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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)

September 2009

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LIST OF ABBREVIATIONS AND ACRONYMS

ACO	acyl-CoA oxidase
ACP	acid phosphatase
AHF	altered hepatic foci
AIC	Akaike 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
BSA	bovine serum albumin
CACT	carnitine acetyl-CoA transferase
CAT	catalase
CASRN	Chemical Abstracts Service Registry Number
CPF	ciprofibrate
CpG	cytosine-guanine dinucleotide
CPK	creatine phosphokinase
CYP450	cytochrome P450
DCA	dichloroacetic acid
DEHP	di(2-ethylhexyl)phthalate
DEN	diethylnitrosamine
DMR-2	differentially methylated region-2
DMSO	dimethyl sulfoxide
EC_{50}	median effective concentration
ED_{10}	exposure dose at 10% extra risk
ENU	ethylnitrosourea
EPA	U.S. Environmental Protection Agency
FMU	first morning urine
GC/MS	gas chromatography/mass spectrometry
GD	gestation day
GGT	gamma-glutamyl transpeptidase
GR	glutathione reductase
GSH	glutathione
GST	glutathione S-transferase
GTPase	guanosine triphosphatase
HPLC	high performance liquid chromatography
IAP	intracisternal A particle
IGF	insulin-like growth factor
IL	interleukin
i.p.	intraperitoneal(ly)
IPCS	International Programme on Chemical Safety

IPRL	isolated perfused rat liver
IRIS	Integrated Risk Information System
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LED_{10}	lower 95% bound on exposure dose at 10% extra risk
LINE	long interspersed nucleotide element
LOAEL	lowest-observed-adverse-effect level
LOH	loss of heterozygosity
LTR	long terminal repeat
MCA	monochloroacetic acid
MCP	methylclofenapate
MDA	malondialdehyde
MDD	mean daily dose
5MeC	5-methylcytosine
M_1G	MDA-derived deoxyguanosine
MNU	N-methyl-N-nitrosourea
MOA	mode of action
MTase	methyltransferase
MTD	maximum tolerated dose
NAF	nafenopin
NIOSH	National Institute for Occupational Safety and Health
NLM	National Library of Medicine
NOAEL	no-observed-adverse-effect level
NPC	non-parenchymal cell
NRC	National Research Council
NTD	neural tube development
8-OHdG	8-hydroxy-2′-deoxyguanosine
PAS	periodic acid-Schiff's reagent
PB	phenobarbital
PBPK	physiologically based pharmacokinetic
PCNA	proliferating cell nuclear antigen
PCO	palmitoyl-CoA oxidase
PCR	polymerase chain reaction
PFOA	perfluorooctanoic acid
PG	prostaglandin
PH	partial hepatectomy
POD	point of departure
POR	prevalence odds ratio
PP-A	peroxisome proliferation-associated
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RA	retinoic acid
RDS	replicative DNA synthesis
RfC	reference concentration
RfD	reference dose
RT-PCR	reverse transcription PCR
SA	superoxide anion
SAM	S-adenosylmethionine

SD	standard deviation
SOD	superoxide dismutase
SSB	single-strand break
SSCP	single-stranded conformation polymorphism
SuDH	succinate dehydrogenase
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
TCE	trichloroethylene
TGF	transforming growth factor
UF	uncertainty factor

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 ($\leq$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 (1983). 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, 2000a), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000b), *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000c), *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002), *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b), *Science Policy Council Handbook: Peer Review* (U.S. EPA, 2006a), and *A Framework for Assessing Health Risks of Environmental Exposures to Children* (U.S. EPA, 2006b).

The literature search strategy employed for this compound was based on the Chemical Abstracts Service Registry Number (CASRN) and at least one common name. Any pertinent scientific information submitted by the public to the IRIS Submission Desk was also considered in the development of this document. The relevant literature was reviewed through March 2009.

2. CHEMICAL AND PHYSICAL INFORMATION

Trichloroacetic acid (TCA) is a colorless to white crystalline solid with a sharp, pungent odor (National Institute for Occupational Safety and Health [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 for trichloroacetic acid include TCA, trichloroethanoic acid, and trichloro-methanecarboxylic acid. The structure of TCA is shown in Figure 2-1.

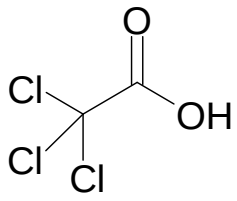

Figure 2-1. Trichloroacetic acid.

Selected physical and chemical properties of TCA (CASRN 76-03-9):

Empirical 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)
Partition coefficient (log K_{ow})	1.33 (Hansch et al., 1995)
Vapor pressure	0.16 mmHg at 25°C (Liley et al., 1984)
Log dissociation constant (pKa)	0.51 at 25°C (Serjeant and Dempsey, 1979)
Henry's law constant	1.35×10^{-8} atm-m^3/mol at 25°C (Bowden et al., 1998)
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)

TCA is used as a soil sterilizer and laboratory intermediate or reagent in the synthesis of a variety of medicinal products and organic chemicals (National Library of Medicine [NLM], 2003). 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 (Al-Waiz and Al-Sharqi, 2002; Lee et al., 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, 2002).

TCA can be formed as a combustion by-product 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 (Mower and Nordin, 1987). TCA could be a photooxidation product of tetrachloroethylene and 1,1,1-trichloroethane in the atmosphere (Juuti and Hoekstra, 1998; Reimann et al., 1996; Sidebottom and Franklin, 1996). 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., 1996).

TCA is formed from organic material during water chlorination (International Programme on Chemical Safety [IPCS], 2000; Coleman et al., 1980) and has been detected in groundwater, surface water distribution systems, and swimming pool water. Human exposure to TCA directly occurs through consumption and use of tap water disinfected with chlorine-releasing disinfectants (U.S. EPA, 2005c). TCA was detected in vegetables, fruits, and grains (Reimann et al., 1996) and can be taken up into foodstuffs from the cooking water (U.S. EPA, 2005c). 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 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, 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, 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., 1988a) and male $B6C3F_1$ mice (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 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 was 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 ng to 1,183 ng, whereas postexposure amounts ranged from 294 ng 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- to 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 ranged from about 10 to 50 ng/m^2 and 60 to 160 ng/m^2, respectively. The results from this study indicate that dermal absorption and subsequent urinary elimination of TCA are rapid but were 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 [1-^{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) in plasma, red blood cells, muscle, and fat; (2) moderate elimination (rate constants between 0.064 and 0.077) in kidney and skin; and (3) slow elimination (rate constants between 0.037 and 0.063) 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 less than 20% of the administered dose. Results from in vitro binding studies indicated that noncovalent, reversible binding of TCA in rat plasma (presumably to proteins) 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., 1988b, 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 in vivo and in vitro studies (Lumpkin et al., 2003; Toxopeus and Frazier, 2002, 1998; Yu et al., 2000; Schultz et al., 1999; 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 weight >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 below 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

	6 nmol/mL	61 nmol/mL	612 nmol/mL
Mouse	55%	52%	34%
Rat	53.5%	48.9%	38.3%
Dog	64.8%	58.5%	54.2%
Human	84.3%	83.3%	74.8%

Note: Values are expressed as percent of [^{14}C]-TCA associated with protein fraction, expressed as mean value for two replications of pooled samples.

Source: Templin et al. (1995).

Toxopeus and Frazier (1998) investigated the kinetics of TCA in isolated perfused rat liver (IPRL) from male F344 rats. The IPRL 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 (BSA) 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 BSA) 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 BSA, and the free TCA concentration averaged 15.4 µM at 5 minutes of exposure and 14.9 µM at 120 minutes. At the low concentration, 96% of the TCA was bound to protein and the free TCA concentration was approximately constant at 0.9 to 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 BSA 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), more than 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 than for rodent plasmas. Decreases in the proportion of bound to free TCA at concentrations exceeding 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 were 2.97, 1.49, and 0.17, respectively. The low number of binding sites observed for mice may indicate 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 correlated with 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 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 trichloroethylene (TCE) and has been frequently measured in the urine or blood of humans exposed to TCE as a result of environmental contamination (Bruning 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 to high concentrations of TCE or tetrachloroethylene (presumably 1,100–1,200 ppm) (Ghantous et al., 1986). 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 metabolized to only 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 less than 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 (i.e., dichloroacetate) 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 by-products 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.

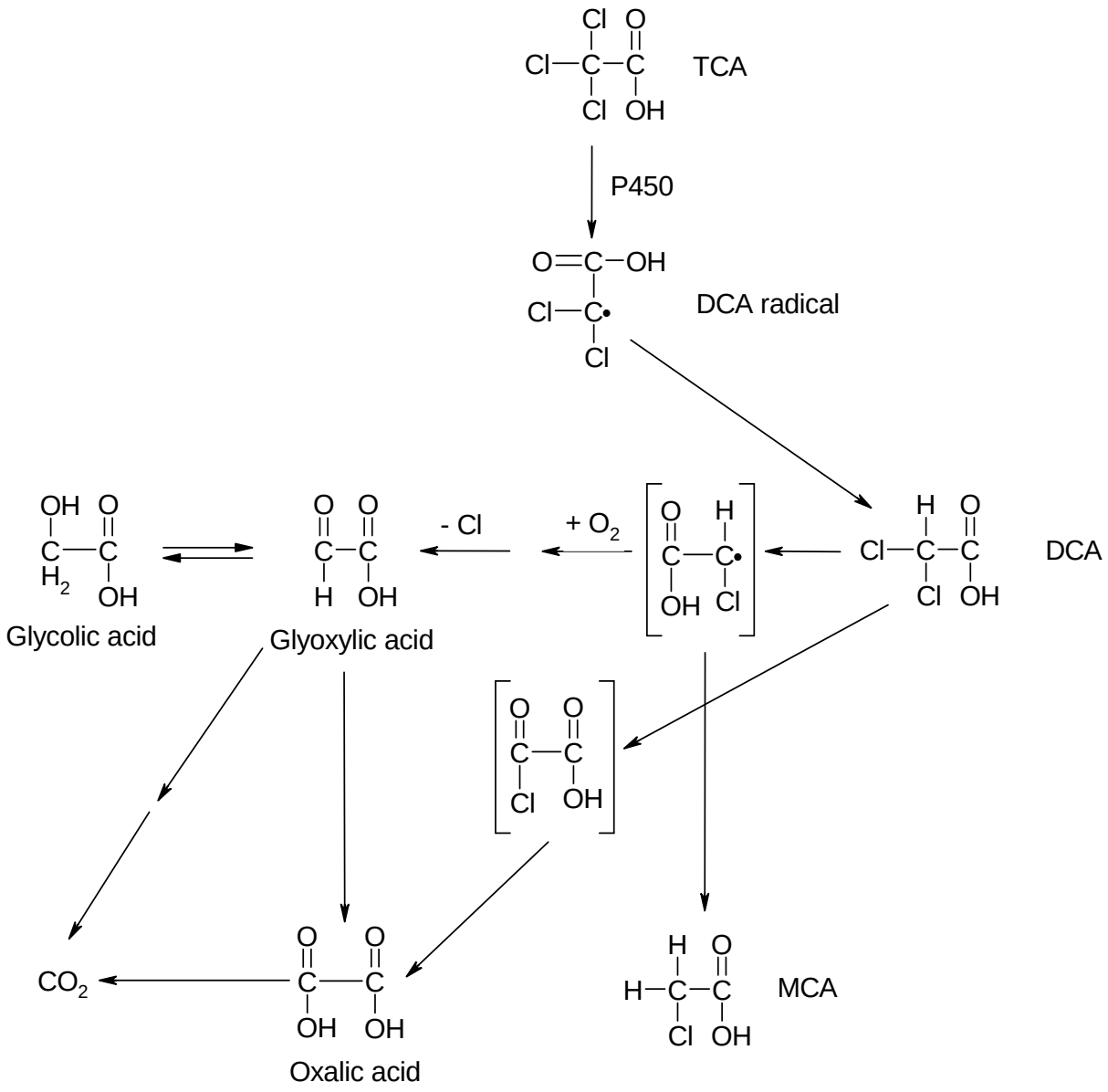

Figure 3-1. Proposed metabolic scheme for TCA.

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).

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 (GC/MS) 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 and Stacpoole, 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., 1998a, b).

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 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 NADPH and GSH dependent (e.g., Cornett et al. [1999, 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 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, and 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 [1,2-^{14}C]-labeled TCA (Gonzalez-Leon et al., 1999). Pretreated mice 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 (Merdink et al., 2000, 1998; Yu et al., 2000; 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 by-products. 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 more than 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 (FMU) samples and normalized to creatinine concentration to adjust for differences in FMU 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. A study of urinary excretion of TCA following inhalation exposure to perchloroethylene (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 perchloroethene (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 GC/MS 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 perchloroethylene 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 of 2 weeks of exposure to tap water containing TCA (Froese et al., 2002) or cessation of a 6-hour inhalation exposure to perchloroethylene (Volkel 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, 1998; Yu et al., 2000; 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; Volkel 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.

3.5. PHYSIOLOGICALLY BASED AND OTHER TOXICOKINETIC MODELS

Physiologically based toxicokinetic models have not been developed for TCA.

[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).

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 by-products. These complex mixtures of disinfectant by-products have been associated with increased potential for bladder, rectal, and colon cancer in humans (reviewed by Boorman et al. [1999]; Mills et al. [1998]) and adverse effects on reproduction (reviewed by Nieuwenhuijsen et al. [1999]; Mills et al. [1998]).

Most of the studies of human health effects following exposure to water disinfectant by-products have used trihalomethanes and haloacetic acid concentrations as the exposure metric (Hinckley et al., 2005; King et al., 2005; Porter et al., 2005). For example, a population-based case-control study conducted by Klotz and Pyrch (1999) examined the relationship between drinking water exposure to haloacetic acids (and other disinfection by-products, including trihalomethanes and haloacetonitriles) and neural tube defects. The study included 112 eligible cases of neural tube defects in 1993 and 1994 that were identified through the New Jersey Birth Defect and Fetal Death Registries. A total of 248 controls were selected randomly from all New Jersey births, with approximately 10 controls selected for each month over 24 months. A statistically significant difference between cases and controls was observed when cases were restricted to subjects with known residency at conception and to those with isolated neural tube defects (i.e., cases where no other birth defects were present). A prevalence odds ratio (POR) of 2.1 was reported (95% confidence interval 1.1–4.0) for the highest tertile (third) of trihalomethane exposure. However, only a slight (not statistically significant) excess risk (POR 1.2, 95% confidence interval 0.5–2.6) was found for the highest tertile (≥35 ppb) of the 5 regulated haloacetic acids (HAA5). The specific haloacetic acids that were measured as part of the total haloacetic acid exposure estimate were not reported. Based on the results of the study, the authors concluded that haloacetic acid concentration did not exhibit a clear association with neural tube defects.

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; Chiarello et al., 1996; Moy et al., 1996; Tse et al., 1996; Witheiler et al., 1996; Rubin, 1995). Concentrations of 45% and higher have an increased risk of causing scarring. The skin peeling procedure results in a pink erythema and swelling for the first few days posttreatment and is followed by exfoliation of the dead skin. Histologic studies (Moy et al., 1996; Tse 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 was reported where a 35% TCA solution inadvertently entered the eye of a patient receiving a dermal peel, resulting in marked conjunctivitis and abrasions that involved 25% of the cornea (Fung et al., 2002). Complete corneal healing was reported within 72 hours of initiation of supportive care and no lasting effects were evident, 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. The surface of each wart was coated with TCA (concentration not reported). Initially the patients complained of burning, which was short-lived. After a second TCA treatment a week later, the patients reported continual soreness or burning. On clinical examination, marked erythema and tenderness in the vulvar and vestibular areas were noted. The symptoms in these patients lasted for 2 to 15 weeks. 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 (number of patients treated with TCA was not reported); however, the study was not specifically designed to identify adverse side effects in treated patients.

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

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

4.2.1. Subchronic Studies

4.2.1.1. *Subchronic Oral Studies*

4.2.1.1.1. *Rats.* 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, creatinine phosphokinase, and gamma-glutamyl transpeptidase [GGT]). In addition, the following immune function parameters were evaluated: antibody production, delayed hypersensitivity, natural killer cell cytotoxicity, and production of prostaglandin (PG) 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.

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. (1991) 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 daily 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, may be more likely a frank effect level rather than a LOAEL for 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, creatine phosphokinase [CPK], acid phosphatase [ACP], ALP, and LDH), erythrocytes and tissue antioxidant defense systems (GSH, glutathione reductase [GR], superoxide dismutase [SOD], GST catalase [CAT]), and malondialdehyde (MDA) (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 CAT were significantly increased in the brain, liver, and kidney. However, no changes in GSH, GR, and GST activities were found in all tissues. Celik (2007) concluded 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 CAT activities in the tissues after TCA treatment were probably due to increased generation of reactive oxygen species.

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-day)	LOAEL (mg/kg-day)	Comments
Rats								
Mather et al. (1990)	Sprague-Dawley rats (males, 10/dose)	Oral, drinking water	90 days	0, 4.1, 36.5, or 355 mg/kg-day	Increased absolute spleen weight; increased relative liver and kidney weights; increased liver, kidney, and spleen sizes; peroxisome proliferation	36.5	355	
Bhat et al. (1991)	Sprague-Dawley rats (males, five/group)	Oral, drinking water	90 days	0 or 825 mg/kg-day	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.
Acharya et al. (1997, 1995)	Wistar rats (males, five to six/dose)	Oral, drinking water	10 weeks	0 or 3.8 mg/kg-day	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-day is judged as an equivocal LOAEL because the observed severity of the observed liver changes was considered minimal.
Davis (1990)	Sprague-Dawley rats (six/sex /dose)	(A) Oral, drinking water	(A) 14 days	(A) 5.2–309 mg/kg-day	(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-day study (A).
		(B) Oral, gavage	(B) 3 doses over 24 h	(B) 0, 0.15, or 0.4 mg/kg	(B) Decreased plasma and liver lactate levels	(B) Not determined	(B) 0.15	

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-day)	LOAEL (mg/kg-day)	Comments
DeAngelo et al. (1989)	Sprague-Dawley, F344, and Osborne-Mendel rats (males, six/group /strain)	Oral, drinking water	14 days	0, 212, 327, or 719 mg/kg-day	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.
Goldsworthy and Popp (1987)	F344 rats (males, six/group)	Oral, gavage	10 days	0 or 500 mg/kg 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.
Celik (2007)	Sprague-Dawley rats (female)	Oral, drinking water	50 days	0 or 300 mg/kg-day	Increase in serum AST, ALT, CPK, and ACP activities; increase in SOD and CAT activities in brain, liver, and kidney tissues	Not determined	300	
Mice								
Kato-Weinstein et al. (2001)	$B6C3F_1$ mice (males, five/dose)	Oral, drinking water	(A) 4 or 8 weeks (B) 12 weeks	(A) 750 mg/kg-day (B) 0, 75, 250, or 750 mg/kg-day	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.

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-day)	LOAEL (mg/kg-day)	Comments
Parrish et al. (1996)	$B6C3F_1$ mice (males, six/group)	Oral, drinking water	3 or 10 weeks	0, 25, 125, 500 mg/kg-day	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-week evaluations; 8-OHdG[b] levels were not affected by TCA.
Austin et al. (1995)	$B6C3F_1$ mice (males, six/group)	(A) Oral, drinking water (B) Oral, gavage	(A) 14 days (B) Single dose	(A) 0 or 250 mg/kg-day (B) 0 or 300 mg/kg	(A) Increased relative liver weight (B) Decreased TBARS[c]; increased PCO, CAT, 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-day pretreatment period.
DeAngelo et al. (1989)	$B6C3F_1$, C3H, Swiss-Webster, C57BL/6 mice (n = 6)	Oral, drinking water	14 days	0, 261, or 442 mg/kg-day	Increased relative liver weight, peroxisome proliferation (PCO activity)	Not determined	261	C57BL/6 mice were more sensitive than the other strains to peroxisome proliferation.
Sanchez and Bull (1990)	$B6C3F_1$ mice (males, 12/group)	Oral, drinking water	14 days	0, 75, 250, or 500 mg/kg-day	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-day, there was a slightly increased hepatocyte diameter because of increased glycogen deposition.

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-day)	LOAEL (mg/kg-day)	Comments
Dees and Travis (1994)	$B6C3F_1$ mice (five/sex /dose)	Oral, gavage	11 days	0, 100, 250, 500, or 1,000 mg/kg-day	Increased absolute and relative liver weight; increased hepatocyte labeling	Not determined	100	
Goldsworthy and Popp (1987)	$B6C3F_1$ mice (males, seven to eight /group)	Oral, gavage	10 days	0 or 500 mg/kg in corn oil	Induction of hepatic and renal peroxisome proliferation; increased relative liver weight	Not determined	500	The cyanide-insensitive PCO activity assay was used to measure the proliferative response. Liver:body weight ratios were also significantly increased in both.
Austin et al. (1996)	$B6C3F_1$ mice (males, six/group)	Oral, gavage	Single dose	0, 30, 100, or 300 mg/kg	Oxidative stress (increased 8-OHdG[b] levels)	Not reported	Not reported	Doses were estimated based on default drinking water intake values for male $B6C3F_1$ mice; 8-OHdG[b] levels at 30 or 100 mg/kg were not reported.
Laughter et al. (2004)	SV129 wild-type mice; PPAR[d]α-null mice (males, three to five/group)	Oral, drinking water	7 days	0, 57.5, 115, 230, or 460 mg/kg-day	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 high dose exhibited centrilobular hepatocyte hypertrophy	115	230	No reported or default data were available for estimation of average daily doses.

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

Source: Adapted from U.S. EPA (2005c).

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 (SuDH), 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. SuDH activity was increased by roughly 30% compared with controls. Liver triglyceride and cholesterol levels were significantly decreased, while liver-glycogen levels were dramatically increased (roughly 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 SuDH, 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 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, 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 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).

Davis (1990) investigated the effects of TCA on weight gain, urine volume and osmolality, and plasma glucose and liver lactate levels in Sprague-Dawley rats in a 14-day study. Groups of rats (six animals/sex and dose group) received TCA in drinking water at concentrations of 0, 0.04, 0.16, 0.63, or 2.38 g/L (equivalent to approximate dose levels of 0, 5.2, 20.8, 81.9, or 309 mg/kg-day, based on a water consumption factor of 0.13 L/kg-day for Sprague-Dawley rats from U.S. EPA [1988]). High-dose rats consumed less food and water and lost weight during the first few days of exposure. Weight gain was similar to that in controls at subsequent time points. Urine volume and osmolality were not affected except for a temporary lesser increase in osmolality to match decrease in urine volume on day 7 in high-dose females. No clearly adverse effects or dose-related trends were demonstrated. No effects on plasma glucose or liver lactate levels occurred after the 14-day exposure period. EPA has not determined the NOAEL for Davis (1990) since limited endpoints were monitored in this study.

Additional information collected by Davis (1990) suggests that TCA may have transient effects on plasma glucose and plasma and liver lactate levels in rats. Three gavage doses of 0.92 μmol/kg or 2.45 μmol/kg TCA (approximately 0.15 and 0.40 mg/kg, respectively) were administered to Sprague-Dawley rats (five/sex/dose) over 24 hours. The rats were killed 3 hours after the last dose. Significantly reduced plasma (45%) and liver lactate (48%) levels were observed in females. Plasma lactate level was significantly reduced in males (30%) at the high dose. Plasma glucose level was significantly reduced (25%) in females given the high dose. These data suggest that TCA can affect intermediary metabolism, although the absence of effects

on plasma lactate or glucose levels in the 14-day study conducted by Davis (1990) suggests that the effect may be transient.

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 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.

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 (CACT) activity (another peroxisomal enzyme marker) was determined only in Sprague-Dawley rats, and induction of the peroxisome proliferation-associated (PP-A) 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. CACT 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 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. Effects were observed at doses as low as 0.45 mg/kg-day (decreased liver and plasma lactate levels) (Davis, 1990). 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, 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.*** 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 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, CAT activity was increased by 461% above controls in $B6C3F_1$ mice at the high dose, with accompanying increases in the level of PP-A 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).

Miyagawa et al. (1995) tested for acute toxicity as part of a dose-range finding study on hepatocyte replicative DNA synthesis (RDS) for 41 putative Ames-negative mouse hepatocarcinogens. Groups of male $B6C3F_1$ mice (four or five/dose) were administered a single gavage dose of TCA to determine the maximum tolerated dose (MTD), which was set at about half the LD_{50}. The MTD for TCA was estimated to be 500 mg/kg.

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). 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) contrasted the profile of oxidative DNA damage induced by TCA in this study with TCA-induced levels of thiobarbituric acid-reactive substances (TBARS) (an indicator of lipid peroxidation) reported in a previous study (Larson and Bull, 1992). In the earlier study, Larson and Bull (1992) reported a maximum concentration of TBARS at 9 hours post-dosing in the livers of mice given 2,000 mg/kg TCA. The Larson and Bull (1992) study also 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 standard measures of liver or systemic toxicity were reported. A limitation of this study is that a high single dose was used.

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 roughly 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 daily 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 CA activity, and changes in microsomal 12-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, CAT, and CYP4A activities were increased by 4.5-fold, 1.7-fold, 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 reduction in the TBARS response observed in TCA-pretreated animals resulted from activities associated with peroxisome proliferation and might be related to a shift in the expression of P450 isoforms, such as CYP4A. 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, CAT, and CYP4A activities), the single dose tested of 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 (Austin et al., 1996, 1995; Parrish et al., 1996; Larson and Bull, 1992). TCA induces both lipid peroxidation (TBARS) and oxidative DNA damage (8-OHdG) following administration of single oral doses. However, these increases appear transient, 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 are 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 daily 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 (PAS)-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 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 11 daily gavage doses of 0, 100, 250, 500, or 1,000 mg/kg-day TCA in corn oil. 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. 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% or less at all doses, indicating 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 and

higher for female mice and 100 mg/kg-day and higher 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 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 daily 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 PAS 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 to 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 PAS staining) and glycogen-poor (low PAS staining) cells were mixed in each hepatic zone, with slightly higher numbers of glycogen-rich cells in the portal area. In comparison, PAS 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 than in centrilobular portions of the liver acinus.

Laughter et al. (2004) exposed wild-type SV129 mice and a mouse strain lacking a functional form of peroxisome proliferator activated receptor (PPAR)α (PPARα-null mice) 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 daily doses of approximately 0, 57.5, 115, 230, or 460 mg/kg-day, respectively, based on a reference water intake value of 0.23 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 liver effects through activation of PPARα.

4.2.1.2. *Subchronic Inhalation Studies*

No short-term 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 4-2a (noncancer data) and Table 4-2b (cancer and tumor promotion data).

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 daily doses (MDDs) of 0, 3.6, 32.5, or 364 mg/kg 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. An MTD 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 the 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 19–24/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 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.

Table 4-2a. Summary of longer-term 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	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Comments
Rats									
DeAngelo et al. (1997)	F344 rats (males, 50/group)	Oral, drinking water	104 weeks	0, 3.6, 32.5, or 364 mg/kg-day	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 weeks Studies 2 and 3: 104 weeks	Study 1: 0, 8, 68, or 602 mg/kg-day; Study 2: 0 or 572 mg/kg-day; Study 3: 0, 6, or 58 mg/kg-day	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 necropies; complete histopathologic examination on 5 mice from the high-dose and control groups	Decreased body weight, increased absolute and relative liver weight in the 68 and 602 mg/kg-day groups, hepatic inflammation and necrosis, increased LDH activity in the 68 and 602 mg/kg-day groups at 30 weeks, increased liver PCO activity in the 68 and 602 mg/kg-day groups, increased labeling index for nuclei outside of hepatic proliferative lesions, and testicular tubular degeneration at 602 mg/kg-day	8	68	Time-weighted average daily doses were calculated by the study authors; a comprehensive set of tissues was examined microscopically.

Table 4-2a. Summary of longer-term 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	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Comments
Pereira (1996)	$B6C3F_1$ mice (females, 38–134/ group)	Oral, drinking water	51 or 82 weeks	0, 78, 262, or 784 mg/kg-day	Body and liver weight, liver histopathology	Increased relative liver weight	78	262	Increased liver weight was observed after 82 weeks at 262 mg/kg-day; 262 mg/kg-day was judged to be an equivocal LOAEL in the absence of other measures of liver toxicity.
Bull et al. (1990)	$B6C3F_1$ mice (A) (5–35 mice/dose /time point, see text) (B) (11 males/ dose)	Oral, drinking water	(A) 52 weeks (w/ interim sacrifices at 15, 24, and 37 weeks) (B) 37 weeks + 15-week recovery	(A) 0, 164, or 329 mg/kg-day (B) 0 or 309 mg/kg-day	Liver and kidney weight and histopathology	Increased absolute and relative liver weight, cytomegaly, modest glycogen accumulation	Not achieved	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 weeks	0, 500, or 1,250 mg/kg-day	Liver weight and histopathology	Increased absolute and relative liver weight	Not achieved	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).

Source: Adapted from U.S. EPA (2005c).

Table 4-2b. 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
Rats							
DeAngelo et al. (1997)	F344 rats (males, 50/group)	Cancer assay, multiple organs	Oral, drinking water	104 weeks	0, 3.6, 32.5, or 364 mg/kg-day	Negative	A comprehensive set of tissues was microscopically examined; only about 30 animals/ concentration were exposed for >60 weeks.
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 months	0, 2.9, 29.6, and 277 mg/kg-day at 6 months	GGT-positive foci in liver	TCA promoted GGT-positive foci in diethylnitrosamine-initiated rats at all doses evaluated, but only one rat showed a liver carcinoma. TCA showed no evidence as an initiator.
Mice							
DeAngelo et al. (2008)	$B6C3F_1$ mice (males, 27–30/dose at terminal sacrifice; five/dose at interim sacrifices	Cancer bioassay	Oral, drinking water	Study 1: 60 weeks; interim sacrifices at 4, 15, 30, and 45 weeks Studies 2 and 3: 104 weeks	Study 1: 0, 8, 68, or 602 mg/kg-day; Study 2: 0 or 572 mg/kg-day; Study 3: 0, 6, or 58 mg/kg-day	Positive for liver tumors starting at 45 weeks	Liver, kidneys, spleen, and testes were evaluated microscopically for tumors; complete histopathologic evaluation was conducted on other organs for 5 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 weeks	0, 78, 262, and 784 mg/kg-day	Positive at 51 and 82 weeks	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 weeks	0, 120, or 480 mg/kg-day	Increased incidence of liver tumors	Only the liver was microscopically examined; doses were estimated based on a default water intake of 0.25 L/kg-day.

Table 4-2b. 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
Bull et al. (1990)	$B6C3F_1$ mice (5–35/dose)	Chronic toxicity study with microscopic examination of the liver	Oral, drinking water	(A) 52 weeks (interim sacrifices at 15, 24, and 37 weeks) (B) 37 weeks + 15-week recovery	(A) 0, 164, or 329 mg/kg-day (B) 0 or 309 mg/kg-day	Positive for cancer, and increased absolute and relative liver weight, cytomegaly, apparent glycogen accumulation	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.
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 days of age; tumors evaluated 12 or 20 months of age	1,000 or 2,000 nmol (16 or 32 mg/kg) total dose over a 2-day period (at 8 and 15 days of age)	Negative for tumor induction	TCA induced oxidative stress but not a significant increase in tumors in the neonatal mouse.
Herren-Freund et al. (1987)	$B6C3F_1$ mice (males, 22–33/group)	Cancer assay and tumor promotion, liver	Oral, drinking water	61 weeks	0, 400, or 1,000 mg/kg-day	Positive for tumor production and for tumor promotion	Only the liver was microscopically examined; liver tumors were observed either with or without ethylnitrosourea pretreatment.
Pereira and Phelps (1996)	$B6C3F_1$ mice (females, 8–40/ group)	Cancer assay and tumor promotion	Oral, drinking water	Up to 52 weeks	0, 78, 262, or 784 mg/kg-day	Positive with or without MNU[a] initiation	Only the liver was examined for tumors.
Pereira et al. (2001)	$B6C3F_1$ mice (14–16/sex)	Tumor promotion	Oral, drinking water	31 weeks	0 or 960 mg/kg-day	Positive for liver and kidney tumor promotion	Only the liver and kidneys were examined for tumors; MNU[a] was used as an initiator; statistically significant increases in tumor yield were only observed in males.
Pereira et al. (1997)	$B6C3F_1$ mice (females, 20–45/ dose)	Tumor promotion	Oral, drinking water	44 weeks	0, 235, or 980 mg/kg-day	Positive, liver tumors	MNU[a] was used as an initiator; only the liver was microscopically examined.

[a]MNU = N-methyl-N-nitrosourea.

Source: Adapted from U.S. EPA (2005c).

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 (PH) 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 PH, 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 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. None of the groups that received initiation doses of TCA or the associated controls exhibited significant induction of GGT-positive foci. Thus, TCA does 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 (PH/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 (PH alone, PH/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 PH/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 PH and TCA. There was no study group that treated sham-operated rats with DEN, followed by TCA. PH 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 (50/dose at study initiation) were exposed to 0.05, 0.5, or 5 g/L TCA in the drinking water for 60 weeks (Study 1); to 4.5 g/L TCA (58 animals/group) for 104 weeks (Study 2); or to 0.05 and 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 (NaCl) 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 necropsies, livers, kidneys, spleens, and testes were examined for gross lesions and microscopically for proliferative and nonneoplastic lesions. At the termination of the studies, a complete necropsy was performed, and pathological examination was conducted on gross lesions, liver, kidney, spleen, and testis. A complete pathological examination was performed on five mice from the high-dose and control 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 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 bromodeoxyuridine (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 MDDs of 8, 68, and 602 mg/kg-day were calculated by the study authors from concentration and consumption data for the low-, mid-, and high-dose groups. Animals in the mid- and high-dose groups consumed significantly less water than the controls. No significant differences in animal survival were noted for any treatment group. An

MDD of 572 mg/kg-day was calculated by the study authors for 4.5 g/L TCA (Study 2) and 6 and 58 mg/kg-day for 0.05 and 0.5 mg/kg-day (Study 3). With the exception of liver neoplasia, all data presented by DeAngelo et al. (2008) were from the 60-week study (Study 1).

No decrease in animal survival was found at any TCA dose in all studies. Exposure to TCA in the 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 4-3 and 4-4). 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 4-4). 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 4-3). No treatment-related changes were observed in the spleen or kidney.

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 and hepatic necrosis) 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).

Table 4-3. 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	7%	0	14%[e]	21%[e]
	Severity	0.10 ± 0.40	0	0.17 ± 0.47	0.21 ± 0.41

[a]Time-weighted MDD (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 4-4. 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-day)	**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 MDD.
[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).

Exposure to TCA induced tumors in the liver at 60 weeks (Table 4-5). 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 0.5g/L group and one hepatocellular adenoma was found in the 0.05 g/L group. No induction of tumors was reported in other organs.

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 4-6). Neoplastic lesions observed at organ sites other than the liver were considered spontaneous for the male mice and did not exceed the tumor incidences when compared to a historical control database.

