumor count, microscopic examination of tissues, and histopathologic diagnoses. There was no treatment-

related mortality in assay A or B. A marginal increase (not statistically significant) in liver tumors was observed in TCA-treated males in Assay A (4/24) when compared with the control group (1/24). The incidence of liver tumors in TCA-treated males in Assay B (5/23) was less than in the control group (7/23). No tumors were observed in dimethyl sulfoxide-treated control females in either assay. The study authors concluded that TCA did not induce significant tumor incidences when compared with the dimethyl sulfoxide controls. In contrast, 4-aminobiphenyl (the positive control substance) induced tumors in mice (liver tumors in all male mice in Assays A and B, lung tumors in 9/22 male mice in Assay B, and liver tumors in 9/23 female mice in Assay B; no tumors were diagnosed in female mice in Assay A).

In a related mechanistic study, von Tungeln et al. (2002) dosed an additional group of male neonatal B6C3F_1 mice with TCA to evaluate TCA-induced formation of MDA-derived deoxyguanosine adducts and 8-OHdG in hepatic DNA in relation to TCA tumorigenicity. This study was conducted because previous results from the same laboratory had shown that in vitro metabolism of TCA by hepatic microsomes isolated from adult mice results in lipid peroxidation, with subsequent production of MDA (Ni et al., 1996) (see Section 3.3), and metabolism of TCA in the presence of calf thymus DNA resulted in the formation of MDA-derived deoxyguanosine adducts [Ni et al. (1995), as cited in von Tungeln et al. (2002)]. In addition, TCA induces formation of 8-OHdG (see Section 4.2.1.1), and induction of elevated levels of 8-OHdG may induce tumors (Wagner et al., 1992).

Male neonatal B6C3F_1 mice (number of animals treated not stated) were given a total dose of 2,000 nmol TCA by i.p. injection as described for the neonatal mice cancer assays summarized above (Von Tungeln et al., 2002). The test animals were sacrificed 1, 2, or 7 days after the final TCA treatment at 15 days of age, and liver tissue was collected for extraction of DNA and determination of levels of MDA-derived deoxyguanosine and 8-OHdG. TCA induced a significant ($p < 0.05$) increase in MDA-derived deoxyguanosine adduct formation in liver DNA at 24 and 48 hours (but not at 7 days) after the final dose. The increase was approximately 190% of the control value at each time point. TCA treatment also resulted in a significant ($p < 0.05$) increase in 8-OHdG formation in liver DNA at 24 and 48 hours and at 7 days after administration of the final dose. The magnitude of the increase was approximately 2.5-fold greater than the control values. Because TCA was not carcinogenic in the neonatal cancer bioassays conducted by von Tungeln et al. (2002), these results suggest that neonatal B6C3F_1 mice are not sensitive to either TCA-induced lipid peroxidation or oxidative stress as an MOA for tumor induction under the experimental conditions used in these studies. The study authors speculated that TCA was negative in their neonatal cancer bioassays because it may act as a cell proliferator. According to this hypothesis, liver cells were already replicating at a very high rate in the neonatal mice when TCA was administered; therefore, any additional cell proliferation induced by TCA may have been negligible in comparison with the existing rate of proliferation.

4.3. REPRODUCTIVE/DEVELOPMENTAL STUDIES – ORAL AND INHALATION

4.3.1. Reproductive Studies

The effect of TCA on in vitro fertilization was examined in $B6D2F_1$ mouse gametes (Cosby and Dukelow, 1992). TCA was constituted in a culture medium to yield concentrations of 100, 250, or 1,000 ppm on a volume/volume basis (approximately 0.98, 2.4, and 9.8 mM) and incubated with mouse oocytes and sperm for 24 hours. Each culture dish was subsequently scored for percentage of oocytes fertilized. The percent of oocytes fertilized was significantly decreased from 82% for controls to 77.3% when oocytes and sperm were placed in a medium containing 2.4 mM TCA. At 9.8 mM TCA, only 53.1% of the oocytes were fertilized; the decrease was statistically significant ($p < 0.001$).

4.3.2. Developmental Studies

4.3.2.1. *Oral Developmental Studies*

Seven studies have evaluated the potential of TCA to induce developmental toxicity in rats (Table 4-10). In addition, one study has been conducted to identify embryonic genes, which undergo changes in expression (up- or down-regulation) in response to maternal TCA exposure. No studies in other test species (e.g., mice or rabbits) were located.

Table 4-10. Summary of developmental studies evaluating effects of TCA after oral administration in rats

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects[a]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Smith et al. (1989)	Long-Evans rats (20–21/dose)	Oral, gavage	GDs 6–15	0, 330, 800, 1,200, or 1,800 mg/kg-d in distilled water, adjusted to pH 7 with NaOH	Decreased fetal weight, decreased crown-rump length, increased incidence of soft-tissue malformations and cardiovascular malformations, increased maternal spleen and kidney weights	Maternal: not determined Developmental: not determined	Maternal: 330 Developmental: 330	
Johnson et al. (1998)	Sprague-Dawley rats (55 controls and 11 TCA treated)	Oral, drinking water	GDs 1–22	0 or 291 mg/kg-d in distilled water, titrated to pH 7 with NaOH	Increased cardiac malformations, number of implantation sites/litter, number of resorption sites/litter, and total number of resorptions among treated dams	Maternal: not determined Developmental: not determined	Maternal: 291 Developmental: 291	Dose estimated by the authors, based on the average amount of water consumed by the animals on a daily basis. The tested concentration/dose was also a maternal LOAEL for decreased weight gain. Study was not adequately designed and/or reported, and a complete array of standard developmental endpoints was not assessed.
Fisher et al. (2001)	Sprague-Dawley rats (19/dose)	Oral, gavage	GDs 6–15	0 or 300 mg/kg-d in water, adjusted to pH 7.5	Decreased maternal weight gain, reduced fetal body weight	Maternal: not determined Developmental: not determined	Maternal: 300 Developmental: 300	Cardiac defects were the only visceral malformation evaluated; maternal toxicity indicated by decreased body weight gain for GDs 7–15 and 18–21; mean uterine weight was also significantly less ($p < 0.05$) than controls.

Table 4-10. Summary of developmental studies evaluating effects of TCA after oral administration in rats

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects[a]	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Singh (2005a)	Inbred Charles Foster rats (6–12/group)	Oral, gavage	GDs 6–15	0, 1,000, 1,200, 1,400, 1,600, or 1,800 mg/kg-d in distilled water, adjusted to pH 7.0–7.5	Increase in post-implantation loss, decreased fetal testes weight, reduction in the length and diameter of the seminiferous tubules, increased apoptosis of the gonocytes	Developmental: not determined	Developmental (increase in post-implantation loss): 1,000	Only evaluated effects on fetal testes. The most sensitive effect was postimplantation loss. Maternal toxicity was not reported.
Singh (2005b)	Inbred Charles Foster rats (6–12/group)	Oral, gavage	GDs 6–15	0, 1,000, 1,200, 1,400, 1,600, or 1,800 mg/kg-d in distilled water, adjusted to pH 7.0–7.5	Decrease in fetal ovaries weight with increasing dose; decrease in the number of oocytes and the size of the ovaries, apoptosis of oocytes	Developmental: 1,200	Developmental (effect on fetal ovary): 1,400	Only evaluated effects on fetal ovaries. Maternal toxicity was not reported.
Singh (2006)	Inbred Charles Foster rats (6–12/group)	Oral, gavage	GDs 6–15	0, 1,000, 1,200, 1,400, 1,600, or 1,800 mg/kg-d in distilled water, adjusted to pH 7.0–7.5	Decrease in maternal weight gains; decrease in fetal weight and fetal brain weight; hydrocephalus, vacuolation, and hemorrhages in fetal brains	Maternal: 1,000 Developmental: not determined	Maternal: 1,200 Developmental (effect on fetal brain): 1,000	Focused only on effects of TCA on fetal brains. Maternal toxicity was not reported.
Warren et al. (2006)	Sprague-Dawley Crl:CDR (SD) BR rats	Oral, gavage	GDs 6–15	0 or 300 mg/kg-d in water	No significant decrease in maternal body weight; significant decrease in fetal body weight, no eye malformation, no significant reductions in lens area, globe area, medial canthus distance, or interocular distance	Developmental: not determined	Developmental: 300	Focused on eye malformations and microphthalmia in fetal rats.

[a]The effects listed in this table may have occurred either at the LOAEL or at higher doses.

Smith et al. (1989) dosed pregnant Long-Evans rats (20–21/dose) with 0, 330, 800, 1,200, or 1,800 mg/kg-day TCA (adjusted to pH 7 by NaOH) by gavage on GDs 6–15. Clinical signs of toxicity and body weight gain were monitored throughout the exposure period. The dams were sacrificed on GD 20. The liver, spleen, and kidneys were removed and weighed. The uterine horns were examined for the number and location of fetuses or resorption sites. The fetuses were subsequently removed and weighed, measured, sexed, and evaluated for external malformations. Two-thirds of each litter was preserved for evaluation of visceral abnormalities. The remaining one-third of the fetuses was reserved and processed for evaluation of skeletal abnormalities.

Evidence of maternal toxicity was observed in all TCA treatment groups as indicated by a significant ($p < 0.05$) increase in spleen (up to 74% increase) and kidney (up to 24% increase) weights when compared with the control group. Unadjusted mean terminal (GD 20) body weights were significantly reduced (5–12%; $p < 0.05$) at all doses, but no statistically significant differences were observed in average percent maternal weight gain when adjusted for gravid uterine weight. Dams exposed to 800, 1,200, or 1,800 mg/kg-day had significantly ($p < 0.05$) decreased body weight gains on GDs 6–9 and 15–20 (up to a 54% decrease). The weight change for GDs 15–20 may have been influenced by reductions in fetal body weight. The number of litters totally resorbed was significantly increased (5/21 and 12/20, respectively), and the number of viable litters (14/21 and 8/20, respectively) was significantly decreased at 1,200 and 1,800 mg/kg-day. Developmental effects were observed at all doses (Table 4-11) and included significant ($p \leq 0.05$) decreases in mean fetal weight per fetus (up to a 33% decrease in males and females); significant decreases in fetal crown-rump length (up to a 15% decrease in males and females); and increased percentages of fetuses affected per litter with total soft-tissue malformations. Most of the total soft tissue malformations were cardiovascular malformations, and in particular levocardia (or extremely left-sided heart). The authors noted that the Long-Evans strain of rat appears somewhat susceptible to this alteration. They also observed that levocardia is an ill-defined malformation and of trivial appearance as found in Bouins fixed sections. The maternal and developmental LOAELs in this study are 330 mg/kg-day. Maternal and developmental NOAEL values for TCA could not be determined because adverse effects were observed at all tested doses.

Table 4-11. Selected data for fetal anomalies, showing dose-related trends following exposure of female Long-Evans rats to TCA on GDs 6–15

Type	Dose (mg/kg-d)				
	0	**330**	**800**	**1,200**	**1,800**
Maternal body weight on GD 20	344.97 ± 19.04[a]	327.12 ± 25.42[a]	323.87 ± 19.04[a]	306.88 ± 29.09[a]	303.40 ± 27.03[a]
Malformations: mean % fetuses affected per litter ± SD (number of litters affected/number examined)					
Total soft tissue (visceral)	3.50 ± 8.7 (4/26)	9.06 ± 12.9[a] (8/19)	30.37 ± 28.1[a] (15/17)	55.36 ± 36.1[a] (12/14)	96.88 ± 8.8[a] (8/18)
Cardiovascular	0.96 ± 4.9 (1/26)	5.44 ± 10.0[a] (6/19)	23.59 ± 28.0[a] (12/17)	46.83 ± 36.5[a] (11/14)	94.79 ± 9.9[a] (8/8)
Levocardia: number of fetuses or litters affected/number examined					
Fetal incidence	0/196	9/151	20/111	24/69	17/22
Litter incidence	0/26	6/19	12/17	10/14	7/8
Intraventricular septal defect: number of fetuses or litters affected/number examined					
Fetal incidence	0/196	0/151	6/111	3/69	5/22
Litter incidence	0/26	0/19	4/17	3/14	5/8
Fetal crown-rump length (cm): mean ± SD					
Male	3.71 ± 0.12	3.58 ± 0.10[a]	3.46 ± 0.10[a]	3.36 ± 0.15[a]	3.16 ± 0.12[a]
Female	3.64 ± 0.15	3.53 ± 0.09[a]	3.38 ± 0.12[a]	3.33 ± 0.16[a]	3.15 ± 0.15[a]
Mean fetal body weight (g): mean ± SD					
Male	3.70 ± 0.24	3.20 ± 0.26[a]	2.98 ± 0.17[a]	2.74 ± 0.30[a]	2.49 ± 0.16[a]
Female	3.54 ± 0.20	3.08 ± 0.27[a]	2.83 ± 0.18[a]	2.67 ± 0.29[a]	2.36 ± 0.15[a]

[a]Significantly different from control ($p \leq 0.05$) as reported by Smith et al. (1989).

Source: Smith et al. (1989).

Johnson et al. (1998) evaluated the teratogenicity of TCA (adjusted to pH 7 by NaOH) by exposing pregnant Sprague-Dawley rats to 0 (n = 55) or 2,730 (n = 11) mg/L TCA in neutralized drinking water on GDs 1–22. The authors estimated the doses to be 0 or 291 mg/kg-day, based on the average amount of water consumed by the animals on a daily basis and measured body weights. Maternal toxicity was evaluated by clinical observation and maternal weight gain. Dams were sacrificed on GD 22, and implantation sites, resorption sites, fetal placements, fetal weights, placental weights, fetal crown-rump lengths, gross fetal abnormalities, and abnormal fetal abdominal organs were recorded. In addition, the fetal hearts were removed, dissected, and examined microscopically for abnormalities by using a detailed microdissection cardiac evaluation technique.

No signs of maternal toxicity were reported. Although the authors reported that the weight gain during pregnancy of treated females was not significantly different from controls, the average maternal weight gain for TCA-exposed animals was 84.6 g as compared with 122 g for control animals, representing a 30% decrease in maternal body weight gain. Average

drinking water consumption was reported as 38 mL/day in treated rats as compared with 46 mL/day in control rats.

Statistically significant increases were reported in the average number of resorption sites (2.7 resorptions/litter in treated animals, compared with 0.7 in the controls), total number of resorptions (30 resorptions reported among 11 treated females as compared with 40 resorptions among 55 control females), and average number of implantation sites (defined as sites where the fetus was implanted but did not mature) (1.1 implantation sites/litter, compared with 0.2 in the controls). In treated groups, the total number of fetuses reported was 115 in 11 rats, resulting in an average number of fetuses/litter of 10.5. In the control group, the total number of fetuses was reported as 605 in 55 rats, with an average number of fetuses/litter of 11.3. These differences were not reported as statistically significant. The number of maternal rats with abnormal fetuses was 7 out of 11 for TCA-treated animals as compared with 9 out of 55 for controls. No significant differences were reported in the numbers of live or dead fetuses, fetal weight, placental weight, fetal crown-rump length, fetal external morphology, or fetal gross external or noncardiac internal congenital abnormalities; however, data for these endpoints were not reported in the paper and could not be independently assessed.

Cardiac abnormalities were evident in 10.5% of the fetuses in the TCA group, compared with 2.15% of the controls. Although these results were not reported in terms of the more appropriate measure of number of affected litters, Johnson et al. (1998) stated that the incidence of cardiac malformations was significantly greater in treated rats as compared with control rats on both a per-fetus basis ($p = 0.0001$) and a per-litter basis ($p = 0.0004$). Complete fetal examinations for internal or skeletal abnormalities were not conducted, and the study is limited by the small size of the exposed group and the use of only one dosed group. Based on the toxicologically significant decrease in maternal body weight, 291 mg/kg-day is considered to be a maternal LOAEL. Based on an increase in cardiac malformations occurring at a maternally toxic dose, the developmental LOAEL is 291 mg/kg-day. The maternal and developmental NOAELs could not be determined because adverse effects were observed at the only dose tested.

In contrast to the results of Smith et al. (1989) and Johnson et al. (1998), Fisher et al. (2001) did not observe significant differences in the fetal or litter incidence of heart malformations following administration of neutralized TCA in distilled water to groups of pregnant Sprague-Dawley rats (n = 19). Doses of 0 or 300 mg/kg-day were given by gavage on GDs 6–15. Vehicle control animals (n = 19) received distilled water. Positive control animals (n = 12) received all-trans retinoic acid (15 mg/kg-day) dissolved in soybean oil. On GD 21, body weight, uterine weight, number and viability of fetuses, and number of implantation and resorption sites were recorded for each pregnant animal. All treated rats were then sacrificed, full-term fetuses were removed, and the following parameters were recorded: sex, fetal weight (per fetus and per litter), percent of dams with an early resorption, and number of fetuses per dam. The heart of each full-term fetus was thoroughly examined in situ and then removed,

sectioned, and microscopically examined for cardiac malformations by using a detailed cardiac microdissection technique that included staining of fetal heart tissue for detection of malformations.

The single dose evaluated produced maternal toxicity as indicated by decreased body weight gain on GDs 7–15 and 18–21 ($p \leq 0.05$, approximately 17% relative to controls). Mean uterine weight was significantly less than controls ($p \leq 0.05$, 9%). The number of implantations, percent of dams with an early resorption, and number of fetuses per litter were similar to control values. Mean fetal body weight (per litter and per fetus) on GD 21 was significantly less than that of controls ($p \leq 0.05$, approximately 8%). The heart malformation incidence in the TCA-treated group was similar to that of controls; 3.3% (9/269) of the fetuses and 42% (8/19) of the litters from TCA-treated animals were affected compared with 2.9% (8/273) of fetuses and 37% (7/19) of litters from control animals. Maternal exposure to the positive control (all-trans retinoic acid) significantly increased the incidence of cardiac defects when analyzed on a per-fetus (32.9%) or per-litter basis (92%) when compared with the corresponding soybean oil vehicle fetal and litter control incidences (6.5 and 52%, respectively). These data identify a maternal LOAEL of 300 mg/kg-day based on significantly reduced body weight gain and uterine weight. A developmental LOAEL of 300 mg/kg-day was identified, based on significantly reduced mean fetal body weight on a per-litter and per-fetus basis. Maternal and developmental NOAEL values were not identified in this single dose study because adverse effects were noted at the only dose tested.

Singh (2006, 2005b, a) treated pregnant inbred Charles Foster rats (6–12 rats/dose group; control group = 25) with 0, 1,000, 1,200, 1,400, 1,600, or 1,800 mg/kg-day TCA by gavage on GDs 6–15 and examined the effect of TCA on the developing testis (Singh, 2005a), developing ovary (Singh, 2005b), and developing brain (Singh, 2006). TCA was neutralized by sodium hydroxide to pH 7.0–7.5 before administration to rats. Control animals received distilled water by gavage. The pregnant rats were sacrificed on GD 19, and the fetuses and placenta were collected for examination. The testes of each pup of different dose groups were dissected out, weighed, and subjected to histological examination (Singh, 2005a). Other than reporting maternal weight gain on GD 19, maternal toxicity findings were not reported. Percentage of postimplantation loss was significantly increased in a dose-related manner (22% at 1,000 mg/kg-day versus 3% for control group). No external abnormalities were observed. The average weights of the fetal testes were significantly reduced when compared to the control at ≥1,200 mg/kg-day. Histological examination of fetal rat testes of the 1,200 mg/kg-day dose group revealed a reduction in the diameter of the seminiferous tubules, which only occupied the peripheral region. This effect was more pronounced in the higher dosed groups. At the higher doses, reduction in length of the seminiferous tubules was also reported. Examination of the testes at higher magnification revealed increased apoptosis of the gonocytes as well as the Sertoli cells within the seminiferous tubules in comparison to the controls at ≥1,200 mg/kg-day.

The rat fetal ovaries of each pup of different dose groups from the above study were also dissected out, weighed, and subjected to histological examination (Singh, 2005b). The average weights of the ovaries were significantly reduced for the dose groups ≥1,400 mg/kg-day. Histological examination of the fetal ovaries showed small size cells with less prominent nuclei at the coelomic epithelium with ≥1,400 mg/kg-day TCA. The cortical cords proliferating from the coelomic epithelium traversing the gonads were either shortened or lacking. Oocytes in the ovarian stroma showed shrinkage in size with distorted cell membrane and indistinct nucleus, suggestive of cell apoptosis. The number of oocytes and the size of ovary were reduced. Singh (2005b) suggested that the gonadal changes were due to anoxia and oxidative stress resulting from TCA exposure.

The rat fetal brains of different dose groups from the above study were evaluated (Singh, 2006). Maternal weight gains were statistically significantly decreased at TCA doses ≥1,200 mg/kg-day (38–46%). Mean fetal weight and fetal brain weight decreased significantly at TCA doses ≥1,000 mg/kg-day; while the length of the fetal brain increased significantly at 1,000 and 1,200 mg/kg-day (about 10% at 1,000 mg/kg-day) but decreased significantly (8–16%) at TCA doses ≥1,400 mg/kg-day when compared with controls. At doses ≥1,000 mg/kg-day, the fetal brains showed hydrocephalus with breech of the ependymal lining, altered choroids plexus architecture, and increased apoptosis. Vacuolation of the neutrophil was a prominent feature with TCA exposure, with an incidence of 26% at 1,000 mg/kg-day (0% in controls) and reached 100% in the 1,600 and 1,800 mg/kg-day dose groups. The incidence of brain hemorrhages increased to 30% at TCA doses ≥1,200 mg/kg-day (0% in controls) and reached 100% at 1,800 mg/kg-day. The infarcts were mainly concentrated in the periventricular zone. Singh (2006) concluded that the rat fetal brain was susceptible to the toxic effects of TCA.

In a study that evaluated if TCE, TCA, and DCA affect eye development in the Sprague-Dawley rat (Warren et al., 2006), pregnant Sprague-Dawley Crl:CDR (SD) BR rats were administered 0 or 300 mg/kg-day TCA by gavage on GDs 6–15. Retinoic acid (15 mg/kg-day) was used as a positive control. A subset of the fetuses evaluated in the Fisher et al. (2001) study was selected for ocular examination (1,185 fetuses [71%] from 108 dams). The number of fetuses undergoing ocular examination was reduced further to approximately 30% compared to the cardiac study. Heads of GD 21 fetuses were fixed in Bouin's solution, examined for gross external malformations, sectioned, and subjected to computerized morphometry. For detection of subtle eye anomalies, the following measurements on head sections were determined: interocular distance, total area of the cut surface, areas of left and right lenses, and areas of left and right globes.

Mean fetal body weight was statistically significantly reduced in the TCA and retinoic acid treatment groups. Mean maternal body weight was not reduced in these treatment groups (Warren et al., 2006). Fetuses with exencephaly, anophthalmia, or microphthalmia were found only in the retinoic acid treatment group. Mean fetal lens and globe areas were statistically

significantly reduced in the retinoic acid treatment group. However, mean lens and globe areas and mean medial canthus and interocular distances were not reduced in the TCA-exposed fetuses when compared with values from the control group. Thus, TCA did not appear to affect eye development in the Sprague-Dawley rat at 300 mg/kg-day.

Collier et al. (2003) investigated the effects of TCA on gene expression in embryos collected on GDs 10.5–11 from pregnant Sprague-Dawley rats exposed to 0, 1.63, or 16.3 mg/mL (0, 10, or 100 mM, respectively) TCA in drinking water on GDs 0–11. Estimated intakes were 0, 200, and 2,000 mg/kg-day, based on a body weight of 0.35 kg and drinking water rate of 0.046 L/day (U.S. EPA, 1988). The objective of the study was to identify altered expression of genes (using a subtractive hybridization technique) that might be used as markers of exposure to TCE or its metabolites (i.e., TCA) in the developing rat heart. Exposure to TCA down-regulated rat ribosomal protein S10 (a housekeeping gene) and rat chaperonin 10 (a stress response gene) and up-regulated rat Ca^{2+}-ATPase (a calcium-responsive gene) and rat gC1qBP (function not reported). The expression of the up-regulated genes was found to be strongly heart specific on embryonic days 10.5–11. However, no correlation between up-regulation of these genes and occurrence of TCA-mediated cardiac defects has yet been identified.

4.3.2.2. *Inhalation Developmental Studies*

No studies on the developmental toxicity of TCA were identified for exposure by the inhalation route.

4.3.2.3. *In Vitro Studies*

TCA has also been tested in a number of alternative screening assays for assessment of developmental toxicity. Hunter et al. (1996) conducted a 24-hour exposure of 3–6 somite stage CD-1 mice embryos to 11 haloacetic acids, including TCA. TCA was tested at concentrations of 0, 0.5, 1, 2, 3, 4, or 5 mM. Effects on neural tube development were observed at concentrations lower than effects on other morphological processes. Other statistically significant dysmorphology included eye defects, pharyngeal arch defects, and heart defects. TCA produced abnormal embryonic development at concentrations ≥2 mM, with a very steep dose-response slope from 2 to 5 mM. No adverse effects were observed at ≤1 mM, and defects of the eyes, arches, and heart were seen only in embryos that also had very high rates of neural tube development abnormalities. The observed effects did not result from low pH in the culture medium, since they were not seen when HCl was added to adjust the culture medium to similar pH values.

The potential developmental toxicity of TCA was studied in vitro by using a rat whole-embryo culture system by Saillenfait et al. (1995). Groups of 10–20 explanted embryos from Sprague-Dawley rats on GD 10 were cultured for 46 hours in 0, 0.5, 1, 2.5, 3.5, 5, or 6 mM TCA. TCA induced statistically significant, concentration-related decreases in the growth and

development parameters of conceptuses. Yolk sac diameter was significantly decreased, beginning at a concentration of 1 mM. Other developmental measures, including crown-rump length, head length, somite (embryonic segment) number, protein content, and DNA content, were significantly decreased at ≥2.5 mM. The total number of malformed embryos was increased beginning at 2.5 mM. At 2.5 mM, 55% of the embryos had brain defects, 50% had eye defects, 32% had reduced embryonic axes, 55% had reductions in the first branchial arch, and 36% had otic (auditory) system defects.

TCA has also been evaluated in developmental toxicity screening assays in nonmammalian systems. TCA was evaluated using the FETAX assay in a study that assessed the developmental toxicity of TCE and its metabolites (Fort et al., 1993). Early *Xenopus laevis* embryos were exposed to a range of TCA concentrations for 96 hours. The culture stock solution was buffered to pH 7.0. The median lethal concentration was 4,060 mg/L (24.8 mM) and the median effective concentration (EC_{50}) for malformations was 1,740 mg/L (10.6 mM). Malformations were observed at concentrations >1,500 mg/L (9.2 mM) and included gut miscoiling, craniofacial defects, microphthalmia, microcephaly, and various types of edema. The assay was conducted with high TCA concentrations, and is of limited value for evaluating developmental toxicity of TCA.

4.4. OTHER DURATION- OR ENDPOINT-SPECIFIC STUDIES

4.4.1. Immunological Studies

The available information on the potential for TCA to affect the immune system is limited. Mather et al. (1990) (described in Section 4.2) did not observe any effects on several immunotoxicity parameters, including antibody production, delayed hypersensitivity, natural killer cell cytotoxicity, and production of prostaglandin E2 and IL-2 in male Sprague-Dawley rats (10 males/dose) exposed to TCA in drinking water at up to 355 mg/kg-day for 90 days. However, Tang et al. (2002) reported that TCA was positive in the guinea pig maximization test. A 58% sensitization rate (7/12) was observed in animals given an intradermal injection (2% solution) and topical application (5% solution), then challenged with a topical application of a 2% TCA solution 21 days after the first intradermal induction. The following scale was used to grade the reactions: 0 = no reaction, 1 = scattered mild redness, 2 = moderate and diffuse redness, and 3 = intensive erythema and swelling. The mean score for redness in this study was 1.1, and the mean score for swelling was 0.0. Histological examination of the affected skin revealed that TCA induced allergenic transformation. These limited data suggest that TCA could induce a mild allergenic response on exposure to sub-irritating doses.

4.5. MECHANISTIC DATA AND OTHER STUDIES IN SUPPORT OF THE MODE OF ACTION

4.5.1. Mechanistic Studies

Several studies have been conducted for the primary purpose of evaluating the potential mechanisms by which TCA induces tumors in laboratory animals. These studies can be divided into five types: peroxisome proliferation, oncogene activation, cell proliferation, DNA hypomethylation, and inhibition of intercellular communication. Histochemical properties of TCA-induced tumors have also been characterized in a number of studies, and these properties have been compared with the same properties in DCA-induced tumors in order to compare the potential mechanisms of tumor induction.

4.5.1.1. *Peroxisome Proliferation*

The ability of TCA to induce peroxisome proliferation has been demonstrated in several studies in rats (DeAngelo et al., 1997; Mather et al., 1990; DeAngelo et al., 1989; Goldsworthy and Popp, 1987; Elcombe, 1985) and mice (DeAngelo et al., 2008; Laughter et al., 2004; Parrish et al., 1996; Austin et al., 1995; DeAngelo et al., 1989; Goldsworthy and Popp, 1987). These studies and the evidence for peroxisome proliferation are summarized in Sections 4.2.1.1.1, 4.2.1.1.2, 4.2.2.1.1.1, and 4.2.2.1.2.1.

4.5.1.2. *Oncogene Activation*

Ferreira-Gonzalez et al. (1995) studied the K- and H-*ras* proto-oncogene mutation patterns in TCA-induced tumors in male B6C3F_1 mice. The *ras* gene encodes a plasma membrane-bound guanosine triphosphatase. Guanosine triphosphatase activates kinase cascades that regulate cell proliferation. The *ras* gene was studied because changes in the rate and spectrum of mutations in the *ras* proto-oncogene have been linked to the carcinogenic mechanism of various liver carcinogens.

Mice (number per group not reported) were exposed to 0 or 4,500 mg/L [1,080 mg/kg-day based on default water intake values in U.S. EPA (1988)] TCA in drinking water for 104 weeks. The incidence of liver carcinomas was 19% in the untreated mice and 73.3% in the TCA-exposed group. DNA samples were extracted from 32 spontaneous liver tumors from the control group and from 11 liver tumors in mice treated with TCA. DNA samples containing point mutations in exons 1, 2, and 3 of the K- and H-*ras* genes were detected by the presence of single-stranded conformation polymorphisms. The single-stranded conformation polymorphism analysis involved amplification of DNA from the control or tumor tissue to generate DNA fragments containing normal or mutated *ras* gene fragments. Since single-stranded DNA fragments containing base-pair changes have different mobility's when run in polyacrylamide gels (gel electrophoresis), the pattern of bands observed following gel electrophoresis served to indicate the presence of a mutated base.

In the spontaneous tumors from control mice, *ras* mutations were detected only at the H-61 codon (i.e., the mutation was in the H-*ras* gene, in the 61st codon, which is in the second exon); 58% of the spontaneous liver carcinomas showed mutations in H-61, compared with 45% of the tumors from TCA-treated mice. One TCA-induced tumor showed a mutation in K-61 (i.e., in the K-*ras* gene, in the second exon). Identification of the specific base-pair change was done by sequencing of the DNA fragment obtained in the single-stranded conformation polymorphism analysis. Comparative sequence analysis of exon 2 mutations from spontaneous and TCA-induced tumors revealed that mutations detected in the TCA tumors matched the mutation spectrum seen in the spontaneous tumors from control mice. Therefore, TCA changed neither the rate of *ras* mutations nor the type of mutations occurring at codon 61.

These results were confirmed in a more recent study. Bull et al. (2002) (described in Section 4.2) exposed male B6C3F$_1$ mice (20–40/group) at 125–500 mg/kg-day in the drinking water for 52 weeks. A decrease in the mutation frequency in H-*ras* codon 61 in TCA-induced tumors compared with spontaneous tumors from control animals was observed, confirming the observations of Ferreira-Gonzalez et al. (1995). Also, the type of H-*ras* codon 61 mutations was similar to the spectra of mutations observed in spontaneous tumors from control animals.

Based on the absence of an effect on mutation rate, the authors indicated that it was not clear if TCA was acting through a genotoxic or nongenotoxic mechanism (Ferreira-Gonzalez et al., 1995). However, the number of tumors with *ras* mutations was slightly decreased in TCA-treated animals, consistent with TCA acting through a nongenotoxic mechanism. Because of the large proportion of tumors carrying a *ras* mutation, the authors concluded that *ras* mutations are important for the development of carcinogen-induced tumors as well as spontaneous tumors. TCA increased the tumor yield but did not change mutations in *ras*, leading the study authors to conclude that TCA might facilitate the growth of preneoplastic lesions that arise from spontaneously initiated (i.e., *ras* mutated) hepatocytes.

Tao et al. (1996) investigated whether liver tumors initiated by MNU and promoted by TCA exhibited loss of heterozygosity in four polymorphic loci on chromosome 6. According to the authors, inactivation of one or more of the polymorphic alleles at these loci may be related to the inactivation of an, as yet, unidentified tumor-suppressor gene, resulting in oncogene activation that may be a key event in the pathogenesis of some liver tumors. This hypothesis is supported by the results of a study by Davis et al. (1994), in which 20% of hepatic tumors induced by tetrachloroethylene exhibited loss of heterozygosity on chromosome 6, suggesting the presence of a tumor suppressor gene at this site. In this study, 15-day-old female B6C3F$_1$ mice were pretreated with 25 mg/kg MNU via i.p. injection and administered TCA in drinking water at a concentration of 20.0 mmol/L (3,268 mg/L) for 52 weeks. The authors did not provide a dose estimate, but the approximate dose is 784 mg/kg-day, based on the default drinking water intake value for female B6C3F$_1$ mice (U.S. EPA, 1988). Thirty-seven liver tumors promoted by TCA were examined for loss of heterozygosity by using four polymorphic loci on chromosome

6. Ten of 37 tumors (7/27 carcinomas and 3/10 adenomas) promoted by TCA showed evidence of loss of heterozygosity for at least two loci on chromosome 6. The C57BL/6J alleles at both the D6mit9 and D6mit323 loci were lost in all 10 tumors exhibiting loss of heterozygosity, and 2 of these 10 tumors also lost at least one of the C3H/HeJ alleles. No loss of heterozygosity on chromosome 6 was observed in 24 DCA-promoted liver tumors. The observed loss of heterozygosity on chromosome 6 in many of the tumors suggests the presence of an unidentified tumor-suppressor gene on this chromosome. However, as the majority of tumors in TCA-treated mice did not exhibit loss of heterozygosity on chromosome 6, the authors concluded that other molecular activity is probably involved in the hepatocarcinogenesis of TCA.

4.5.1.3. *Cell Proliferation*

Investigations of the effects of TCA on cell growth rates have produced conflicting results. Miyagawa et al. (1995) examined the effect of TCA (and a battery of putative nongenotoxic liver carcinogens and noncarcinogens) on replicative DNA synthesis to assess the utility of measurement of cell proliferation as a screening assay for detecting nongenotoxic carcinogens. Groups of male $B6C3F_1$ mice (four or five per dose) were administered a single gavage dose of TCA in an acute toxicity test to determine the maximum tolerated dose. The maximum tolerated dose for TCA was reported to be approximately one-half of the LD_{50}. Groups of four or five animals were administered a single gavage dose of one-half of the maximum tolerated dose (250 mg/kg, as estimated from data provided by the authors) or the maximum tolerated dose (500 mg/kg, as estimated from data provided by the authors), and incorporation of [^{3}H]thymidine in harvested hepatocytes was measured 24, 39, or 48 hours after dosing. For TCA, positive responses were observed at 250 mg/kg at 24 and 39 hours (6.5- and 4.9-fold above controls) and at 500 mg/kg (9.8-fold above controls). Although the mean increase in replicative DNA synthesis met the criteria for a positive response, the increases did not appear to be statistically significant based on the SDs supplied in the summary table.

In contrast to the increased cell proliferation observed by Miyagawa et al. (1995), Channel and Hancock (1993) found that TCA can decrease the rate of progression through S-phase of the cell cycle. WB344 cells, a non-tumorigenic epithelial rat hepatocyte cell line, were exposed to TCA-free medium or medium containing 100 µg/mL TCA. Cell growth rates were assessed by cell counting, and transition through the cell cycle was monitored by labeling nascent DNA with BrdU. The resulting labeling data were used to identify fractions of cells in various stages of the cell cycle and to model transit times through each phase. The transit time through S-phase was estimated to be 5.20 hours for treated and 5.02 hours for control cells ($p < 0.05$). As further support for this effect, cells in S-phase were elevated by approximately 5–20% for the first 6 hours after release from TCA-treatment but returned to control values after this initial period. In contrast to these results, indicating slowing of S-phase transit, relative movement plots (also related to S-phase transit time) did not differ from controls. The authors

suggested, however, that this might reflect the insensitivity of relative movement plots for detection of small treatment-related changes, such as those observed for TCA. The authors suggested that the observed pattern of cell cycle perturbation (i.e., a slightly extended period of S-phase) would be consistent with a sublethal effect of cytotoxicity and would be less serious than a decrease in transit time through G_2M phase (which could potentially increase chromosomal mismatches and rearrangements, due to an insufficient time spent in mitosis). The toxicological significance of these results by Miyagawa et al. (1995) and Channel and Hancock (1993) are difficult to interpret, since they might not reflect the cell growth conditions of normal hepatocytes in vivo. For this reason, these studies are of limited use in evaluating the effects of TCA on cell growth in vivo.

Pereira (1996) evaluated cell proliferation in the liver of female $B6C3F_1$ mice (10/group) treated with 0, 2, 6.67, or 20 mmol/L TCA in drinking water for 5, 12, or 33 days by estimating hepatocyte BrdU-labeling index. TCA statistically significantly increased the BrdU-labeling index after 5 days of exposure by approximately 2.5–3-fold at all three concentrations [values estimated from Figure 4 in Pereira (1996)]; however, BrdU-labeling indices were not increased after 12 or 33 days of exposure. Thus, cell proliferation was enhanced by 5 days exposure to TCA but not for longer exposures of 12 or more days.

In a cell proliferation study reported by Stauber and Bull (1997), male $B6C3F_1$ mice were pretreated with 2,000 mg/L of TCA [480 mg/kg-day based on default water-intake values in U.S. EPA (1988)] in drinking water for 50 weeks. The mice were then given drinking water containing 0, 20, 100, 500, 1,000, or 2,000 mg/L TCA [estimated doses of 0, 5, 23, 115, 230, or 460 mg/kg-day, based on default water intake values in U.S. EPA (1988)] for 2 additional weeks to assess whether cell proliferation induced by TCA in either normal liver cells or tumors was dependent on continued treatment. All dose groups contained 12 animals, except for the 2,000 mg/L group, which consisted of 22 mice. Five days prior to sacrifice, DNA in replicating hepatocytes was labeled in vivo by administering BrdU via subcutaneously implanted pumps. Liver tissue was stained, and dividing nuclei were counted. Cell division rates were evaluated separately in normal hepatocytes, in tumors, and in altered hepatic foci (AHF).

A transient but significant elevation (about twofold) in normal hepatocyte division rates was evident in mice consuming 2,000 mg/L TCA for 14 or 28 days (apparently as part of the pretreatment phase), but continued treatment for 52 weeks resulted in a significant decrease (about 70%) in hepatocyte division rate. In the mice treated for 50 weeks with 2,000 mg/L and then shifted to the lower concentrations for 2 weeks, the cell division rate in normal liver cells was elevated (but not statistically significantly so) at 100 and 500 mg/L, but in mice exposed to 1,000 or 2,000 mg/L for 2 weeks, there was a significant decrease (about 1.5-fold) in cell division. Cell division rates in TCA-induced AHFs and tumors were high at all doses. Rates of cell division in AHFs and tumors remained high in mice whose exposure was terminated during

the last 2 weeks of the study, indicating that these rates were independent of continued TCA treatment.

TCA-induced lesions were histochemically stained with anti-*c-jun* and anti-*c-fos* antibodies, component proteins of the AP-1 transcription factor that up-regulates expression of genes required for DNA synthesis. No differences were observed in the levels of proteins reacting with *c-jun* and *c-fos* antibodies in either liver AHFs or tumors, relative to normal hepatocytes, indicating that TCA produces little, if any, direct stimulation of the replication of initiated cells through this pathway. However, three tumors induced by TCA each contained a nodule that stained heavily for *c-fos*, and cell-division rates within these nodules were very high, suggesting a transition to an aggressive tumor. The low frequency of this marker (3/52 tumors) suggested that its presence in these nodules was not due to a direct effect of TCA.

Based on these results, Stauber and Bull (1997) proposed a mechanism for TCA-induced hepatocarcinogenesis. They proposed that the initial growth stimulation induced by TCA causes normal cells to compensate by increasing signals that inhibit cell proliferation, which ultimately results in the TCA-induced growth inhibition observed with chronic treatment. Pre-initiated cells refractory to this growth inhibition would then have a selective growth advantage. The authors noted that the lack of effect on *c-jun* by TCA was consistent with tumor characteristics of other peroxisome proliferators in rats, as demonstrated by Rao et al. (1986). Because cell replication in AHFs was independent of TCA (i.e., discontinued TCA treatment did not alter AHFs or tumor-cell labeling), it was proposed by Stauber and Bull (1997) and Ferreira-Gonzalez et al. (1995) that TCA might enhance growth of initiated cells by suppressing apoptosis in such cells, as has been demonstrated for other peroxisome proliferators and is consistent with agonism of PPARα receptor playing an important role in TCA-induced carcinogenesis. Cell proliferation has also been observed in short-term studies (Dees and Travis, 1994; Sanchez and Bull, 1990) that are described in Section 4.2. The results of these studies were consistent with the results described by Stauber and Bull (1997).

4.5.1.4. *DNA Hypomethylation*

The hypomethylation of DNA in response to TCA exposure was investigated by Tao et al. (1998) as a potential nongenotoxic mechanism involved in TCA-induced tumor promotion and carcinogenesis. Mammalian DNA naturally contains the methylated base 5-methylcytosine (5MeC), which plays a role in regulation of gene expression and DNA imprinting (Razin and Kafri, 1994). An overall decrease in the content of 5MeC in DNA is often found in tumors and has been considered to represent an important event in the clonal expansion of premalignant cells during neoplastic progression (Counts and Goodman, 1995, 1994).

In the Tao et al. (1998) study, female B6C3F_1 mice were injected i.p. with 25 mg/kg of MNU at 15 days of age. When the mice were 6 weeks of age, TCA, neutralized to a concentration of 25 mmol/L (4,085 mg/L), was administered in drinking water for 44 weeks.

This concentration corresponds to approximately 980 mg/kg-day, based on a default water factor of 0.24 L/kg-day for female B6C3F$_1$ mice for chronic exposure (U.S. EPA, 1988). Control mice received only MNU.

To test the effects of short-term treatment with TCA on DNA methylation, mice not administered MNU were given 0 or 25 mmol/L TCA in drinking water for 11 days, corresponding to approximately 1,062 mg/kg-day, based on the strain-specific water factor for a short-term study (U.S. EPA, 1988). DNA extracted from liver tissue and tumors were hydrolyzed, and 5MeC and the four DNA bases were separated and quantified by HPLC.

After 11 days of exposure to TCA (without pretreatment with MNU), the level of 5MeC in total-liver DNA was decreased (about 60%) relative to untreated controls. After 44 weeks of TCA treatment, 5MeC levels were not different from controls that had received only MNU. No difference in DNA methylation was observed between the control groups in the short-term (drinking water control) and long-term (MNU only control) experiments. These results indicate that TCA caused only a transient decrease in DNA methylation in the liver.

In TCA-promoted hepatocellular adenomas and carcinomas, the level of 5MeC in DNA was decreased 40 and 51% when compared with either noninvolved tissue from the same animal and liver tissue from control animals given only MNU, respectively. Termination of TCA treatment 1 week prior to sacrifice did not change the levels of 5MeC in either adenomas or carcinomas; however, they remained lower than in noninvolved tissue. 5MeC levels in DNA from carcinomas were lower than in DNA from adenomas, suggesting that DNA methylation is further decreased with tumor progression. DNA hypomethylation tends to favor gene expression, which may drive cell-proliferation responses. Therefore, based on the change observed in the adenomas and carcinoma tissue compared with the uninvolved tissue, Tao et al. (1998) suggested that hypomethylation of DNA, as indicated by decreased 5MeC in tumor DNA, is involved in the carcinogenic and tumor-promoting activity of TCA.

The marked increase in hypomethylated DNA in mouse liver tumors observed by Tao et al. (1998) indicated that the methylation of numerous genes was decreased. Tao et al. (2004; 2000a, b) investigated the methylation status and expression of specific genes in mouse liver tumors and uninvolved liver tissue, as well as in livers of mice initiated with MNU but not exposed to TCA, in a series of studies described below.

Tao et al. (2000b) evaluated the methylation and expression of *c-jun* and *c-myc* protooncogenes in mouse liver after short-term exposure to TCA. Female B6C3F$_1$ mice (four/group) were dosed by gavage for 5 days with 500 mg/kg-day TCA in water neutralized with sodium hydroxide to pH 6.5–7.5. This dose was selected because it was reported to increase liver growth, cell proliferation, and lipid peroxidation in mice (Dees and Travis, 1994; Larson and Bull, 1992). Vehicle-control mice received the same volume of water or corn oil. At 30 minutes after each dose of TCA or vehicle, the mice received 0, 30, 100, 300, or 450 mg/kg methionine by i.p. injection. The mice were sacrificed 100 minutes after the last dose and the

livers were excised. Methylation status in the promoter region for *c-jun* and *c-myc* protooncogenes was evaluated by using methylation-sensitive restriction endonuclease *HpaII* digestion, followed by Southern blot analysis of DNA. *HpaII* does not cut CCGG sites when the internal cytosine is methylated, and Southern blots, probed for the promoter region of these two genes, would only contain extra bands in *HpaII* digested hypomethylated DNA. Expression of mRNA for *c-jun* and *c-myc* protooncogenes and *c-jun* and *c-myc* proteins were also analyzed.

Decreased methylation in the promoter regions of the *c-jun* and *c-myc* genes and increased levels of their mRNA and proteins were found in the livers of TCA-treated mice. Methionine prevented the decreased methylation of the two genes in a dose-dependent manner, with the effective dose ≥100 mg/kg. Methionine also prevented the increased levels of the mRNA and proteins from the two genes at 450 mg/kg. Tao et al. (2000b) concluded that the prevention of TCA-induced DNA hypomethylation by methionine suggested that the decrease in the formation of 5MeC in DNA is due to a decrease in the concentration of S-adenosyl-methionine substrate, and the dose of TCA must be sufficient to decrease the level of S-adeno-sylmethionine in order for it to be active as a carcinogen.

In another study, Tao et al. (2000a) examined the methylation of *c-jun* and *c-myc* genes, expression of both genes, and activity of DNA methyltransferase in mouse liver tumors initiated by MNU and promoted by TCA in female B6C3F_1 mice. The tumors were obtained from test animals used in the promotion study described by Pereira and Phelps (1996) (see Section 4.2.2.1). Briefly, the test animals were given either 25 mg/kg MNU or the saline vehicle control at 15 days of age. Starting at 6 weeks of age, animals were given neutralized TCA in drinking water at 20 mmol/L (3,268 mg/L) continuously until 52 weeks of age. Dose estimates were not reported by the study authors, but the concentration provided in drinking water would result in a dose of approximately 784 mg/kg-day based on the default drinking water value of 0.24 L/kg-day for female B6C3F_1 mice (U.S. EPA, 1988). TCA-promoted liver tumors and noninvolved liver tissue, as well as liver tissue from MNU-initiated mice not exposed to TCA, were collected when the animals were euthanized at 52 weeks of age.

Methylation status in the promoter regions of the *c-jun* and *c-myc* genes was determined by Southern blot analysis of DNA extracted from the three types of harvested tissues and digested with the methylation-sensitive restriction endonuclease *HpaII*. Expression of the *c-jun* and *c-myc* genes was determined by northern blot analysis of mRNA levels and western blot analysis of protein levels. DNA methyltransferase activity was determined in nuclear extracts prepared from the harvested liver tumors or the other two types of liver tissues described previously. Tao et al. (2000a) concluded that the promoter regions of *c-jun* and *c-myc* in tumors were hypomethylated relative to the promoter regions in noninvolved liver tissue from TCA-promoted animals. The expression of the mRNA and protein for each of these genes was also increased in TCA-promoted tumors relative to noninvolved liver tissue. DNA methyltransferase activity was significantly increased in liver tumors from TCA-promoted mice when compared

with noninvolved liver from the same mice. Collectively, these results suggest that TCA-promoted carcinogenesis involves decreased methylation and increased expression of the *c-jun* and *c-myc* protooncogenes in the presence of increased DNA methyltransferase activity.

In a related study, Tao et al. (2004) investigated DNA hypomethylation and the methylation status and expression of the insulin-like growth factor (IGF)-II gene[5] in TCA-promoted mouse liver tumors and noninvolved liver tissue, as well as in liver tissue samples from MNU-initiated mice that were not exposed to TCA. Expression of the IGF-II gene was investigated because increased hepatic cell proliferation is associated with increased expression of growth-related genes, such as IGF-II (Fürstenberger and Senn, 2002; Werner and Le Roith, 2000). Loss of imprinting[4] and increased expression of IGF-II have been observed in liver tumors (Scharf et al., 2001; Khandwala et al., 2000).

In the study by Tao et al. (2004), mouse liver tumors and tissues were obtained from female B6C3F$_1$ mice as described above. At necropsy, no liver tumors were found in mice that were treated with MNU alone or TCA alone. The levels of 5MeC in DNA extracted from tumors and liver tissues were quantified by a dot blot analysis procedure that used a mouse monoclonal primary antibody specific for 5MeC. Methylation status of 28 cytosine-guanine dinucleotide sites[6] in the differentially methylated region-2 of the mouse IGF-II gene was determined by a bisulfite-modified DNA sequencing procedure. In this procedure, DNA extracted from tumors and liver tissues was incubated with sodium metabisulfite to convert unmethylated (but not methylated) cytosine to uracil to enable detection of unmethylated sites in the sequencing analysis. Bisulfite-modified DNA was recovered, and the differentially methylated region-2 of the IGF-II gene was amplified by polymerase chain reaction for sequencing. Expression of IGF-II mRNA was determined by reverse transcription polymerase chain reaction. The level of 5MeC in DNA from noninvolved liver tissue in mice treated with TCA was decreased relative to that in DNA from mice initiated with MNU but not exposed to TCA. The level of 5MeC in TCA-promoted tumors was further decreased relative to the noninvolved liver tissue, indicating hypomethylation. These observations confirm the previous results of Tao et al. (1998) for DNA hypomethylation obtained by using HPLC analysis.

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[5]IGF-II is involved in cell division, differentiation, and apoptosis. According to information presented in Tao et al. (2004), the IGF-II gene is imprinted with the paternal allele being expressed, and the maternal allele is methylated and silent in normal adult tissue, including the mouse liver, while in tumors, the imprinting is lost. Loss of imprinting is accompanied by increased expression of its mRNA in tumors.

[6]Cytosine-guanine dinucleotide sites are regions in DNA where a cytosine nucleotide (C) is situated next to a guanine nucleotide (G). The "p" denotes the phosphodiester bond that links the nucleotides. Cytosine-guanine dinucleotide sites are relatively rare in eukaryotic genomes except in regions near the promoter regions of genes. Methylation of the cytosine nucleotide at cytosine-guanine dinucleotide sites to form 5MeC is believed to play a critical role in regulation of gene expression. Decreased methylation or hypomethylation is associated with gene expression, while increased methylation has an inhibitory effect on gene expression. Aberrant promoter methylation has been proposed as a possible mechanism for increased protooncogene expression in cancer.

Sequencing of the differentially methylated region-2 of the IGF-II gene promoter revealed that 21–24 cytosine-guanine dinucleotide sites were methylated in initiated liver, compared with 15–17 sites in noninvolved liver tissue from TCA-promoted mice. Thus, exposure to TCA reduced the percentage of cytosine-guanine dinucleotide sites that were methylated from approximately 79 to 58%. The number of methylated cytosine-guanine dinucleotide sites was further reduced to 0–7 (approximately 11%) in liver tumors promoted by TCA. mRNA expression was significantly increased (5.1-fold) in liver tumors relative to noninvolved liver tissue from mice treated with TCA. mRNA expression was not increased in noninvolved liver tissue from TCA-promoted animals when compared to level of expression in the MNU-initiated control. These results demonstrated that TCA treatment caused hypomethylation of DNA and increased expression of the IGF-II gene in the TCA-promoted liver tumors. Thus, the hypothesis that DNA hypomethylation is involved in the mechanism for tumorigenicity of TCA is supported.

The temporal association of DNA methylation and cell proliferation in mice treated with TCA has been investigated by Ge et al. (2001b). Female B6C3F$_1$ mice were given gavage doses of 500 mg/kg-day TCA and sacrificed at 24, 36, 48, 72, and 96 hours after the first dose. (TCA was neutralized to pH 6–7 with NaOH.) The liver, kidney, and urinary bladder were removed and weighed, and subsamples were processed for extraction of DNA and determination of methylation status in the promoter region of the *c-myc* protooncogene. Methylation status was determined by Southern blot analysis following digestion of the isolated and purified DNA with a methyl-sensitive restriction enzyme. Liver and kidney tissues were collected for measurement of cell proliferation by determination of proliferating cell nuclear antigen labeling and mitotic indices.

Relative liver weights were significantly increased at the 36-, 72-, and 96-hour time points; there was no effect of TCA on relative kidney weights. The proliferating cell nuclear antigen labeling index was significantly increased in liver cells at 72 and 96 hours relative to controls. The mitotic index was significantly elevated in liver cells at 96 hours after the first dose. Southern blot analysis indicated that the tumor promoter region of the *c-myc* protooncogene in the liver was hypomethylated at the 72- and 96-hour time points. These data indicate that TCA caused simultaneous enhancement of cell proliferation and decreased methylation in liver cells starting at 72 hours after exposure. TCA also decreased methylation in the promoter region of the *c-myc* gene in the kidney and urinary bladder after 72 and 96 hours of treatment, but the response was less pronounced than in liver. Cell proliferation data for the kidney were not reported. The study authors proposed that TCA induces hypomethylation by inducing DNA replication and preventing the methylation of the newly synthesized strands of DNA.

Pereira et al. (2001) examined the effect of chloroform (a disinfection byproduct present as a co-contaminant with TCA in drinking water) on DCA- or TCA-induced hypomethylation

and expression of the c-*myc* protooncogene in female B6C3F$_1$ mice. Chloroform has been reported to cause hypomethylation of DNA and of the *c-myc* gene by preventing the methylation of hemimethylated DNA formed when DNA is replicated (Coffin et al., 2000). Six mice per treatment group were exposed to 0, 400, 800, or 1,600 mg/L chloroform in the drinking water for 17 days. A DCA or TCA dose of 500 mg/kg was administered daily by gavage on the last 5 days of the exposure period. At sacrifice, livers were removed and processed for extraction of DNA. Methylation of the promoter region was evaluated by using *HpaII* restriction enzyme digestion followed by Southern blot analysis. Expression of *c-myc* mRNA was evaluated using reverse transcription-polymerase chain reaction followed by northern blot analysis. Both DCA and TCA decreased methylation in the promoter region of the *c-myc* gene and increased expression of *c-myc* mRNA. Coadministration of chloroform did not affect the extent of TCA-induced hypomethylation or mRNA expression or the incidence or multiplicity of liver tumors promoted by TCA. By contrast, coadministration of chloroform prevented the hypomethylation and mRNA expression of the c-myc gene and the promotion of liver tumors by DCA. This study suggests that the ability of chloroform, DCA, and TCA to hypomethylate *c-myc* and increase *c-myc* mRNA expression in the liver was correlated with their effect on liver tumor promotion.

4.5.1.5. *Inhibition of Intercellular Communication*

Benane et al. (1996) assessed the effects of TCA on gap junctional intercellular communication (GJIC) in Clone 9 (ATCC CRL 1439), a normal liver epithelial cell line from a 4-week-old Sprague-Dawley male rat. The cells were grown in a nutrient mixture, plated, and exposed to TCA at concentrations of 0, 0.5, 1.0, 2.5, and 5 mM (1, 4, 6, 24, 48, or 168 hours) for varying time periods. Lucifer yellow scrape-load dye transfer was used as a measure of GJIC. At a concentration of 0.5 mM, there were no statistically significant differences in dye transfer among control and treated cells at any of the time points. At a concentration of 1.0 mM, statistically significant differences were found for all time periods except 4 and 168 hours. At concentrations of 2.5 and 5 mM, the level of dye transfer was statistically decreased as compared with controls for all time points. The lowest concentration and shortest time to reduce dye transfer was 1 mM over a 1-hour period. The reduction in dye transfer increased with higher concentrations and longer treatment time. 12-O-tetradecanoylphorbol 13-acetate, a tumor promoter and a known disruptor of intercellular communication, used as positive control, caused a rapid reduction in dye transfer.

Klaunig et al. (1989) performed a series of experiments to determine the effects of TCA on GJIC in primary cultured B6C3F$_1$ mouse and F344 rat hepatocytes. Mouse and rat hepatocytes were isolated from 6–8-week-old male mice and rats by two-stage collagenase perfusion and plated in glass Petri dishes or flasks. Following preliminary experiments to identify cytotoxic concentrations, 24-hour-old hepatocytes were treated with 0, 0.1, 0.5, or 1 mM TCA dissolved in dimethyl sulfoxide for up to 24 hours. The controls included “no treatment”

and solvent controls in sealed and unsealed culture vessels. PB was used as the positive control. Effects on GJIC were evaluated by the iontophoretic microinjection of fluorescent Lucifer yellow CH dye into one hepatocyte and observation of the dye spread to adjacent hepatocytes. Adjacent cells that fluoresced were designated as dye coupled (i.e., communicating through gap junctions). The experimental results were expressed as the number of coupled/noncoupled recipient cells and a percentage of coupled cells. TCA inhibited dye transfer in both 24-hour-old and freshly plated mouse hepatocytes. The inhibitory effect in 24-hour-old cultures was transient; dye coupling was significantly reduced at all tested concentrations after 4 hours of treatment but not after 8 or 24 hours. PB, the positive control, significantly reduced dye transfer in cells treated with 1 or 2 mM after 4 or 8 hours of treatment but not after 24 hours. In an experiment to compare the response of freshly plated and 24-hour-old mouse hepatocytes, all tested concentrations of TCA significantly inhibited dye transfer in both types of culture after 3 and 6 hours of treatment. The inhibitory effect on dye transfer in mouse cells was unaffected by treatment with SKF-525A, a CYP450 inhibitor.

Dye transfer in 24-hour-old primary rat hepatocytes was unaffected by treatment with TCA at concentrations up to 1 mM for as long as 24 hours. Dye transfer in freshly plated rat primary rat hepatocytes was unaffected by treatment with concentrations up to 1 mM TCA for as long as 6 hours. PB, the positive control, significantly reduced the percentage of coupled cells in cultures treated with 1 or 2 mM after 4 or 8 hours of treatment but not after 24 hours. The results obtained for primary F344 rat hepatocytes by Klaunig et al. (1989) differ from those reported in rat cell cultures by Benane et al. (1996), who observed inhibition of dye transfer in cells from a Sprague-Dawley rat epithelial cell line treated with 1 mM for durations of 1–168 hours. The reason for the differential response in rat liver cells is unknown but may be related to differences in the originating strain or in the type of cultured cell tested (primary cultured hepatocytes versus established cell line).

4.5.1.6. *Oxidative Stress*

The ability of TCA to induce oxidative-stress responses, such as lipid peroxidation and oxidative DNA damage, and the relationship between these responses and indicators of peroxisome proliferation or altered CYP450 activities have been tested in a series of studies following acute or short-term TCA dosing in mice (Parrish et al., 1996; Austin et al., 1996; Austin et al., 1995; Larson and Bull, 1992). TCA induced both lipid peroxidation (TBARS) and oxidative DNA damage (8-OHdG) following administration of single oral doses. These studies are described in Section 4.2.

A potential mechanism of TCA-induced oxidative stress via macrophage activation was investigated by Hassoun and Ray (2003). Studies have shown that macrophages can be activated and become a source of reactive oxygen species that may produce damage to surrounding tissues (Karnovsky et al., 1988; Briggs et al., 1986). In this study, the ability of TCA to activate

cultured macrophages (J744A.1 cell line) in vitro to become a source of reactive oxygen species was evaluated. Oxidative stress was evaluated by time- and concentration-dependent production of superoxide anion in response to TCA; resulting cytotoxicity, as indicated by effects on SOD activity and cell viability; and release of LDH by the cells into cultured media. Cells were exposed to TCA at 8–32 mM for 24–60 hours (pH of TCA solution was adjusted to pH 7.0 by NaOH).

Incubation with TCA caused a significant decrease in cell viability as assessed by trypan blue staining at all concentrations tested, although at 8 mM cell viability was only significantly reduced compared with controls at the 60-hour incubation. Reduced cell viability results correlated well with increased LDH activity in media. Twenty-four hour incubation with TCA did not cause increases in superoxide anion levels; however, incubations of 36 and 60 hours caused significant increases in superoxide anion levels at 16, 24, and 32 mM ($p < 0.05$). SOD activity was also affected by TCA treatment. Significant increases in SOD activity occurred at lower TCA concentrations (8–24 mM) compared with controls, but SOD activity at the highest concentration (32 mM) for 24–36 hours was similar to that of controls. Incubation of cells with 32 mM TCA for 60 hours resulted in 100% cell death. These results indicate that incubation with TCA at 8–32 mM for 24–60 hours induces macrophage activation, which resulted in cytotoxicity due to oxidative stress. Hassoun and Ray (2003) noted that, although TCA exposure concentrations were high, they were comparable to those used in animal studies (Austin et al., 1996; Larson and Bull, 1992; Sanchez and Bull, 1990; Bull et al., 1990).

The activation of phagocytic cells was supported by in vivo studies (Hassoun and Dey, 2008). Groups of male B6C3F_1 mice (8 animals/group) were administered 300 mg/kg TCA by gavage and sacrificed after 6 or 12 hours. Because obtaining pure Kupffer cells from liver is difficult, peritoneal lavage cells were isolated to examine the production of superoxide anion as the indication of phagocytic activation. Hepatic tissues were isolated to assay superoxide anion, lipid production, and DNA single-strand breaks (SSBs). At 6 hours, none of the biomarkers was induced by TCA. At 12 hours, the superoxide anion increased 62.5% in peritoneal lavage cells and 17.6% in hepatic tissue. Lipid peroxidation and DNA single-strand breaks increased 29.4 and 167%, respectively, in hepatic tissue.

The same group of scientists further conducted subacute (4 weeks) and subchronic (13 weeks) studies in mice to investigate the possible role of oxidative stress induced by macrophage activation in TCA hepatocarcinogenicity (Hassoun et al., 2010a; Hassoun et al., 2010b). Groups of male B6C3F_1 mice (7 animals/group) were administered 7.7, 77, 154, and 410 mg/kg-day TCA by gavage for 4 and 13 weeks. Hepatic tissues were examined for the production of superoxide anion, lipid peroxidation, and DNA single-strand breaks. Peritoneal lavage cells, a surrogate for liver Kupffer cells, were collected and tested for biomarkers of phagocytic activation, including superoxide anion, tumor necrosis factor-alpha (TNF-α), and myeloperoxidase. The results showed dose- and time-dependent increases in the production of

superoxide anion (increases of 30 and 167% at doses of 154 and 410 kg/mg-day at 4 weeks; 20, 100, 133, and 200% at doses of 7.7, 77, 154, and 410 mg/kg-day at 13 weeks), lipid peroxidation (increases of 67, 80, and 567% at doses of 77, 154, and 410 mg/kg-day at 4 weeks; 33, 400, 500, and 733% at doses of 7.7, 77, 154, and 410 mg/kg-day at 13 weeks), and DNA single-strand breaks (increases of 75, 125, and 300% at doses of 77, 154, and 410 mg/kg-day at 4 weeks; 125, 200, and 310% at doses of 77, 154, and 410 mg/kg-day) in the liver, indicating the production of reactive oxygen species and their associated effects on hepatic cellular components. Because the doses administered in these studies are comparable to the doses inducing hepatocarcinogenicity, but the treatment was a shorter period, the authors considered the significant increases of oxidative stress as initial events that may lead to later production of long-term effects.

These studies also showed that TCA induced increases of biomarkers of phagocytic activation. Interestingly, the biomarkers of phagocytyic activation in peritoneal lavage cells showed dose-dependent increases after 4 weeks of treatment, but not at 13 weeks of treatment. The production of superoxide anion at 4 weeks increased 56, 106, and 175% at doses of 77, 154, and 410 mg/kg-day, whereas at 13 weeks, the production increased significantly (60%) in the group treated at 77 mg/kg-day only. Similarly, the increase of myeloperoxidase activity was robust at 4 weeks (increased by 15-, 20-, 29, and 7.5-fold at doses of 7.7, 77, 154, and 410 mg/kg-day) and was modest at 13 weeks (increased by 2.5-, 5-, and 2-fold at doses of 7.7, 77, and 154 mg/kg-day). TNF-α, released by peritoneal lavage cells, increased dose-dependently at 4 weeks (increased by 2-, 3.2-, and 9-fold at 77, 154, and 410 mg/kg-day), whereas at 13 weeks, the increase (1.8-fold) was only found at 77 mg/kg-day. The findings, i.e., more increases in the biomarkers at the 4-week treatment period than at the 13-week treatment period, and in response to the lower doses to a greater extent than the higher doses (carcinogenic doses), indicate that TCA-induced phagocytic activation may be an initial adaptive response to protect against TCA-induced damage.

4.5.1.7. *Histochemical Characteristics of TCA-Induced Tumors*

Biomarkers of cell growth, differentiation, and metabolism in proliferative hepatocellular lesions promoted by TCA were investigated by Latendresse and Pereira (1997) to further determine differences in DCA and TCA carcinogenesis. Female B6C3F_1 mice were initiated with an i.p. injection of MNU at 15 days of age and treated with TCA in drinking water at a concentration of 20 mmol/L from age 49 days to 413 days. The authors did not provide a dose estimate, but the approximate dose is 784 mg/kg-day, based on the default drinking water intake value for female B6C3F_1 mice (U.S. EPA, 1988). At 413 days of age, the mice were sacrificed and liver tissues were examined histologically. A panel of histochemical markers was evaluated, including transforming growth factor-α (a growth factor that stimulates cell proliferation and is expressed in tumor cells), transforming growth factor-β (a growth factor that is inhibitory to hepatocyte proliferation), *c-jun* and *c-fos* (component proteins of the AP-1 transcription factor

that regulates expression of genes involved in DNA synthesis), *c-myc* (a regulator of gene transcription induced during cell proliferation), CYP2E1 (potentially involved in TCA metabolism) and CYP4A1 (induced by peroxisome proliferation signaling), and GST-π (a marker for certain tumor types).

TCA-induced foci of altered hepatocytes and tumors tended to be predominantly basophilic and stained variably for the histochemical markers examined. In TCA-treated mice, none of the markers stained positive in >50% of the cells/tumor, except *c-jun*, which was observed in >50% of cells from 9 of the 13 tumors evaluated. This profile of marker expression contrasts with the tumors from DCA-treated mice for which more than half of the examined tumors expressed transforming growth factor-α, *c-myc*, CYP2E1, CYP4A1, and GST-π in >50% of the cells. The contrasting histochemical-marker profiles induced by DCA and TCA provide evidence for a different MOA for these two haloacetic acids. In a recent study, Bull et al. (2002) (described in Section 4.2) observed that TCA-induced tumors were uniformly lacking in *c-jun* expression, but DCA-induced tumors often expressed *c-jun*, providing further evidence of a different MOA for TCA and DCA induction of liver tumors.

In the case of the TCA-promoted tumors, the minimal immunostaining for most markers (with the exception of *c-jun*) suggested that these proteins are not particularly important in TCA-induced tumor promotion. On the other hand, Latendresse and Pereira (1997) pointed out that the regional staining variability within the lesions for *c-jun* and *c-myc* proteins is consistent with localized clonal expansion and/or tumor progression. Non-tumor hepatocytes in TCA-treated animals were generally negative for transforming growth factor-β and GST-π staining and positive for CYP2E1 (centrilobular region) and CYP4A1 (panlobular region). CYP4A1 is an enzymatic marker for peroxisome proliferation, since its expression precedes peroxisomal response, and is coordinated with the transcription of the peroxisomal β-oxidation enzymes. The expression of CYP4A1 in normal hepatocytes in TCA-treated animals is consistent with TCA-induced peroxisome proliferation. However, CYP4A1 was not highly expressed in the tumor cells. This result suggests that, if PPARα agonism is involved in TCA-induced cancer, it is likely that the effect occurs earlier in the tumorigenic process than was evaluated in this study.

Pereira (1996) studied the characteristics of the lesions in female B6C3F_1 mice to evaluate differences in MOA of DCA and TCA. AHFs and tumors induced by DCA were reported as being predominantly eosinophilic. AHFs induced by TCA were equally distributed between basophilic and eosinophilic, whereas hepatic tumors induced by TCA were predominantly basophilic, including all observed hepatocellular carcinomas (n = 11), and lacked GST-π expression. These characteristics for TCA-induced tumors were also reported by Pereira et al. (1997) (described in Section 4.2). Tumors in control mice were also mostly basophilic or mixed basophilic and eosinophilic. Since comparable numbers of the foci of TCA-treated animals were basophilic and eosinophilic, the author suggested that the basophilic foci induced by TCA treatment may be more likely to progress to tumors. Based on differences in the shape

of the tumor dose-response curves and staining characteristics of tumors, Pereira (1996) concluded that DCA and TCA act through different mechanisms. The characteristics of the foci and tumors induced by TCA were described as being consistent with the predominant basophilic staining observed in tumors induced by peroxisome proliferators, suggesting that this pathway might be involved in the observed hepatocarcinogenicity of TCA.

Similarly, Bull et al. (1990) (described in Section 4.2) also presented evidence that the mechanisms of TCA and DCA carcinogenesis are different. In this study, DCA-treated mice showed marked cytomegaly, substantial glycogen accumulation, and necrosis of the liver. The dose-response relationship between proliferative liver lesions and DCA treatment followed a "hockey stick" pattern. In contrast, these effects were either minimal or absent in TCA-treated mice, and accumulation of lipofuscin (an indication of lipid peroxidation) was observed only in TCA-treated mice. In contrast to the dose-response curve for DCA, the dose-response curve for TCA and proliferative lesions was linear. Based on these data, the authors suggested that DCA may induce tumors by stimulating cell division through cytotoxicity, while TCA may induce tumors via lipid peroxidation.

4.5.2. Genotoxicity Studies

4.5.2.1. *In Vitro Studies*

TCA has been evaluated in a number of in vitro test systems (Table 4-12). The mutagenicity of TCA has been assessed in several variations of the Ames test. Among the *Salmonella typhimurium* strains that have been evaluated (i.e., TA98, TA100, TA104, TA1535, and RSJ100), the available studies have produced mixed results. Rapson et al. (1980) reported negative results for TCA in strain TA100 in the absence of metabolic activation (S9). Similarly, Nelson et al. (2001) reported negative results in strain TA104 with or without the addition of S9 or rat cecal homogenate. In an assay designed to investigate the genotoxicity of the volatile organic solvent tetrachloroethylene and its metabolites, TCA was also negative in *S. typhimurium* TA100 at up to cytotoxic concentrations of 600 ppm (without S9) and ~80 ppm (with S9). The assay utilized the vaporization technique, which permits the evaluation of volatile agents as vapors within a closed system (DeMarini et al., 1994). In this system, agar cultures on Petri dishes were inserted into a sealed Tedlar bag, and various amounts of the test compound were injected through a septum on the bag into the inverted top of the Petri dish. In a more recent study by Kargalioglu et al. (2002), TCA (0.1–100 mM) was not mutagenic when tested in TA98, TA100, and RSJ100 with or without S9.

Table 4-12. Summary of available genotoxicity data on TCA

Endpoint	Test system	Metabolic activation[a]	Concentration/dose	Results	Reference
In vitro studies					
Reverse mutations	*S. typhimurium* (TA98)	+/–	10–80 mM	Negative	Kargalioglu et al. (2002)
	S. typhimurium (TA100)	+/–	5–100/0.5–80 mM	Negative	Kargalioglu et al. (2002)
	S. typhimurium (TA100)	–	0.1–1,000 µg/plate	Negative	Rapson et al. (1980)
	S. typhimurium (TA104)	+/–	1 mg/mL	Negative	Nelson et al. (2001)
	S. typhimurium TA100 (TCA vapors were tested in a closed system)	+/–	0–600 mg/L	Negative	DeMarini et al. (1994)
	S. typhimurium TA100 (fluctuation assay)	+/–	+ S9: 3,000–7,500 µg/mL; –S9: 1,750–2,250 µg/mL	Positive, addition of S9 decreased mutagenicity. Toxic concentration: 10,000 µg/mL with S9; 2,500 µg/mL without S9	Giller et al. (1997)
	S. typhimurium RSJ100	+/–	0.1–100/5–80 mM	Negative	Kargalioglu et al. (2002)
Prophage induction	*Escherichia coli* microscreen assay	+/–	0–10 mg/mL	Negative	DeMarini et al. (1994)
SOS repair induction	*S. typhimurium* (TA1535)	+	58.5 µg/mL	Positive	Ono et al. (1991)
SOS chromotest	*E. coli* (PQ37)	+/–	10–10,000 µg/mL	Negative	Giller et al. (1997)
Forward mutations	Cultured mammalian cells (Chinese hamster ovary K1 cells) HGPRT assay	–	200–10,000 µM	Negative	Zhang et al. (2010)
	Cultured mammalian cells (L5178Y/TK+/– mouse lymphoma cells)	+/–	+S9: 0–3,400 µg/mL; –S9: 0–2,150 µg/mL	+ S9: weakly positive –S9: equivocal	Harrington-Brock et al. (1998)
Chromosomal aberrations	Mouse lymphoma cells	+/–	0–2,500 µg/mL	Weakly positive	Harrington-Brock et al. (1998)
Chromosomal damage	Cultured human peripheral lymphocytes	+/–	2,000 and 5,000 µg/mL	TCA as free acid: positive; neutralized TCA: negative	Mackay et al. (1995)
DNA strand breaks	Chinese hamster ovary AS52 cells	–	0.1–3 mM	Negative	Plewa et al. (2002)

Table 4-12. Summary of available genotoxicity data on TCA

Endpoint	Test system	Metabolic activation[a]	Concentration/dose	Results	Reference
In vivo studies					
Chromosomal aberration	Swiss mice, bone marrow	NA	0, 125, 250, or 500 mg/kg i.p.; 500 mg/kg orally (TCA not neutralized before administration)	Positive	Bhunya and Behera (1987)
Micronucleus induction	Swiss mice, bone marrow	NA	0, 125, 250, or 500 mg/kg-day i.p. (two doses) (TCA not neutralized before administration)	Positive	Bhunya and Behera (1987)
	C57BL mice, bone marrow evaluated	NA	337–1,300 mg/kg-day i.p. (25–80% of LD_{50}) (neutralized TCA was administered)	Negative	Mackay et al. (1995)
	Newt larvae (*Pleurodeles waltl*), erythrocytes	NA	40, 80, or 160 µg/mL (TCA not neutralized before treatment)	Weakly positive at 80 µg/mL	Giller et al. (1997)
DNA strand breaks (alkaline unwinding assay)	$B6C3F_1$ mice and Sprague-Dawley rats	NA	0.6 mmol/kg oral (TCA not neutralized)	Positive	Nelson and Bull (1988)
	$B6C3F_1$ mice	NA	500 mg/kg p.o. in one, two, or three daily doses (TCA neutralized)	Negative	Styles et al. (1991)
	$B6C3F_1$ mice and F344 rats	NA	Mice: 10 mmol/kg, oral Rats: 5 mmol/kg (TCA neutralized)	Negative	Chang et al. (1992)
Oxidative DNA damage (8-OHdG adducts)	$B6C3F_1$ mice	NA	300 mg/kg, single dose (TCA neutralized)	Positive	Austin et al. (1996)
	$B6C3F_1$ mice	NA	0–3 g/L TCA oral, for 21 or 71 d	Negative	Parrish et al. (1996)

[a]NA = not applicable; +/– = with and without S9.

In contrast, Giller et al. (1997) reported that TCA demonstrated mutagenic activity in an Ames fluctuation test in *S. typhimurium* TA100 in the absence of S9 at noncytotoxic concentrations ranging from 1,750 to 2,250 µg/mL. The addition of S9 decreased the mutagenic response, and genotoxic effects were observed at 3,000–7,500 µg/mL. Cytotoxic concentrations in the Ames fluctuation assay were 2,500 and 10,000 µg/mL without and with microsomal activation, respectively. Similarly, TCA induced a weak increase in "SOS DNA repair" (an inducible error-prone repair system) in *S. typhimurium* strain TA1535 in the presence of S9 (Ono et al., 1991).

In other bacterial test systems, TCA was negative in the SOS chromotest (which measures DNA damage and induction of the SOS repair system) in *Escherichia coli* PQ37, +/–

S9 (Giller et al., 1997). The test evaluated concentrations of TCA ranging from 10 to 10,000 µg/mL. Similarly, TCA was not genotoxic in the Microscreen prophage-induction assay in *E. coli* with TCA concentrations ranging from 0 to 10,000 µg/mL, with and without S9 activation (DeMarini et al., 1994).

TCA mutagenicity has also been tested in cultured mammalian cells. The potential of TCA to induce mutations in L5178Y/TK$^{+/-}$ –3.7.2C mouse lymphoma cells was examined by Harrington-Brock et al. (1998). The mouse lymphoma cells were incubated in culture medium treated with TCA concentrations up to 2,150 µg/mL without S9 metabolic activation and up to 3,400 µg/mL with S9. TCA was in free acid form when evaluated without S9. When it was evaluated with S9, the sodium salt form was used to maintain neutral pH. A positive response was defined as a doubling of the background mutant frequency[7]. In the absence of S9, TCA increased the mutant frequency by twofold or greater in one experiment only at concentrations resulting in ≤11% survival (≥2,000 µg/mL). In a repeat experiment, cultures producing the same level of cytotoxicity were not positive. Therefore, the authors considered the mutagenicity of TCA without activation to be equivocal. In the presence of S9, a doubling of mutant frequency was seen at concentrations of ≥2,250 µg/mL, including several concentrations with survival >10%. The response was considered by the study authors to be very weakly positive. Because of the weak mutagenic response, cytogenetic analysis was not conducted with TCA-treated cells. However, the study authors noted that the mutants included both large-colony and small-colony mutants. The small-colony mutants are indicative of chromosomal damage, which cannot be attributed to low pH, since the authors stated that no pH change was observed in the presence of S9. Harrington-Brock et al. (1998) noted that TCA (with S9 activation) was one of the least potent mutagens evaluated in this in vitro system and that the weight of evidence suggested that TCA was unlikely to be mutagenic. Other mutagenicity/genotoxicity studies support this conclusion (Zhang et al., 2010). It is noteworthy that in vitro mutagenicity tests mentioned in this section were not designed specifically to detect genotoxic endpoints induced by oxidative DNA damage.

Plewa et al. (2002) evaluated the induction of DNA strand breaks by TCA (0.1–3 mM) in Chinese hamster ovary cells. TCA was found to be not genotoxic in this assay. Mackay et al. (1995) investigated the ability of TCA to induce chromosomal DNA damage in an in vitro assay by using cultured human lymphocytes. Treatment with TCA as free acid, with and without metabolic activation, induced chromosome damage in cultured human peripheral lymphocytes

[7]As an outcome of the Mouse Lymphoma Assay Workgroup of the International Workshop on Genotoxicity Testing (Moore et al., 2006), the criteria for calling a response positive in the mouse lymphoma assay has changed. A twofold response is no longer considered to be positive. Rather, there is a requirement that the induced mutant frequency (i.e., the response above the background mutant frequency) should exceed a global factor of 90×10^{-6}. Application of the new criterion does not change the overall determination for TCA in Harrington-Brock et al. (1998).

only at concentrations (2,000 and 3,500 µg/mL) that significantly reduced the pH of the medium. Neutralized TCA had no effect in this assay even at a cytotoxic concentration of 5,000 µg/mL, suggesting that reduced pH was responsible for the TCA-induced clastogenicity in this study. To further evaluate the role of pH changes in the induction of chromosome damage, isolated liver-cell nuclei from $B6C3F_1$ mice were suspended in a buffer at various pH levels and were stained with chromatin-reactive (fluorescein isothiocyanate) and DNA-reactive (propidium iodide) fluorescent dyes. Chromatin staining intensity decreased with decreasing pH, suggesting that pH changes alone can alter chromatin conformation. Thus, Mackay et al. (1995) concluded that TCA-induced pH changes were likely to be responsible for the chromosome damage induced by un-neutralized TCA.

4.5.2.2. *In Vivo Studies*

TCA has been tested for genotoxicity in several in vivo test systems (Table 4-12). Bhunya and Behera (1987) treated Swiss mice with 0, 125, 250, or 500 mg/kg unneutralized TCA i.p. (the highest dose, 500 mg/kg, was also administered orally for the chromosome aberration assay). Three different cytogenetic assays—bone marrow chromosomal aberrations and micronucleus and sperm-head abnormalities—were carried out. TCA induced chromosomal aberrations and micronuclei in bone-marrow and altered sperm morphology of treated mice. In a later study, Mackay et al. (1995) utilized the study design of Bhunya and Behera (1987), including an extra sampling time at 24 hours to investigate the ability of TCA to induce chromosomal DNA damage in the in vivo bone-marrow micronucleus assay in mice. C57BL mice were given neutralized TCA at i.p. doses of 0, 337, 675, or 1,080 mg/kg-day for males and 0, 405, 810, or 1,300 mg/kg-day for females for 2 consecutive days, and bone-marrow samples were collected 6 and 24 hours after the last dose. The administered doses represented 25, 50, and 80% of the LD_{50}. No significant treatment-related increase in micronucleated polychromatic erythrocytes was observed. Mackay et al. (1995) concluded that the positive results previously observed by Bhunya and Behera (1987) may have been due to a non-genotoxic mechanism, possibly caused by physicochemically induced stress resulting from i.p. pH changes. In another study, unneutralized TCA induced a small increase in the frequency of micronucleated erythrocytes at 80 µg/mL in a newt (*Pleurodeles waltl* larvae) micronucleus test (Giller et al., 1997).

Studies on the ability of TCA to induce single-strand breaks have produced mixed results (Chang et al., 1992; Styles et al., 1991; Nelson and Bull, 1988). Nelson and Bull (1988) evaluated the ability of TCA to induce single-strand breaks in vivo in Sprague-Dawley rats and $B6C3F_1$ mice. Single oral doses of unneutralized TCA in 1% Tween were administered to three groups of three animals, with an additional group as a vehicle control. Animals were sacrificed after 4 hours, and 10% liver suspensions were analyzed for single-strand breaks by the alkaline unwinding assay. Dose-dependent increases in single-strand breaks were induced in both rats

and mice, with mice being more susceptible than rats. The lowest doses of TCA that produced significant single-strand breaks were 0.6 mmol/kg (98 mg/kg) in rats and 0.006 mmol/kg (0.98 mg/kg) in mice.

Styles et al. (1991) tested TCA for its ability to induce strand breaks in male B6C3F_1 mice in the presence and absence of liver growth induction. The test animals were given one, two, or three daily doses of neutralized TCA (500 mg/kg) by gavage and killed 1 hour after the final dose. Additional mice were given a single 500 mg/kg gavage dose and sacrificed 24 hours after treatment. Liver nuclei DNA were isolated, and the induction of single-strand breaks was evaluated by using the alkaline unwinding assay. Exposure to TCA did not induce strand breaks under the conditions tested in this assay. In a study by Chang et al. (1992), administration of single oral doses of neutralized TCA (1–10 mmol/kg) to B6C3F_1 mice did not induce DNA strand breaks in a dose-related manner as determined by the alkaline unwinding assay. No DNA damage (as strand breakage) was detected in F344 rats administered by gavage up to 5 mmol/kg (817 mg/kg) neutralized TCA. In evaluating these studies, the reason for the inconsistent results among studies may be related to whether TCA was administered as sodium salt (neutralized) or as free acid (not neutralized). The different results did not appear to be related to the method chosen to measure strand breakage. Although Chang et al. (1992) used a different unwinding assay, Nelson and Bull (1988) and Styles et al. (1991) employed the same unwinding assay and obtained contrasting results.

Two related studies were conducted to evaluate the relationship between TCA-induced lipid peroxidation and oxidative DNA damage (Parrish et al., 1996; Austin et al., 1996) (described in detail in Section 4.2.1.1). In the acute study by Austin et al. (1996), male B6C3F_1 mice (six/group) were treated with a single oral dose of TCA (0, 30, 100, or 300 mg/kg), and 8-OHdG adducts were measured in liver DNA. A significant increase of about one-third in 8-OHdG levels was observed in the 300 mg/kg group at 8–10 hours post-dosing. Parrish et al. (1996) expanded on this study by evaluating TCA-induced oxidative DNA damage following repeated dosing. Male B6C3F_1 mice (six/group) were exposed to 0, 100, 500, or 2,000 mg/L TCA in drinking water for either 3 or 10 weeks (approximate doses of 0, 25, 125, or 500 mg/kg-day). The levels of 8-OHdG were unchanged at both time periods. Thus, oxidative damage to genomic DNA as measured by 8-OHdG adducts did not occur with prolonged TCA treatment.

In summary, these data collectively provide limited evidence regarding the genotoxicity of TCA. No mutagenicity was reported in *S. typhimurium* strain TA100 in the absence of metabolic activation (Rapson et al., 1980) or in an alternative protocol using a closed system (DeMarini et al., 1994), but a mutagenic response was induced in this same strain in the Ames fluctuation test reported by Giller et al. (1997). On the other hand, mutagenicity in mouse lymphoma cells was only induced at cytotoxic concentrations (Harrington-Brock et al., 1998). Measures of DNA-repair responses in bacterial systems have been similarly inconclusive, with induction of DNA repair reported in *S. typhimurium* by Ono et al. (1991) but not by Giller et al.

(1997) in *E. coli*. Although positive results were reported for unneutralized TCA in three in vivo cytogenetic assays by Bhunya and Behera (1987), later in vitro studies by Mackay et al. (1995), using neutralized TCA, reported negative results, suggesting that TCA-induced clastogenicity may occur secondary to pH changes. TCA-induced hepatic DNA strand breaks and chromosome damage have been observed in two studies (Giller et al., 1997; Nelson and Bull, 1988) and were suggested by the results of Harrington-Brock et al. (1998). However, these effects have not been uniformly reported (Chang et al., 1992; Styles et al., 1991) and may be related to low pH when TCA was not neutralized. TCA induced oxidative DNA damage in the livers of mice following a single dose (Austin et al., 1996) but not following repeated dosing over 3 or 10 weeks (Parrish et al., 1996).

4.6. SYNTHESIS OF MAJOR NONCANCER EFFECTS

No epidemiologic data that evaluate TCA alone for noncancer effects in humans are available. The experimental database for animals includes acute, short-term, subchronic, and chronic studies conducted in rats and mice. A major limitation of the experimental database is that few studies have examined toxic effects in organs other than the liver. Based on the currently available data, oral exposure of rats and mice to TCA induces systemic, noncancer effects in animals that can be grouped into three general categories: metabolic alterations, liver toxicity, and developmental toxicity. These effects are described below.

4.6.1. Oral

4.6.1.1. *Metabolic Alterations*

Chronic exposure to TCA results in accumulation of lipofuscin in areas surrounding hepatoproliferative lesions in the liver of mice (Bull et al., 1990). Lipofuscin is a complex of lipid-protein substances derived from lipid peroxidation of membranes and hence provides evidence of lipid peroxidation initiated by a free radical species generated from its metabolism. Alternatively, Bull et al. (1990) suggested that accumulation of lipofuscin could be related to the ability of TCA to induce peroxisomal oxidative enzymes. TCA also demonstrated its ability to induce lipid peroxidation by the formation of TBARS in the liver of rats and mice when administered acutely (Austin et al., 1996; Larson and Bull, 1992). This lipid peroxidation response was reduced with pretreatment of TCA for 14 days (Austin et al., 1995). Decreased liver triglyceride and cholesterol levels were observed in Wistar rats treated with 25 ppm TCA in drinking water for 10 weeks, while serum triglyceride level increased (Acharya et al., 1995).

Exposure to TCA has been reported to alter liver glycogen content secondary to alterations of lipid and carbohydrate homeostasis (Acharya et al., 1995; Sanchez and Bull, 1990; Bull et al., 1990). TCA significantly increased glycogen content in the livers of rats exposed to 25 mg/L in the drinking water (neutralization not reported) for 10 weeks, as assessed by analysis of liver homogenates (Acharya et al., 1995). Bull et al. (1990) reported that "TCA-treated

animals displayed less evidence of glycogen accumulation [than DCA-treated animals] and it was more prominent in periportal than centrilobular portions of the liver acinus" as assessed by periodic acid-Schiff's reagent staining in a 52-week study of mice exposed to 1 or 2 g/L in drinking water. In a study where mice were exposed to 0.3, 1.0, or 2.0 g/L TCA in neutralized drinking water for 14 days, Sanchez and Bull (1990) reported that glycogen, as detected by periodic acid-Schiff's reagent-staining in hepatic sections from animals receiving the highest concentrations of TCA, "displayed a much less intense staining [than DCA-treated mice] that was confined to periportal areas." In contrast, Kato-Weinstein et al. (2001) reported significantly decreased glycogen content, especially in the central lobular region in mice treated with 3.0 g/L in neutralized drinking water for 4 or 8 weeks and in mice treated with 0.3, 1.0, or 3.0 g/L for 12 weeks, as measured chemically in liver preparations and verified histologically by periodic acid-Schiff's reagent staining. The reason for the discrepancy is unknown, but does not appear to be related to differences in study duration or administered dose.

4.6.1.2. *Liver Toxicity*

The liver has consistently been identified as a target organ for TCA toxicity in short-term (Sanchez and Bull, 1990; DeAngelo et al., 1989; Goldsworthy and Popp, 1987) and longer-term (Bull et al., 1990; Mather et al., 1990; Bhat et al., 1990) studies. Collective analysis of the available studies reveals a common spectrum of liver effects that includes changes in lipid and carbohydrate homeostasis, increased liver weight, increased hepatic DNA labeling, and hepatocyte necrosis. The ability of TCA to induce peroxisome proliferation has been a primary endpoint evaluated (DeAngelo et al., 2008; DeAngelo et al., 1997; Parrish et al., 1996).

TCA induced peroxisome proliferation (in the absence of effects on liver weight) in $B6C3F_1$ mice exposed for 3 or 10 weeks to drinking water concentrations as low as 0.5 g/L (approximately 125 mg/kg-day) (Parrish et al., 1996). The NOAEL in this study was 25 mg/kg-day. In rats exposed to TCA for up to 104 weeks (DeAngelo et al., 1997), peroxisome proliferation was observed at 364 mg/kg-day but not at 32.5 mg/kg-day. Peroxisome proliferation has also been demonstrated in a number of other short-term and long-term exposure studies in both rats and mice (DeAngelo et al., 2008; Austin et al., 1995; Mather et al., 1990; DeAngelo et al., 1989; Parnell et al., 1988; Goldsworthy and Popp, 1987). Increased liver weight and significant increases in hepatocyte proliferation have been observed in short-term studies in mice at doses as low as 100 mg/kg-day (Dees and Travis, 1994), but no increase in hepatocyte proliferation was noted in rats given TCA at up to 364 mg/kg-day (DeAngelo et al., 1997). More clearly adverse liver toxicity endpoints, including increased serum levels of liver enzymes (indicating leakage from cells) or histopathologic evidence of necrosis, have been reported in rats but generally only at high doses. For example, increased hepatocyte necrosis was observed at a dose of 364 mg/kg-day in a rat chronic drinking water study (DeAngelo et al.,

1997). Increased hepatic necrosis was observed in male B6C3F$_1$ mice treated with ≥68 mg/kg-day TCA in a chronic drinking water study (DeAngelo et al., 2008).

Rats are less sensitive than mice to the peroxisome-proliferating effects of TCA. For example, PCO activity was measured by DeAngelo et al. (1989) (described in Section 4.2) in four strains of male mice and three strains of male rats exposed to TCA in drinking water for 14 days. PCO activity was increased by 648–2,500% over controls in the mouse strains compared with increases of up to 138% over controls in rats at the same drinking water concentrations (31 mM), demonstrating the greater response in exposed mice. The relevance of TCA effects associated with peroxisome proliferation to human health is presently uncertain. Further information on this issue is presented in Section 4.7.3.1.1.4.

4.6.1.3. *Developmental Toxicity*

Six published studies have addressed the developmental toxicity of TCA in rats exposed via the oral route. Some of these studies were conducted with excessively high TCA concentrations (Singh, 2006, 2005b, a) or with a single dose of TCA (Fisher et al., 2001; Johnson et al., 1998), and therefore provide limited information useful for informing the dose-response relationship for TCA in the low-dose region.

Nevertheless, available data indicate that TCA is a developmental toxicant in the pregnant rat at doses of ≥300 mg/kg-day. TCA decreased fetal weight and length and increased cardiovascular malformations at 330 mg/kg-day in Long-Evans rats (Smith et al., 1989). In a study focused on cardiac teratogenicity, Fisher et al. (2001) observed significantly reduced fetal body weights on GD 21 following treatment of Sprague-Dawley rats with 300 mg/kg-day of TCA. In contrast to the Smith et al. (1989) study, Fisher et al. (2001) did not observe treatment-related effects on the incidence of cardiac malformations. The reason for the inconsistent findings is unknown. Smith et al. (1989) considered levocardia to be an ill-defined malformation and possibly of trivial appearance as found in Bouin's fixed slices. Thus, the finding of possible cardiac malformations is of uncertain biological relevance. Overall, the available data do not permit identification of NOAEL values for the developmental or maternal toxicity of TCA, since in each study, adverse effects were observed at the lowest or only dose tested.

Mouse and rat whole embryo cultures have been used to assess the potential for developmental toxicity of TCA (Hunter et al., 1996; Saillenfait et al., 1995). TCA induces a variety of morphologic changes in mouse and rat whole embryo cultures, supporting the appearance of soft-tissue malformations observed in vivo at maternally toxic doses. The xenopus assay system (frog embryo teratogenesis assay) (Fort et al., 1993) provided positive results for developmental toxicity of TCA. Because of the high concentrations used in these assays, however, in vitro test systems are limited in their utility to predict adverse developmental effects and associated toxic potencies in intact organisms.

4.6.2. Inhalation

No inhalation studies of TCA are available.

4.6.3. Mode-of-Action Information – Non-Cancer

Target organs for the toxicity of TCA in humans have not been specifically identified. The experimental database for MOA in animals is limited to studies in rats and mice, and few studies have evaluated events in organs other than the liver. Based on currently available data, systemic, noncancer effects induced in animals can be grouped into three general categories: metabolic alterations, liver toxicity, and developmental toxicity.

4.6.3.1. *Metabolic Alterations*

Exposure to TCA causes disturbances in lipid homeostasis. TCA is a PPARα agonist. An associated event with the activation of PPARα receptor by TCA is proliferation of peroxisomes [reviewed in Bull (2000); Austin et al. (1996; 1995); Parrish et al. (1996)]. Peroxisomes contain hydrogen peroxide and fatty acid oxidation systems important in lipid metabolism. Activation of the peroxisome proliferation pathway induces the transcription of genes that encode enzymes responsible for fatty acid metabolism (Lapinskas, 1999), suggesting that lipid homeostasis might be affected through this mechanism. Alternatively, metabolism of TCA might generate free radical species that initiate lipid peroxidation (Bull et al., 1990). The appearance of DCA in the urine of TCA-exposed animals provided evidence for a free radical-generating, reductive dechlorination metabolic pathway (Larson and Bull, 1992).

TCA has been reported to induce glycogen accumulation in rats (Acharya et al., 1995) and possibly in mice (Sanchez and Bull, 1990; Bull et al., 1990). The data are not fully consistent, however, since Kato-Weinstein et al. (2001) observed decreased glycogen content in mice treated with TCA. Although TCA-induced changes in glycogen storage have not been well studied, examination of DCA effects on the same endpoint can be informative. DCA-induced glycogen accumulation is potentially pathological, because chronic treatment might result in glycogen stores becoming difficult to mobilize (Kato-Weinstein et al., 1998). The mechanism for glycogen accumulation is not known, but it may be associated with inhibition of glycogenolysis, since the observed effects resemble those observed in glycogen storage disease, an inherited deficiency or alteration in any one of the enzymes involved in glycogen metabolism. In this regard, the enzymatic basis for increased hepatic glycogen accumulation was studied by Kato-Weinstein et al. (1998). TCA was not evaluated as part of this study. However, TCA might act similarly to DCA, since both compounds induce glycogen accumulation (Acharya et al., 1995), although the degree of accumulation is less with TCA. Kato-Weinstein et al. (1998) reported that DCA concentrations that induced glycogen accumulation did not alter glycogen synthase activity and had no effect on glycogen phosphorylase (which degrades glycogen) or the activity of glucose-6-phosphatase (which converts glucose-6-phosphate to glucose) from liver

homogenates. In an in vitro study using purified enzyme, DCA did not alter the activity of glycogen synthase kinase-3β (which down-regulates glycogen synthase activity and up-regulates glycogen phosphorylase activity). Based on the absence of an effect on enzymes that regulate glycogen synthesis rates and decreased glycogen degradation observed in fasted mice, the authors concluded that glycogen accumulation was related to a decrease in degradation rate. There are currently no data on TCA to show that it acts via a similar MOA.

4.6.3.2. *Liver Toxicity*

Increased liver weight is typically observed concurrently with or at lower doses than other endpoints following oral dosing with TCA. Changes in liver weight can reflect increases in cell size, cell number, or both. TCA appears to induce both hepatocellular enlargement (Acharya et al., 1997; Mather et al., 1990) and cell proliferation as assessed by differences in hepatocyte DNA labeling (Dees and Travis, 1994; Sanchez and Bull, 1990). Increased cell proliferation in normal cells may, however, be transient, with no change or even decreased growth observed after chronic exposure (DeAngelo et al., 1997; Pereira, 1996). Both cytomegaly and increased cell proliferation might be explained by TCA-induced peroxisome proliferation (Lapinskas, 1999). There is little evidence that increased cell proliferation is secondary to hepatocyte cytotoxicity, as previously discussed in Section 4.5.1.2, although TCA can induce hepatic necrosis at high doses (DeAngelo et al., 1997).

Oxidative stress may also contribute to the toxicity of TCA in the liver. Several studies have shown that TCA induces oxidative-stress responses (e.g., lipid peroxidation and oxidative DNA damage) in the liver in single-dose or short-term studies (Parrish et al., 1996; Austin et al., 1996; Austin et al., 1995; Larson and Bull, 1992). Oxidative stress may contribute to the short-term toxicity of TCA; however, the contribution of oxidative stress to the chronic toxicity of TCA is uncertain because the response is transient and is not observed in longer-term studies (Parrish et al., 1996).

4.6.3.3. *Developmental Toxicity*

The mechanisms for developmental toxicity are unknown. However, TCA was found to accumulate in amniotic fluid when pregnant rodents were exposed to TCE or tetrachloroethylene (Ghantous et al., 1986b). Thus, TCA may also have accumulated in amniotic fluid when pregnant rodents were exposed to this chemical, because most of the parent compound remains unmetabolized. TCA accumulated in the amniotic fluid may be transported through fetal skin or swallowed then excreted by the fetus. Singh (2006) suggested that TCA in the amniotic fluid may be circulated several times, which would contribute to the long-term retention in the fetus. Since TCA is a strong acid with high protein binding and was reported to cause placental lesion (Ghantous et al., 1986b), developmental toxicities may be related to anoxia resulting from toxic effect on the placenta and from apoptosis resulting from oxidative stress, as observed in studies

by Singh (2006, 2005b, a). On the other hand, Selmin et al. (2008) reported that TCA disrupted the expression of genes involved in processes important during embryonic development. A microarray study conducted on P19 mouse embryonic carcinoma cells treated with TCA provided evidence that TCA altered the expressions of several genes implicated in calcium regulation and heart development (Selmin et al., 2008). Real-time polymerase chain reaction analysis confirmed the effect of TCA on genes involved in calcium regulation (CamK and RyR), glucose/insulin signaling (Dok3), and ubiquitin-mediated cell proliferation (Ubec2).

4.7. EVALUATION OF CARCINOGENICITY

4.7.1. Summary of Overall Weight of Evidence

Under the *Guidelines for Carcinogen Risk Assessment* (Cancer Guidelines) (U.S. EPA, 2005c), there is *suggestive evidence of carcinogenic potential* for TCA based on significantly increased incidences of liver tumors in male B6C3F$_1$ mice exposed via drinking water for 52–104 weeks (DeAngelo et al., 2008; Bull et al., 2002; Bull et al., 1990; Herren-Freund et al., 1987) and female B6C3F$_1$ mice exposed for 51 or 82 weeks (Pereira, 1996), and lack of treatment-related tumors in a study of male F344/N rats following lifetime exposure in drinking water (DeAngelo et al., 1997).

There are no studies of TCA in humans. In animals, the scope of carcinogenicity testing has been limited. The only lifetime studies (104 weeks) are of TCA administered in drinking water to male F344/N rats and to male B6C3F$_1$ mice. TCA did not induce tumors at any site in male rats (DeAngelo et al., 1997), but in mice TCA induced a statistically significant increase in hepatocellular adenomas and carcinomas at the high dose (0.5 g/L in drinking water) (DeAngelo et al., 2008).

There are several less-than-lifetime studies (51–82 weeks) of TCA-induced liver cancer following administration in drinking water to male and female B6C3F$_1$ mice. In all studies in male mice, TCA induced hepatocellular carcinomas (DeAngelo et al., 2008; Bull et al., 2002; Bull et al., 1990). It is noteworthy that the high background rate of liver tumors observed in male B6C3F$_1$ mice at 104 weeks was not reported in these less-than-lifetime studies. Bull et al. (1990) reported no liver tumors in female mice in a 52-week study. However, this result is outweighed by an 82-week study (Pereira, 1996) that found no tumors in female mice at a comparable dose administered in drinking water for 51 weeks but reported hepatocellular carcinomas at a higher dose at 51 weeks and at the highest two doses by 82 weeks.

Taking the results of these studies together, TCA: 1) has consistently tested positive in males in one strain of mouse in one lifetime and several less-than-lifetime studies; 2) has not been tested in lifetime studies in females, and was shown to induce tumors in one less-than-lifetime study but did not produce tumors in another; and 3) has tested negative in one lifetime study that was conducted in male rats only. Therefore, there are consistent observations of tumor

formation in male mice, however, the overall weight of evidence is tempered due to a lack of studies on female animals in general and the negative results in male rats.

EPA's Cancer Guidelines (U.S. EPA, 2005c) emphasize the importance of weighing all of the evidence in reaching conclusions about the human carcinogenic potential of agents. Each cancer descriptor may be applicable to a variety of potential data sets and represent points along a continuum of evidence. The available tumorigenic evidence for TCA could be considered a borderline case between two descriptors—*likely to be carcinogenic to humans* and *suggestive evidence of carcinogenic potential*. For example, TCA has tested positive in more than one sex of $B6C3F_1$ mice, which minimally corresponds to one of the examples provided in EPA's Cancer Guidelines (U.S. EPA, 2005c) for the descriptor *likely to be carcinogenic to humans*. The example states that supporting data for this descriptor may include "an agent that has tested positive in animal experiments in more than one species, sex, strain, site, or exposure route, with or without evidence of carcinogenicity in humans."

In evaluating this borderline case, EPA considered Section 2.5 of the Cancer Guidelines which states that the descriptor *likely to be carcinogenic to humans* is appropriate when "the weight of evidence is adequate to demonstrate carcinogenic potential to humans but does not reach the weight of evidence for the descriptor *carcinogenic to humans*." The Cancer Guidelines further state that the descriptor *suggestive evidence of carcinogenic potential* is appropriate when "the weight of evidence is suggestive of carcinogenicity, a concern for potential carcinogenic effects is raised, but the data are not judged sufficient for a stronger conclusion."

Thus, although either descriptor is plausible and the consistent positive evidence in $B6C3F_1$ mice raises a concern for carcinogenic effects in humans, this assessment attaches greater weight to the lack of evidence in other strains or species than to the replication of positive results in this one strain. Accordingly, this assessment concludes that there is *suggestive evidence of carcinogenic potential* for TCA.

In choosing a cancer descriptor, consideration was also given to the nature of the only tumor type induced by TCA, i.e., liver tumors (hepatocellular adenomas and carcinomas). The mouse, and in particular the $B6C3F_1$ mouse, is relatively susceptible to liver tumors, and the background incidence of this tumor is generally high. For these reasons, use of mouse liver tumor data in risk assessment has been a subject of controversy (King-Herbert and Thayer, 2006). The less-than-lifetime drinking water bioassays of TCA in the $B6C3F_1$ mouse (DeAngelo et al., 2008; Bull et al., 2002; Bull et al., 1990; Pereira, 1996) reported relatively low incidences of liver adenomas and carcinomas in control animals (ranging from 0 to 13%), thereby minimizing the possible confounding of compound-related liver tumors. In the only lifetime (104-week) study in the male $B6C3F_1$ mouse (females were not tested), however, the incidence of spontaneous liver tumors was 55%, an incidence that was higher than the liver tumor incidence in the low-dose group in this study.

EPA's Cancer Guidelines (U.S. EPA, 2005c) indicate that for tumors occurring at a site other than the initial point of contact, the weight of evidence for carcinogenic potential may apply to all routes of exposure that have not been adequately tested, unless there is convincing toxicokinetic data that absorption does not occur by other routes. For TCA, systemic tumors were observed in mice following oral exposure, but carcinogenicity studies of TCA by the inhalation or dermal routes in humans or animals have not been conducted. There is some evidence of rapid absorption of TCA through the skin, but no studies of uptake following inhalation exposure (see Section 3.1). Because TCA is highly soluble in water, it is reasonable to assume that TCA can be absorbed and taken up into the blood via the inhalation route. Moreover, the drinking water studies demonstrate that TCA acts systemically rather than only at the site of first contact. In the absence of information to indicate otherwise, there is *suggestive evidence of carcinogenic potential* for TCA by all routes of exposure.

In view of widespread human exposure to TCA as a water chlorination byproduct and as a metabolite of several commonly used chlorinated solvents, there is a need for further testing of TCA in experimental models other than the $B6C3F_1$ mouse.

As discussed in more detail in Section 4.7.3, the MOA for TCA-induced liver carcinogenesis has not been established. The available data collectively provide limited evidence of genotoxic potential of TCA. In mouse liver tumor promotion assays, also conducted exclusively in the $B6C3F_1$ strain, TCA induced liver tumors with and without pre-treatment with an initiator (see Table 4-3). GGT-positive foci (closely linked to the subsequent development of tumors) were observed in DEN-initiated male Sprague-Dawley rats following promotion with TCA (Parnell et al., 1988); however, the attribution of this effect to TCA is confounded by the fact that the treated rats also received a partial hepatectomy, which can itself act as a promoter.