Table 4-5. Prevalence and multiplicity of hepatocellular neoplasia in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks

	Treatment	Control	0.05 g/L TCA	0.5 g/L TCA	5 g/L TCA
	Dose[a]	**0**	**8**	**68**	**602**
Neoplasia type	Number[b]	30 (30)	27 (30)	29 (30)	29 (30)
HA[c]	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[c]	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[c]	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 MDD (mg/kg-day).
[b]Number of animals examined. Parentheses = number of animals/group scheduled for terminal necropsy.
[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).

Table 4-6. Incidence of hepatocellular neoplasia in male B6C3F$_1$ mice exposed to TCA in drinking water for 104 weeks

Neoplasia type	Treatment	Control			4.5 g/L TCA
	Dose[a]	**0**			**572**
	Number[b]	25 (32)			36 (43)
HA[c]	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+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+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 MDD calculated over 104 weeks (mg/kg-day).
[b]Animals surviving ≥78 weeks, parentheses = number of animals/group scheduled for terminal necropsy.
[c]HA = adenoma, HC = carcinoma, HA or HC = either adenoma or carcinoma.
[d]Number of animals with a lesion/number of animals examined.
[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 when compared with control values. The range of PCO activity for mice exposed to 0.5 g/L and 5g/L was 129–260% and 326–575%, respectively, above the control value. Autoradiographs of the livers from animals exposed to 5 g/L TCA showed significantly increased labeling of hepatocyte nuclei at 30 weeks (about threefold) 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. Bull et al. (1990) examined the induction of tumors in the liver of 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 daily doses of 0, 164, and 329 mg/kg-day as calculated from data for total dose provided in the

study report. The approximate average daily 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 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 after exposure to 2 g/L for 37 weeks, hepatocellular carcinomas developed in 3/11 (30%) male mice, but hepatic adenomas had not occurred by that date. 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 7–8 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 NaCl. 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 roughly 40% over controls at 360 days, and liver weights for the mid- and high-dose groups increased by roughly 25 and 60% over controls, respectively, after 576 days. EPA determined the increase in liver weight to be 2.0 mmol/L (78 mg/kg-day) and the LOAEL to be 6.67 mmol/L (262 mg/kg-day). However, this study was not designed to evaluate noncancer effects of TCA.

The adversity of the liver weight increase at 6.67 mmol/L is supported by short-term studies in $B6C3F_1$ mice that have reported some evidence for glycogen accumulation (Sanchez and Bull, 1990), increased hepatocyte labeling (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 360 days (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 576 days 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%). 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%), 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%).

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 NaCl 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 NaCl control. Absolute and relative liver weights were significantly increased (by 41–73%) in all TCA treatment groups relative to the corresponding NaCl control group. The incidences of hepatocellular adenomas (8/22, 36%) and hepatocellular carcinomas (7/22, 32%) were significantly increased in the uninitiated group receiving 5 g/L TCA when compared with the uninitiated NaCl control group (hepatocellular adenomas: 2/22, 9%; hepatocellular carcinoma: 0/22, 0%). The incidences of hepatocellular adenomas (NaCl control: 1/22, 5%; TCA 2 g/L: 11/33, 33%; 5 g/L: 6/23, 26%) and hepatocellular carcinomas (NaCl control: 1/22, 5%; TCA 2 g/L: 16/33, 48%; 5 g/L: 11/23, 48%) 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 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.

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 increasing 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. 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 more than 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 less than 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.

Bannasch et al. (2003, 2001) have presented detailed information about phenotype for foci of altered hepatocytes observed in the rat following treatment with classic peroxisome proliferators and the changes that occur as foci progress to liver tumors. The phenotype for altered hepatic foci (AHFs) induced by TCA in mice (mixed basophilic and eosinophilic) and progressed to basophilic in tumors is inconsistent with the peroxisome proliferator phenotype (amphophilic-basophilic) described for hepatic preneoplastic lesions in rats. The analysis presented by these authors has potential implications for evaluation of the MOA, leading to tumors in mice treated with TCA and their potential relevance to humans. However, there is, at present, no pattern of gene expression to serve as a template for agents that are PPARα agonists that could be used to compare the phenotypes described by Bannasch et al. (2003, 2001) with those observed for TCA; the existing data for TCA do not include the detailed characterization of phenotype required to support such a comparison. In addition, the patterns of tumor phenotype and differences between the primary lineages observed in preneoplastic foci and those induced by peroxisome proliferators have not been as well studied in the mouse. Consequently, the implications of the work of Bannasch et al. (2003, 2001) for analysis of foci and lesions produced by TCA are unclear.

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 (DMSO) 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 (22–24 mice/sex/dose) and total doses of 1,000 and 500 nmol were given by i.p. injection for Assays A and B, respectively. DMSO solvent control groups (23–24 mice/sex) were included in each assay. Body weight (at 28-day intervals) and mortality were evaluated in all treatment groups. At sacrifice, all test animals were necropsied for gross tumor count, microscopic examination of tissues, and histopathologic diagnoses. No unscheduled deaths occurred in Assay A. In Assay B, one mouse each died in the male and female solvent control groups and in the female TCA group. 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 DMSO-treated control females in either assay. The study authors concluded that TCA did not induce significant tumor incidences when compared with the DMSO controls. In contrast, all male mice treated with 4-aminobiphenyl (the positive control substance) in Assays A and B developed liver tumors, and 9/22 male mice in Assay B also developed lung tumors. Nine of 23 female mice treated with 4-aminobiphenyl in Assay B developed liver tumors; no tumors were diagnosed in female mice dosed with 4-aminobiphenyl 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 (M_1G) 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 for a summary of this study), and metabolism of TCA in the presence of calf thymus DNA resulted in the formation of M_1G 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 M_1G and 8-OHdG. TCA induced a significant ($p < 0.05$) increase in M_1G 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 AND DEVELOPMENTAL STUDIES

4.3.1. Reproductive Studies

One in vitro study was identified that suggested that TCA might decrease fertilization. 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 160, 400, or 1,600 mg/L) and incubated with mouse oocytes and sperm for 24 hours. Each culture dish was subsequently scored for percentage oocytes fertilized. The percent of oocytes fertilized was significantly decreased from 82% for controls to 53% for oocytes exposed to 1,000 mg/L TCA ($p < 0.001$).

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 4-7). 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 4-7. Summary of developmental studies evaluating effects of TCA after oral administration in rats

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	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-day	Decreased fetal weight, decreased crown-rump length, increased incidence of soft-tissue malformations and cardiovascular malformations, increased maternal spleen and kidney weights	Maternal: None Developmental: None	Maternal: 330 Developmental: 330	Critical study for 1994 RfD
Johnson et al. (1998)	Sprague-Dawley rats (55 controls and 11 TCA treated)	Oral, drinking water	GDs 1–22	0 or 291 mg/kg-day	Increase in cardiac malformations, number of implantation sites/litter, number of resorption sites/litter, and total number of resorptions among treated dams	Maternal: None Developmental: None	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 end points was not assessed.
Fisher et al. (2001)	Sprague-Dawley rats (19/dose)	Oral, gavage	GDs 6–15	0 or 300 mg/kg-day	Decreased maternal weight gain, reduced fetal body weight	Maternal: none Developmental: none	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 in controls.

Table 4-7. Summary of developmental studies evaluating effects of TCA after oral administration in rats

Reference	Species	Exposure route	Exposure duration	Doses evaluated	Effects	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	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-day	Increase in post-implantation loss, decreased fetal testes weight, reduction in the diameter of the seminiferous tubules, increased apoptosis of the gonocytes	Developmental (increase in implantation loss): none Effect on fetal testes: 1,000	Developmental: 1,000 Effect on fetal testes: 1,200	Only evaluated effects on fetal testes
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-day	Decrease in fetal ovaries weight, decrease in the number of oocytes and the size of the ovaries, apoptosis of oocytes	Effect on fetal ovary: 1,200	Effect on fetal ovary: 1,400	Only evaluated effects on fetal ovaries
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-day	Decrease in maternal weight gains; decrease in fetal weight and fetal brain weight; hydrocephalus, vacuolation, and hemorrhages in fetal brains	Maternal: 1,000 Effect on fetal brain: none	Maternal: 1,200 Effect on fetal brain: 1,000	Focused only on effects of TCA on fetal brains
Warren et al. (2006)	Sprague-Dawley Crl:CDR (SD) BR rats	Oral, gavage	GDs 6–15	0 or 300 mg/kg-day	Decrease in fetal weight, no eye malformation, no significant reductions in lens area, globe area, medial canthus distance, and interocular distance	Developmental: none	Development: 300	Focused on eye malformations and microphthalmia in fetal rats

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 by gavage on gestation days (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 GDs 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 4-8) 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); increased percentages of fetuses affected per litter with cardiovascular malformations, particularly levocardia and interventricular septal defects; and increased percentages of fetuses affected per litter for total soft-tissue malformations. 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 4-8. 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-day)				
	0	330	800	1,200	1,800
Malformations: mean % fetuses affected per litter ± SD (number of litters affected/number examined)[a]					
Total soft tissue (visceral)	3.50 ± 8.7 (4/26)	9.06 ± 12.9[b] (8/19)	30.37 ± 28.1[b] (15/17)	55.36 ± 36.1[b] (12/14)	96.88 ± 8.8[b] (8/18)
Cardiovascular	0.96 ± 4.9 (1/26)	5.44 ± 10.0[b] (6/19)	23.59 ± 28.0[b] (12/17)	46.83 ± 36.5[b] (11/14)	94.79 ± 9.9[b] (8/8)
Levocardia: number of fetuses or litters affected/number examined[c]					
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[c]					
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[d]					
Male	3.71 ± 0.12	3.58 ± 0.10[b]	3.46 ± 0.10[b]	3.36 ± 0.15[b]	3.16 ± 0.12[b]
Female	3.64 ± 0.15	3.53 ± 0.09[b]	3.38 ± 0.12[b]	3.33 ± 0.16[b]	3.15 ± 0.15[b]
Mean fetal body weight (g): mean ± SD[c]					
Male	3.70 ± 0.24	3.20 ± 0.26[b]	2.98 ± 0.17[b]	2.74 ± 0.30[b]	2.49 ± 0.16[b]
Female	3.54 ± 0.20	3.08 ± 0.27[b]	2.83 ± 0.18[b]	2.67 ± 0.29[b]	2.36 ± 0.15[b]

[a]Table 5 of Smith et al. (1989).
[b]Mean is significantly different from control mean ($p \leq 0.05$) as reported by Smith et al. (1989).
[c]Table 6 of Smith et al. (1989).
[d]Table 4 of Smith et al. (1989).

Source: Smith et al. (1989).

Johnson et al. (1998) evaluated the teratogenicity of TCA 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. No measure of variation around the mean (e.g., SD or standard error) was reported, and it is not clear why this reduction was not

reported as statistically significant. Nonetheless, a decrease of this magnitude in body weight gain during pregnancy is considered to be toxicologically significant. Average daily drinking water consumption was reported as 38 mL/day in treated rats as compared with 46 mL/day in control rats; this difference was not reported as statistically significant, but it was unclear from the publication whether a statistical analysis was performed.

Statistically significant increases were reported in 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. A limitation of this study is that 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 (RA) (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 from 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 RA) 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, 2005a, b) 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 euthanized 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 histologic examination (Singh, 2005a). 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 and higher. Histologic 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. 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 and higher.

The rat fetal ovaries of each pup of different dose groups from the above study were also dissected out, weighed, and subjected to histologic examination (Singh, 2005b). The average weights of the ovaries were significantly reduced for the dose groups ≥1,400 mg/kg-day. Histologic 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 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 decreased at TCA doses ≥1,200 mg/kg-day (38% at 1,200 mg/kg-day). 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. RA (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 day 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 RA treatment groups. Mean maternal body weight was also reduced in these treatment groups, but the reduction was not significant (Warren et al., 2006). Fetuses with exencephaly, anophthalmia, or microphthalmia were found only in the RA treatment group. Mean fetal lens and globe areas were statistically significantly reduced in the RA treatment group. However, mean lens and globe areas and mean medial canthus and interocular distances were reduced by only 1–9 %, and the reductions were not statistically significant. 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) TCA in drinking water on GDs 0–11. 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 such that these genes may be used to explain the gross cardiac effects associated with exposure. 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 (NTD) 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 greater than or equal to 2 mM, with a very steep dose-response slope from 2 to 5 mM. No adverse effects were observed at 1 mM or below, and defects of the eyes, arches, and heart were seen only in embryos that also

had very high rates of NTD 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 to 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 beginning at 2.5 mM and above. 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 and the median effective concentration (EC_{50}) for malformations was 1,740 mg/L. Malformations were observed at concentrations greater than 1,500 mg/L and included gut miscoiling, craniofacial defects, microphthalmia, microcephaly, and various types of edema.

Fu et al. (1990) studied the developmental toxicity potential of TCA by using a regeneration assay from reaggregated *Hydra attenuata* cells. The hydra system is an in vitro assay that determines the degree to which a test chemical can perturb embryonic development at maternally subtoxic doses and thus is considered to be useful as a prescreening assay for developmental toxicity (Fu et al., 1990). In this study, both intact adult hydra and artificial "embryos" (pellets of the disassociated and randomly reaggregated, terminally differentiated, and pluripotent stem cells of hydra) were treated with TCA at concentrations ranging from 10^{-3} to 10^{3} mg/L. The minimal effective toxic concentrations for adults (A) and artificial embryos (D) were determined, and the A/D ratio was evaluated as a developmental toxicity hazard index. The TCA treatment resulted in an A/D ratio of 1.0. This result suggested that the developing hydras are no more sensitive to TCA than adult hydras and indicates that in this test system TCA does not selectively interfere with embryonic development at adult subtoxic doses. According to the authors (Fu et al., 1990), the hydra system is designed to overestimate developmental hazard potential and is considered to be more sensitive to developmental toxicity than most in vitro mammalian test systems; its primary utility is to identify compounds for in vivo developmental

toxicity testing. Based on these results, TCA would not be considered a high-priority compound for further testing in vivo.

4.4. OTHER 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 PG 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. Histologic 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 four types: 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. A number of studies have also been conducted that evaluate the induction of peroxisome proliferation by TCA; these studies are described in Section 4.2.

4.5.1.1. *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 (GTPase). This GTPase 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 (SSCPs). The SSCP 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 mobilities 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 SSCP 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 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.

The authors further suggested that TCA was not enhancing growth of preneoplastic lesions through increased cell proliferation, since TCA has not been demonstrated to be mitogenic, a statement the authors based on the results of DeAngelo et al. (1989). More recent studies seem to confirm this result. Although TCA might induce hepatocyte proliferation following short-term dosing in mice (Stauber and Bull, 1997; Dees and Travis, 1994), chronic exposure of mice to TCA decreased normal hepatocyte proliferation and the high proliferation rate in AHFs was not TCA-dependent (Stauber and Bull, 1997).

As an alternative to increased cell growth signaling to explain enhanced growth of pre-initiated cells, Ferreira-Gonzalez et al. (1995) suggested that TCA might be blocking pathways that suppress cell growth, such as intercellular communication (Benane et al., 1996; Klaunig et al., 1989). Another possible nongenotoxic mechanism might be mediated by increased peroxisomal proliferation, which, based on current knowledge of other peroxisomal proliferators, has an inhibitory effect on apoptosis that might facilitate the growth of initiated cells (Stauber and Bull, 1997).

Tao et al. (1996) investigated whether liver tumors initiated by MNU and promoted by TCA exhibited loss of heterozygosity (LOH) 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 perchloroethene exhibited LOH 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 LOH 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 LOH 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 LOH, and 2 of these 10 tumors also lost at least one of the C3H/HeJ alleles. No LOH on chromosome 6 was observed in 24 DCA-promoted liver tumors. The observed LOH 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 LOH on chromosome 6, the authors concluded that other molecular activity is probably involved in the hepatocarcinogenesis of TCA.

4.5.1.2. *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 RDS 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 MTD. The MTD 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 MTD (250 mg/kg, as estimated from data provided by the authors) or the MTD (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 RDS 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 but are summarized here for completeness.

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 for 5, 12, or 33 days by estimating hepatocyte BrdU-labeling index. TCA increased the BrdU-labeling index after 5 days of exposure for all three concentrations but not for exposures of 12 or 33 days. 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 AHFs.

A transient but significant elevation 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 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 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), Stauber and Bull (1997) proposed 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).

4.5.1.3. *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. (2000a) 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 TCA in water neutralized with sodium hydroxide to pH 6.5 to 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 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. (2000a) concluded that the

prevention of TCA-induced DNA hypomethylation by methionine suggested that the decrease in the formation of 5-MeC in DNA is due to a decrease in the concentration of S-adenosylmethionine (SAM) as substrate, and the dose of TCA must be sufficient to decrease the level of SAM in order for it to be active as a carcinogen.

In another study, Tao et al. (2000b) examined the methylation of *c-jun* and *c-myc* genes, expression of both genes, and activity of DNA methyltransferase (MTase) 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 20.0 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 MTase 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. (2000b) 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 MTase 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 MTase 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[4] in TCA-promoted mouse liver tumors and noninvolved liver tissue, as well as in liver tissue samples

[4]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.

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 (Furstenberger 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. (2000), 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 5-MeC 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 5-MeC. Methylation status of 28 cytosine-guanine dinucleotide (CpG) sites[5] in the differentially methylated region-2 (DMR-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 DMR-2 of the IGF-II gene was amplified by polymerase chain reaction (PCR) for sequencing. Expression of IGF-II mRNA was determined by reverse transcription PCR (RT-PCR). The level of 5-MeC 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 5-MeC 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.

Sequencing of the DMR-2 of the IGF-II gene promoter revealed that 21 to 24 CpG sites were methylated in initiated liver, compared with 15 to 17 sites in noninvolved liver tissue from TCA-promoted mice. Thus, exposure to TCA reduced the percentage of CpG sites that were methylated from approximately 79 to 58%. The number of methylated CpG sites was further reduced to 0 to 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 of the

[5]CpG 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. CpG sites are relatively rare in eukaryotic genomes except in regions near the promoter regions of genes. Methylation of the cytosine nucleotide at CpG 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.

IGF-II gene in the noninvolved mouse liver tissue and 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. (2001a). Female B6C3F_1 mice were given daily gavage doses of 500 mg/kg 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 tissue were collected for measurement of cell proliferation by determination of proliferating cell nuclear antigen (PCNA)-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 PCNA 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 by-product present as a co-contaminant with TCA in drinking water) on 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 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 RT-PCR followed by Northern blot analysis. 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. The ability of chloroform and TCA to hypomethylate *c-myc* and increase *c-myc* mRNA expression in the liver was correlated with their effect on liver tumor promotion.

4.5.1.4. *Inhibition of Intercellular Communication*

Benane et al. (1996) assessed the effects of TCA on gap junction intercellular communication 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 a range of concentrations for varying time periods. Lucifer yellow scrape-load dye transfer was used as a measure of gap junction intercellular communication. Following an initial screen to identify the lowest concentration at which TCA affected dye transfer, the main study was conducted at concentrations of 0, 0.5, 1.0, 2.5, and 5 mM. Cells were treated for 1, 4, 6, 24, 48, or 168 hours. 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 gap junction intracellular communication in primary cultured $B6C3F_1$ mouse and F344 rat hepatocytes. Mouse and rat hepatocytes were isolated from 6- to 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 DMSO 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 gap junction intercellular communication 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).

4.5.1.5. *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 (Austin et al., 1996, 1995; Parrish et al., 1996; 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 was investigated by Hassoun and Ray (2003). Studies are available that report that macrophages could 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 (SA) 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 SA levels; however, incubations of 36 and 60 hours caused significant increases in SA 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; Bull et al., 1990; Sanchez and Bull, 1990).

4.5.1.6. *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 (TGF)-α (a growth factor that stimulates cell proliferation and is expressed in tumor cells), TGF-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 more than 50% of the cells/tumor, except c-jun, which was observed in greater than 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 TGF-α, c-myc, CYP2E1, CYP4A1, and GST-π in greater than

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 TGF-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.

Pereira (1996) also evaluated cell proliferation following 5, 12, or 33 days of treatment with TCA. TCA increased the BrdU-labeling index after 5 days of exposure but not after the longer exposure durations; the degree of increase was similar for all three of the doses tested. The author found that the tumorigenic activity of TCA was linearly related to the concentration in drinking water. Bull et al. (1990) (described in Section 4.2) also observed this linear relationship. Based on differences in the shape of the tumor dose-response curve 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 4-9). 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 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 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 4-9. 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	*E. 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 (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)
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)

Table 4-9. Summary of available genotoxicity data on TCA

Endpoint	Test system	Metabolic activation[a]	Concentration/dose	Results	Reference
Sperm-head abnormalities	Swiss mice	NA	0, 125, 250, 500 mg/kg i.p. divided into 5 daily doses (TCA not neutralized before administration)	Positive	Bhunya and Behera (1987)
Micronucleus induction	Swiss mice, bone marrow	NA	0, 125, 250, or 500 mg/kg i.p. (2 daily doses) (TCA not neutralized before administration)	Positive	Bhunya and Behera (1987)
	C57BL mice, bone marrow evaluated	NA	337–1,300 mg/kg i.p. (25–80% of LD_{50}) (neutralized TCA was administered)	Negative	Mackay et al. (1995)
	Newt larvae (*Pleurodeles waltl*), erythrocytes	NA	40, 80, 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 1, 2, or 3 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 days or 71 days	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. In the absence of S9, TCA increased the mutant frequency by twofold or greater only at concentrations resulting in ≤11% survival (2,000 μg/mL or higher), leading the study authors to characterize the mutagenicity of TCA as equivocal. In the presence of S9, a doubling of mutant frequency was seen at concentrations of 2,250 μg/mL and higher, including several concentrations with survival >10%. No statistical evaluation of these data was conducted. Due to 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.

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 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 4-9). 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 (SSBs) 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 SSBs 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 SSBs by the alkaline unwinding assay. Dose-dependent increases in SSBs were induced in both rats and mice, with mice being more susceptible than rats. The lowest dose of TCA that produced significant SSBs was 0.6 mmol/kg (98 mg/kg) in rats but 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 1, 2, or 3 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 SSBs 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 to 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 (Austin et al., 1996; Parrish 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 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 in rats. 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 PAS 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 PAS-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 PAS 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 (Bhat et al., 1991; Bull et al., 1990; Mather 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. Peroxisome proliferation has been a primary endpoint evaluated (DeAngelo et al., 1997; Parrish et al., 1996), with mice reported to be more sensitive to this effect than rats.

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 (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).

One commonly observed histopathologic change associated with alteration of lipid and carbohydrate homeostasis is glycogen accumulation in the liver (Acharya et al., 1995; Bull et al., 1990; Sanchez and Bull, 1990). Acharya et al. (1995) reported decreased levels of liver triglyceride and liver cholesterol and increased liver glycogen in rats given TCA for 10 weeks. Serum triglyceride levels, glucose levels, and SuDH levels were increased.

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), clearly 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. The available data indicate that TCA is a developmental toxicant. TCA significantly increased resorptions, decreased implantations, increased cardiovascular malformations at 291 mg/kg-day in Sprague-Dawley rats (Johnson et al., 1998), 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 previous studies, Fisher et al. (2001) did not observe treatment-related effects on the incidence of cardiac malformations. The reason for this discrepancy is unknown but might be related to purity of the test material, differences in test strains among laboratories, differences in experimental design, methods used to detect cardiac abnormalities, and/or route of administration (gavage versus drinking water). 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.

TCA was also demonstrated to cause toxicity in the developing testis (Singh, 2005a), developing ovary (Singh, 2005b), and developing brain (Singh, 2006) when pregnant inbred Charles Foster rats were treated with 1,000–1,800 mg/kg-day TCA on GDs 6–15. However, these studies were limited by the administration of a higher dose range of TCA to rats than in the previous studies by Smith et al. (1989) and Johnson et al. (1998).

Although in vitro test systems are limited in their utility to predict adverse developmental effects and associated toxic potencies in intact organisms, they are useful in generating mechanistic hypotheses. 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. In contrast, testing using hydra (freshwater invertebrate hydrozoa) as a model has given negative results (Fu et al., 1990).

4.6.2. Inhalation

No inhalation studies are available.

4.6.3. Mode-of-Action Information

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 and Corton, 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 (Bull et al., 1990; Sanchez and Bull, 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. Therefore, the study has implications for the mechanism of TCA-induced glycogen accumulation. 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 et al., 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 and Corton, 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 (Austin et al., 1996, 1995; Parrish et al., 1996; 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., 1986). 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. Accumulated TCA in the amniotic fluid may be transported through fetal skin and swallowing and excreted by the fetus. Singh (2006) suggested TCA in the amniotic fluid may be circulated for several times and contributes 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., 1986), developmental toxicities may be related to anoxia resulting from toxic effect on the placenta, and apoptosis resulted from oxidative stress, as observed in studies by

Singh (2006, 2005a, b). 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 PCR 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

Based on the observations summarized in Section 4.2.2 and criteria outlined in the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), as well as MOA considerations, TCA is described as "likely to be carcinogenic to humans." The selection of this descriptor for TCA is based on positive results for liver carcinogenicity in male and female mice in multiple studies, development of liver tumors in mice with less than life-time exposure, no positive evidence of carcinogenicity in rats, and no data on carcinogenicity in humans.

U.S. EPA's *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a) 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. For TCA, systemic tumors were observed in mice following oral exposure. Information on carcinogenic effects via the inhalation or dermal routes in humans or animals is absent. Data evaluating absorption by the inhalation route are unavailable and limited data are reported for dermal absorption (Kim and Weisel, 1998). However, based on the observance 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. Therefore, TCA is "likely to be carcinogenic to humans" by all routes of exposure.

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

There are no epidemiologic studies of TCA carcinogenicity in humans. The experimental database for carcinogenicity of TCA consists of studies in rats and mice. The results of the mice studies indicate that, in mice, TCA is a complete carcinogen that significantly increased the incidence of liver tumors in male and female $B6C3F_1$ mice exposed via drinking water for 52–82 weeks (DeAngelo et al., 2008; Bull et al., 2004, 2002, 1990; Pereira, 1996; Pereira and Phelps, 1996; Herren-Freund et al., 1987). Incidence of tumors increased with increasing TCA concentrations (DeAngelo et al., 2008; Bull et al., 2002, 1990; Pereira, 1996). These results

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 (Pereira et al., 2001, 1997) 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.

The MOA for TCA-induced liver carcinogenesis has not been established. The available data collectively provide limited evidence regarding the genotoxicity of TCA. 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 methylation, and/or reduced intracellular communication. Of these, PPARα agonism has been advanced as the most likely MOA contributing to the development of liver tumors. However, significant gaps in knowledge exist in the hypothesized PPARα MOA (Yang et al., 2007; Ito et al., 2007), such that the formation of liver tumors cannot be sufficiently accounted for by this proposed MOA and the existence of other contributing MOA(s) is assumed.

4.7.3. Mode-of-Action Information

Exposure to TCA in drinking water has induced increased incidence of liver tumors in $B6C3F_1$ mice exposed for 52–82 weeks (Pereira et al., 2001, 1996; Bull, 2000; Bull et al., 1990; Herren-Freund et al., 1987) but did not increase incidence of tumors in male F344 rats exposed to TCA up to 102 weeks (DeAngelo et al., 1997). At the present time, the events leading to development of liver cancer in mice exposed to TCA have not been fully characterized, although

several MOAs have been postulated. As discussed below, many of the experimental data for TCA are consistent with a PPARα-mediated MOA for development of liver tumors in mice. However, because it is possible that more than one MOA is operative in the development of mouse liver tumors, it is important to consider whether other MOAs could contribute to the observed pattern of response following TCA exposure. Events that may be related to hepatocarcinogenesis are illustrated in Figure 4-1.

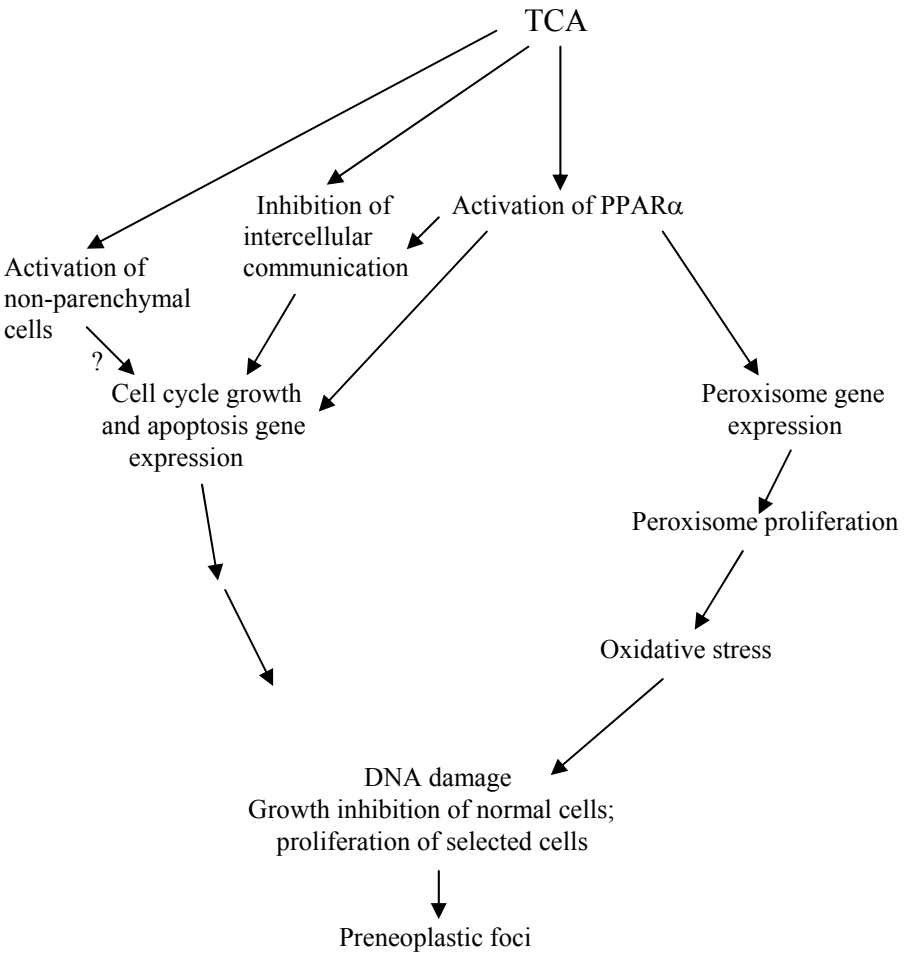

Figure 4-1. Possible key events in the MOA(s) for TCA carcinogenesis.

4.7.3.1. *Hypothesized Modes of Action*

Tumor induction for TCA appears to include events such as perturbation of cell growth and/or reduced intercellular communication. There is support for the involvement of PPARα agonism; however, whether there is a single or multiple MOA(s) capable of producing TCA induced tumors is unknown.

4.7.3.1.1. ***PPARα agonism.*** Peroxisome proliferation has been proposed as a possible MOA for development of tumors in mice exposed to TCA (e.g., Bull [2000]). Peroxisome proliferation refers to an increase in the number and volume fraction of peroxisomes (subcellular organelles) in the cytoplasm of mammalian and other eukaryotic cells. Peroxisomes are known to proliferate under a variety of altered physiological and metabolic states and in response to exposure to a wide array of xenobiotic compounds generally referred to as peroxisome proliferators (Klaunig et al., 2003). 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 rats and mice (Klaunig et al., 2003). At one time, peroxisome proliferation was proposed as a causative factor in the development of liver tumors. However, increased knowledge of the molecular events leading to peroxisome proliferation suggests that it is an associative rather than a causal event in 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[6] 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 peroxisome proliferators, as demonstrated by using the prototypical PPARα agonist Wy-14643 (Klaunig et al., 2003). 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.

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 (Klaunig et al., 2003). 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 (Klaunig et al., 2003). PPARα is known to interact with other transcription factors (e.g., the RA receptor and thyroid hormone receptor), co-activators, and co-repressors to regulate gene expression.

[6]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).

4.7.3.1.1.1. ***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 DNA-damaged cells to persist and proliferate, resulting in preneoplastic hepatic foci and ultimately in tumors via selective clonal expansion. 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. PPARα agonists also inhibit gap junction intracellular communication and stimulate Kupffer cells, the resident macrophages of the liver. Kupffer cells were identified as playing a role in peroxisome-proliferator-induced effects, independently of PPARα. Specifically, Kupffer cells were reported to mediate acute effects of peroxisome proliferators on cell proliferation and production of oxidants in liver (Parzefall et al., 2001; Rusyn et al., 2001, 2000; Hasmall et al., 2000), though, as discussed below, the contribution of Kupffer and other non-parenchymal cells (NPCs) to the chronic effects of PPARα agonists, including hepatocarcinogenesis, is not well known.

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 key events postulated for PPARα-induced hepatocarcinogenesis, activation of PPARα is highly specific for this MOA. While alterations in cell proliferation and apoptosis and clonal expansion are common to other MOAs, recent findings by Shah et al. (2007) indicate that regulation of cell proliferation by peroxisome proliferators may also be PPARα specific. Oxidative stress occurring in conjunction with peroxisomal proliferation is regarded as a general phenomenon and is not considered to be a highly specific marker of PPARα-induced liver carcinogenesis. Moreover, while it is known that activation of PPARα leads to 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.

The understanding of the PPARα agonism MOA has been expanded with recent findings. First, 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-14643, including significantly decreased serum fatty acids and

marked induction of PPARα target genes encoding fatty acid oxidation enzymes, suggesting the transgene functions in the same manner as peroxisome proliferators to regulate fatty acid 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 genes expression—were not sufficient to induce liver tumors. However, it should be noted that, while most PPARα target genes were activated in the LAP-VP16 PPARα mice, several genes (e.g., *c-myc*) were not activated without ligand treatment.

Second, progress has been made as to the involvement of NPCs, which include Kupffer cells, hepatic stellate cells, and sinusoid endothelial cells, in peroxisome-proliferator-induced liver tumors, though many questions remain. Yang et al. (2007) suggest that activation of NPCs plays an important role in peroxisome-proliferator-induced hepatocarcinogenesis. Specifically, induction of proliferation of NPCs was only observed in wild-type mice upon Wy-14643 treatment but not in the transgenic mice. Yang et al. (2007) suggested that lack of tumor induction in transgenic mice as compared to Wy-14643-treated wild-type mice may be associated with the differences of NPC activation. However, Shah et al. (2007) (see next paragraph) suggested that PPARα agonists, such as Wy-14643, may regulate other genes in addition to the gene for the VP16 PPARα fusion protein. These possibilities are being investigated by researchers. To examine the role of Kupffer-cell-derived oxidants in the MOA for liver carcinogenesis, Woods et al. (2007) treated NADPH-oxidase-deficient mice (their Kupffer cells cannot produce oxidants), along with wild-type and PPARα knockout mice, with Wy-14643 for 1 week, 5 weeks, or 5 months. Wy-14643 treatment induced similar levels of hepatocyte proliferation and DNA damage in NADPH-oxidase-deficient mice and wild-type mice, while both were abolished in PPARα knockout mice. Woods et al. (2007) concluded that Kupffer-cell-derived oxidants may play a limited, if any, role in long-term effects of peroxisome proliferators, such as hepatocarcinogenesis.