Tumor induction appears to include perturbation of cell growth, both through growth inhibition of normal cells and proliferation of selected cell populations. Specific mechanisms of altered growth control that have been investigated for TCA include activation of the PPARα pathway, global DNA hypomethylation, reduced intercellular communication, and oxidative stress. Of these, PPARα agonism has been advanced as the most likely MOA contributing to the development of liver tumors. However, significant gaps in the understanding of the hypothesized PPARα MOA exist. Specifically, Ito et al. (2007) showed that the peroxisome proliferator, DEHP, induced liver tumors in PPARα-null mice. Yang et al. (2007) demonstrated that transgenic mice with PPARα activation constitutively in hepatocytes did not development liver tumor. These data indicated that PPARα agonism is neither necessary nor sufficient in hepatocarcinogenesis. As such, the formation of liver tumors cannot be sufficiently accounted for by the proposed PPARα MOA and the existence of other contributing MOA(s) is assumed.

4.7.2. Synthesis of Human, Animal, and Other Supporting Evidence

There are no epidemiologic studies of TCA carcinogenicity in humans. Most of the human health data for chlorinated acetic acids concern components of complex mixtures of water disinfectant byproducts. These complex mixtures of disinfectant byproducts have been associated with increased potential for bladder, rectal, and colon cancer in humans [reviewed by Boorman et al. (1999) and Mills et al. (1998)].

The experimental database for carcinogenicity of TCA consists of studies in rats and mice. Studies in mice indicate that TCA is a complete carcinogen that significantly increased the incidence of liver tumors in male B6C3F$_1$ mice exposed via drinking water for 52–104 weeks (DeAngelo et al., 2008; Bull et al., 2002; Bull et al., 1990; Herren-Freund et al., 1987) and female B6C3F$_1$ mice exposed for 51 or 82 weeks (Pereira, 1996). Incidence of tumors increased with increasing TCA concentrations (DeAngelo et al., 2008; Bull et al., 2002; Pereira, 1996; Bull et al., 1990). Results from the less-than-lifetime studies were obtained under conditions where the background incidence of tumors in control animals was generally low. The development of tumors in animals exposed to TCA progressed rapidly, as evident from the observation of significant numbers of tumors in less-than-lifetime studies of 82 weeks or less. Positive evidence for tumor promotion by TCA (following exposure to known tumor initiators) has been reported for liver tumors in B6C3F$_1$ mice (Bull et al., 2004; Pereira et al., 2001; Pereira et al., 1997; Pereira and Phelps, 1996; Herren-Freund et al., 1987) and for GGT-positive foci in livers of partially hepatectomized Sprague-Dawley rats (Parnell et al., 1988).

In contrast to the results observed for mice, treatment-related tumors were not observed in a study of male F344/N rats exposed to TCA via drinking water for 104 weeks (DeAngelo et al., 1997). The carcinogenicity of TCA has not been evaluated in female rats or in other species of experimental animals. However, treatment of primary cultures of male Long-Evans rat hepatocytes with 0.01–1.0 mM TCA for 10–40 hours did not induce proliferation of the cultured hepatocytes (Walgren et al., 2005).

A significant limitation of the experimental database for carcinogenicity is the limited number of studies that included microscopic examination of a comprehensive set of organs in addition to the liver. The most complete evaluations were conducted by DeAngelo et al. (2008), who examined a comprehensive set of organs in B6C3F$_1$ mice from the high-dose and control groups. The kidney, liver, spleen, and testes were examined in all dose groups. DeAngelo et al. (1997) also examined a comprehensive set of organs in F344 rats receiving the highest dose of TCA and selected tissues (kidney, liver, spleen, testes) in the remainder of the treatment groups.

Evidence for genotoxic activity of TCA is inconclusive. No mutagenicity was reported in *S. typhimurium* strain TA100 in the absence of metabolic activation (Rapson et al., 1980) or in an alternative protocol using a closed system (DeMarini et al., 1994), but a mutagenic response was induced in this same strain in the Ames fluctuation test reported by Giller et al. (1997). Mutagenicity in mouse lymphoma cells was only induced at cytotoxic concentrations

(Harrington-Brock et al., 1998). Measures of DNA-repair responses in bacterial systems are similarly inconclusive, with induction of DNA repair reported in *S. typhimurium* (Ono et al., 1991), but not in *E. coli* (Giller et al., 1997). Although positive results were reported for unneutralized TCA in three in vivo cytogenetic assays by Bhunya and Behera (1987), later in vitro studies by Mackay et al. (1995), using neutralized TCA, reported negative results, suggesting that TCA-induced clastogenicity may occur secondary to pH changes. Some evidence for TCA-induction of hepatic DNA strand breaks and chromosome damage has been reported (Harrington-Brock et al., 1998; Giller et al., 1997; Nelson and Bull, 1988); however, these effects have not been uniformly reported (Chang et al., 1992; Styles et al., 1991) and may be related to low pH when TCA was not neutralized. TCA induced oxidative DNA damage in the livers of mice following a single dose (Austin et al., 1996), but not following repeated dosing over 3 or 10 weeks (Parrish et al., 1996).

4.7.3. Mode-of-Action Information - Cancer

Multiple studies have demonstrated that exposure to TCA in drinking water for periods of 52–104 weeks can produce an increased incidence of liver tumors in B6C3F$_1$ mice (DeAngelo et al., 2008; Bull et al., 2002; Pereira, 1996; Bull et al., 1990; Herren-Freund et al., 1987). In the only available chronic study in rats, TCA did not increase tumor incidence in male F344 rats exposed to TCA for up to 102 weeks (DeAngelo et al., 1997). The events leading to the development of liver cancer in mice exposed to TCA have not been fully characterized, although several MOAs have been postulated.

Analysis of the available MOA information reveals that the cancer MOA for TCA is complex, and more than one MOA may be operative in the development of mouse liver tumors. This section will discuss the evidentiary support for several hypothesized modes of action for liver carcinogenicity (including peroxisome proliferation, as well as several additional proposed hypotheses and key events with limited evidence or inadequate experimental support), following the framework outlined in the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005c, b).[8]

Specific cancer MOAs for TCA addressed in the following sections include PPARα-agonism (Section 4.7.3.1), Kupffer cell activation and subsequent release of cytokines and oxidants (Section 4.7.3.2), DNA hypomethylation (Section 4.7.3.3), decreased intercellular

[8] As recently reviewed (Guyton et al., 2008) the approach to evaluating mode of action information described in EPA's *Guidelines for Carcinogen Risk Assessment* (2005c) considers the issue of human relevance of a hypothesized mode of action in the context of hazard evaluation. This excludes, for example, consideration of toxicokinetic differences across species; specifically, the Cancer Guidelines state, "the toxicokinetic processes that lead to formation or distribution of the active agent to the target tissue are considered in estimating dose but are not part of the mode of action." In addition, information suggesting quantitative differences in the occurrence of a key event between test species and humans are noted for consideration in the dose-response assessment, but is not considered in human relevance determination.

communication (Section 4.7.3.4), and genotoxicity (Section 4.7.3.5). Possible key events in these hypothesized MOAs for hepatocarcinogenesis are illustrated in Figure 4-1.

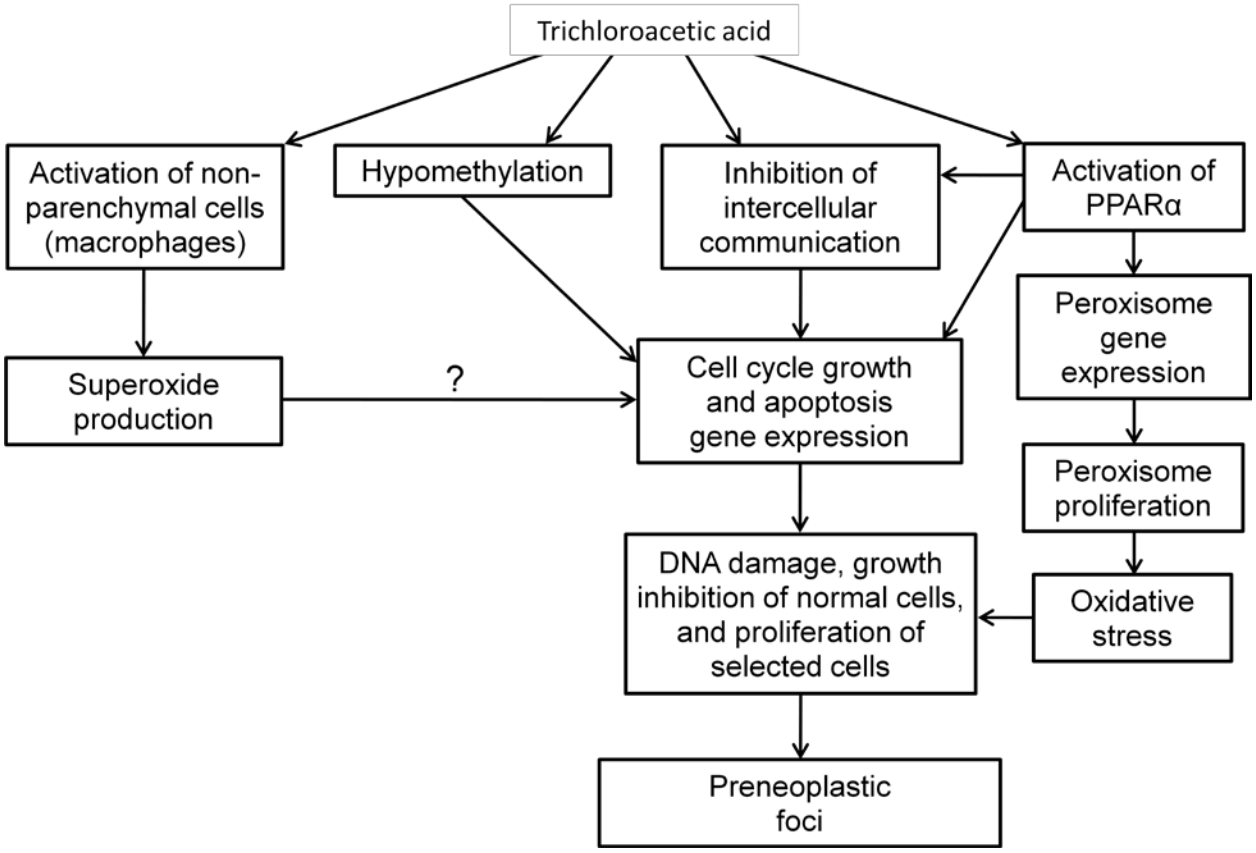

Figure 4-1. Possible key events in the MOA(s) for TCA carcinogenesis.

4.7.3.1. *PPARα agonism*[9].

The hypothesis is that TCA acts by a PPARα agonism MOA in inducing mouse liver tumors. Three key events are proposed in this MOA: activation of the receptor, perturbation of hepatocellular apoptosis and proliferation, and selective clonal expansion.

Peroxisome proliferators are a structurally diverse group of nonmutagenic or weakly mutagenic chemicals that induce a predictable suite of pleiotropic (multiple) responses, including the induction of tumors in rodents (Reddy and Menon, 1979). At one time, peroxisome proliferation, i.e., an increase in the number and volume fraction of peroxisomes (subcellular organelles) in the cytoplasm of mammalian and other eukaryotic cells, was proposed as a causative factor in the development of liver tumors. However, increased knowledge of the

[9]The data related to PPARα-agonism is relatively extensive, as this has been well-studied in order to inform the human relevance of PPARα-agonism in hepatocarcinogenesis (e.g., Klaunig et al., 2003). Therefore, this hypothesized MOA is discussed in the following section in relatively more detail than other hypothesized MOA topics.

molecular events leading to peroxisome proliferation suggests that it is an associative event, rather than a causal event, in the development of liver tumors (Klaunig et al., 2003).

Current understanding of the events leading to peroxisome proliferation indicates that peroxisome proliferating chemicals initiate the pleiotropic response by interacting with PPARs. PPARs are ligand-activated transcription factors that belong to the nuclear receptor "superfamily." When activated[10] by peroxisome proliferators (agonists), PPARs bind to response elements in the promoter regions of genes and elicit changes in gene expression. Three PPAR isoforms have been identified to date and are designated PPARα, PPARβ/δ, and PPARγ. Gene disruption experiments in mice indicate that PPARα is required for the pleiotropic response (including development of liver tumors) observed following exposure to the prototypical PPARα agonist Wy-14,643 (Escher and Wahli, 2000; Peters et al., 1997; Issemann and Green, 1990). However, peroxisome-proliferation-like events have been observed in PPARα-null mice treated with extremely high doses of ligands specific for other PPAR family members (Klaunig et al., 2003), suggesting possible cross talk between PPAR isoforms. Moreover, development of liver tumors was observed in PPARα-null mice treated with the peroxisome proliferator, di(2 ethyl-hexyl)phthalate (DEHP), suggesting that PPARα may not be a key event in the MOA for liver tumors of some peroxisome proliferators (Ito et al., 2007).

PPARα is highly expressed in cells that have active fatty acid oxidation capacity, including hepatocytes, cardiomyocytes, enterocytes, and the proximal tubule cells of the kidney, and it is well accepted that PPARα plays a central role in lipid metabolism (Dreyer et al., 1992; Göttlicher et al., 1992). Ligand or pharmaceutical activation of PPARα facilitates increased mobilization, transport, and oxidation of fatty acids, which serve as energy substrates during periods of starvation or activation, by hypolipidemic drugs such as clofibrate (Göttlicher et al., 1992). PPARα is known to interact with other transcription factors (e.g., the retinoic acid receptor and thyroid hormone receptor), co-activators, and co-repressors to regulate gene expression (Aranda and Pascual, 2001).

4.7.3.1.1. ***Identification of key events.*** Klaunig et al. (2003) have proposed an MOA hypothesis for induction of liver tumors by PPARα agonists that incorporates the following key events. PPARα ligands activate PPARα, which subsequently alters the transcription of genes involved in peroxisome proliferation, cell cycling/apoptosis, and lipid metabolism. The changes in gene expression lead to perturbations in cell proliferation and apoptosis and to peroxisome proliferation. Suppression of apoptosis coupled with increased cell proliferation allows

[10]The term "activation" refers to an alteration of the three-dimensional structure of the receptor protein or receptor complex, resulting in altered response element binding potential. The alterations initiated by ligand binding may include events such as loss of heat shock and chaperone proteins, nuclear translocation, and protein turnover (Klaunig et al., 2003).

DNA-damaged cells to persist and proliferate, resulting in preneoplastic hepatic foci and ultimately in tumors via selective clonal expansion.

In describing this progression of events, Klaunig et al. (2003) distinguish between what they consider to be causal events (i.e., required for this MOA) and associative events (i.e., markers of PPARα agonism but not shown to be directly involved with formation of liver tumors). Among the causal events postulated for PPARα-induced hepatocarcinogenesis, activation of PPARα is highly specific for this MOA. Alterations in cell proliferation and apoptosis and clonal expansion were also postulated causal events, but are common to other MOAs, and hence not specific to this MOA. Moreover, while it is known that activation of PPARα leads to an increase in cell proliferation and inhibition of apoptosis, it is uncertain whether this is due to a direct interaction with an unidentified target gene or occurs through secondary or tertiary events. Oxidative stress occurring in conjunction with peroxisomal proliferation is regarded as a general phenomenon and is considered neither a causal event nor a highly specific marker of PPARα-induced liver carcinogenesis. Peroxisome proliferation may lead to oxidative stress, which potentially contributes to the proposed MOA by causing indirect DNA damage and/or by contributing to the stimulation of cell proliferation. This section will focus on those key events considered required for this MOA (e.g., PPARα activation and alterations in proliferation, gene expression, and oxidative stress) with associated key events described in subsequent sections.

PPARα activation. The understanding of the PPARα agonism MOA has been expanded with recent findings. As reviewed by Guyton et al. (2009), recent data strongly suggest that PPARα and key events hypothesized by Klaunig et al. (2003) are not sufficient for carcinogenesis induced by the purported prototypical agonist Wy-14643. Therefore, the proposed PPARα MOA is likely "incomplete" in the sense that the sequence of key events[11] necessary for cancer induction has not been identified. It has been demonstrated in a transgenic mouse model that activation of PPARα alone in hepatocytes was not sufficient to induce hepatocellular tumors (Yang et al., 2007). In this mouse model, the potent viral transcriptional activator VP16 was fused to the mouse PPARα cDNA to create a transcription factor that constitutively activates PPARα-responsive genes in the absence of ligands. The transgenic mice demonstrated responses that mimic wild-type mice when treated with peroxisome proliferator Wy-14,643, a potent PPARα agonist, including significantly decreased serum fatty acids and marked induction of PPARα target genes encoding fatty acid oxidation enzymes, suggesting that the transgene functions in the same manner as peroxisome proliferators to regulate fatty acid

[11] As defined by the EPA *Guidelines for Carcinogen Risk Assessment* (2005c) a "key event" is "an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element," and the term "mode of action" (MOA) is defined as "a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation." Therefore, a single key event alone is necessary, but not necessarily sufficient for carcinogenesis; however, the *sequence* of key events constituting a MOA needs to be sufficient for carcinogenesis.

metabolism. In addition, while these transgenic mice demonstrated increased hepatocellular proliferation (Yang et al., 2007), no liver tumors were observed. Therefore, it appeared that many of the hepatocellular responses commonly associated with PPARα agonism—fatty acid oxidation, peroxisome proliferation, hepatocellular proliferation, and cell-cycle control gene expression—were not sufficient to induce liver tumors. However, it should be noted that, while most genes associated with exposure to PPARα agonists were activated in the LAP-VP16 PPARα mice, several genes (e.g., *c-myc*) were not activated without treatment with Wy-14643. Thus, it appears that this PPARα agonist regulates genes in addition to those of the LAP-VP16 PPARα fusion protein.

Alterations in proliferation. Several studies have observed hepatocyte proliferation in response to TCA in mice (e.g., DeAngelo et al., 2008; Stauber and Bull, 1997; Pereira, 1996; Dees and Travis, 1994; Sanchez and Bull, 1990). For instance, Dees and Travis (1994) observed relatively small (two- to threefold) but statistically significant increases in [^{3}H]thymidine incorporation in hepatic DNA in mice exposed for 11 days at TCA doses (100−1,000 mg/kg) that increased relative liver weight. Increased hepatic DNA labeling was seen at doses lower than those associated with evidence of necrosis, suggesting that TCA-induced cell proliferation is not due to regenerative hyperplasia. PPARα-null mice exposed to 2-g/L TCA in drinking water for 7 days do not show the characteristic responses of ACO, PCO, and CYP4A induction associated with PPARα activation and peroxisome proliferation in wild-type mice (Laughter et al., 2004). In addition, the livers from wild-type but not PPARα-null mice exposed to TCA developed centrilobular hepatocyte hypertrophy, although no significant increase in relative liver weight was observed. Therefore, while there are data associating TCA exposure, PPARα activation, and cell proliferation, it is not clear the extent to which PPARα activation is the cause of the observed cell proliferation.

Gene expression alterations. A novel mechanism by which PPARα regulates gene expression, hepatocellular proliferation, and tumorigenesis was uncovered by Shah et al. (2007). Activated PPARα was demonstrated to be a major regulator of hepatic microRNA (miRNA)[12] expression, especially let-7C, an miRNA found to be a potential tumor suppressor (Zhang et al., 2007; Lee and Dutta, 2007) and to inhibit the expression of the *ras* oncogene (Johnson et al., 2005). Let-7C was inhibited following treatment with 0.1% Wy-14,643 in wild-type mice for 4 hours, 2 weeks, or 11 months. No decrease in let-7C miRNA was observed in the PPARα-null mice that underwent the same treatment. In addition, expression of the longer primary let-7C transcript (pri-let-7C) was also decreased following 4-hour and 2-week Wy-14,643 treatments. Moreover, pri-let-7C, AK033222, and pri-mir-99a were regulated in a PPARα-dependent manner, since Wy-14,643 had no effect on pri-let-7C, AK033222, or pri-mir-99a in PPARα-null

[12]miRNAs are noncoding RNAs that are transcribed in the nucleus as single primary transcripts (pri-miRNAs) or large polycistronic transcripts encoding several miRNAs. Mature miRNA molecules are partially complementary to one or more mRNA molecules, and they function to down-regulate gene expression.

mice treated for 4 hours or 2 weeks. The chromosomal positional relationship of let-7C was found to be downstream of mir-99a and EMBL transcript AK033222 (Shah et al., 2007).

Shah et al. (2007) observed that let-7C regulated *c-myc* gene expression via direct interaction with the 3′-untranslated region of *c-myc* mRNA, causing mRNA degradation. Increasing let-7C expression in the mouse hepatoma cell line Hepa-1 decreased *c-myc* expression in a dose-dependent manner. PPARα-mediated induction of *c-myc* via let-7C subsequently increased expression of the oncogenic mir-17-92 polycistronic cluster, which has been implicated in enhanced cell cycle progression, blockade of tumor cell apoptosis, and increased neovascularization. These events did not occur in PPARα-null mice (Shah et al., 2007). When Hepa-1 cells were transfected with 5–25 nM let-7C, at 72 hours post-transfection, cell growth was inhibited in a dose-dependent manner. Let-7C decreased BrdU incorporation in a dose-dependent manner, but had no effect on cell apoptosis. In addition, co-transfection of let-7C and *c-myc* increased cell proliferation in Hepa-1 cells compared with cells transfected with let-7C alone, suggesting that *c-myc* is a critical downstream effector of let-7C.

No difference in basal let-7C expression was observed between wild-type mice and the LAP-VP16 PPARα transgenic mice mentioned previously, even though PPARα was activated in the hepatocytes of transgenic mice. However, Shah et al. (2007) reported that Wy-14,643 treatment decreased let-7C expression in these transgenic mice (which still possessed native PPARα), suggesting either that ligand treatment is needed for inhibition of let-7C, indicating that PPARα agonists may regulate other genes in addition to the gene for the VP16 PPARα fusion proteins, or that activation of non-parenchymal cells is critical for tumorigenesis and let-7C expression. Moreover, let-7C was not suppressed in humanized PPARα mice, which were resistant to Wy-14,643-induced hepatocellular proliferation and liver tumor formation (Shah et al., 2007). Wy-14,643 treatment of humanized PPARα mice also did not induce *c-myc* and mir-17 expression. These findings suggest that the let-7C signaling cascade may be critical for PPARα agonist-induced liver proliferation and tumorigenesis. Interestingly, however, the LAP-VP16 PPARα mice described above showed liver proliferation with neither changes in let-7C expression nor tumorigenesis, thus suggesting that proliferation itself is a poor marker for tumorigenicity.

Another mechanism, hypomethylation of DNA, has been proposed by Pogribny et al. (2007) as an important link between hepatocellular proliferation and hepatocarcinogenesis in the MOA of peroxisome proliferators. Hypomethylation of DNA is an early event to most cancers, including liver (Yamada et al., 2005; Baylin et al., 1998; Counts and Goodman, 1995; Gama-Sosa et al., 1983), and has been postulated to be a secondary mechanism involved in carcinogenesis (Watson, 2002). DNA hypomethylation is associated with opening of the chromatin configuration and transcriptional activation, leading to chromosomal instability and aberrant gene expression (Dunn, 2003; Baylin et al., 2001; Baylin et al., 1998; Jones and Gonzalgo, 1997).

When male SV129 mice were fed a control diet or Wy-14,643-containing diet (1,000 ppm) for 1 week, 5 weeks, or 5 months, treatment with Wy-14,643 led to progressive global hypomethylation of liver DNA as determined by *HpaII*-cytosine extension assay, reaching the maximum effect of >200% at 5 months. Trimethylation of histone H4 lysine 20 and H3 lysine 9 was significantly decreased at all time points. Since the majority of cytosine methylation in mammals resides in repetitive DNA sequences, Pogribny et al. (2007) measured the effect of Wy-14,643 on the methylation status of major and minor satellites, as well as in the intracisternal A particle (IAP) of long terminal repeat (LTR) retrotransposone, and long interspersed nucleotide elements (LINE) 1 and 2 (representing the non-LTR retrotransposons) in liver DNA and found that exposure to Wy-14,643 resulted in a gradual loss of cytosine methylation in major and minor satellites, IAP, LINE1, and LINE2 elements. Previously, gavage of female B6C3F_1 mice with 50 mg/kg Wy-14,643 for up to 4 days resulted in hypomethylation of the *c-myc* gene in the liver and temporally correlated with an earlier burst of cell proliferation (Ge et al., 2001a). No effect on *c-myc* promoter methylation was observed with long-term treatment (Pogribny et al., 2007). Pogribny et al. (2007) concluded that alterations in the genome methylation patterns with long-term exposure to Wy-14,643 may not be confined to specific cell-proliferation-related genes. It has been demonstrated that genome-wide hypomethylation in cancer, including liver cancer, largely involves repetitive DNA elements (Schulz et al., 2006; Chalitchagorn et al., 2004).

Pogribny et al. (2007) also found that Wy-14,643 had no effect on DNA or histone methylation status in PPARα-null mice at any of the evaluated time points. Previously, treatment of PPARα-null mice with Wy-14,643 for 11 months had produced no liver tumors, whereas treatment of wild-type mice with 1,000 ppm Wy-14,643 had resulted in 100% incidence of hepatocellular adenomas and carcinomas (Peters et al., 1997). In addition, Wy-14,643 had no effect on liver cell proliferation in PPARα-null mice (Woods et al., 2007a; Peters et al., 1997). Therefore, these epigenetic alterations were PPARα-dependent and may play a key role in hepatocarcinogenesis of peroxisome proliferators. It was suggested that peroxisome-proliferator-induced increases in hepatocellular proliferation prevented the methylation of newly synthesized strands of DNA (Ge et al., 2001a), since a temporal relationship between increased cell proliferation and DNA hypomethylation of the *c-myc* gene was observed after a single dose of Wy-14,643 to mice. Long-term treatment of wild-type mice with Wy-14,643 in Pogribny et al. (2007) demonstrated gradual worsening dysregulation of normal methylation patterns in genomic DNA.

In a subsequent study (Pogribny et al., 2008), male F344 rats were treated with 1.2% w/w DEHP, a peroxisome proliferator, in their diet for 5 months; DNA methylation in liver was unchanged. In another group of male F344 rats treated with 0.1% w/w Wy-14,643 in their diet for 5 months, global hypomethylation of DNA in liver occurred, along with a significant (twofold) increase in DNA single-strand breaks. It is unclear why the effects on DNA

hypomethylation differ between the two PPARα agonists. While these results are not consistent with hypomethylation of DNA being a key event in the PPARα MOA, such a conclusion is complicated by the fact that DEHP can induce mouse liver tumors in a PPARα-independent manner.

4.7.3.1.2. ***Biological plausibility, consistency, specificity of association.*** TCA is classifiable as a peroxisome proliferator based on morphologic and biochemical evidence from multiple studies. With respect to peroxisome proliferation, microscopic examination of responses consistent with peroxisome proliferation (e.g., induction of lipid metabolism enzymes such as ACO and PCO, increased liver weight) has been observed in male F344 rats exposed to TCA by gavage for 14 days (Goldsworthy and Popp, 1987), in male F344 rats exposed to TCA in drinking water for 14 days (DeAngelo et al., 1989) or 104 weeks (DeAngelo et al., 1997), in male Osborne-Mendel rats exposed to TCA in drinking water for 14 days (DeAngelo et al., 1989), and in male Sprague-Dawley rats treated with TCA in the drinking water for 90 days (Mather et al., 1990). In mice, peroxisome proliferation or changes consistent with peroxisome proliferation have been reported in male $B6C3F_1$ mice exposed to TCA in drinking water for 2–10 weeks (Parrish et al., 1996; Austin et al., 1995; Sanchez and Bull, 1990; DeAngelo et al., 1989), in male $B6C3F_1$ mice exposed by gavage for 10 days (Goldsworthy and Popp, 1987), and in male C57BL/6 and Swiss-Webster mice exposed to TCA in the drinking water for 14 days (DeAngelo et al., 1989). Furthermore, PPARα-null mice exposed to 2 g/L TCA in drinking water for 7 days do not show the characteristic responses of ACO, PCO, and CYP4A induction associated with PPARα activation and peroxisome proliferation in wild-type mice (Laughter et al., 2004). In addition, the livers from wild-type but not PPARα-null mice exposed to TCA developed centrilobular hepatocyte hypertrophy, although no significant increase in relative liver weight was observed.

In addition, PPARα agonism in response to treatment with TCA has been demonstrated in vitro in COS-1 cells transfected with human and mouse PPARα expression plasmids together with a peroxisome proliferator response element-luciferase reporter (Maloney and Waxman, 1999). Cells were treated for 24 hours with 0.1–5 mM TCA. TCA activated human and mouse PPARα with no difference between species in receptor sensitivity or maximal responsiveness.

Third, TCA has been shown to increase hepatocyte proliferation in DNA-labeling experiments in mice (Dees and Travis, 1994). Relatively small (two- to threefold) but statistically significant increases in [^{3}H]thymidine incorporation in hepatic DNA were observed in mice exposed to 100–1,000 mg/kg-day TCA for 11 days at doses that increased relative liver weight. Dees and Travis (1994) observed increased hepatic DNA labeling at doses lower than those associated with evidence of necrosis, suggesting that TCA-induced cell proliferation is not due to regenerative hyperplasia. The study authors reached this conclusion based on the pattern of observed histopathologic changes, which indicated nodular areas of cellular proliferation, and the results of liver DNA labeling experiments, which showed incorporation of [^{3}H]thymidine in

extracted liver DNA but no difference in total liver DNA content (mg DNA/g liver). Dees and Travis (1994) concluded that their results were consistent with an increase in DNA synthesis and cell division in response to TCA treatment. The authors further suggested that the absence of histopathologic effects makes it unlikely that the increased radiolabel was secondary to tissue repair.

Hepatocyte proliferation in response to treatment with TCA has also been demonstrated in studies by Stauber and Bull (1997), Pereira (1996), and Sanchez and Bull (1990). Details of these studies were provided in Sections 4.5.1.3 and 4.2.2.1. A dose-related increase in incorporation of [^{3}H]thymidine into hepatic DNA was observed in B6C3F$_1$ mice treated with 0.3–2 g/L TCA for 5 or 14 days (Sanchez and Bull, 1990). This increase was significant at 2 g/L TCA. No increases in labeled hepatocytes as seen by autoradiography were apparent at 2 or 5 days. Thus, an increase in incorporation of [^{3}H]thymidine did not correlate with replicative synthesis of DNA measured by autoradiography up to 5 days of treatment. Pereira (1996) reported that TCA increased the BrdU-labeling index (calculated as the percentage of hepatocytes with labeled nuclei) in mice exposed to 0.33–3.3 g/L TCA for 5 days but not after 12 or 33 days. Stauber and Bull (1997) reported a statistically significant two- to threefold elevation in division rate in normal hepatocytes after male B6C3F$_1$ mice were treated for 14 or 28 days with 2 g/L TCA. However, continued treatment for 52 weeks resulted in a decrease in division rate in normal hepatocytes. Cell division rates in TCA-induced AHFs and tumors were high at all TCA doses administered in the last 2 weeks of the study.

DeAngelo et al. (2008) reported hepatocyte proliferation in B6C3F$_1$ mice exposed to 5 g/L TCA at 30 and 40 weeks, with mice exposed to 0.5 g/L TCA demonstrating hepatocyte proliferation at 60 weeks. Therefore, DeAngelo et al. (2008) observed hepatocyte proliferation in mice after long-term TCA treatment in contrast to Stauber and Bull (1997), who observed it as a transient event. This result was in agreement with the observation by Woods et al. (2007a) that the robust proliferative effect of Wy-14,643 in rodent livers extended beyond the short time frame that was traditionally considered. Hepatocyte proliferation has been demonstrated in chronic studies with other peroxisome proliferators (Woods et al., 2007a; Yeldandi et al., 1989; Ward et al., 1988). It should also be noted that TCA did not induce hepatocyte proliferation or tumors in F344 rats after 104 weeks of exposure (DeAngelo et al., 1997), consistent with the hypothesis that cell proliferation is a causal event in tumorigenesis under the PPARα MOA.

Moreover, as presented previously, whereas PPARα-null mice treated with 2 g/L TCA in drinking water for 7 days did not develop centrilobular hepatocyte hypertrophy, treated wild-type mice did (Laughter et al., 2004). Thus, TCA-induced hepatocyte hypertrophy is PPARα dependent.

A 2006 report by the NRC of the National Academy of Sciences, *Assessing the Human Health Risks of Trichloroethylene: Key Scientific Issues* (NRC, 2006), stated that "[t]here is sufficient weight of evidence to conclude that the mode of action of trichloroacetic acid as a

rodent liver carcinogen is principally as a liver peroxisome proliferator in a specific strain of mouse, B6C3F$_1$." However, the NRC (2010) panel reviewing EPA's 2008 external review draft of tetrachloroethylene did not, as a whole, support the view that the only MOA of TCA is peroxisome proliferation. They judged that the relevance of the peroxisome proliferator MOA to mouse and human hepatic cancer remains hypothetical and requires further rigorous testing. Hence, the report concludes that is premature to draw conclusions on the relevance of the PPARα MOA to human hepatic carcinogenesis (NRC, 2010).

The evidence described above supports the involvement of PPARα agonism in the overall cancer MOA for TCA; however, some studies of PPARα published since NRC (2006), notably Ito et al. (2007) (see discussion below), suggest that the mechanism by which TCA induces liver tumors in mice is more complex than that presented in NRC (2006). Indeed, the 2011 Science Advisory Board reviewing EPA's draft trichloroethylene IRIS assessment cited studies in PPARα-null mice (Eveillard et al., 2009; Takashima et al., 2008; Ito et al., 2007), PPARα humanized transgenic mice (Morimura et al., 2006), and hepatocyte-specific constitutively-activated PPARα transgenic mice (Yang et al., 2007) in concluding that activation of PPARα is an important factor, but not a limiting factor for the development of mouse liver tumors and that additional molecular events may be involved. Inconsistencies and gaps in the data with respect to consistency and specificity of PPARα agonism as an MOA are also discussed further below.

PPARα-independent tumor induction by DEHP. Ito et al. (2007) recently reported that the peroxisome proliferator, DEHP, induces hepatic tumorigenesis through a PPARα-independent pathway. Specifically, the authors administered relatively low doses of DEHP (0, 0.01, and 0.05% in diet) to wild-type and PPARα knockout mice for 22 months and found a higher incidence of liver tumors in treated PPARα knockout than in treated wild-type mice at the higher dose. (This was the first published study using PPARα knockout mice that were treated for over 1 year, allowing for the full expression of tumor development.) DEHP treatment also dose-dependently increased 8-OHdG levels in mice of both genotypes, although the degree of increase was higher in PPARα knockout mice. Ito et al. (2007) suggested that increases in oxidative stress induced by DEHP exposure may lead to induction of inflammation, resulting in a higher incidence of liver tumors in PPARα knockout mice and a potential PPARα-independent pathway for DEHP-induced liver tumors. The NRC (2008) report entitled *Phthalates and Cumulative Risk Assessment: The Tasks Ahead* states that the Ito et al. (2007) results "suggest that DEHP might cause hepatic cancer in rodents through a mechanism that is independent of PPARa, as has been suggested by others [see, for example, Takashima et al. (2008)]." A separate NRC (2009) report entitled *Science and Decisions: Advancing Risk Assessment* states that the Ito et al. (2007) study "calls into question" the conclusion regarding DEHP carcinogenicity that is based on the PPARa activation MOA. Similar conclusions were reached by NRC (2010) in their review of EPA's external review draft of tetrachloroethylene.

Another possibility is the involvement of other nuclear receptors. A comprehensive evaluation of the ability of DEHP to activate gene expression through nuclear receptors other than PPARα (Ren et al., 2010) demonstrated that exposure to DEHP activated multiple nuclear receptors, including PPARα, constitutive activated/androstane receptor (CAR), and pregnane X receptor, in the rodent liver. Although direct evidence for TCA is not available, studies have shown that other PPARα agonists, such as Wy-14,643, perfluorooctanoic acid (PFOA), ciprofibrate, and clofibrate, regulate gene expression via CAR (Cheng and Klaassen, 2008; Guo et al., 2007). It should be noted that DEHP induced liver tumors in rats and mice, but TCA induced liver tumors only in mice. Therefore, the MOA for hepatocarcinogenesis for DEHP and TCA may not be comparable. However, the above findings for DEHP suggest that demonstration of many of the key events proposed for a PPARα MOA are insufficient to preclude existence of a PPARα-independent pathway for tumorigenesis. Previously, Melnick (2001) suggested PPARα-independent pathways for tumorigenesis by DEHP.

let-7C miRNA mediated signaling cascade. Researchers have explored other possible key events for a PPARα agonism MOA, including the possible roles of let-7C miRNA on hepatocarcinogenesis of PPARα agonists in mice. The expression of *c-myc* mRNA was increased in TCA-treated female B6C3F$_1$ mice (Pereira et al., 2001). *c-myc* has been demonstrated to be a critical downstream effector of let-7C (Shah et al., 2007). Thus, increased expression of *c-myc* mRNA in TCA-treated mice is consistent with the proposed let-7C miRNA mediated signaling cascade in alteration of gene expression, hepatocellular proliferation, and tumorigenesis in TCA-treated mice. However, it has not been shown that TCA-induced increases in *c-myc* expression are PPARα-dependent, since increased expression of *c-myc* is common to both carcinogens and noncarcinogenic mitogens (Hasmall et al., 1997).

Differences in species response to hepatocarcinogenicity. While TCA induces peroxisome proliferation (a marker for PPARα agonism) in both rats and mice, to date, TCA has been shown to be tumorigenic in B6C3F$_1$ mice but not F344 rats (DeAngelo et al., 1997) (the only strains tested for carcinogenicity). No complete explanation for this species difference has been developed, although the NRC (2006) suggested that, at the same doses, rats and mice have different responsiveness to peroxisome proliferation. For instance, Bull (2000) noted that, under similar dosing regimens, a two- to threefold increase in peroxisome proliferation was observed in F344 rats compared with a 10-fold increase over controls in mice (strains not specified). However, this relationship may not hold for all mouse and rat species and strains and may be chemical specific. For example, Elcombe (1985) reported that Wistar rats displayed a higher induction of peroxisome proliferation than mice in response to TCA, as measured by increases in cyanide insensitive palmitoyl CoA oxidation in both species. Moreover, evidence from other peroxisome proliferators suggests that the degree of peroxisome proliferation and hepatocarcinogenic potency are not well correlated (Marsman et al., 1988). The finding that hepatocyte proliferation only occurred in TCA-treated mice (DeAngelo et al., 2008) but not in

treated rats (DeAngelo et al., 1997) is consistent with it being a key event in tumorigenesis under the PPARα agonism MOA. However, it still does not provide an explanation as to the species difference, given that the prototypical PPARα agonist Wy-14,643 is hepatocarcinogenic in both rodent species.

Another possible explanation for the lack of TCA-induced tumors in rats is that the binding of TCA to total plasma protein may be higher in rats than in mice, reducing its bioavailability in the liver. However, the extent of these differences in binding is not clear. For instance, at around 600 µM, Lumpkin et al. (2003) reported the plasma-bound fraction of TCA in rats to be about four- to fivefold higher than in mice, while Templin et al. (1995; 1993) reported this difference to be only about 1.1-fold.

Therefore, overall, the lack of explanation for the absence of liver tumors in TCA-treated rats that demonstrate peroxisome proliferation raises some questions about PPARα agonism as the only MOA for liver tumor induction by TCA.

Phenotypic characteristics of tumors. TCA has also been associated with a PPARα-mediated MOA based on evidence that the phenotypic characteristics of TCA-induced tumors appear similar to those of tumors induced by other peroxisome proliferators (NRC, 2006). However, on closer examination, certain characteristics of TCA-induced foci and tumors, including mutation frequencies and spectra, phenotypic characteristics, and immunostaining characteristics, are different from those induced by other peroxisome proliferators, and those characteristics that are similar may be relatively nonspecific to peroxisome proliferators. This suggests that PPARα agonism may not be the sole MOA for TCA-induced tumors in mice.

Specifically, with respect to mutations in TCA-induced foci and tumors, both Ferreira-Gonzalez et al. (1995) and Bull et al. (2002) observed that the H-*ras* codon 61 mutation frequency and spectrum of TCA-induced tumors were similar to historical controls, while peroxisome proliferators ciprofibrate (Hegi et al., 1993) and methylclofenapate (Stanley et al., 1994) have lower H-*ras* codon 61 mutation frequency than do spontaneous tumors in B6C3F_1 mice (11/46 versus 85/130 for methylclofenapate; 8/39 versus 32/50 for ciprofibrate) and their mutation spectrums differed from those of spontaneous tumors. The lower frequency and distinct pattern of H-*ras* mutation observed in methylclofenapate and ciprofibrate would suggest that the activation of H-*ras* protooncogene in spontaneous liver lesions is not involved in hepatocarcinogenesis by these two peroxisome proliferators. Since the H-*ras* codon 61 mutation frequency and the spectrum of TCA-induced tumors were similar to historical controls, a similar conclusion as to the role of H-*ras* activation cannot be drawn for TCA-induced tumors. On the other hand, Ferreira-Gonzalez et al. (1995) reported K-*ras* codon 61 mutations in 1/11 TCA-induced liver tumors and none in 32 spontaneous tumors from control animals. Both Hegi et al. (1993) and Stanley et al. (1994) found such a rare mutation in 1/23 ciprofibrate-induced and one methylclofenapate-induced hepatocarcinoma (the number of samples examined was not provided), suggesting that such a rare mutation may be caused by indirect DNA damage induced

by treatment (Hegi et al., 1993). Reynolds et al. (1987) reported K-*ras* mutations in mouse liver tumors induced by the peroxisome proliferators furfural and furan, but the mutations were not at codon 61. However, it should be noted that, in all cases, the overall rates of K-*ras* mutations are low (<10% of tumors), so their reliability as indicators of MOA is likely to be low.

With respect to tumor phenotype[13], although Stauber and Bull (1997) reported TCA-induced foci and tumors to be predominantly basophilic, Pereira (1996) reported that the foci of altered hepatocytes in mice treated with TCA were half basophilic and half eosinophilic, with liver tumors predominantly basophilic. DeAngelo et al. (2008) reported that cytoplasmic alterations in hepatocytes of TCA-treated mice were characterized by intense eosinophilic cytoplasm with deep basophilic granularity (microsomes). By contrast, it has been suggested that peroxisome proliferators selectively promote basophilic foci generally (Cattley et al., 1995). Furthermore, Weber et al. (1988) and Bannasch et al. (2001) reported that foci of altered hepatocytes in rats treated with peroxisome proliferators are amphophilic-basophilic (amphophilic: increased granular acidophilia and randomly scattered cytoplasmic basophilia), suggesting a phenotype that also has increased mitochondrial proliferation and peroxisome proliferation. Thus, the phenotype of TCA hepatic preneoplastic lesions may be different than that induced by peroxisome proliferators.

Kraupp-Grasl et al. (1991; 1990) noted a difference in the ability of a peroxisome proliferator to promote tigroid foci, which are characterized by large basophilic bodies on a clear or eosinophilic cytoplasmic background, and weakly basophilic foci, which are characterized by weak diffuse basophilia and some eosinophilia (equivalent to amphophilic foci described earlier). In their experiments, using PB or the peroxisome proliferator nafenopin as promoters, only nafenopin and not PB promoted the weakly basophilic foci. In addition, a substantial number of spontaneous foci (the number of which were actually decreased by nafenopin) were tigroid. Both tigroid and weakly basophilic foci may appear to be basophilic at the light microscopic level; thus, it is not clear from Stauber and Bull (1997) and Pereira (1996) whether the reported "basophilic" foci from TCA treatment are actually "tigroid" or "weakly basophilic." Moreover, because of the natural progression of several lineages of preneoplastic lesions, including those

[13]According to the extensive published literature (Bannasch et al., 2001; Bannasch, 1996; Weber et al., 1988), altered hepatic foci in hepatocarcinogenesis generally fall into three types: (1) glycogenotic-basophilic lineage: glycogenotic clear and acidophilic (smooth endoplasmic reticulum-rich) hepatocytes that progress to glycogen-poor, homogenously basophilic (ribosome rich) phenotype in undifferentiated hepatocellular carcinomas; (2) tigroid-basophilic lineage: tigroid foci, a variant of glycogenotic foci (probably occurring at low dose), contain large basophilic bodies on a clear or eosinophilic cytoplasmic background; (3) amphophilic-basophilic cell lineage: amphophilic cells consist of glycogen-poor cytoplasm containing both abundant granular-aci xzdophilic (mitochondria and peroxisomes) and basophilic (ribosomes) components. Amphophilic cells occur when rats are treated with nongenotoxic peroxisome proliferators. All three types of foci can progress to a basophilic phenotype as tumors progress.

not induced by peroxisome proliferators, to basophilic neoplasms (Bannasch, 1996), basophilic tumors themselves are nonspecific to peroxisome proliferators.

Immunostaining characteristics. With respect to immunostaining characteristics, the foci and tumors induced by peroxisome proliferators have been noted to not express GGT and GST-π (Rao et al., 1986). It has been shown by Parnell et al. (1988) that TCA promotes GGT-positive foci in partially hepatectomized rats initiated with DEN, which is the opposite of that expected for peroxisome proliferators. (However, it is not known if TCA promotes GGT-positive foci in rats that were not partially hepatectomized.) With respect to GST-π, Pereira and Phelps (1996), Pereira et al. (1997), and Latendresse and Pereira (1997) found most tumors in their initiation-promotion studies of MNU+TCA to be lacking in GST-π, consistent with that expected from peroxisome proliferators. However, basophilic foci that are both GGT negative and GST-π negative are not specific to peroxisome proliferators. For instance, Kraupp-Grasl et al. (1990) and Grasl-Kraupp et al. (1993) reported that tigroid foci, which display basophilia, were predominantly GGT negative regardless of whether they were found in control rats or rats given AfB1 only, AfB1 plus the peroxisome proliferator nafenopin, or AfB1 plus the non-peroxisome proliferator PB. Ittrich et al. (2003) stated that GST-π is negative in preneoplastic and neoplastic cell populations with increased basophilic components.

With respect to immunostaining characteristic for *c-jun*, Stauber and Bull (1997) suggested that their observation that all TCA-induced tumors were *c-jun* negative, a characteristic also found by Bull et al. (2002), was consistent with peroxisome proliferators. However, tumors promoted by TCA in the experiments of Latendresse and Pereira (1997) variably stained for *c-jun*. Furthermore, although spontaneous and some chemically-induced foci and tumors have been reported to express or stain for *c-jun* (Sakai et al., 1995; Nakano et al., 1994; Suzuki et al., 1990) both induction (Tharappel et al., 2003) and suppression (Yokoyama et al., 1993) of *c-jun* by short-term exposure to peroxisome proliferators have been reported in the liver or in vitro, with no studies located that report *c-jun* immunostaining of peroxisome proliferator-induced foci or tumors. Therefore, the use of immunostaining characteristic for *c-jun* as an indicator for the PPARα MOA is questionable.

Summary. In summary, proposed key events in the hypothesized PPARα agonism MOA have been shown to occur with TCA treatment, including PPARα activation and hepatocellular proliferation. The available data are insufficient, however, to confirm the PPARα MOA as a sole causative factor for TCA hepatocarcinogenesis. Studies of PPARα published since NRC (2006) indicate that the TCA MOA is more complex than that presented in NRC (2006). Specifically, a study by Yang et al. (2007) showed that ligand-independent PPARα activation in hepatocytes evokes the MOA, but not hepatocarcinogenesis in a transgenic mouse model. In addition, while other data associated PPARα agonism with DEHP hepatocarcinogenesis, a second recent study found that DEHP induces liver tumors in PPARα-null mice (Ito et al., 2007). Together, these studies demonstrate that PPARα activation is neither sufficient for carcinogenesis nor necessary

for DEHP-induced liver tumors. While prior reviews (e.g., Klaunig et al., 2003) have proposed that PPARα agonism and its sequelae constitute an MOA for hepatocarcinogenesis as a sole causative factor, these newer data have raised considerable doubt about the validity of this hypothesis for DEHP[14]. In addition, effects of TCA, including increased *c-myc* expression and hypomethylation of DNA, are not specific to the PPARα activation MOA, and other data also contribute uncertainty as to whether a PPARα-independent MOA explains TCA-induced tumors in mice.

4.7.3.1.3. *Dose-response concordance*. Clear dose-response concordance between proposed key events and tumor response is lacking. The doses that induce peroxisome proliferation in mice are similar to tumorigenic doses of TCA (Bull, 2000). B6C3F_1 and other strains of mice treated with 1–5 g/L TCA in drinking water for 14 days showed dose-dependent increases in hepatic peroxisomal enzyme carnitine acetyl-CoA transferase activity and cyanide-insensitive PCO activity (DeAngelo et al., 1989). Dose-dependent increases in relative liver weights were also observed. Similarly, dose-related increases in hepatic cyanide-insensitive ACO activity and 12-hydroxylation of lauric acid were observed in male B6C3F_1 mice treated with 0.1–2 g/L TCA in drinking water for 3 or 10 weeks.

Peroxisome proliferation was evaluated in only one chronic bioassay in mice (DeAngelo et al., 2008). PCO activity was increased in mice treated with 0.5 g/L (68 mg/kg-day) or 5 g/L (602 mg/kg-day) of TCA, dose levels that were carcinogenic, providing support that PPARα agonism is related to tumor formation. As stated above, however, peroxisome proliferation is an associative event and marker of PPARα agonism and not correlated with carcinogenic potency of PPARα agonists.

The doses that induce hepatocellular proliferation in mice corresponded to tumorigenic doses of TCA in DeAngelo et al. (2008). An increase in incidence of hepatocellular adenomas and carcinomas was observed in male B6C3F_1 mice exposed to 0.5 or 5 g/L TCA for 30–60 weeks but not at 0.05 g/L TCA. A significant increase in hepatocellular proliferation was found in mice exposed to 5 g/L TCA at 30 and 45 weeks and in 0.5 g/L TCA group at 60 weeks. A small increase in hepatocyte proliferation was found in the 0.05 g/L TCA group at 78 weeks. Doses of 0.3–3.3 g/L TCA that caused hepatocellular proliferation in short-term studies (Pereira, 1996; Sanchez and Bull, 1990) were similar to the tumorigenic doses.

[14]The NRC (2008) report entitled *Phthalates and Cumulative Risk Assessment: The Tasks Ahead* states that the Ito et al. (2007) results "suggest that DEHP might cause hepatic cancer in rodents through a mechanism that is independent of PPARα, as has been suggested by others [see, for example, Takashima et al. (2008)]." A separate NRC (2009) report entitled *Science and Decisions: Advancing Risk Assessment* states that the Ito et al. (2007) study "calls into question" the conclusion regarding DEHP carcinogenicity that is based on the PPARα activation MOA.

4.7.3.1.4. *Human relevance*. In its framework for making conclusions about human relevance, EPA's *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005c) outline the following elements to evaluate: (1) identifying critical similarities and differences between test animals and humans regarding the sequence of key precursor events; (2) flagging quantitative differences for consideration in dose-response assessment, such as the potential for different internal doses of the active agent or differential occurrence of a key precursor event; and (3) considering all populations and life stages, including special attention to whether tumors can arise from childhood exposure.

With respect to the first element, it was originally believed that there is no evidence for functional differences between rodents and humans in the key events described above for the proposed PPARα MOA, and humans possess PPARα at sufficient levels to mediate the human hypolipidemic response to peroxisome-proliferating fibrate drugs (Klaunig et al., 2003). Klaunig et al. (2003) reached a conclusion [reiterated in NRC (2006)] that the key events are plausible in humans in the sense that "a point in the rat/mouse key events cascade where the pathway is biologically precluded in humans cannot be identified, in principle." This was supported by an early in vitro study (Maloney and Waxman, 1999), in which the human and mouse forms of PPARα are comparable in their affinity for TCA. The results from in vitro studies should be interpreted cautiously because cultured human hepatocytes could lose or gain biological characteristics in the process of immortalization, and the microenvironment of cultured cells is different from the in situ hepatocytes in terms of the three dimensional cell-cell contact, cell heterogenicity, and endocrine feedback in the intact animal. Recent studies suggested that there might be functional differences between human and mouse PPARα. Studies on PFOA and ammonium perfluorooctanoate showed that a lower concentration of PFOA and ammonium perfluorooctanoate was required to activate mouse PPARα than to activate human PPARα (Nakamura et al., 2009; Takacs and Abbott, 2007). The activation of mouse PPARα by PFOA and ammonium perfluorooctanoate was generally higher compared to that of human PPARα (Wolf et al., 2008). Altogether, human PPARα may have a weaker affinity for PFOA than does mouse PPARα. No direct evidence exists to show that this is also true for TCA.

With respect to the second question, the limited available data suggest that there are quantitative differences between rodents and humans in the occurrence of events following PPARα activation. However, these data do not appear sufficient for use in dose-response analysis. Walgren et al. (2000) found that TCA did not increase palmitoyl CoA oxidation and caused a decrease in DNA synthesis in primary and long-term human hepatocytes cultures (in contrast to rodents). Palmer et al. (1998) and Holden and Tugwood (1999) reported about 10-fold less PPARα mRNA in human liver as compared with rat or mouse, but mRNA levels are not necessarily indicative of protein levels. Walgren et al. (2000) found, on average, lower levels of PPARα protein in human livers compared with rodents, but expression levels were highly variable among individuals and, at least in one case, were comparable to rodents' levels.

Moreover, expression levels may not be related to potency, since the hypolipidemic response to PPARα agonists is similar in humans and rodents. On the other hand, humans and nonhuman primates appear less sensitive than rodents to the PPARα-mediated peroxisome proliferation response and its associated changes in regulation of peroxisomal genes and proteins. None of these effects, however, are thought to be causally related to hepatocarcinogenesis (Klaunig et al., 2003), and it appears that carcinogenic potency and degree of peroxisomal response are not well correlated (Marsman et al., 1988).

Lack of induction of cell proliferation or increased apoptosis have been observed in vitro with human hepatocytes, but no method for quantitative extrapolation of these results to the in vivo situation is available. Moreover, these assay systems remove the non-parenchymal cells (e.g., Kupffer cells) during preparation, which has been shown to prevent the proliferative response to PPARα agonists (Parzefall et al., 2001; Hasmall et al., 2000b). In vivo, no increase in cell proliferation was observed in nonhuman primates treated with PPARα agonists (Doull et al., 1999), but no human data are available. Hoivik et al. (2004) noted that fenofibrate and ciprofibrate induced treatment-related increases in liver weight, hypertrophy, numbers of peroxisomes, and numbers of mitochondria and smooth endoplasmic reticulum in cynomolgus monkeys at 15 days of exposure; however, no cell proliferation was found.

While the observed species differences in the occurrence of key events may be explained partially by differences in expression levels of PPARα in liver, recent studies (Shah et al., 2007; Morimura et al., 2006; Cheung et al., 2004) using PPARα-humanized (hPPARα) mice fed Wy-14,643 examine the hypothesis that structural differences in human and mouse PPARα receptors may be responsible. A hPPARα mouse line in which the human PPARα was expressed in liver under control of the tetracycline responsive regulatory system was used in these studies. The hPPARα mice were fed the prototype peroxisome proliferator, Wy-14,643, or the lipid-lowering drug, fenofibrate. Decreased serum triglycerides were observed in both the wild-type and hPPARα mice, with no difference in basal serum triglyceride levels between the two types of mice. In addition, a robust induction of the expression of genes encoding enzymes involved in peroxisomal, mitochondrial, and microsomal fatty acid catabolism and those involved in fatty acid synthesis and transport was found in hPPARα mice after 2 weeks of Wy-14,643 or fenofibrate feeding. Hepatomegaly and increases in hepatocyte size were observed in mice fed Wy-14,643 for 2 weeks; however, the extent of cell size and hepatomegaly was markedly less in hPPARα mice when compared with wild-type mice, especially after 8 weeks of Wy-14,643 feeding.

Cheung et al. (2004) also evaluated peroxisome-proliferator-induced replicative DNA synthesis by measuring BrdU incorporation into hepatocyte nuclei in hPPARα mice and wild-type mice after 8 weeks of feeding with Wy-14,643. In wild-type mouse livers, Wy-14,643 treatment resulted in a BrdU labeling index of 57.9% compared with 1.6% in untreated controls. However, in hPPARα mice, Wy-14,643 treatment did not increase the incorporation of BrdU

with average labeling indices of 2.8 and 1.6% in Wy-14,643-treated and control mice, respectively. In addition, Wy-14,643 treatment resulted in a marked induction in the expression of various genes involved in cell cycle control (proliferating cell nuclear antigen, *c-myc*, CDK1, and CDK4 and cyclins A2, D1, and E) in the livers of wild-type mice. By contrast, the expression of these genes was unchanged with Wy-14,643 treatment in hPPARα mice. However, the lack of induction of these cell cycle regulated genes in hPPARα mice may be due to differences in binding of activated hPPARα to mouse co-activators or to certain mouse peroxisome proliferator response elements. On the other hand, genes encoding peroxisomal, mitochondrial, and microsomal fatty acid oxidation enzymes were still markedly induced in hPPARα mice following 8 weeks of Wy-14,643 feeding. Therefore, whereas human PPARα in mice regulates induction of fatty acid catabolism and lipid lowering, it does not stimulate the cell proliferative response that is thought to contribute to liver carcinogenesis. In addition, as discussed above, Shah et al. (2007) reported that miRNA let-7C was not suppressed in Wy-14,643-treated hPPARα mice. Wy-14,643 treatment of hPPARα mice also did not induce *c-myc* and mir-17 expression.

Decreased susceptibility of hPPARα mice to Wy-14,643-induced liver tumorigenesis was shown by Morimura et al. (2006). When the feeding study of 0.1% Wy-14,643 was extended to 44 weeks for hPPARα mice and 38 weeks for wild-type mice, the incidence of liver tumors, including hepatocellular carcinoma, was 71% in wild-type mice (five adenomas and two carcinomas out of seven mice; 3/10 treated mice died of toxicity); by contrast, only 5% of Wy-14,643-treated hPPARα mice developed liver tumors (one adenoma out of 20 mice; the adenoma resembled spontaneous tumor). In addition, up-regulation of cell cycle regulated genes, such as cyclin D1 (*cd1*) and cyclin-dependent kinases (*Cdks*) 1 and 4, were observed in non-tumorous liver tissues of Wy-14,643-treated wild-type mice. The *c-myc* mRNA was also significantly overexpressed in the Wy-14,643-treated wild-type mice. On the other hand, expression of the tumor suppressor gene, p53, was increased only in the livers of Wy-14,643-treated hPPARα mice.

Morimura et al. (2006) concluded that these data in hPPARα mice are consistent with toxicodynamic differences between humans and mice being due to structural differences between human and mouse PPARα. It should be noted, however, that only Wy-14,643 has been tested in hPPARα mice for carcinogenicity to date, and the duration of treatment was <1 year. Therefore, more studies need to be conducted, especially with TCA, before definitive conclusions can be made regarding human relevance using data from hPPARα mice.

As discussed previously, toxicokinetic differences also exist between humans and mice. Binding of TCA to plasma proteins was found to be higher in humans than in mice in two in vitro studies (Lumpkin et al., 2003; Templin et al., 1995). Thus, plasma levels of free TCA would be expected to be lower in humans than in mice administered the same dose of TCA. However, the extent to which administration of the same dose in humans and mice would yield a

relatively smaller tissue dose in humans is not directly related to plasma binding, due to differences in clearance between species (humans having a longer half-life). Due to lack of the PBPK model, the extent to which such toxicokinetic differences would impact species differences has not been quantified.

With respect to the final question, little data on population variability and lifestages, particularly with respect to childhood exposures and susceptibility, are available either for TCA or PPARα agonists in general.

A number of other reports have also reached conclusions as to the human relevance of PPARα-agonist-induced hepatocarcinogenesis, both in general and with respect to specific chemicals. The NRC (2006) report reiterated the position of Klaunig et al. (2003) that "[w]hereas the mode of action is plausible in humans, the weight of evidence suggests that this mode of action is not likely to occur in humans based on differences in several key steps when taking into consideration kinetic and dynamic factors." NRC (2006) also stated "[i]nduction of peroxisome proliferation in human liver is not a prominent feature; therefore, this key event related to trichloroacetic acid liver carcinogenesis is not likely to occur in humans." In the framework for MOA used here (U.S. EPA, 2005c), human relevance is considered in the context of hazard characterization. As discussed above, both humans and rodents share the ability for PPARα receptor activation but with similarities and differences in a number of responses. In addition, in this analysis (U.S. EPA, 2005c), quantitative differences due to "kinetic and dynamic factors" are flagged for consideration in dose-response assessment. Toxicokinetics of TCA are discussed earlier in this document. With respect to toxicodynamics, as discussed above, data suitable for use in dose-response analysis of TCA hepatocarcinogenic risk are lacking.

In the Science Advisory Board's review of EPA's draft risk assessment of potential human health effects associated with PFOA and its salts (U.S. EPA, 2006b), it was concluded that PFOA-induced liver tumors in rats were considered relevant to humans based on the following considerations: (1) "uncertainties still exist as to whether PPARα agonism constitutes the sole mode of action for perfluorooctanoic acid effects on liver"; (2) "[u]ncertainties exist with respect to the relevance to exposed fetuses, infants and children of the PPARα agonism mode of action for induction of liver tumors in adults"; and (3) "the interplay between PPARα agonism and Kupffer cells (resident macrophages in the liver) has not been characterized. Kupffer cells do not express PPARα, but are activated by peroxisome proliferators. Prevention of Kupffer cell activation by glycine inhibited, although not completely, the development of liver tumors by the potent peroxisome proliferator Wy-14,643. There are no data available on the effects of peroxisome proliferators on human Kupffer cells." These conclusions regarding human relevance are similar to those reached here for TCA.

4.7.3.1.5. *Summary*. The data for TCA, while supportive of the involvement of PPARα in hepatocarcinogenesis, are not sufficient to conclude that it is the sole MOA. Moreover, issues

with respect to biological consistency and specificity of association for this proposed MOA have been identified. Thus, the current data do not rule out the possibility that TCA could induce cancer in humans by a MOA not associated with PPARα agonism. To the extent that PPARα is involved, the key events in the proposed MOA by Klaunig et al. (2003) are biologically plausible in humans, so this MOA is assumed to be relevant to humans. On the other hand, data are consistent with toxicokinetic and toxicodynamic differences between species in the responses to the prototypical PPARα agonists, Wy-14,643, but data are lacking for TCA specifically. The available data on such differences are not suitable for use in dose-response analysis of TCA hepatocarcinogenic risk. Further studies with various types of PPARα agonists need to be conducted before definitive conclusions can be drawn regarding the relative human sensitivity to the hepatocarcinogenic effects of PPARα agonists.

4.7.3.2. *Additional Proposed Hypotheses and Key Events with Limited Evidence or Inadequate Experimental Support*

Several effects that been hypothesized to be associated with liver cancer induction are discussed in more detail below, including Kupffer cell activation, DNA hypomethylation, decreased intercellular communication, and genotoxicity.

4.7.3.2.1. ***Kupffer cell activation: release of cytokines and oxidants.*** The hypothesis is that Kuppfer cell activation plays a critical role in hepatocarcinogenesis. This MOA entails the following key events leading to TCA-induced liver tumor formation: following activation of Kupffer cells, oxidants and cytokines are released; the resultant oxidative stress and cytokines advance acquisition of the multiple critical traits contributing to carcinogenesis.

The liver consists of the hepatic parenchyma (hepatocytes) and non-parenchymal cells, including sinusoidal endothelial cells, Ito cells, and Kupffer cells. Kupffer cells are dedicated hepatic macrophages. Investigation of the role of non-parenchymal cells in mediating hepatocarcinogenesis has focused mainly on Kupffer cells, which have been proposed as important mediators of cell proliferation by tumor promoters (Hasmall et al., 2000a; Rusyn et al., 1998; Rose et al., 1997). The role of Kupffer cell activation in the induction of a proliferative response has been documented for peroxisome proliferators more generally, although evidence specific to TCA is limited.

Progress has been made in understanding the involvement of non-parenchymal cells, specifically Kupffer cells (i.e., liver-specific macrophages), in peroxisome-proliferator-induced liver tumors, though many questions remain. Yang et al. (2007) suggested that activation of non-parenchymal cells, which is independent of PPARα activation, plays an important role in peroxisome-proliferator-induced hepatocarcinogenesis. Specifically, induction of proliferation of non-parenchymal cells was observed in wild-type mice upon Wy-14,643 treatment, but not in transgenic mice. Yang et al. (2007) suggested that lack of tumor induction in transgenic mice as

compared to Wy-14,643-treated wild-type mice may be associated with the differences of non-parenchymal cell activation. To examine the role of Kupffer-cell-derived oxidants in the MOA for liver carcinogenesis, Woods et al. (2007a) treated NADPH-oxidase-deficient mice (their Kupffer cells cannot produce oxidants), along with wild-type and PPARα knockout mice, with Wy-14,643 for 1 week, 5 weeks, or 5 months. Wy-14,643 treatment induced similar levels of hepatocyte proliferation and DNA damage in NADPH-oxidase-deficient and wild-type mice, while both were abolished in PPARα knockout mice. By contrast, evidence of suppressed apoptosis by Wy-14,643 was absent in both NADPH-oxidase-deficient and PPARα knockout mice. Thus, NADPH oxidase was not required for chronic proliferative response or DNA damage, although it played a role in the suppression of apoptosis along with PPARα. Woods et al. (2007a) concluded that Kupffer-cell-derived oxidants may play a limited, if any, role in long-term effects of peroxisome proliferators, such as hepatocarcinogenesis.

Activation of Kupffer cells by toxic agents can result in the release a wide range of biologically active products, including reactive oxygen and nitrogen species, cytokines (Decker, 1990), such as TNF-α and ILs, proteases, and lipid metabolites, such as prostaglandins and thromboxane. The mediators released from Kupffer cells can initiate a variety of downstream events that may initially stimulate survival and protection but with continued or higher dose exposure, may ultimately contribute to hepatic injury. In particular, TNF-α has been linked to the stimulation of hepatocellular growth by tumor promoting compounds (Hasmall et al., 2000b). Roberts et al. (2007) hypothesized that activation of Kupffer cells caused the release of cellular growth regulatory signaling molecules that resulted in an increase in the proliferation of hepatocytes; this is expected to be transient in normal hepatocytes but sustained in preneoplastic, initiated hepatocytes, ultimately resulting in selective clonal expansion of the preneoplastic hepatocytes (i.e., hepatic tumor promotion).

Activation of Kupffer cells by peroxisome proliferators is PPARα independent (Peters et al., 2000), involves generation of reactive oxygen species, and leads to production of mitogenic cytokines (Rusyn et al., 2000). Peroxisome proliferators appear to directly activate Kupffer cells through mechanisms involving oxygen radicals, protein kinase C, and the transcription factor nuclear factor-kappa B (NF-κB) (Rose et al., 1999).

Activation of Kupffer cells resulted in production of super oxide anion via NADPH oxidase (Decker, 1990). It was suggested that Kupffer cell-derived oxidants play a role in signaling rapid and robust increases in cell proliferation caused by peroxisome proliferators in rodent liver via a mechanism that also involves activation of NF-κB and production of TNF-α (Rose et al., 2000).

Recent studies (Woods et al., 2007b; Woods et al., 2007a) have revealed that NADPH oxidase-dependent events in the Kupffer cells in response to peroxisome proliferators (Wy-14,643 and DEHP) may only be transient. As peroxisome proliferator treatment is continued, there appeared to be a shift of the cellular source of the radicals, from Kupffer cells to

hepatocytes. This is in line with the study findings from Hassoun and colleagues (Hassoun et al., 2010a; Hassoun et al., 2010b; Hassoun and Dey, 2008), who showed an increase in biomarkers of phagocytic activation, including superoxide anion and lipid peroxidation, in mice exposed to TCA once and for 4 weeks, but not in mice exposed for 13 weeks (see Section 4.5.1.6).

In summary, Kupffer cells, the resident macrophages of the liver, are mediators of acute phase responses to peroxisomal proliferators, including TCA. The release of cellular growth regulatory signaling molecules and oxidants from Kupffer cells results in an increase in the proliferation of hepatocytes, which may play a role in TCA-induced hepatocarcinogenesis.

4.7.3.2.2. ***Hypomethylation of DNA*.** The hypothesis is that TCA induces hepatocarcinogenesis via the induction of epigenetic changes, particularly DNA methylation. Key events in this MOA comprise the induction of epigenetic alterations that advance acquisition of the multiple critical traits contributing to carcinogenesis. Experimental evidence supports the hypothesis that hypomethylation of DNA may be related to the carcinogenicity of TCA in mice. In female $B6C3F_1$ mice that received an i.p. injection of MNU and were then administered TCA in drinking water at 25 mmol/L (4,085 mg/L) for 44 weeks, the level of 5MeC in the DNA of hepatocellular adenomas and carcinomas was decreased by 40 and 51%, respectively, as compared with noninvolved liver tissue from the same animal and control animals given only MNU; termination of TCA treatment 1 week prior to sacrifice did not change the levels of 5MeC in either adenomas or carcinomas (Tao et al., 1998). In another experiment, female $B6C3F_1$ mice treated with 25 mmol/L (1,062 mg/kg-day) TCA for 11 days in their drinking water also showed a 60% decrease in the level of 5MeC in total liver DNA (Tao et al., 1998).

The decrease in the level of 5MeC in these studies indicated that many genes may be hypomethylated. For example, Tao et al. (2000a) reported that the promoter regions of the *c-jun* and *c-myc* genes were hypomethylated in the livers of mice exposed to 500 mg/kg-day TCA for 5 days. Expression of the mRNA and proteins of these two protooncogenes were increased. This is in line with the studies by Latendresse and Pereira (1997) and Nelson et al. (1990), which reported increased mRNA and proteins of *c-jun* and *c-myc* protooncogenes in TCA-induced foci of altered hepatocytes and liver tumors. In another study (Tao et al., 2000b), the expression of the mRNA and proteins of the two protooncogenes were found to be increased in MNU-initiated and TCA-promoted mouse liver tumors. DNA methyltransferase activity was increased in tumors and decreased in noninvolved liver tissue. Increased expression of *c-jun* and *c-myc* has been associated with increased cell proliferation (Fausto and Webber, 1993; Saeter and Seglen, 1990). Therefore, increased expression and decreased methylation of the *c-jun* and *c-myc* genes could be involved in the carcinogenic activity of TCA by facilitating cell proliferation.

The same group of scientists (Tao et al., 2004) also demonstrated that a region of the IGF-II gene was hypomethylated in the livers of mice initiated with MNU and subsequently exposed to TCA. [The IGF-II gene is growth-related and is associated with hepatic cell

proliferation (Fürstenberger and Senn, 2002; Werner and Le Roith, 2000).] In TCA-exposed mice, the percentage of cytosine-guanine dinucleotide sites that were methylated was reduced from 79.3 to 58% in noninvolved liver tissue and further reduced to 10.7% in liver tumors. mRNA expression increased by 5.1-fold in liver tumors relative to noninvolved liver tissue from mice treated with TCA.

An association between hypomethylation and cell proliferation in liver of TCA-treated mice was demonstrated by Ge et al. (2001a). An increase in DNA replication (evidenced by increased proliferating cell nuclear antigen labeling index and mitotic labeling index) was observed 72 and 96 hours after the first gavage dose of 500 mg/kg-day TCA. Hypomethylation of the internal cytosine of CCGG sites in the promoter region of the *c-myc* gene began between 48 and 72 hours from the initiation of treatment with TCA and continued to 96 hours.

These experimental findings suggest that TCA induces global and locus-specific DNA hypomethylation in mouse liver. Given the recent finding discussed in Section 4.7.3.1.1.1 that the DNA hypomethylation by the potent PPARα agonist, Wy-14,643, was PPARα-dependent (Pogribny et al., 2007), but hypomethylation of DNA by DEHP-treated rats was not observed., hypomethylation of DNA may not be a key event in PPARα MOA. Moreover, hypomethylation is a relatively ubiquitous phenomenon in carcinogenesis and it has not been demonstrated that TCA-induced hypomethylation is PPARα-dependent. Therefore, the possibility of hypomethylation of DNA as a PPARα-independent MOA cannot be discounted.

4.7.3.2.3. ***Decreased intercellular communication.*** Inhibition of intercellular communication has been identified as a contributor to tumor induction by some peroxisome proliferators (Klaunig et al., 2003; Klaunig et al., 1988). However, similar inhibition has been reported with nongenotoxic liver carcinogens that are not peroxisome proliferators. Thus, this hypothesized MOA is not specific to peroxisome proliferators and PPARα agonism. This MOA has not been well characterized with respect to the component key events.

From a physiological perspective, the formation of gap junctions with short half-lives in cell membranes can be considered a regulatory control factor for tumor formation (Benane et al., 1996). Transfer of molecules from neighboring normal cells to transformed cells via intercellular communication allows growth suppression of transformed cells. Blocking intercellular communication on a repetitive basis releases the "initiated" cells from the growth control constraint exerted by neighboring cells and facilitates tumor formation. Studies by Benane et al. (1996) and Klaunig et al. (1989) (see Section 4.5.1) suggest that TCA-induced inhibition of gap junction intercellular communication could potentially play a role in regulation of cell differentiation, growth and homeostasis, and tumor promotion.

4.7.3.2.4. ***Genotoxicity.*** A hypothesized mutagenic MOA entails the following key events leading to TCA-induced liver tumor formation: TCA alters the genetic material in a manner that

causes changes to be transmitted during cell division through one or more mechanisms (gene mutations, deletions, translocations, or amplification). TCA has been tested for genotoxicity in a variety of in vitro and in vivo assays as described in Section 4.5.2. Most, but not all, studies (Kargalioglu et al., 2002; Nelson et al., 2001; DeMarini et al., 1994; Rapson et al., 1980) report negative results for mutagenicity in *S. typhimurium* in the absence of cytotoxicity. Mutagenicity in mouse lymphoma cells was only induced at cytotoxic concentrations (Harrington-Brock et al., 1998). Both positive and negative responses have been observed in vivo. TCA-induced DNA strand breaks and chromosome damage were observed in the liver in several studies (Giller et al., 1997; Nelson and Bull, 1988; Bhunya and Behera, 1987) and were suggested by the results of Harrington-Brock et al. (1998), although these effects have not been uniformly reported (Chang et al., 1992; Styles et al., 1991). However, some evidence indicates that TCA-induced chromosome damage assayed in vitro and in vivo may be secondary to pH changes rather than a direct effect of TCA (Mackay et al., 1995), underscoring the need to carefully evaluate assay conditions.

In other studies of potential genotoxicity, DNA-repair responses to TCA in bacterial systems have been inconsistent, with induction of DNA repair reported in *S. typhimurium* (Ono et al., 1991) but not in *E. coli* (Giller et al., 1997). TCA induced oxidative DNA damage in the livers of mice following a single dose (Austin et al., 1996) but not following repeated dosing over 3 or 10 weeks (Parrish et al., 1996), possibly suggesting either effective DNA repair and/or adaptation to repeated TCA exposures. Ferreira-Gonzalez et al. (1995) found that the mutation frequency and mutation spectrum in the H-*ras* gene were similar in tumors from control and TCA-treated mice, suggesting that TCA was not inducing tumors through direct DNA damage at this locus. The pattern of TCA-induced tumors in mice does not support a mutagenic MOA. Tumors were observed only in livers of TCA-exposed mice and no tumors were found in TCA-treated rats.

In summary, there is some evidence that TCA is weakly mutagenic. Therefore, the hypothesis that mutagenicity contributes to the MOA for TCA-induced liver tumors cannot be ruled out.

4.7.3.3. *Conclusions About the Hypothesized Mode of Action*

In summary, TCA is carcinogenic in mice (DeAngelo et al., 2008; Bull et al., 2002; Pereira, 1996; Bull et al., 1990; Herren-Freund et al., 1987). Studies of the mechanism by which TCA induces liver tumors reveal that the MOA for TCA is complex and that TCA may induce tumors by multiple MOAs that may not be mutually exclusive. While PPARα-related events represent some of the major components of the overall mechanism of toxicity and carcinogenicity, it is premature to conclude that this is the only MOA for TCA-induced carcinogenicity. In addition, in light of new evidence that challenges the hypothesis that PPARα is absolutely required for hepatocarcinogenesis of peroxisome proliferators in mice (Ito et al.,

2007), the strength of this linkage becomes more uncertain. Tumor induction by TCA appears to involve perturbation of cell growth, reduced intercellular communication, release of cytokines and oxidants by activated Kupffer cells, and hypomethylation of DNA. The data do not support a major role for a mutagenic MOA (Bull, 2000; Moore and Harrington-Brock, 2000).

4.8. SUSCEPTIBLE POPULATION AND LIFE STAGES

4.8.1. Possible Childhood Susceptibility

Age-dependent differences in susceptibility to TCA have not been investigated in systemic toxicity studies. The dose spacing in the available developmental toxicity studies (Table 4-10) is inadequate to determine the relative fetal and maternal toxicity of TCA. The LOAELs for developmental toxicity range from 291 mg/kg-day (Johnson et al., 1998) to 1,000 mg/kg-day (Singh, 2005a). Most developmental LOAELs occurred at maternally toxic doses. Therefore, these developmental toxicity data are too limited to draw any conclusions on whether developing organisms might be a sensitive subpopulation. In subchronic toxicity studies, LOAEL and NOAEL values of 355 and 36.5 mg/kg-day, respectively, were observed in male rats exposed to TCA in drinking water for 90 days (Mather et al., 1990). In the Parrish et al. (1996) 10-week drinking water study with male mice, the LOAEL and NOAEL values were 125 and 50 mg/kg-day, respectively. The LOAELs observed in the subchronic toxicity studies suggest that systemic effects are observed at doses similar to, or less than, those at which developmental toxicity has been observed; however, no developmental NOAELs are available for comparison with the subchronic systemic NOAELs. Given the lack of a developmental NOAEL, it is uncertain what dose would be protective for developmental toxicity.

The data are also insufficient to determine whether there are age-dependent differences in the toxicokinetics (e.g., plasma binding and metabolism) of TCA that might lead to differences in health risk. There are no published comparative data for plasma binding of TCA in young and old animals. The enzymes responsible for the metabolism of TCA have not been conclusively identified. Even in the cases where relevant metabolizing enzymes have been identified, no information on age-dependent changes in the expression or activity of these enzymes has been identified. The health implications of any differences between children and adults in metabolic capacity are also difficult to determine for the haloacetic acids, since the toxic form of each compound has not been identified. The mechanisms involved in haloacetic acid toxicity are not sufficiently understood to make this determination. The preliminary results of Hunter and Rogers (1999) in whole embryo culture suggest that, at least for developmental effects, the parent compound may be involved in the toxicity of MCA, while for TCA, a metabolite may be involved. However, in vitro studies such as whole embryo culture have limited utility for predicting the developmental toxicity of chemical agents in intact organisms and are considered to be useful only for hypothesis generation not for hypothesis testing. Further in vivo studies are

needed to determine whether there are age-related differences in susceptibility to toxic effects of TCA.

The cancer potency of TCA in very young animals has been investigated in a mouse neonatal cancer assay (Von Tungeln et al., 2002). In this study, neonatal male and female B6C3F$_1$ mice were given i.p. injections of TCA in dimethyl sulfoxide at 1,000 or 2,000 nmol (total dose, which corresponds to approximately 16 or 32 mg/kg) in split doses delivered at 8 and 15 days of age. The test animals were sacrificed and evaluated for liver tumors at 12 (high dose) or 20 (low dose) months of age. The incidence of hepatic tumors in TCA-treated animals did not differ significantly from tumor incidences observed in the solvent controls.

4.8.2. Possible Gender Differences

The available animal data, although limited, suggest that males may be more sensitive to the carcinogenicity of TCA than females. Only one cancer bioassay was located that concurrently exposed both male and female mice to TCA (Bull et al., 1990) (described in Section 4.2). In this study, male B6C3F$_1$ mice exposed to TCA in drinking water for 52 weeks showed a clear dose-related increase in animals with proliferative lesions (hyperplastic nodules, adenomas, or carcinomas), whereas the incidence of proliferative lesions in females was not increased. Although no other studies were available that evaluated the carcinogenicity of TCA in males and females concurrently, the available single-sex cancer bioassays conducted in separate laboratories also suggest that males may be more sensitive than females to TCA carcinogenicity. For example, Pereira et al. (2001) (described in Section 4.2) observed a tumor incidence of 25% in female B6C3F$_1$ mice exposed to TCA in the drinking water at a dose of 784 mg/kg-day for 51 weeks. In contrast, tumor incidences ranging from 55 to 83% have been reported in males exposed to lower TCA doses (309–480 mg/kg-day) in the drinking water for a comparable duration (Bull, 2000; Bull et al., 1990).

Although males appear to be more sensitive than females to carcinogenicity of TCA, the available data suggest that males and females are about equally sensitive to noncancer effects induced by TCA. For example, Bull et al. (1990) observed that the type and magnitude of the noncancer liver effects induced by TCA were similar in male and female B6C3F$_1$ mice exposed to TCA in the drinking water at comparable doses for 52 weeks. Davis (1990) did not observe marked differences in the susceptibility of males and females to TCA-induced noncancer effects in a short-term toxicity study. Although both of these studies were limited by the scope of toxicological parameters evaluated, they suggest that male and female animals are similar in their sensitivity to TCA-induced noncancer effects.

4.8.3. Other

Limited information was identified regarding other factors (e.g., genetic polymorphisms, enzyme deficiencies, or altered health states) that might influence susceptibility to TCA. Some

data are available for DCA and may be relevant to TCA. Several genetic polymorphisms have been identified in GST-ζ, a key enzyme involved in DCA metabolism. As noted previously, it is unclear whether TCA is metabolized to DCA (Lash et al., 2000; Bull, 2000); these polymorphisms would be relevant to TCA susceptibility only if DCA is a metabolite of TCA.

As noted previously, TCA induces glycogen accumulation. Kato-Weinstein et al. (1998) suggested that prolonged glycogen accumulation can become irreversible. These data suggest that individuals with glycogen storage disease (an inherited deficiency or alteration in any one of the enzymes involved in glycogen metabolism) constitute another group that may be more susceptible to TCA toxicity.

No quantitative evaluation has been conducted on the health impact of environmental exposures for individuals harboring polymorphisms in genes related to glycogen storage or antioxidant response. In each of these cases, a significant background load of the stressor may be present; thus, the excess risk associated with low doses of TCA is not clear.

5. DOSE-RESPONSE ASSESSMENTS

5.1. ORAL REFERENCE DOSE (RfD)

The RfD[15] for TCA was derived through a three-step process consistent with EPA guidance (U.S. EPA, 2002): (1) evaluating all toxicity studies and selecting the critical effects from these studies that occur at the lowest dose; (2) selecting the dose or point of departure[16] (POD) at which the critical effect either is not observed or would be predicted to occur at a relatively low incidence (e.g., 10%); and (3) dividing this POD by uncertainty factors (UFs) to reflect uncertainties in extrapolating from study conditions to conditions of human environmental exposure.

5.1.1. Choice of Principal Study and Critical Effect—Rationale and Justification

Chronic, subchronic, and developmental animal toxicity studies considered for derivation of the oral RfD are summarized in Table 5-1. Two of the available chronic oral drinking water studies (DeAngelo et al., 2008; DeAngelo et al., 1997) were identified as potential candidates from which to derive the RfD. The study in rats by DeAngelo et al. (1997) identified a NOAEL of 32.5 mg/kg-day and a LOAEL of 364 mg/kg-day based on significantly decreased body weight, a statistically significant and dose-related increase in serum ALT activity, and histopathologic changes in the liver. The study in mice by DeAngelo et al. (2008) identified a NOAEL of 8 mg/kg-day and a LOAEL of 68 mg/kg-day for increased liver weight, liver peroxisome proliferation, hepatic necrosis, and testicular tubular degeneration. Histopathologic examinations were conducted on organs other than the liver in both DeAngelo et al. (1997) and DeAngelo et al. (2008); other chronic mouse studies have only evaluated the liver. In a cancer study in mice by Pereira (1996), only a limited number of endpoints were evaluated, but a higher NOAEL of 78 mg/kg-day for liver effects was identified. Two other chronic-duration drinking water studies (Bull et al., 1990; Herren-Freund et al., 1987) were not further considered for derivation of the RfD because they examined only a limited number of endpoints in the liver and used higher administered doses than those employed by DeAngelo et al. (2008; 1997).

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[15]The RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious effects during a lifetime. It can be derived from a NOAEL, LOAEL, or benchmark dose (BMD), with UFs generally applied to reflect limitations of the data used. The RfD is expressed in terms of mg/kg-day of exposure to an agent.

[16]The POD denotes a dose at the lower end of the observed dose-response curve where extrapolation to lower doses begins. For effects other than cancer, the POD is either a NOAEL, a LOAEL if no NOAEL can be identified, or a modeled point (for example, a 95% lower bound on exposure dose or concentration at 10% extra risk) if the data are suitable for dose-response modeling.

Table 5-1. Candidate studies for derivation of the RfD for TCA

Reference	Species	Exposure route	Exposure duration	Doses evaluated (mg/kg-d)	Observed effects	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Chronic studies								
DeAngelo et al. (1997)	F344 rats (males, 50/group)	Oral, drinking water	104 wks	0, 3.6, 32.5, or 364	Decreased body weight, increased serum ALT activity; increased peroxisome proliferation	32.5	364	Time-weighted average daily doses were calculated by the authors; a comprehensive set of tissues was microscopically examined.
DeAngelo et al. (2008)	B6C3F$_1$ mice (males, 50/group)	Oral, drinking water	60 wks	0, 8, 68, or 602	Increase in liver weight, increase in liver peroxisome proliferation, hepatic necrosis, testicular tubular degeneration	8	68	Time-weighted average daily doses were calculated by the authors; a comprehensive set of tissues was microscopically examined for the control and high-dose groups.
Pereira (1996)	B6C3F$_1$ mice (females, 38–134/group)	Oral, drinking water	51 or 82 wks	0, 78, 262, or 784	Increased relative liver weight	78	262	Increased liver weight was observed after 82 wks at 262 mg/kg-d; 262 mg/kg-d was judged to be an equivocal LOAEL in the absence of other measures of liver toxicity.
Bull et al. (1990)	B6C3F$_1$ mice (11–35/sex and dose)	Oral, drinking water	(A) 52 wks (B) 37 wks + 15-wk recovery	(A) 0, 164, or 329 (B) 0 or 309	Increased absolute and relative liver weight, cytomegaly, glycogen accumulation	Not determined	164	Only the liver and kidneys were evaluated; dose was estimated by the authors.
Herren-Freund et al. (1987)	B6C3F$_1$ mice (males, 22–33/group)	Oral, drinking water	61 wks	0, 500, or 1,250	Increased absolute and relative liver weight	Not determined	500	Only the liver was microscopically examined.

Table 5-1. Candidate studies for derivation of the RfD for TCA

Reference	Species	Exposure route	Exposure duration	Doses evaluated (mg/kg-d)	Observed effects	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Subchronic studies								
Mather et al. (1990)	Sprague-Dawley rats (males, 10/dose)	Oral, drinking water	90 d	0, 4.1, 36.5, or 355	Decreased absolute spleen weight; increased relative liver and kidney weights; peroxisome proliferation	36.5	355	
Bhat et al. (1990)	Sprague-Dawley rats (males, 5/group)	Oral, drinking water	90 d	0 or 825	Decreased body weight gain; minor changes in liver morphology; collagen deposition; perivascular inflammation of the lungs	Not determined	825	1/4 of the LD_{50} (3,300 mg/kg) was administered daily.
Developmental studies								
Smith et al. (1989)	Long-Evans rats (20–21/dose)	Oral, gavage	GDs 6–15	0, 330, 800, 1,200, or 1,800	Maternal: decreased body weight; increased spleen and kidney weights Developmental: decreased fetal weight, decreased crown-rump length, increased incidence of soft-tissue and cardiovascular malformations (mainly levocardia); increased maternal spleen and kidney weights	Maternal: Not determined Develop-mental: Not determined	Maternal: 330 Develop-mental: 330	Critical study for 1994 RfD. The developmental LOAEL was also a maternal LOAEL. Cardiovascular malformations were not confirmed by Fisher et al. (2001).

Table 5-1. Candidate studies for derivation of the RfD for TCA

Reference	Species	Exposure route	Exposure duration	Doses evaluated (mg/kg-d)	Observed effects	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	Comments
Fisher et al. (2001)	Sprague-Dawley rats (19/dose)	Oral, gavage	GDs 6–15	0 or 300	Maternal: decreased body weight gain on GDs 7–15 and 18–21; decreased uterine weight Developmental: Decreased fetal body weight (per litter and per fetus)	Maternal: Not determined Develop-mental: Not determined	Maternal: 300 Develop-mental: 300	A limited number of fetal endpoints were evaluated, including sex, fetal weight, and incidence of heart malformations.
Johnson et al. (1998)	Sprague-Dawley rats (55 controls and 11 TCA treated rats)	Drinking water	GDs 1-22	0 or 291	Maternal: decreased body weight Developmental: Increase in cardiac malformations; increase in number of implantation sites/litter, number of resorption sites/litter, and total resorptions	Maternal: None Develop-mental: None	Maternal: 291 Develop-mental: 291	Dose estimated by the authors, based on the average amount of water consumed by the animals on a daily basis. Study was not adequately designed and/or reported, and a complete array of standard developmental endpoints was not assessed.

Subchronic toxicity data were available from studies conducted in rats by Mather et al. (1990) and Bhat et al. (1990). The 90-day drinking water study by Mather et al. (1990) established NOAEL and LOAEL values of 36.5 and 355 mg/kg-day, respectively, for effects on relative liver and kidney weights and peroxisome proliferation. These values are similar to and support the NOAEL and LOAEL values obtained for hepatic effects in the chronic study of DeAngelo et al. (1997) in rats. Bhat et al. (1990) observed decreased body weight gain, minor changes in liver morphology, and inflammation of the lungs in rats administered a dose equivalent to one-fourth of the LD_{50} of 3,300 mg/kg (or approximately 825 mg/kg-day).

Three developmental toxicity studies (Fisher et al., 2001; Johnson et al., 1998; Smith et al., 1989) were also evaluated as potential candidates for use in the derivation of the RfD. Smith et al. (1989) identified a developmental LOAEL of 330 mg/kg-day (the lowest dose tested) for increased incidence of fetal cardiac malformations and significantly reduced fetal body weight and crown-rump length in Long-Evans rats dosed by gavage on GDs 6–15. Johnson et al. (1998) identified a developmental LOAEL of 291 mg/kg-day for fetal cardiac malformations in a single-dose study where Sprague-Dawley rats were dosed via drinking water on GDs 1–22. Fisher et al. (2001) observed decreased fetal body weight, but saw no evidence of cardiac malformations in a single-dose study where Sprague-Dawley rats were dosed with 300 mg/kg-day by gavage on GDs 6–15. Because of inconsistent findings of cardiac malformations (in particular levocardia) across the three developmental toxicity studies and questions of interpretation raised by Smith et al. (1989) (see Section 4.6.1.3), cardiac malformation was not considered a candidate critical effect. Furthermore, because the incidence of total soft tissue (visceral) malformations as reported by Smith et al. (1989) was attributable largely to the incidence of cardiac malformations, total soft tissue malformations were similarly not considered a candidate critical effect. Fisher et al. (2001) and Johnson et al. (1998) were single-dose studies, and as such, provide less useful information for dose-response analysis than does Smith et al. (1989). Johnson et al. (1998) also suffers from issues related to adequacy of reporting and limited examination of endpoints. Therefore, Fisher et al. (2001) and Johnson et al. (1998) were not considered as candidate principal studies.

The chronic drinking water study in mice by DeAngelo et al. (2008) was considered the most appropriate choice among the available studies for derivation of the RfD. In this study, the route of exposure was oral, both a LOAEL and NOAEL were identified for liver effects that were both lower than the corresponding values identified in the chronic drinking water study in rats (DeAngelo et al., 1997), and the data in this chronic mouse study were consistent with the findings in both chronic drinking water studies in rats (DeAngelo et al., 1997; Mather et al., 1990). In addition, complete histopathologic examinations were conducted for all organs for the control and high-dose groups, whereas other studies in mice only evaluated the liver. Moreover, the incidence data in DeAngelo et al. (2008) were amenable to benchmark dose (BMD) modeling.

Selected data sets from the developmental toxicity study conducted by Smith et al. (1989), specifically data on mean fetal body weight and fetal crown-rump length, were analyzed by BMD modeling for comparison with the candidate PODs derived for endpoints from the DeAngelo et al. (2008) study.

5.1.2. Methods of Analysis—Including Models (e.g, PBPK and BMD)

5.1.2.1. *BMD Modeling of Liver and Testicular Effects from DeAngelo et al. (2008)*

BMD modeling was used to analyze liver and testicular effects in male mice exposed to TCA in drinking water (DeAngelo et al., 2008). Incidence data for hepatocellular inflammation, hepatocellular necrosis, and testicular tubular degeneration are summarized in Table 5-2 and mean PCO activity (a marker of peroxisome proliferation) is summarized in Table 5-3. The incidence of hepatocellular cytoplasmic alteration was elevated in TCA-exposed mice (see Table 4-4); however, because the incidence deviated from a monotonic dose-response relationship, this endpoint was not subject to BMD modeling.

Table 5-2. Incidence of nonneoplastic lesions in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks

Lesion	Control	0.05 g/L TCA (8 mg/kg-d)[a]	0.5 g/L TCA (68 mg/kg-d)[a]	5 g/L TCA (602 mg/kg-d)[a]
Hepatocellular inflammation	3/30	0/27	2/29	7/29[b]
Hepatocellular necrosis	0/10	0/10	3/10	5/10[b]
Testicular tubular degeneration	2/30	0/27	4/29[b]	6/29[b]

[a]Time-weighted mean daily dose in mg/kg-day.
[b]Statistically significant from the control group, $p \leq 0.05$.

Source: DeAngelo et al. (2008).

Table 5-3. Mean PCO activity in male B6C3F$_1$ mice exposed to TCA in drinking water for up to 60 weeks

	Control	0.05 g/L TCA (8 mg/kg-d)[b]	0.5 g/L TCA (68 mg/kg-d)[b]	5 g/L TCA (602 mg/kg-d)[b]
Mean PCO activity (nmol NAD reduced/min/mg protein)[a]	2.59 ± 1.04	2.85 ± 0.86	4.75 ± 1.16	11.99 ± 3.04

[a]Mean PCO activity ± SD was calculated as an arithmetic mean of the PCO activity for mice sacrificed at weeks 4, 15, 30, 45, and 60. PCO activity for each time point was based on five mice/group/time point. The total number of mice for each concentration was 25 (with the exception of 24 mice for the 5 g/L TCA group).
[b]Time-weighted mean daily dose in mg/kg-day.

Source: DeAngelo et al. (2008) and email dated March 12 2010, from Anthony DeAngelo, NHEERL, ORD, U.S. EPA, to Diana Wong, NCEA, ORD, U.S. EPA.

All of the available dichotomous models in U.S. EPA's benchmark dose software (BMDS) (version 1.4.1) were fit to incidence data for hepatocellular inflammation, hepatocellular necrosis, and testicular tubular degeneration. Doses (i.e., benchmark dose [BMD_{10}] and 95% lower confidence limit on the BMD [$BMDL_{10}$]) associated with a benchmark response (BMR) of 10% extra risk were calculated. A BMR of 10% is generally used in the absence of information regarding what level of change is considered biologically significant, and also to facilitate a consistent basis of comparison across assessments (U.S. EPA, 2000a). In addition, the minimal to mild severity of the endpoints (see Table 4-4) and the relatively low sensitivity of the principal study (i.e., resulting from the use of 10 mice/group) did not warrant the use of a BMR less than 10%. All of the continuous models in BMDS (version 2.1.1) were fit to mean PCO activity data. A BMR of 1 SD from the control mean was used to calculate the BMD_{1SD} and $BMDL_{1SD}$ for mean PCO activity. A BMR of 1 SD is generally used as the BMR for continuous data in the absence of knowledge of what level of response to consider as biologically significant, and to facilitate a consistent basis of comparison across assessments where continuous data are used (U.S. EPA, 2000a).

Details of the BMD modeling conducted for each data set from DeAngelo et al. (2008) are provided in Appendix B. In general, model fit was assessed by a χ^2 goodness-of-fit test (i.e., models with $p < 0.1$ failed to meet the goodness-of-fit criterion) and the Akaike's Information Criterion (AIC) value (i.e., a measure of the deviance of the model fit that allows for comparison across models for a particular endpoint). Of the models exhibiting adequate fit, the lowest BMDL was selected as the POD when the BMDLs estimated from these models varied by more than threefold; otherwise, the BMDL from the model with the lowest AIC was chosen. If more than one model shared the lowest AIC, $BMDL_{10}$ values from these models were averaged to obtain a POD (U.S. EPA, 2000a). The model results for the best fitting model for each data set from DeAngelo et al. (2008) are summarized in Table 5-4.

Table 5-4. BMD modeling results for data sets from DeAngelo et al. (2008)

Endpoint	Best fitting model	BMR	BMD (mg/kg-d)	BMDL (mg/kg-d)
Hepatocellular inflammation in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 wks[a]	Logistic and log-probit	Extra risk 10%	393.0[c]	260.5[c]
Incidence of hepatocellular necrosis in male B6C3F$_1$ mice exposed to TCA in drinking water for 30–45 wks[a]	Log-logistic	Extra risk 10%	40.7	17.9
Incidence of testicular tubular degeneration in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 wks[a]	Log-logistic	Extra risk 10%	298.2	127.4
Cyanide-insensitive PCO activity in male B6C3F$_1$ mice exposed to TCA in drinking water for up to 60 wks[b]	Polynomial (2°)	1 SD	28.4	21.1

[a]All dichotomous dose-response models were fit using BMDS, version 1.4.1. The best-fit model is presented here. See Appendix B for the results of all dichotomous models in BMDS.
[b]All continuous dose-response models were fit using BMDS, version 2.1.1. The best-fit model is presented here. See Appendix B for the results of all continuous models in BMDS.
[c]Because the logistic and log-probit models shared the lowest AIC value (i.e., 74.19), the BMD_{10} and $BMDL_{10}$ values from these two models were averaged.

For hepatocellular inflammation, the logistic, one-stage multistage, probit, and log-probit models all exhibited adequate fit. Because the logistic and log-probit models shared the lowest AIC value (i.e., 74.19), the $BMDL_{10}$ values from these two models were averaged to yield a candidate POD of 260.5 mg/kg-day. Four of the seven dichotomous models in BMDS fit to the incidence of hepatocellular necrosis in male mice exhibited adequate fit. These four models were the gamma, log-logistic, one-stage multistage, and Weibull models. Among these four models, the gamma, one-stage multistage, and Weibull models yielded identical fits, essentially reducing the number of adequately fitting models to two. The log-logistic model yielded the lowest AIC value (i.e., 30.42) of the two adequately fitting models. Thus, the $BMDL_{10}$ of 17.9 mg/kg-day estimated by the log-logistic model was selected as a candidate POD. All of the models fit to the incidence of testicular tubular degeneration exhibited adequate fit. Of these seven models, the gamma, one-stage multistage, and Weibull models yielded identical fits, essentially reducing the number of adequately fitting models to five. The log-logistic model yielded the lowest AIC (i.e., 76.08). Therefore, the $BMDL_{10}$ estimate of 127.4 mg/kg-day from the log-logistic model was selected as a candidate POD. For mean PCO activity, only the second-degree polynomial model of the four continuous models in BMDS showed adequate fit. Thus, the $BMDL_{1SD}$ of 21.1 mg/kg-day estimated by the second-degree polynomial model was selected as a candidate POD for this endpoint.

Of the four endpoints evaluated by DeAngelo et al. (2008) for which dose-response modeling was performed, hepatocellular necrosis was the most sensitive, as it yielded the lowest

POD of 17.9 mg/kg-day. Therefore, 17.9 mg/kg-day was selected as a candidate POD for use in derivation of the RfD.

5.1.2.2. *BMD Modeling of Developmental Toxicity Data from Smith et al. (1989)*

As discussed in Section 5.1.1, selected data from the developmental toxicity study conducted by Smith et al. (1989)—fetal body weight and fetal crown-rump length—were analyzed by BMD modeling for comparison with the POD derived from DeAngelo et al. (2008). These data sets are summarized in Table 5-5.

Table 5-5. Dose-response data for developmental endpoint in TCA-treated Long-Evans rats

Endpoint	Dose (mg/kg-d)				
	0	**330**	**800**	**1,200**	**1,800**
Mean fetal crown-rump length (cm)					
Male	3.71 ± 0.12	3.58 ± 0.10[a]	3.46 ± 0.10[a]	3.36 ± 0.15[a]	3.16 ± 0.12[a]
Female	3.64 ± 0.15	3.53 ± 0.09[a]	3.38 ± 0.12[a]	3.33 ± 0.16[a]	3.15 ± 0.15[a]
Mean fetal body weight (g)					
Male	3.70 ± 0.24	3.20 ± 0.26[a]	2.98 ± 0.17[a]	2.74 ± 0.30[a]	2.49 ± 0.16[a]
Female	3.54 ± 0.20	3.08 ± 0.27[a]	2.83 ± 0.18[a]	2.67 ± 0.29[a]	2.36 ± 0.15[a]

[a]Mean is significantly different from control mean ($p \leq 0.05$) as reported by Smith et al. (1989).

Source: Smith et al. (1989).

All of the continuous models in BMDS (version 2.1.1) provided by U.S. EPA's were fit to the data for fetal body weight and fetal crown rump length data. Doses (i.e., BMD_{05} and $BMDL_{05}$) associated with a BMR of 5% extra risks were calculated. A BMR of 5% extra risk was selected for developmental endpoints to assure protection of the sensitive developing fetus. This selection is consistent with the EPA's BMD technical guidance (U.S. EPA, 2000a).

Details of the BMD modeling conducted for each data set from Smith et al. (1989) are provided in Appendix C. As with the endpoints from DeAngelo et al. (2008), model fit was assessed by a χ^2 goodness-of-fit test (i.e., models with $p < 0.1$ failed to meet the goodness-of-fit criterion) and the AIC value. The model results for the best fitting model for each data set from Smith et al. (1989) are summarized in Table 5-6.

Table 5-6. BMD modeling results for data sets from Smith et al. (1989)

Endpoint	Best fitting model	BMR	BMD (mg/kg-d)	BMDL (mg/kg-d)
Fetal body weight				
Male[a]	Hill	Relative deviation 5%	121.4	84.0
Female[a]	Hill		126.5	87.7
Fetal crown-rump length				
Male[a]	Exponential model 2	Relative deviation 5%	600.7	534.4
Female[a]	Exponential model 2		650.9	562.9

[a]All continuous dose-response models were fit using BMDS, version 2.1.1. The best-fit model is presented here. See Appendix C for the results of all continuous models in BMDS.

For body weight in male and female fetuses, two of the continuous models in BMDS exhibited adequate fit—the exponential model 4 and the Hill model. For both male and female data, the Hill model yielded the lowest AIC values (i.e., -151.07 and -158.80) of the adequately fitting models. The $BMDL_{05}$ of 84.0 mg/kg-day estimated by the Hill model for male fetal body weight was smaller than the $BMDL_{05}$ of 87.7 mg/kg-day for female fetal body weight and was therefore selected as a candidate POD. It should be noted that this value is well below the lowest tested dose of 330 mg/kg-day.

For fetal crown-rump length in male and female fetuses, all continuous models in BMDS exhibited adequate fit. For both male and female fetal data, exponential model 2 had the lowest AIC value (i.e., -273.53 and -250.59, respectively). The $BMDL_{05}$ of 534.4 mg/kg-day estimated by exponential model 2 for male fetal crown-rump length was smaller than the $BMDL_{05}$ of 562.9 mg/kg-day for females and was therefore selected as a candidate POD.

5.1.2.3. *Selection of POD*

Comparison of the candidate PODs based on endpoints from the DeAngelo et al. (2008) study with PODs based on developmental endpoints reported by Smith et al. (1989) reveal that the liver endpoints are more sensitive than testicular or developmental endpoints. Therefore, the $BMDL_{10}$ of 17.9 mg/kg-day based on hepatocellular necrosis was selected as the POD for use in deriving the TCA RfD.

5.1.3. RfD Derivation—Including Application of Uncertainty Factors (UFs)

The chronic mouse drinking water study by DeAngelo et al. (2008) was selected as the principal study for derivation of the oral RfD as discussed in Section 5.1.1. The RfD for TCA was calculated from the POD of 17.9 mg/kg-day (based on the incidence of hepatocellular necrosis) and application of a composite UF of 1,000, as follows:

RfD = POD ÷ UF
= 17.9 mg/kg-day ÷ 1,000
= 0.0179 mg/kg-day, rounded to 0.02 mg/kg-day

The UFs, selected based on EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002; Section 4.4.5), address five areas of uncertainty. The UFs applied to the selected POD to derive an RfD are as follows:

- *Human variation*. An UF of 10 was selected for interindividual variability to account for human-to-human variability in susceptibility in the absence of quantitative information to assess the toxicokinetics and toxicodynamics of TCA in humans.

- *Animal-to-human extrapolation*. An UF of 10 was selected for interspecies extrapolation to account for uncertainty in extrapolating from laboratory animals to humans (i.e., interspecies variability) because information was unavailable to quantitatively assess toxicokinetic or toxicodynamic differences between animals and humans for TCA.

- *Database deficiences*. An UF of 10 was used to account for database deficiences. There are no TCA-specific systemic toxicity data in humans. Although subchronic and chronic animal studies of TCA have been conducted in rats and mice, most studies have focused primarily or exclusively on liver lesions and have not examined other organs for microscopic lesions. DeAngelo et al. (2008) is the only study in mice that included histopathological examination of organs other than the liver; however, complete histopathologic examinations were performed on only five mice from the high-dose and control groups. Other data gaps include lack of a multigeneration reproductive toxicity study. Available developmental studies were conducted at high doses, and did not allow identification of a NOAEL.

- *Subchronic-to-chronic extrapolation*. An UF for study duration was not required in this assessment because the principal study was of chronic duration.

- *LOAEL-to-NOAEL extrapolation*. An UF for LOAEL-to-NOAEL extrapolation was not applied because the current approach is to address this factor as one of the considerations in selecting a BMR for BMD modeling. In this case, a BMR of 10% increase in the incidence of hepatocellular necrosis was selected under an assumption that it represents a minimally biologically significant change.

5.1.4. RfD Comparison Information

The RfD derived using liver as an endpoint (specifically hepatocellular necrosis) based on data from the DeAngelo et al. (2008) mouse study was compared with potential reference values that would result from the use of alternative critical effects in target organs other than the liver, specifically testicular effects identified in the rat (DeAngelo et al., 2008) and developmental effects (decreased fetal body weight) in the rat (Smith et al., 1989). The potential reference values derived from these studies are presented in Figure 5-1.