Third, 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)[7] expression, especially let-7C, an miRNA found to be a potential tumor suppressor (Zhang et al., 2007; Lee and Dutta, 2006) and to inhibit the expression of the *ras* oncogene (Johnson et al., 2005). Let-7C was inhibited following treatment with 0.1% Wy-14643, a potent PPARα agonist, in wild-type mice for 4 hours, 2 weeks, or 11 months. No decrease in let-7C miRNA was observed in the PPARα-

[7] MicroRNAs (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.

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-14643 treatments. Moreover, pri-let-7C, AK033222, and pri-mir-99a were regulated in a PPARα-dependent manner, since Wy-14643 had no effect on pri-let-7C, AK033222, or pri-mir-99a in PPARα-null 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-14643 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 NPCs is critical for tumorigenesis and let-7C expression. Moreover, let-7C was not suppressed in humanized PPARα mice, which were resistant to Wy-14643-induced hepatocellular proliferation and liver tumor formation (Shah et al., 2007). Wy-14643 treatment of humanized PPARα mice also did not induce *c-myc* and mir-17 expression. These findings suggested the let-7C signaling cascade may be critical for PPARα agonist-induced liver proliferation and tumorigenesis.

Fourth, 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 and Goodman, 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, 1998; Jones and Gonzalgo, 1997).

When male SV129 mice were fed a control diet or Wy-14643-containing diet (1,000 ppm) for 1 week, 5 weeks, or 5 months, treatment with Wy-14643 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-14643 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-14643 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-14643 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., 2001b). 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-14643 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-14643 had no effect on DNA and histone methylation status in PPARα-null mice at any of the evaluated time points. Previously, treatment of PPARα-null mice with Wy-14643 for 11 months had produced no liver tumors, whereas treatment of wild-type mice with 1,000 ppm Wy-14643 had resulted in 100% incidence of hepatocellular adenomas and carcinomas (Peters et al., 1997). In addition, Wy-14643 had had no effect on liver cell proliferation in PPARα-null mice (Woods et al., 2007; 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., 2001b), since a temporal relationship between increased cell proliferation and DNA hypomethylation of the *c-myc* gene was observed after a single dose of Wy-14643 to mice. Long-term treatment of wild-type mice with Wy-14643 in Pogribny et al.

(2007) demonstrated gradual worsening dysregulation of normal methylation patterns in genomic DNA.

4.7.3.1.1.2. ***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., enzyme induction, 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 (PPRE)-luciferase reporter (Maloney and Waxman, 1999). Cells were treated for 24 hours with 0.1 to 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 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 4.5.1.2 and 4.2.1.1. 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, 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. (2007) that the robust proliferative effect of Wy-14643 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., 2007; Ward et al., 1988; Yeldandi et al., 1989). 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 recent report by the National Research Council (NRC) of the National Academy of Science, *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, Ito et al. (2007) recently reported that the peroxisome proliferator di(2-ethylhexyl)phthalate (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 higher incidence of liver tumors in PPARα knockout mice and a potential PPARα-independent pathway for DEHP-induced liver tumors. 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, this finding for DEHP does show that demonstration of many of the key events proposed for a PPARα MOA is insufficient to preclude existence of a PPARα-independent pathway for tumorigenesis. Previously, Melnick (2001) has suggested PPARα-independent pathways for tumorigenesis by DEHP.

Researchers have explored other possible key events for a PPARα agonism MOA, including the possible roles of let-7C micro-RNA and hypomethylation of DNA on hepatocarcinogenesis of PPARα agonists in mice. These are discussed with respect to available data on TCA below.

First, 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 micro-RNA 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).

Second, experimental evidence supports the hypothesis that hypomethylation of DNA may be related to the carcinogenicity of TCA in mice. In female B6C3F$_1$ mice initiated by an i.p. injection of MNU and then administered TCA in drinking water at 25 mmol/L (4,085 mg/L) for 44 weeks, the level of 5MeC in DNA of hepatocellular adenomas and carcinomas was decreased 40 and 51%, respectively, as compared with noninvolved 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 that were treated with 25 mmol/L (1,062 mg/kg-day) of 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 substantial decrease in the level of 5MeC in these studies indicated that many genes may be involved. Increased mRNA and proteins of *c-jun* and *c-myc* protooncogenes have been reported in TCA-induced foci of altered hepatocytes and liver tumors in studies by Latendresse and Pereira (1997) and Nelson et al. (1990). Accordingly, Tao et al. (2000a, b) investigated the methylation of DNA in the promoter regions of *c-jun* and *c-myc* protooncogenes.

Using methylation-sensitive restriction enzymes followed by Southern blot analysis, Tao et al. (2000a) reported that the promoter regions of the *c-jun* and *c-myc* genes were hypomethylated in mice exposed to 500 mg/kg TCA for 5 days. Expression of the mRNA and proteins of these two protooncogenes were increased. 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 MTase activity was increased in tumors and decreased in noninvolved liver.

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.

TCA-induced hypomethylation is supported by a study using a bisulfite-modified DNA sequencing procedure (Tao et al., 2004) that demonstrated that the DMR-2 region of the IGF-II gene was hypomethylated in liver and tumors from mice initiated with MNU and treated with TCA. The percentage of CpG sites that were methylated was reduced from 79.3 to 58% in liver and further reduced to 10.7% in tumors promoted by TCA.

An association between hypomethylation and cell proliferation in liver of TCA-treated mice was demonstrated by Ge et al. (2001b). Increase in DNA replication (evidenced by increased PCNA labeling index and mitotic labeling index) was observed 72 hours and 96 hours after the first gavage daily dose of 500 mg/kg 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.

Based on the above experimental results, TCA induced global and locus-specific DNA hypomethylation in mouse liver. Given the recent finding, discussed above, that the DNA hypomethylation by the potent PPARα agonist Wy-14643 was PPARα-dependent (Pogribny et al., 2007), the data on TCA is consistent with a PPARα MOA. However, because hypomethylation is a relatively ubiquitous phenomenon in carcinogenesis and it has not been

demonstrated that TCA-induced hypomethylation is PPARα-dependent, alternative mechanisms cannot be discounted.

There are a number of inconsistencies and data gaps that reduce the confidence in the conclusion that TCA induced hepatocarcinogenesis through a PPARα MOA. First, while TCA induces peroxisome proliferation (a marker for PPARα agonism) in both rats and mice, to date, it has only 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). Another finding that may explain liver tumors only occurring in mice but not in rats is that hepatocyte proliferation only occurred in TCA-treated mice (DeAngelo et al., 2008) but not in treated rats (DeAngelo et al., 1997). Since cell proliferation is a critical event in tumorigenesis under the PPARα agonism MOA, this may be the main reason that tumors were not found in exposed rats.

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) report 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.

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 (CPF) (Hegi et al., 1993) and methylclofenapate (MCP) (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 MCP; 8/39 versus 32/50 for CPF) and their mutation spectrums differed from those of spontaneous tumors. The lower frequency and distinct pattern of H-*ras* mutation observed in MCP and CPF would suggest 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 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 rare mutation in 1/23 CPF- induced and one MCP-induced hepatocarcinoma (the number of samples examined was not provided), suggesting that such 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 (less than 10% of tumors), so their reliability as indicators of MOA is likely to be low.

With respect to tumor phenotype, 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. 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.

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-acidophilic (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.

Experimental support for these three altered hepatocyte lineages is available. Kraupp-Grasl et al. (1991, 1990) noted a difference in the ability of a peroxisome proliferator to promote tigroid foci 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 (NAF) as promoters, only NAF and not PB promoted the weakly basophilic foci. In addition, a substantial number of spontaneous foci (the number of which were actually decreased by NAF) 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 not induced by peroxisome proliferators, to basophilic neoplasms (Bannasch, 1996), basophilic tumors themselves are nonspecific to peroxisome proliferators.

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 partial 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 NAF, 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, it is questionable to use immunostaining characteristic for c-Jun as an indicator for the PPARα MOA.

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. However, the available data are insufficient to discern whether the PPARα MOA is a sole causative factor for TCA hepatocarcinogenesis. Studies on PPARα published since NRC (2006) indicate that the TCA mechanism of action 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[8]. 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 may be involved in TCA-induced tumors in mice.

4.7.3.1.1.3. *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 CACT 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

[8] 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.

lauric acid were observed in male B6C3F$_1$ mice treated with 0.1 to 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, the dose levels that were carcinogenic, providing support that PPARα agonism is related to tumor formation. However, as stated above, 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). 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 0.05g/L TCA. 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.

4.7.3.1.1.4. *Human relevance*. In its framework for making conclusions about human relevance, the EPA cancer guidelines (U.S. EPA, 2005a) 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; (3) considering all populations and life stages, including special attention to whether tumors can arise from childhood exposure.

With respect to the first element, there is no evidence for *qualitative* differences between rodents and humans in the key events described above for the proposed PPARα MOA. 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." The human and mouse forms of PPARα are comparable in their affinity for TCA, as shown in vitro by Maloney and Waxman (1999). Therefore, the PPARα MOA described above should be relevant to humans.

With respect to the second question, the limited available data suggest 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.

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. However, none of these effects 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 in vitro to in vivo of results from these systems is available. Moreover, these assay systems remove the NPCs (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., 2000). 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 CPF 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 mice fed Wy-14643 suggested that structural differences in human and mouse PPARα receptors may be more critical. A PPARα-humanized 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 PPARα-humanized mice were fed the prototype peroxisome proliferator Wy-14643 or lipid-lowering drug fenofibrate. Decreased serum triglycerides were observed in both the wild-type and PPARα-humanized (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-14643 or fenofibrate feeding. Hepatomegaly and increases in hepatocyte

size were observed in mice fed Wy-14643 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-14643 feeding.

Cheung et al. (2004) also evaluated peroxisome-proliferator-induced RDS by measuring BrdU incorporation into hepatocyte nuclei in hPPARα mice and wild-type mice after 8 weeks of feeding with Wy-14643. In wild-type mouse livers, Wy-14643 treatment resulted in a BrdU labeling index of 57.9% compared with 1.6% in untreated controls. However, in hPPARα mice, Wy-14643 treatment did not increase the incorporation of BrdU with average labeling indices of 2.8 and 1.6% in Wy-14643-treated and control mice, respectively. In addition, Wy-14643 treatment resulted in a marked induction in the expression of various genes involved in cell cycle control (PCNA, c-myc, CDK1, and CDK4 and cyclins A2, D1, and E) in the livers of wild-type mice. However, the expression of these genes was unchanged with Wy-14643 treatment in hPPARα mice. 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-14643 feeding. Therefore, whereas human PPARα in mice regulates induction of fatty acid catabolism and lipid lowering, it does not stimulate the adverse cell proliferative response that is thought to contribute to liver carcinogenesis. In addition, as discussed above, Shah et al. (2007) reported that microRNA let-7C was not suppressed in Wy-14643-treated hPPARα mice. Wy-14643 treatment of hPPARα mice also did not induce *c-myc* and mir-17 expression.

Decreased susceptibility of hPPARα mice to Wy-14643-induced liver tumorigenesis was shown by Morimura et al. (2006). When the feeding study of 0.1% Wy-14643 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 7 mice; 3/10 treated mice died of toxicity). However, only 5% of Wy-14643-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-14643-treated wild-type mice. The c-myc mRNA was also significantly overexpressed in the Wy-14643-treated wild-type mice. On the other hand, expression of the tumor suppressor gene, p53, was increased only in the livers of Wy-14643-treated hPPARα mice. Morimura et al. (2006) concluded that structural differences between human and mouse PPARα were responsible for the differential susceptibility to the peroxisome-proliferator-induced hepatocarcinogenesis.

These data in hPPARα mice are consistent with toxicodynamic differences between humans and mice and are due to structural differences between human and mouse PPARα. However, it should be noted that only Wy-14643 has been tested in hPPARα mice for

carcinogenicity so far, and the duration of treatment was less than 1 year, so more studies need to be conducted, especially with TCA, before definitive conclusions can be made regarding human relevance using hPPARα mice.

As discussed previously, toxicokinetic differences also exist between human and mouse. 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, consistent with less susceptibility of humans than mice to TCA-induced liver tumors.

With respect to the final question, little data on population variability and life-stages, 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 made conclusions as to the human relevance of the PPARα-agonist-induced hepatocarcinogenesis, both in general and with respect to specific chemicals. The recent 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 "Induction 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, 2005a), 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, 2005a), 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.

Another recent report is the Science Advisory Board's review of EPA's draft risk assessment of potential human health effects associated with perfluorooctanoic acid (PFOA) and its salts (U.S. EPA, 2006c). The Science Advisory Board 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 PFOA 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-14643. There are no data available on the effects of peroxisome proliferators on human Kupffer cells." These conclusions are similar to those above for TCA.

4.7.3.1.1.5. ***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, there is substantial uncertainty and inconsistency with this proposed MOA. Thus, the current data do not rule out the possibility that TCA could induce cancer in humans by an 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) to be causally related to carcinogenesis are biologically plausible in humans, so this MOA would be considered relevant to humans. On the other hand, toxicokinetic and toxicodynamic differences between species exist in the responses to PPARα agonists and specifically to TCA, although the available data on such differences are not suitable for use in dose-response analysis of TCA hepatocarcinogenic risk. While tremendous progress has been made on the knowledge of the PPARα MOA, 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.

4.7.3.1.2. ***Decreased intercellular communication.*** Inhibition of intercellular communication has been attributed to tumor induction by some peroxisome proliferators (Klaunig et al., 2003, 1988). However, similar inhibition has been reported with nongenotoxic liver carcinogens that are not peroxisome proliferators. Thus, this proposed MOA is not specific to peroxisome proliferators and PPARα agonism. This MOA is not well characterized.

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.

4.7.3.1.3. ***Altered cell proliferation.*** TCA-induced changes in cell growth regulation have also been suggested as a mechanism for the formation of liver tumors. As discussed previously, TCA-induced cell proliferation may be PPARα dependent, since centrilobular hepatocyte hypertrophy (cell proliferation itself was not measured) was observed only in the livers of wild-type mice treated with up to 2.0 g/L TCA in drinking water for 7 days but not in PPARα-null

mice treated with the same dose of TCA (Laughter et al., 2004). The discussion here evaluates other possible pathways.

There is little evidence that hepatocyte cytotoxicity followed by regenerative hyperplasia is associated with TCA exposure. As described above for noncarcinogenic liver effects of TCA, increased liver weight has been consistently reported as a low-dose effect in numerous studies, but liver necrosis is generally either not reported or occurs only at higher doses (Parrish et al., 1996; Pereira, 1996; Acharya et al., 1995; Dees and Travis, 1994).

In vitro studies also support the conclusion that TCA does not induce tumors through cell growth secondary to necrosis, because TCA does not appear to be highly toxic to hepatocytes. Pravacek et al. (1996) evaluated the hepatotoxicity of DCA and TCA in liver slices from male $B6C3F_1$ mice and the metabolic capacity of the liver for these two compounds. In the cytotoxicity studies, the liver slices were exposed for up to 8 hours at concentrations of TCA ranging from 0 to 86 mM. Cytotoxicity was dependent on the duration of exposure, with a greater effect observed at 8 hours than at 3 or 6 hours. Estimated EC_{50} values were reported for each of four measures of cytotoxicity, including potassium leakage, LDH, AST, and ALT activities in the medium. Estimated EC_{50} values ranged from 64 to 72 mM for potassium leakage, LDH activity, and AST activity, while no dose response was observed for ALT activity. In another in vitro study using hepatocyte suspensions from male $B6C3F_1$ mice and Sprague-Dawley rats, the possible role of cytotoxic effects in contributing to TCA-induced hepatocarcinogenicity was evaluated (Bruschi and Bull, 1993). Cytotoxicity was measured by the release of LDH and by trypan blue exclusion in the exposed cells, as well as by depletion of intracellular reduced GSH. No effects were seen in TCA-treated cells at concentrations up to 5.0 mM and exposure times up to 240 minutes, suggesting that little cytotoxicity occurs from exposure to TCA as measured by the biomarkers employed. Thus, the in vitro results suggest that TCA is not highly cytotoxic to hepatocytes.

Rather than regenerative hyperplasia, differential effects on growth of normal and initiated cells have been suggested as an alternative MOA of TCA, although the underlying mechanism is unclear, and may involve PPARα. Bull (2000) suggested that TCA acts by increasing the clonal expansion of initiated cells while decreasing growth of normal cells. Data from Stauber and Bull (1997) were cited as evidence for this MOA. In this experiment, mice were exposed to a high concentration of TCA for 50 weeks and then removed from treatment or continued at the same exposure for an additional 2 weeks. Evaluation of cell proliferation found that the growth of TCA-initiated tumor cells was high and similar levels were seen in mice taken off TCA treatment and in animals maintained on TCA for the entire experiment. By contrast, replication was inhibited in normal hepatocytes. Thus, initiated cells would have a growth advantage over growth-inhibited normal cells following continuous treatment.

Bull (2000) argued that TCA might not only inhibit growth of normal cells but may also enhance growth of initiated cells with certain phenotypes, based on the results of Stauber et al. (1998). Stauber et al. (1998) demonstrated that TCA increases cell proliferation of c-jun negative hepatocytes in vitro. These investigators treated isolated hepatocytes from naïve 5- to 8-week-old mice with TCA at concentrations ranging from 0 to 2.0 mM and plated the cells to allow them to form colonies. Exposure of the cells to 0.5 mM TCA and above significantly increased colony formation in the absence of cytotoxicity as compared with controls. Anchorage-independent colonies were induced by TCA in a dose-dependent manner and were c-jun negative, which is the same phenotype observed in TCA-induced liver tumors in mice exposed in vivo to TCA. The expression of c-jun was not induced when isolated hepatocytes were cultured as monolayers in the presence of 2.0 mM TCA, indicating that TCA selectively affects subpopulations of anchorage-independent hepatocytes. The authors concluded that the results of this study demonstrated that TCA promotes the survival and growth of different populations of initiated hepatocytes. The ability of TCA to act as a tumor promoter (Latendresse and Pereira, 1997; Pereira and Phelps, 1996; Parnell et al., 1988) supports the selective growth MOA described in Bull (2000).

4.7.3.1.4. ***Genotoxicity.*** 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 can 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. No tumors were found in TCA treated rats.

In summary, there is some evidence that TCA is weakly mutagenic; however, the overall evidence for the MOAs for carcinogenicity is inconclusive.

4.7.3.2. *Conclusions About the Hypothesized Mode of Action*

In summary, TCA is clearly carcinogenic in mice (Bull et al., 2004, 2002, 1990; Bull, 2000; Pereira, 1996). Numerous recent studies have investigated the mechanism by which TCA induces liver tumors. The data do not support a mutagenic mechanism (Bull, 2000; Moore and Harrington-Brock, 2000). Rather, tumor induction appears to involve perturbation of cell growth and/or reduced intercellular communication (Benane et al., 1996). There is support for the involvement of PPARα; however, uncertainties remain if PPARα agonism is the sole carcinogenic MOA of TCA in mice.

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 4-7) 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, a LOAEL and NOAEL 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 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 the 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 DMSO 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

No data directly relevant to the evaluation of the effects of gender on TCA toxicity in humans were located. 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 and female $B6C3F_1$ mice were exposed to TCA in the drinking water at concentrations that resulted in doses of up to approximately 329 mg/kg-day for 52 weeks. A clear dose-related increase in animals with proliferative lesions (hyperplastic nodules, adenomas, or carcinomas) was observed in males (incidence of up to 19/24, which occurred at 329 mg/kg-day). In contrast, the incidence of proliferative lesions in females was not increased (data not reported). 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 to 480 mg/kg-day) in the

drinking water for a comparable duration (Bull, 2000; Bull et al., 1990). These data indicate that TCA is a more potent carcinogen in male than in female mice.

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 Factors Influencing Susceptibility

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 (Bull, 2000; Lash et al., 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[9] for TCA was derived through a three-step process: (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[10] (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—with 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, 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 mice 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).

[9]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, 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.

[10]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-day)	Observed effects	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Comments
Chronic studies								
DeAngelo et al. (1997)	F344 rats (males, 50/group)	Oral, drinking water	104 weeks	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 weeks	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 weeks	0, 78, 262, or 784	Increased relative liver weight	78	262	Increased liver weight was observed after 82 weeks at 262 mg/kg-day; 262 mg/kg-day was judged to be an equivocal LOAEL in the absence of other measures of liver toxicity.
Bull et al. (1990)	B6C3F$_1$ mice (11–24/sex and dose)	Oral, drinking water	(A) 52 weeks (B) 37 weeks + 15-week recovery	(A) 0, 164, or 329 (B) 0 or 309	Increased absolute and relative liver weight, cytomegaly, glycogen accumulation	None	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 weeks	0, 500, or 1,250	Increased absolute and relative liver weight	None	500	Only the liver was microscopically examined.
Subchronic studies								
Mather et al. (1990)	Sprague-Dawley rats (males, 10/dose)	Oral, drinking water	90 days	0, 4.1, 36.5, or 355	Decreased absolute spleen weight; increased relative liver and kidney weights; peroxisome proliferation	36.5	355	

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

Reference	Species	Exposure route	Exposure duration	Doses evaluated (mg/kg-day)	Observed effects	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Comments
Bhat et al. (1991)	Sprague-Dawley rats (males, 5/group)	Oral, drinking water	90 days	0 or 825	Decreased body weight gain; minor changes in liver morphology; collagen deposition; perivascular inflammation of the lungs	None	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; increased maternal spleen and kidney weights	Maternal: None Develop-mental: None	Maternal: 330 Develop-mental: 330	Critical study for 1994 RfD. The developmental LOAEL was also a maternal LOAEL. Values of 28 and 31 mg/kg-day for the 95% lower bound of the effective dose at 10% extra risk (LED_{10}) were obtained for reduced fetal body weight and litter incidence of levocardia, respectively, by benchmark dose modeling. (See Tables 5-3 and 5-4.)
Johnson et al. (1998)	Sprague-Dawley rats (55 controls and 11 TCA-treated rats)	Oral, drinking water	GDs 1–22	0 or 291	Maternal: Toxicologically significant decrease in maternal 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 end points was not assessed.

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

Reference	Species	Exposure route	Exposure duration	Doses evaluated (mg/kg-day)	Observed effects	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	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: None Develop-mental: None	Maternal: 300 Develop-mental: 300	A limited number of fetal endpoints were evaluated, including sex, fetal weight, and incidence of heart malformations.

Source: Adapted in part from U.S. EPA (2003). Additional details on these studies are provided in Section 4 of this document.

Subchronic toxicity data were available from studies conducted in rats by Mather et al. (1990) and Bhat et al. (1991). The 90-day drinking water study by Mather et al. (1990) established NOAEL and LOAEL values of 36.5 and 355 mg/kg-day 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. (1991) 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. These studies were considered for use in the derivation of an oral RfD. Although both Smith et al. (1989) and Johnson et al. (1998) observed increased incidences of cardiac defects following treatment of pregnant rats with TCA, Fisher et al. (2001) observed no significant increase in cardiac anomalies, despite using a sensitive staining technique for analysis of fetal cardiac tissues.

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 from the developmental toxicity study conducted by Smith et al. (1989) were analyzed by BMD modeling for comparison with the POD for liver effects (DeAngelo et al., 2008) selected for derivation of the RfD. The developmental data analyzed were incidence data for fetuses with visceral malformations (of which levocardia was the principal lesion), data on fetal body weight and fetal crown-rump length, and litter incidence data for levocardia.

5.1.2. Methods of Analysis

5.1.2.1. *Benchmark Dose 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 Tables 4-3 and 4-4. All of the available dichotomous models in U.S. EPA's BMDS (version 1.4.1) were fit to these incidence data. Doses (i.e., benchmark dose [BMD_{10}] and 95% lower confidence limit on the benchmark dose [$BMDL_{10}$]) associated with a benchmark response (BMR) of 10% extra risk were calculated and are presented in Tables 5-2 through 5-4. 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.

Details of the BMD modeling conducted for each endpoint presented in Tables 5-2 through 5-4 are provided in Appendix B. In general, model fit was assessed by a chi-square 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 model yielding the lowest AIC value was selected as the best-fit model. If more than one model shared the lowest AIC, $BMDL_{10}$ values from these models were averaged to obtain a POD (U.S. EPA, 2000b).

For hepatocellular inflammation, Table 5-2 shows that 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 $BMDL_{10}$s from these two models were averaged to yield a candidate POD of 260.5 mg/kg-day. As shown in Table 5-3, 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 adequate fit models. Thus, the $BMDL_{10}$ of 18 mg/kg-day estimated by the log-logistic model was selected as a candidate POD. Finally, as shown in Table 5-4, 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. Of the three endpoints under consideration, hepatocellular necrosis was the

most sensitive, as it yielded the lowest POD of 18 mg/kg-day. Therefore, 18 mg/kg-day was selected as the POD for use in derivation of the RfD.

Table 5-2. BMD modeling results based on incidence of hepatocellular inflammation in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks (DeAngelo et al., 2008)

Fitted Dichotomous Model[a]	Chi-Square Goodness-of-Fit Test *p*-Value[b]	AIC[c]	BMD_{10}[d] (mg/kg-day)	$BMDL_{10}$[e] (mg/kg-day)
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 chi-square 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 = Akaike's Information Criterion, 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} = Benchmark dose at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the benchmark dose at 10% extra risk.

Table 5-3. BMD modeling results based on incidence of hepatocellular necrosis in male B6C3F$_1$ mice exposed to TCA in drinking water for 30 to 45 weeks (DeAngelo et al., 2008)

Fitted Dichotomous Model[a]	Chi-Square Goodness-of-Fit Test *p*-Value[b]	AIC[c]	BMD_{10}[d] (mg/kg-day)	$BMDL_{10}$[e] (mg/kg-day)
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 model is indicated in boldface type.
[b]*p*-Value from the chi-square 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 = Akaike's Information Criterion, 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} = Benchmark dose at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the benchmark dose at 10% extra risk.

Table 5-4. BMD modeling results based on incidence of testicular tubular degeneration in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks (DeAngelo et al., 2008)

Fitted Dichotomous Model[a]	Chi-Square Goodness-of-Fit Test *p*-Value[b]	AIC[c]	BMD_{10}[d] (mg/kg-day)	$BMDL_{10}$[e] (mg/kg-day)
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 model is indicated in boldface type.
[b]*p*-Value from the chi-square 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 = Akaike's Information Criterion, 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} = Benchmark dose at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the benchmark dose at 10% extra risk.

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

Selected data from the developmental toxicity study conducted by Smith et al. (1989) (Table 5-5) were analyzed by BMD modeling for comparison with the POD derived from DeAngelo et al. (2008). Nested developmental toxicity models were employed in order to account for interindividual correlation of toxicity endpoints within litters. Supporting information for the BMD analyses is provided in Appendix C. The fetal data analyzed were quantal incidence data for fetuses with visceral malformations (of which levocardia was the principal lesion) and continuous data for fetal body weight and fetal crown-rump length. These endpoints were selected based on the availability of individual animal data, which is required for the nested analysis used to account for interindividual correlation within litters. To facilitate comparison of BMDs across endpoints, individual data for fetal body weight and crown-rump length were converted into quantal form, as discussed in the next paragraph.

The continuous data were converted into quantal form (i.e., incidence of the number of responders per number of members in a group) for analysis. Conversion involved an assumption that the data (either body weight or crown-rump length) were normally distributed and the use of the estimated distribution of the controls to define a response. Responders were defined as displaying a measured value $\leq$ a critical value = the overall control mean $-z_\alpha \times$ SD, where z_α is the percentage point of the standard normal distribution at a probability level of α (conversions were calculated with $\alpha = 0.05$; for large numbers of samples, z_α is approximately equal to 1.645), and SD is the standard deviation of the mean of the control group.

Table 5-5. Dose response data for developmental endpoints in TCA-treated Long-Evans rats (Smith et al., 1989)

Endpoint	Dose (mg/kg-day)				
	0	330	800	1,200	1,800
Quantal data					
Fetuses with visceral malformations					
Fetal incidence[a,b]	6/176	14/140	27/111	29/65	19/20
Litter incidence[b,c]	4/26	8/19	15/17	11/14	8/8
Mean % fetuses affected per litter[c]	3.50 ± 8.7	9.06 ± 12.9[d]	30.4 ± 28.1	55.4 ± 36.1[d]	96.98 ± 8.8[d]
Fetuses with cardiovascular malformations					
Fetal incidence	NR[e]	NR	NR	NR	NR
Litter incidence	NR	NR	NR	NR	NR
Mean % fetuses affected per litter[f]	0.96 ± 4.9	5.44 ± 10.0[d]	23.6 ± 28.0[d]	46.8 ± 36.5[d]	94.8 ± 9.9[d]
Fetuses with levocardia					
Fetal incidence[f]	0/196	9/151	20/111	24/69	17/22
Litter incidence[f]	0/26	6/19	12/17	10/14	7/8
Mean % fetuses affected per litter	NR	NR	NR	NR	NR
Continuous data					
Mean fetal crown-rump length in cm[g]					
Male	3.71 ± 0.12	3.58 ± 0.10[d]	3.46 ± 0.10[d]	3.36 ± 0.15[d]	3.16 ± 0.12[d]
Female	3.64 ± 0.15	3.53 ± 0.09[d]	3.38 ± 0.12[d]	3.33 ± 0.16[d]	3.15 ± 0.15[d]
Mean fetal body weight in g[g]					
Male	3.70 ± 0.24	3.20 ± 0.26[d]	2.98 ± 0.17[d]	2.74 ± 0.30[d]	2.49 ± 0.16[d]
Female	3.54 ± 0.20	3.08 ± 0.27[d]	2.83 ± 0.18[d]	2.67 ± 0.29[d]	2.36 ± 0.15[d]

[a]Fetal incidence = number of fetuses affected/number of fetuses examined.
[b]Unpublished data provided to Dr. R. Kavlock, EPA, by Dr. K. Smith.
[c]Litter incidence = number of litters with ≤1 affected fetus/number of litters examined.
[d]Mean is significantly different from control mean ($p \leq 0.05$) as reported by Smith et al. (1989).
[e]NR = not reported or able to be calculated from available sources.
[f]From Tables 5 or 6, Smith et al. (1989).
[g]From Table 4, Smith et al. (1989).

This conversion method assumes that the control group has a 5% background response rate (i.e., 5% of individuals in the control population have body weight or crown-rump length below the critical value). SDs used in this method were derived from all fetal body weights or crown-rump lengths in the control group without regard to litter. These estimates, therefore, contain both between-litter and within-litter variations. The control group mean body weight was 3.64 g (SD = 0.287; n = 284); the calculated critical value for $\alpha = 0.05$ was 3.16 g. The control group mean crown-rump length was 3.7 cm (SD = 0.163; n = 282); the calculated critical value for $\alpha = 0.05$ was 3.4 cm. Thus, for the two continuous variable endpoints, the quantilization process classified each fetus in each litter as either a responder (e.g., body weight ≤3.16 g or crown-rump length ≤3.4 cm) or a nonresponder (body weight value >3.16 g or crown-rump length >3.4 cm).

Three nested models, each of which included dose and litter size as explanatory variables and accounted for intralitter correlation by assuming a β-binomial distribution for individual fetal responses (see eq. 5-1), were used to model each data set. The models were as follows: (1) a log-logistic model as described by Kupper et al. (1986); (2) the model described by Rai and van Ryzin (1985); and (3) the modified model described by Kodell et al. (1991). Computer programs (TERALOG, TERAVAN, and TERAMOD) (developed based on the three papers cited above by Richard Howe from ICF Kaiser International, 1208 Gaines Street, Ruston, LA, 71270)[11] were used to fit these models by maximum likelihood methods to the Smith et al. (1989) data sets. The following equations represent the models (d = dose; s = litter size; d_0 = threshold dose set to zero for these data sets; α = background response parameter; β = dose rate parameter; Θ_1, Θ_2 = litter size parameters):

$$\text{TERALOG: } P(d,s) = \alpha + \Theta_1 \times s + \{1 - \alpha - \Theta_1 \times s\} / \{1 + \exp[\beta + \Theta_2 \times s - \gamma \log (d-d_0)]\},$$
where $0 \leq \alpha + \Theta_1 \times s \leq 1$ and γ = log dose coefficient, restricted to ≤ 1

$$\text{TERAVAN: } P(d,s) = \{1 - \exp[-\alpha - \beta(d-d_0)^{\gamma}]\} \times \exp\{- s [\Theta_1 + \Theta_2(d-d_0)]\},$$
where γ = Weibull power parameter, restricted to $1 \leq \gamma \leq 18$

$$\text{TERAMOD: } P(d,s) = 1 - \exp\{ - [\alpha + \Theta_1 \times s + (\beta + \Theta_2 \times s)(d-d_0)^{\gamma}]\},$$
where γ = Weibull power parameter, restricted to $1 \leq \gamma \leq 18$ (5-1)

The data were modeled by using BMRs of 5 and 10% extra risk. The results obtained by applying the above three models to the data sets for fetal body weight, fetal visceral malformations, and fetal crown-rump length are summarized in Table 5-5. Within each of the data sets, all model fits were comparable as judged by the chi-square goodness-of-fit test and log-likelihood values (see Appendix C).

The modeling results for fetal data (Table 5-6) suggest that fetal body weight was the most sensitive endpoint among those examined in the Smith et al. (1989) study. Quantal responses for body weight decrease are estimated to occur at lower doses than those producing equivalent responses for increased visceral malformations or crown-rump length decrease. For example, using the $\alpha_{0.05}$ critical values of 3.16 g and 3.4 cm to define response of body weight and crown-rump length, the BMD_{05} values for increased incidence of fetuses with decreased body weight were 72, 25, and 23 mg/kg-day for the three models, respectively, compared with BMD_{05} values of 399, 369, and 320 mg/kg-day for increased visceral malformations and 391, 375, and 345 mg/kg-day for increased incidence of fetuses with decreased crown-rump length (Table 5-6). Corresponding $BMDL_{05}$ values were 41, 21, and 21 mg/kg-day for decreased body weight compared with 220, 218, and 212 mg/kg-day for visceral malformations and 278, 272,

[11] These programs are essentially equivalent to the nested logistic, Rai and van Ryzin (1985), and National Center for Toxicological Research models, respectively, included in BMDS (version 1.3.1) (U.S. EPA, 2000b).

and 241 mg/kg-day for crown-rump length ≤3.4 cm. The average BMD_{05} and $BMDL_{05}$ (calculated from the values obtained by using each of the three models) for fetal body weight were 40 and 28 mg/kg-day, respectively. It should be noted that these values are well below the lowest tested dose of 330 mg/kg-day. The use of the $BMDL_{05}$ for decreased fetal body weight as a potential POD for the RfD is discussed in Section 5.1.3.