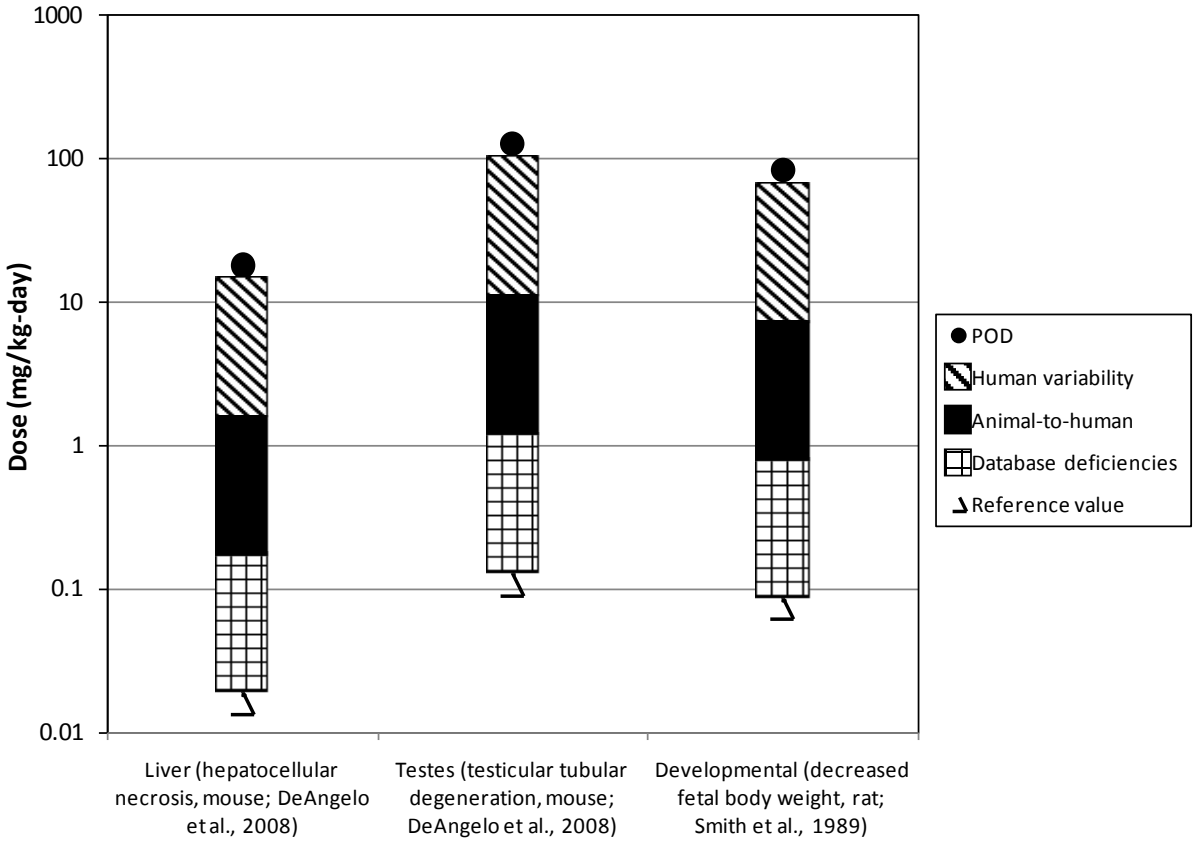

Figure 5-1. PODs (mg/kg-day) with corresponding potential oral reference values that would result if alternative endpoints were used as the critical effect.

5.1.5. Previous RfD Assessment

The previous IRIS assessment for TCA did not provide an RfD.

5.2. INHALATION REFERENCE CONCENTRATION (RfC)

No inhalation studies adequate for the derivation of an RfC[17] were located. The respiratory tract has not been examined in oral studies of TCA. Because the liver is the critical target organ for oral toxicity and first-pass effect by the liver is expected following oral administration, the route of exposure may influence the hepatic response to TCA. PBPK models that would support route-to-route extrapolation for TCA have not been published. Thus, the available information is inadequate for extrapolation of oral toxicity data to the inhalation pathway. For these reasons, an RfC for TCA was not derived.

[17]The RfC is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious effects during a lifetime. It can be derived from a NOAEL, LOAEL, or benchmark concentration, with UFs generally applied to reflect limitations of the data used.

5.3. UNCERTAINTIES IN THE RfD

The following discussion identifies uncertainties associated with the RfD for TCA. As presented in Section 5.1.3, the UF approach, following EPA methodology for RfD development (U.S. EPA, 2002), was applied to a POD. For the RfD, the POD was determined as the $BMDL_{10}$ for hepatocellular necrosis in treated mice. Factors accounting for uncertainties associated with a number of steps in the analyses were adopted to account for extrapolating the POD, the starting point in the analysis, to a diverse population of varying susceptibilities. These extrapolations are carried out with default approaches instead of from data on TCA, given the limited experimental TCA data to inform individual steps.

Selection of principal study and critical effect for reference value determination. The selected principal study (DeAngelo et al., 2008) was the most complete study in mice, with a well-defined NOAEL/LOAEL and data that were amenable to dose-response modeling. Complete histopathologic examination was conducted for the high-dose and control groups, although examination was limited to only five mice from each group, and female mice were not studied. Most subchronic and chronic animal studies of TCA conducted in rats and mice have focused primarily or exclusively on liver lesions and, other than DeAngelo et al. (2008), have not examined other organs for microscopic lesions. Nevertheless, liver toxicity appears to be the most consistent and most sensitive effect in rats and mice. Liver toxicity, specifically hepatocellular necrosis, was selected as the critical effect for the RfD. The uncertainty associated with the relevance of this effect to humans is therefore considered low.

Animal-to-human extrapolation. No human exposure studies are available for derivation of the RfD. For derivation of the RfD, extrapolating dose-response data from animals to humans is a source of uncertainty. Uncertainties pertaining to unknown interspecies differences in toxicokinetics and toxicodynamics were addressed by application of an UF of 10.

Dose-response modeling. BMD modeling was used to estimate the POD for the RfD. While models with better biological support may exist, the selected models provided adequate mathematical fits to the experimental data sets. BMD modeling has advantages over a POD based on a NOAEL or LOAEL because the latter are a reflection of the particular exposure concentration or dose at which a study was conducted, they lack characterization of the dose-response curve, and they do not address the variability of the study population. NOAELs and LOAELs also are less amenable to quantitative uncertainty analysis.

Interhuman variability. Heterogeneity among humans is another source of uncertainty. Although male mice appear to be more sensitive than female mice to the carcinogenicity of TCA, available data suggest that males and females are about equally sensitive to noncancer effects induced by TCA. Limited information was identified regarding other factors (e.g., genetic polymorphism) that might influence susceptibility to TCA (see Section 4.8.3). An UF of 10 was

used to account for interhuman variability. A factor of 10 was found to be generally sufficient to account for human variability (Renwick and Lazarus, 1998).

5.4. CANCER ASSESSMENT

As discussed in Section 4.1.1, no epidemiologic studies currently exist that have evaluated the carcinogenicity of TCA in humans. The carcinogenicity of TCA has been evaluated, however, in several studies in both rats and mice. In mice, bioassay results provide evidence that TCA is a complete carcinogen, as exposure to TCA in drinking water for periods of 52–104 weeks significantly increased the incidence of liver tumors in male and female $B6C3F_1$ mice (DeAngelo et al., 2008; Bull et al., 2002; Pereira, 1996; Bull et al., 1990; Herren-Freund et al., 1987). In several of these studies, a clear monotonic dose-response relationship was evident (DeAngelo et al., 2008; Bull et al., 2002; Pereira, 1996; Bull et al., 1990). Moreover, the development of tumors in animals exposed to TCA progressed rapidly, as evident from the appearance of significant numbers of tumors in several of the less-than-lifetime studies (i.e., ≤82 weeks). Positive evidence for tumor promotion by TCA (following exposure to known tumor initiators) has been reported for liver tumors in $B6C3F_1$ mice (Bull et al., 2004; Pereira et al., 2001; Pereira et al., 1997; Pereira and Phelps, 1996; Herren-Freund et al., 1987) and for GGT-positive foci in livers of partially hepatectomized Sprague-Dawley rats (Parnell et al., 1988). In contrast to the results observed in mice, TCA was not carcinogenic in a study of male F344/N rats exposed via drinking water for 104 weeks (DeAngelo et al., 1997). The carcinogenicity of TCA has not been evaluated in female rats or in other species of experimental animals.

5.4.1. Choice of Study/Data—Rationale and Justification

Five bioassays in $B6C3F_1$ mice exposed to TCA in drinking water were selected for analysis and derivation of an oral slope factor for TCA because they: (1) included adequate numbers of animals for statistical analyses; (2) showed statistically significant increased incidences of liver tumors (i.e., combined incidences of adenomas and carcinomas) compared with control values; and (3) included multiple TCA exposure levels to support characterization of the dose-response relationship. These five bioassays consisted of two 52-week studies in male mice (Bull et al., 2002; Bull et al., 1990), a 60-week study in male mice (DeAngelo et al., 2008), an 82-week study in female mice (Pereira, 1996), and a 104-week study in male mice (DeAngelo et al., 2008).

5.4.2. Dose-Response Data

Dose-response data for the combined incidence of hepatocellular adenomas and carcinomas from five bioassays of TCA are shown in Tables 5-7 through 5-11.

Table 5-7. Incidence of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in male B6C3F$_1$ mice exposed to TCA in drinking water for 52 weeks

TCA concentration (g/L)	Estimated intake[a] (mg/kg-d)	Human equivalent lifetime dose[b] (mg/kg-d)	Incidence of adenomas	Incidence of carcinomas	Incidence of adenomas or carcinomas[c]
0	0	0	0/20	0/20	0/20
0.5	120	2.3	5/20	3/20	6/20
2	480	9.0	6/20	3/20	8/20

[a]Doses were calculated using reference water intakes of 0.24 L/kg-day for male B6C3F$_1$ mice (U.S. EPA, 1988).
[b]See Appendix D for conversion of mouse daily intakes to human equivalent lifetime doses.
[c]Bull et al. (2002) reported combined incidences of adenomas or carcinomas for each dose group.

Source: Bull et al. (2002).

Table 5-8. Incidence of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in male B6C3F$_1$ mice exposed to TCA in drinking water for 52 weeks

TCA concentration[a] (g/L)	Estimated intake[b] (mg/kg-d)	Human equivalent lifetime dose[c] (mg/kg-d)	Incidence of adenomas	Incidence of carcinomas	Incidence of adenomas or carcinomas[d]
0	0	0	0/35	0/35	0/35
1	164	3.1	2/11	2/11	4/11
2	329	6.2	1/24	4/24	5/24

[a]An experimental design that included a control group and one dose group (2 g/L) using female mice was also part of this study, but the data were deemed inadequate for modeling because a response at a single dose was considered insufficient for properly characterizing a dose-response relationship.
[b]Calculated using total doses (g/kg) reported by Bull et al. (1990).
[c]See Appendix D for conversion of mouse daily intakes to human equivalent lifetime doses.
[d]Bull et al. (1990) did not report combined incidences for adenomas and carcinomas, so this total assumes that each animal had either adenomas or carcinomas, but not both.

Source: Bull et al. (1990).

Table 5-9. Incidences of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in male B6C3F$_1$ mice exposed to TCA in drinking water for up to 60 weeks

TCA concentration (g/L)	Estimated intake[a] (mg/kg-d)	Human equivalent lifetime dose[b] (mg/kg-d)	Incidence of adenomas[c]	Incidence of carcinomas[c]	Incidence of adenomas or carcinomas[c]
0	0	0	2/30	2/30	4/30
0.05	7.7	0.2	1/32	5/32	5/32
0.5	68.2	2.0	7/34	6/34	12/34
5	602.1	17.4	13/34	13/34	19/34

[a]Estimated daily intakes were calculated with the mean measured TCA concentrations reported by DeAngelo et al. (2008) where available; if not, the nominal concentration for the dose group was used (see Appendix D, Table D-1 for details).
[b]See Appendix D, Table D-1 for conversion of mouse estimated daily intake to human equivalent lifetime dose.
[c]Individual animal data were obtained from the study author (email dated April 26, 2010, from Anthony DeAngelo, NHEERL, ORD, U.S. EPA, to Diana Wong, NCEA, ORD, U.S. EPA). Because the first liver tumor occurred at 45 weeks for 0.05, 0.5, 5 g/L dose groups, adenoma or carcinoma data for all mice examined histopathologically between weeks 45–60 were included for those dose groups. For the control group, the first tumor occurred at 60 weeks, so adenoma or carcinoma data for all mice examined histopathologically on and after 52 weeks were included.

Source: DeAngelo et al. (2008).

Table 5-10. Incidence of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in female B6C3F$_1$ mice exposed to TCA in drinking water for 82 weeks

TCA concentration (mmol/L)	Estimated intake[a] (mg/kg-d)	Human equivalent lifetime dose[b] (mg/kg-d)	Incidence of adenomas	Incidence of carcinomas	Incidence of adenomas or carcinomas[c]
0	0	0	2/90	2/90	4/90
2	78	5.7	4/53	0/53	4/53
6.67	262	19.3	3/27	5/27	8/27
20	784	57.6	7/18	5/18	12/18

[a]Intakes were calculated using reference water intake of 0.24 L/kg-day for female B6C3F$_1$ mice (U.S. EPA, 1988).
[b]See Appendix D for conversion of mouse daily intakes to human equivalent lifetime doses.
[c]Pereira (1996) did not report combined incidences for adenomas and carcinomas, so this total assumes that each animal had either adenomas or carcinomas, but not both.

Source: Pereira (1996).

Table 5-11. Incidence of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in male B6C3F$_1$ mice exposed to TCA in drinking water for up to 104 weeks

TCA concentration (g/L)	Estimated intake[a] (mg/kg-d)	Human equivalent lifetime dose[b] (mg/kg-d)	Incidence of adenomas[c]	Incidence of carcinomas[c]	Incidence of adenomas or carcinomas[c]
0	0	0	10/56	26/56	31/56
0.05	6.7	1	10/48	15/48	21/48
0.5	81.2	12.8	20/51	32/51	36/51

[a]Estimated daily intakes were calculated with the mean measured TCA concentrations reported by DeAngelo et al. (2008) where available; if not, the nominal concentration for the dose group was used (see Appendix D, Table D-1 for details).
[b]See Appendix D, Table D-2 for conversion of mouse estimated daily intake to human equivalent lifetime dose.
[c]Individual animal data were obtained through the study author (email dated February 1, 2010, from Anthony DeAngelo, NHEERL, ORD, U.S. EPA, to Diana Wong, NCEA, ORD, U.S. EPA). Because the first liver tumors were found at the interim sarcrifice (52 weeks), adenoma or carcinoma data for all mice examined histopathologically between weeks 52 and 104 were included.

Source: DeAngelo et al. (2008).

5.4.3. Dose Conversion

Before fitting the multistage model to the combined incidence data for adenomas and carcinomas in Tables 5-7 through 5-11, estimated daily intakes of TCA from the mouse studies were converted to human equivalent doses for continuous lifetime exposure using an interspecies body weight scaling factor and continuous exposure time adjustment factors (see Appendix D for the equations and calculations). The human equivalent lifetime doses used in the dose-response modeling are shown in the third column of Tables 5-7 through 5-11.

5.4.4. Extrapolation Methods

As discussed in Section 4.7.3, studies of the mechanism by which TCA induces liver tumors reveal that the MOA for TCA is complex and that TCA may induce tumors by multiple MOAs that may not be mutually exclusive. While PPARα-related events represent some of the major components of the overall mechanism of toxicity and carcinogenicity, it is premature to conclude that this is the only MOA for TCA-induced carcinogenicity. The data do not support a major role for a mutagenic MOA (Bull, 2000; Moore and Harrington-Brock, 2000). Because the MOA for TCA-induced liver carcinogenesis has not been established, the cancer dose-response modeling is carried out using linear extrapolation (U.S. EPA, 2005c). In addition, data to identify dose-response relationships for possible precursor events for TCA-induced liver tumors are not available. Therefore, data from mouse studies are too limited for the application of a biologically-based dose-response model.

The multistage model in U.S. EPA's BMDS (version 2.1.1) was fit to liver tumor incidence data for the five data sets described in Section 5.4.2. The multistage model has been

used by EPA in the vast majority of quantitative cancer assessments because it is thought to reflect the multistage carcinogenic process. Furthermore, this model can accommodate a wide variety of dose-response shapes and its use provides consistency with previous quantitative dose-response assessments for cancer.

The multistage model was restricted to two stages or less for the 52-week Bull et al. (2002; 1990) and the 104-week DeAngelo et al. (2008) data sets employing three dose groups (including controls), and to three stages or less for the 82-week Pereira (1996) and the 60-week DeAngelo et al. (2008) data sets employing four dose groups (including controls). For each of the five data sets, the best-fit model was selected by comparing AIC values, as well as by examining the visual fit of the model to the data. The $BMDL_{10}$ estimates from the best-fit models were used as the POD for deriving the candidate oral cancer slope factors (Table 5-12). Additional model details, including model outputs from BMDS, are provided in Appendix D.

Table 5-12. Candidate oral cancer slope factors derived from cancer bioassays in $B6C3F_1$ mice

Study reference (study duration)	BMD_{10} (mg/kg-d)[a]	$BMDL_{10}$ (mg/kg-d)[a]	χ^2 goodness-of-fit *p*-value	Slope of linear extrapolation from BMD_{10}[b] $(mg/kg\text{-}d)^{-1}$	Oral cancer slope factor[c] $(mg/kg\text{-}d)^{-1}$
Male mice					
Bull et al. (2002) (52 wks)	1.34	0.89	0.17	7.5×10^{-2}	1.1×10^{-1}
Bull et al. (1990) (52 wks)	1.87	1.13	0.12	5.3×10^{-2}	8.8×10^{-2}
DeAngelo et al. (2008) (60 wks) (Study 1)	2.67	1.67	0.22	3.7×10^{-2}	6.0×10^{-2}
DeAngelo et al. (2008) (104 wks) (Study 3)	5.71	1.50	0.23	1.8×10^{-2}	6.7×10^{-2}
Female mice					
Pereira (1996) (82 wks)	6.73	4.67	0.51	1.5×10^{-2}	2.1×10^{-2}

[a]BMD_{10} and $BMDL_{10}$ were derived from the best-fit multistage model.
[b]The slope of a linear extrapolation from the BMD_{10} is calculated as $0.1/BMD_{10}$.
[c]The oral cancer slope factor is derived by linear extrapolation from the $BMDL_{10}$ (i.e., $0.1/BMDL_{10}$).

5.4.5. Time-to-tumor Modeling

Individual animal data (specifying when liver tumors were detected in each animal) for the three bioassays conducted by DeAngelo et al. (2008) were obtained from the study author (emails dated February 1 and April 26, 2010, from Anthony DeAngelo, NHEERL, ORD, U.S. EPA, to Diana Wong, National Center for Environmental Assessment (NCEA), ORD, U.S. EPA). The availability of individual animal data permitted the application of more sophisticated dose-response modeling approaches (i.e., time-to-tumor modeling) to estimate lifetime cancer risks based on both the TCA dose and the liver tumor appearance time. These bioassays

included the 60- and 104-week studies considered in Section 5.4.2 and a third (104-week) study that used only one dose group and a control.

Consideration was also given to whether the liver tumor incidence data from these three bioassays could be combined to derive an oral cancer slope factor. A statistical analysis was conducted employing a generalized likelihood ratio test (Stiteler et al., 1993), after both individual and combined data sets were fitted by the multistage Weibull (MSW) time-to-tumor model (U.S. EPA, 2009). This statistical analysis for data set compatibility is presented in Appendix E. The analysis revealed that two liver tumor data sets from DeAngelo et al. (2008) (i.e., the 60-week study and the multi-dose 104-week study) were statistically compatible to be combined for MSW time-to-tumor modeling.

The results of the MSW time-to-tumor modeling for both individual and combined data sets from DeAngelo et al. (2008) are presented in Appendix E and are summarized in Table 5-13. For the individual studies, the cancer slope factors derived using the MSW time-to-tumor model and those derived with the multistage model in BMDS were similar. In the case of the 60-week study, the multistage model in BMDS yielded a cancer slope factor fivefold higher than the value derived from the MSW time-to-tumor model. In the case of the 104-week study, the multistage model in BMDS yielded a cancer slope factor 21% lower than the MSW time-to-tumor model.

Table 5-13. Candidate oral cancer slope factors derived from liver tumor data sets in B6C3F$_1$ male mice (DeAngelo et al., 2008) using MSW time-to-tumor modeling and comparison to slope factors derive using the multistage model in BMDS

	Model	AIC	BMR	BMD_{10}[a]	$BMDL_{10}$[b]	Slope of linear extrapolation from BMD_{10}[c]	Cancer slope factor from $BMDL_{10}$[d]
Study 1[e]	MSW time-to-tumor (Stage 1)	158.9	0.1	13.5	8.4	7.4×10^{-3}	1.2×10^{-2}
	BMDS multistage (Stage 1)	149.0	0.1	2.7	1.7	3.7×10^{-2}	6.0×10^{-2}
Study 3[f]	MSW time-to-tumor (Stage 2)	226.4	0.1	5.0	1.2	2.0×10^{-2}	8.5×10^{-2}
	BMDS multistage (Stage 2)	210.0	0.1	5.7	1.5	1.8×10^{-2}	6.7×10^{-2}
Study 1+3	MSW time-to-tumor (Stage 1)	381.0	0.1	2.2	1.4	4.5×10^{-2}	7.2×10^{-2}

[a]BMD_{10} = dose at 10% cancer risk.
[b]$BMDL_{10}$ = dose at 95% lower bound with 10% cancer risk.
[c]Slope of linear extrapolation from $BMD_{10} = 0.1/BMD_{10}$.
[d]Cancer slope factor = $0.1/BMDL_{10}$.
[e]60-week study using drinking water concentrations of 0, 0.05, 0.5, and 5 g/L.
[f]104-week study using drinking water concentrations of 0, 0.05, and 0.5 g/L.

The cancer slope factor derived from the combined data set was similar to the cancer slope factors derived from the individual study data sets. As shown in Table 5-13, the cancer

slope factor for the combined data set (7.2×10^{-2} [mg/kg-day]$^{-1}$) fell between the values based on the individual study data sets (1.2×10^{-2} [mg/kg-day]$^{-1}$ and 8.5×10^{-2} [mg/kg-day]$^{-1}$). Also as shown in Table 5-13, the cancer slope factors derived using the MSW time-to-tumor modeling and the multistage model were similar, especially when applied to tumor incidence data from the 104-week DeAngelo et al. (2008) study (i.e., 8.5×10^{-2} [mg/kg-day]$^{-1}$ and 6.7×10^{-2} [mg/kg-day]$^{-1}$).

For consistency with the dose-response analyses conducted for the tumor data sets from Bull et al. (2002; 1990) and Pereira (1996) and because application of the MSW time-to-tumor model to the DeAngelo et al. (2008) data yielded cancer slope factors similar to the multistage model, further evaluation of candidate cancer slope factor derived for all five TCA tumor data sets was based on model results using the multistage model.

5.4.6. Oral Cancer Slope Factor and Inhalation Unit Risk

The oral cancer slope factor is an upper-bound estimate of risk per increment of dose that can be used to estimate lifetime cancer risk from different TCA exposure levels. The candidate oral cancer slope factors derived from the five bioassays in mice with exposure durations of 52–104 weeks ranged from 2.1×10^{-2} to 1.1×10^{-1} (mg/kg-day)$^{-1}$ (see Table 5-12).

In the conversion of animal doses to human equivalent doses for continuous lifetime exposure, exposure time adjustment factors (i.e., [duration of experiment/duration of animal life]^3) were used. For the 104-week study of DeAngelo et al. (2008), this factor was equal to 1. Because of the uncertainty inherent in applying this adjustment factor, the slope factor derived from the study of longest duration is generally preferred. Moreover, TCA may be a more potent carcinogen in male mice than in female mice, as discussed previously in Section 4.8.2. In addition, the four slope factors derived from the incidence data in male mice varied by about twofold. In light of these considerations, the slope factor of 6.7×10^{-2} (mg/kg-day)$^{-1}$ derived from the study of longest duration [i.e., the 104-week mouse bioassay by DeAngelo et al. (2008)] was selected as the cancer slope factor for TCA.

The slopes of the linear extrapolation from the BMD_{10}, the central estimate of exposure associated with 10% extra cancer risk, were also derived (Table 5-12) from the same studies used to derive the oral cancer slope factors (DeAngelo et al., 2008; Bull et al., 2002; Pereira, 1996; Bull et al., 1990). Based on the study of longest duration [the 104-week data from DeAngelo et al. (2008)], the slope of the linear extrapolation from the BMD_{10} is 1.8×10^{-2} (mg/kg-day)$^{-1}$.

No inhalation unit risk (IUR) for TCA was derived. Cancer bioassays involving inhalation exposure to TCA are not currently available, and PBPK models that could be used to support route-to-route extrapolation for TCA have not been published. In the absence of a PBPK model, route-to-route extrapolation (from oral to inhalation) is not recommended because the liver is the critical target organ for oral toxicity, and first-pass effect by the liver is expected

following oral administration. Furthermore, the respiratory tract has not been evaluated in oral exposure studies.

5.4.7. Previous Cancer Assessment

In the previous cancer assessment of TCA posted to the IRIS database in 1996, TCA was classified as a "C," or "possible human carcinogen." This classification was based on a lack of human data, limited evidence of an increased incidence of liver neoplasms in both sexes of one strain of mice, and no evidence of carcinogenicity in rats. The previous IRIS assessment did not provide quantitative estimates of carcinogenic risk from oral or inhalation exposure to TCA.

6. MAJOR CONCLUSIONS IN THE CHARACTERIZATION OF HAZARD AND DOSE RESPONSE

6.1. HUMAN HAZARD POTENTIAL

TCA (CASRN 76-03-9) has the chemical formula $C_2HCl_3O_2$ and a molecular weight of 163.39 g/mol. At room temperature, TCA is a colorless to white crystalline solid with a sharp, pungent odor. It is used as a soil sterilant and as a laboratory reagent in the synthesis of medicinal products and organic chemicals. TCA is used in industry as an etching and pickling agent. Medical applications of TCA include use as an antiseptic, as a reagent for detection of albumin, and as a skin peeling agent. TCA is formed as a combustion byproduct of organic compounds in the presence of chlorine. TCA is also formed by the interaction of organic material with chlorine during drinking water disinfection. TCA has been detected in water distribution systems, tap water used for drinking and household activities, and swimming pools.

Direct human exposure to TCA occurs via ingestion of disinfected tap water, inhalation, and dermal contact. TCA is also formed as a metabolite in the human body after exposure to the environmental contaminants TCE, tetrachloroethylene, and chloral hydrate.

TCA is readily absorbed by the oral route in rats and by the dermal and oral routes in humans. Once absorbed, TCA is available for systemic distribution, based on the appearance of TCA in blood after oral exposure in rodents. Tissue distribution of TCA appears to be dependent on the time of measurement following dosing. TCA binds to plasma proteins, which is an important determinant of the extent to which TCA partitions from plasma into target tissues. No studies were identified that investigated the tissue distribution of TCA in humans, but the appearance of TCA in the blood and urine of humans exposed to chlorinated solvents or orally administered chloral hydrate indicates that it is present in the systemic circulation as a downstream metabolite. No studies investigating the kinetics or degree of maternal-to-fetus or blood-to-breast-milk transfer of TCA were located.

TCA is not readily metabolized, as indicated by minimal first-pass metabolism in the liver following oral dosing with TCA and by limited amounts of radioactivity excreted in exhaled air or present as non-extractable radioactivity in plasma and liver following intravenous administration of [1-^{14}C]-TCA. Results from animal studies indicate that TCA is not as extensively metabolized as other chlorinated acids, such as DCA, and that TCA is metabolically converted to DCA. However, with exposure to TCA, levels of DCA in blood, liver, and urine are low or not detectable, presumably due to rapid metabolism of DCA. The metabolic conversion of TCA to DCA via reductive dehalogenation is likely catalyzed by CYP450 enzymes through the dichloroacetate radical intermediate, but, in general, enzymes involved in TCA metabolism are poorly characterized. The primary route of excretion of TCA is in the urine, with exhalation of CO_2 and fecal excretion contributing to a lesser extent.

The available human data do not provide a definitive picture of the possible noncancer effects of long-term human exposure to TCA. No human epidemiology or occupational studies of TCA were located. Case reports and accounts of the medical use of TCA for skin treatments demonstrate its potential for skin corrosion and eye irritation. However, no information on systemic toxicity following dermal exposure of humans to TCA was identified.

In animals, TCA induces systemic, noncancer effects that can be grouped into three general categories: liver toxicity, metabolic alterations, and developmental toxicity. Studies in rats and mice indicate that TCA primarily affects the liver, although effects on the lungs and kidneys have also been noted in rats. Observed hepatic effects in rodents include increased size and weight, collagen deposition, indications of altered lipid and carbohydrate metabolism, histopathologic changes, peroxisome proliferation, evidence of lipid peroxidation, and oxidative damage to hepatic DNA. TCA may influence intermediary carbohydrate metabolism, as shown by altered glycogen content in the livers of mice treated with TCA. Administration of TCA to female rats during pregnancy induced developmental effects in six studies at doses that also resulted in maternal toxicity. Two of these studies are single-dose studies. The observed effects include fetal cardiac malformations, decreased crown-rump length, and reduced fetal body weight. The pattern of observed fetal cardiac malformation effects is not consistent across the available studies.

There appear to be different MOAs for the liver toxicity, metabolic alterations, and developmental effects induced by TCA. For liver effects, some changes such as cytomegaly and cell proliferation may be explained by TCA-induced peroxisome proliferation. Oxidative stress responses such as lipid peroxidation and/or oxidative DNA damage may also contribute to the hepatotoxicity of TCA. The cellular mechanisms underlying changes in lipid and carbohydrate homeostasis have not been conclusively identified. It has been proposed that TCA may alter carbohydrate and lipid homeostasis by activation or inhibition of key liver enzymes; by activation of the peroxisome proliferation pathway, which in turn induces transcription of genes that encode enzymes responsible for fatty acid metabolism; and/or by suppression of one or more steps of the glycogen degradation process. The MOA for developmental toxicity is unknown. It has been suggested that TCA, as a strong acid, might induce developmental toxicity by causing lesions in the placenta, resulting in anoxia, oxidative stress, and apoptosis in the developing fetus or embryo.

The genotoxicity of TCA has been evaluated in assays of mutagenicity, DNA repair, clastogenicity, micronucleus induction, and DNA strand breaks. The weight of evidence from these studies suggests that TCA is, at most, weakly genotoxic.

No human oral or inhalation cancer data are available specifically for TCA. In animals, the carcinogenic potential of TCA has been evaluated in oral bioassays conducted in mice and rats. TCA induced tumors in the livers of male and female mice in multiple bioassays, but

treatment-related tumors of the liver or other organs were not observed in a chronic drinking water bioassay in rats.

Under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005c), there is *suggestive evidence of carcinogenic potential* for TCA based on increased incidences of liver tumors in male B6C3F_1 mice in multiple drinking water studies and in female B6C3F_1 mice in one drinking water study, and no treatment-related tumors in a drinking water study in male F344/N rats.

Studies to investigate potential susceptibility to the toxic effects of TCA as a result of age, gender, health status, or genetic factors have not been conducted. The developmental toxicity data on TCA are too limited to draw any conclusions on whether developing organisms might be a sensitive subpopulation. The LOAELs observed in subchronic toxicity studies suggest that systemic effects are observed at doses similar to or less than those at which developmental toxicity has been observed; however, no developmental NOAELs are available for comparison with the subchronic systemic NOAELs. Given the lack of a developmental NOAEL, it is uncertain what dose would be protective for developmental toxicity. The existing data on TCA are also insufficient to determine whether there are age-dependent differences (e.g., plasma binding and metabolism) in the toxicokinetics of TCA that might lead to differences in health risk. There are no published comparative data for plasma binding of TCA in young and old animals. In the only study to evaluate the cancer potency of TCA in young animals, the incidence of liver tumors in mice injected with TCA as neonates did not differ significantly from solvent controls when evaluated at 15 or 20 months of age.

No data on gender effects of TCA toxicity in humans were located. Studies in mice and rats where males and females were tested concurrently suggest that both sexes are about equally susceptible to the noncancer effects of TCA. In contrast, male mice appear to be more susceptible to the carcinogenic effects of TCA, based on the observation of a dose-related increase in proliferative lesions in males but not females when both sexes were tested concurrently. Other factors that might confer greater susceptibility to the toxic effects of TCA include a medical history of glycogen storage disease or genetic deficiencies in glyoxylate-metabolizing enzymes or antioxidant response.

6.2. DOSE RESPONSE

6.2.1. Noncancer/Oral

No human data were available for oral dose-response analysis; therefore, the oral RfD is based on data from laboratory animals. An estimated $BMDL_{10}$ of 18 mg/kg-day derived using BMD modeling based on the increased incidence of hepatocellular necrosis in male $B6C3F_1$ mice exposed to TCA via drinking water for 30–45 weeks (DeAngelo et al., 2008) was selected as the POD for calculation of the RfD. This value was divided by a composite UF of 1,000 that includes individual factors of 10 each to account for variability among humans, extrapolation from laboratory animal data to humans, and database limitations. The oral RfD is therefore 18 mg/kg-day/1,000 = 0.02 mg/kg-day.

Confidence in the principal study (DeAngelo et al., 2008) is medium. The study was well designed and conducted, with a study duration of 60 weeks. Only male mice were included in this study. Quantitative data for the incidence and severity of the various endpoints were included in the published paper. Complete histopathologic examination was conducted for control and high-dose groups. Confidence in the database is medium. Human data are limited primarily to case reports of skin or eye effects associated with medical treatments, and information on systemic toxicity is lacking. Significant gaps in the animal database include absence of a multigeneration reproductive toxicity study. Overall confidence in the RfD is medium, reflecting these considerations.

6.2.2. Noncancer/Inhalation

No inhalation studies adequate for the derivation of an RfC were located. The respiratory tract has not been examined in oral studies of TCA. Because the liver is the critical target organ for oral toxicity and first-pass effect by the liver is expected following oral administration, the route of exposure may influence the hepatic response to TCA. PBPK models that would support route-to-route extrapolation for TCA have not been published. Thus, the available information is inadequate for extrapolation of oral toxicity data to the inhalation pathway. For these reasons, an RfC for TCA was not derived.

6.2.3. Cancer/Oral and Inhalation

In the absence of a well-characterized MOA that could explain dose-response relationships at doses lower than those leading to observed effects, the cancer dose-response modeling was carried out using linear extrapolation (U.S. EPA, 2005c). No data were found that were suitable for accounting for interspecies differences in toxicokinetics or toxicodynamics in dose-response modeling.

Candidate oral cancer slope factors were derived from liver tumor incidence data from male $B6C3F_1$ mice exposed to TCA in drinking water for 52 weeks (Bull et al., 2002; Bull et al., 1990), 60 weeks (DeAngelo et al., 2008), or 104 weeks (DeAngelo et al., 2008) and from female

$B6C3F_1$ mice exposed to TCA in drinking water for 82 weeks (Pereira, 1996). The slope factors derived from these studies were 1.1×10^{-1}, 8.8×10^{-2}, 6.0×10^{-2}, 6.7×10^{-2}, and 2.1×10^{-2} $(mg/kg\text{-}day)^{-1}$, respectively. These candidate oral slope factors varied by less than one order of magnitude. The oral cancer slope factor derived from the 104-week bioassay in male $B6C3F_1$ mice (DeAngelo et al., 2008), or 6.7×10^{-2} $(mg/kg\text{-}day)^{-1}$, was selected as the oral cancer slope factor for TCA. This bioassay is the only lifetime study of TCA, and an exposure time adjustment factor (i.e., [duration of experiment/duration of animal life]^3) is not required. Because of the uncertainty inherent in applying this adjustment factor, the slope factor derived from the study of longest duration is generally preferred.

No IUR for TCA was derived. Cancer bioassays involving inhalation exposure to TCA are not currently available, and PBPK models that could be used to support route-to-route extrapolation for TCA have not been published. In the absence of a PBPK model, route-to-route extrapolation (from oral to inhalation) is not recommended because the liver is the critical target organ for oral toxicity, and first-pass effect by the liver is expected following oral administration. Furthermore, the respiratory tract has not been evaluated in oral exposure studies.

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APPENDIX A. SUMMARY OF EXTERNAL PEER REVIEW AND PUBLIC COMMENTS AND DISPOSITION

The *Toxicological Review of Trichloroacetic Acid* (dated September 2009) has undergone a formal external peer review performed by scientists in accordance with the EPA guidance on peer review (U.S. EPA, 2006c, 2000c). An external peer review workshop was held December 10, 2009. The external peer reviewers were tasked with providing written answers to general questions on the overall assessment and on chemical-specific questions in areas of scientific controversy or uncertainty. A summary of significant comments made by the external reviewers and EPA's responses to these comments arranged by charge question follow. In many cases the comments of the individual reviewers have been synthesized and paraphrased in development of Appendix A. EPA received no scientific comments on this assessment from the public.

EXTERNAL PEER REVIEW PANEL COMMENTS

The reviewers made several editorial suggestions to clarify specific portions of the text. These changes were incorporated in the document as appropriate and are not discussed further. When the external peer reviewers commented on decisions and analyses in the Toxicological Review under multiple charge questions, these comments were organized under the most appropriate charge question.

General Comments

1. Is the Toxicological Review logical, clear and concise? Has EPA clearly synthesized the scientific evidence for noncancer and cancer hazard?

Comments: Reviewers generally considered the Toxicological Review to be logical and clear. One reviewer observed that the document would benefit from less use of acronyms. Two reviewers observed that the document was not concise, largely as a function of the complexity of the subject and the standard structure of the Toxicological Review.

Response: The content of the *Toxicological Review of Trichloroacetic Acid* is consistent with the current outline for IRIS Toxicological Reviews; however, the Toxicological Review was revised to reduce redundancy. Acronyms used infrequently in the Toxicological Review were spelled out.

Comments: Three reviewers offered comments on the MOA section. One reviewer considered the MOA section difficult to follow. A second reviewer considered the issues associated with the PPARα activation MOA to be adequately described, but suggested that a table showing

consistencies/inconsistencies and data gaps regarding this MOA would provide greater clarity for the current review of TCA as well as for future assessments of other potential peroxisome proliferators. A third reviewer commented that the conclusions derived from the literature review were speculative, especially with respect to MOAs other than peroxisome proliferation.

Response: These comments regarding the MOA section are addressed in EPA's response to comments for Charge Questions C2 and C3.

Comments: One reviewer suggested that additional justification be provided for the selection of dose-response models (when multiple models provided an adequate fit) and a BMR of 10%. The same reviewer also questioned why human equivalent doses were not estimated in the derivation of the oral RfD.

Response: These comments are addressed in EPA's response to comments for Charge Question A3.

2. Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of TCA.

Comments: Five reviewers did not identify any additional studies. One reviewer recommended that the following studies be included:

Allen, B; Fisher, J. (1993) Pharmacokinetic modeling of trichloroethylene and trichloroacetic acid in humans. Risk Anal 13:71–86.

Breimer, DD; Ketelaars, HCJ; Van Rossum, JM. (1974) Gas chromatographic determination of chloral hydrate, trichloroethanol and trichloroacetic acid in blood and in urine employing head-space analysis. J Chromatography 88:55–63.

Muller, G; Spassovaki, M; Henschler, D. (1972) Trichloroethylene exposure and trichloroethylene metabolites in urine and blood. Arch Toxikol 29:335–340.

Muller, G; Spassovaki, M; Henschler, D. (1974) Metabolism of trichloroethylene in man. II. Pharmacokinetics of metabolites. Arch Toxicol 32:283–295.

Response: Information from these studies was added to Sections 3.4 and 3.5 of the Toxicological Review.

Comments: Four reviewers recommended updating the literature on the MOA of PPARα agonists. Specific papers that the reviewers suggested be added or expanded upon were:

Elcombe, CR. (1985) Species differences in carcinogenicity and peroxisome proliferation due to trichloroethylene: a biochemical human hazard assessment. Arch Toxicol Suppl 8:6–17.

Ren, H; Akeksunes, LM; Wood, C; et al. (2010) Characterization of peroxisome proliferator-activated receptor α (PPARα) – independent effects of PPARα activators in the rodent liver: Di-(2-ethylhexyl) phthalate also activates the constitutive activated receptor. Toxicol Sci 113:45–59.

One reviewer observed that the toxicology of TCA and dieldrin were similar; both are PPARα agonists, peroxisome proliferators, and cause liver tumors in mice but not rats. One reviewer commented that the document needs to include literature pertaining to the histopathology and molecular biology of the tumors induced by other PPARα agonists and a discussion of the similarity between these tumors and those found in TCA-treated mice. This reviewer suggested that the following review articles be included:

Corton, JC. (2008) Evaluation of the role of peroxisome proliferator-activated receptor α (PPARα) in mouse liver tumor induction by trichloroethylene and metabolites. Crit Rev Toxicol 38:857–875.

Gonzalez, FJ; Shah, YM. (2008) PPARα: mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. Toxicology 246:2–8.

Köhle, C; Schwarz, M; Bock, KW. (2008) Promotion of hepatocarcinogenesis in humans and animal models. Arch Toxicol 82:623–631.

International Agency for Research on Cancer (IARC). (2004) IARC Monographs on the evaluation of carcinogenic risks to humans. Vol 84. Some drinking-water disinfectants and contaminants, including arsenic. p. 403–440.

Response: The description of the study findings of Elcombe (1985) in Section 4.2.1.1.1 was expanded. Information from the Ren et al. (2010) study related to PPARα agonism was added to Section 4.7.3.1.1. The studies on dieldrin were not included. Unlike dieldrin, TCA exposure does not result in mammary gland tumors in rats and mice. The four review papers identified by the reviewers were not included; however, original key studies from these papers that are relevant to the assessment of TCA are discussed in the Toxicological Review. Other comments on revision of the MOA section are addressed in EPA's response to comments for Charge Questions C2 and C3.

Comments: One reviewer asked that data from Study 2 of DeAngelo et al. (2008) be included in the cancer assessment.

Response: All three studies presented in DeAngelo et al. (2008) were included in the cancer assessment. The data from Study 2 of DeAngelo et al. (2008) were included in the time-to-tumor analysis found in Section 5.4.

Comments: One reviewer recommended that the maximum possible exposure of TCA to humans be presented in the Toxicological Review. This reviewer stated that if maximum

possible exposure to humans is below the calculated cancer risk, then TCA should be classified as noncarcinogenic to humans following environmental exposure.

Response: In general, the scope of an IRIS assessment is the evaluation of hazard and dose-response analysis for a chemical substance. An exposure analysis has not been performed as a part of the development of this Toxicological Review. The recommendation offered by this reviewer involves risk characterization, which is not within the scope of an IRIS assessment. As a general rule, the magnitude of risk at a given level of exposure to a chemical carcinogen is not a factor used in assigning the cancer descriptor according to EPA's Cancer Guidelines (U.S. EPA, 2005c).

Chemical-Specific Charge Questions

(A) Oral Reference Dose (RfD) for Trichloroacetic Acid

1. A 60-week drinking water study in mice (DeAngelo et al., 2008) was selected as the basis for derivation of the RfD for TCA. Please comment on whether the selection of DeAngelo et al. (2008) as the principal study is scientifically justified. Please identify and provide the rationale for any other studies that should be selected as the principal study.

Comments: The reviewers generally agreed with the selection of the 60-week study in male $B6C3F_1$ mice by DeAngelo et al. (2008) as the principal study for RfD derivation. One reviewer commented that a deficiency of DeAngelo et al. (2008) is that complete histopathologic examinations were reportedly performed on only five mice from the high-dose and control groups, and recommended that EPA clarify with the study authors the extent of histopathologic examinations that were performed at the interim sacrifices and at the termination of the 60-week study. This reviewer also recommended further discussion of study limitations (i.e., that effects at sites other than those examined microscopically or in female mice might have been missed). Two reviewers commented that the developmental study by Smith et al. (1989) should also be considered, and a third reviewer observed that the Smith et al. (1989) study provided sufficient information for deriving an RfD.

Response: EPA agrees with the majority of the reviewers in retaining DeAngelo et al. (2008) as the principal study. As stated on page 1058 of DeAngelo et al. (2008), gross lesions, liver, kidney, spleen, and testis were examined by a board-certified veterinary pathologist at the interim and terminal necropsies. For all other tissues, a complete pathologic examination was performed on five mice from the high-dose and control groups. If the number of any histopathologic lesion in a tissue was significantly increased above that in the control animals, then that tissue was examined in all TCA dose groups. This information was included in Section

4.2.2.1.2.1. A discussion of limitations in the DeAngelo et al. (2008) study was added to Sections 5.1.3 and 5.3. Data from Smith et al. (1989) were also modeled to compare the results with that obtained by using the DeAngelo et al. (2008) study as the basis for the RfD (Section 5.1.2.2).

2. Liver toxicity (hepatocellular necrosis) was selected as the critical effect for the determination of the POD. Please comment on whether the selection of this critical effect is scientifically justified. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.

Comments: Five reviewers agreed with the selection of hepatocellular necrosis as the critical effect. One of these reviewers provided qualified support, noting that hepatocellular necrosis is an appropriate endpoint to be used as a critical effect, assuming that the MOA by which it occurs is relevant to humans. This reviewer further observed that if necrosis in mice is a consequence of a PPARα agonism MOA leading to tumors, then one or more of the endpoints identified in the long-term rat study, testicular effects in mice, or developmental effects in rats would be more appropriate. A second of these reviewers recommended that both liver and developmental toxicity endpoints, which yielded similar candidate RfDs, be emphasized throughout the review to strengthen the confidence in the final RfD. Two reviewers (including one who supported hepatocellular necrosis as a critical effect) also recommended consideration of increased liver weight as a candidate critical effect because continuous data often are more sensitive than quantal data and liver weight is less subjective a measure than pathologist ratings. However, one of the reviewers determined that the $BMDL_{10}$ based on the liver weight endpoint was 58 mg/kg-day, which was higher than the $BMDL_{10}$ of 18 mg/kg-day for hepatocellular necrosis.
One reviewer did not consider hepatocellular necrosis to be the best choice for the critical effect because similar mild centrilobular necrosis has been reported for some PPARα agonists. This reviewer proposed the use of testicular tubular degeneration in mice as the critical effect with alternative UFs applied to both hepatocellular necrosis and testicular tubular degeneration such that testicular effects would yield a lower (more sensitive) RfD.

Finally, several reviewers suggested that markers of peroxisome proliferation be used as the critical effect. One of the reviewers commented that evaluation of necrosis was subjective, and that a more appropriate endpoint would be one with a more dynamic range and relevant to the MOA (e.g., a marker of peroxisome proliferation). This reviewer suggested cyanide-insensitive PCO activity as a possible critical effect because it shows consistent dose- and time-responsive changes and because it is amenable to statistical analysis. A second of these reviewers suggested a biomarker of peroxisome proliferation as a possible critical effect because it represents a more upstream effect in the mechanistic chain of responses.

Response: EPA agrees with the majority of the reviewers who suggested hepatocellular necrosis as the critical effect. In response to the reviewers suggestions, developmental endpoints, i.e., fetal body weight and crown rump length, were remodeled as continuous data sets (Appendix C, Sections C.3 and C.4) using 5% extra risk as the BMR. The lowest candidate POD derived from these two endpoints was 84 mg/kg-day (fetal body weight), indicating that these endpoints represented less sensitive effects than the hepatocellular necrosis that was used as the critical endpoint for the derivation of the POD. The $BMDL_{10}$ for testicular tubular degeneration in mice was calculated to be 127 mg/kg-day. This information has also been added. This value is also higher than the potential PODs based on any of the observed liver effects. EPA concurs with the $BMDL_{10}$ of 58 mg/kg-day for liver weight data reported by one of the reviewers, and that a comparison of the $BMDL_{10}$s for liver weight and liver necrosis indicate that liver weight is less sensitive. Of the four endpoints pertaining to liver effects, liver inflammation, liver weight, cyanide-insensitive PCO activity, and liver necrosis, necrosis remains the most sensitive endpoint. The comments related to selection of the POD and UFs are addressed in response to comments on Charge Questions A3 and A4.

Cyanide-insensitive PCO activity was added as a candidate critical effect, and dose-response modeling was performed using BMD modeling methods (BMDS, version 2.1.1). The results of this BMD modeling were added to Appendix B and to a summary of the analysis to Section 5.1.2.1. These continuous data were best fit using a second-degree polynomial model, which yielded a $BMDL_{1SD}$ of 21 mg/kg-day. The candidate POD associated with cyanide-insensitive PCO is higher than the $BMDL_{10}$ of 18 mg/kg-day for hepatocellular necrosis. Therefore, hepatocellular necrosis was retained as the critical effect.

3. Benchmark dose (BMD) modeling was conducted on the liver and testicular effects in male mice exposed to TCA in the drinking water study by DeAngelo et al. (2008) in order to determine the POD. Has the BMD modeling been appropriately conducted? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., 10% extra risk of hepatocellular necrosis) scientifically justified? Please identify and provide the rationale for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.

Comments: The reviewers generally agreed that the modeling was appropriately conducted. Two reviewers commented that modeling should also be conducted on one or more of the endpoints that identified the NOAEL in the long-term rat drinking water study. Three reviewers recommended conducting BMD modeling of continuous endpoints, including liver weight, from the principal study in the mouse.

Response: EPA considered conducting dose-response modeling for the other endpoints suggested by the reviewers. Data from the rat drinking water study (DeAngelo et al., 1997) were not used to derive a candidate POD because the NOAEL (32.5 mg/kg-day) and LOAEL (364 mg/kg-day) identified in this study were higher than those identified in the chronic mouse study (DeAngelo et al., 2008). Comments on the consideration of continuous endpoints as the basis for the RfD are addressed in response to Charge Question A2.

Comments: One reviewer specifically agreed with defining the BMR as an extra risk of 10%. Two reviewers commented that better justification for the selection of a 10% BMR was needed. Two reviewers recommended the use of a BMR of 5% on the grounds that the BMD is intended to approximate the NOAEL and that the BMR level should be close to the lower end of the observed data. The rationales for a BMR of 5% presented by these two reviewers included: 1) a 10% response rate above background in a well-designed study with reasonable statistical power would seldom be seen as a NOAEL, especially if the control rate is zero and the response is clearly adverse, as is the case for the hepatocellular necrosis and inflammation endpoints in the study by DeAngelo et al. (2008); 2) extensive experience with this type of analysis suggests that the 5% response rate yields a more appropriate benchmark for quantal data in animal studies, if the aim is to select a benchmark which is similar to a NOAEL for extrapolation purposes; 3) for dichotomous data, the appropriate basis for selecting a BMR should be the distribution of the data; and 4) the BMR_{05} is within the range of doses for which observations are reported.

Response: In general, the BMR should be selected based on both biological (e.g., endpoint severity) and statistical (e.g., study sensitivity) considerations. For TCA, the severity of the critical effect from the DeAngelo et al. (2008) study used to derive the RfD, incidence of hepatocellular necrosis, was characterized as minimal (i.e., severity score of 1) to mild (severity score of 2) by the study authors (with average severity scores in the mid- and high-dose groups, where necrosis was observed, of 0.5 and 1.3, respectively, on a 4-point scale). In addition, the study authors noted that necrosis was present in the mid- and high-dose groups between 30 and 45 weeks, but this necrosis had abated by 60 weeks. Furthermore, in characterizing hepatic necrosis, DeAngelo et al. (2008) examined only 10 animals per dose group at 30 to 45 weeks, limiting the sensitivity of this study. As noted in EPA's draft *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000b), a 10% response is at or near the limit of sensitivity in most cancer bioassays. Most cancer bioassays employ 50 animals per dose group. Thus, given the minimal to mild severity of the endpoint identified as the critical effect, and the relatively low sensitivity of the principal study, the EPA concluded that use of a BMR of less than 10% was not warranted, and the BMR of 10% was retained. However, in response to reviewer

comments on this issue, text was added to Section 5.1.2.1 to clarify the justification for selection of a 10% BMR.

Comments: Three reviewers suggested that the Toxicological Review be revised to provide clearer documentation of guidance used for model selection. One reviewer commented that minor differences in AIC are not meaningful and should not be over-interpreted.

Response: The procedures used in the selection of the best-fit model are described in Section 5.1.2.1 and are consistent with the guidance in EPA's *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000a). With regard to the use of AICs in model selection, the application of the BMD approach recognizes that more than one model can adequately fit a dose-response data set. Thus, a reasonable and practical procedure for selecting amongst these models is needed in order to support estimation of a BMD and BMDL. The recommended procedure described in Section 5.1.2.1 of selecting the model with the lowest AIC when the BMDLs from all adequately fitting alternative models are within a factor of 3 of each other provides a clear-cut decision rule to arrive at a needed BMDL value -- under circumstances where the model choice does not have a large impact on the POD.

Comments: One reviewer observed that DeAngelo et al. (2008) reported severity as well as incidence data for the various histological endpoints, and suggested that EPA consider the possibility of treating the overall incidence and severity data as a pseudo-continuous variable. One reviewer observed that in Study 1 of DeAngelo et al. (2008) at the 60-week time point, the low-dose group (0.05 g/L TCA) showed a considerably higher incidence and greater severity of hepatocellular cytoplasmic alterations than the mid-dose group (0.5 g/L), but incidence and severity of hepatocellular inflammation and testicular tubular degeneration were reported as zero. This raised the question of whether there was something anomalous in the treatment or analysis of the low-dose group at 60 weeks and, by extension, of the hepatocellular necrosis findings in this group at 30–45 weeks.

Response: Hepatocellular necrosis was reported in mid- and high-dose mice in the DeAngelo et al. (2008) study. Average severity in these groups was characterized as minimal to mild; data on severity of necrosis in individual animals were not provided. In light of the similarity in severity scores in the two groups and the lack of more detailed severity information, a more rigorous analysis using both incidence and severity data would not be supported. The lack of a monotonic dose-related increase in the incidence of centrilobular cytoplasmic alteration is acknowledged and was added to Section 5.1.2.1. It should be noted that this endpoint was not considered as a candidate data set for derivation of the RfD. As the peer reviewer observed, anomalous dose-

response findings may simply reflect normal fluctuations in response and, in this instance, did not necessarily reduce confidence in other data sets from this study.

Comments: One reviewer commented that the BMD modeling in the Toxicological Review should reflect the latest version of BMDS.