Table 5-6. BMD modeling results for fetal incidence data (Smith et al., 1989)

Model	BMD_{05}[a] (mg/kg-day)	$BMDL_{05}$[b] (mg/kg-day)	BMD_{10}[a] (mg/kg-day)	$BMDL_{10}$[b] (mg/kg/day)
Fetal body weight				
TERALOG	72	41	107	67
TERAMOD	25	21	50	42
TERAVAN	23	21	48	43
Fetal crown-rump length				
TERALOG	391	278	530	417
TERAMOD	375	272	525	420
TERAVAN	345	241	510	439
Fetal visceral malformations				
TERALOG	399	220	537	352
TERAMOD	369	218	518	358
TERAVAN	320	212	485	397

Note: Continuous data (body weight and crown-rump length) were converted to quantal data before modeling, as discussed in text.
[a]BMD_{05}, BMD_{10} = maximum likelihood estimates of dose associated with 5% or 10% extra risk of fetuses with decreased body weight, decreased crown-rump length, or visceral malformations.
[b]$BMDL_{05}$, $BMDL_{10}$ = 95% lower confidence limits for the respective BMD_{05} or BMD_{10} values.

Litter incidence data (number of affected litters/number of litters examined) for levocardia (Table 5-7) were modeled using U.S. EPA's BMDS (version 1.3.1) in accordance with U.S. EPA (2000d) recommendations. The data were analyzed using dichotomous models (gamma, logistic and log-logistic, probit and log-probit, multistage, and Weibull) in the BMDS program. Use of nested models was not required because the data analyzed were reported on a per litter basis, and thus no adjustment was required for intralitter correlation. Note, however, that the extent of levocardia within each litter is not captured in this incidence measure. The BMD and BMDL values were calculated based on BMRs of 5 and 10% extra risk that a litter would have at least one fetus affected with levocardia. Confidence bounds calculated by BMDS used a maximum likelihood profile method. Output from the BMDS program was evaluated by using the criteria described in U.S. EPA (2000d).

The best fits to the data were obtained with the multistage and gamma models (Table 5-7), as judged by AIC. The results from these models were identical (as were the forms of the models based on the data input). Figure 5-1 plots predicted (from the fitted gamma

model) and observed incidence of levocardia as a function of administered dose, as well as the BMD_{05} and the $BMDL_{05}$. The BMD_{05} and $BMDL_{05}$ values estimated for the litter incidence of levocardia by these models were 42 and 31 mg/kg-day (rounded values), respectively. It should be noted that these values are well below the lowest tested dose. The use of the $BMDL_{05}$ for increased incidence of litters with levocardia as a potential POD for the RfD is discussed in Section 5.1.3.

Table 5-7. BMD modeling results for litter incidence of levocardia (Smith et al., 1989)

Model	Goodness-of-fit *p* value	AIC	BMD_{05}	$BMDL_{05}$	BMD_{10}	$BMDL_{10}$
Multistage	0.9430	69.8459	42	31[a]	86	64[a]
Gamma	0.9430	69.8459	42	31[a]	86	64[a]
Log-logistic	0.9106	71.6069	74	17	122	36
Log-probit	0.9069	71.6259	87	9	130	20
Weibull	0.8648	71.8203	36	1	76	5
Logistic	0.0520[b]	80.642	144	101	253	187
Probit	0.0449[b]	80.6568	136	99	244	185

[a]Preferred model(s) based on criteria described in U.S. EPA (2000d).
[b]Because goodness-of-fit *p* values were below the recommended minimum value of 0.1, the results of these models were not further considered for estimation of the BMD.

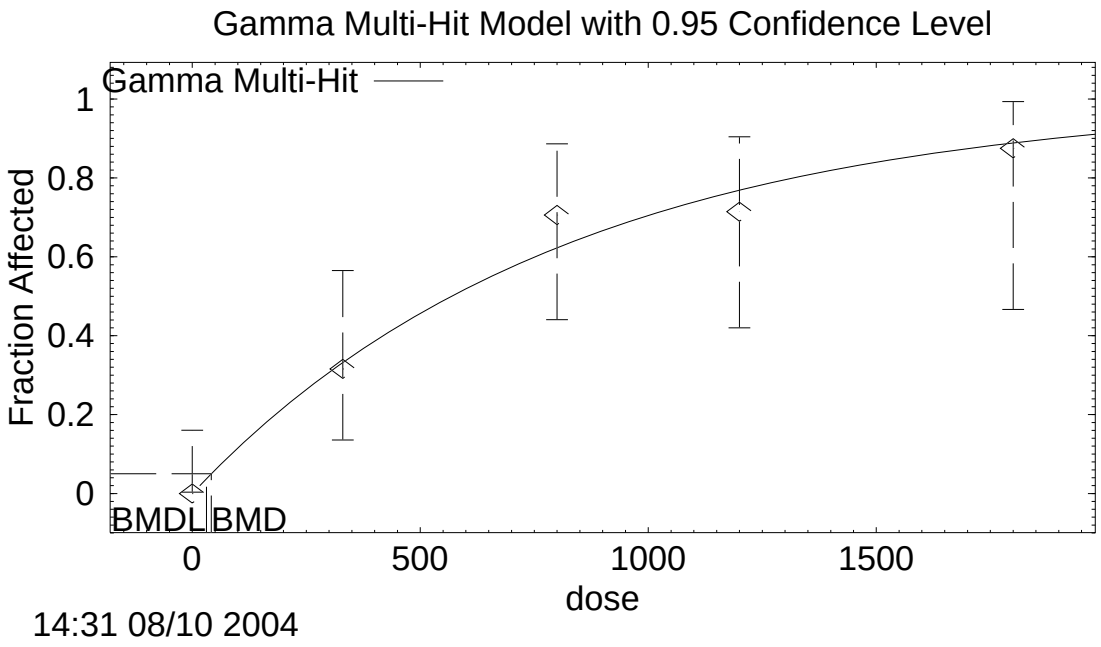

Figure 5-1. Plot of predicted and observed litter incidence of levocardiain offspring of female Long-Evans rats exposed to TCA on GDs 6–15.

Note: The BMD and BMDL are the predicted dose and lower 95% confidence limit associated with a 5% extra risk for litters with at least one fetus with levocardia.

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 is calculated by using the POD based on the incidence of hepatocellular necrosis identified in the principal study (eq. 5-2).

$$\begin{aligned} \text{RfD} &= \text{POD} \div \text{UF} \\ &= 18 \text{ mg/kg-day} \div 1{,}000 \\ &= 0.018 \text{ mg/kg-day, rounded to } 0.02 \text{ mg/kg-day} \end{aligned} \qquad (5\text{-}2)$$

where 18 mg/kg-day = POD for the incidence of hepatocellular necrosis in mice exposed to TCA via drinking water for 30 to 45 weeks (DeAngelo et al., 2008) and 1,000 = composite UF chosen to account for extrapolation from animals to humans, interindividual variability in humans, and insufficiencies in the database (see below).

For developmental endpoints, $BMDL_{05}$ values were used as the POD. Reproductive and developmental studies having nested study designs often have greater sensitivity, and a BMR of 5% has typically been used for such studies (U.S. EPA, 2000b). Use of the $BMDL_{05}$ value for

either reduced fetal body weight (28 mg/kg-day) or litter incidence of levocardia (31 mg/kg-day) (Smith et al., 1989) as an alternative POD and the composite UF of 1,000 would result in an RfD of 0.03 mg/kg-day (i.e., a value 50% higher than the one obtained by using the POD based on hepatocellular necrosis). Because these alternative derivations are based on results extrapolated about an order of magnitude below the observed data, however, they are relatively uncertain compared with the POD derived from the principal study. Thus, the RfD for TCA was derived from the POD for hepatocellular necrosis observed by DeAngelo et al. (2008).

The following UFs were applied in the calculation of the RfD to address extrapolation from animal study conditions to conditions of human environmental exposure: 10 for consideration of intraspecies (human) variability, 10 for extrapolation from an animal study to humans (animal-to-human), and 10 to account for deficiencies in the TCA database. The total UF = 10 × 10 × 10 = 1,000.

The UFs used in the calculation of the RfD were selected for the following reasons:

- *Human variation*. A default UF value of 10 is used to account for human variability and protection of potentially sensitive subpopulations. This value was selected because there are no data on human variability in the toxicokinetics or toxicodynamics of TCA and because information on differences in human susceptibility to TCA as a consequence of age, sex, health, or genetic factors is lacking.
- *Animal-to-human extrapolation*. A default UF of 10 is used to account for extrapolation from an animal study to humans. No suitable data on the toxicity of TCA to humans exposed by the oral route were identified. Insufficient information is currently available to assess rat-to-human differences in TCA toxicokinetics or toxicodynamics.
- *Database insufficiencies*. An UF of 10 is used to account for database insufficiencies. 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. Other data gaps include lack of a multigeneration reproductive toxicity study.
- *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 adjustment was not required in this assessment because the current approach is to address this extrapolation as one of the considerations in selecting a BMR for BMD modeling. In this case, a BMR corresponding to a 10% increase in the incidence of hepatocellular necrosis was selected under the assumption that it represents a minimally biologically significant change.

5.1.4. RfD Comparison Information

The RfD derived from the DeAngelo et al. (2008) mouse study was compared with potential RfDs derived from the DeAngelo et al. (1997) rat study and the Smith et al. (1989) rat study. The RfDs derived from these studies are similar (Figure 5-2).

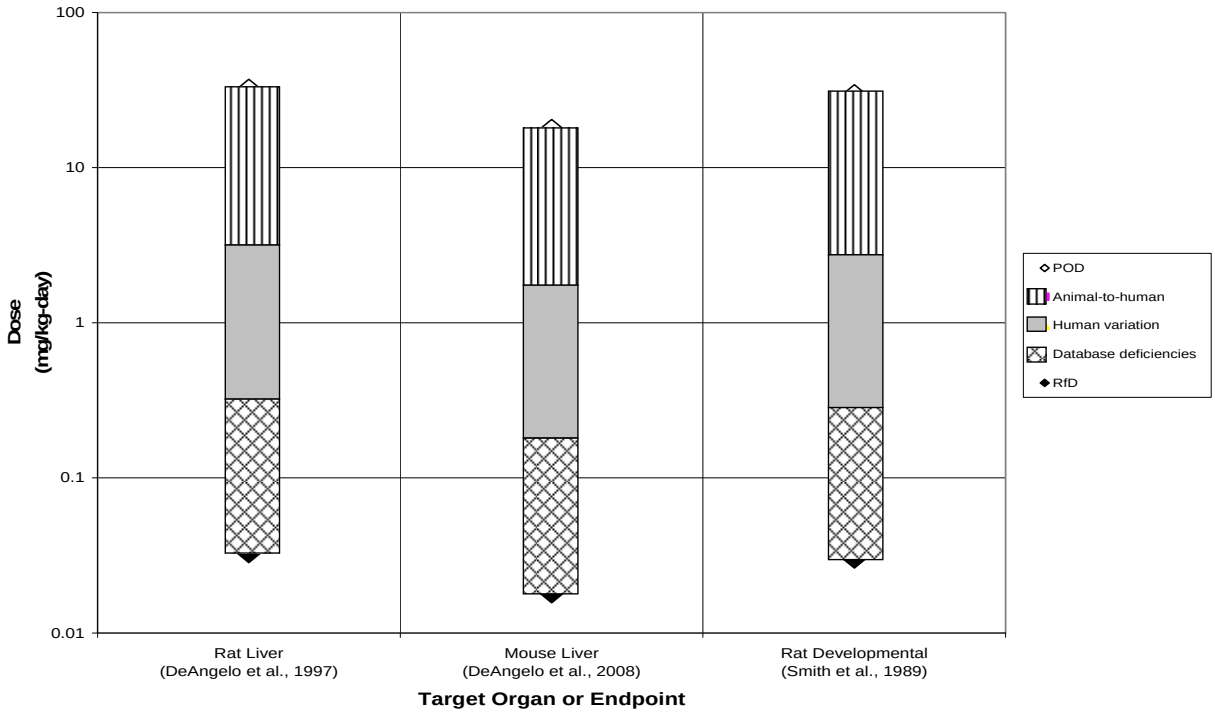

Figure 5-2. Comparison of RfDs across target organs or endpoints.

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[12] were located. The available information was inadequate for a route-to-route extrapolation from the oral pathway to the inhalation pathway. Physiologically based toxicokinetic models, which might be useful for route-to-route extrapolation, have not been developed for TCA.

[12]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 ORAL REFERENCE DOSE

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 paucity of 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 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. Liver toxicity, specifically hepatocellular necrosis, was selected as the critical effect for the RfD. Liver toxicity was the most consistent and sensitive effect in rats and mice. Thus, there is little uncertainty that this effect is relevant to humans.

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 a 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. Uncertainty related to human variation needs consideration, also, in extrapolation from a small subset of presumably healthy humans to a larger, more diverse population. 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). A 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 of both rats and mice. In mice, the results of these studies provide evidence that TCA is a complete carcinogen, as exposure to TCA in drinking water for periods of from 52 to 104 weeks significantly increased the incidence of liver tumors in male and female $B6C3F_1$ mice (Bull et al., 2002, 1990; Pereira, 1996; Pereira and Phelps, 1996; DeAngelo et al., 2008; Herren-Freund et al., 1987). In several of these studies, a clear monotonic dose-response relationship was evident (Pereira, 1996; Bull et al., 2002, 1990; DeAngelo et al., 2008). 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 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 (Pereira et al., 2001, 1997) 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.

As discussed in Section 4.7.3, data from recent TCA studies that have investigated the MOA for hepatocarcinogenesis do not support a direct mutagenic mechanism. Instead, tumor induction appears to result from perturbation of cell growth and/or reduced intracellular communication, possibly through a PPARα MOA. Considerable debate currently exists about the mechanism by which peroxisome proliferators cause liver tumors in rodents, and whether these chemicals represent a human cancer risk (NRC, 2006). Much of the experimental data on TCA is consistent with a PPARα-mediated MOA (NRC, 2006). In this document, two alternative interpretations of available data were considered in order to evaluate current scientific uncertainties with respect to dose-response assessment and peroxisome proliferator liver tumor induction.

The first possible interpretation is that the MOA or MOAs for TCA-induced liver tumors are unknown. Data suggest a number of potentially interrelated MOAs. While PPARα-mediated

effects appear to play a role in the induction of some rodent liver tumors, certain inconsistencies in the data exist. Unresolved issues for PPARα as a MOA for TCA-induced liver tumors include: inconsistencies in experimental results across species, sex, and PPARα agonists; some proposed key events are not specific to PPARα; the lack of clear dose concordance between proposed key events and tumor response; PPARα activation by itself was insufficient to induce liver tumors (Yang et al., 2007); and PPARα activation was not necessary for tumor induction by DEHP (Ito et al., 2007). While progress has been made recently in filling gaps in the understanding of this potential MOA, further studies, especially with TCA, are needed. Based on these concerns, it seemed premature to conclude that PPARα is the sole operative MOA for TCA-induced liver tumors. This interpretation would imply a weight of evidence determination that TCA is "likely to be carcinogenic to humans," with subsequent use of the default linearly extrapolated dose-response analysis.

An alternative interpretation proposes that PPARα is the significant MOA in mouse liver tumor induction by TCA, and the determination of human relevance is likely to depend on comparison of cross-species dose-response relationships. Under this interpretation, a weight of evidence determination could be either "likely or unlikely to be carcinogenic to humans" depending on the relative cross-species (mouse to human) differences in toxicokinetic or toxicodynamic sensitivity. Humans have functional PPARα receptors as evidenced by PPARα-mediated responses to the therapeutic fibrate class of drugs. Data from chemicals other than TCA suggest that humans are refractory to some, but not all, PPARα activation effects. Careful consideration should be given to how kinetic and dynamic factors control human vs. animal response. While this assessment has evaluated some of these possible kinetic and dynamic factors, this effort is by no means comprehensive. Further efforts in this regard are outside the scope of this TCA Toxicological Review.

As new data become available, conclusions regarding the MOA for TCA-induced liver tumors may change. For instance, if key events are identified that support a nonlinear dose-response relationships below those doses leading to observed effects, then nonlinear extrapolation could be utilized in the dose-response assessment. If key causal events were identified that were both well correlated with cancer potency and for which cross-species sensitivity were known quantitatively, then the dose-response assessment could be conducted to account for the relative sensitivity between humans and mice to TCA-induced tumors. Finally, if it were shown that one or more key events in TCA-induced tumorigenesis were precluded in humans, then the weight-of-evidence determination would be changed to "not likely to be carcinogenic in humans."

In conclusion, TCA has been determined to be "likely to be carcinogenic to humans" under EPA's *Guidelines for Carcinogen Assessment* (U.S. EPA, 2005a). Three lines of evidence support this determination: 1) TCA is carcinogenic in the liver in multiple studies conducted in

$B6C3F_1$ mice of both sexes; 2) the tumor response was robust, occurring at substantially less-than-lifetime exposures at which tumor rates in control animals were relatively low; and 3) there are data gaps that preclude a decision that the MOA for hepatocarcinogenesis in mice is not relevant to humans. Finally, two significant limitations of the database for TCA carcinogenicity are: 1) limited number of mouse studies that included microscopic evaluation of a comprehensive set of organs in addition to the liver; and 2) the absence of epidemiologic studies of TCA carcinogenicity in humans.

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

5.4.1. Choice of Study/Data—with Rationale and Justification

Using U.S. EPA Benchmark Dose Software (BMDS, version 1.4.1), the multistage model was fit to liver tumor incidence data (i.e., adenomas and carcinomas combined) from bioassays in $B6C3F_1$ mice exposed to TCA in drinking water for 52 weeks (two studies in male mice, Bull et al., 2002, 1990), 60 weeks (one study in male mice, DeAngelo et al., 2008), 82 weeks (one study in female mice, Pereira, 1996), and 104 weeks (one study in male mice, DeAngelo et al., 2008). The tumor incidence data from these studies for adenomas, carcinomas, and adenomas and carcinomas combined are presented in the next section.

These studies in mice cited above 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 allowing for a better characterization of the dose-response relationship, especially at low dose.

5.4.2. Dose-Response Data

The dose-response data (i.e., incidence of hepatocellular adenomas and carcinomas combined and human equivalent lifetime dose) from the five bioassays referenced above are shown in Tables 5-8 through 5-12, and were fit using the multistage model in BMDS (version 1.4.1).

Table 5-8. Incidences of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in male B6C3F$_1$ mice exposed to TCA in drinking water for 52 weeks (Bull et al., 2002)

TCA concentration (g/L)	Estimated daily intake[a] (mg/kg-day)	Human lifetime equivalent dose[b] (mg/kg-day)	Incidence of adenomas	Incidence of carcinomas	Incidence of adenomas or carcinomas[c]
0	0	0	0/20	0/20	0/20
0.5	120	2.38	5/20	3/20	6/20
2	480	9.5	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 text 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.

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 52 weeks (Bull et al., 1990)

TCA concentration[a] (g/L)	Estimated daily intake[b] (mg/kg-day)	Human lifetime equivalent dose[c] (mg/kg-day)	Incidence of adenomas	Incidence of carcinomas	Incidence of adenomas or carcinomas[d]
0	0	0	0/35	0/35	0/35
1	164	3.25	2/11	2/11	4/11
2	329	6.51	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 text 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.

Table 5-10. Incidences of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks (DeAngelo et al., 2008)

TCA concentration (g/L)	Estimated daily intake[a] (mg/kg-day)	Human lifetime equivalent dose[b] (mg/kg-day)	Incidence of adenomas[c]	Incidence of carcinomas[c]	Incidence of adenomas or carcinomas[d]
0	0	0	2/30	2/30	4/30
0.05	8	0.24	4/27	1/27	4/27
0.5	68	2.07	6/29	6/29	11/29
5	602	18.3	11/29	11/29	16/29

[a] Intakes were reported by DeAngelo et al. (2008).
[b] See text for conversion of mouse daily intakes to human equivalent lifetime doses.
[c] Calculated from reported percentages of mice with adenomas or carcinomas.
[d] DeAngelo et al. (2008) reported combined incidences of adenomas or carcinomas for each dose group.

Table 5-11. Incidences of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in female B6C3F$_1$ mice exposed to TCA in drinking water for 82 weeks (Pereira, 1996)

TCA concentration (mmol/L)	Estimated daily intake[a] (mg/kg-day)	Human life-time equivalent dose[b] (mg/kg-day)	Incidence of adenomas	Incidence of carcinomas	Incidence of adenomas or carcinomas[c]
0	0	0	2/90	2/90	4/90
2	78	6.1	4/53	0/53	4/53
6.67	262	20.4	3/27	5/27	8/27
20	784	61.1	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 text 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.

Table 5-12. Incidences of hepatocellular adenomas, carcinomas, or adenomas and carcinomas combined in male B6C3F$_1$ mice exposed to TCA in drinking water for 104 weeks (DeAngelo et al., 2008)

TCA concentration (g/L)	Estimated daily intake[a] (mg/kg-day)	Human lifetime equivalent dose[b] (mg/kg-day)	Incidence of adenomas[c]	Incidence of carcinomas[c]	Incidence of adenomas or carcinomas[d]
0	0	0	9/42	23/42	27/42
0.05	5.6	0.84	8/35	14/35	20/35
0.5	58	8.7	19/37	29/37	32/37

[a] Intakes were reported by DeAngelo et al. (2008).
[b] See text for conversion of mouse daily intakes to human equivalent lifetime doses.
[c] Calculated from reported percentages of mice with adenomas or carcinomas.
[d] DeAngelo et al. (2008) reported combined incidences of adenomas or carcinomas for each dose group.

5.4.3. Dose Conversion

Before fitting the multistage model to the incidence data for adenomas and carcinomas combined in Tables 5-8 through 5-12, estimated daily intakes of TCA from the mouse studies were converted to human equivalent doses for continuous lifetime exposure using an interspecies scaling factor of 0.15 (i.e., [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) and exposure duration scaling factors of 0.132, 0.203, or 0.520 to adjust the 52-, 60-, or 82-week exposure durations, respectively, to equivalent lifetime exposure durations (i.e., [duration of experiment/duration of lifetime]^3 = [52/102]^3 = 0.132, or = [60/102]^3 = 0.203, or [82/102]^3=0.520). These factors for adjusting to lifetime equivalent durations are 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). An exposure duration scaling factor was not used in converting animal doses to human equivalents

in the 104-week study of DeAngelo et al. (2008) (Table 5-12) because 104 weeks represents a lifetime exposure in mice. The human equivalent lifetime doses used in the dose-response modeling are shown in the third column of Tables 5-8 through 5-12.

Individual animal data (specifying when tumors were detected in each animal with a liver tumor) from the five bioassays were not available, precluding application of more sophisticated dose-response modeling approaches to estimating lifetime cancer risks (e.g., by fitting models that predict tumor incidence as a function of two explanatory variables, dose and time, and using these models to predict tumor incidences for lifetime exposure). 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, a one-stage multistage model provided the best fit to the data as determined by the chi-square goodness-of-fit statistic and Akaike's information criterion (AIC), as well as by examining the visual fit of the model to the data. Plots of model predictions compared with observed incidences are shown in Figures D-1, D-2, D-3, D-4, and D-5 in Appendix D.

5.4.4. Extrapolation Methods

Adequacy of fit of the multistage model to each of the data sets was evaluated through use of the chi-square goodness-of-fit statistic (see Table 5-13 for a summary and the BMDS computer outputs in Appendix D for further details). For those models that did not exhibit significant lack of fit (chi-square *p*-value > 0.1), the fitted model was used to estimate the human equivalent lifetime dose associated with 10% extra risk (ED_{10}), and its corresponding 95% lower bound (LED_{10}) (Table 5-13). Candidate oral cancer slope factors were derived by linear extrapolation from the LED_{10}, i.e., $0.1/LED_{10}$. Slopes from the linear extrapolation from the ED_{10} were also calculated, i.e., $0.1/ED_{10}$ (Table 5-13).

Table 5-13. Candidate oral cancer slope factors derived from cancer bioassays in B6C3F$_1$ mice

Study Reference (study duration)	ED_{10} (mg/kg-day)[a]	LED_{10} (mg/kg-day)[a]	χ^2 goodness-of-fit *p*-value	Slope of linear extrapolation from ED_{10}[b] $(mg/kg\text{-}day)^{-1}$	Oral cancer slope factor[c] $(mg/kg\text{-}day)^{-1}$
Male Mice					
Bull et al., 2002 (52 weeks)	1.41	0.93	0.16	7.1×10^{-2}	1.1×10^{-1}
Bull et al., 1990 (52 weeks)	1.97	1.19	0.12	5.1×10^{-2}	8.4×10^{-2}
DeAngelo et al., 2008 (60 weeks)	2.83	1.71	0.15	3.5×10^{-2}	5.8×10^{-2}
DeAngelo et al., 2008 (104 weeks)	0.89	0.50	0.32	1.1×10^{-1}	2.0×10^{-1}
Female Mice					
Pereira, 1996 (82 weeks)	7.14	4.96	0.5	1.4×10^{-2}	2.0×10^{-2}

[a]ED_{10} and LED_{10} were derived from the one-stage multistage model.
[b]The slope of a linear extrapolation from the ED_{10} is calculated as follows: $0.1/ED_{10}$.
[c]The oral cancer slope factor is derived by linearly extrapolating from the LED_{10} (i.e., $0.1/LED_{10}$).

As discussed in Section 4.7.3, studies investigating the mode of action for TCA-induced liver tumors do not provide strong evidence for genotoxicity (Bull, 2000; Moore and Harrington-Brock, 2000). Rather, tumor induction appears to involve perturbation of cell growth, through activation of the PPARα pathway (Bull, 2000; Austin et al., 1996; Parrish et al., 1996), and reduced intracellular communication (Benane et al., 1996). However, the existing evidence is not sufficient to determine which, if any, of these mechanisms are causally related to the observed tumor responses. In addition, data are not available to identify dose-response relationships for possible precursor events for TCA-induced liver tumors. Therefore, data from these mouse studies are too limited for the application of biologically-based dose-response models, or other more sophisticated methods of analysis. Moreover, based on dose-response modeling, both Pereira (1996) and Bull et al. (1990) concluded that the tumorigenic response of TCA exhibited a linear relationship with increasing dose. Therefore, linear extrapolation from the LED_{10} for liver tumors was used for deriving an oral slope factor for TCA.

5.4.5. 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 slope factor is typically derived by linear extrapolation from the LED_{10} (i.e., $0.1/LED_{10}$) (U.S. EPA, 2005a). The estimated oral cancer slope factors based on the tumor responses in male mice in the Bull et al. (2002, 1990) and DeAngelo et al. (2008) studies, and the tumor responses in female mice in the Pereira (1996) study, ranged from 2×10^{-2} to 2×10^{-1} per mg/kg-day (Table 5-13).

Candidate oral cancer slope factors were derived from male mice studies with durations ranging from 52 to 104 weeks. During conversion of animal doses to human equivalent doses for continuous lifetime exposure, cross-time scaling factors of [duration of experiment/duration of animal life]^3 were used for all studies except the 104-week study of DeAngelo et al. (2008). Due to the uncertainty inherent in applying this scaling factor, the slope factor derived from the study of longest duration is generally preferred. Moreover, TCA may be a more potent carcinogen in male than in female mice, as discussed previously in Section 4.8.2. Also, the four slope factors derived from the incidence data in male mice varied by about three-fold. Based on these considerations, the slope factor derived from the study of longest duration (i.e., the 104-week data from DeAngelo et al., 2008) is recommended, i.e., 2×10^{-1} $(mg/kg\text{-}day)^{-1}$.

The slopes of the linear extrapolation from the ED_{10}, the central estimate of exposure associated with 10% extra cancer risk, were also derived. Five such slopes (7.1×10^{-2}, 5.1×10^{-2}, 3.5×10^{-2}, 1.1×10^{-1}, and 1.4×10^{-2}) were derived from the same studies used to derive the oral cancer slope factors (Bull et al. 2002, 1990; DeAngelo et al., 2008; Pereira, 1996). Based on the study of longest duration (the 104-week data from DeAngelo et al., 2008), the slope of the linear extrapolation from the ED_{10} is 1×10^{-1} $(mg/kg\text{-}day)^{-1}$.

No inhalation unit risk for TCA was derived. Cancer bioassays involving inhalation exposure to TCA are not currently available, and a route-to-route extrapolation (from oral to inhalation) is not recommended at this time because the currently available physiologically-based toxicokinetic models, which might be useful for route-to-route extrapolation, do not include an inhalation pathway.

5.4.6. 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 by-product 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 metabolic transformation of DCA into other metabolites. 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 adverse 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, reduced fetal body weight, decreased fetal testes weight, decreased fetal ovary weight, increased apoptosis of gonocytes, and decreased fetal brain weight. The pattern of observed fetal cardiac malformation effects has not been completely consistent across the available studies. The reason for this inconsistency is unknown but may be related to factors such as the dosing method, differences in the strain or source of the test animals, and/or the method used for evaluation of cardiac malformations.

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 has 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 of rats.

Using the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), TCA is determined to be "likely to be carcinogenic to humans" by all routes of exposure. In the previous IRIS assessment of TCA, TCA was classified as a "C," or a "possible human carcinogen."

Three lines of evidence support the weight-of-evidence descriptor of "likely to be carcinogenic to humans": (1) TCA is carcinogenic in the liver in multiple studies conducted in $B6C3F_1$ mice of both sexes; (2) tumor response was robust, occurring at substantially less-than-lifetime exposures at which tumor rates in control animals were relatively low; and (3) there are data gaps that preclude a determination that the MOA for hepatocarcinogenesis in mice is not relevant to humans. Finally, two significant limitations of the database for TCA carcinogenicity are the limited number of mouse studies that included microscopic evaluation of a comprehensive set of organs in addition to the liver and the absence of epidemiologic studies of TCA carcinogenicity in humans.

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 is carried out using default linear extrapolation (U.S. EPA, 2005a). In addition, no data were found that were suitable for accounting for interspecies differences in toxicokinetics or toxicodynamics in dose-response modeling.

It is possible that there are segments of the human population that are especially susceptible to the toxic effects of TCA as a result of age, gender, health status, or genetic factors, but there are no studies specifically on TCA to fully evaluate this possibility. Age-dependent differences in susceptibility to noncancer effects of TCA have not been investigated in systemic toxicity studies. 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 for gender effects on 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 to 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. Alternative RfDs derived from the $BMDL_{05}$ for developmental effects in rats (Smith et al., 1989) and from the NOAEL for liver effects in rats (DeAngelo et al., 1997) support this RfD derived for liver effects in mice. Figure 5-2 shows a comparison of these three candidate RfDs, and how they were derived from their respective PODs that illustrate the similarity between these toxicity values.

Confidence in the principal study chosen for the RfD is medium. The study appears to have been well designed and well conducted; quantitative data for the incidence and severity of the various endpoints were included in the published paper. Study duration was up to 104 weeks. The observed hepatocellular neoplasia correlated well with peroxisome proliferation, and 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

An inhalation RfC has not been calculated for TCA. No inhalation studies in humans or animals that were adequate for the derivation of RfC were located. Route-to-route extrapolation and use of physiologically based pharmacokinetic (PBPK) modeling techniques were considered as alternative approaches for derivation of the RfC. However, the existing information on the toxicokinetics of TCA was inadequate for a route-to-route extrapolation from the oral pathway to the inhalation pathway and validated PBPK models are not currently available for TCA.

6.2.3. Cancer/Oral and Inhalation

Under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), TCA is determined to be "likely to be carcinogenic to humans" for all routes of exposure. Five candidate oral cancer slope factors (1.1×10^{-1}, 8.4×10^{-2}, 5.8×10^{-2}, 2.0×10^{-1}, and 2.0×10^{-2} per mg/kg-day) 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, 1990), 60 weeks (DeAngelo et al., 2008), or 104 weeks (DeAngelo et al., 2008) or from female $B6C3F_1$ mice exposed to TCA in drinking water for 82 weeks (Pereira, 1996). These candidate oral slope factors vary over one order of magnitude, with the 104-week tumor incidence data from DeAngelo et al. (2008) yielding the highest potency. The oral cancer slope factor of 2×10^{-1} per mg/kg-day derived from the 104-week bioassay in male $B6C3F_1$ mice (DeAngelo et al., 2008) is recommended as the oral cancer slope factor for TCA.

To derive these oral cancer slope factors, the average daily intakes of TCA from the mouse studies were converted to human equivalent lifetime doses by using an interspecies scaling factor based on equivalence of $(mg/kg)^{3/4}$ per day (U.S. EPA, 1992), and a cross-time scaling factor based on the assumption that the age-specific rate for cancer increased by at least the third power of age (U.S. EPA, 1980). A cross-time scaling factor was not used for the 104-week mouse study (DeAngelo et al., 2008) because exposure duration was for a full lifetime. Using BMDS (version 1.4.1), the multistage model was fit to mouse liver tumor incidence data (i.e., combined adenomas and carcinomas) and associated human equivalent lifetime TCA doses. Oral cancer slope factors were calculated by linear extrapolation from the lower 95% confidence limit on model-predicted human equivalent lifetime doses associated with 10% extra risk for liver tumors (LED_{10}s).

The default linear low-dose extrapolation method was selected because the shape of cancer dose-response curves is linear, and current understanding of the MOA whereby TCA induces liver tumors is not sufficient to rule out the possibility of a linear slope at low doses. In addition, data from mouse studies are too limited for other more sophisticated methods of analysis (i.e., biologically based dose-response modeling). Moreover, available data do not provide strong evidence for a direct mutagenic MOA and suggest that tumor induction may involve perturbation of cell growth through PPARα agonism and reduced intercellular

communication. However, current understanding is insufficient to determine which, if any, of these MOAs may be causally related to the observed tumor responses, and data are not available to characterize dose-response relationships for as yet unidentified precursor events for TCA-induced liver tumors.

No inhalation unit risk for TCA was derived. Cancer bioassays involving inhalation exposure to TCA are not currently available, and a route-to-route extrapolation (from oral to inhalation) is not recommended at this time because the currently available physiologically based toxicokinetic models, which might be useful for route-to-route extrapolation, do not include an inhalation pathway.