Response: EPA BMDS version 1.4.1 was used in the analysis of dichotomous data sets. BMDS version 2.0 includes more functions, but the core calculation remains the same, and as such, use of the newer version would not change the modeling results. Therefore, the modeling based on BMDS version 1.4.1 was retained.

Comments: One reviewer stated that because hepatocellular effects in the mouse result from peroxisome proliferation, these effects do not represent a toxic response in humans and as such, the UF for mouse-to-human extrapolation should be 1 and not 10. This reviewer further observed that the POD for testicular tubular degeneration divided by an UF of 10 would result in a lower RfD (0.127 mg/kg-day) than that derived for liver inflammation using an UF of 1 (0.18 mg/kg-day).

Response: The MOA for TCA-induced liver effects has not been established. As such, none of the hepatic effects reported in animals can be excluded as candidate critical effects based on lack of human relevance. Therefore, all liver endpoints were considered for selection of the critical effect. Responses related to the selection of the critical effect are also addressed under Charge Question A2; responses related to UFs are addressed under Charge Question A4.

4. Please comment on the rationale for the selection of the uncertainty factors applied to the POD for the derivation of the RfD. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s).

Comments: Eight reviewers agreed with the UF for interspecies extrapolation of 10. One reviewer did not consider hepatocellular effects originating from peroxisome proliferation to be related to a toxic response in humans and that a more appropriate UF for mouse-to-human extrapolation would be 1 or 3.

Response: EPA agrees with the majority of the reviewers in applying an UF of 10 for interspecies extrapolation. Insufficient information is currently available to assess mouse-to-human differences in TCA toxicokinetics or toxicodynamics. The MOA by which TCA induces liver toxicity is complex; while peroxisome proliferation is likely a component of the overall mechanism of toxicity, the final conclusion is that it is not established as the sole MOA of TCA

toxicity. Reduction of the UF based on issues of human relevance was considered but was not thought to be supported.

Comments: Eight reviewers agreed with the UF for human variation of 10. One reviewer commented that the use of an UF of 3 would be better justified because of limited TCA metabolism and a peroxisome proliferation MOA that suggests that human susceptibility to TCA would not vary significantly. Another reviewer commented that a number of publications and guidance documents suggest that the default value of 10 for human variability is insufficient. particularly when the range of human metabolic capabilities and the need to protect children and other sensitive subpopulations are considered; however, this reviewer further observed that because the extent of TCA metabolism is minor and not a major determinant of TCA toxicity or clearance, an UF for human diversity of 10 is probably sufficient.

Response: EPA agrees with the majority of the reviewers in applying an UF of 10 for human variation, including protection of sensitive subpopulations, in light of the lack of information to support a chemical-specific factor.

Comments: Eight reviewers agreed with the database UF of 10. Two reviewers commented that the discussion of deficiencies in the database should be expanded to consider limitations of the available developmental studies, the failure of the principal study (DeAngelo et al., 2008) to include female mice, and questions about the completeness of the histopathological examinations. One reviewer stated that a case could be made for the use of a database UF of 1 or 3 rather than 10 because the DeAngelo et al. (2008; 1997) studies were conducted in two species (mice and rats) and developmental data are available.

Response: EPA agrees with the majority of the reviewers in applying a database UF of 10. The justification for the database UF in Section 5.1.3 was expanded to include the additional database deficiencies identified by the reviewers.

Comments: Most reviewers agreed with the application of a LOAEL-to-NOAEL UF of 1. Two reviewers commented on the LOAEL-to-NOAEL UF in the context of selection of the BMR. One of these reviewers suggested an additional UF of 3–10 be applied with a BMR of 10% extra risk. The second reviewer recommended the use of a $BMDL_{05}$ instead of a $BMDL_{10}$, but further stated that if the $BMDL_{10}$ was retained, a LOAEL-to-NOAEL UF is required.

Response: EPA agrees with the majority of the reviewers and has retained an UF of 1 for LOAEL-to-NOAEL extrapolation since BMD modeling was used to estimate the POD. In response to the reviewers who provided alternative recommendations, it may be helpful to note

that EPA considers biological significance of the endpoint when selecting a BMR and chooses a BMR that is intended to represent a minimally biologically significant change, if data are available to inform the choice as per EPA guidance (U.S. EPA, 2000a). When data are not available or informative, as is generally the case for TCA, a 10% BMR is chosen for consistency across endpoints.

Comments: One reviewer discussed evidence for inter-strain differences in adsorption, distribution, metabolism, and excretion and response to chemicals in mice and recommended an UF for within-mouse (or rat) variability.

Response: EPA recognizes the variability within and among mouse (and rat) strains; however, information to characterize the magnitude of this variability, particular for TCA, is not available. The general practice of selecting the most sensitive species and strain tested from among adequately conducted studies reduces the uncertainty associated with this variability. For these reasons, an additional UF for inter-strain variability was not included in the derivation of the RfD.

(B) Inhalation Reference Concentration (RfC) for Trichloroacetic Acid

1. An RfC was not derived for TCA. Has the scientific justification for not deriving an RfC been clearly described in the document? Please identify and provide the rationale for any studies that should be selected as the principal study.

Comments: Four reviewers agreed that not deriving an RfC for TCA was justified. Three reviewers commented that the decision for not deriving an RfC should be better justified (e.g., by using EPA's (1994b) *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry*). Two reviewers acknowledged that there is no sophisticated PBPK model for TCA exposure by inhalation or other exposure routes. However, these reviewers suggested that an RfC could be derived by route-to-route extrapolation from oral data and the use of a simple set of assumptions such as 100% absorption by inhalation, followed by systemic distribution via the bloodstream. One reviewer commented that given the possibility of respiratory-specific toxicity and the lack of data that can address respiratory-specific toxicity, it may be more appropriate to simply provide the exposure relationship that would allow an estimate of cumulative (ingestion plus inhalation) exposure under standard exposure assumptions.

Response: No inhalation studies of TCA are available. Available information suggests that a simple set of assumptions might not account for possible differences in route-specific toxicity. The respiratory tract has not been studied in the available oral studies. In addition, the liver is

the critical target organ for oral toxicity, and a first-pass effect by the liver is expected following oral administration. Consistent with guidance for route-to-route extrapolation in EPA's RfC Methodology (p. 4–5) (U.S. EPA, 1994b), which specifies that PBPK modeling is the preferred methodology for extrapolation, simple route-to-route extrapolation was not performed and an RfC for TCA was not derived. Justification for not deriving an RfC for TCA in Section 5.2 was expanded.

(C) Carcinogenicity of Trichloroacetic Acid

1. Under the EPA's (2005c) *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgr-d.htm), the Agency concluded that TCA is *likely to be carcinogenic to humans* by all routes of exposure. Please comment on the cancer weight of evidence characterization. Is the weight of evidence characterization scientifically justified?

Comments: Two reviewers considered the carcinogenicity data for TCA to be consistent with the descriptor of *likely to be carcinogenic to humans*. One of these reviewers stated that the characterization was carefully balanced and scientifically justified. The second of these two reviewers observed that TCA is one of the weakest among the chemicals in the IRIS database with this descriptor, and recommended that EPA provide some perspective on the extent of evidence relative to other chemicals that share this descriptor.

Four reviewers provided qualified support for the descriptor of *likely to be carcinogenic to humans*. One of the four observed that the document did a good job of presenting scientific justification for the characterization of weight of the evidence, but did not directly indicate his support for the descriptor. A second observed that TCA meets the definition of *likely to be carcinogenic to humans* within the requirements of the 2005 Cancer Guidelines, but considered *suggestive evidence of carcinogenic potential* to be an appropriate descriptor given that TCA tested positive in only one strain of one species (albeit both sexes); that the tumor response, while statistically significant, was not extraordinary; that liver tumors are relatively common in rodents; and that supporting evidence for events associated with tumor formation were somewhat speculative. One reviewer observed that the "likely" descriptor was largely based on lack of evidence to the contrary. Another reviewer commented that the "likely" descriptor appears to be appropriate for high doses; this reviewer offered no views on the descriptor at low doses.

Three reviewers did not view the characterization of *likely to be carcinogenic to humans* to be supported. One of these reviewers commented that the classification for TCA is at best *suggestive evidence of carcinogenic potential* but should more likely be *not likely to be carcinogenic to humans*. A second of the reviewers commented that TCA is not likely to be carcinogenic to humans because the increase in liver tumors occurred in a strain of mice with a

high background incidence, TCA did not induce tumors in rats, and TCA induces tumors by a nongenotoxic MOA related to peroxisome proliferation that is not relevant to humans. A third reviewer did not consider the characterization of "likely" to be justified, noting that induction of liver tumors in multiple studies in a single species (mouse) and both sexes following administration of a PPARα agonist and lack of concordance in the rat did not provide a biologically significant signal of the likelihood of human carcinogenicity. This reviewer did not suggest an alternative descriptor.

Response: In light of the peer reviewer comments, EPA changed the cancer descriptor for TCA from *likely to be carcinogenic to humans* to *suggestive evidence of carcinogenic potential*. The broad range of views on the weight of evidence for TCA carcinogenicity expressed by the peer reviewers reflects the challenges in weighing the evidence for TCA carcinogenic potential. As noted in the Cancer Guidelines, choosing a descriptor is a matter of judgment that cannot be reduced to a formula. Cancer descriptors represent points along a continuum of evidence, and consequently there are gradations and border line cases. Such is the situation for TCA. Although the carcinogenicity data for TCA is consistent with at least one example used in the Cancer Guidelines to illustrate the classification of *likely to be carcinogenic to humans* (i.e., "an agent that has tested positive in animal experiments in more than one ..., sex,..."), TCA meets this criterion only minimally: for more than one sex but not for more than one species, strain, site, or exposure route (although TCA has not been tested in multiple strains or exposure routes). In re-evaluating the selection of a cancer descriptor, EPA also considered the nature of the only tumor type induced by TCA, i.e., liver tumors (hepatocellular adenomas and carcinomas). The mouse, and in particular the $B6C3F_1$ mouse, is relatively susceptible to liver tumors, and the background incidence of this tumor is generally high. For these reasons, use of mouse liver tumor data in risk assessment has been a subject of controversy (King-Herbert and Thayer, 2006).

Thus, although either the descriptor of *likely to be carcinogenic to humans* or *suggestive evidence of carcinogenicity* is plausible, EPA attached greater weight in the re-evaluation of the weight of evidence for TCA carcinogenicity to the lack of effects outside the $B6C3F_1$ mouse than to the replication of positive results in this one strain. Taking into consideration this weighing of the data and the controversy surrounding mouse liver tumors, the final assessment was revised to present a descriptor of *suggestive evidence of carcinogenic potential.* Section 4.7.1 was revised accordingly.

Comments: One reviewer concurred that the descriptor is applicable to all routes of exposure because absorbed TCA from any route of administration will likely be systemically distributed, a major portion of inhaled TCA would likely be absorbed given its high water solubility, and

because TCA undergoes minimal metabolism, it is likely that carcinogenic effects observed with oral exposure would also occur with dermal or inhalation exposure. Another reviewer disagreed with EPA's conclusion of *likely to be carcinogenic to humans* by all routes of exposure, and stated that only "…by the oral and dermal routes of exposure" is supported. This reviewer further observed that there are no inhalation studies to determine whether or not TCA is absorbed following inhalation exposure, and no scientific argument is offered to support the conclusion that uptake into the blood of intact parent TCA could occur by the inhalation route.

Response: EPA's *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005c) indicate that for tumors occurring at a site other than the initial point of contact, the weight of evidence for carcinogenic potential may apply to all routes of exposure that have not been adequately tested at sufficient doses. An exception occurs when there is convincing toxicokinetic data that absorption does not occur by other routes. As stated by one reviewer, data evaluating absorption by the inhalation route are unavailable; however, TCA is highly soluble in water and, as such, it is reasonable to assume that it can be absorbed and taken up into the blood via the inhalation route. Moreover, based on the observation of systemic tumors following oral exposure, and in the absence of information to indicate otherwise, it is assumed that an internal dose will be achieved regardless of the route of exposure. The cancer descriptor narrative in Section 4.7.1 was revised to better describe this decision.

Comments: One reviewer expressed reservations about the characterization of the genetic toxicity information for TCA. The reviewer agreed with the conclusion that TCA is, at best, a weak mutagen, but did not consider the case for mutagenesis related to oxidative stress to have been made. This reviewer suggested that for the most part, the tests utilized, particularly those in vitro, either lacked the ability to detect oxidative effects or, even if they had such sensitivity, the likely metabolic or cellular mechanisms for generating activated oxygen species were absent from the test systems.

Response: EPA agrees that in vitro tests of TCA genotoxicity do not include tests designed specifically to detect genotoxic endpoints induced by oxidative DNA damage. This information has been added to the text. Studies investigating the ability of TCA to induce oxidative stress, including DNA damage, are included in Sections 4.2 and 4.5.1.6.

2. Have the studies supporting the discussion of the mode(s) of carcinogenic action been clearly described?

Comments: Six reviewers generally concurred with the cancer MOA discussion as written; comments included statements that the available studies were adequately described and that the

review was extensive and comprehensive. One reviewer commented that the document suffered from inconsistencies in listing the components of the MOA in different sections, which detracted from driving the message home that the MOA is complex, and suggested refocusing such discussions to strengthen the MOA analysis. Two reviewers did not consider the cancer MOA section to be clearly described or complete. One of these reviewers commented that the MOA discussion needed to be restructured in order to achieve adequate clarity. This reviewer commented that the analysis did not do a good job of applying EPA's framework from the Cancer Guidelines for evaluating the hypothesized MOA. This reviewer also suggested that a second possible MOA (direct damage to DNA) undergo a "formal" MOA analysis, and that there should be a separate section for other possible MOAs (including the role of nonparenchymal cells such as Kupffer cells, the role of other nuclear receptors, and GJIC-intercellular communication) that currently do not have sufficient information to be subjected to a formal MOA analysis. Two reviewers suggested the use of tables in the MOA section. One of these reviewers suggested adding a table that identifies consistencies/inconsistencies in a series of experimental observations and data gaps for other well studied peroxisome proliferators (e.g., DEHP and Wy-14643) and for TCA. The second reviewer suggested that tables organized around proposed key events and the modified Hill criteria be added.

Response: Section 4.7.3 was revised to improve the clarity of the MOA discussion and to make the discussion more concise. Included in the revisions is a paragraph that was added at the start of the MOA section to lay out the potential MOAs to be discussed. Because PPARα-related events represent some of the major component of the MOA, the PPARα agonism hypothesis was evaluated following EPA's MOA framework from the 2005 Cancer Guidelines. A second possible MOA (direct damage to DNA) was not evaluated using a "formal" MOA analysis because the available information was too limited. Discussions of other MOAs (Kupffer cell activation, hypomethylation of DNA, and reduced intercellular communication) were added as a new Section 4.7.3.2. Data gaps related to consistency and specificity of PPARα agonism as the sole MOA for liver tumor induction were discussed from multiple perspectives in Section 4.7.3.1.1. An analysis of the data for other well-studied peroxisome proliferators and comparison to TCA was considered, but was determined to be outside the scope of this Toxicological Review. EPA agrees that tables can be an effective tool for evaluating MOA information. Much of the data supporting cancer MOAs for TCA, however, comes from in vitro studies with doses that were not comparable to those used in two-year bioassays. Therefore, it was not possible to develop a table (or tables) of TCA MOA information organized around proposed key events that would be useful for the examination of dose or temporal concordance.

Comments: One reviewer provided the following references for discussion in the MOA section:

Preston, RJ; Williams, GM. (2005) DNA-reactive carcinogens: mode of action and human cancer hazard. Crit Rev Toxicol 33:673–683.

Roberts, RA; et al. (2007) Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. Toxicol Sci 96(1):2–15.

Woods, CG; et al. (2007b) Sustained formation of POBN radical adducts in mouse liver by peroxisome proliferators is dependent upon PPARα but not NADPH oxidase. Free Radic Biol Med 42(3):335–342.

Woods, CG; et al. (2007c) Time-course investigation of PPARα- and Kupffer cell-dependent effects of Wy-14643 in mouse liver using microarray gene expression. Toxicol Appl Pharmacol 225(3):267–277.

Guo, D; et al. (2007) Induction of nuclear translocation of constitutive androstane receptor by peroxisome proliferator-activated receptor α synthetic ligands in mouse liver. J Biol Chem 282(50): 36766–36776.

Zhen, Y; et al. (2007) Metabolomic and genetic analysis of biomarkers for peroxisome proliferator-activated receptor α expression and activation. Mol Epidemiol 21(9):2136–2151.

The reviewer also provided six references on PFOA, another PPARα agonist, that this reviewer thought could be helpful.

Response: The references were added to the Toxicological Review, with the exception of Preston and Williams (2005), Zhen et al. (2007), and the papers on PFOA, which were of interest but did not directly relate to TCA toxicity.

Comments: One reviewer recommended that a more complete description be provided of the early report by Elcombe (1985), and the studies by Ito et al. (2007) and Ren et al. (2010). Another reviewer suggested that EPA: (1) strengthen the quantitative assessment of the relative potency of PPARα activation by TCA in comparison to other chlorinated solvents; and (2) compare the potency indicators for mouse hepatocarcinogenicity of various peroxisome proliferators, including chloroacetic acids, with common short-term markers of PPARα activation and in vitro transactivation of PPARα. One reviewer commented that the short section on decreased cell-cell communication seemed speculative.

Response: The study description for Elcombe (1985) was expanded in the Toxicological Review as suggested. A summary of Ren et al. (2010) was added to support the possibility of activation of other nuclear receptors by PPARα agonists, but a detailed description of this study was not included as the study investigated DEHP and not TCA. For the same reason, the Ito et al. (2007) study description was not expanded. As indicated above, an analysis of the data for other well-studied peroxisome proliferators and comparison to TCA was considered, but was determined to be outside the scope of this Toxicological Review. The MOA of decreased cell-cell communication has not been well-characterized in the scientific literature, and is characterized as such in Section 4.7.3.1.4.

Comments: One reviewer commented that potential carcinogenic MOAs other than PPARα agonist-induced peroxisome proliferation are speculative and should either be identified as such or not discussed. This reviewer believed that "TCA could not have produced any tumors by a MOA similar to a non-PPARα agonist" because: (1) foci and tumors found in mouse liver tumors produced by chemicals that are not PPARα agonists are eosinophilic and GST-π positive, which is not the characteristic of TCA-induced tumors; and (2) the histopathology, biology, and molecular biology of the liver tumors in TCA-treated mice are completely consistent with those found in mice treated with other inducers of peroxisome proliferation.

Response: Hypothesized MOAs other than PPARα agonism were based on studies of TCA as well as other classic PPARα agonists such as DEHP and Wy-14643. Section 4.7.3 was revised to clarify when data for PPARα agonists other than TCA provided the major support for alternative potential MOAs for TCA-induced hepatocarcinogenesis.

3. EPA has concluded that the available data do not support any specific MOA. In addition, EPA has determined that the data are not supportive of PPARα agonist-induced peroxisome proliferation as the sole MOA leading to tumor formation. Please comment on whether these determinations are scientifically justified.

Comments: Six reviewers generally agreed with EPA's conclusions regarding cancer MOA. Several of these reviewers further observed that PPARα-related events represent some major components of the MOA, but that there are likely multiple MOAs that may not be mutually exclusive. One reviewer suggested that the statement that the data are not supportive of PPARα induced peroxisome proliferation as the sole MOA was too strong; this reviewer preferred the statement that the data do not identify any specific MOA (including peroxisome proliferation) as the sole MOA.

Three reviewers disagreed with the conclusion that there are insufficient data to establish PPARα agonism as a MOA. It was the judgment of two of these reviewers that the data supported a PPARα agonist MOA as the sole MOA; one of these reviewers commented that MOAs other than PPARα were speculative and without support. One reviewer commented that if more than one MOA is involved, then it is necessary to describe which MOA could occur under what circumstances/conditions.

Response: EPA agrees that the MOA for TCA carcinogenicity is complex, that multiple MOAs that are not mutually exclusive may be involved, and that while PPARα-related events represent some of the major components of the overall MOA, it is premature to conclude that this is the only MOA for TCA. Discussion of data gaps with respect to consistency and specificity of PPARα agonism as the sole MOA for TCA carcinogenicity in Section 4.7.3.1.1 was re-structured

to improve the clarity. The MOAs other than PPARα agonism are hypotheses based on experimental evidence. Discussions of these MOAs in Sections 4.7.3.1.2–4.7.3.1.4 were revised to clarify the nature and extent of scientific support.

Comments: Two reviewers indicated that the discussion of human relevance of the PPARα agonist MOA was incomplete. One of these reviewers stated that the growing body of evidence that reveals qualitative differences between the rodent and human PPARα cascade needed to be summarized. The second reviewer recommended that differences in mouse and human binding of activated hPPARα and the reliability of studies of primary human hepatocyte cultures be included in the discussion of human relevance.

Response: The discussion of human relevance has been expanded. New studies related to the possible differences between human and mouse PPARα and a discussion of the weakness of in vitro studies were added to Section 4.7.3.1.1.4.

4. A 104-week drinking water study in mice (DeAngelo et al., 2008) was selected as the basis for quantification of the oral cancer slope factor. Please comment on whether the selection of this study is scientifically justified.

Comments: Five reviewers considered the selection of DeAngelo et al. (2008) as the basis for the oral cancer slope factor to be justified. One reviewer disagreed with the application of an exposure duration adjustment factor for the 82-week study (Pereira, 1996). This reviewer suggested that the slope factor from this study be recalculated without the exposure duration factor, and re-compared to the slope factor based on the 104-week study before reaching a final decision as to which of the two studies provides the best data set for quantitative assessment. This reviewer also suggested excluding oral slope factors calculated from the 52- and 60-week studies because of the uncertainty introduced by the exposure duration factor. One reviewer expressed concern that there were a large number of animals identified as unscheduled deaths in the DeAngelo et al. (2008) study that were not examined for hepatocellular neoplasia and suggested obtaining an explanation from the study author as to why these animals were not examined and the impact of the missing data on the cancer potency estimate. This reviewer also asked for an explanation for the difference in the liver tumor incidence in the control group from Study 2 (12%) versus the control group from Study 3 (64%). One reviewer commented that the 104-week study does not appear to be a good choice for modeling of the cancer slope factor because: (1) the incidence of combined adenomas and carcinomas at the lower of the two doses in this study is less than the incidence in the control mice (so that the POD is based essentially on the observation of a single dose group); and (2) the 104-week duration study had a very high incidence of combined adenomas and carcinomas in the control group (64%) when compared

with other studies in the male B6C3F$_1$ mouse. Two reviewers commented that the development of an oral cancer slope factor is not justified since the liver tumors induced by TCA are not sufficient to classify TCA as a potential human carcinogen.

Response: Because the 82-week study by Pereira (1996) was not a lifetime study for the mouse, applying an exposure time adjustment factor as $(82/104)^{0.25} = 0.49$ is considered to be appropriate. Without this adjustment, the candidate cancer slope factor derived from this study would be approximately half of the current value (i.e., 2.1×10^{-2}), which is lower than the oral cancer slope factor derived from the 104-week study by DeAngelo et al. (2008). The cancer slope factors from the 52- and 60-week studies were calculated for purpose of comparison. The final slope factor was based on the 104-week lifetime study.

In the 104-week study from DeAngelo et al. (2008), the animals identified as unscheduled deaths without pathology vs. unscheduled deaths in the 0, 0.05, and 0.5 g/L TCA dose groups were 9/17, 17/24, and 14/24, respectively. According to the study author, Dr. Anthony DeAngelo (email dated April 6, 2010, from Anthony DeAngelo, NHEERL, ORD, U.S. EPA, to Diana Wong, NCEA, ORD, U.S. EPA), most of the pathology data were missing because the tissues of the animals found dead were autolyzed or the remains were eaten by cage mates. One or two animals might also have escaped over the 2-year period of study. The possible impact of missing pathology could be an underestimation of the liver tumor incidence and a lower oral cancer slope factor; however, the difference among the five candidate cancer slope factors derived from five independent studies was approximately fivefold, which lends some confidence to the oral slope factor derived from the DeAngelo et al. (2008) study (see Table 5-12).

For B6C3F$_1$ mice, the higher rate of adenomas and carcinomas in the control group in the 104-week study is not unexpected. For the cancer quantification, percent increase in tumors compared with the control was used in BMD modeling, not absolute percent tumors.

Variable incidence of spontaneous liver tumors in the male B6C3F$_1$ mouse is not unexpected. For example, Haseman et al. (1985) reported that the incidence of liver tumors (adenoma or carcinoma) in male B6C3F$_1$ mice (untreated controls) from National Toxicology Program bioassays ranged from 14 to 58%. Further, the study 2 and 3 control groups were treated with different control vehicles—one was neutralized acetic acid and the second was deionized water. The experiments were conducted in different labs at different times. These factors might contribute to the differences in tumor incidence in the two control groups.

Comments related to the choice of studies for BMD modeling are addressed in responses to Charge Question C5.

5. The oral cancer slope factor was calculated by linear extrapolation from the POD (lower 95% confidence limit on the dose associated with 10% extra risk for liver tumors). Has the

modeling approach been appropriately conducted? Please identify and provide the rationale for any alternative approaches for the determination of the slope factor and discuss whether such approaches are preferred to EPA's approach.

Comments: Four reviewers generally agreed that modeling of the cancer dose response and derivation of the oral cancer slope factor was appropriately undertaken. One of these reviewers observed that the assumption used in the exposure duration scaling of a mouse lifetime of 104 weeks underestimates the true lifetime of a $B6C3F_1$ mouse and that longer lifetimes should be used for this scaling. This reviewer questioned why the multistage model was the only model in BMDS used to fit liver tumor data and recommended that other models be fit and evaluated for goodness-of-fit. This reviewer also suggested that male mouse liver tumor data from the 60- and 104-week studies by DeAngelo et al. (2008) be combined and used for the determination of the $BMLD_{10}$ and the oral cancer slope factor. A second of these reviewers observed that the response at the low dose in the 104-week DeAngelo et al. (2008) study was essentially the same as the control, so that there was only one positive value for tumor incidence. In this case, the reviewer questioned the use of BMD modeling.

One reviewer commented that the margin-of-exposure approach would be more appropriate for a chemical with a "suggestive" weight-of-evidence characterization, and that no quantitative assessment is necessary for an "unlikely" characterization. Three reviewers did not comment on this charge question.

Response: EPA considers 104 weeks to be the standard lifetime for rats and mice. Therefore, exposure duration scaling for the 104-week mouse study is not warranted. The rationale for use of the multistage model is provided in Section 5.4.4. As noted in this section, the multistage model has been used by EPA in the vast majority of quantitative cancer assessments because it is thought to reflect the multistage carcinogenic process. This model can accommodate a wide variety of dose-response shapes and its use provides consistency with previous quantitative dose-response assessments for cancer.

An analysis using combined tumor data from the DeAngelo et al. (2008) study was conducted. Because the 60- and 104-week studies used different study time frames, dose-response analysis of combined data sets based on the summary incidence data in the published paper could not be performed with the models in BMDS. EPA obtained the individual animal data from the study authors (Dr. Anthony DeAngelo), including when each liver tumor was identified in individual animals. The MSW time-to-tumor model, which models both the exposure dose and appearance time of the tumor, was used to model combined tumor data sets. A statistical analysis (generalized likelihood ratio test) was first applied to determine which of the three studies reported in DeAngelo et al. (2008) were compatible for combined analysis. A summary of the MSW time-to-tumor modeling was added to Section 5.4.5, and a detailed

discussion of the modeling, including model outputs, was provided in a new Appendix E. This analysis revealed that oral cancer slope factors for the individual study and combined data sets were not substantially different, nor were there substantial differences in the slope factors derived with the MSW time-to-tumor model or the multistage model in BMDS. The 104-week study in DeAngelo et al. (2008) was selected for deriving the cancer slope factor because it is the only lifetime study of TCA. The BMDL derived from this study was within 2.5-fold of BMDL values from other TCA cancer bioassays, including 52-week studies in male mice (Bull et al., 2002; 1990), a 60-week study in male mice (DeAngelo et al., 2008), and an 82-week study in female mice (Pereira, 1996). This consistency in BMDL values supports the use of data from the 104-week study for slope factor derivation.

6. An inhalation unit risk (IUR) for cancer was not derived for TCA. Is the determination that the available data for TCA do not support derivation of an IUR scientifically justified?

Comments: Most reviewers agreed that the decision not to derive an IUR for TCA was justified. One reviewer considered the justification for not deriving an IUR to be inadequate. Another reviewer observed that, in view of the relatively minor importance of metabolism in TCA toxicity and the water-soluble nature of the chemical, an IUR could be derived by route-to-route extrapolation by assuming 100% absorption by the inhalation route followed by systemic distribution via the bloodstream. One reviewer questioned how the Toxicological Review could support the conclusion that inhaled TCA is carcinogenic in laboratory animals and humans if no inhalation data exist.

Response: In the absence of inhalation studies of TCA and given the lack of a PBPK model for TCA to support route-to-route extrapolation, an IUR for TCA was not derived. Available information suggests that a simple set of assumptions might not account for possible differences in the magnitude of response across routes. The liver is the critical target organ for oral toxicity, and a first-pass effect by the liver is expected following oral administration. Justification for not deriving an IUR in Section 5.4.6 was expanded. The rationale for extending the cancer descriptor to all routes of exposure is addressed in response to comments on Charge Question C1.

APPENDIX B. BENCHMARK DOSE MODELING RESULTS FOR LIVER DATA SETS FROM DeANGELO ET AL. (2008)

B.1. INCIDENCE OF HEPATOCELLULAR INFLAMMATION

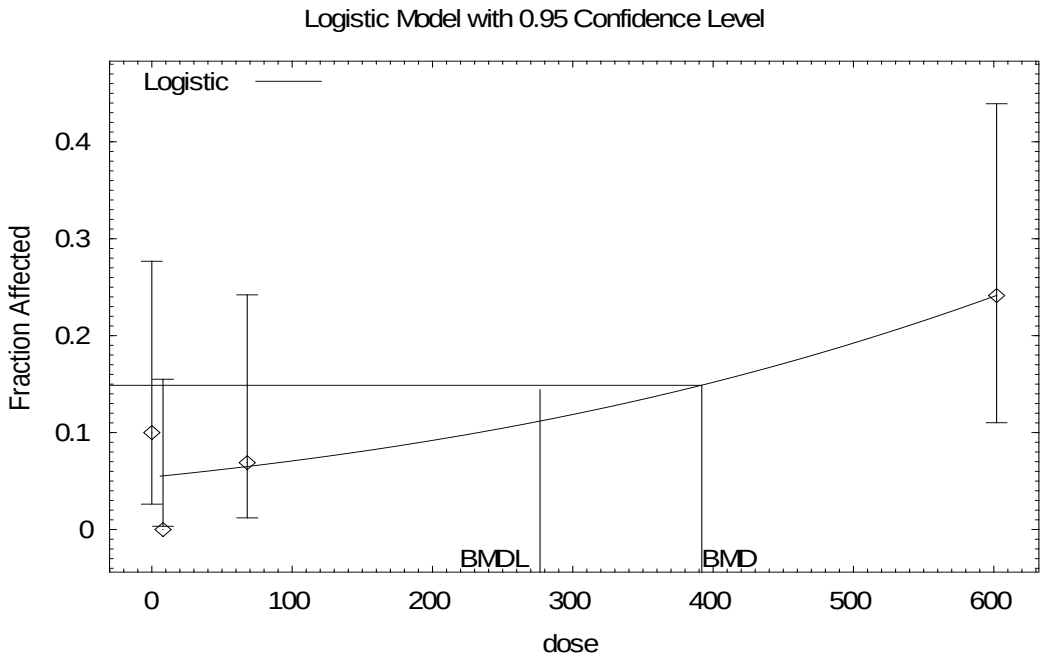
Table B-1. BMD modeling results based on incidence of hepatocellular inflammation in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks

Fitted dichotomous model[a]	χ^2 goodness-of-fit test *p*-value[b]	AIC[c]	BMD_{10}[d] (mg/kg-d)	$BMDL_{10}$[e] (mg/kg-d)
Gamma	0.096	76.15	354.2	151.6
Logistic	**0.24**	**74.19**	**391.9**	**276.6**
Log-logistic	0.096	76.16	351.0	132.1
Multistage (1°)	0.22	74.29	292.0	149.4
Probit	0.24	74.20	376.1	257.1
Log-probit	**0.26**	**74.19**	**394.1**	**244.4**
Weibull	0.096	76.16	361.9	151.6

[a]All dichotomous dose-response models were fit using BMDS, version 1.4.1. The best-fit models are indicated in boldface type.
[b]*p*-Value from the χ^2 goodness-of-fit test for the selected model. Values <0.1 suggest that the model exhibits a significant lack of fit, and a different model should be chosen.
[c]AIC is a value useful for evaluating model fit. For those models exhibiting adequate fit, lower values of the AIC suggest better model fit.
[d]BMD_{10} = BMD at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the BMD at 10% extra risk.

Source: DeAngelo et al. (2008).

Of the seven models fit, four (i.e., logistic, one-stage multistage, probit, and log-probit) showed adequate fit. The BMDS outputs from the two models with the best fit (based on lowest AIC value), the logistic and log-probit models, are provided below.



12:07 09/05 2008

```
 ================================================================
         Logistic Model. (Version: 2.10; Date: 09/23/2007)
         Input Data File: M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.(d)
         Gnuplot Plotting File:  M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.plt
                                                  Fri Sep 05 12:07:17 2008
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = 1/[1+EXP(-intercept-slope*dose)]


   Dependent variable = Response
   Independent variable = Dose
   Slope parameter is not restricted

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     background =            0   Specified
                      intercept =     -2.90541
                          slope =   0.00303299


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1        -0.76
```

slope -0.76 1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
intercept	-2.85931	0.482625	-3.80523	-1.91338
slope	0.00284529	0.00109927	0.000690752	0.00499983

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-33.0575	4			
Fitted model	-35.0966	2	4.07833	2	0.1301
Reduced model	-38.4712	1	10.8276	3	0.0127

AIC: 74.1932

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0542	1.626	3	30	1.108
8.0000	0.0554	1.495	0	27	-1.258
68.0000	0.0650	1.886	2	29	0.086
602.0000	0.2411	6.993	7	29	0.003

Chi^2 = 2.82 d.f. = 2 P-value = 0.2444

Benchmark Dose Computation

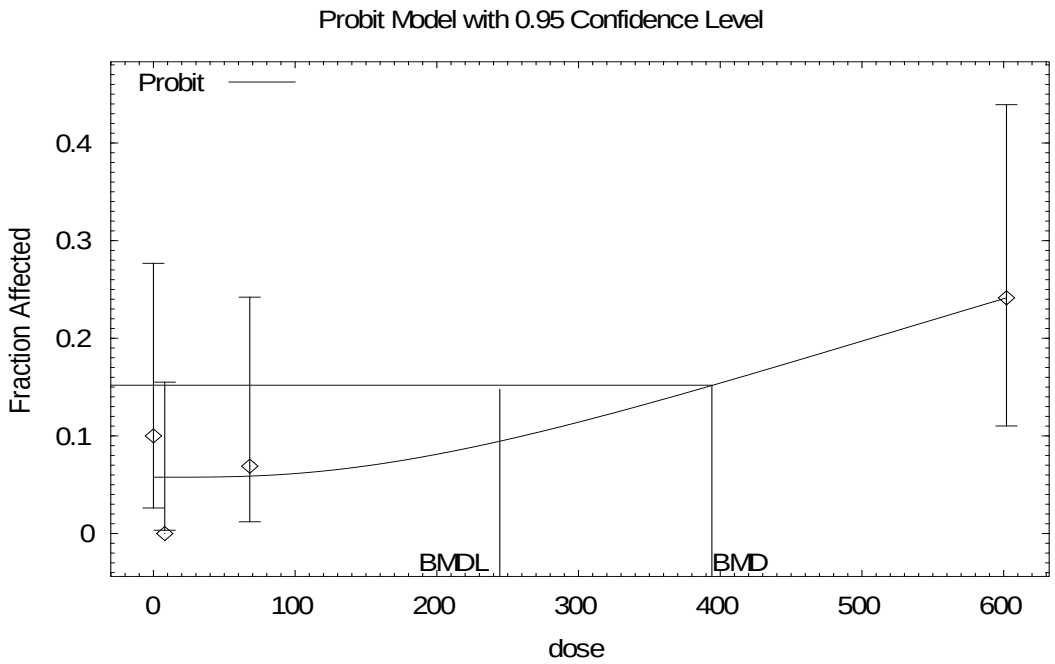
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 391.918

BMDL = 276.646




```
 ======================================================================
         Probit Model. (Version: 2.9;  Date: 09/23/2007)
         Input Data File: M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.(d)
         Gnuplot Plotting File:  M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.plt
                                                  Fri Sep 05 12:14:41 2008
 ======================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = Background
               + (1-Background) * CumNorm(Intercept+Slope*Log(Dose)),

   where CumNorm(.) is the cumulative normal distribution function

   Dependent variable = Response
   Independent variable = Dose
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model

                 Default Initial (and Specified) Parameter Values
                    background =          0.1
                     intercept =      -7.0776
                         slope =            1

            Asymptotic Correlation Matrix of Parameter Estimates

            ( *** The model parameter(s)  -slope
                  have been estimated at a boundary point, or have been specified by the user,
                  and do not appear in the correlation matrix )
```

	background	intercept
background	1	-0.26
intercept	-0.26	1

Parameter Estimates

Variable	Estimate	95.0% Wald Confidence Interval Std. Err.	Lower Conf. Limit	Upper Conf. Limit
background	0.0576569	0.0253479	0.00797583	0.107338
intercept	-7.25815	0.31762	-7.88067	-6.63563
slope	1	NA		

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-33.0575	4			
Fitted model	-35.0974	2	4.07991	2	0.13
Reduced model	-38.4712	1	10.8276	3	0.0127

AIC: 74.1948

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0577	1.730	3	30	0.995
8.0000	0.0577	1.557	0	27	-1.285
68.0000	0.0588	1.705	2	29	0.233
602.0000	0.2419	7.014	7	29	-0.006

Chi^2 = 2.70 d.f. = 2 P-value = 0.2597

Benchmark Dose Computation

Specified effect = 0.1
Risk Type = Extra risk
Confidence level = 0.95
BMD = 394.098
BMDL = 244.412

B.2. INCIDENCE OF HEPATOCELLULAR NECROSIS

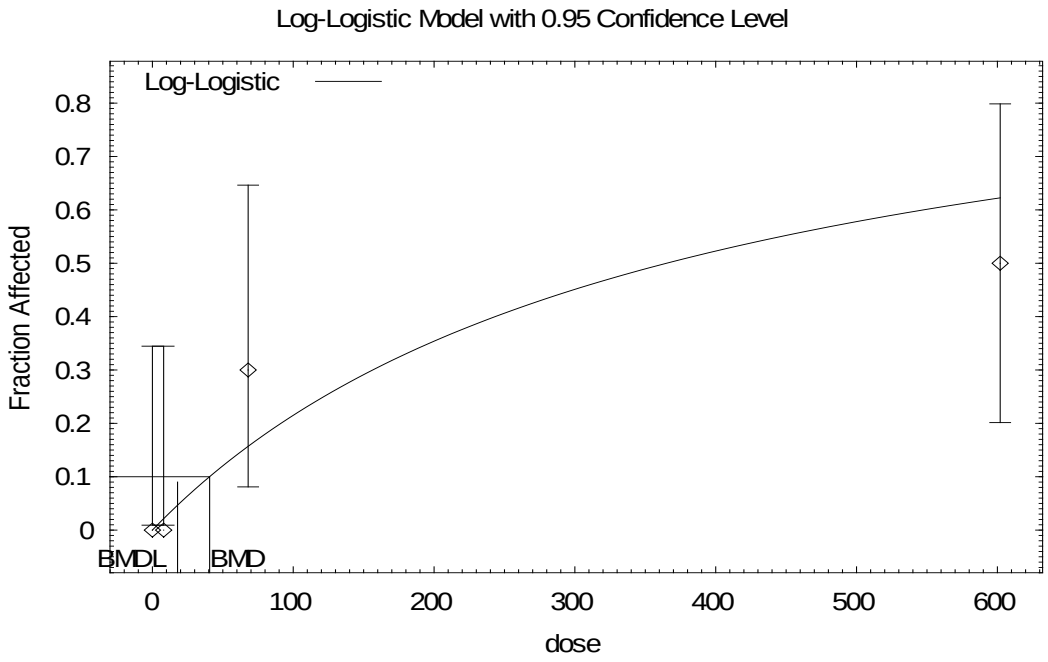
Table B-2. BMD modeling results based on incidence of hepatocellular necrosis in male B6C3F$_1$ mice exposed to TCA in drinking water for 30–45 weeks

Fitted dichotomous model[a]	χ^2 goodness-of-fit test *p*-value[b]	AIC[c]	BMD_{10}[d] (mg/kg-d)	$BMDL_{10}$[e] (mg/kg-d)
Gamma, multistage (1°), and Weibull	0.18	31.85	64.9	37.6
Logistic	0.058	36.39	205.1	128.4
Log-logistic	**0.49**	**30.42**	**40.7**	**17.9**
Probit	0.060	36.26	188.0	120.0
Log-probit	0.036	36.84	158.7	54.3

[a]All dichotomous dose-response models were fit using BMDS, version 1.4.1. The best-fit models are indicated in boldface type.
[b]*p*-Value from the χ^2 goodness-of-fit test for the selected model. Values <0.1 suggest that the model exhibits a significant lack of fit, and a different model should be chosen.
[c]AIC is a value useful for evaluating model fit. For those models exhibiting adequate fit, lower values of the AIC suggest better model fit.
[d]BMD_{10} = BMD at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the BMD at 10% extra risk.

Source: DeAngelo et al. (2008).

Of the seven models fit, four (i.e., gamma, log-logistic, one-stage multistage, and Weibull) showed adequate fit. The BMDS output for the best fitting model of the four (based on lowest AIC value), the log-logistic model, is provided below.




```
 ======================================================================
         Logistic Model. (Version: 2.10; Date: 09/23/2007)
         Input Data File: M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_HEPATOCELLULAR_NECROSIS_DEANGELO_2008 .(d)
         Gnuplot Plotting File:  M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_HEPATOCELLULAR_NECROSIS_DEANGELO_2008.plt
                                                    Fri Sep 05 14:21:36 2008
 ======================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = Response
   Independent variable = Dose
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


   User has chosen the log transformed model


                 Default Initial Parameter Values
                    background =           0
                     intercept =    -5.96722
                         slope =           1
          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -background    -slope
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

             intercept

 intercept           1
```

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
background	0	*	*	*
intercept	-5.90256	*	*	*
slope	1	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-13.0401	4			
Fitted model	-14.2076	1	2.33493	3	0.5059
Reduced model	-20.0161	1	13.952	3	0.002971

AIC: 30.4152

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0.000
8.0000	0.0214	0.214	0	10	-0.468
68.0000	0.1567	1.567	3	10	1.247
602.0000	0.6219	6.219	5	10	-0.795

Chi^2 = 2.40 d.f. = 3 P-value = 0.4927

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 40.6639

BMDL = 17.8767

B.3. INCIDENCE OF TESTICULAR TUBULAR DEGENERATION

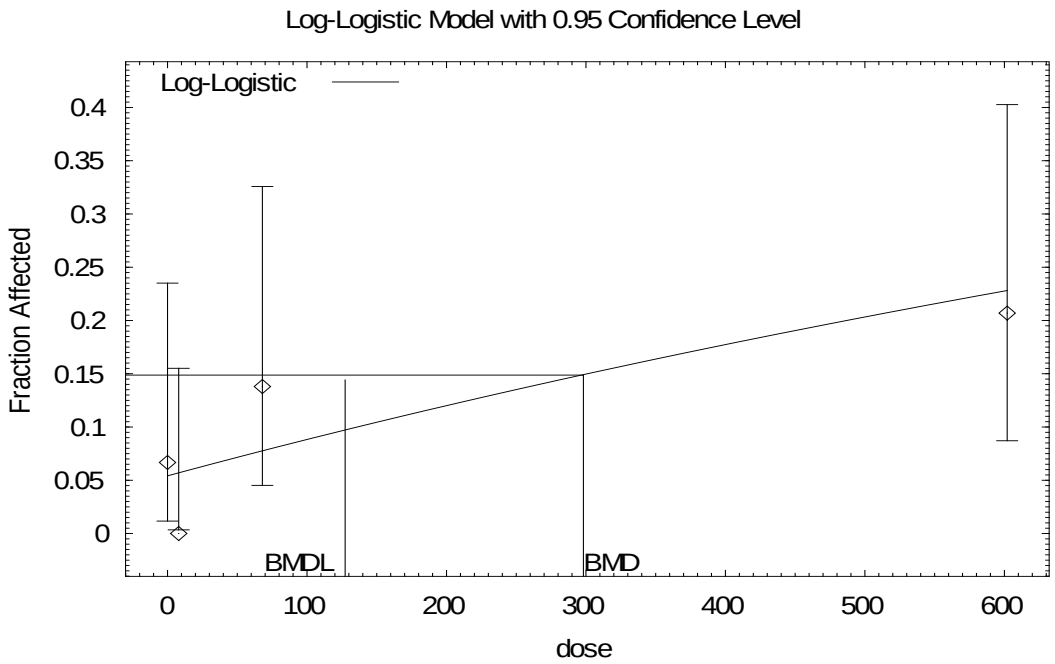
Table B-3. BMD modeling results based on incidence of testicular tubular degeneration in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks

Fitted dichotomous model[a]	χ^2 goodness-of-fit test *p*-value[b]	AIC[c]	BMD$_{10}$[d] (mg/kg-d)	BMDL$_{10}$[e] (mg/kg-d)
Gamma, multistage (1°), and Weibull	0.19	76.16	321.9	153.3
Logistic	0.16	76.59	439.7	290.3
Log-logistic	**0.19**	**76.08**	**298.2**	**127.4**
Probit	0.17	76.54	425.3	271.2
Log-probit	0.13	77.06	471.6	276.8

[a]All dichotomous dose-response models were fit using BMDS, version 1.4.1. The best-fit models are indicated in boldface type.
[b]*p*-Value from the χ^2 goodness-of-fit test for the selected model. Values <0.1 suggest that the model exhibits a significant lack of fit, and a different model should be chosen.
[c]AIC is a value useful for evaluating model fit. For those models exhibiting adequate fit, lower values of the AIC suggest better model fit.
[d]BMD$_{10}$ = BMD at 10% extra risk.
[e]BMDL$_{10}$ = 95% lower confidence limit on the BMD at 10% extra risk.

Source: DeAngelo et al. (2008).

All seven models showed adequate fit. The BMDS output from the model that provided the best fit of the seven (based on lowest AIC value), the log-logistic model, is provided below.




```
 ================================================================
         Logistic Model. (Version: 2.10; Date: 09/23/2007)
         Input Data File: M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_TESTICULAR_DEGENERATION_60_WKS_DEANGELO_2008.(d)
         Gnuplot Plotting File:  M:\TCA DOSE-RESPONSE
MODELING\MALE_MOUSE_TESTICULAR_DEGENERATION_60_WKS_DEANGELO_2008.plt
                                                  Fri Sep 05 13:50:29 2008
 ================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = Response
   Independent variable = Dose
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


   User has chosen the log transformed model

                 Default Initial Parameter Values
                    background =    0.0666667
                     intercept =     -7.67626
                         slope =            1
          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -slope
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

            background    intercept

background            1        -0.47
```

intercept -0.47 1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
background	0.0540864	*	*	*
intercept	-7.89489	*	*	*
slope	1	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-33.7671	4			
Fitted model	-36.0406	2	4.54705	2	0.1029
Reduced model	-38.4712	1	9.40833	3	0.02433

AIC: 76.0812

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0541	1.623	2	30	0.305
8.0000	0.0569	1.536	0	27	-1.276
68.0000	0.0775	2.246	4	29	1.218
602.0000	0.2274	6.595	6	29	-0.263

Chi^2 = 3.27 d.f. = 2 P-value = 0.1945

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 298.169

BMDL = 127.35

B.4. CYANIDE-INSENSITIVE PCO ACTIVITY

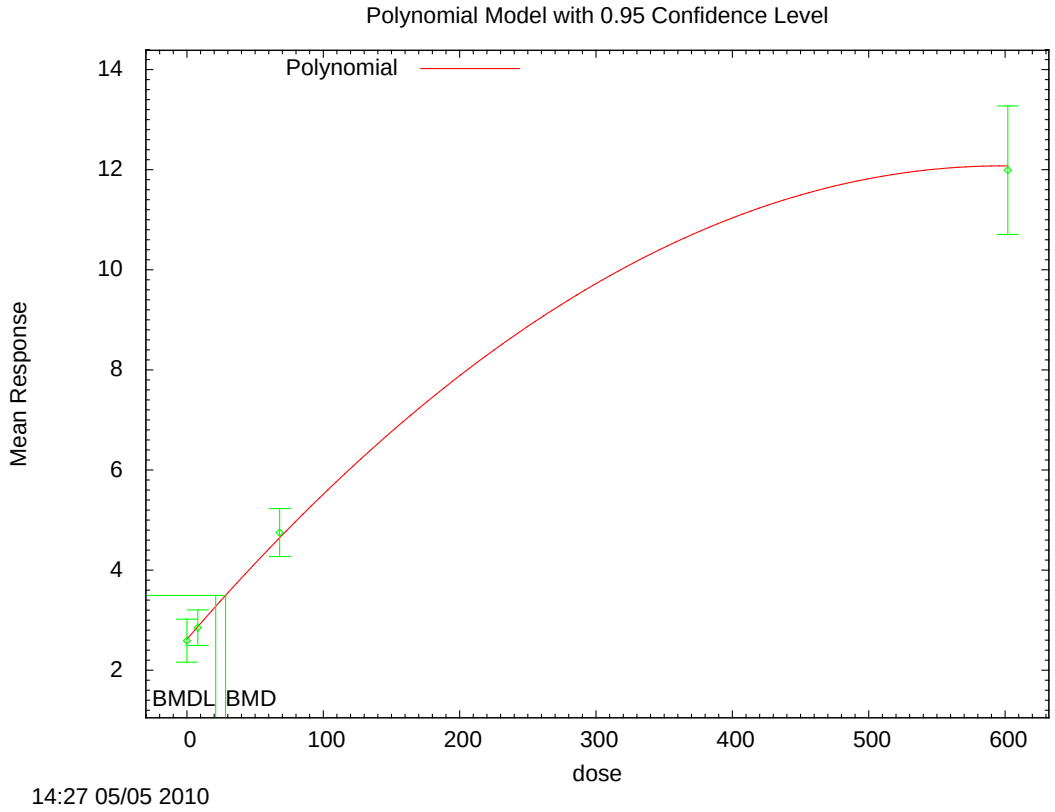
Table B-4. BMD modeling results based on cyanide-insensitive PCO activity in male B6C3F$_1$ mice exposed to TCA in drinking water for up to 60 weeks

Fitted continuous model[a]	Test of homogeneity of variances *p*-value	χ^2 goodness-of-fit test *p*-value[b]	AIC[c]	BMD_{1SD}[d] (mg/kg-d)	$BMDL_{1SD}$[e] (mg/kg-d)
Hill	0.18	NA	165.52	33.5	20.0
Linear	0.18	0.003	173.39	61.7	50.2
Polynomial (2°)	**0.18**	**0.66**	**163.71**	**28.4**	**21.1**
Power	0.18	0.003	173.39	61.7	50.2

[a]All continuous dose-response models were fit using BMDS, version 2.1.1. The variances were determined to be non-homogeneous across dose groups, so, in each case, the non-constant variance version of the model was fit to the data. The best-fit model is indicated in boldface type.
[b]*p*-Value from the χ^2 goodness-of-fit test for the selected model. Values <0.1 suggest that the model exhibits a significant lack of fit, and a different model should be chosen.
[c]AIC is a value useful for evaluating model fit. For those models exhibiting adequate fit, lower values of the AIC suggest better model fit.
[d]BMD_{1SD} = BMD estimated at 1 SD from the control mean.
[e]$BMDL_{1SD}$ = estimated 95% lower confidence limit on the BMD at 1 SD from the control mean.

Source: DeAngelo et al. (2008).