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

[to be added]

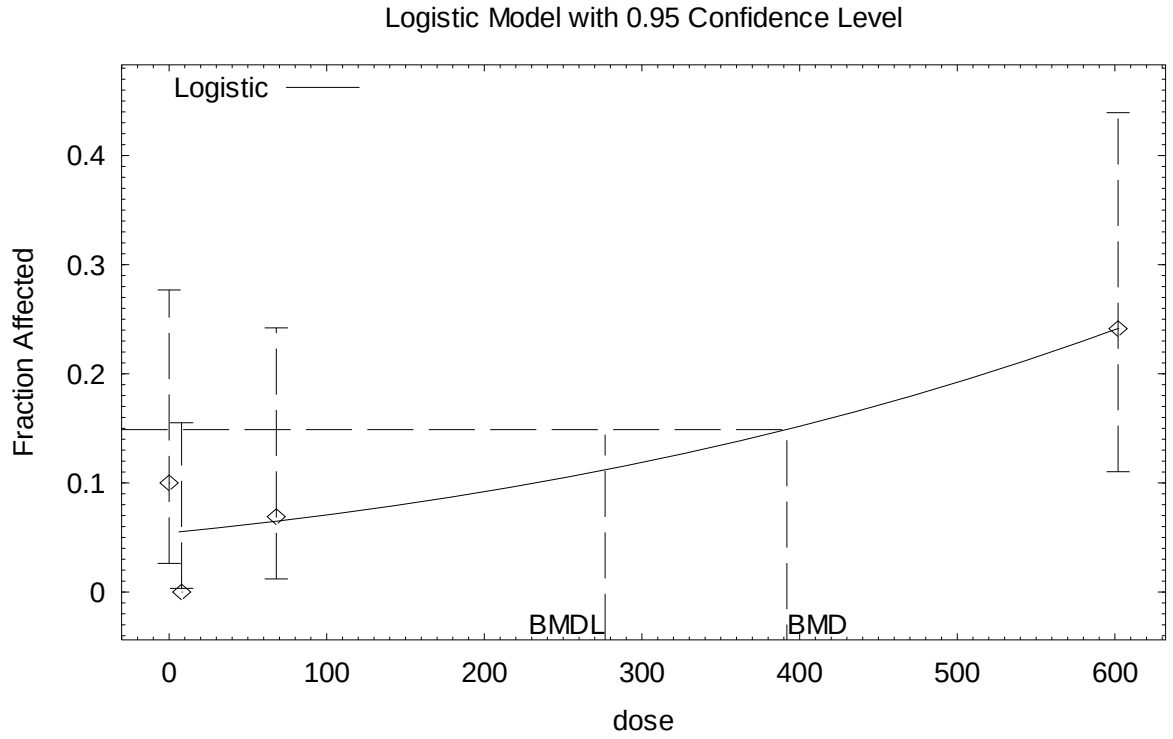
APPENDIX B. BENCHMARK DOSE MODELING RESULTS FOR THE INCIDENCE OF HEPATOCELLULAR INFLAMMATION, HEPATOCELLULAR NECROSIS, AND TESTICULAR TUBULAR DEGENERATION IN MICE EXPOSED TO TCA IN DRINKING WATER FOR USE IN DERIVATION OF THE REFERENCE DOSE

Table B-1.1. Benchmark dose modeling results based on incidence of hepatocellular inflammation in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks (DeAngelo et al., 2008)

Fitted Dichotomous Model[a]	Chi-Square Goodness-of-Fit Test *p*-Value[b]	AIC[c]	BMD_{10}[d] (mg/kg-day)	$BMDL_{10}$[e] (mg/kg-day)
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 chi-square 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 = Akaike's Information Criterion, 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} = Benchmark dose at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the benchmark dose at 10% extra risk.

Of the seven models fit, four (i.e., logistic, one-stage multistage, probit, and log-probit) showed adequate fit, and thus the BMDS outputs from these four models are provided below.



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MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.plt
                                          Fri Sep 05 12:07:17 2008
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 BMDS MODEL RUN
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   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

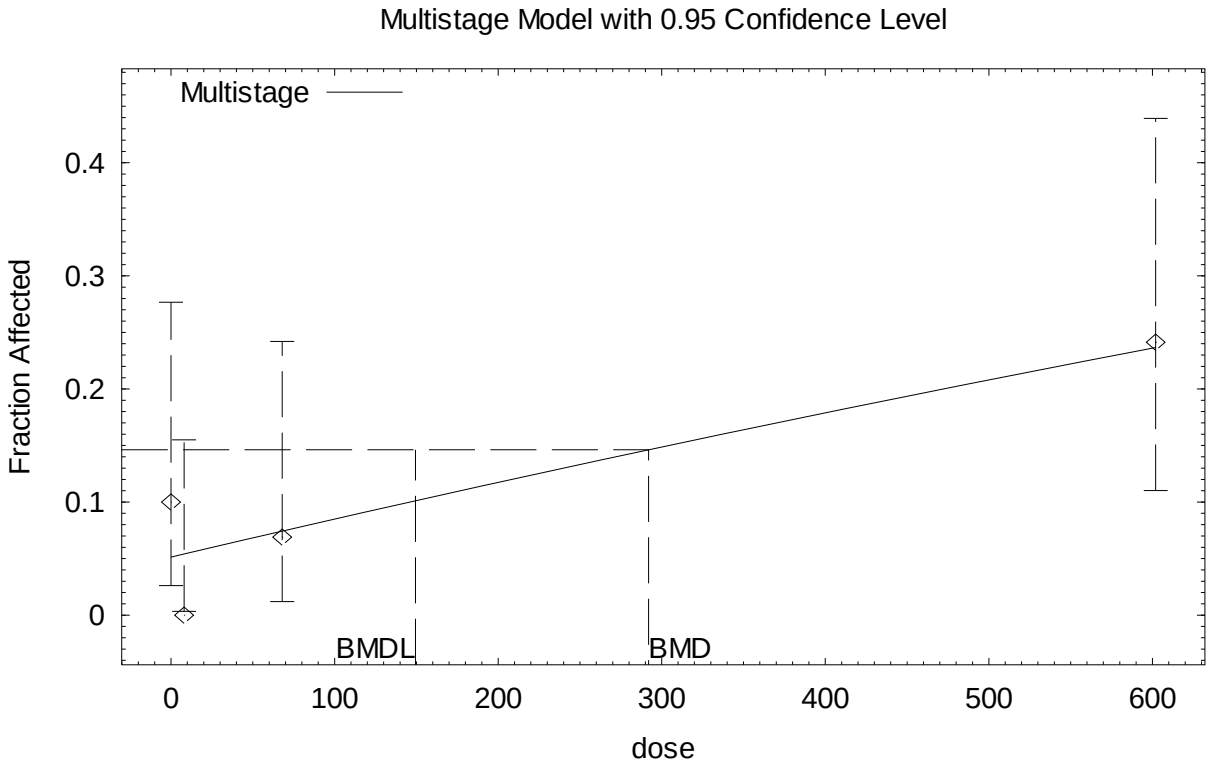
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 391.918

BMDL = 276.646



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MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.plt
                                          Fri Sep 05 12:11:21 2008
 ======================================================================

 BMDS MODEL RUN
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   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 =    0.0486161
                        Beta(1) =  0.000374222
```

Asymptotic Correlation Matrix of Parameter Estimates

	Background	Beta(1)
Background	1	-0.52
Beta(1)	-0.52	1

Parameter Estimates

Variable	Estimate	95.0% Wald Confidence Interval Std. Err.	Lower Conf. Limit	Upper Conf. Limit
Background	0.051295	*	*	*
Beta(1)	0.000360853	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-33.0575	4			
Fitted model	-35.1449	2	4.17486	2	0.124
Reduced model	-38.4712	1	10.8276	3	0.0127

AIC: 74.2898

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0513	1.539	3	30	1.209
8.0000	0.0540	1.459	0	27	-1.242
68.0000	0.0743	2.154	2	29	-0.109
602.0000	0.2365	6.860	7	29	0.061

Chi^2 = 3.02 d.f. = 2 P-value = 0.2209

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

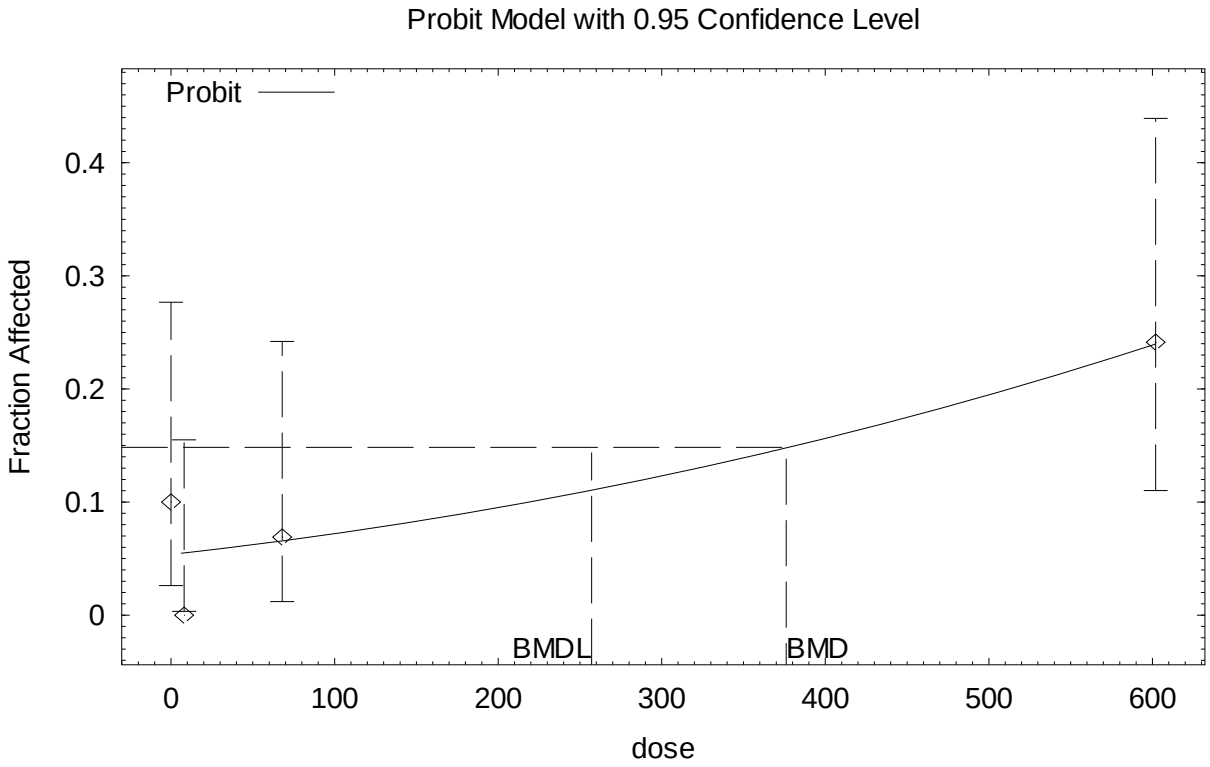
Confidence level = 0.95

BMD = 291.976

BMDL = 149.431

BMDU = 928.712

Taken together, (149.431, 928.712) is a 90 % two-sided confidence interval for the BMD



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MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.plt
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 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = CumNorm(Intercept+Slope*Dose),

   where CumNorm(.) is the cumulative normal distribution function


   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 (and Specified) Parameter Values
                   background =            0   Specified
                    intercept =      -1.7688
                        slope =    0.0018081
          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	-0.69
slope	-0.69	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
intercept	-1.60927	0.227286	-2.05474	-1.1638
slope	0.00150498	0.000580302	0.000367607	0.00264235

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-33.0575	4			
Fitted model	-35.0988	2	4.08263	2	0.1299
Reduced model	-38.4712	1	10.8276	3	0.0127

AIC: 74.1975

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0538	1.613	3	30	1.122
8.0000	0.0551	1.488	0	27	-1.255
68.0000	0.0659	1.912	2	29	0.066
602.0000	0.2409	6.987	7	29	0.005

Chi^2 = 2.84 d.f. = 2 P-value = 0.2419

Benchmark Dose Computation

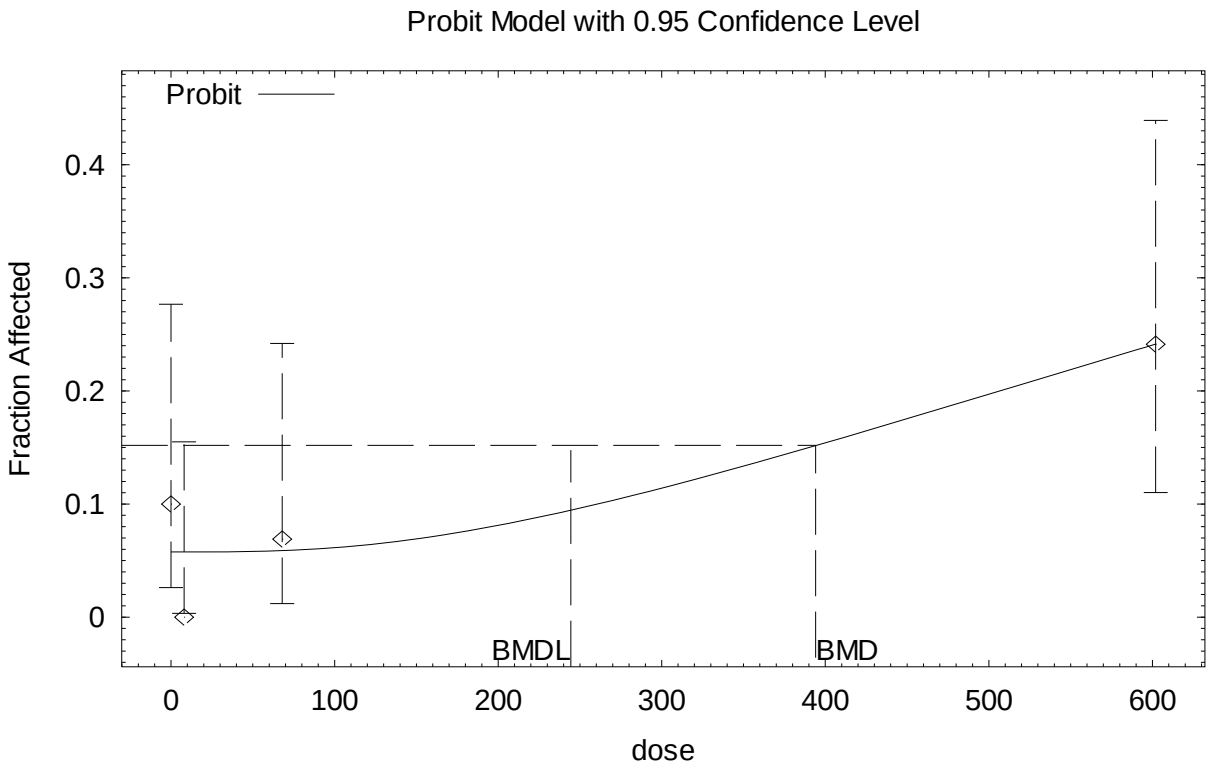
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 376.053

BMDL = 257.089



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MODELING\MALE_MOUSE_HEPATOCELLULAR_INFLAMMATION_60_WKS_DEANGELO_2008.plt
                                        Fri Sep 05 12:14:41 2008
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 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	Std. Err.	95.0% Wald Confidence Interval 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

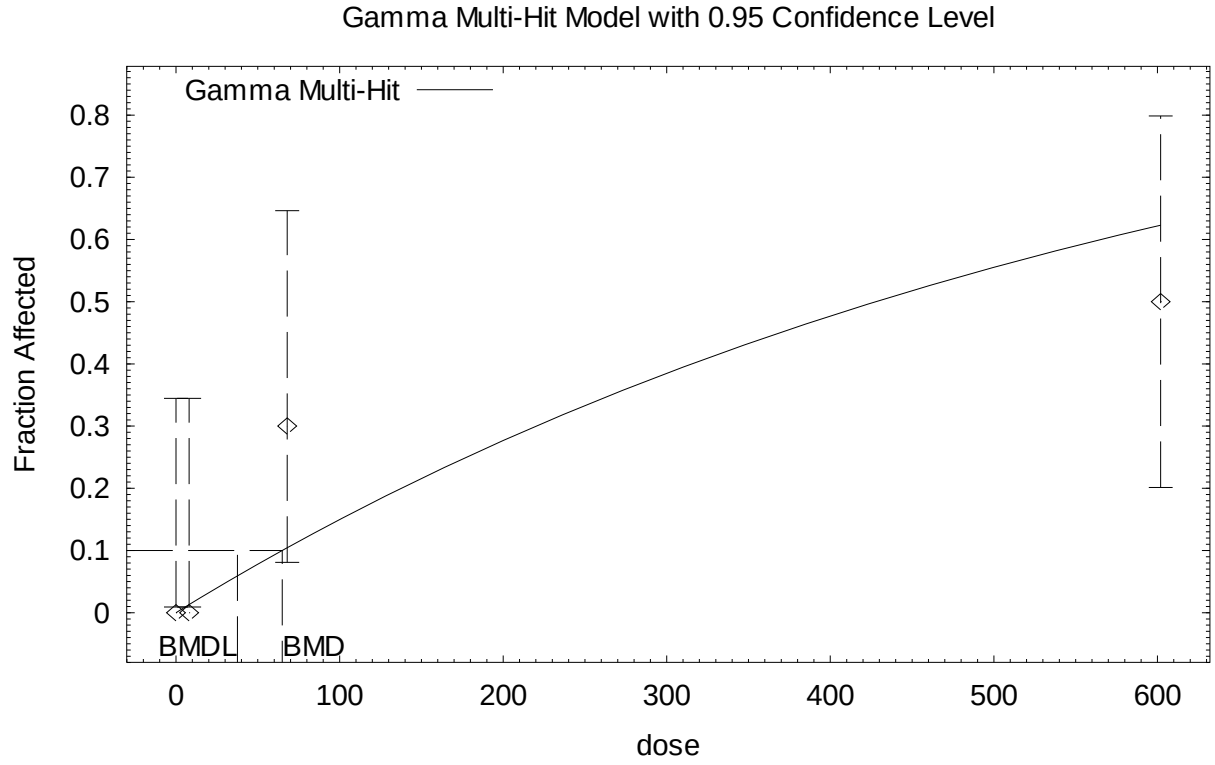
Specified effect = 0.1
Risk Type = Extra risk
Confidence level = 0.95
BMD = 394.098
BMDL = 244.412

Table B-1.2. Benchmark dose modeling results based on incidence of hepatocellular necrosis in male B6C3F$_1$ mice exposed to TCA in drinking water for 30 to 45 weeks (DeAngelo et al., 2008)

Fitted Dichotomous Model[a]	Chi-Square Goodness-of-Fit Test *p*-Value[b]	AIC[c]	BMD_{10}[d] (mg/kg-day)	$BMDL_{10}$[e] (mg/kg-day)
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 chi-square 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 = Akaike's Information Criteria, 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} = Benchmark dose at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the benchmark dose at 10% extra risk.

Of the seven models fit, four (i.e., gamma, log-logistic, one-stage multistage, and Weibull) showed adequate fit, and thus the BMDS outputs from these four models are provided below.

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MODELING\MALE_MOUSE_HEPATOCELLULAR_NECROSIS_DEANGELO_2008.(d)
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MODELING\MALE_MOUSE_HEPATOCELLULAR_NECROSIS_DEANGELO_2008.plt
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 BMDS MODEL RUN
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   The form of the probability function is:

   P[response]= background+(1-background)*CumGamma[slope*dose,power],
   where CumGamma(.) is the cumulative Gamma distribution function


   Dependent variable = Response
   Independent variable = Dose
   Power parameter is restricted as power >=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


                 Default Initial (and Specified) Parameter Values
                    Background =    0.0454545
                         Slope =   0.00722137
                         Power =          1.3
          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -Background    -Power
```

have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

Slope

Slope 1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0	NA		
Slope	0.00162275	0.000587954	0.000470383	0.00277512
Power	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	-13.0401	4			
Fitted model	-14.925	1	3.76969	3	0.2874
Reduced model	-20.0161	1	13.952	3	0.002971

AIC: 31.8499

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0.000
8.0000	0.0129	0.129	0	10	-0.361
68.0000	0.1045	1.045	3	10	2.021
602.0000	0.6235	6.235	5	10	-0.806

Chi^2 = 4.87 d.f. = 3 P-value = 0.1818

Benchmark Dose Computation

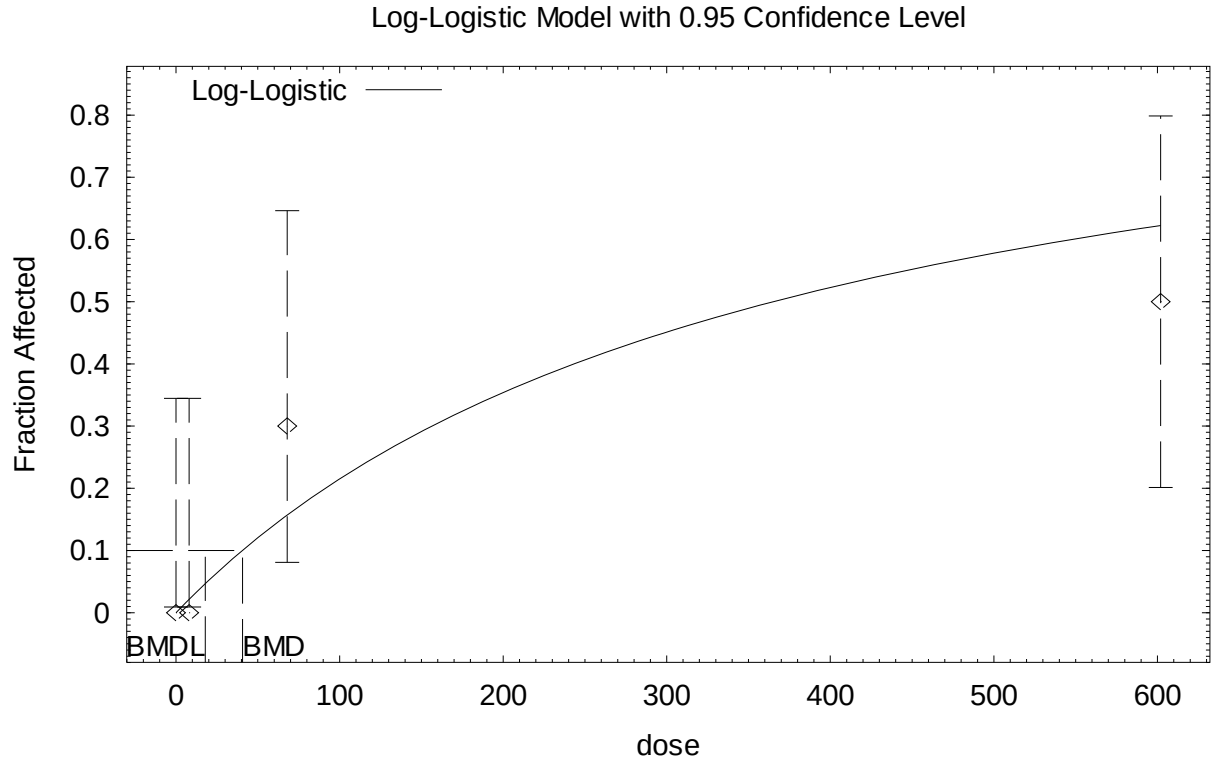
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 64.9271

BMDL = 37.5509



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 ================================================================
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MODELING\MALE_MOUSE_HEPATOCELLULAR_NECROSIS_DEANGELO_2008.plt
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 ================================================================

 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

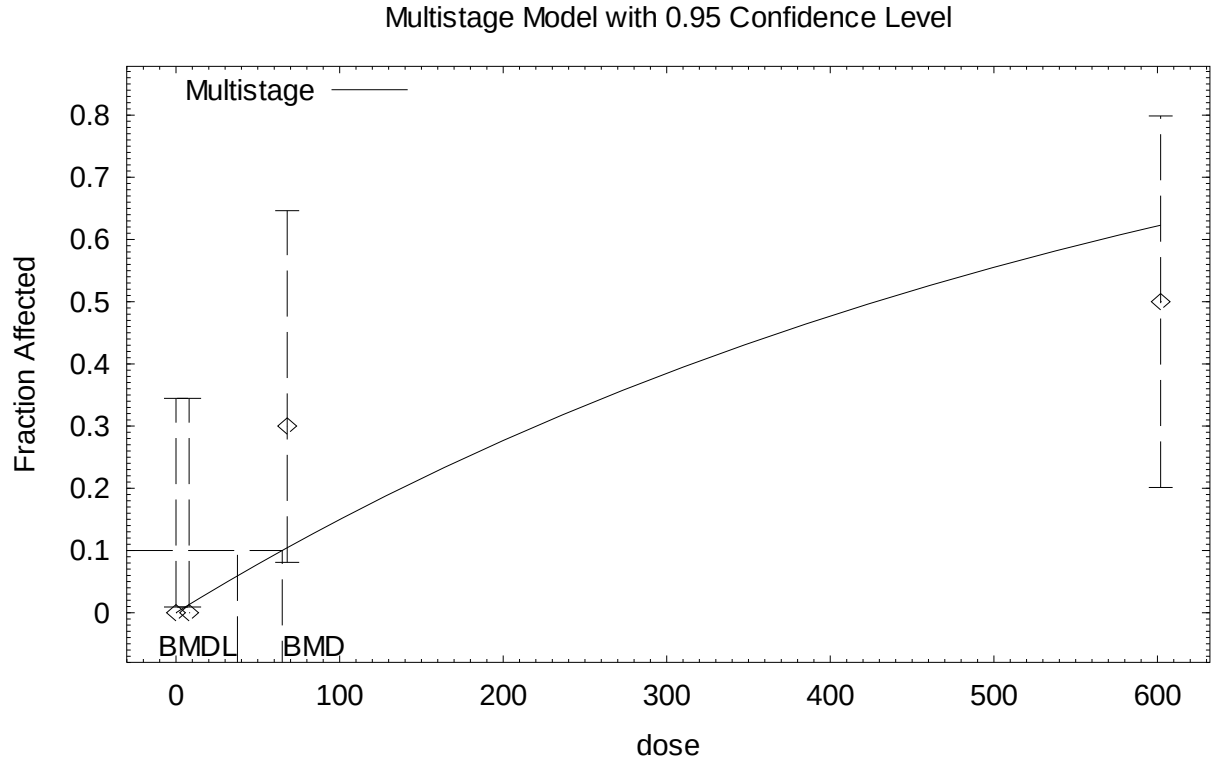
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 40.6639

BMDL = 17.8767



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        Multistage Model. (Version: 2.8;  Date: 02/20/2007)
        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 14:23:03 2008
 ================================================================

 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 = 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 =    0.0817489
                        Beta(1) =   0.00104526
```

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)	0.00162275	*	*	*

* - 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	-14.925	1	3.76969	3	0.2874
Reduced model	-20.0161	1	13.952	3	0.002971

AIC: 31.8499

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0.000
8.0000	0.0129	0.129	0	10	-0.361
68.0000	0.1045	1.045	3	10	2.021
602.0000	0.6235	6.235	5	10	-0.806

Chi^2 = 4.87 d.f. = 3 P-value = 0.1818

Benchmark Dose Computation

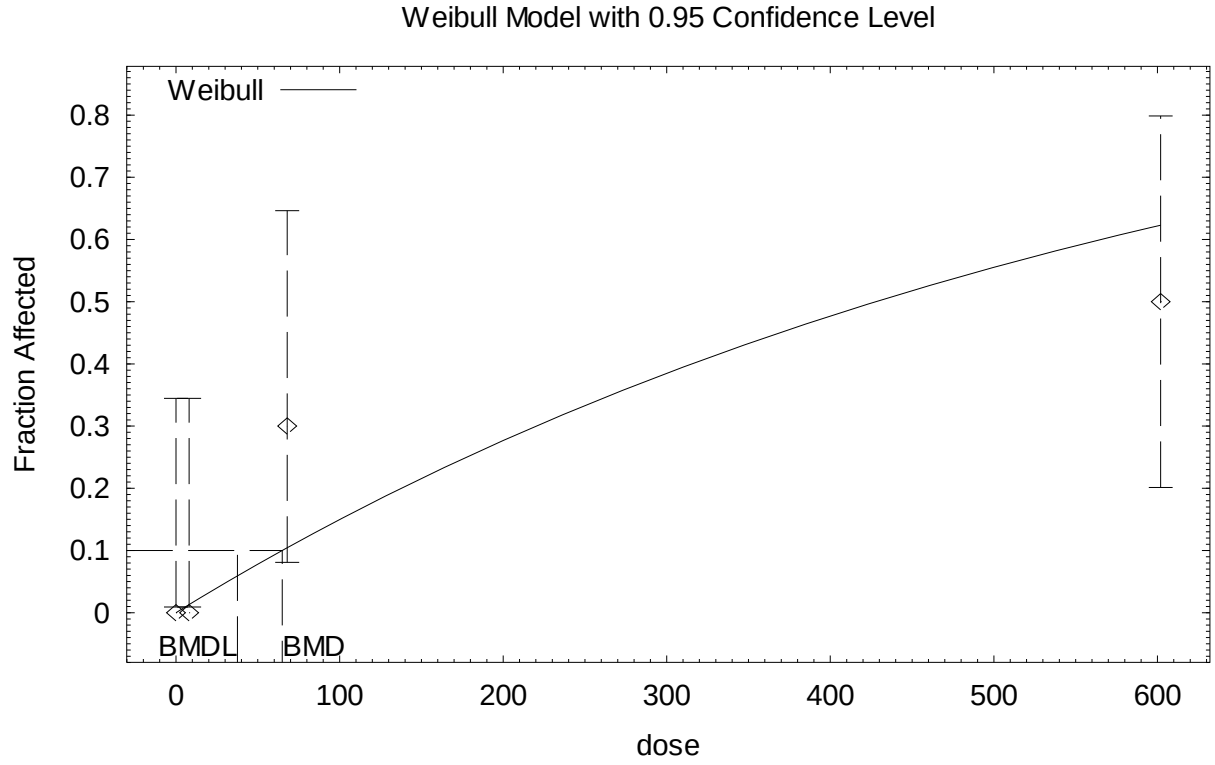
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 64.9271
BMDL = 37.5509
BMDU = 167.542

Taken together, (37.5509, 167.542) is a 90 % two-sided confidence interval for the BMD



14:28 09/05 2008

```
 ======================================================================
        Weibull Model using Weibull Model (Version: 2.10;  Date: 10/31/2007)
        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 14:28:13 2008
 ======================================================================

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

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^power)]


   Dependent variable = Response
   Independent variable = Dose
   Power parameter is restricted as power >=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



                  Default Initial (and Specified) Parameter Values
                     Background =    0.0454545
                          Slope =   0.00107413
                          Power =            1

           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Background    -Power
```

have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

Slope

Slope 1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0	NA		
Slope	0.00162275	0.000587954	0.000470384	0.00277512
Power	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	-13.0401	4			
Fitted model	-14.925	1	3.76969	3	0.2874
Reduced model	-20.0161	1	13.952	3	0.002971

AIC: 31.8499

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0.000
8.0000	0.0129	0.129	0	10	-0.361
68.0000	0.1045	1.045	3	10	2.021
602.0000	0.6235	6.235	5	10	-0.806

Chi^2 = 4.87 d.f. = 3 P-value = 0.1818

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 64.9271

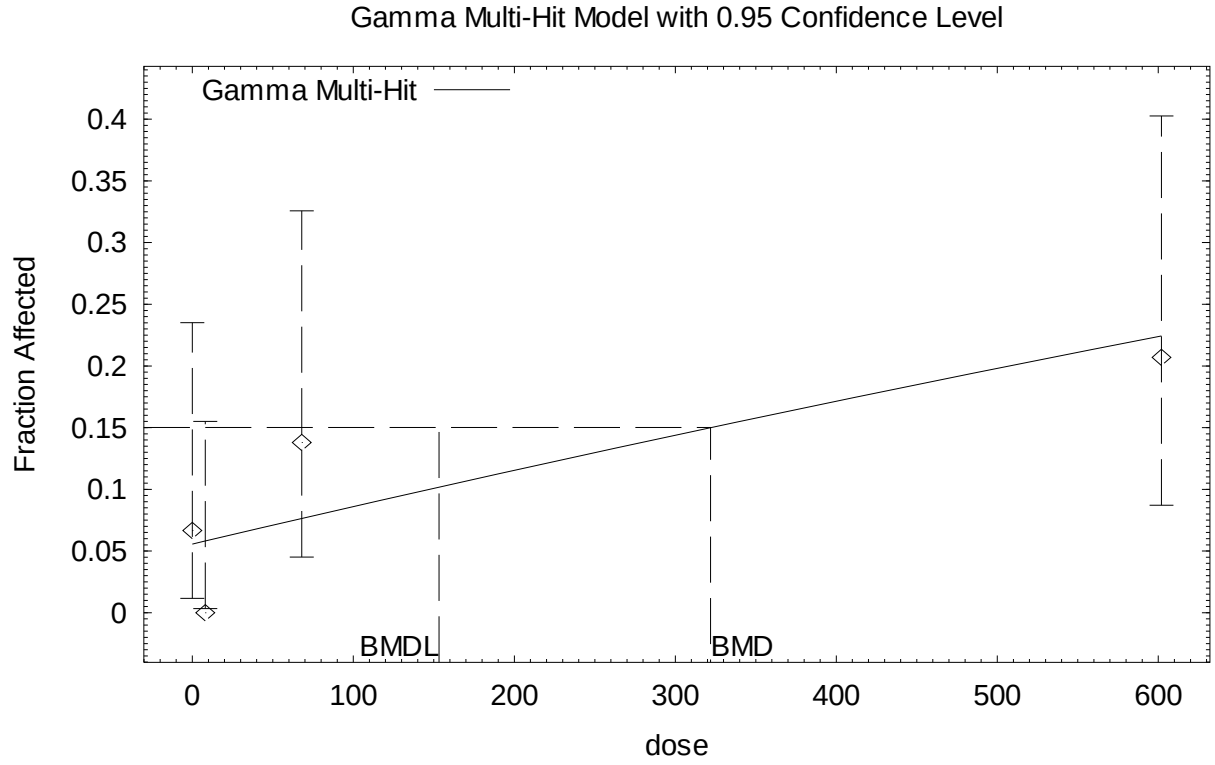
BMDL = 37.5509

Table B-1.3. Benchmark dose modeling results based on incidence of testicular tubular degeneration in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks (DeAngelo et al., 2008)

Fitted Dichotomous Model[a]	Chi-Square Goodness-of-Fit Test *p*-Value[b]	AIC[c]	BMD_{10}[d] (mg/kg-day)	$BMDL_{10}$[e] (mg/kg-day)
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 chi-square 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 = Akaike's Information Criterion, 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} = Benchmark dose at 10% extra risk.
[e]$BMDL_{10}$ = 95% lower confidence limit on the benchmark dose at 10% extra risk.

All seven models showed adequate fit. The BMDS outputs from these seven models are provided below.



13:47 09/05 2008

```
 ================================================================
        Gamma Model. (Version: 2.11;  Date: 10/31/2007)
        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 13:47:08 2008
 ================================================================

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

   The form of the probability function is:

   P[response]= background+(1-background)*CumGamma[slope*dose,power],
   where CumGamma(.) is the cumulative Gamma distribution function


   Dependent variable = Response
   Independent variable = Dose
   Power parameter is restricted as power >=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


                 Default Initial (and Specified) Parameter Values
                    Background =    0.0806452
                         Slope =   0.00135334
                         Power =          1.3
          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -Power
```

have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Background	Slope
Background	1	-0.45
Slope	-0.45	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.0556454	0.028903	-0.0010035	0.112294
Slope	0.000327288	0.000185399	-3.60877e-005	0.000690665
Power	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	-33.7671	4			
Fitted model	-36.0814	2	4.62871	2	0.09883
Reduced model	-38.4712	1	9.40833	3	0.02433

AIC: 76.1628

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0556	1.669	2	30	0.263
8.0000	0.0581	1.569	0	27	-1.291
68.0000	0.0764	2.216	4	29	1.247
602.0000	0.2245	6.511	6	29	-0.228

Chi^2 = 3.34 d.f. = 2 P-value = 0.1882

Benchmark Dose Computation

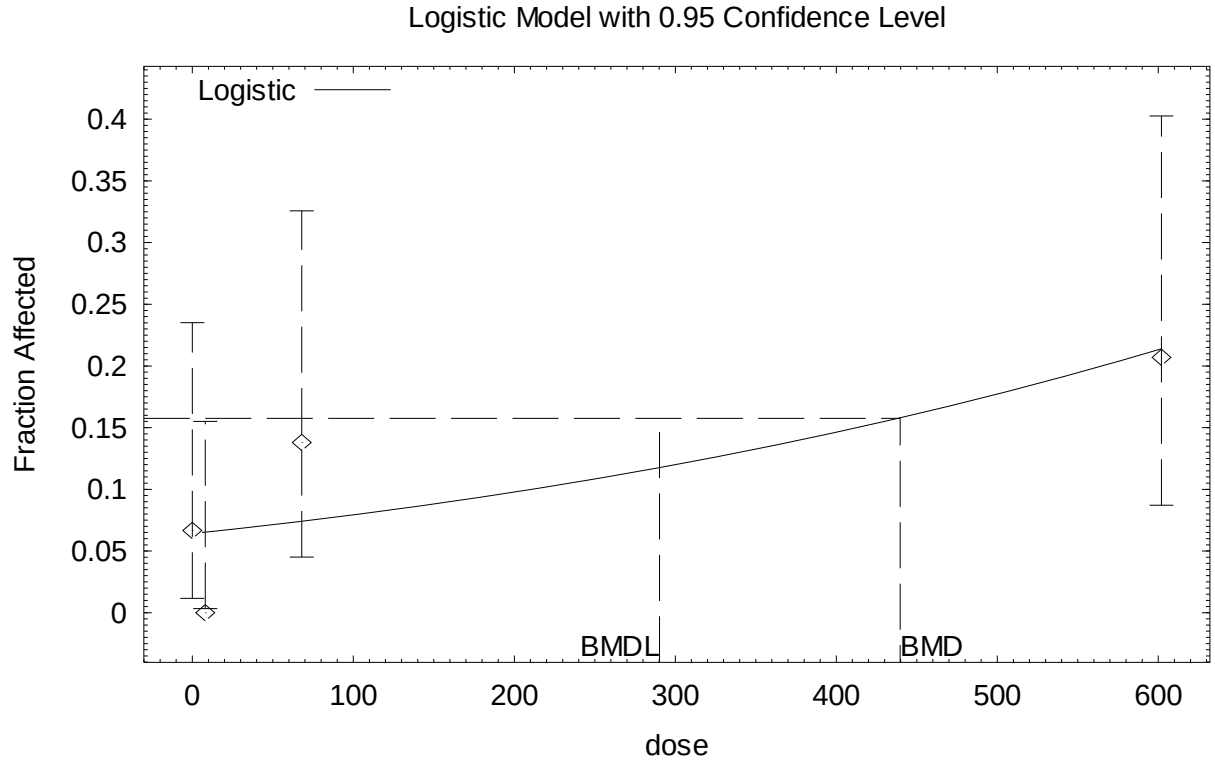
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 321.919

BMDL = 153.274



13:48 09/05 2008

```
 ================================================================
        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 13:48:30 2008
 ================================================================

 BMDS MODEL RUN
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   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 =     -2.82219
                         slope =   0.00269617

          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	-0.72
slope	-0.72	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
intercept	-2.68463	0.449806	-3.56623	-1.80303
slope	0.00229179	0.00108339	0.000168388	0.00441519

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-33.7671	4			
Fitted model	-36.2929	2	5.05173	2	0.07999
Reduced model	-38.4712	1	9.40833	3	0.02433

AIC: 76.5859

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0639	1.917	2	30	0.062
8.0000	0.0650	1.755	0	27	-1.370
68.0000	0.0739	2.142	4	29	1.319
602.0000	0.2133	6.187	6	29	-0.085

Chi^2 = 3.63 d.f. = 2 P-value = 0.1630

Benchmark Dose Computation

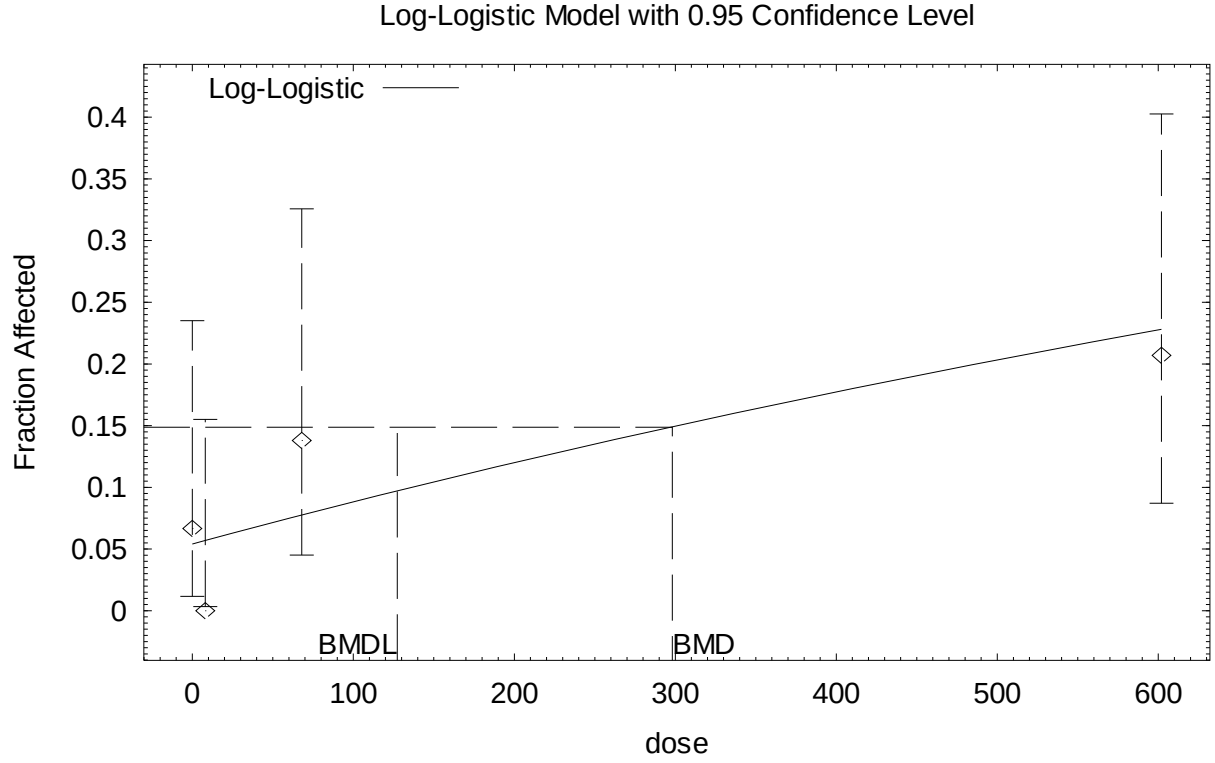
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 439.685

BMDL = 290.255



13:50 09/05 2008

```
 ================================================================
        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
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   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

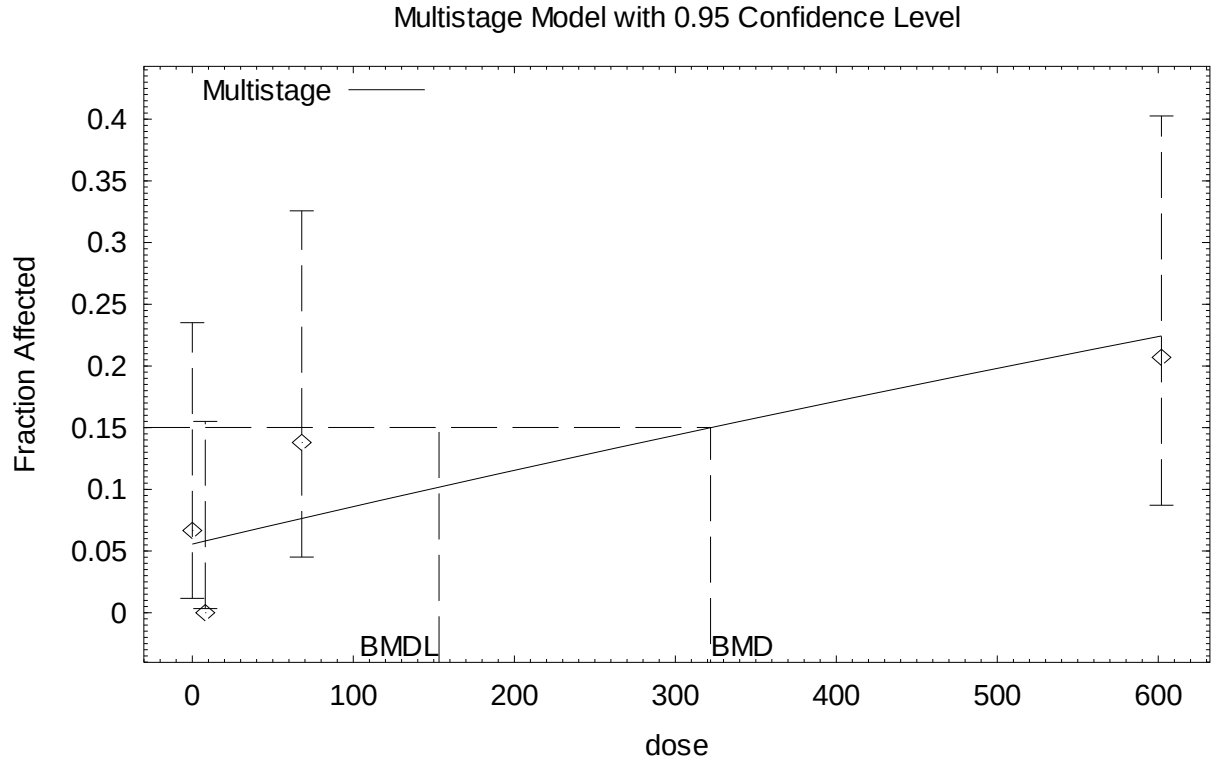
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 298.169

BMDL = 127.35



13:51 09/05 2008

```
 ================================================================
        Multistage Model. (Version: 2.8;  Date: 02/20/2007)
        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 13:51:55 2008
 ================================================================

 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 = 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 =    0.0609653
                        Beta(1) =   0.00029145
```

Asymptotic Correlation Matrix of Parameter Estimates

	Background	Beta(1)
Background	1	-0.56
Beta(1)	-0.56	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.0556454	*	*	*
Beta(1)	0.000327288	*	*	*

* - 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	-36.0814	2	4.62871	2	0.09883
Reduced model	-38.4712	1	9.40833	3	0.02433

AIC: 76.1628

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0556	1.669	2	30	0.263
8.0000	0.0581	1.569	0	27	-1.291
68.0000	0.0764	2.216	4	29	1.247
602.0000	0.2245	6.511	6	29	-0.228

Chi^2 = 3.34 d.f. = 2 P-value = 0.1882

Benchmark Dose Computation

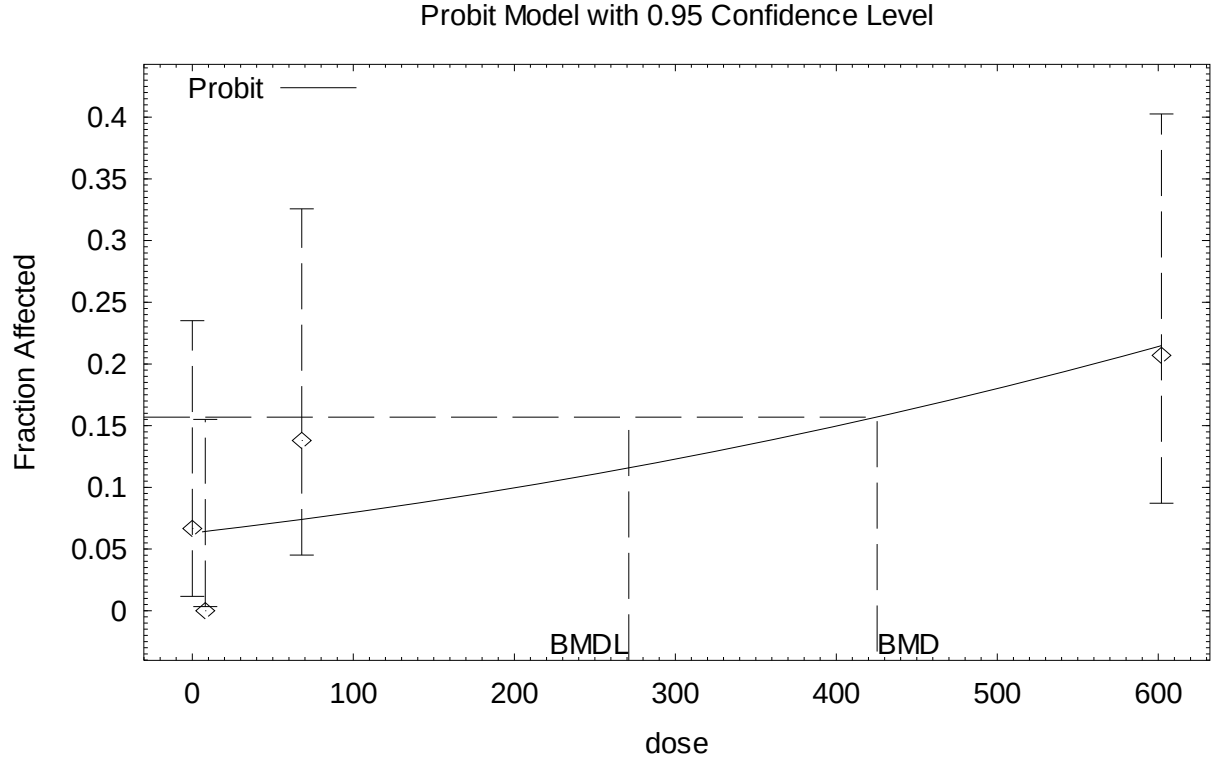
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 321.92
BMDL = 153.274
BMDU = 1517.45

Taken together, (153.274, 1517.45) is a 90 % two-sided confidence interval for the BMD



13:53 09/05 2008

```
 ================================================================
        Probit Model. (Version: 2.9;  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 13:53:07 2008
 ================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = CumNorm(Intercept+Slope*Dose),

   where CumNorm(.) is the cumulative normal distribution function


   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 (and Specified) Parameter Values
                    background =            0   Specified
                     intercept =     -1.72179
                         slope =   0.00160607
          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	-0.67
slope	-0.67	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
intercept	-1.52928	0.217945	-1.95644	-1.10211
slope	0.00122623	0.000581105	8.72829e-005	0.00236517

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-33.7671	4			
Fitted model	-36.2697	2	5.00537	2	0.08186
Reduced model	-38.4712	1	9.40833	3	0.02433

AIC: 76.5395

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0631	1.893	2	30	0.080
8.0000	0.0643	1.737	0	27	-1.362
68.0000	0.0741	2.149	4	29	1.312
602.0000	0.2144	6.219	6	29	-0.099

Chi^2 = 3.59 d.f. = 2 P-value = 0.1658

Benchmark Dose Computation

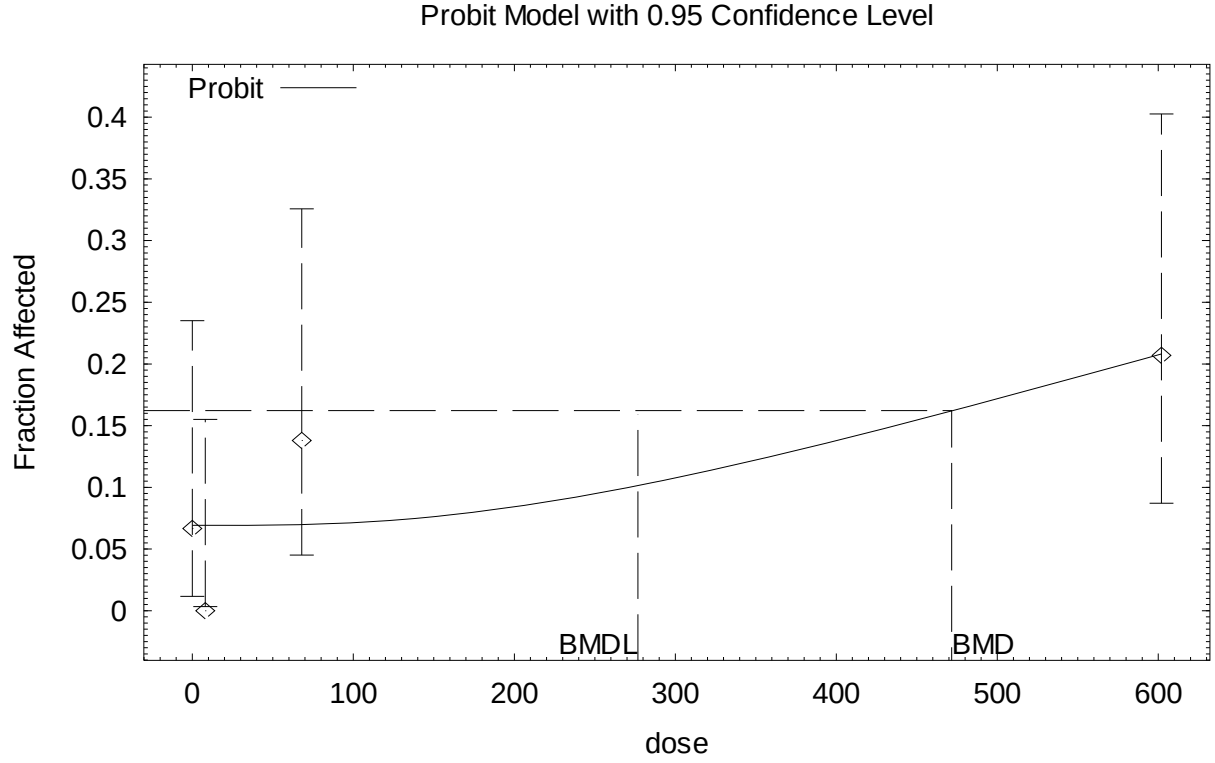
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 425.313

BMDL = 271.161



13:54 09/05 2008

```
 ================================================================
        Probit Model. (Version: 2.9;  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 13:54:25 2008
 ================================================================

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

   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 =    0.0666667
                     intercept =     -6.86605
                         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	-0.31
intercept	-0.31	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
background	0.0691801	0.0275874	0.0151099	0.12325
intercept	-7.43777	0.370612	-8.16415	-6.71138
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	-33.7671	4			
Fitted model	-36.5279	2	5.52164	2	0.06324
Reduced model	-38.4712	1	9.40833	3	0.02433

AIC: 77.0558

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0692	2.075	2	30	-0.054
8.0000	0.0692	1.868	0	27	-1.417
68.0000	0.0698	2.024	4	29	1.440
602.0000	0.2086	6.049	6	29	-0.022

Chi^2 = 4.09 d.f. = 2 P-value = 0.1297

Benchmark Dose Computation

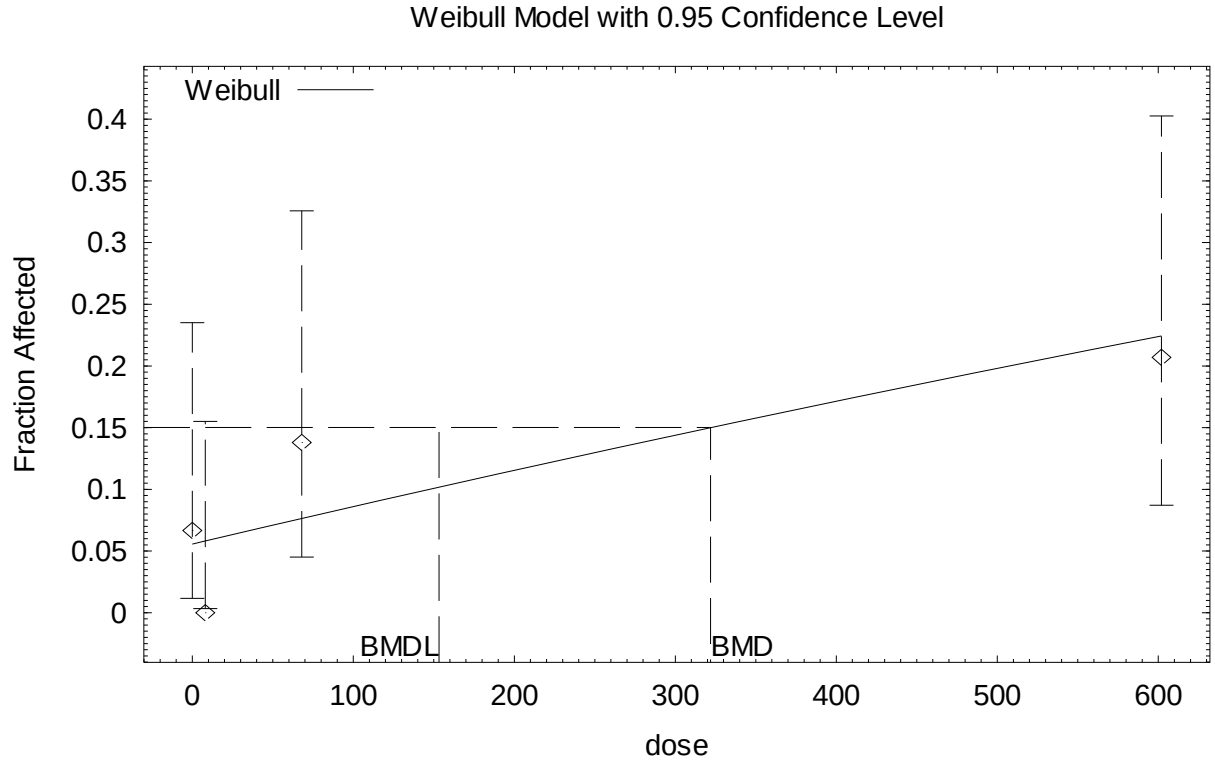
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 471.64

BMDL = 276.75



13:56 09/05 2008

```
 ================================================================
        Weibull Model using Weibull Model (Version: 2.10;  Date: 10/31/2007)
        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 13:56:04 2008
 ================================================================

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

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^power)]


   Dependent variable = Response
   Independent variable = Dose
   Power parameter is restricted as power >=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