Of the four models fit to the data, only the second-degree polynomial model showed adequate fit, and thus, the BMDS output from this model is provided below.




```
 ======================================================================
         Polynomial Model. (Version: 2.13;  Date: 04/08/2008)
         Input Data File:
M:\TCA_Dose_Response_Modeling\ply_Male_Mice_PCO_Activity_60_Weeks_Setting.(d)
         Gnuplot Plotting File:
M:\TCA_Dose_Response_Modeling\ply_Male_Mice_PCO_Activity_60_Weeks_Setting.plt
                                                  Wed May 05 14:26:53 2010
 ======================================================================

 BMDS Model Run
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = Mean
   Independent variable = Dose
   Signs of the polynomial coefficients are not restricted
   The variance is to be modeled as Var(i) = exp(lalpha + log(mean(i)) * rho)

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                        lalpha =      1.11103
                           rho =            0
                        beta_0 =      2.58577
                        beta_1 =      0.03388
                        beta_2 = -3.03295e-005
```

Asymptotic Correlation Matrix of Parameter Estimates

	lalpha	rho	beta_0	beta_1	beta_2
lalpha	1	-0.92	0.041	-0.011	0.00089
rho	-0.92	1	-0.014	-0.041	0.054
beta_0	0.041	-0.014	1	-0.54	0.49
beta_1	-0.011	-0.041	-0.54	1	-0.98
beta_2	0.00089	0.054	0.49	-0.98	1

Parameter Estimates

			95.0% Wald Confidence Interval	
Variable	Estimate	Std. Err.	Lower Conf. Limit	Upper Conf. Limit
lalpha	-1.74224	0.364233	-2.45612	-1.02835
rho	1.52897	0.223741	1.09045	1.9675
beta_0	2.6204	0.137476	2.35095	2.88985
beta_1	0.0315656	0.00523455	0.0213061	0.0418251
beta_2	-2.64058e-005	8.69347e-006	-4.34447e-005	-9.36686e-006

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Est Mean	Obs Std Dev	Est Std Dev	Scaled Res.
0	25	2.59	2.62	1.04	0.874	-0.174
8	25	2.85	2.87	0.86	0.937	-0.113
68	25	4.75	4.64	1.16	1.35	0.389
602	24	12	12.1	3.04	2.81	-0.111

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = exp(lalpha + rho*ln(Mu(i)))
Model A3 uses any fixed variance parameters that were specified by the user

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	# Param's	AIC
A1	-102.454268	5	214.908535
A2	-75.063486	8	166.126972
A3	-76.759109	6	165.518219
fitted	-76.853814	5	163.707628
R	-190.262801	2	384.525601

Explanation of Tests

Test 1: Do responses and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)

(Note: When rho=0 the results of Test 3 and Test 2 will be the same.)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	230.399	6	<.0001
Test 2	54.7816	3	<.0001
Test 3	3.39125	2	0.1835
Test 4	0.189409	1	0.6634

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels It seems appropriate to model the data

The p-value for Test 2 is less than .1. A non-homogeneous variance model appears to be appropriate

The p-value for Test 3 is greater than .1. The modeled variance appears to be appropriate here

The p-value for Test 4 is greater than .1. The model chosen seems to adequately describe the data

Benchmark Dose Computation

Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 28.3615

BMDL = 21.0843

APPENDIX C. BENCHMARK DOSE MODELING RESULTS FOR DEVELOPMENTAL DATA SETS FROM SMITH ET AL. (1989)

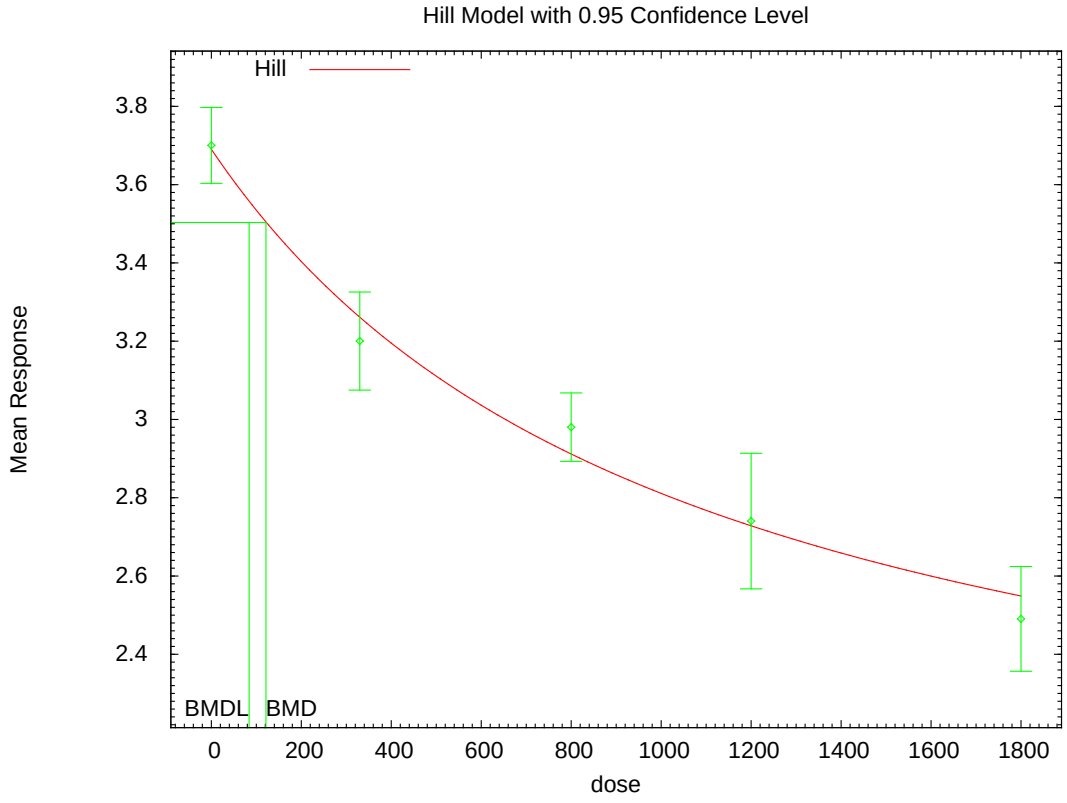
C.1. FETAL BODY WEIGHT

Table C-1. BMD modeling results based on fetal body weight in Long-Evans rats exposed to TCA by gavage on GDs 6–15—male fetuses

Model	*p*-value	AIC	Largest residual (mg/kg-d)	BMD_{1SD} (mg/kg-d)	$BMDL_{1SD}$ (mg/kg-d)	BMD_{05} (mg/kg-d)	$BMDL_{05}$ (mg/kg-d)
Exponential (model 2)	0.0061	-144.03	-2.6	307.2	258.5	221.3	197.8
Exponential (model 3)	0.0061	-144.03	-2.6	307.2	258.5	221.3	197.8
Exponential (model 4)	0.1050	-149.93	-1.5	182.4	133.0	138.3	103.4
Exponential (model 5)	0.1050	-149.93	-1.5	182.4	133.0	138.3	103.4
Hill (constant variance)	**0.1861**	**-151.07**	**-1.1**	**160.0**	**109.0**	**121.4**	**84.0**
Polynomial-linear	0.0005	-138.89	-2.7	373.0	317.9	261.0	236.7
Polynomial (degree ≥2)	0.0363	-147.80	-2.1	224.2	180.4	168.4	141.9
Power	0.0005	-138.89	-2.7	373.0	317.9	261.0	236.7

BMD_{05} = BMD at 5% change in mean relative to the control mean; BMD_{1SD} = BMD at 1 SD change in mean from the control mean

Source: Smith et al. (1989).




```
==========================================================================
         Hill Model. (Version: 2.14;  Date: 06/26/2008)
         Input Data File: C:\USEPA\BMDS21\Data\TCA\Body-weight\body-weight-m\hil_body-weight-
m_hil-5%.(d)
         Gnuplot Plotting File:  C:\USEPA\BMDS21\Data\TCA\Body-weight\body-weight-m\hil_body-
weight-m_hil-5%.plt
==========================================================================
BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
   The form of the response function is:
 Y[dose] = intercept + v*dose^n/(k^n + dose^n)

   Dependent variable = Mean
   Independent variable = Dose
   rho is set to 0
   Power parameter restricted to be greater than 1
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008

                  Default Initial Parameter Values
                          alpha =     0.056562
                            rho =            0   Specified
                      intercept =          3.7
                              v =        -1.21
                              n =      1.15572
                              k =      554.318

           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -rho    -n
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

                   alpha    intercept           v           k
     alpha             1     4.5e-008     1.1e-007    -9.6e-008
```

intercept	4.5e-008	1	0.2	-0.45
v	1.1e-007	0.2	1	-0.95
k	-9.6e-008	-0.45	-0.95	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.0553685	0.00854354	0.0386234	0.0721135
intercept	3.68734	0.0465166	3.59617	3.77851
v	-1.81631	0.35395	-2.51004	-1.12258
n	1	NA		
k	1074.7	449.461	193.777	1955.63

NA - Indicates that this parameter has hit a bound implied by some inequality constraint and thus has no standard error.

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Est Mean	Obs Std Dev	Est Std Dev	Scaled Res.
0	26	3.7	3.69	0.24	0.235	0.274
330	19	3.2	3.26	0.26	0.235	-1.12
800	17	2.98	2.91	0.17	0.235	1.19
1200	14	2.74	2.73	0.3	0.235	0.172
1800	8	2.49	2.55	0.16	0.235	-0.722

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2
Model A3 uses any fixed variance parameters that were specified by the user

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	# Param's	AIC
A1	81.219029	6	-150.438057
A2	84.930660	10	-149.861321
A3	81.219029	6	-150.438057
fitted	79.537298	4	-151.074597
R	21.120977	2	-38.241954

Explanation of Tests

Test 1: Do responses and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)
(Note: When rho=0 the results of Test 3 and Test 2 will be the same.)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	127.619	8	<.0001
Test 2	7.42326	4	0.1151
Test 3	7.42326	4	0.1151
Test 4	3.36346	2	0.1861

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is greater than .1. A homogeneous variance model appears to be appropriate here

The p-value for Test 3 is greater than .1. The modeled variance appears to be appropriate here

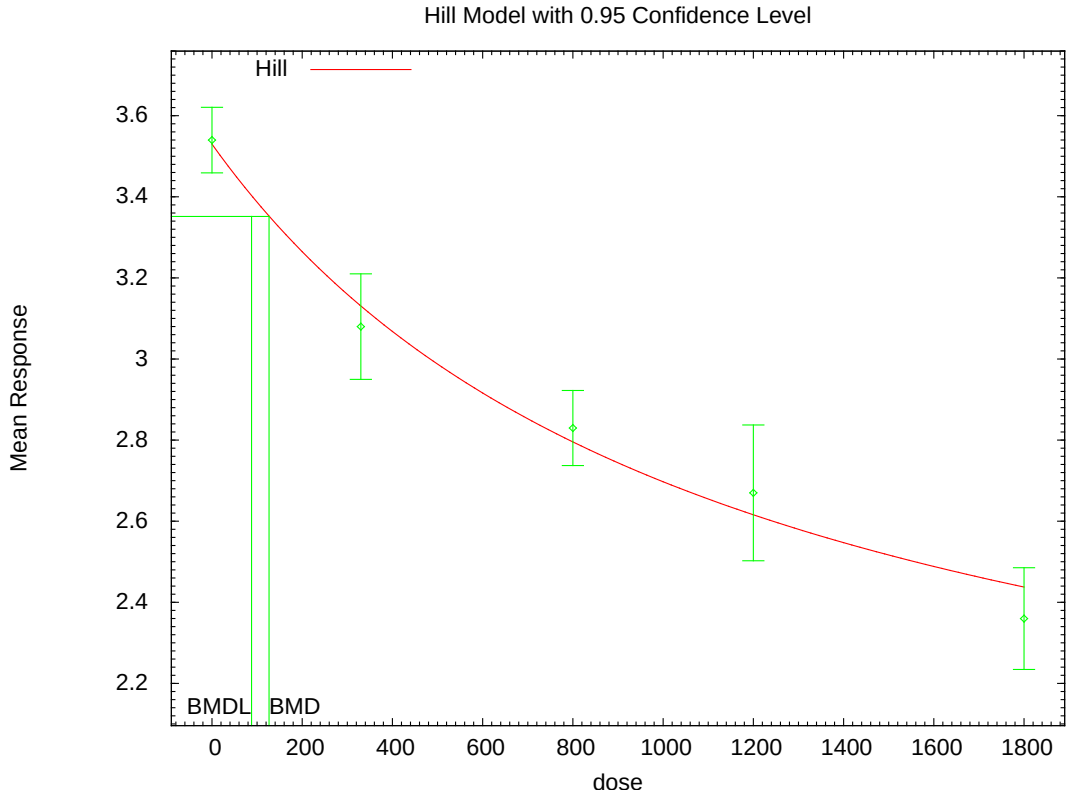
```
The p-value for Test 4 is greater than .1.  The model chosen seems
to adequately describe the data

        Benchmark Dose Computation
Specified effect =          0.05
Risk Type        =     Relative risk
Confidence level =           0.95
             BMD =        121.414
            BMDL =       84.0299
```

Table C-2. BMD modeling results based on fetal body weight in Long-Evans rats exposed to TCA by gavage on GDs 6–15—female fetuses

Model	*p*-value	AIC	Largest residual (mg/kg-d)	BMD_{1SD} (mg/kg-d)	$BMDL_{1SD}$ (mg/kg-d)	BMD_{05} (mg/kg-d)	$BMDL_{05}$ (mg/kg-d)
Exponential (model 2)	0.0106	-152.82	-2.359	305.9	257.5	222.5	199.0
Exponential (model 3)	0.0106	-152.82	-2.359	305.9	257.5	222.5	199.0
Exponential (model 4)	0.1113	-157.66	-1.328	188.2	136.9	143.1	106.7
Exponential (model 5)	0.1113	-157.66	-1.328	188.2	136.9	143.1	106.7
Hill (constant variance)	**0.1970**	**-158.80**	**-1.000**	**166.1**	**113.4**	**126.5**	**87.7**
Polymomial-linear	0.0011	-148.04	-2.470	369.7	315.2	261.8	237.5
Polynomial (degree ≥2)	0.0372	-155.46	-1.880	230.7	185.0	173.6	145.7
Power	0.0011	-148.04	-2.470	369.7	315.2	261.8	237.5

BMD_{05} = BMD at 5% change in mean relative to the control mean; BMD_{1SD} = BMD at 1 SD change in mean from the control mean

Source: Smith et al. (1989).




```
==============================================================================
         Hill Model. (Version: 2.14;  Date: 06/26/2008)
         Input Data File: C:\USEPA\BMDS21\Data\TCA\Body-weight\body-weight-f\hil_body-weight-
f_hil-5%.(d)
         Gnuplot Plotting File:  C:\USEPA\BMDS21\Data\TCA\Body-weight\body-weight-f\hil_body-
weight-f_hil-5%.plt
==============================================================================
 BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
    The form of the response function is:
  Y[dose] = intercept + v*dose^n/(k^n + dose^n)

   Dependent variable = Mean
   Independent variable = Dose
   rho is set to 0
   Power parameter restricted to be greater than 1
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008

                  Default Initial Parameter Values
                          alpha =    0.0516633
                            rho =            0   Specified
                      intercept =         3.54
                              v =        -1.18
                              n =      1.15142
                              k =        574.4

           Asymptotic Correlation Matrix of Parameter Estimates
           ( *** The model parameter(s)  -rho    -n
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

                    alpha    intercept            v            k
        alpha           1     3.3e-009    -4.8e-009     1.1e-008
    intercept     3.3e-009            1         0.22        -0.45
```

v	-4.8e-009	0.22	1	-0.95
k	1.1e-008	-0.45	-0.95	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.0505044	0.00779299	0.0352304	0.0657783
intercept	3.52807	0.0444292	3.44099	3.61515
v	-1.78616	0.368915	-2.50922	-1.0631
n	1	NA		
k	1154.27	497.126	179.921	2128.62

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Est Mean	Obs Std Dev	Est Std Dev	Scaled Res.
0	26	3.54	3.53	0.2	0.225	0.271
330	19	3.08	3.13	0.27	0.225	-0.988
800	17	2.83	2.8	0.18	0.225	0.608
1200	14	2.67	2.62	0.29	0.225	0.872
1800	8	2.36	2.44	0.15	0.225	-1

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2
Model A3 uses any fixed variance parameters that
were specified by the user

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	# Param's	AIC
A1	85.023823	6	-158.047645
A2	88.973191	10	-157.946383
A3	85.023823	6	-158.047645
fitted	83.399219	4	-158.798439
R	25.160911	2	-46.321822

Explanation of Tests

Test 1: Do responses and/or variances differ among Dose levels?
(A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)
(Note: When rho=0 the results of Test 3 and Test 2 will be the same.)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	127.625	8	<.0001
Test 2	7.89874	4	0.09536
Test 3	7.89874	4	0.09536
Test 4	3.24921	2	0.197

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .1. Consider running a
non-homogeneous variance model

The p-value for Test 3 is less than .1. You may want to consider a

```
different variance model

The p-value for Test 4 is greater than .1.  The model chosen seems
to adequately describe the data

        Benchmark Dose Computation
Specified effect =          0.05
Risk Type        =     Relative risk
Confidence level =           0.95
             BMD =        126.489
            BMDL =       87.7222
```

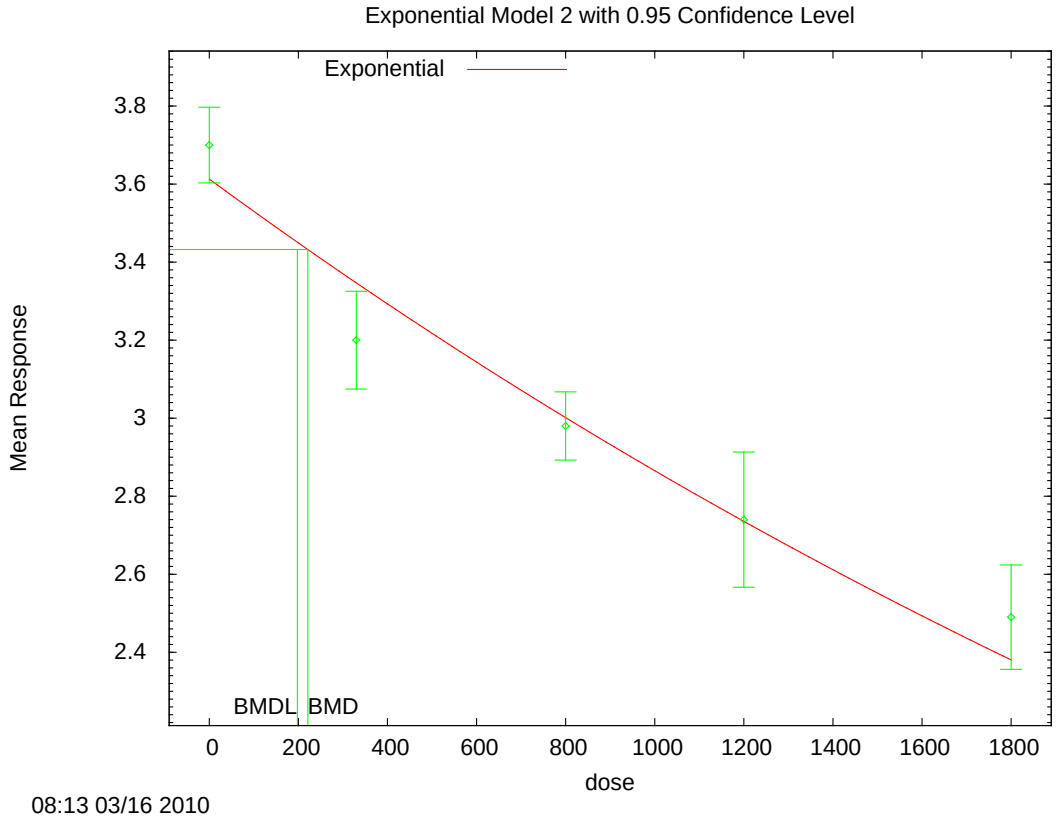
C.2. FETAL CROWN-RUMP LENGTH

Table C-3. BMD modeling results based on fetal crown-rump length in Long-Evans rats exposed to TCA by gavage on GDs 6–15—male fetuses

Model	*p*-value	AIC	Largest residual (mg/kg-d)	BMD_{1SD} (mg/kg-d)	$BMDL_{1SD}$ (mg/kg-d)	BMD_{05} (mg/kg-d)	$BMDL_{05}$ (mg/kg-d)
Exponential (model 2)	**0.762**	**-273.53**	**-0.740**	**369.1**	**311.7**	**600.7**	**534.4**
Exponential (model 3)	0.762	-273.53	-0.740	369.1	311.7	600.7	534.4
Exponential (model 4)	0.762	-273.53	-0.740	369.1	271.8	600.7	468.4
Exponential (model 5)	0.559	-271.53	-0.740	369.1	271.8	600.7	468.4
Hill (constant variance)	0.562	-271.54	-0.756	373.0	265.2	605.7	460.3
Polymomial-linear	0.751	-273.49	-0.816	388.5	330.2	625.4	560.7
Polynomial (degree ≥2)	0.560	-271.53	-0.766	375.2	282.2	608.4	482.6
Power	0.751	-273.49	-0.816	388.5	330.2	625.4	560.7

BMD_{05} = BMD at 5% change in mean relative to the control mean; BMD_{1SD} = BMD at 1 SD change in mean from the control mean

Source: Smith et al. (1989).



08:13 03/16 2010

```
================================================================================
         Exponential Model. (Version: 1.61;  Date: 7/24/2009)
         Input Data File: C:\USEPA\BMDS21\Data\TCA\Crownrump-length\crownrump-length-
m\exp_Crownrump-length-m_exp-5%.(d)
         Gnuplot Plotting File:
================================================================================
 BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

    The form of the response function by Model:
      Model 2:     Y[dose] = a * exp{sign * b * dose}
      Model 3:     Y[dose] = a * exp{sign * (b * dose)^d}
      Model 4:     Y[dose] = a * [c-(c-1) * exp{-b * dose}]
      Model 5:     Y[dose] = a * [c-(c-1) * exp{-(b * dose)^d}]

    Note: Y[dose] is the median response for exposure = dose;
          sign = +1 for increasing trend in data;
          sign = -1 for decreasing trend.

      Model 2 is nested within Models 3 and 4.
      Model 3 is nested within Model 5.
      Model 4 is nested within Model 5.
   Dependent variable = Mean
   Independent variable = Dose
   Data are assumed to be distributed: normally
   Variance Model: exp(lnalpha +rho *ln(Y[dose]))
   rho is set to 0.
   A constant variance model is fit.

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008

   MLE solution provided: Exact

                              Initial Parameter Values

     Variable         Model 2          Model 3          Model 4          Model 5
     --------         -------          -------          -------          -------
```

lnalpha	-4.34162	-4.34162	-4.34162	-4.34162
rho(S)	0	0	0	0
a	3.33708	3.33708	3.8955	3.8955
b	8.54483e-005	8.54483e-005	0.000919238	0.000919238
c	--	--	0.772564	0.772564
d	--	1	--	1

(S) = Specified

Parameter Estimates by Model

Variable	Model 2	Model 3	Model 4	Model 5
lnalpha	-4.32778	-4.32778	-4.32778	-4.32778
rho	0	0	0	0
a	3.70235	3.70235	3.70235	3.70235
b	8.53849e-005	8.53849e-005	8.53849e-005	8.53849e-005
c	--	--	0	0
d	--	1	--	1

Table of Stats From Input Data

Dose	N	Obs Mean	Obs Std Dev
0	26	3.71	0.12
330	19	3.58	0.1
800	17	3.46	0.1
1200	14	3.36	0.15
1800	8	3.16	0.12

Estimated Values of Interest

Model	Dose	Est Mean	Est Std	Scaled Residual
2	0	3.702	0.1149	0.3393
	330	3.599	0.1149	-0.7395
	800	3.458	0.1149	0.07542
	1200	3.342	0.1149	0.5931
	1800	3.175	0.1149	-0.3669
3	0	3.702	0.1149	0.3393
	330	3.599	0.1149	-0.7395
	800	3.458	0.1149	0.07542
	1200	3.342	0.1149	0.5931
	1800	3.175	0.1149	-0.3669
4	0	3.702	0.1149	0.3393
	330	3.599	0.1149	-0.7395
	800	3.458	0.1149	0.07542
	1200	3.342	0.1149	0.5931
	1800	3.175	0.1149	-0.3669
5	0	3.702	0.1149	0.3393
	330	3.599	0.1149	-0.7395
	800	3.458	0.1149	0.07542
	1200	3.342	0.1149	0.5931
	1800	3.175	0.1149	-0.3669

Other models for which likelihoods are calculated:

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = exp(lalpha + log(mean(i)) * rho)

Model R: Yij = Mu + e(i)
Var{e(ij)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	140.3479	6	-268.6958
A2	142.1334	10	-264.2668
A3	140.3479	6	-268.6958
R	90.80382	2	-177.6076
2	139.7669	3	-273.5337
3	139.7669	3	-273.5337

4	139.7669	3	-273.5337
5	139.7669	4	-271.5337

Additive constant for all log-likelihoods = -77.19. This constant added to the above values gives the log-likelihood including the term that does not depend on the model parameters.

Explanation of Tests

Test 1: Does response and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A2 vs. A1)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does Model 2 fit the data? (A3 vs. 2)

Test 5a: Does Model 3 fit the data? (A3 vs 3)
Test 5b: Is Model 3 better than Model 2? (3 vs. 2)

Test 6a: Does Model 4 fit the data? (A3 vs 4)
Test 6b: Is Model 4 better than Model 2? (4 vs. 2)

Test 7a: Does Model 5 fit the data? (A3 vs 5)
Test 7b: Is Model 5 better than Model 3? (5 vs. 3)
Test 7c: Is Model 5 better than Model 4? (5 vs. 4)

Tests of Interest

Test	-2*log(Likelihood Ratio)	D. F.	p-value
Test 1	102.7	8	< 0.0001
Test 2	3.571	4	0.4672
Test 3	3.571	4	0.4672
Test 4	1.162	3	0.7621
Test 5a	1.162	3	0.7621
Test 5b	0	0	N/A
Test 6a	1.162	3	0.7621
Test 6b	0	0	N/A
Test 7a	1.162	2	0.5593
Test 7b	0	1	1
Test 7c	0	1	1

The p-value for Test 1 is less than .05. There appears to be a Difference between response and/or variances among the dose levels, it seems appropriate to model the data.

The p-value for Test 2 is greater than .1. A homogeneous variance model appears to be appropriate here.

The p-value for Test 3 is greater than .1. The modeled variance appears to be appropriate here.

The p-value for Test 4 is greater than .1. Model 2 seems to adequately describe the data.

The p-value for Test 5a is greater than .1. Model 3 seems to adequately describe the data.

Degrees of freedom for Test 5b are less than or equal to 0. The Chi-Square test for fit is not valid.

The p-value for Test 6a is greater than .1. Model 4 seems to adequately describe the data.

Degrees of freedom for Test 6b are less than or equal to 0. The Chi-Square test for fit is not valid.

The p-value for Test 7a is greater than .1. Model 5 seems To adequately describe the data.

The p-value for Test 7b is greater than .05. Model 5 does not seem to fit the data better than Model 3.

The p-value for Test 7c is greater than .05. Model 5 doesnot seem to fit the data better than Model 4.

Benchmark Dose Computations:
Specified Effect = 0.050000

Risk Type = Relative deviation
Confidence Level = 0.950000

BMD and BMDL by Model

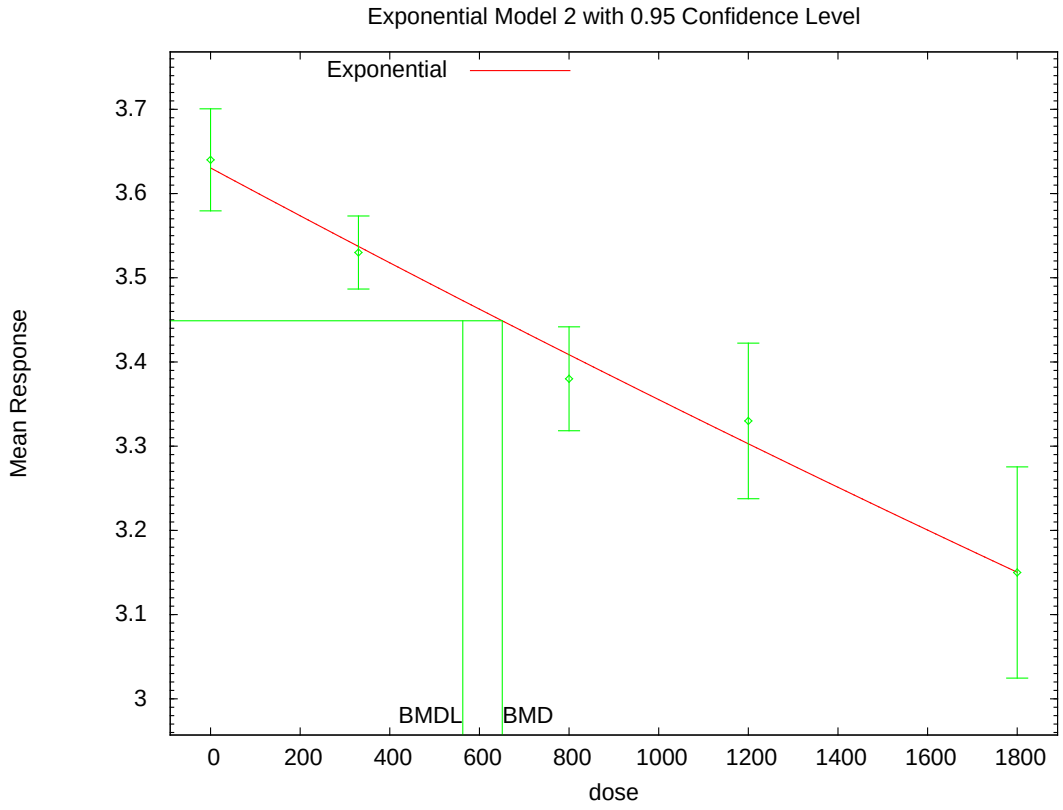
Model	BMD	BMDL
2	600.73	534.4
3	600.73	534.4
4	600.73	468.389
5	600.73	468.389

Table C-4. BMD modeling results based on fetal crown-rump length in Long-Evans rats exposed to TCA by gavage on GDs 6–15—female fetuses

Model	*p*-value	AIC	Largest residual (mg/kg-d)	BMD_{1SD} (mg/kg-d)	$BMDL_{1SD}$ (mg/kg-d)	BMD_{05} (mg/kg-d)	$BMDL_{05}$ (mg/kg-d)
Exponential (model 2)	**0.658**	**-250.59**	**-0.897**	**468.9**	**387.8**	**650.9**	**562.9**
Exponential (model 3)	0.658	-250.59	-0.897	468.9	387.8	650.9	562.9
Exponential (model 4)	0.515	-248.88	0.887	421.5	287.8	596.7	428.6
Exponential (model 5)	0.515	-248.88	0.887	421.5	287.8	596.7	428.6
Hill (constant variance)	0.521	-248.90	0.883	417.2	274.2	592.4	414.2
Polymomial-linear	0.595	-250.31	0.702	491.4	409.6	675.7	589.5
Polynomial (degree ≥2)	0.503	-248.83	0.891	429.6	312.7	605.2	458.9
Power	0.595	-250.31	0.702	491.4	409.6	675.7	589.5

BMD_{05} = BMD at 5% change in mean relative to the control mean; BMD_{1SD} = BMD at 1 SD change in mean from the control mean

Source: Smith et al. (1989).




```
==============================================================================
         Exponential Model. (Version: 1.61;  Date: 7/24/2009)
         Input Data File: C:\USEPA\BMDS21\Data\TCA\Crownrump-length\crownrump-length-
f\exp_Crownrump-length-f_exp-5%.(d)
         Gnuplot Plotting File:
==============================================================================
 BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
  The form of the response function by Model:
      Model 2:     Y[dose] = a * exp{sign * b * dose}
      Model 3:     Y[dose] = a * exp{sign * (b * dose)^d}
      Model 4:     Y[dose] = a * [c-(c-1) * exp{-b * dose}]
      Model 5:     Y[dose] = a * [c-(c-1) * exp{-(b * dose)^d}]

    Note: Y[dose] is the median response for exposure = dose;
          sign = +1 for increasing trend in data;
          sign = -1 for decreasing trend.

      Model 2 is nested within Models 3 and 4.
      Model 3 is nested within Model 5.
      Model 4 is nested within Model 5.

   Dependent variable = Mean
   Independent variable = Dose
   Data are assumed to be distributed: normally
   Variance Model: exp(lnalpha +rho *ln(Y[dose]))
   rho is set to 0.
   A constant variance model is fit.

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008

   MLE solution provided: Exact

                              Initial Parameter Values
     Variable         Model 2          Model 3          Model 4          Model 5
     --------         -------          -------          -------          -------
```

lnalpha	-4.07384	-4.07384	-4.07384	-4.07384
rho(S)	0	0	0	0
a	3.29932	3.29932	3.822	3.822
b	7.84982e-005	7.84982e-005	0.000906122	0.000906122
c	--	--	0.784929	0.784929
d	--	1	--	1

(S) = Specified

Parameter Estimates by Model

Variable	Model 2	Model 3	Model 4	Model 5
lnalpha	-4.0547	-4.0547	-4.05805	-4.05805
rho	0	0	0	0
a	3.63044	3.63044	3.63687	3.63687
b	7.87996e-005	7.87996e-005	0.000271529	0.000271529
c	--	--	0.665739	0.665739
d	--	1	--	1

Table of Stats From Input Data

Dose	N	Obs Mean	Obs Std Dev
0	26	3.64	0.15
330	19	3.53	0.09
800	17	3.38	0.12
1200	14	3.33	0.16
1800	8	3.15	0.15

Estimated Values of Interest

Model	Dose	Est Mean	Est Std	Scaled Residual
2	0	3.63	0.1317	0.3701
	330	3.537	0.1317	-0.2401
	800	3.409	0.1317	-0.8969
	1200	3.303	0.1317	0.7706
	1800	3.15	0.1317	-0.007621
3	0	3.63	0.1317	0.3701
	330	3.537	0.1317	-0.2401
	800	3.409	0.1317	-0.8969
	1200	3.303	0.1317	0.7706
	1800	3.15	0.1317	-0.007621
4	0	3.637	0.1315	0.1213
	330	3.533	0.1315	-0.08889
	800	3.4	0.1315	-0.612
	1200	3.299	0.1315	0.8873
	1800	3.167	0.1315	-0.3633
5	0	3.637	0.1315	0.1213
	330	3.533	0.1315	-0.08889
	800	3.4	0.1315	-0.612
	1200	3.299	0.1315	0.8873
	1800	3.167	0.1315	-0.3633

Other models for which likelihoods are calculated:

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = exp(lalpha + log(mean(i)) * rho)

Model R: Yij = Mu + e(i)
Var{e(ij)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	129.1014	6	-246.2027
A2	132.5454	10	-245.0907
A3	129.1014	6	-246.2027
R	91.75225	2	-179.5045
2	128.2973	3	-250.5946
3	128.2973	3	-250.5946

4	128.4379	4	-248.8759
5	128.4379	4	-248.8759

Additive constant for all log-likelihoods = -77.19. This constant added to the above values gives the log-likelihood including the term that does not depend on the model parameters.

Explanation of Tests

Test 1: Does response and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A2 vs. A1)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does Model 2 fit the data? (A3 vs. 2)

Test 5a: Does Model 3 fit the data? (A3 vs 3)
Test 5b: Is Model 3 better than Model 2? (3 vs. 2)

Test 6a: Does Model 4 fit the data? (A3 vs 4)
Test 6b: Is Model 4 better than Model 2? (4 vs. 2)

Test 7a: Does Model 5 fit the data? (A3 vs 5)
Test 7b: Is Model 5 better than Model 3? (5 vs. 3)
Test 7c: Is Model 5 better than Model 4? (5 vs. 4)

Tests of Interest

Test	-2*log(Likelihood Ratio)	D. F.	p-value
Test 1	81.59	8	< 0.0001
Test 2	6.888	4	0.1419
Test 3	6.888	4	0.1419
Test 4	1.608	3	0.6575
Test 5a	1.608	3	0.6575
Test 5b	5.684e-014	0	N/A
Test 6a	1.327	2	0.5151
Test 6b	0.2813	1	0.5958
Test 7a	1.327	2	0.5151
Test 7b	0.2813	1	0.5958
Test 7c	0	0	N/A

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels, it seems appropriate to model the data.

The p-value for Test 2 is greater than .1. A homogeneous variance model appears to be appropriate here.

The p-value for Test 3 is greater than .1. The modeled variance appears to be appropriate here.

The p-value for Test 4 is greater than .1. Model 2 seems to adequately describe the data.

The p-value for Test 5a is greater than .1. Model 3 seems to adequately describe the data.

Degrees of freedom for Test 5b are less than or equal to 0. The Chi-Square test for fit is not valid.

The p-value for Test 6a is greater than .1. Model 4 seems to adequately describe the data.

The p-value for Test 6b is greater than .05. Model 4 does not seem to fit the data better than Model 2.

The p-value for Test 7a is greater than .1. Model 5 seems to adequately describe the data.

The p-value for Test 7b is greater than .05. Model 5 does not seem to fit the data better than Model 3.

Degrees of freedom for Test 7c are less than or equal to 0. The Chi-Square test for fit is not valid.

Benchmark Dose Computations:
  Specified Effect = 0.050000
         Risk Type = Relative deviation
  Confidence Level = 0.950000

            BMD and BMDL by Model

Model	BMD	BMDL
2	650.934	562.874
3	650.934	562.874
4	596.729	428.617
5	596.729	428.617

APPENDIX D. MODELING OF LIVER TUMOR INCIDENCE DATA FOR MICE EXPOSED TO TCA IN DRINKING WATER

Five tumor data sets for combined incidence of hepatocellular adenomas and carcinomas in B6C3F$_1$ mice are shown in Tables 5-7 to 5-11 in Section 5.4.2. The estimated daily intakes of TCA from the mouse studies were converted to human equivalent lifetime doses using an interspecies body weight scaling factor and exposure time adjustment factor, which was based on the assumption that the age-specific rate for cancer in humans will increase by at least the third power of age (U.S. EPA, 1980).

For studies by Bull et al. (2002; 1990) and Pereira (1996), the body weight scaling factor was calculated as [male B6C3F$_1$ mouse reference body weight/human reference body weight]$^{0.25}$ = [0.0373/70]$^{0.25}$ = 0.15 (U.S. EPA, 1992, 1988). The exposure time adjustment factor was calculated as: [duration of experiment/duration of animal lifetime (i.e., 104 weeks)]^3. For the 52-week study by Bull et al. (2002; 1990), the exposure time adjust factor was calculated as (52/104)^3 = 0.125; for the 82-week study by Pereira (1996), the factor was calculated as (82/104)^3 = 0.49.

For studies by DeAngelo et al. (2008), the dose conversion is detailed in Tables D-1 and D-2.

Table D-1. Dose conversion for 60-week study

TCA dose group (g/L)	Mean water consumption (mL/kg-d)[a]	Mean measured TCA concentration (mg/mL)[a]	Estimated mean intake (mg/kg-d)[b]	Continuous exposure time adjustment factor[c]	Average animal lifetime weight (g)[d]	Body weight scaling factor[e]	Human equivalent lifetime dose (mg/kg-d)[f]
0.00	171	NA	0	0.19	38.0	0.15	0
0.05	153	NA	7.70	0.19	38.0	0.15	0.2
0.50	142	0.48	68.16	0.19	37.7	0.15	2.0
5.00	119	5.06	602.14	0.19	36.0	0.15	17.4

[a]Reported by DeAngelo et al. (2008).
[b]Estimated mean daily intake = mean water consumption × mean measured TCA concentration; where the mean measured TCA concentration was not reported, the nominal concentration for the dose group was used to calculate the estimated mean daily intake.
[c]Continuous exposure time adjustment factor = [duration of experiment/duration of animal lifetime]^3, or [60/104]^3
[d]Calculated using animal body weights at different weeks reported by DeAngelo et al. (2008).
[e]Body weight scaling factor = [average animal lifetime weight (kg)/human reference body weight (i.e., 70 kg)]$^{0.25}$.
[f]Human equivalent lifetime dose = estimated mean daily intake × exposure time adjustment factor × body weight scaling factor.

Source: DeAngelo et al. (2008).

Table D-2. Dose conversion for 104-week study

TCA dose group (g/L)	Mean water consumption (mL/kg-d)[a]	Mean measured TCA concentration (mg/mL)[a]	Estimated mean intake (mg/kg-d)[b]	Continuous exposure time adjustment factor[c]	Average animal lifetime weight (g)[d]	Body weight scaling factor[e]	Human equivalent lifetime dose (mg/kg-d)[f]
0.00	112	Not applicable	0	1	42.2	0.16	0
0.05	111	0.06	6.66	1	42.5	0.16	1.0
0.50	116	0.70	81.20	1	42.6	0.16	12.8

[a]Reported by DeAngelo et al. (2008).
[b]Estimated mean daily intake = mean water consumption × mean measured TCA concentration; where the mean measured TCA concentration was not reported, the nominal concentration for the dose group was used to calculate the estimated mean daily intake.
[c]Continuous exposure time adjustment factor = [duration of experiment/duration of animal lifetime]^3, or [104/104]^3.
[d]Calculated using animal body weights at different weeks reported by DeAngelo et al. (2008).
[e]Body weight scaling factor = [average animal lifetime weight (kg)/human reference body weight (i.e., 70 kg)]$^{0.25}$.
[f]Human equivalent lifetime dose = estimated mean daily intake × exposure time adjustment factor × body weight scaling factor.

Source: DeAngelo et al. (2008).

In the cancer dose-response analysis, EPA used tumor incidence values that differed from those presented in the publication by DeAngelo et al. (2008). Tumor incidence values were derived by EPA from individual animal data obtained from the study author (emails dated February 1 and April 26, 2010, from Anthony DeAngelo, NHEERL, ORD, U.S. EPA, to Diana Wong, NCEA, ORD, U.S. EPA). DeAngelo et al. (2008) based their tumor incidence values on terminal sacrifice animals only. In EPA's analysis, the sample sizes represented the number of animals at risk for tumor development (i.e., the number of mice included in the study when the first tumor was discovered, which was week 45 in the 60-week study [Study 1] and week 52 in the 104-week study [Study 3]). Animals that died before the first tumor was discovered were excluded. The estimated mean daily intakes used in EPA's BMD analysis were calculated as a product of mean daily water consumption data and measured TCA concentrations as reported in DeAngelo et al. (2008), and not the nominal concentration presented DeAngelo et al. (2008). Table D-3 compares the nominal doses and tumor incidence values presented in DeAngelo et al. (2008) and the values used in the cancer dose-response analysis for TCA.

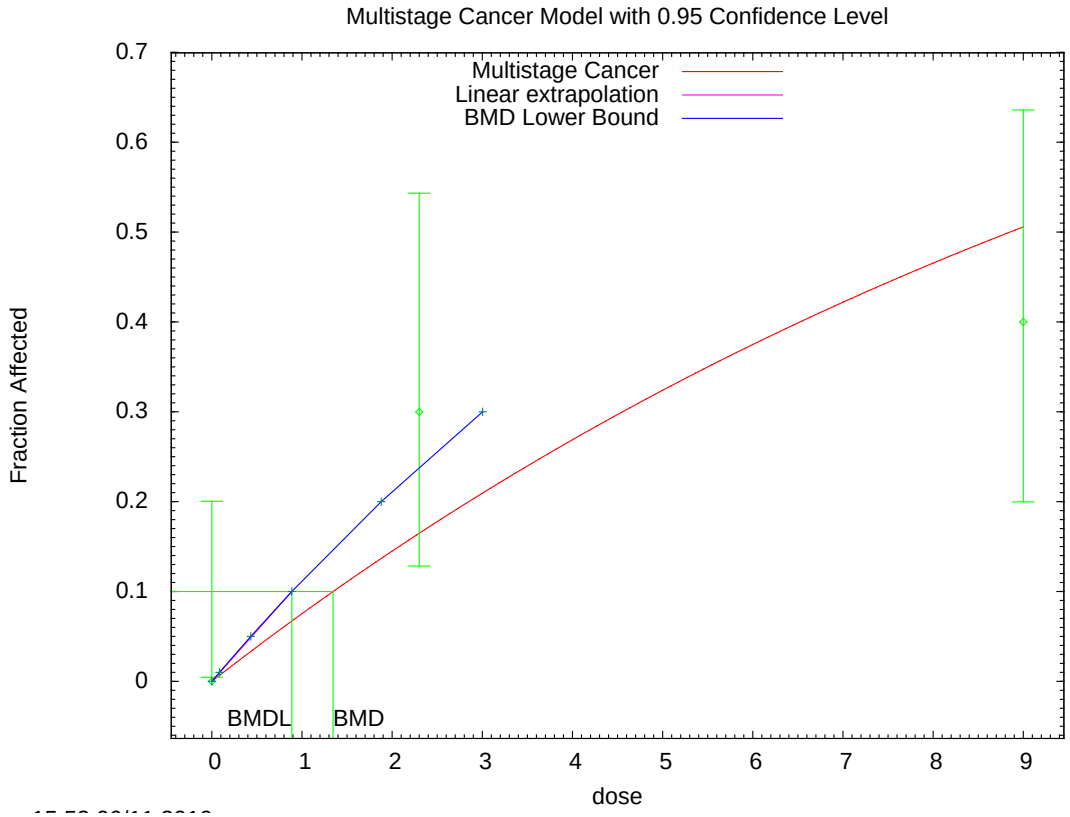
Table D-3. Comparison of average daily dose, sample size, and tumor incidence from DeAngelo et al. (2008) as reported by study authors and as recalculated by EPA

Endpoints	Study duration	Nominal drinking water concentration (g/L)	DeAngelo et al. (2008)	EPA re-calculation
Average dose (mg/kg-d)	60 wks (study 1)	0	0	0
		0.05	8	7.7
		0.5	68	68.2
		5	602	602.1
	104 wks (study 3)	0	0	0
		0.05	6	6.7
		0.5	58	81.2
Sample size	60 wks (study 1)	0	30	35
		0.05	27	32
		0.5	29	34
		5	29	34
	104 wks (study 3)	0	42	56
		0.05	35	48
		0.5	37	51
Tumor incidence	60 wks (study 1)	0	4/30[a]	4/35
		0.05	4/27[a]	5/32
		0.5	11/29[a]	12/34
		5	16/29[a]	19/34
	104 wks (study 3)	0	27/42[a]	31/56
		0.05	20/35[a]	21/48
		0.5	32/37[a]	36/51

[a]Tumor incidence was estimated based on data from DeAngelo et al. (2008), which reported only the number of animals examined and percent of animals with tumors.

Using the EPA BMDS (version 2.1.1), the multistage model was fit to the combined incidence of hepatocellular adenomas and carcinomas. Output files from BMDS are provided in Sections D.1–D.5.

D.1. FIFTY-TWO-WEEK STUDY FROM BULL ET AL. (2002) WITH THREE DOSE GROUPS



Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day.

```
=================================================================
         Multistage Cancer Model. (Version: 1.7;  Date: 05/16/2008)
         Input Data File: C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_52wkBull2002_st1.(d)
         Gnuplot Plotting File:  C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_52wkBull2002_st1.plt
=================================================================

 BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                -beta1*dose^1)]

   The parameter betas are restricted to be positive


   Dependent variable = Incidence
   Independent variable = Dose

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values
Background = 0.0987263
Beta(1) = 0.0491736

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Background
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(1)
Beta(1)	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0	*	*	*
Beta(1)	0.0783449	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-25.6775	3			
Fitted model	-27.2494	1	3.14381	2	0.2076
Reduced model	-32.5964	1	13.8377	2	0.000989

AIC: 56.4988

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0.000	20	0.000
2.3000	0.1649	3.298	6.000	20	1.628
9.0000	0.5059	10.119	8.000	20	-0.948

Chi^2 = 3.55 d.f. = 2 P-value = 0.1695

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.34483

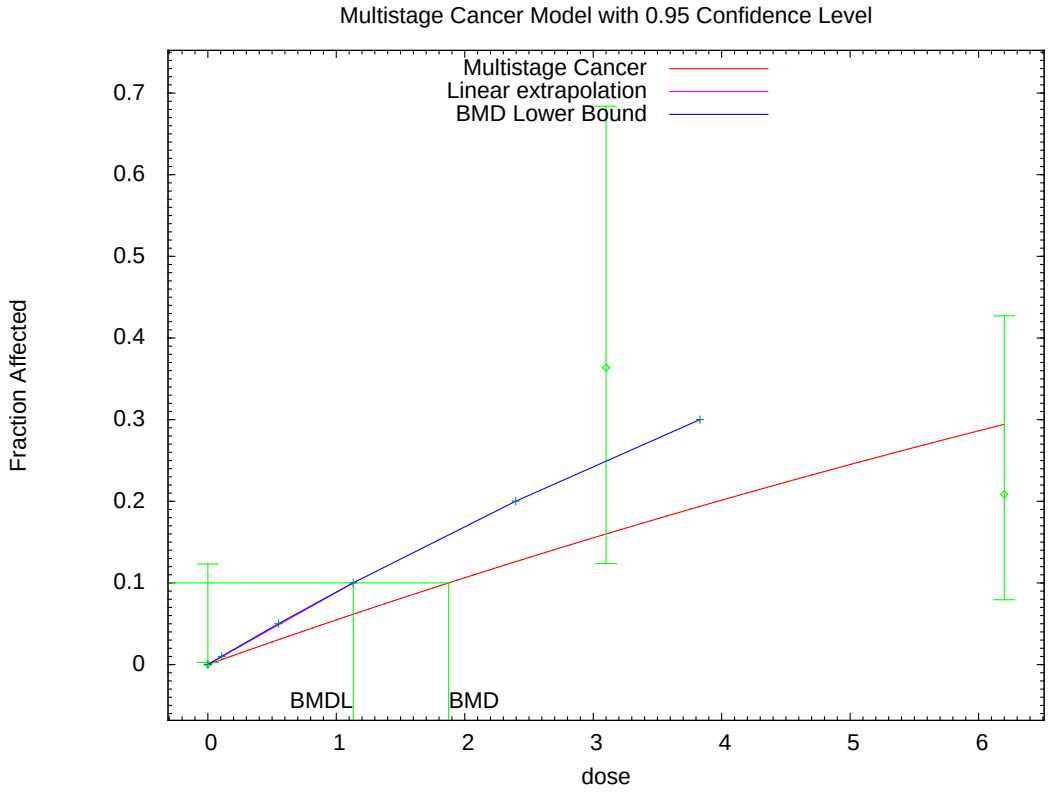
BMDL = 0.887265

BMDU = 2.61396

Taken together, (0.887265, 2.61396) is a 90% two-sided confidence
interval for the BMD

Multistage Cancer Slope Factor = 0.112706

D.2. FIFTY-TWO-WEEK STUDY FROM BULL ET AL. (1990) WITH THREE DOSE GROUPS



Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day.