                 Default Initial (and Specified) Parameter Values
                    Background =    0.0806452
                         Slope =   0.00026597
                         Power =            1
           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Power
                 have been estimated at a boundary point, or have been specified by
the user,
```

and do not appear in the correlation matrix)

	Background	Slope
Background	1	-0.45
Slope	-0.45	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.0556454	0.0289026	-0.00100271	0.112293
Slope	0.000327288	0.000185396	-3.60816e-005	0.000690658
Power	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	-33.7671	4			
Fitted model	-36.0814	2	4.62871	2	0.09883
Reduced model	-38.4712	1	9.40833	3	0.02433

AIC: 76.1628

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0556	1.669	2	30	0.263
8.0000	0.0581	1.569	0	27	-1.291
68.0000	0.0764	2.216	4	29	1.247
602.0000	0.2245	6.511	6	29	-0.228

Chi^2 = 3.34 d.f. = 2 P-value = 0.1882

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 321.919

BMDL = 153.274

APPENDIX C. INPUT AND OUTPUT DATA FOR BENCHMARK DOSE MODELING OF DEVELOPMENTAL DATA FROM SMITH ET AL. (1989)

A. Data sets for modeling rat fetal response to exposure to trichloroacetic acid in drinking water during GDs 6–15 (Smith et al., 1989).

A.1. Data for fetal body weights ≤3.16 g ($\alpha = 0.05$)

As summarized from the individual animal data sheets (see section B). Each triplet of numbers represents a distinct set of litters within a dose group; in order, the numbers in each triplet represent number of fetuses with body weight <3.16 g, number of fetuses in the litter, number of litters.

Dose = 0
0 6 1,1 6 1,1 7 1,1 8 1,0 9 2,2 10 1, 3 10 1,0 11 4, 2 11 1,0 12 7,1 12 1, 2 12 1,0 13 1, 0 14 2,3 15 1

Dose = 330 mg/kg-day
0 1 1,5 6 1,0 9 1,1 10 1,8 10 1,6 11 1, 8 11 1,0 12 1, 5 12 1, 11 12 1,7 13 1, 8 13 1, 11 13 1, 13 13 1,
1 14 1, 12 14 1, 0 15 1, 12 15 1, 16 16 1

Dose = 800 mg/kg-day
1 2 1,2 3 1,4 4 1,6 7 1, 7 7 1,5 8 1,9 9 1,8 10 1,4 11 1,8 11 1,11 11 3,12 12 1,12 13 1,13 13 1, 8 14 1

Dose = 1,200 mg/kg-day
1 1 2,4 4 1,4 6 1,6 6 1,2 7 1,6 7 2,7 7 1,9 9 1,10 11 1,11 11 1,13 13 1

Dose = 1,800 mg/kg-day
1 1 2,2 2 2,3 3 1,6 6 2,8 8 1

A.2. Data for fetal visceral malformations

As summarized from the individual pathology reports by R. Kavlock.
Each triplet of numbers represents a distinct set of litters within a dose group; in order, the numbers in each triplet represent number of malformed fetuses, number of fetuses in the litter, number of litters.

Dose = 0
0 4 1,1 4 1,0 5 1,0 6 3,0 7 1,2 7 1,0 8 12,1 8 1,2 8 1,0 9 1,0 10 3,

Dose = 330 mg/kg-day
0 1 1,0 4 1,1 6 1,0 7 2,0 8 3,1 8 1,2 8 1,0 9 3,2 9 1,3 9 1,0 10 1,1 10 2,3 10 1

Dose = 800 mg/kg-day
1 1 1,0 2 1,0 3 1,1 5 1,2 5 1,1 6 1,2 6 1,1 7 1,1 8 3,3 8 1,4 8 1,5 8 1,1 9 1,3 9 1,2 10 1

Dose = 1,200 mg/kg-day
1 1 2,1 4 1, 2 4 1, 3 4 1,0 5 2, 2 5 1, 4 5 1, 5 5 1,4 6 1,3 8 2,3 9 1

Dose = 1,800 mg/kg-day

1 1 4,2 2 1,3 4 1, 4 4 1,6 6 1

A.3. Data for fetal crown-rump length ≤3.4 cm g ($\alpha = 0.05$)

As summarized from the individual animal data sheets (see section B). Each triplet of numbers represents a distinct set of litters within a dose group; in order, the numbers in each triplet represent number of fetuses with crown-rump length <3.4 cm, number of fetuses in the litter, number of litters.

Dose = 0
0 6 1 1 6 1 1 7 1 0 8 1
0 9 2 0 10 1 2 10 1
0 11 4 1 11 1
0 12 9 0 13 1
0 14 2 1 15 1

Dose = 330 mg/kg-day
0 1 1 1 6 1
0 9 1 0 10 1 2 10 1
0 11 1 1 11 1
0 12 3 0 13 2 1 13 2
0 14 1 1 14 1
0 15 1 1 15 1 7 16 1

Dose = 800 mg/kg-day
1 2 1 0 3 1 0 4 1
0 7 1 1 7 1
5 8 1 5 9 1
2 10 1
0 11 2 1 11 1 4 11 1 6 11 1
11 12 1
0 13 1 5 13 1 1 14 1

Dose = 1,200 mg/kg-day
1 1 2 4 4 1
0 6 1 6 6 1
1 7 1 2 7 3 5 7 1
2 9 1 0 11 1 10 11 1
3 13 1

Dose = 1,800 mg/kg-day
0 1 1 1 1 1
1 2 1 2 2 1
3 3 1
6 6 2 8 8 1

B. Individual fetal body weight and crown-rump length data from the Smith et al. (1989) rat developmental study.

KEY:

Column 1 Dam ID
Column 2 Pup ID
Column 3 Dose (mg/kg)
Column 4 Litter Size
Column 5 Sex
Column 6 Weight (g)
Column 7 Crown-Rump Length (cm)

532	4	1200	6	M	3.32	3.5
532	5	1200	6	F	2.93	3.5
532	8	1200	6	F	2.94	3.4
532	10	1200	6	M	2.71	3.4
532	11	1200	6	M	3.21	3.5
532	13	1200	6	M	3.00	3.5
535	2	1800	6	F	2.14	3.1
535	3	1800	6	F	2.24	3.0
535	5	1800	6	M	2.17	3.0
535	11	1800	6	F	2.29	3.2
535	12	1800	6	M	2.54	3.1
535	16	1800	6	F	2.46	3.2
536	1	800	11	F	2.47	3.2
536	3	800	11	M	2.79	3.5
536	4	800	11	M	2.62	3.2
536	5	800	11	F	2.59	3.2
536	6	800	11	M	2.94	3.5
536	7	800	11	M	2.76	3.5
536	10	800	11	F	2.68	3.4
536	11	800	11	F	2.59	3.1
536	13	800	11	F	2.91	3.2
536	14	800	11	M	2.95	3.4
536	15	800	11	F	2.97	3.2
537	7	1800	1	M	2.79	3.4
538	4	1800	6	M	2.51	3.3
538	6	1800	6	M	2.19	3.2
538	9	1800	6	F	2.40	3.2
538	10	1800	6	M	2.38	3.1
538	16	1800	6	F	2.21	3.1
538	17	1800	6	M	2.41	3.2
539	1	1200	7	F	2.86	3.3
539	2	1200	7	M	3.31	3.5
539	3	1200	7	F	2.86	3.3
539	4	1200	7	M	2.70	3.3
539	6	1200	7	F	2.82	3.4

539	9	1200	7	M	2.86	3.3
539	10	1200	7	M	2.76	3.3
540	1	800	9	M	2.77	3.3
540	2	800	9	F	2.79	3.3
540	3	800	9	M	3.01	3.4
540	4	800	9	M	2.94	3.5
540	7	800	9	M	2.93	3.4
540	8	800	9	M	2.82	3.4
540	11	800	9	F	2.43	3.1
540	12	800	9	F	2.58	3.1
540	13	800	9	M	2.86	3.3
541	2	1200	11	M	2.37	3.0
541	3	1200	11	F	2.56	3.1
541	4	1200	11	M	2.58	3.4
541	6	1200	11	M	2.64	3.2
541	7	1200	11	M	2.47	3.1
541	10	1200	11	M	2.84	3.3
541	12	1200	11	F	2.30	3.1
541	13	1200	11	M	2.73	3.3
541	14	1200	11	F	2.54	3.2
541	15	1200	11	M	2.45	3.2
541	16	1200	11	M	2.51	3.3
542	3	0	6	F	3.42	3.4
542	4	0	6	M	3.67	3.5
542	6	0	6	M	4.06	3.8
542	9	0	6	M	3.71	3.6
542	10	0	6	M	3.07	3.0
542	11	0	6	M	3.68	3.7
543	1	800	11	M	2.88	3.5
543	2	800	11	M	2.94	3.3
543	3	800	11	M	2.85	3.4
543	5	800	11	M	2.86	3.5
543	6	800	11	F	2.62	3.2
543	7	800	11	F	2.97	3.4
543	8	800	11	F	3.10	3.3
543	9	800	11	M	3.09	3.3
543	10	800	11	F	3.14	3.4
543	11	800	11	M	2.92	3.4
543	12	800	11	M	3.00	3.5
544	2	0	7	F	3.46	3.4
544	3	0	7	F	3.55	3.5
544	5	0	7	F	3.48	3.5
544	6	0	7	M	3.76	3.7
544	8	0	7	M	3.57	3.5
544	9	0	7	F	3.04	3.2
544	10	0	7	M	3.61	3.6
545	1	800	13	M	2.91	3.4
545	2	800	13	F	2.43	3.3
545	3	800	13	M	3.09	3.3
545	5	800	13	F	2.70	3.3
545	6	800	13	M	2.97	3.4

545	7	800	13	M	2.68	3.4
545	8	800	13	M	2.72	3.2
545	9	800	13	F	2.39	3.1
545	10	800	13	M	2.74	3.4
545	11	800	13	F	2.76	3.4
545	12	800	13	M	2.99	3.5
545	13	800	13	F	2.97	3.4
545	14	800	13	M	2.85	3.4
546	1	0	11	F	3.11	3.5
546	2	0	11	F	3.07	3.3
546	3	0	11	M	3.47	3.6
546	4	0	11	F	3.19	3.4
546	5	0	11	M	3.29	3.4
546	6	0	11	M	3.68	3.7
546	7	0	11	M	3.42	3.4
546	8	0	11	M	3.19	3.5
546	9	0	11	M	3.36	3.6
546	10	0	11	F	3.22	3.5
546	11	0	11	F	3.47	3.5
547	1	1800	2	F	2.30	3.0
547	3	1800	2	M	2.41	3.1
548	1	0	12	F	3.75	3.8
548	2	0	12	M	3.51	3.9
548	3	0	12	M	3.35	3.7
548	4	0	12	F	3.46	3.7
548	5	0	12	F	3.90	3.7
548	7	0	12	F	3.73	3.5
548	8	0	12	F	3.83	3.7
548	9	0	12	M	3.76	3.6
548	10	0	12	F	3.58	3.7
548	11	0	12	F	3.63	3.5
548	12	0	12	F	3.37	3.7
548	13	0	12	M	3.53	3.8
549	4	1200	6	F	2.23	3.0
549	6	1200	6	M	2.66	3.3
549	7	1200	6	M	2.08	2.9
549	8	1200	6	M	2.35	3.1
549	9	1200	6	M	2.49	3.2
549	12	1200	6	M	2.25	3.0
550	1	0	12	M	3.47	3.7
550	2	0	12	M	3.85	3.7
550	3	0	12	F	3.58	3.6
550	5	0	12	F	3.71	3.5
550	6	0	12	F	3.52	3.5
550	7	0	12	M	3.52	3.6
550	8	0	12	M	3.31	3.6
550	9	0	12	M	3.38	3.5
550	11	0	12	F	3.55	3.5
550	12	0	12	M	3.33	3.6
550	13	0	12	M	3.56	3.7
550	14	0	12	F	3.39	3.5
551	1	800	8	F	2.75	3.3

551	3	800	8	M	3.35	3.5
551	4	800	8	M	3.07	3.3
551	6	800	8	F	2.96	3.3
551	7	800	8	F	2.99	3.2
551	9	800	8	M	3.20	3.5
551	10	800	8	M	3.26	3.4
551	11	800	8	F	3.12	3.3
552	1	800	12	F	2.63	3.1
552	2	800	12	F	2.68	3.3
552	3	800	12	M	2.69	3.1
552	4	800	12	M	2.71	3.2
552	6	800	12	M	2.49	3.2
552	7	800	12	F	2.78	3.3
552	8	800	12	F	2.38	3.2
552	9	800	12	M	2.49	3.1
552	10	800	12	M	2.40	3.0
552	12	800	12	M	2.82	3.4
552	13	800	12	M	2.87	3.3
552	14	800	12	M	2.59	3.2
553	1	0	14	F	3.46	3.6
553	2	0	14	F	3.77	3.4
553	3	0	14	M	3.97	3.6
553	4	0	14	F	3.76	3.6
553	5	0	14	M	3.58	3.4
553	6	0	14	F	3.73	3.5
553	7	0	14	M	3.91	3.6
553	8	0	14	M	3.86	3.7
553	9	0	14	M	3.58	3.6
553	10	0	14	F	3.73	3.5
553	11	0	14	F	3.65	3.5
553	12	0	14	M	3.71	3.8
553	13	0	14	M	3.84	3.5
553	14	0	14	M	3.66	3.7
554	1	1800	8	M	2.70	3.3
554	3	1800	8	M	3.14	3.0
554	6	1800	8	M	2.34	3.1
554	7	1800	8	M	2.33	3.1
554	8	1800	8	M	2.48	3.0
554	10	1800	8	M	2.82	3.2
554	11	1800	8	M	2.50	3.1
554	12	1800	8	M	2.42	3.2
555	13	1800	1	F	2.36	3.0
560	1	0	10	M	3.35	3.5
560	2	0	10	F	3.43	3.5
560	3	0	10	M	2.94	3.3
560	4	0	10	F	3.33	3.4
560	5	0	10	M	3.21	3.5
560	6	0	10	M	3.30	3.6
560	7	0	10	F	3.25	3.4
560	8	0	10	F	3.14	3.3
560	9	0	10	M	3.38	3.5
560	10	0	10	F	3.42	3.5

561	1	800	4	M	2.95	3.5
561	2	800	4	M	3.12	3.5
561	5	800	4	M	2.99	3.6
561	7	800	4	F	2.95	3.4
562	7	1200	4	F	2.49	3.1
562	8	1200	4	M	2.76	3.2
562	11	1200	4	F	2.68	3.2
562	13	1200	4	M	2.50	3.2
564	13	1200	1	M	2.54	3.3
567	7	800	3	M	3.04	3.6
567	8	800	3	M	2.64	3.4
567	11	800	3	M	3.18	3.6
568	3	800	11	M	2.59	3.3
568	4	800	11	F	2.82	3.4
568	5	800	11	M	2.93	3.5
568	6	800	11	F	2.73	3.4
568	7	800	11	M	2.83	3.4
568	8	800	11	F	2.72	3.4
568	9	800	11	M	2.86	3.4
568	10	800	11	F	2.79	3.4
568	11	800	11	F	2.94	3.4
568	12	800	11	F	2.90	3.4
568	13	800	11	M	3.02	3.5
569	1	0	10	M	3.55	3.7
569	2	0	10	F	3.35	3.6
569	4	0	10	F	3.40	3.6
569	5	0	10	F	3.16	3.7
569	6	0	10	F	3.70	3.6
569	7	0	10	M	3.76	3.6
569	8	0	10	M	3.67	3.5
569	9	0	10	M	2.88	3.5
569	10	0	10	F	3.30	3.6
569	12	0	10	F	3.06	3.5
572	1	800	7	M	3.25	3.7
572	2	800	7	M	3.07	3.5
572	6	800	7	F	2.50	3.4
572	7	800	7	F	2.89	3.5
572	8	800	7	M	3.12	3.5
572	11	800	7	F	3.05	3.5
572	12	800	7	M	3.15	3.6
573	1	0	12	F	4.16	3.9
573	2	0	12	M	3.90	3.8
573	3	0	12	F	4.05	3.9
573	4	0	12	M	4.09	3.8
573	5	0	12	F	3.92	4.0
573	6	0	12	M	4.39	4.0
573	7	0	12	M	4.37	3.9
573	8	0	12	F	3.95	4.0
573	9	0	12	M	3.85	3.8

573	11	0	12	F	3.84	3.9
573	12	0	12	M	3.73	3.8
573	13	0	12	F	3.51	
574	1	0	9	M	3.78	3.9
574	2	0	9	F	3.71	3.9
574	3	0	9	F	3.89	3.8
574	4	0	9	F	3.71	3.9
574	5	0	9	F	3.66	3.9
574	7	0	9	M	3.93	3.7
574	8	0	9	M	4.21	3.9
574	9	0	9	M	3.85	3.8
574	10	0	9	M	3.74	3.8
576	1	0	12	M	3.52	3.6
576	2	0	12	F	3.38	3.6
576	3	0	12	F	3.40	3.6
576	4	0	12	M	3.86	3.7
576	5	0	12	M	3.51	3.7
576	6	0	12	M	3.69	3.8
576	7	0	12	F	2.72	3.4
576	10	0	12	M	3.75	3.8
576	11	0	12	M	2.67	3.5
576	12	0	12	M	3.90	4.0
576	13	0	12	M	3.51	3.7
576	14	0	12	M	3.67	3.7
577	3	1800	3	M	2.37	3.2
577	5	1800	3	F	2.28	3.2
577	14	1800	3	M	2.37	3.1
578	4	1200	7	M	2.68	3.8
578	6	1200	7	M	2.58	3.7
578	9	1200	7	F	2.29	3.5
578	11	1200	7	M	2.78	3.5
578	13	1200	7	M	2.42	3.3
578	14	1200	7	M	2.74	3.5
578	15	1200	7	F	2.52	3.3
580	1	0	12	M	3.90	4.0
580	2	0	12	F	3.56	3.7
580	3	0	12	F	3.70	3.6
580	4	0	12	M	4.10	3.8
580	5	0	12	F	3.68	3.8
580	6	0	12	F	3.91	3.8
580	7	0	12	F	3.90	3.8
580	8	0	12	F	3.65	3.7
580	9	0	12	M	3.75	3.6
580	10	0	12	F	3.66	3.8
580	11	0	12	F	3.77	3.8
580	12	0	12	M	3.68	3.7
581	10	1800	2	M	2.57	3.1
581	12	1800	2	F	2.66	3.4
582	2	1200	11	M	2.92	3.6
582	4	1200	11	F	2.91	3.5

582	5	1200	11	M	3.12	3.5
582	6	1200	11	M	2.81	3.4
582	7	1200	11	M	3.10	3.5
582	8	1200	11	F	2.97	3.6
582	9	1200	11	M	3.08	3.4
582	10	1200	11	M	3.00	3.7
582	11	1200	11	M	2.82	3.4
582	13	1200	11	M	3.13	3.6
582	14	1200	11	M	3.22	3.6
583	1	0	12	F	3.65	3.8
583	2	0	12	M	3.83	3.8
583	3	0	12	M	3.71	3.8
583	4	0	12	M	3.85	3.8
583	5	0	12	F	3.63	3.8
583	6	0	12	M	3.80	3.8
583	7	0	12	F	3.50	3.7
583	8	0	12	F	3.52	3.7
583	9	0	12	M	3.87	3.8
583	10	0	12	M	3.80	3.8
583	11	0	12	F	3.66	3.8
583	12	0	12	F	3.79	3.8
585	1	0	13	M	3.55	3.8
585	2	0	13	M	3.44	3.9
585	3	0	13	F	3.46	3.8
585	4	0	13	M	3.79	3.9
585	5	0	13	M	3.52	3.7
585	6	0	13	F	3.56	3.8
585	7	0	13	M	3.86	3.9
585	8	0	13	M	3.86	3.9
585	9	0	13	M	3.77	3.9
585	10	0	13	M	3.60	3.8
585	11	0	13	F	3.53	3.7
585	12	0	13	F	3.49	3.7
585	13	0	13	M	3.34	3.7
587	1	800	13	M	3.09	3.7
587	2	800	13	F	2.91	3.5
587	3	800	13	F	3.05	3.6
587	5	800	13	F	2.99	3.5
587	6	800	13	F	2.75	3.5
587	7	800	13	M	3.12	3.6
587	8	800	13	F	2.67	3.5
587	9	800	13	F	2.73	3.5
587	10	800	13	M	3.18	3.5
587	11	800	13	F	2.51	3.4
587	12	800	13	M	3.05	3.5
587	13	800	13	M	2.81	3.5
587	14	800	13	F	2.66	3.4
588	1	800	11	M	3.06	3.5
588	2	800	11	M	2.88	3.4
588	3	800	11	M	3.21	3.5
588	5	800	11	M	3.29	3.6
588	6	800	11	M	3.19	3.6
588	7	800	11	F	2.96	3.5

588	9	800	11	F	2.97	3.4
588	10	800	11	F	2.94	3.4
588	11	800	11	F	3.03	3.4
588	12	800	11	M	3.16	3.5
588	13	800	11	F	3.16	3.7
589	1	0	11	F	3.34	3.7
589	2	0	11	M	3.72	3.8
589	3	0	11	F	3.52	3.8
589	4	0	11	F	3.41	3.8
589	5	0	11	F	3.51	3.7
589	6	0	11	M	3.72	3.7
589	7	0	11	F	3.62	3.8
589	8	0	11	M	3.73	3.7
589	9	0	11	F	3.44	3.7
589	10	0	11	F	3.46	3.8
589	11	0	11	M	3.68	3.8
591	1	1200	13	M	3.07	3.4
591	2	1200	13	F	2.66	3.3
591	3	1200	13	M	2.91	3.3
591	4	1200	13	M	2.83	3.4
591	5	1200	13	F	2.69	3.5
591	8	1200	13	M	3.12	3.4
591	9	1200	13	M	2.92	3.4
591	10	1200	13	F	2.98	3.5
591	11	1200	13	F	2.91	3.6
591	12	1200	13	M	2.78	3.4
591	13	1200	13	F	2.60	3.3
591	14	1200	13	F	2.79	3.4
591	15	1200	13	M	2.77	3.5
594	1	1200	7	F	2.67	3.5
594	3	1200	7	M	2.91	3.4
594	4	1200	7	F	2.58	3.3
594	5	1200	7	F	2.82	3.4
594	6	1200	7	M	2.64	3.3
594	7	1200	7	M	3.24	3.5
594	10	1200	7	M	2.90	3.4
595	1	1200	7	F	2.73	3.5
595	3	1200	7	F	2.64	3.4
595	4	1200	7	M	2.76	3.5
595	7	1200	7	F	2.39	3.1
595	8	1200	7	M	3.07	3.4
595	11	1200	7	F	2.42	3.0
595	12	1200	7	M	2.76	3.4
596	1	0	15	F	3.02	3.3
596	2	0	15	M	3.39	3.9
596	3	0	15	F	3.07	3.5
596	4	0	15	F	3.39	3.7
596	5	0	15	M	3.38	3.7
596	6	0	15	F	3.22	3.5
596	7	0	15	M	3.22	3.5
596	8	0	15	M	3.15	3.7
596	9	0	15	F	3.30	3.7

596	10	0	15	F	3.45	3.7
596	11	0	15	F	3.28	3.6
596	12	0	15	M	3.36	3.8
596	13	0	15	M	3.48	3.8
596	14	0	15	F	3.19	3.9
596	15	0	15	M	3.58	
597	7	1200	7	M	3.31	3.4
597	8	1200	7	M	3.20	3.4
597	11	1200	7	F	3.33	3.6
597	12	1200	7	F	3.28	3.5
597	13	1200	7	M	3.38	3.6
597	14	1200	7	F	3.12	3.3
597	15	1200	7	M	3.16	3.4
599	2	800	7	M	2.73	3.3
599	3	800	7	M	2.94	3.6
599	4	800	7	F	2.44	3.4
599	5	800	7	M	2.68	3.5
599	10	800	7	M	2.65	3.5
599	11	800	7	F	2.64	3.5
599	12	800	7	M	2.68	3.7
600	1	800	10	F	2.41	3.3
600	3	800	10	F	3.01	3.6
600	5	800	10	M	3.19	3.5
600	6	800	10	M	2.78	3.2
600	7	800	10	F	2.70	3.4
600	9	800	10	M	3.42	3.7
600	10	800	10	M	2.98	3.7
600	11	800	10	F	2.85	3.6
600	12	800	10	F	2.67	3.5
600	13	800	10	M	3.06	3.6
601	1	0	8	F	3.34	3.8
601	2	0	8	F	3.60	3.9
601	4	0	8	M	3.47	3.7
601	5	0	8	M	3.57	3.8
601	6	0	8	M	3.46	3.6
601	9	0	8	F	3.15	3.5
601	10	0	8	F	3.57	3.7
601	11	0	8	M	3.20	3.8
602	2	1200	9	F	2.50	3.5
602	4	1200	9	F	2.66	3.4
602	5	1200	9	M	2.44	3.3
602	9	1200	9	M	2.63	3.5
602	10	1200	9	F	2.21	3.2
602	11	1200	9	M	2.56	3.5
602	12	1200	9	M	2.62	3.6
602	13	1200	9	F	2.36	3.4
602	14	1200	9	M	2.46	3.5
603	3	800	11	F	3.16	3.6
603	4	800	11	M	3.20	3.6
603	5	800	11	F	3.15	3.4
603	6	800	11	M	3.19	3.5

603	7	800	11	M	3.07	3.5
603	9	800	11	M	3.28	3.7
603	11	800	11	M	3.20	3.6
603	12	800	11	F	3.28	3.4
603	13	800	11	M	3.24	3.5
603	14	800	11	M	3.22	3.6
603	15	800	11	F	3.03	3.4
604	1	0	9	M	4.03	3.9
604	2	0	9	F	3.89	3.8
604	4	0	9	F	3.94	3.9
604	6	0	9	M	4.39	4.0
604	7	0	9	M	4.12	3.9
604	8	0	9	F	3.72	3.6
604	9	0	9	F	3.81	3.7
604	10	0	9	M	3.98	4.0
604	11	0	9	M	3.98	3.9
605	2	0	12	F	3.86	3.7
605	3	0	12	M	4.50	3.9
605	4	0	12	F	3.93	3.8
605	5	0	12	M	4.27	3.8
605	6	0	12	M	4.39	3.8
605	7	0	12	M	4.01	3.9
605	8	0	12	F	3.64	3.9
605	9	0	12	M	4.12	3.9
605	10	0	12	M	3.98	3.8
605	11	0	12	F	3.57	3.5
605	12	0	12	M	4.36	3.8
605	13	0	12	M	3.98	3.8
606	1	0	11	M	3.59	3.7
606	2	0	11	F	3.39	3.5
606	3	0	11	M	3.60	3.6
606	4	0	11	F	3.33	3.5
606	5	0	11	F	3.29	3.4
606	6	0	11	M	3.94	3.7
606	7	0	11	M	3.90	3.6
606	8	0	11	F	3.52	3.7
606	9	0	11	M	3.78	3.8
606	11	0	11	M	3.67	3.9
606	12	0	11	F	3.49	3.7
608	11	1200	1	M	2.16	3.1
611	6	800	2	F	2.96	3.3
611	8	800	2	F	3.17	3.7
612	1	800	14	M	3.26	3.6
612	2	800	14	M	2.93	3.4
612	3	800	14	F	2.95	3.6
612	4	800	14	F	3.22	3.7
612	5	800	14	M	3.18	3.5
612	6	800	14	M	3.03	3.5
612	7	800	14	F	2.63	3.2
612	8	800	14	M	3.07	3.4
612	9	800	14	M	3.28	3.6
612	10	800	14	M	3.39	3.5

612	11	800	14	F	2.95	3.5
612	12	800	14	F	3.18	3.5
612	13	800	14	M	2.99	3.5
612	14	800	14	M	3.10	3.5
613	1	330	13	F	2.63	3.4
613	2	330	13	M	3.29	3.6
613	4	330	13	F	2.84	3.4
613	5	330	13	F	2.92	3.5
613	6	330	13	M	2.87	3.6
613	7	330	13	F	2.89	3.5
613	8	330	13	F	3.08	3.6
613	9	330	13	M	3.36	3.8
613	10	330	13	M	2.76	3.4
613	11	330	13	F	3.04	3.6
613	12	330	13	M	3.07	3.7
613	13	330	13	F	3.01	3.4
613	14	330	13	F	2.84	3.4
614	1	330	1	M	3.34	3.8
615	1	330	14	M	3.23	3.8
615	2	330	14	F	3.01	3.5
615	3	330	14	M	3.01	3.8
615	4	330	14	F	3.15	3.7
615	5	330	14	M	3.12	3.5
615	6	330	14	M	3.44	3.7
615	7	330	14	M	3.08	3.6
615	8	330	14	F	3.02	3.6
615	9	330	14	F	3.13	3.7
615	11	330	14	F	3.02	3.7
615	12	330	14	M	3.02	3.5
615	13	330	14	F	3.15	3.6
615	14	330	14	M	2.97	3.6
615	15	330	14	M	2.95	3.6
616	1	330	12	M	3.04	3.5
616	2	330	12	M	3.08	3.5
616	3	330	12	M	2.87	3.5
616	4	330	12	F	3.17	3.5
616	5	330	12	F	2.99	3.4
616	7	330	12	F	3.39	3.6
616	8	330	12	F	3.24	3.6
616	9	330	12	M	3.20	3.6
616	10	330	12	F	3.50	3.7
616	11	330	12	F	2.95	3.6
616	12	330	12	M	3.40	3.7
616	14	330	12	F	3.36	3.6
617	1	330	11	F	2.92	3.4
617	2	330	11	M	3.22	3.5
617	3	330	11	F	2.87	3.5
617	4	330	11	F	3.24	3.6
617	6	330	11	F	3.23	3.6
617	7	330	11	F	2.89	3.5
617	8	330	11	F	2.65	3.4
617	9	330	11	F	2.85	3.5

617	10	330	11	M	3.15	3.6
617	11	330	11	M	3.20	3.6
617	12	330	11	M	3.60	3.7
618	2	330	6	M	3.28	3.6
618	3	330	6	F	2.88	3.8
618	4	330	6	F	3.08	3.6
618	6	330	6	F	3.00	3.5
618	8	330	6	F	3.00	3.5
618	9	330	6	M	3.00	3.3
619	1	330	13	M	3.00	3.5
619	3	330	13	F	3.04	3.5
619	4	330	13	F	2.70	3.5
619	5	330	13	M	2.81	3.5
619	6	330	13	M	2.90	3.5
619	7	330	13	M	1.88	3.5
619	8	330	13	F	3.01	3.8
619	9	330	13	F	2.65	3.5
619	10	330	13	M	2.56	3.4
619	11	330	13	M	2.86	3.5
619	12	330	13	M	3.13	3.7
619	13	330	13	M	2.80	3.5
619	14	330	13	F	2.68	3.3
620	1	330	13	M	3.11	3.8
620	2	330	13	F	3.05	3.8
620	3	330	13	M	3.14	3.7
620	5	330	13	M	3.33	3.6
620	6	330	13	F	3.31	3.6
620	7	330	13	F	3.17	3.6
620	8	330	13	F	2.79	3.4
620	9	330	13	M	3.39	3.8
620	10	330	13	F	3.06	3.8
620	11	330	13	F	3.22	3.5
620	12	330	13	F	3.05	3.5
620	13	330	13	F	3.23	3.6
620	15	330	13	M	3.15	3.6
621	1	330	12	F	3.27	3.5
621	2	330	12	M	3.55	3.4
621	3	330	12	M	3.57	3.6
621	4	330	12	F	3.37	3.6
621	5	330	12	M	3.45	3.6
621	6	330	12	M	3.49	3.6
621	7	330	12	M	3.62	3.8
621	8	330	12	M	3.76	3.7
621	9	330	12	F	3.77	3.8
621	10	330	12	F	3.61	3.8
621	11	330	12	M	3.61	3.7
621	12	330	12	F	3.60	3.7
622	1	330	13	F	2.98	3.3
622	3	330	13	F	3.21	3.5
622	4	330	13	M	3.44	3.7
622	5	330	13	M	3.13	3.7
622	6	330	13	F	3.01	3.5

622	7	330	13	M	2.86	3.4
622	8	330	13	F	2.99	3.4
622	9	330	13	M	3.50	3.6
622	10	330	13	F	2.83	3.4
622	11	330	13	M	3.40	3.6
622	12	330	13	F	2.95	3.4
622	13	330	13	M	3.56	3.6
622	14	330	13	M	3.16	3.6
623	1	0	12	M	3.58	3.5
623	2	0	12	M	3.77	3.8
623	3	0	12	M	3.69	3.6
623	4	0	12	F	3.75	3.8
623	5	0	12	M	3.82	3.7
623	6	0	12	F	3.63	3.5
623	7	0	12	F	3.66	3.6
623	8	0	12	M	3.28	3.5
623	9	0	12	F	2.94	3.4
623	10	0	12	M	3.58	3.6
623	11	0	12	F	3.66	3.7
623	13	0	12	M	3.64	3.6
624	1	0	12	M	3.93	3.9
624	2	0	12	F	3.84	3.9
624	3	0	12	M	3.98	3.9
624	4	0	12	M	3.92	3.8
624	5	0	12	M	3.82	3.8
624	6	0	12	F	3.64	3.8
624	7	0	12	F	3.74	3.7
624	8	0	12	F	3.62	3.7
624	9	0	12	M	4.05	3.8
624	10	0	12	M	4.10	3.9
624	11	0	12	F	3.70	3.8
624	12	0	12	M	3.80	3.9
625	1	330	15	M	3.46	3.7
625	2	330	15	F	3.38	3.5
625	3	330	15	F	3.46	3.5
625	4	330	15	F	3.40	3.5
625	5	330	15	M	3.66	3.6
625	6	330	15	F	3.58	3.6
625	7	330	15	M	3.68	3.8
625	8	330	15	F	3.54	3.6
625	9	330	15	M	3.64	3.5
625	10	330	15	M	3.59	3.6
625	11	330	15	F	3.57	3.5
625	12	330	15	M	3.94	3.7
625	13	330	15	M	3.63	3.6
625	14	330	15	F	3.28	3.5
625	15	330	15	F	3.69	3.6
626	1	330	10	F	3.09	3.5
626	2	330	10	F	2.63	3.5
626	3	330	10	M	2.98	3.2
626	4	330	10	M	3.30	3.5
626	5	330	10	M	2.86	3.4

626	6	330	10	M	2.61	3.3
626	7	330	10	F	2.86	3.4
626	8	330	10	M	3.14	3.5
626	9	330	10	M	3.18	3.6
626	10	330	10	M	3.12	3.5
627	1	330	16	F	2.51	3.1
627	2	330	16	M	3.02	3.5
627	3	330	16	M	2.81	3.4
627	4	330	16	M	2.75	3.4
627	5	330	16	M	3.07	3.5
627	6	330	16	M	2.69	3.3
627	7	330	16	M	2.76	3.3
627	8	330	16	F	2.44	3.4
627	9	330	16	F	2.62	3.4
627	10	330	16	F	2.85	3.3
627	11	330	16	M	2.65	3.4
627	12	330	16	F	2.65	3.3
627	13	330	16	F	2.58	3.3
627	14	330	16	M	2.84	3.5
627	15	330	16	M	2.79	3.4
627	16	330	16	M	2.42	3.3
629	1	330	15	F	2.88	3.5
629	2	330	15	F	2.94	3.4
629	3	330	15	M	2.05	3.0
629	4	330	15	F	2.58	3.4
629	5	330	15	F	2.71	3.5
629	6	330	15	M	2.91	3.6
629	8	330	15	F	2.70	3.5
629	9	330	15	F	3.18	3.5
629	10	330	15	F	2.76	3.6
629	11	330	15	F	2.75	3.5
629	12	330	15	M	3.09	3.6
629	13	330	15	M	3.23	3.7
629	14	330	15	F	2.81	3.5
629	15	330	15	M	2.95	3.4
629	16	330	15	M	3.32	3.6
630	2	330	10	F	3.22	3.6
630	3	330	10	F	3.57	3.8
630	4	330	10	M	3.21	3.5
630	5	330	10	F	3.28	3.6
630	6	330	10	F	3.40	3.6
630	7	330	10	M	3.28	3.6
630	8	330	10	F	3.40	3.5
630	9	330	10	F	3.05	3.5
630	10	330	10	F	3.38	3.6
630	11	330	10	F	3.39	3.5
631	1	330	9	M	3.53	3.6
631	3	330	9	M	3.68	3.8
631	4	330	9	M	3.80	3.7
631	5	330	9	M	3.26	3.5
631	7	330	9	F	3.61	3.6
631	8	330	9	F	3.35	3.5
631	9	330	9	M	3.57	3.6

631	10	330	9	F	3.64	3.7
631	11	330	9	M	3.58	3.7
632	1	0	11	M	3.77	3.8
632	2	0	11	F	3.81	3.7
632	3	0	11	M	3.99	3.8
632	4	0	11	M	3.98	4.0
632	5	0	11	M	3.91	3.6
632	6	0	11	F	3.72	3.7
632	7	0	11	M	3.87	3.9
632	8	0	11	F	3.89	3.8
632	9	0	11	M	3.85	3.7
632	10	0	11	M	3.82	3.7
632	12	0	11	F	3.66	3.6
633	1	330	12	F	2.61	3.8
633	2	330	12	F	3.02	3.6
633	3	330	12	F	2.97	3.4
633	4	330	12	F	3.12	3.5
633	5	330	12	M	2.94	3.4
633	6	330	12	M	3.15	3.5
633	7	330	12	F	3.17	3.6
633	8	330	12	F	2.81	3.4
633	9	330	12	M	3.14	3.5
633	10	330	12	F	3.05	3.5
633	11	330	12	M	2.98	3.6
633	12	330	12	M	2.66	3.4
634	1	0	11	M	3.66	3.7
634	2	0	11	F	3.68	3.6
634	4	0	11	F	3.69	3.7
634	5	0	11	F	3.48	3.7
634	6	0	11	F	3.55	3.6
634	7	0	11	M	3.81	3.6
634	8	0	11	F	3.61	3.7
634	9	0	11	M	3.90	3.8
634	10	0	11	F	3.73	3.6
634	11	0	11	M	3.71	3.7
634	12	0	11	M	3.52	3.5
635	2	0	6	M	3.54	3.5
635	3	0	6	F	3.43	3.4
635	4	0	6	M	3.67	3.6
635	6	0	6	M	3.41	3.4
635	7	0	6	F	3.55	3.6
635	8	0	6	M	3.63	3.5
636	1	0	14	M	3.60	3.6
636	2	0	14	M	3.99	3.8
636	4	0	14	M	3.75	3.7
636	5	0	14	F	3.46	3.8
636	6	0	14	M	3.90	3.8
636	7	0	14	M	4.24	4.0
636	8	0	14	M	3.92	3.8
636	9	0	14	F	3.66	3.7
636	10	0	14	M	3.97	3.8
636	11	0	14	M	3.99	3.8

636	12	0	14	F	3.61	3.6
636	13	0	14	M	3.71	3.7
636	15	0	14	F	3.54	3.6
636	16	0	14	F	3.54	3.6
637	1	330	14	F	3.47	3.6
637	2	330	14	F	3.37	3.5
637	3	330	14	F	3.63	3.5
637	4	330	14	M	3.59	3.7
637	5	330	14	F	3.41	3.6
637	6	330	14	F	3.32	3.6
637	7	330	14	F	2.56	3.3
637	8	330	14	M	3.36	3.5
637	9	330	14	M	3.42	3.7
637	10	330	14	M	3.66	3.7
637	11	330	14	M	3.49	3.8
637	12	330	14	F	3.52	3.8
637	13	330	14	M	3.72	3.7
637	14	330	14	M	3.76	3.9
638	1	330	11	M	3.08	3.6
638	3	330	11	M	3.32	3.7
638	4	330	11	M	3.14	3.5
638	5	330	11	F	2.94	3.5
638	6	330	11	M	3.08	3.4
638	7	330	11	F	3.24	3.6
638	9	330	11	M	2.97	3.4
638	10	330	11	M	3.39	3.7
638	11	330	11	M	3.12	3.6
638	12	330	11	F	2.45	3.3
638	13	330	11	F	2.95	3.5

C. Input Data and Results for BMDS Modeling of Litter Incidence Data for Levocardia (Smith et al., 1989)

Table C-1. BMD modeling results for litter incidence of levocardia (Smith et al., 1989)

Model	Goodness-of-fit *p* value	AIC	BMD_{05}	$BMDL_{05}$	BMD_{10}	$BMDL_{10}$
Multistage	0.9430	69.8459	42	31[a]	86	64[a]
Gamma	0.9430	69.8459	42	31[a]	86	64[a]
Log-logistic	0.9106	71.6069	74	17	122	36
Log-probit	0.9069	71.6259	87	9	130	20
Weibull	0.8648	71.8203	36	1	76	5
Logistic	0.0520[b]	80.642	144	101	253	187
Probit	0.0449[b]	80.6568	136	99	244	185

[a]Preferred model(s) based on criteria described in U.S. EPA (2000d).
[b]Because goodness-of-fit *p* values were below the recommended minimum value of 0.1, the results of these models were not further considered for estimation of the BMD.

Of the seven models fitted, five (i.e., multistage, gamma, log-logistic, log-probit, and Weibull) showed adequate fit, and thus the BMDS outputs from these five models are provided below. BMD and BMDL values associated with a BMR of both 5 and 10% are shown.

```
 ======================================================================
          $Revision: 2.2 $ $Date: 2001/03/14 01:17:00 $
         Input Data File: C:\BMDS\UNSAVED1.(d)
         Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                              Mon Apr 19 14:12:21 2004
 ======================================================================

 BMDS MODEL RUN: GAMMA
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response]= background+(1-background)*CumGamma[slope*dose,power],
   where CumGamma(.) is the cumulative Gamma distribution function

   Dependent variable = COLUMN3
   Independent variable = COLUMN1
   Power parameter is restricted as power >=1

   Total number of observations = 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 (and Specified) Parameter Values
```

```
                    Background =    0.0185185
                         Slope =   0.00171859
                         Power =          1.3

          Asymptotic Correlation Matrix of Parameter Estimates

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

                 Slope

     Slope            1

                         Parameter Estimates

       Variable           Estimate             Std. Err.
     Background                  0               NA
          Slope          0.00122482         0.000223166
          Power                   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)  Deviance  Test DF     P-value
     Full model        -33.5379
   Fitted model        -33.9229      0.770003      4          0.9424
  Reduced model        -57.0522       47.0286      4         <.0001

          AIC:         69.8459


                    Goodness  of  Fit

                                                                    Scaled
    Dose     Est._Prob.    Expected    Observed     Size       Residual
  ---------------------------------------------------------------------
    0.0000      0.0000          0.000          0           26            0
  330.0000      0.3325          6.317          6           19      -0.1545
  800.0000      0.6246         10.619         12           17       0.6918
 1200.0000      0.7700         10.780         10           14      -0.4956
 1800.0000      0.8897          7.118          7            8      -0.1329

 Chi-square =       0.77     DF = 4        P-value = 0.9430


   Benchmark Dose Computation

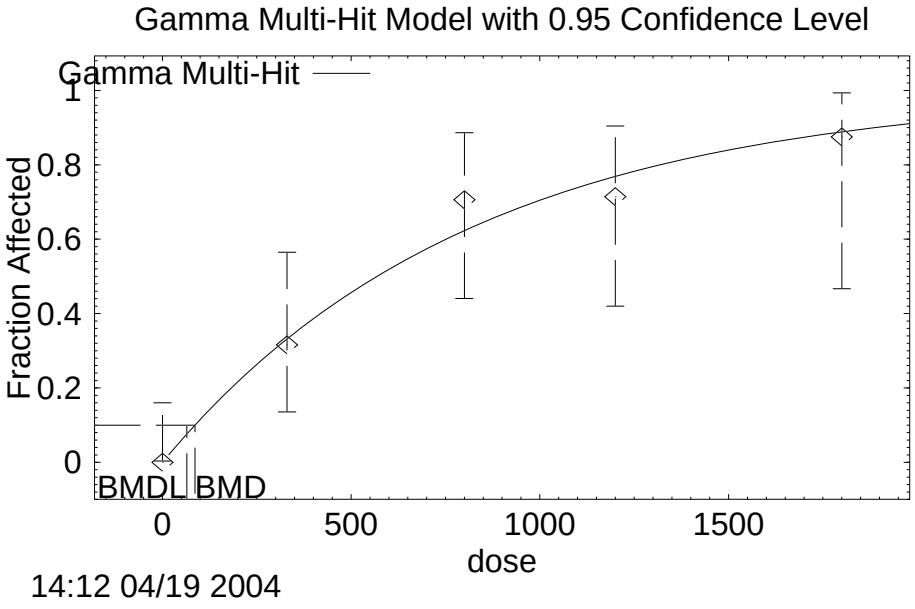
Specified effect =      0.05                  0.1

Risk Type        =      Extra risk

Confidence level =      0.95

             BMD =      41.8783               86.0214

            BMDL =      31.3527               64.4009
```



Graph shows the BMD and BMDL associated with a BMR = 0.1.