```
 ================================================================
         Multistage Cancer Model. (Version: 1.7;  Date: 05/16/2008)
         Input Data File: C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_52wkBull1990_st1.(d)
         Gnuplot Plotting File:  C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_52wkBull1990_st1.plt
 ================================================================

 BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                 -beta1*dose^1)]

   The parameter betas are restricted to be positive


   Dependent variable = Incidence
   Independent variable = Dose

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values
Background = 0.105711
Beta(1) = 0.0376798

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Background
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(1)
Beta(1)	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0	*	*	*
Beta(1)	0.0562073	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-19.4921	3			
Fitted model	-21.2903	1	3.59649	2	0.1656
Reduced model	-26.8563	1	14.7286	2	0.0006335

AIC: 44.5806

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0.000	35	0.000
3.1000	0.1599	1.759	4.000	11	1.844
6.2000	0.2942	7.062	5.000	24	-0.924

Chi^2 = 4.25 d.f. = 2 P-value = 0.1193

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.8745

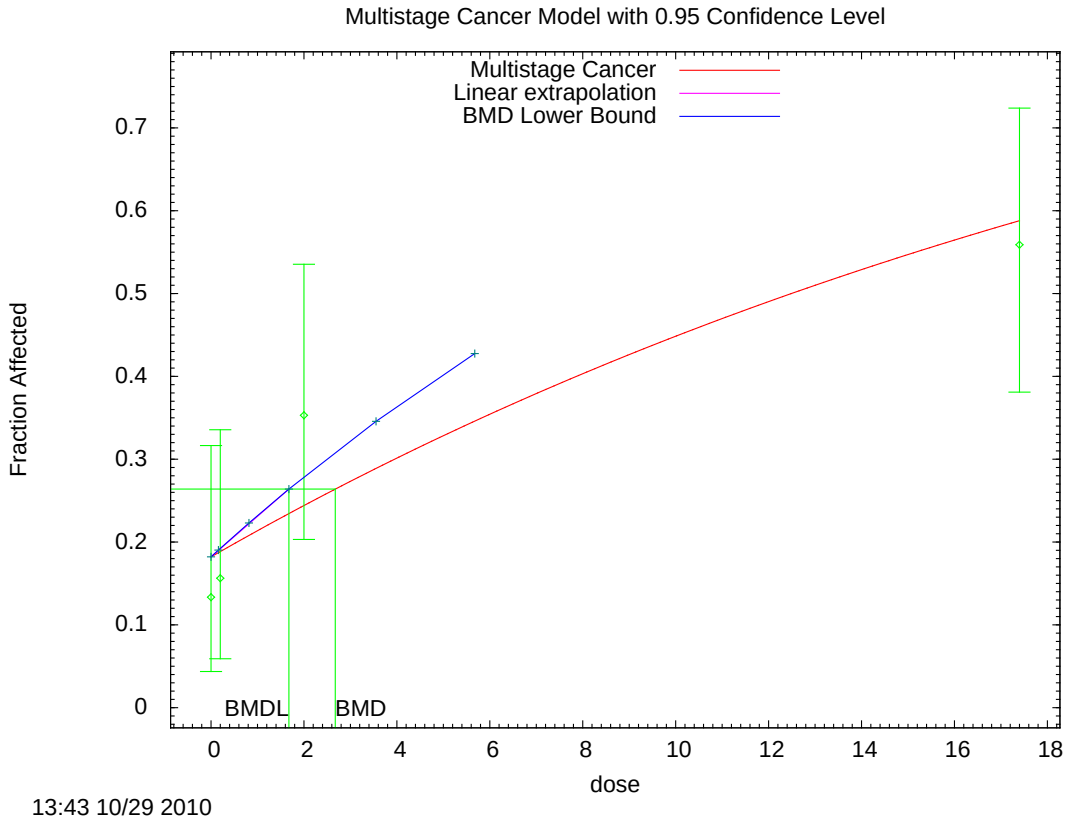
BMDL = 1.13168

BMDU = 3.4391

Taken together, (1.13168, 3.4391) is a 90 % two-sided confidence
interval for the BMD

Multistage Cancer Slope Factor = 0.0883644

D.3. SIXTY-WEEK STUDY FROM DeANGELO ET AL. (2008) WITH FOUR DOSE GROUPS



Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day.

```
 ======================================================================
      Multistage Cancer Model. (Version: 1.9;  Date: 05/26/2010)
      Input Data File:
C:/USEPA/BMDS212/Data/TCA/TCA_Study1/DeAngelo_2008_Tumor_MultiCanc1_ExtraRisk10%.(d)
      Gnuplot Plotting File:
C:/USEPA/BMDS212/Data/TCA/TCA_Study1/DeAngelo_2008_Tumor_MultiCanc1_ExtraRisk10%.plt
======================================================================
   The form of the probability function is:
   P[response] = background + (1-background)*[1-EXP(
                -beta1*dose^1)]

   The parameter betas are restricted to be positive
   Dependent variable = Response
   Independent variable = Dose

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1

 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008

                  Default Initial Parameter Values
                     Background =     0.195924
                        Beta(1) =    0.0354277


           Asymptotic Correlation Matrix of Parameter Estimates
             Background      Beta(1)
Background            1        -0.51
   Beta(1)        -0.51            1
```

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.182037	*	*	*
Beta(1)	0.0393995	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-71.0546	4			
Fitted model	-72.4916	2	2.87397	2	0.2376
Reduced model	-80.2414	1	18.3736	3	0.0003683

AIC: 148.983

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.1820	5.461	4.000	30	-0.691
0.2000	0.1885	6.031	5.000	32	-0.466
2.0000	0.2440	8.297	12.000	34	1.479
17.4000	0.5879	19.989	19.000	34	-0.344

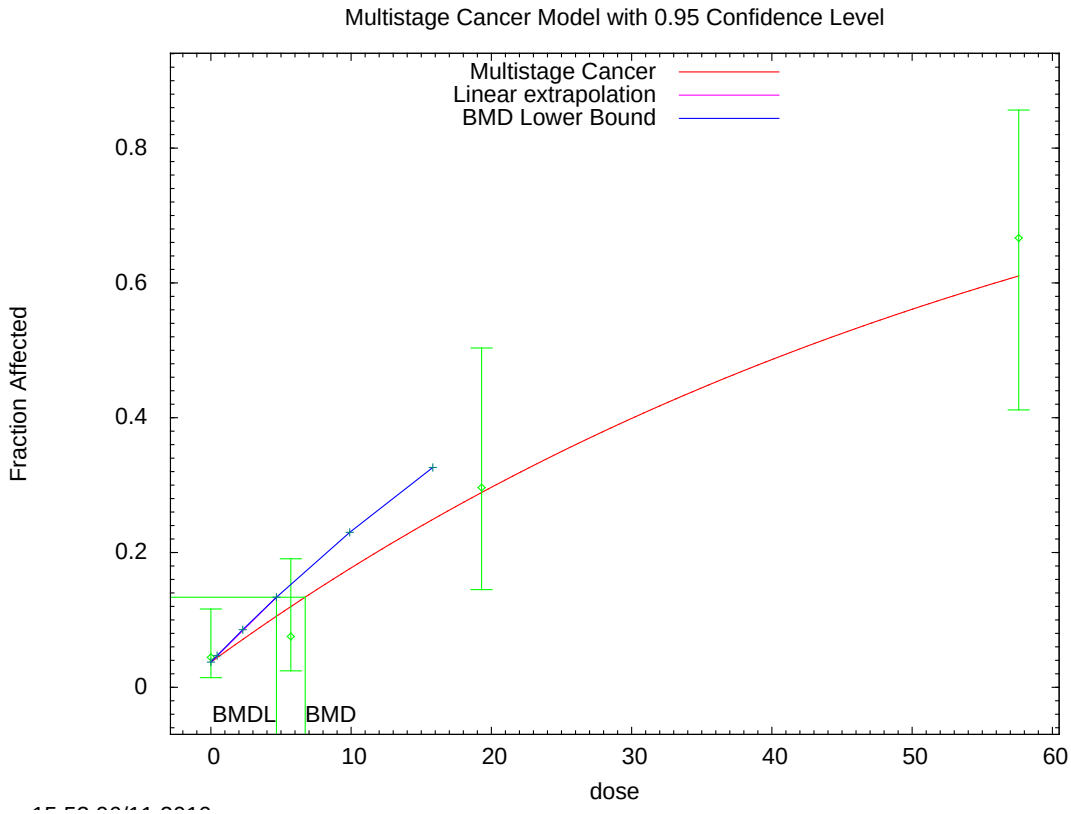
Chi^2 = 3.00 d.f. = 2 P-value = 0.2231

Benchmark Dose Computation

Specified effect = 0.1
Risk Type = Extra risk
Confidence level = 0.95
BMD = 2.67416
BMDL = 1.6767
BMDU = 5.1239

Taken together, (1.6767 , 5.1239) is a 90 % two-sided confidence interval for the BM
Multistage Cancer Slope Factor = 0.0596408

D.4. EIGHTY-TWO-WEEK STUDY FROM PEREIRA (1996) WITH FOUR DOSE GROUPS



Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day.

```
=============================================================================
         Multistage Cancer Model. (Version: 1.7;  Date: 05/16/2008)
         Input Data File: C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_82wkPere1996_st4-1.(d)
         Gnuplot Plotting File:  C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_82wkPere1996_st4-1.plt
 =============================================================================

 BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                 -beta1*dose^1)]

   The parameter betas are restricted to be positive


   Dependent variable = Incidence
   Independent variable = Dose

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =   0.00436735
```

Beta(1) = 0.0188431

Asymptotic Correlation Matrix of Parameter Estimates

	Background	Beta(1)
Background	1	-0.43
Beta(1)	-0.43	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.0373398	*	*	*
Beta(1)	0.0156633	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-58.4099	4			
Fitted model	-59.1538	2	1.48782	2	0.4753
Reduced model	-79.1216	1	41.4233	3	<.0001

AIC: 122.308

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0373	3.361	4.000	90	0.356
5.7000	0.1196	6.337	4.000	53	-0.989
19.3000	0.2885	7.789	8.000	27	0.090
57.6000	0.6095	10.971	12.000	18	0.497

Chi^2 = 1.36 d.f. = 2 P-value = 0.5065

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 6.72658

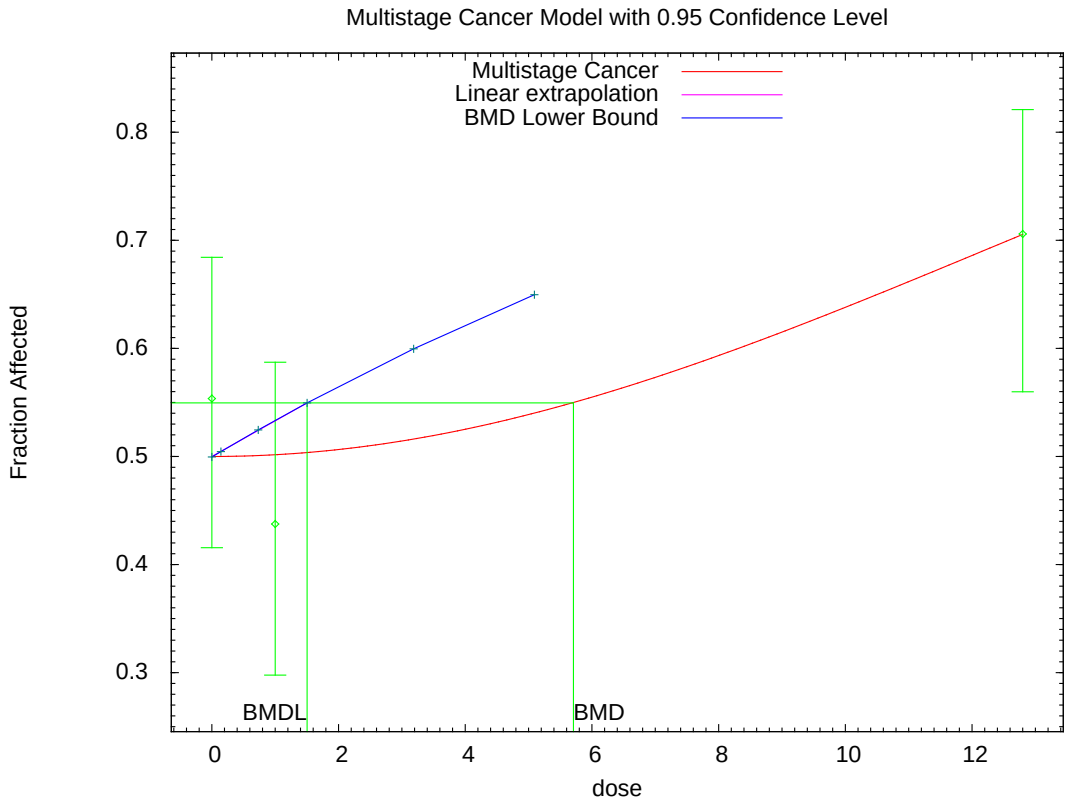
BMDL = 4.67475

BMDU = 10.3673

Taken together, (4.67475, 10.3673) is a 90% two-sided confidence interval for the BMD

Multistage Cancer Slope Factor = 0.0213915

D.5. ONE-HUNDRED-FOUR-WEEK STUDY FROM DeANGELO ET AL. (2008) WITH THREE DOSE GROUPS



Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day.

```
=================================================================
         Multistage Cancer Model. (Version: 1.7;  Date: 05/16/2008)
         Input Data File:
C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_TCALiverTumorStudy3_LiverCacerPol2.(d)
         Gnuplot Plotting File:
C:\USEPA\BMDS21\Data\TCA\LiverCancer\msc_TCALiverTumorStudy3_LiverCacerPol2.plt
=================================================================

 BMDS Model Run
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                 -beta1*dose^1-beta2*dose^2)]

   The parameter betas are restricted to be positive


   Dependent variable = Incidence
   Independent variable = Dose

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 3
 Total number of specified parameters = 0
 Degree of polynomial = 2


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values
Background = 0.498249
Beta(1) = 0
Beta(2) = 0.00325566

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Beta(1)
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Background	Beta(2)
Background	1	-0.48
Beta(2)	-0.48	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.499528	*	*	*
Beta(1)	0	*	*	*
Beta(2)	0.00323379	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-102.285	3			
Fitted model	-103.003	2	1.4352	1	0.2309
Reduced model	-106.011	1	7.4518	2	0.02409

AIC: 210.005

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4995	27.974	31.000	56	0.809
1.0000	0.5011	24.055	21.000	48	-0.882
12.8000	0.7054	35.974	36.000	51	0.008

Chi^2 = 1.43 d.f. = 1 P-value = 0.2314

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 5.70799

BMDL = 1.50402

BMDU = 10.2515

Taken together, (1.50402, 10.2515) is a 90% two-sided confidence
interval for the BMD

Multistage Cancer Slope Factor = 0.0664883

APPENDIX E. MULTISTAGE-WEIBULL (MSW) TIME-TO-TUMOR MODELING OF INDIVIDUAL AND COMBINED LIVER TUMOR INCIDENCE DATA SETS FROM DeANGELO ET AL. (2008)

The findings of three chronic drinking water bioassays of TCA were reported by DeAngelo et al. (2008) in male B6C3F$_1$ mice. Key characteristics of the three bioassays are presented in Table E-1.

Table E-1. Key characteristics of the three drinking water studies

Study number	Study duration (wks)	Dose groups
Study 1	60	0, 0.05, 0.5, 5 g/L TCA
Study 2	104	0, 4.5 g/L TCA
Study 3	104	0, 0.05, 0.5 g/L TCA

Source: DeAngelo et al. (2008).

Consideration was given to combining the liver tumor incidence data from the three bioassays in order to derive an oral cancer slope factor based on a wider range of doses and a larger number of mice. To determine whether the incidence data from these three studies could be combined, a statistical analysis was conducted employing a generalized likelihood ratio test (Stiteler et al., 1993) after both individual and combined data sets were fitted by the MSW time-to-tumor model.

The results of the generalized likelihood ratio test of statistical compatibility are presented in Section E.4 and the results of the dose-response analysis based on combined data sets are presented in Section E.5.

E.1. DOSE CONVERSIONS

Before fitting the MSW time-to-tumor model to the liver tumor incidence data, estimated mean daily intakes of TCA from the mouse studies were converted to human equivalent lifetime doses by adjusting for continuous exposure time (in this case, the adjustment factor was 1because TCA was administered in drinking water) and applying body weight scaling factors (see Tables E-2 to E-4).

Table E-2. Dose adjustments for Study 1

TCA dose group (g/L)	Mean water consumption (mL/kg-d)[a]	Mean measured TCA concentration (mg/mL)[a]	Estimated mean intake (mg/kg-d)[b]	Average animal lifetime weight (g)[c]	Body weight scaling factor[d]	Human equivalent lifetime dose (mg/kg-d)[e]
0	171	Not applicable	0.00	38.0	0.15	0.0
0.05	153	Not applicable	7.70	38.0	0.15	1.2
0.5	141	0.48	68.16	37.7	0.15	10.4
5.0	119	5.06	602.14	36.0	0.15	90.7

[a]Reported by DeAngelo et al. (2008).
[b]Estimated mean daily intake = mean water consumption × mean measured TCA concentration; where the mean measured TCA concentration was not reported, the nominal concentration for the dose group was used to calculate the estimated mean daily intake.
[c]Calculated using animal body weights at different weeks reported by DeAngelo et al. (2008).
[d]Body weight scaling factor = [average animal lifetime weight (kg)/human reference body weight (i.e., 70 kg)]$^{0.25}$.
[e]Human equivalent lifetime dose = estimated mean daily intake × body weight scaling factor.

Table E-3. Dose adjustments for Study 2

TCA dose group (g/L)	Mean water consumption (mL/kg-d)[a]	Mean measured TCA concentration (mg/mL)[a]	Estimated mean intake (mg/kg-d)[b]	Average animal lifetime weight (g)[c]	Body weight scaling factor[d]	Human equivalent lifetime dose (mg/kg-d)[e]
0	132	Not applicable	0.00	37.6	0.15	0
4.5	129	4.43	571.47	63.1	0.15	86.1

[a]Reported by DeAngelo et al. (2008).
[b]Estimated mean daily intake = mean water consumption × mean measured TCA concentration.
[c]Calculated using animal body weights at different weeks reported by DeAngelo et al. (2008).
[d]Body weight scaling factor = [average animal lifetime weight (kg)/human reference body weight (i.e., 70 kg)]$^{0.25}$.
[e]Human equivalent lifetime dose = estimated mean daily intake × body weight scaling factor.

Table E-4. Dose adjustments for Study 3

TCA dose group (g/L)	Mean water consumption (mL/kg-d)[a]	Mean measured TCA concentration (mg/mL)[a]	Estimated mean intake (mg/kg-d)[b]	Average animal lifetime weight (g)[c]	Body weight scaling factor[d]	Human equivalent lifetime dose (mg/kg-d)[e]
0	112	Not applicable	0	42.2	0.16	0.0
0.05	111	0.06	6.66	42.5	0.16	1.0
0.5	116	0.70	81.20	42.6	0.16	12.8

[a]Reported by DeAngelo et al. (2008).
[b]Estimated mean daily intake = mean water consumption × mean measured TCA concentration.
[c]Calculated using animal body weights at different weeks reported by DeAngelo et al. (2008).
[d]Body weight scaling factor = [average animal lifetime weight (kg)/human reference body weight (i.e., 70 kg)]$^{0.25}$.
[e]Human equivalent lifetime dose = estimated mean daily intake × body weight scaling factor.

E.2. DOSE-RESPONSE DATA

Individual animal data for the three bioassays reported in DeAngelo et al. (2008) were obtained from the study author (emails dated February 1 and April 26, 2010, from Anthony DeAngelo, NHEERL, ORD, U.S. EPA, to Diana Wong, NCEA, ORD, U.S. EPA). Before fitting MSW time-to-tumor models, each animal was classified into one of three response categories: "I" (hepatocellular carcinoma and/or adenoma were detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death), "U" (the presence or absence of hepatocellular carcinoma and/or adenoma could not be determined when the mouse was removed from the study due to scheduled sacrifice or unscheduled death or other reasons), and "C" (neither hepatocellular carcinoma nor adenoma was detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death). See Tables E-5 to E-7 for details.

Table E-5. Study 1 liver tumor incidence data; B6C3F$_1$ male mice exposed to TCA in drinking water

Human lifetime equivalent dose (mg/kg-d)	Wk of death	Response category for hepatocellular carcinoma and/or adenoma[a]	Number of animals
0	5	C	5
	15	C	5
	30	C	5
	45	C	5
	60	C	26
	60	I	4
1.2	1	U	1
	2	U	1
	5	C	5
	15	C	5
	30	C	5
	45	C	4
	45	I	1
	60	C	23
	60	I	4
	60	U	1
10.4	5	C	5
	15	C	5
	30	C	5
	45	C	4
	45	I	1
	60	I	11
	60	C	18
90.7	5	C	5
	15	C	5
	30	C	5
	45	C	2
	45	I	3
	60	C	13
	60	I	16
	60	U	1

[a]Response categories:
C: Neither hepatocellular carcinoma nor adenoma was detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death.
U: The presence or absence of hepatocellular carcinoma and/or adenoma could not be determined when the mouse was removed from the study due to scheduled sacrifice or unscheduled death or other reasons.
I: Hepatocellular carcinoma and/or adenoma were detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death.

Table E-6. Study 2 liver tumor incidence data; B6C3F$_1$ male mice exposed to TCA in drinking water

Human lifetime equivalent dose (mg/kg-d)	Wk of death	Response category for hepatocellular carcinoma and/or adenoma[a]	Number of animals
0	16	C	5
	29	U	2
	31	C	5
	45	C	5
	60	C	10
	71	U	1
	95	U	1
	98	U	1
	102	U	1
	105	C	4
	105	I	1
	106	C	18
	106	I	2
86.1	15	C	5
	23	U	1
	27	U	1
	30	C	5
	41	I	1
	45	C	4
	45	I	1
	72	I	1
	82	I	1
	89	I	1
	89	U	1
	92	U	2
	94	U	1
	94	I	1
	104	C	4
	104	I	26

[a]Response categories:
C: Neither hepatocellular carcinoma nor adenoma was detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death.
U: The presence or absence of hepatocellular carcinoma and/or adenoma could not be determined when the mouse was removed from the study due to scheduled sacrifice or unscheduled death or other reasons.
I: Hepatocellular carcinoma and/or adenoma were detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death.

Table E-7. Study 3 liver tumor incidence data; B6C3F$_1$ male mice exposed to TCA in drinking water

Human lifetime equivalent dose (mg/kg-d)	Wk of death	Response category for hepatocellular carcinoma and/or adenoma[a]	Number of animals
0	17	U	1
	26	C	7
	43	U	1
	52	C	5
	52	I	2
	72	U	1
	76	U	1
	78	I	2
	78	C	5
	79	U	1
	80	C	1
	81	I	1
	84	I	1
	86	C	1
	86	U	1
	89	I	1
	91	C	1
	95	I	1
	98	U	1
	99	U	1
	101	U	1
	101	I	1
	104	I	22
	104	C	12
1.0	26	C	7
	38	U	1
	39	U	1
	52	C	6
	52	I	1
	59	U	2
	70	U	2
	72	C	1
	73	U	1
	75	U	1
	76	U	1
	77	U	2
	78	C	4
	78	I	1
	79	U	1
	79	C	1
	80	C	1
	83	I	1
	86	C	1
	89	U	1
	90	U	1

Table E-7. Study 3 liver tumor incidence data; B6C3F$_1$ male mice exposed to TCA in drinking water

Human lifetime equivalent dose (mg/kg-d)	Wk of death	Response category for hepatocellular carcinoma and/or adenoma[a]	Number of animals
	91	I	1
	94	U	1
	100	U	1
	104	C	13
	104	I	17
	104	U	1
12.8	26	C	7
	50	U	1
	52	C	7
	55	U	1
	56	U	1
	61	U	1
	69	U	1
	72	U	1
	78	C	4
	78	I	4
	79	I	1
	80	U	1
	82	U	1
	84	I	1
	86	I	1
	91	U	1
	92	U	1
	92	I	2
	94	U	1
	97	C	1
	99	I	2
	100	I	1
	103	U	1
	104	I	24
	104	C	3
	104	U	2

[a]Response categories:
C: Neither hepatocellular carcinoma nor adenoma was detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death.
U: The presence or absence of hepatocellular carcinoma and/or adenoma could not be determined when the mouse was removed from the study due to scheduled sacrifice or unscheduled death or other reasons.
I: Hepatocellular carcinoma and/or adenoma were detected when the mouse was removed from the study due to scheduled sacrifice or unscheduled death.

E.3. MSW TIME-TO-TUMOR MODELING

MSW time-to-tumor modeling is used to model both the dose and the time of appearance of a detectable tumor. With this model, the probability of observing a tumor prior to some

specific observation time, t, upon exposure to a carcinogen at dose level, d, is given by the function:

$$G(t,d) = G(t,d,c,\beta_0,\beta_1,...,\beta_k) = 1 - \exp\left\{-t^c \sum_{i=0}^{k} \beta_i d^i\right\}$$

MSW time-to-tumor models were fit to three individual liver tumor data sets (i.e., Study 1, Study 2, and Study 3) and four combined data sets (i.e., Study 1+2+3, Study 1+2, Study 1+3, and Study 2+3). For each individual or combined data set, specific n-stage model was selected because of lowest log-likelihoods and/or AIC. A tumor incidental risk of 10% was used for low-dose extrapolation. Critical outputs and plots are provided in Section E.6.

The MSW time-to-tumor modeling software program, available for download from the EPA's BMDS website (U.S. EPA, 2009), was used to conduct the MSW time-to-tumor analysis; EPA's gofplot_msw() was used to produce plots to assess goodness-of-fit for the MSW time-to-tumor models.

E.4. STATISTICAL ANALYSIS FOR DATA COMPATIBILITY

To evaluate whether the three independent studies from DeAngelo et al. (2008) were compatible to be combined for MSW time-to-tumor modeling, a generalized likelihood ratio test described by Stiteler et al. (1993) was used, which has an asymptotic χ^2 distribution:

$$-2Ln\Lambda = 2[\max \ln L(H_0 \cup H_1) - \max \ln L(H_0)]$$

Two hypotheses were tested: (1) the null hypothesis (H_0), i.e., that the data sets from individual studies are compatible to be combined for MSW time-to-tumor modeling; and (2) the alternative hypothesis (H_1), i.e., that the data sets from individual studies are not compatible to be combined for MSW time-to-tumor modeling.

The determination to either to accept or reject the null hypothesis was made by comparing the calculated value of $-2Ln\Lambda$ against the tabulated χ^2 at the level of significance $\alpha = 0.05$ and $\alpha = 0.025$ for one-sided distributions (see Table E-8). Based on this statistical analysis, only Study 1 and Study 3 were determined to be statistically compatible to be combined for MSW time-to-tumor modeling.

Table E-8. Summary of the statistical test for compatibility among the individual studies

	Study 1+2+3	Study 1+3	Study 1+2	Study 2+3
Study 1, log (likelihood)	-76.44	-76.44	-76.44	
Study 2, log (likelihood)	-33.71		-33.71	-33.71
Study 3, log (likelihood)	-109.20	-109.20		-109.20
Combined data sets, log (likelihood)	-238.35	-187.49	-117.36	-158.52
-2 lnΛ	37.99	3.71	14.41	31.21
Degree of freedom	2	1	1	1
Critical $\chi^2_{1,0.05}$, one sided		3.84	3.84	3.84
Critical $\chi^2_{1,0.025}$, one sided		5.02	5.02	5.02
Critical $\chi^2_{2,0.05}$, one sided	5.99			
Critical $\chi^2_{2,0.025}$, one sided	7.38			
Conclusion	**Reject H_0, not compatible**	**Accept H_0, compatible**	**Reject H_0, not compatible**	**Reject H_0, not compatible**

Example for calculation:

Study 1, Log(likelihood) = -76.44
Study 3, Log(likelihood) = -109.20
Study 1+3, Log(likelihood) = -187.49
$-2\text{Ln}\Lambda = 2[187.49 - (76.44 + 109.20)] = 3.7$, degree of freedom (df) = 1
$\chi^2_{1,\,0.05} = 3.84$ (df = 1, $\alpha = 0.05$), $\chi^2_{1,\,0.025} = 5.02$ (df = 1, $\alpha = 0.025$)

The calculated *-2LnΛ* for combining data sets from Study 1 and Study 3 equaled 3.7, which is smaller than the right-sided critical value of χ^2 distribution for $\alpha = 0.05$ or $\alpha = 0.025$ at df = 1. Therefore, the null hypothesis was accepted, indicating that Study 1 and Study 3 were statistically compatible to be combined for MSW time-to-tumor modeling.

E.5. EXTRAPOLATION METHOD AND ORAL CANCER SLOPE FACTOR

As discussed in Section 5.4.4, linear extrapolation was applied in this assessment and $BMDL_{10}$ was used as POD for linear extrapolation. The oral cancer slope factor, the upper-bound estimation of risk, was calculated as $0.1/ BMDL_{10}$. The cancer slope factors derived using MSW time-to-tumor model software are provided in Table E-9.

Table E-9. Candidate oral cancer slope factors derived from liver tumor data sets in B6C3F$_1$ male mice using MSW time-to-tumor modeling

	Best time-to-tumor model for the study	Log (likelihood)	AIC	BMR	BMD_{10}[a]	$BMDL_{10}$[b]	Slope of linear extrapolation from BMD_{10}[c]	Cancer slope factor from $BMDL_{10}$[d]
Study 1	Stage 1 polynomial	-76.4	158.9	0.1	13.5	8.4	7.4×10^{-3}	1.2×10^{-2}
Study 2	Stage 1 polynomial	-33.7	73.4	0.1	5.4	3.8	1.9×10^{-2}	2.6×10^{-2}
Study 3	Stage 2 polynomial	-109.2	226.4	0.1	5.0	1.2	2.0×10^{-2}	8.5×10^{-2}
Study 1+3	Stage 1 polynomial	-187.5	381.0	0.1	2.2	1.4	4.5×10^{-2}	7.2×10^{-2}

[a]BMD_{10} = dose at 10% cancer risk.
[b]$BMDL_{10}$ = dose at 95% lower bound with 10% cancer risk.
[c]Slope of linear extrapolation from $BMD_{10} = 0.1/BMD_{10}$.
[d]Cancer slope factor = $0.1/BMDL_{10}$.

E.6. OUTPUT FILES AND PLOTS FOR MSW TIME-TO-TUMOR MODELS

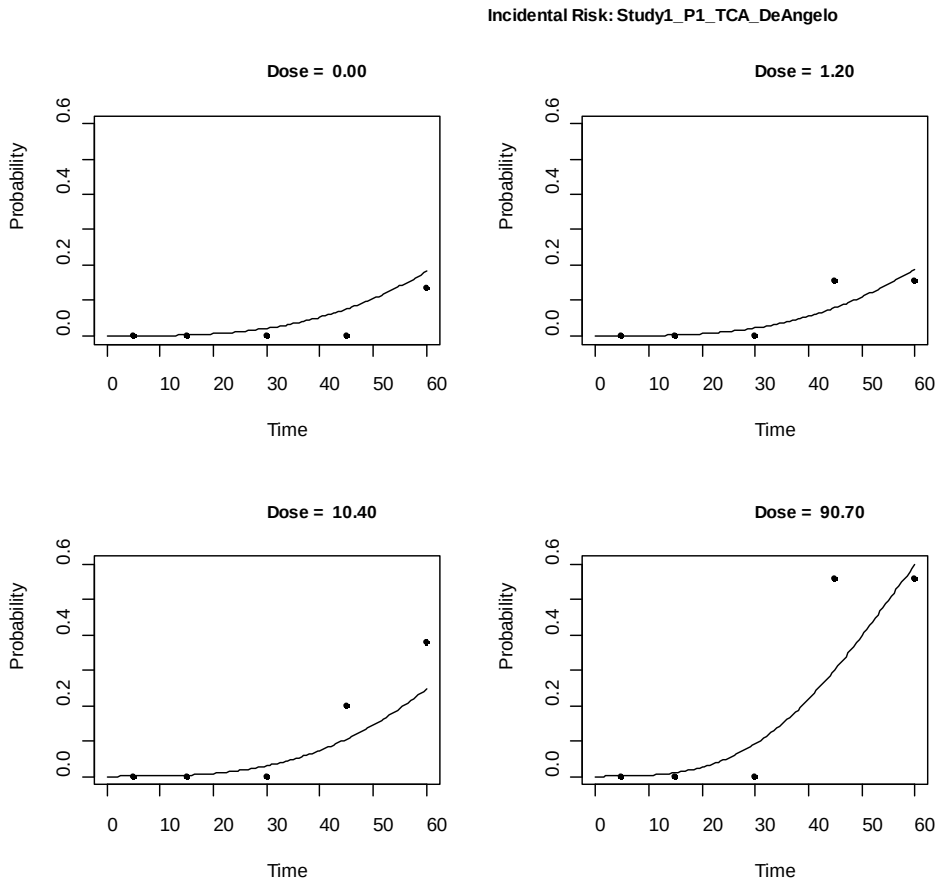
E.6.1. Study 1 from DeAngelo et al. (2008); 60-Week Study with Four Dose Groups

E.6.1.1. *MSW Time-to-Tumor Model Run*

```
 ======================================================================
         Multistage Weibull Model. (Version: 1.6.1;  Date: 11/24/2009)
         Solutions are obtained using donlp2-intv, (c) by P. Spellucci
         Input Data File: TCA-DeAngelo-St1-P1.(d)
======================================================================
 Timer to Tumor Model, TCA, DeAngelo et al, Study 1, Poly 1
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
   The form of the probability function is:
   P[response] = 1-EXP{-(t - t_0)^c *
                 (beta_0+beta_1*dose^1)}

The parameter betas are restricted to be positive
   Dependent variable = CONTEXT
   Independent variables = DOSE, TIME

 Total number of observations = 199
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 1
 Degree of polynomial = 1

   User specifies the following parameters:
        t_0    =          0
 Maximum number of iterations = 64
 Relative Function Convergence has been set to: 2.22045e-016
 Parameter Convergence has been set to: 1.49012e-008

                  Default Initial Parameter Values
                        c      =            3
                        t_0    =            0   Specified
                        beta_0 = 9.21252e-007
                        beta_1 =  3.5896e-008

           Asymptotic Correlation Matrix of Parameter Estimates
           ( *** The model parameter(s)  -t_0
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

                 c            beta_0       beta_1

    c                 1           -1           -1

    beta_0           -1            1         0.99

    beta_1           -1         0.99            1

                                Parameter Estimates
                                                          95.0% Wald Confidence Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
         c               3.23626          1.63392          0.0338329             6.43868
         beta_0     3.53142e-007     2.34711e-006        -4.2471e-006        4.95339e-006
         beta_1     1.37847e-008     9.15217e-008       -1.65594e-007        1.93164e-007

               Log(likelihood)   # Param            AIC
   Fitted Model        -76.4417         3         158.883

        Data Summary
                       CONTEXT
              C      F      I      U  Total  Expected Response
    DOSE
        0     46      0      4      0     50     5.95
      1.2     42      0      5      3     50     5.83
       10     37      0     12      0     49     7.82
       91     30      0     19      1     50    19.96

   Benchmark Dose Computation
Risk Response    =     Incidental
```

```
Risk Type        =          Extra
Specified effect =            0.1
Confidence level =            0.9
Time             =             60
             BMD =        13.4502
            BMDL =         8.4229
            BMDU =         25.589
```

E.6.1.2. *MSW Time-to-Tumor Plots*



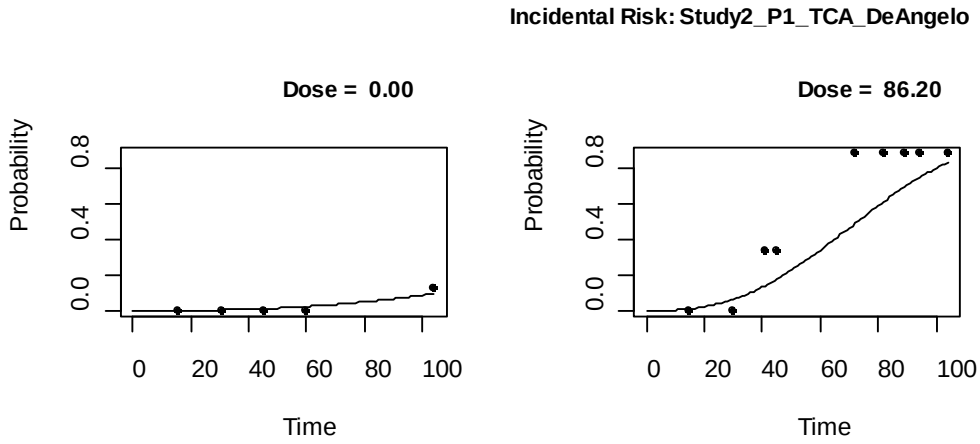
E.6.2. Study 2 from DeAngelo et al. (2008); 104-Week Study with Two Dose Groups

E.6.2.1. *MSW Time-to-Tumor Model Run*

```
=============================================================================
         Multistage Weibull Model. (Version: 1.6.1;  Date: 11/24/2009)
         Solutions are obtained using donlp2-intv, (c) by P. Spellucci
         Input Data File: TCA-DeAngelo-St2-P1.(d)
=============================================================================

 Timer to Tumor Model, TCA, DeAngelo et al, Study 2, Poly 1

   The form of the probability function is:
   P[response] = 1-EXP{-(t - t_0)^c *
                 (beta_0+beta_1*dose^1)}

   The parameter betas are restricted to be positive

   Dependent variable = CONTEXT
   Independent variables = DOSE, TIME
```

```
Total number of observations = 112
Total number of records with missing values = 0
Total number of parameters in model = 4
Total number of specified parameters = 1
Degree of polynomial = 1


  User specifies the following parameters:
        t_0    =          0

Maximum number of iterations = 64
Relative Function Convergence has been set to: 2.22045e-016
Parameter Convergence has been set to: 1.49012e-008


                Default Initial Parameter Values
                       c      =      2.57143
                       t_0    =            0   Specified
                       beta_0 = 6.55001e-007
                       beta_1 = 1.26153e-007


          Asymptotic Correlation Matrix of Parameter Estimates
          ( *** The model parameter(s)  -t_0
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

                c            beta_0       beta_1

   c                 1        -0.99           -1

   beta_0         -0.99            1         0.98

   beta_1            -1         0.98            1


                                Parameter Estimates
                                                        95.0% Wald Confidence Interval
      Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
        c              2.65637          1.11083           0.479187            4.83356
        beta_0     4.44615e-007     2.28279e-006       -4.02958e-006       4.91881e-006
        beta_1     8.57058e-008     4.34281e-007       -7.65469e-007       9.36881e-007


             Log(likelihood)   # Param            AIC
  Fitted Model        -33.7142         3        73.4284


                  Data Summary
                      CONTEXT
            C      F      I      U  Total  Expected Response
   DOSE
       0     47      0      3      6     56     3.02
      86     18      0     32      6     56    31.85


   Benchmark Dose Computation
Risk Response    =     Incidental
Risk Type        =          Extra
Specified effect =            0.1
Confidence level =            0.9

Time             =            104

            BMD =        5.39107
           BMDL =        3.78731
           BMDU =        7.93865
```

E.6.2.2. *MSW Time-to-Tumor Plots*



E.6.3. Study 3 from DeAngelo et al. (2008); 104-Week Study with Three Dose Groups

E.6.3.1. *MSW Time-to-Tumor Model Run*

```
=====================================================================
         Multistage Weibull Model. (Version: 1.6.1;  Date: 11/24/2009)
         Solutions are obtained using donlp2-intv, (c) by P. Spellucci
         Input Data File: TCA-DeAngelo-St3-P2.(d)
=====================================================================

 Timer to Tumor Model, TCA, DeAngelo et al, Study 3, Poly2

   The form of the probability function is:
   P[response] = 1-EXP{-(t - t_0)^c *
                 (beta_0+beta_1*dose^1+beta_2*dose^2)}

   The parameter betas are restricted to be positive

   Dependent variable = CONTEXT
   Independent variables = DOSE, TIME

 Total number of observations = 216
 Total number of records with missing values = 0
 Total number of parameters in model = 5
 Total number of specified parameters = 1
 Degree of polynomial = 2

   User specifies the following parameters:
          t_0    =          0

 Maximum number of iterations = 64
 Relative Function Convergence has been set to: 2.22045e-016
 Parameter Convergence has been set to: 1.49012e-008

                  Default Initial Parameter Values
                         c      =            3
                         t_0    =            0   Specified
                         beta_0 =  7.0609e-007
                         beta_1 = 2.30386e-028
                         beta_2 = 3.14871e-009

           Asymptotic Correlation Matrix of Parameter Estimates
           ( *** The model parameter(s)  -t_0       -beta_1
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

                 c            beta_0       beta_2

    c                 1           -1        -0.99
```

beta_0	-1	1	0.99
beta_2	-0.99	0.99	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
c	4.11251	1.07308	2.00931	6.21571
beta_0	4.40074e-009	2.16313e-008	-3.79957e-008	4.67972e-008
beta_1	0	NA		
beta_2	2.09521e-011	1.02607e-010	-1.80154e-010	2.22058e-010

NA - Indicates that this parameter has hit a
bound implied by some inequality constraint
and thus has no standard error.

	Log(likelihood)	# Param	AIC
Fitted Model	-109.195	4	226.391

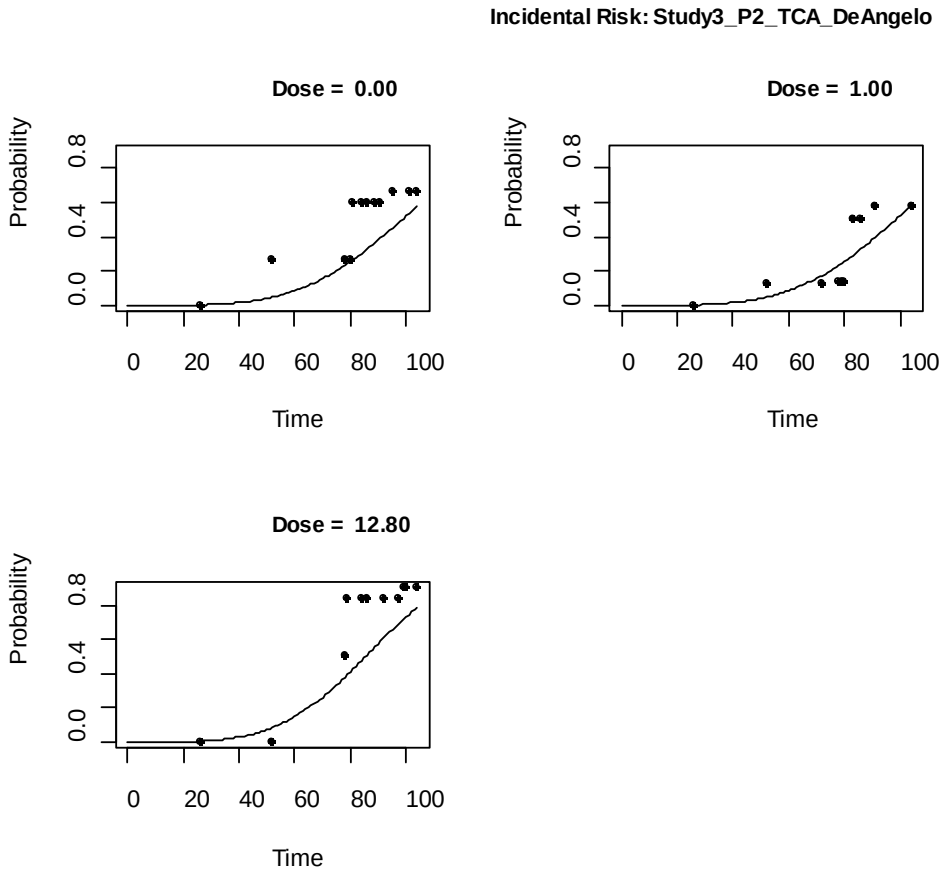
Data Summary

DOSE	CONTEXT C	F	I	U	Total	Expected Response
0	32	0	31	9	72	27.15
1	34	0	21	17	72	24.79
13	22	0	36	14	72	36.35

Benchmark Dose Computation

Risk Response	=	Incidental
Risk Type	=	Extra
Specified effect	=	0.1
Confidence level	=	0.9
Time	=	104
BMD	=	5.04887
BMDL	=	1.17598
BMDU	=	9.08397

E.6.3.2. *MSW Time-to-Tumor Plots*



E.6.4. Combined Dataset (Study 1+3) from DeAngelo et al. (2008)

E.6.4.1. *MSW Time-to-Tumor Model Run*

```
=====================================================================
         Multistage Weibull Model. (Version: 1.6.1;  Date: 11/24/2009)
         Solutions are obtained using donlp2-intv, (c) by P. Spellucci
         Input Data File: Combine1and3-P1-TCA-DeAngelo.(d)
=====================================================================

 Timer to Tumor Model, TCA, DeAngelo et al, Combine Study 1 and 3, Exact Adj. Doses, Poly1

   The form of the probability function is:
   P[response] = 1-EXP{-(t - t_0)^c *
                 (beta_0+beta_1*dose^1)}
   The parameter betas are restricted to be positive

   Dependent variable = CONTEXT
   Independent variables = DOSE, TIME

 Total number of observations = 415
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 1
 Degree of polynomial = 1

   User specifies the following parameters:
          t_0    =          0

 Maximum number of iterations = 64
 Relative Function Convergence has been set to: 2.22045e-016
 Parameter Convergence has been set to: 1.49012e-008
```

Default Initial Parameter Values

c	=	3.6	
t_0	=	0	Specified
beta_0	=	4.78773e-008	
beta_1	=	3.31927e-009	

Asymptotic Correlation Matrix of Parameter Estimates
(*** The model parameter(s) -t_0
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	c	beta_0	beta_1
c	1	-1	-0.99
beta_0	-1	1	0.98
beta_1	-0.99	0.98	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
c	3.1631	0.527188	2.12983	4.19636
beta_0	3.48836e-007	8.3625e-007	-1.29018e-006	1.98786e-006
beta_1	1.993e-008	4.33381e-008	-6.50113e-008	1.04871e-007

	Log(likelihood)	# Param	AIC
Fitted Model	-187.491	3	380.982

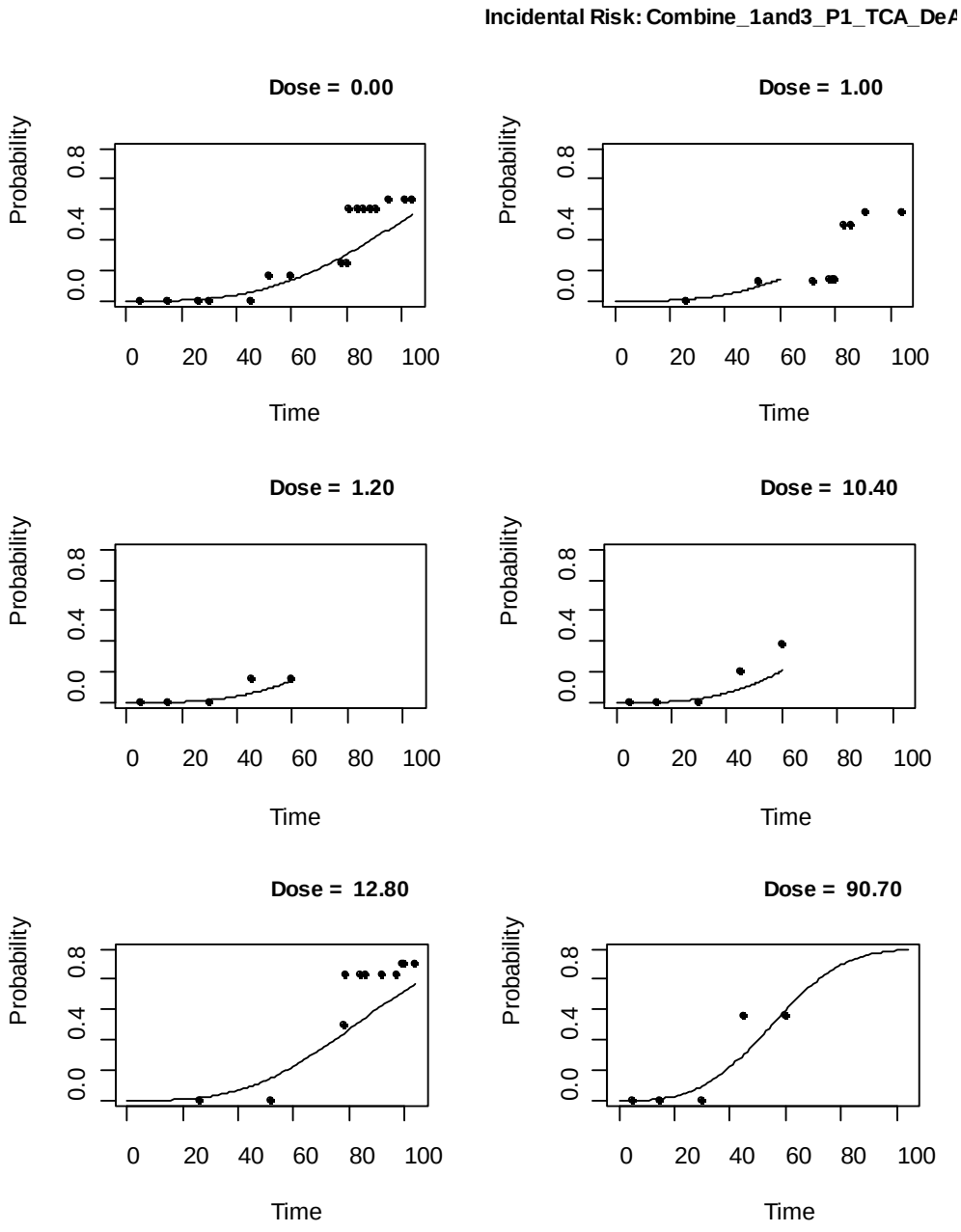
Data Summary

DOSE	CONTEXT C	F	I	U	Total	Expected Response
0	78	0	35	9	122	32.37
1	34	0	21	17	72	26.83
1.2	42	0	5	3	50	4.47
10	37	0	12	0	49	6.65
13	22	0	36	14	72	37.53
91	30	0	19	1	50	19.97

Benchmark Dose Computation

Risk Response	=	Incidental
Risk Type	=	Extra
Specified effect	=	0.1
Confidence level	=	0.9
Time	=	104
BMD	=	2.20344
BMDL	=	1.39087
BMDU	=	3.9828

E.6.4.2. *MSW Time-to-Tumor Plots*



E.6.5. Other Combined Data Sets from DeAngelo et al. (2008)

E.6.5.1. *MSW Time-to-Tumor Model Run for Combining Study 1, Study 2, and Study 3*

```
=====================================================================
         Multistage Weibull Model. (Version: 1.6.1;  Date: 11/24/2009)
         Solutions are obtained using donlp2-intv, (c) by P. Spellucci
         Input Data File: Combine3--P1-TCA-DeAngelo.(d)
=====================================================================

 Timer to Tumor Model, TCA, DeAngelo et al, Combine 3 studies, Poly1
```

```
  The form of the probability function is:
  P[response] = 1-EXP{-(t - t_0)^c *
                (beta_0+beta_1*dose^1)}

  The parameter betas are restricted to be positive

  Dependent variable = CONTEXT
  Independent variables = DOSE, TIME

Total number of observations = 527
Total number of records with missing values = 0
Total number of parameters in model = 4
Total number of specified parameters = 1
Degree of polynomial = 1


  User specifies the following parameters:
        t_0    =          0

Maximum number of iterations = 64
Relative Function Convergence has been set to: 2.22045e-016
Parameter Convergence has been set to: 1.49012e-008


                 Default Initial Parameter Values
                        c      =      2.57143
                        t_0    =            0   Specified
                        beta_0 = 4.20208e-006
                        beta_1 =  1.4128e-007


          Asymptotic Correlation Matrix of Parameter Estimates
          ( *** The model parameter(s)  -t_0
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

                c            beta_0       beta_1

   c                 1           -1        -0.98

   beta_0           -1            1         0.97

   beta_1        -0.98         0.97            1

                                Parameter Estimates
                                                          95.0% Wald Confidence Interval
      Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
        c               2.51012         0.393072             1.73971             3.28052
        beta_0     5.52994e-006     9.77318e-006       -1.36251e-005          2.4685e-005
        beta_1     1.84135e-007      3.1736e-007       -4.37878e-007        8.06149e-007


              Log(likelihood)   # Param            AIC
  Fitted Model        -238.347         3         482.694


                  Data Summary
                      CONTEXT
             C      F      I      U  Total  Expected Response
   DOSE
       0    125      0     38     15    178    44.43
       1     34      0     21     17     72    23.08
     1.2     42      0      5      3     50     4.87
      10     37      0     12      0     49     6.36
      13     22      0     36     14     72    29.94
      86     18      0     32      6     56    36.10
      91     30      0     19      1     50    16.27


   Benchmark Dose Computation
Risk Response    =     Incidental
Risk Type        =          Extra
Specified effect =            0.1
Confidence level =            0.9
```

```
Time             =           104

             BMD =       4.94936
            BMDL =       3.47233
            BMDU =       7.48461
```

E.6.5.2. MSW *Time-to-Tumor Model Run for Combining Study 1 and Study 2*

```
=====================================================================
         Multistage Weibull Model. (Version: 1.6.1;  Date: 11/24/2009)
         Solutions are obtained using donlp2-intv, (c) by P. Spellucci
         Input Data File: Combine1and2-P1-TCA-DeAngelo.(d)
=====================================================================

 Timer to Tumor Model, TCA, DeAngelo et al, Combine Study 1 and 2, Poly1

~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:
   P[response] = 1-EXP{-(t - t_0)^c *
                 (beta_0+beta_1*dose^1)}

   The parameter betas are restricted to be positive

   Dependent variable = CONTEXT
   Independent variables = DOSE, TIME

 Total number of observations = 311
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 1
 Degree of polynomial = 1

   User specifies the following parameters:
          t_0    =          0

 Maximum number of iterations = 64
 Relative Function Convergence has been set to: 2.22045e-016
 Parameter Convergence has been set to: 1.49012e-008


                  Default Initial Parameter Values
                         c      =          1.8
                         t_0    =            0   Specified
                         beta_0 = 6.38638e-005
                         beta_1 = 4.37061e-006


           Asymptotic Correlation Matrix of Parameter Estimates
           ( *** The model parameter(s)  -t_0
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

                 c            beta_0       beta_1

    c                 1        -0.98        -0.99

    beta_0         -0.98            1         0.96

    beta_1         -0.99         0.96            1

                                  Parameter Estimates
                                                          95.0% Wald Confidence Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
         c              1.73322         0.425706           0.898849            2.56759
         beta_0     8.48134e-005      0.000156535        -0.000221989         0.000391616
         beta_1     5.81776e-006     1.07052e-005       -1.51641e-005        2.67997e-005


                Log(likelihood)   # Param            AIC
   Fitted Model        -117.363         3         240.726
```

```
                  Data Summary
                      CONTEXT
             C      F      I      U  Total  Expected Response
    DOSE
        0     93      0      7      6    106    11.58
      1.2     42      0      5      3     50     3.49
       10     37      0     12      0     49     5.52
       86     18      0     32      6     56    34.78
       91     30      0     19      1     50    18.86


   Benchmark Dose Computation
Risk Response    =     Incidental
Risk Type        =          Extra
Specified effect =            0.1
Confidence level =            0.9

Time             =            104

             BMD =        5.78047
            BMDL =        4.12371
            BMDU =        8.37783
```

E.6.5.3. *MSW Time-to-Tumor Model Run for Combining Study 2 and Study 3*

```
================================================================================
         Multistage Weibull Model. (Version: 1.6.1;  Date: 11/24/2009)
         Solutions are obtained using donlp2-intv, (c) by P. Spellucci
         Input Data File: TCA-DeAngelo-Combine2and3-P1.(d)
================================================================================

 Timer to Tumor Model, TCA, DeAngelo et al, Combine study 2 and 3, Poly1

~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:
   P[response] = 1-EXP{-(t - t_0)^c *
                 (beta_0+beta_1*dose^1)}

   The parameter betas are restricted to be positive

   Dependent variable = CONTEXT
   Independent variables = DOSE, TIME

 Total number of observations = 328
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 1
 Degree of polynomial = 1


   User specifies the following parameters:
          t_0    =          0

 Maximum number of iterations = 64
 Relative Function Convergence has been set to: 2.22045e-016
 Parameter Convergence has been set to: 1.49012e-008


                  Default Initial Parameter Values
                        c      =            3
                        t_0    =            0   Specified
                        beta_0 = 5.83762e-007
                        beta_1 = 1.55175e-008


           Asymptotic Correlation Matrix of Parameter Estimates
           ( *** The model parameter(s)  -t_0
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )
```

	c	beta_0	beta_1
c	1	-1	-0.99
beta_0	-1	1	0.99
beta_1	-0.99	0.99	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
c	3.32166	0.765809	1.8207	4.82262
beta_0	1.34723e-007	4.71404e-007	-7.89212e-007	1.05866e-006
beta_1	3.57265e-009	1.25973e-008	-2.11176e-008	2.82629e-008

	Log(likelihood)	# Param	AIC
Fitted Model	-158.516	3	323.031

Data Summary

	CONTEXT					
DOSE	C	F	I	U	Total	Expected Response
0	79	0	34	15	128	38.75
1	34	0	21	17	72	22.24
13	22	0	36	14	72	27.83
86	18	0	32	6	56	33.41

Benchmark Dose Computation

Risk Response	=	Incidental
Risk Type	=	Extra
Specified effect	=	0.1
Confidence level	=	0.9
Time	=	104
BMD	=	5.88546
BMDL	=	3.78614
BMDU	=	10.4269