```
================================================================
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\BMDS\UNSAVED1.(d)
        Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                              Mon Apr 19 14:21:37 2004
 ================================================================

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

   The form of the probability function is:

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


   Dependent variable = COLUMN3
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 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


   User has chosen the log transformed model

                 Default Initial Parameter Values
                    background =            0
                     intercept =     -9.49904
                         slope =      1.51318

           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           -1

     slope           -1            1


                          Parameter Estimates

       Variable           Estimate             Std. Err.
     background                  0                   NA
      intercept            -9.37008             3.27162
          slope             1.49364             0.500096

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)	Deviance	Test DF	P-value
Full model	-33.5379			
Fitted model	-33.8035	0.531045	3	0.912
Reduced model	-57.0522	47.0286	4	<.0001

AIC: 71.6069

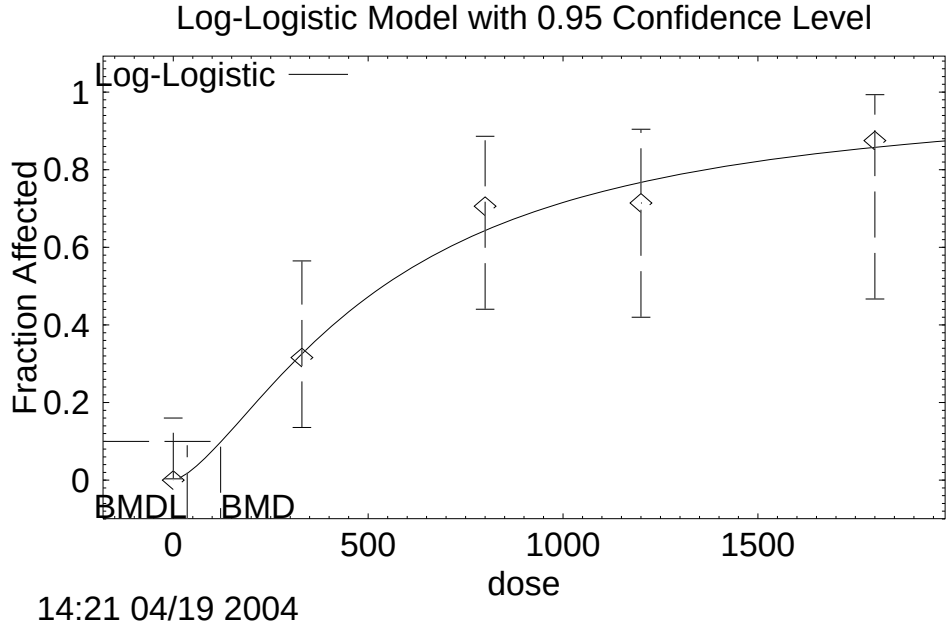
Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	26	0
330.0000	0.3300	6.269	6	19	-0.1315
800.0000	0.6489	11.032	12	17	0.492
1200.0000	0.7721	10.809	10	14	-0.5153
1800.0000	0.8612	6.890	7	8	0.1126

Chi-square = 0.54 DF = 3 P-value = 0.9106

Benchmark Dose Computation

Specified effect =	0.05	0.1
Risk Type =	Extra risk	
Confidence level =	0.95	
BMD =	73.8468	121.785
BMDL =	17.0566	36.0084



Graph shows the BMD and BMDL associated with a BMR = 0.1.

```
 ================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\UNSAVED1.(d)
        Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                              Mon Apr 19 14:30:14 2004
 ================================================================

 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 = COLUMN3
   Independent variable = COLUMN1

 Total number of observations = 5
 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 =    0.0545961
                        Beta(1) =   0.00112706

           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.
     Background                  0                   NA
        Beta(1)          0.00122482          0.000276934

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)  Deviance  Test DF     P-value
     Full model         -33.5379
   Fitted model         -33.9229      0.770003      4          0.9424
  Reduced model         -57.0522       47.0286      4         <.0001
```

AIC: 69.8459

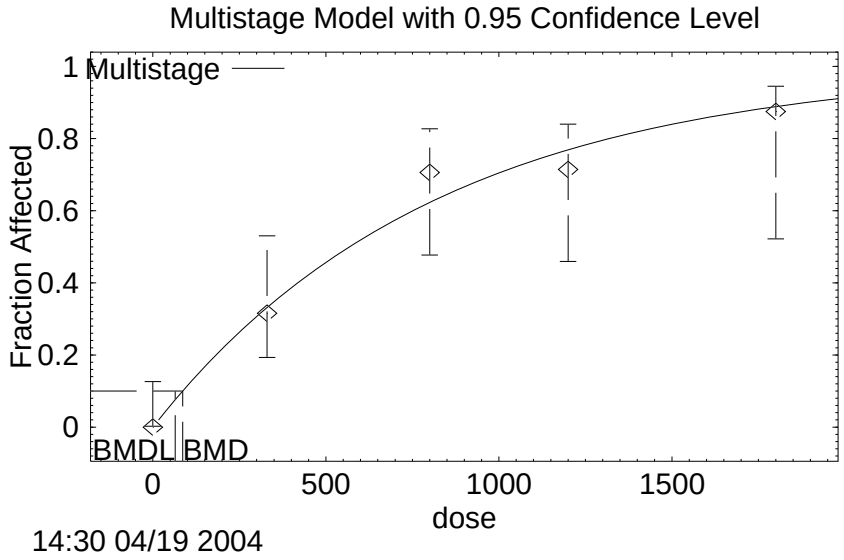
Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.
i: 1					
0.0000	0.0000	0.000	0	26	0.000
i: 2					
330.0000	0.3325	6.317	6	19	-0.075
i: 3					
800.0000	0.6246	10.619	12	17	0.347
i: 4					
1200.0000	0.7700	10.780	10	14	-0.315
i: 5					
1800.0000	0.8897	7.118	7	8	-0.150

Chi-square = 0.77 DF = 4 P-value = 0.9430

Benchmark Dose Computation

Specified effect =	0.05	0.1
Risk Type =	Extra risk	
Confidence level =	0.95	
BMD =	41.8783	86.0214
BMDL =	31.3527	64.4009



Graph shows the BMD and BMDL associated with a BMR = 0.1.

```
 ================================================================
        Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
        Input Data File: C:\BMDS\UNSAVED1.(d)
        Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                              Mon Apr 19 14:34:52 2004
 ================================================================

 BMDS MODEL RUN  Log Probit
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   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 = COLUMN3
   Independent variable = COLUMN1
   Slope parameter is not restricted

   Total number of observations = 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

   User has chosen the log transformed model

                  Default Initial (and Specified) Parameter Values
                     background =            0
                      intercept =     -5.72134
                          slope =     0.911264

           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           -1

     slope           -1            1


                          Parameter Estimates

       Variable           Estimate             Std. Err.
     background                  0                   NA
      intercept           -5.71203             1.93477
          slope           0.909892             0.294137
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)	Deviance	Test DF	P-value
Full model	-33.5379			
Fitted model	-33.813	0.550064	3	0.9078
Reduced model	-57.0522	47.0286	4	<.0001

AIC: 71.6259

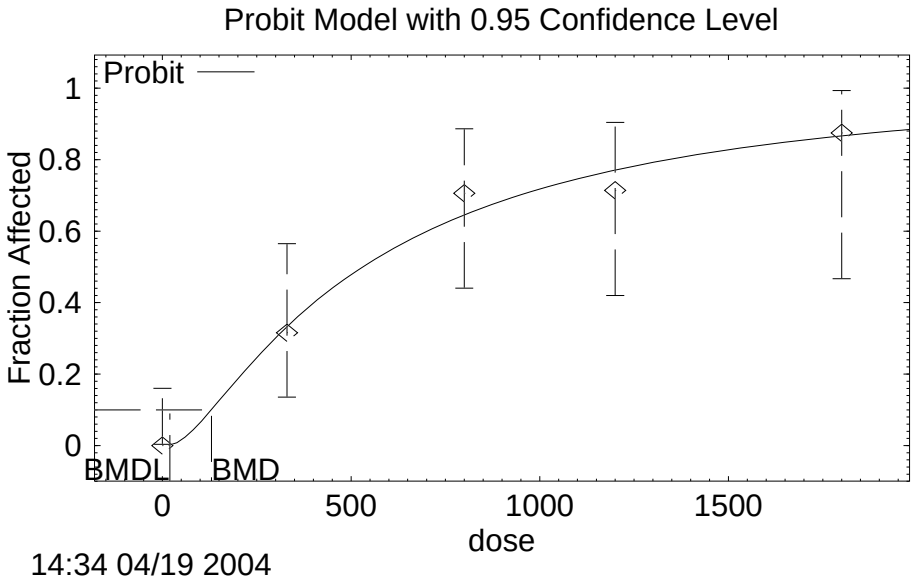
Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	26	0
330.0000	0.3316	6.301	6	19	-0.1464
800.0000	0.6444	10.955	12	17	0.5296
1200.0000	0.7701	10.781	10	14	-0.4963
1800.0000	0.8661	6.929	7	8	0.07399

Chi-square = 0.55 DF = 3 P-value = 0.9069

Benchmark Dose Computation

Specified effect =	0.05	0.1
Risk Type =	Extra risk	
Confidence level =	0.95	
BMD =	87.3528	130.221
BMDL =	8.59815	19.6033



Graph shows the BMD and BMDL associated with a BMR = 0.1.

```
 ================================================================
        Weibull Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\UNSAVED1.(d)
        Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                              Mon Apr 19 14:50:47 2004
 ================================================================

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

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^power)]

   Dependent variable = COLUMN3
   Independent variable = COLUMN1
   Power parameter is not restricted

   Total number of observations = 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 (and Specified) Parameter Values
                     Background =    0.0185185
                          Slope =    0.0025741
                          Power =     0.871847

           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 )

                  Slope        Power

     Slope            1           -1

     Power           -1            1


                          Parameter Estimates

       Variable           Estimate             Std. Err.
     Background                  0                 NA
          Slope          0.00172885          0.00372313
          Power            0.949024            0.317666

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)	Deviance	Test DF	P-value
Full model	-33.5379			
Fitted model	-33.9102	0.74446	3	0.8627
Reduced model	-57.0522	47.0286	4	<.0001

AIC: 71.8203

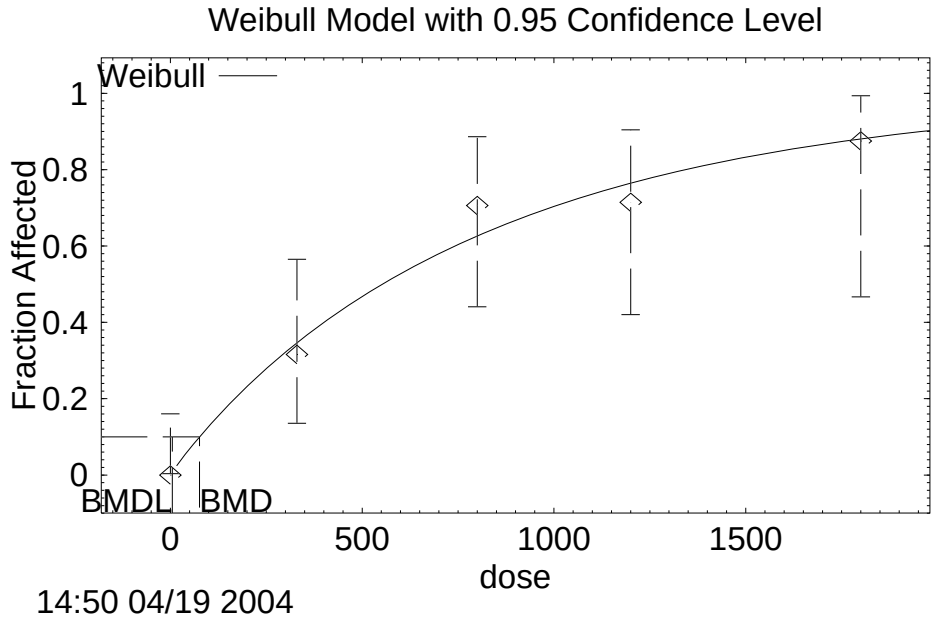
Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	26	0
330.0000	0.3459	6.572	6	19	-0.276
800.0000	0.6261	10.643	12	17	0.6801
1200.0000	0.7643	10.701	10	14	-0.4413
1800.0000	0.8804	7.043	7	8	-0.04715

Chi-square = 0.74 DF = 3 P-value = 0.8648

Benchmark Dose Computation

Specified effect =	0.05	0.1
Risk Type =	Extra risk	
Confidence level =	0.95	
BMD =	35.5948	75.9968
BMDL =	1.01863	5.08236



Graph shows the BMD and BMDL associated with a BMR = 0.1.

APPENDIX D. MODELING OF LIVER TUMOR INCIDENCE DATA FOR MICE EXPOSED TO TRICHLOROACETIC ACID IN DRINKING WATER

Using the EPA BMDS (version 1.4.1), the multistage model was fit to liver tumor incidence data (i.e., adenomas and carcinomas combined) from bioassays in $B6C3F_1$ mice exposed to TCA in drinking water for 52 weeks (two studies in male mice: Bull et al. [2002, 1990]), 60 weeks (one study in male mice: DeAngelo et al. [2008]), 82 weeks (one study in female mice: Pereira [1996]), and 104 weeks (one study in male mice: DeAngelo et al. [2008]). The tumor incidence data for adenomas, carcinomas, and adenomas and carcinomas combined are shown in Tables 5-8 through 5-12 in Section 5.4.2.

Average daily intakes from these mouse studies were converted to human equivalent doses for continuous lifetime exposure by using an interspecies scaling factor of 0.15 ([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) and exposure duration scaling factors of 0.132, 0.203, or 0.520 to adjust the 52-, 60-, or 82-week doses, respectively, to equivalent lifetime exposures ([duration of experiment/duration of life]^3 = [52/102]^3 = 0.132 or = [60/102]^3 = 0.203 or [82/102]^3 = 0.520). These factors for adjusting to lifetime equivalent doses are 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). An exposure duration scaling factor was not used in converting animal doses to human equivalents in the 104-week study of DeAngelo et al. (2008) because 104 weeks represents a lifetime exposure in mice.

Individual animal data (specifying when tumors were detected in each animal with a liver tumor) from the five bioassays were not available, precluding application of more sophisticated approaches to estimating lifetime cancer risks (e.g., by fitting models that predict tumor incidence as a function of two explanatory variables, dose and time). 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 one-stage multistage model provided the best fit to the data as determined by the chi-square goodness-of-fit statistic and AIC. Model predictions compared with observed incidences are shown in Figures D-1, D-2, D-3, D-4, and D-5 of this appendix.

Adequacy of model fit was evaluated for each of the data sets through use of the chi-square goodness-of-fit statistic. The fitted model was used to estimate the ED_{10}, and its corresponding LED_{10}. Candidate oral cancer slope factors were derived by linear extrapolation from the LED_{10} (i.e., $0.1/LED_{10}$).

The slope factors based on the tumor responses in male mice in the Bull et al. (2002, 1990) and DeAngelo et al. (2008) studies and the tumor responses in female mice in the Pereira (1996) study ranged from 2×10^{-2} to 2×10^{-1} per mg/kg-day (Table 5-13). The four slope factors derived from male mice varied by less than fourfold.

The standard output from BMDS (version 1.4.1) is reproduced below for each of the five data sets that were modeled.

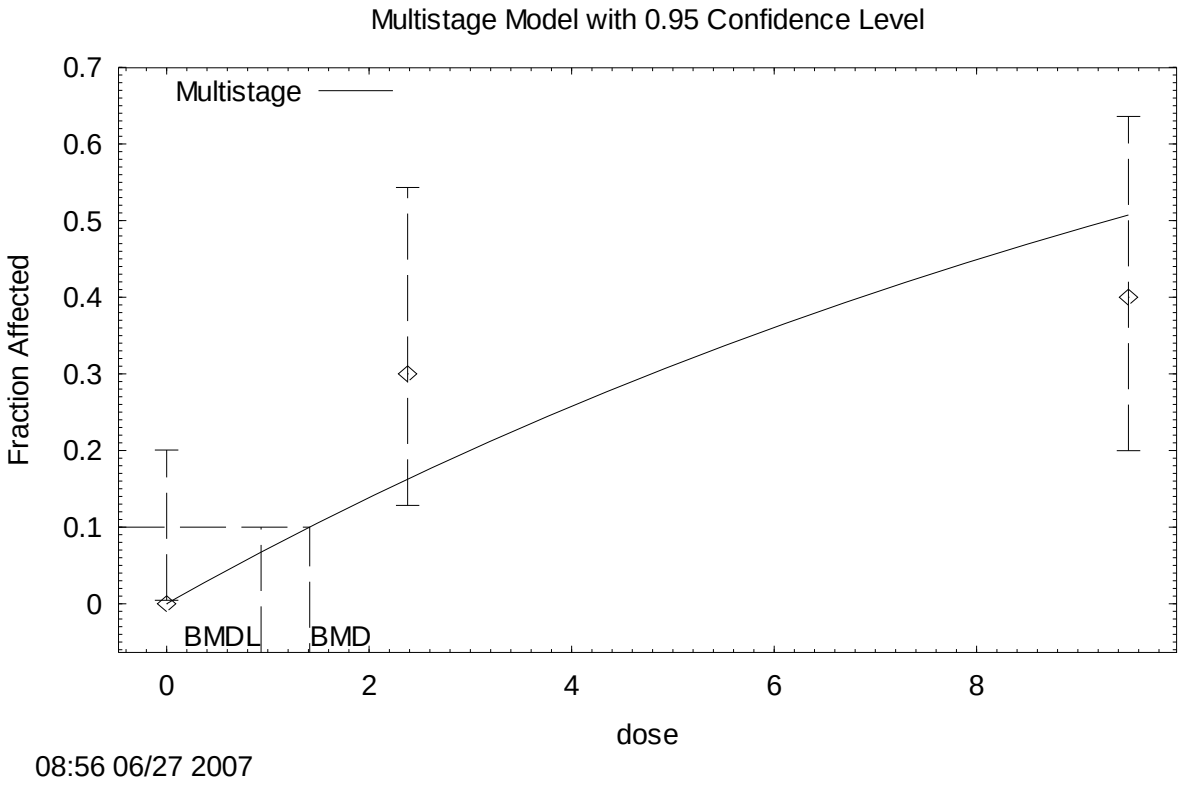


Figure D-1. Observed and predicted combined incidences of hepatocellular adenomas and carcinomas, based on responses in male B6C3F$_1$ mice exposed to TCA in drinking water for 52 weeks.

Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day. BMD and BMDL refer to ED_{10} and LED_{10}, respectively.

Source: Bull et al. (2002).

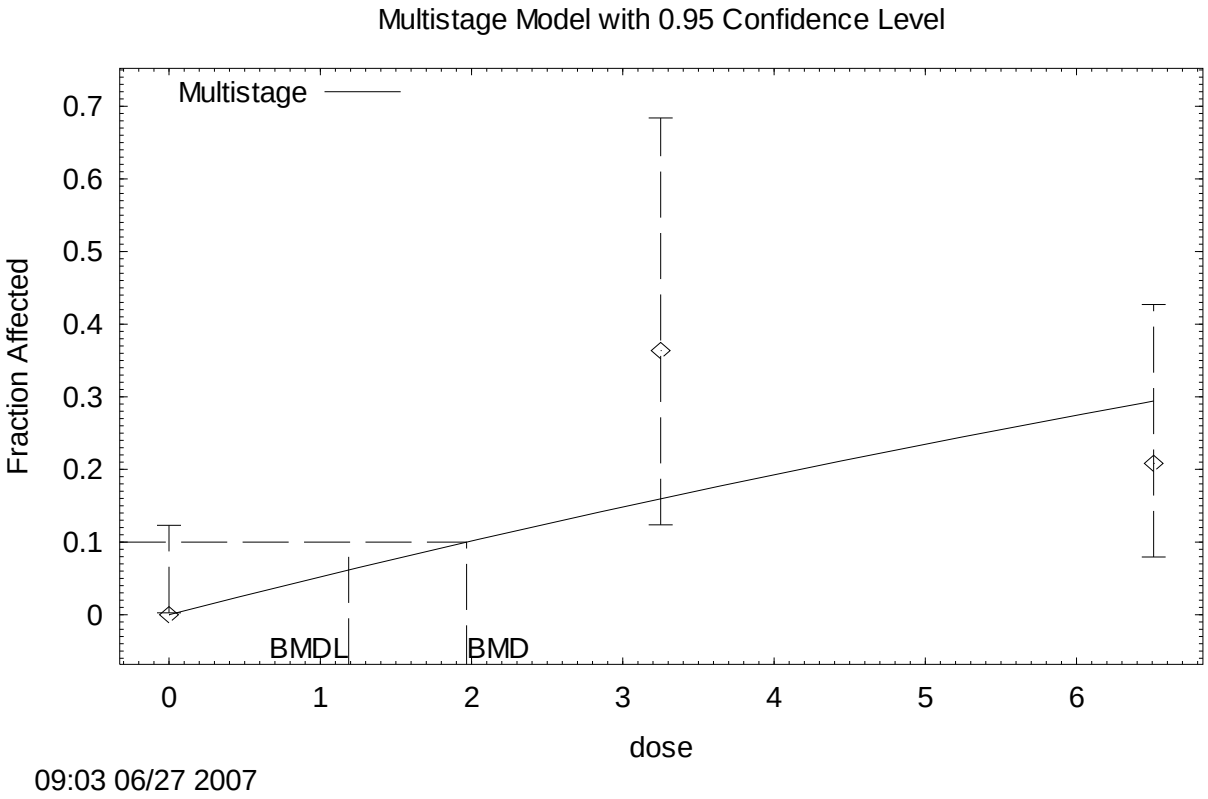


Figure D-2. Predicted and observed combined incidences of hepatocellular adenomas and carcinomas, based on responses in male B6C3F$_1$ mice exposed to TCA in drinking water for 52 weeks.

Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day. BMD and BMDL refer to ED_{10} and LED_{10}, respectively.

Source: Bull et al. (1990).

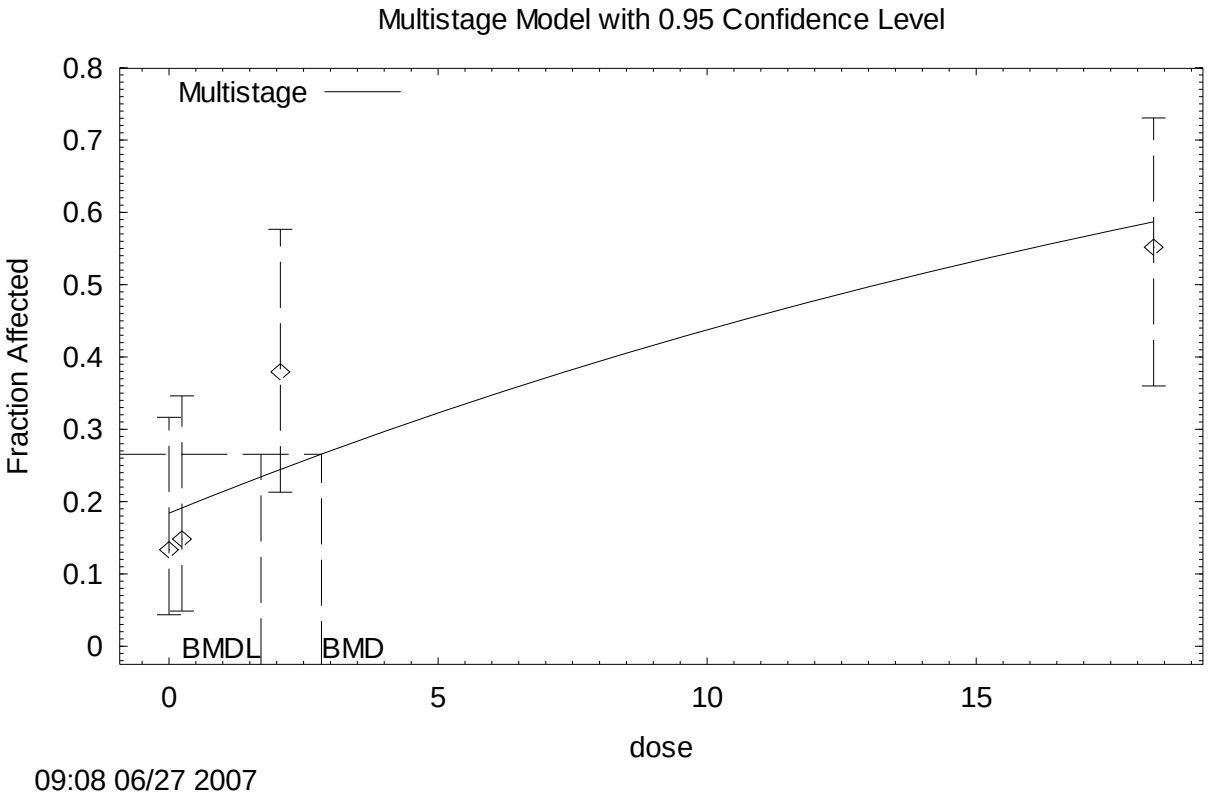


Figure D-3. Predicted and observed combined incidence of hepatocellular adenomas and carcinomas, based on responses in male B6C3F$_1$ mice exposed to TCA in drinking water for 60 weeks.

Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day. BMD and BMDL refer to ED_{10} and LED_{10}, respectively.

Source: DeAngelo et al. (2008).

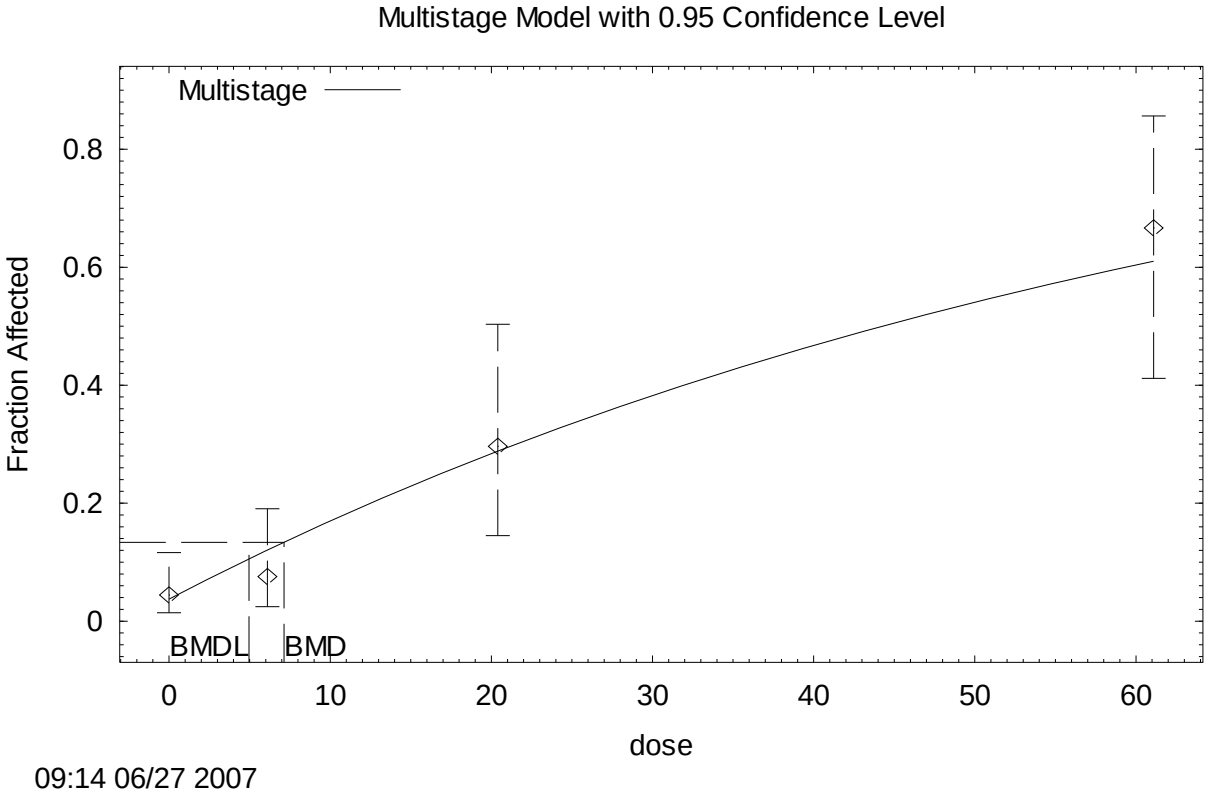


Figure D-4. Predicted and observed combined incidence of hepatocellular adenomas and carcinomas, based on responses in female B6C3F$_1$ mice exposed to TCA in drinking water for 82 weeks.

Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day. BMD and BMDL refer to ED_{10} and LED_{10}, respectively.

Source: Pereira (1996).

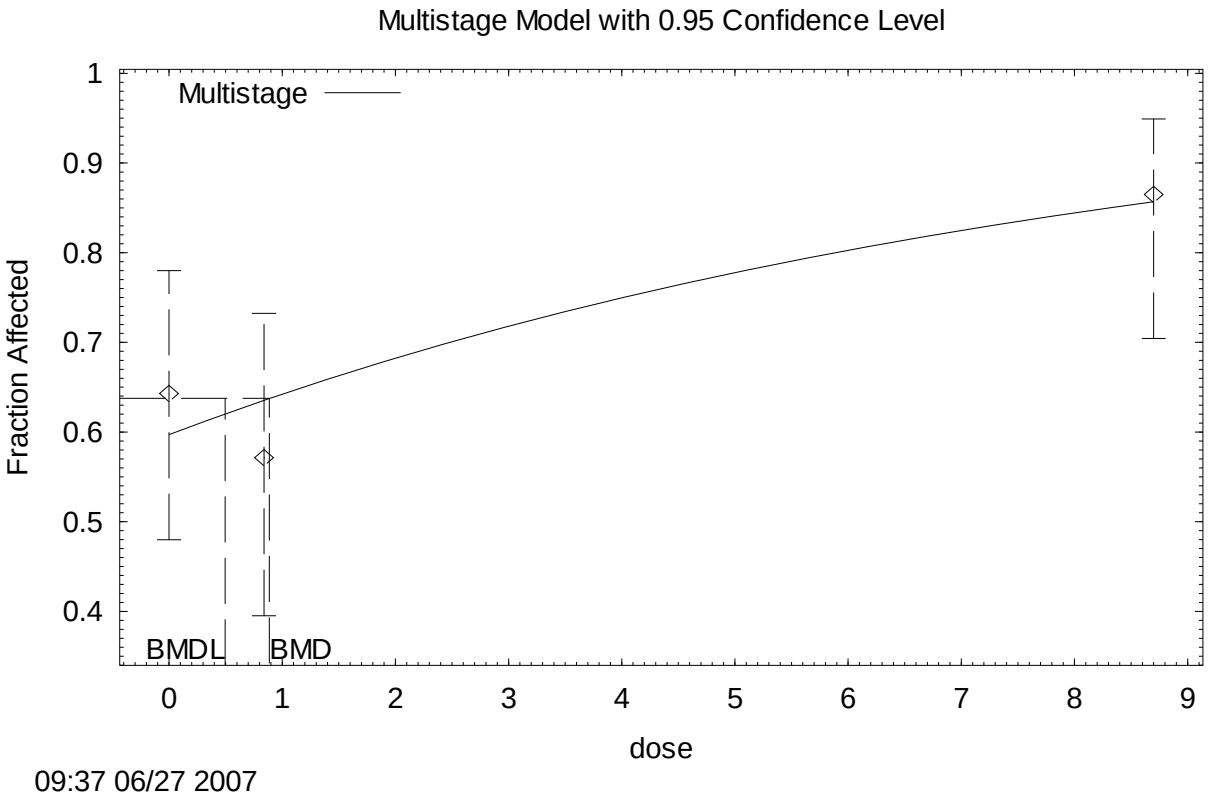


Figure D-5. Predicted and observed combined incidence of hepatocellular adenomas and carcinomas, based on responses in male B6C3F$_1$ mice exposed to TCA in drinking water for 104 weeks.

Note: Doses on x-axis are human equivalent doses for lifetime exposure in units of mg/kg-day. BMD and BMDL refer to ED_{10} and LED_{10}, respectively.

Source: DeAngelo et al. (2008).

BMDS Outputs

Bull et al. (2002)

```
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 = Response
   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 =     0.100138
                        Beta(1) =     0.046377


           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

                                                          95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf.
Limit
     Background                0            *                *                   *
        Beta(1)        0.0745471            *                *                   *

* - 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        -27.3086         1       3.26212      2          0.1957
  Reduced model        -32.5964         1       13.8377      2        0.000989

           AIC:         56.6172


                                  Goodness  of  Fit
                                                                    Scaled
```

```
     Dose     Est._Prob.    Expected    Observed     Size       Residual
  ----------------------------------------------------------------------
    0.0000     0.0000         0.000         0          20        0.000
    2.3800     0.1626         3.251         6          20        1.666
    9.5000     0.5075        10.149         8          20       -0.961

 Chi^2 = 3.70      d.f. = 2        P-value = 0.1573


   Benchmark Dose Computation

Specified effect =           0.1

Risk Type        =      Extra risk

Confidence level =          0.95

             BMD =        1.41334

            BMDL =       0.932428

            BMDU =        2.78979

Taken together, (0.932428, 2.78979) is a 90     % two-sided confidence
interval for the BMD
```

Bull et al. (1990)

```
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 = Response
   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 =     0.105918
                        Beta(1) =    0.0358328


           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)	0.053545	*	*	*

* - 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	-21.2941	1	3.604	2	0.165
Reduced model	-26.8563	1	14.7286	2	0.0006335
AIC:	44.5881				

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	35	0.000
3.2500	0.1597	1.757	4	11	1.846
6.5100	0.2943	7.063	5	24	-0.924

Chi^2 = 4.26 d.f. = 2 P-value = 0.1187

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.9677

BMDL = 1.18795

BMDU = 3.61033

Taken together, (1.18795, 3.61033) is a 90 % two-sided confidence interval for the BMD

DeAngelo et al. (2008) (60 weeks)

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 = 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 =     0.204406
                        Beta(1) =    0.0324139


           Asymptotic Correlation Matrix of Parameter Estimates

             Background      Beta(1)

Background            1         -0.5

   Beta(1)         -0.5            1
                              Parameter Estimates

                                                      95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf.
Limit
     Background         0.183783            *                *                  *
        Beta(1)        0.0372004            *                *                  *

* - Indicates that this value is not calculated.


                        Analysis of Deviance Table

       Model      Log(likelihood)  # Param's  Deviance  Test d.f.   P-value
     Full model        -62.3001         4
   Fitted model        -64.1175         2       3.63465      2          0.1625
  Reduced model        -70.6679         1       16.7355      3        0.000801

           AIC:         132.235


                                  Goodness  of  Fit
                                                                      Scaled
     Dose     Est._Prob.    Expected    Observed     Size       Residual
  ---------------------------------------------------------------------------
    0.0000     0.1838         5.514         4          30       -0.713
    0.2400     0.1910         5.158         4          27       -0.567
    2.0700     0.2443         7.084        11          29        1.692
   18.3000     0.5868        17.017        16          29       -0.384

 Chi^2 = 3.84      d.f. = 2        P-value = 0.1465


   Benchmark Dose Computation

Specified effect =           0.1

Risk Type        =      Extra risk

Confidence level =          0.95
```

```
             BMD =        2.83224

            BMDL =        1.70985

            BMDU =        5.86213

Taken together, (1.70985, 5.86213) is a 90     % two-sided confidence
interval for the BMD
```

Pereira (1996)

```
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 = 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 =   0.00433121
                        Beta(1) =    0.0177692


           Asymptotic Correlation Matrix of Parameter Estimates

             Background      Beta(1)

Background            1        -0.43

   Beta(1)        -0.43            1


                               Parameter Estimates

                                                        95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf.
Limit
     Background        0.0373114            *               *                  *
        Beta(1)        0.0147581            *               *                  *

* - 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        -59.1702         2       1.52058      2          0.4675
  Reduced model        -79.1216         1       41.4233      3         <.0001
```

```
          AIC:          122.34

                                Goodness  of  Fit
                                                                    Scaled
    Dose     Est._Prob.    Expected    Observed     Size       Residual
  ---------------------------------------------------------------------------
   0.0000     0.0373         3.358         4          90        0.357
   6.1000     0.1202         6.370         4          53       -1.001
  20.4000     0.2876         7.765         8          27        0.100
  61.1000     0.6093        10.967        12          18        0.499

 Chi^2 = 1.39      d.f. = 2        P-value = 0.4994


   Benchmark Dose Computation

Specified effect =            0.1

Risk Type        =      Extra risk

Confidence level =           0.95

             BMD =        7.13914

            BMDL =        4.96187

            BMDU =        11.0023

Taken together, (4.96187, 11.0023) is a 90     % two-sided confidence
interval for the BMD
```

DeAngelo et al. (2008) (104 weeks)

```
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 = Response
   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 =     0.590554
                        Beta(1) =     0.125738
           Asymptotic Correlation Matrix of Parameter Estimates

             Background      Beta(1)

Background            1        -0.47
```

```
   Beta(1)        -0.47            1

                                Parameter Estimates

                                                          95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf.
Limit
     Background         0.597398            *                *                  *
        Beta(1)         0.118941            *                *                  *

* - Indicates that this value is not calculated.


                        Analysis of Deviance Table

       Model      Log(likelihood)  # Param's  Deviance  Test d.f.   P-value
     Full model        -65.9288         3
   Fitted model        -66.4266         2      0.995585      1          0.3184
  Reduced model        -70.3031         1       8.74855      2          0.0126

           AIC:         136.853


                                Goodness  of  Fit
                                                                      Scaled
     Dose     Est._Prob.    Expected    Observed     Size       Residual
  ---------------------------------------------------------------------------
    0.0000     0.5974        25.091        27          42        0.601
    0.8400     0.6357        22.249        20          35       -0.790
    8.7000     0.8570        31.707        32          37        0.137

 Chi^2 = 1.00      d.f. = 1        P-value = 0.3164


   Benchmark Dose Computation

Specified effect =           0.1

Risk Type        =      Extra risk

Confidence level =          0.95

              BMD =       0.885825

             BMDL =       0.496499

             BMDU =        2.36969

Taken together, (0.496499, 2.36969) is a 90     % two-sided confidence
interval for the BMD
```