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Executive Summary

Tertiary Butyl Alcohol (TBA) has many industrial and chemical uses (NTP 1995). TBA is used in the manufacture of perfumes and cosmetics, as an additive in gasoline to improve the oxygen content, and is a metabolite of the fuel oxygenate, methyl-tert-butylether (MTBE) (NTP 1995).

The National Toxicology Program (NTP 1995) has conducted a two-year drinking water bioassay with TBA in male and female rats and mice. Results of these studies showed increases in the incidence of renal tubule hyperplasia and renal tubule adenomas in male rats; however, the incidence of renal tubule hyperplasia or adenoma was not significantly increased in female rats. In male and female mice, incidence of thyroid follicular cell hyperplasia was increased, and the incidence of thyroid follicular cell adenoma was increased in female mice. Based on these findings, the NTP (1995) concluded there was some evidence of carcinogenic activity in male rats and female mice, there was equivocal evidence of carcinogenicity in male mice and no evidence of carcinogenicity in female rats.

The purpose of this investigation was to conduct a quantitative risk assessment according to USEPA guidelines (USEPA 2005) in which data on the mode of action by which TBA induced renal tumors in rats and thyroid tumors in mice was considered. When data from animal studies, such as the TBA bioassays, are extrapolated to humans to provide estimates of lifetime cancer risks, then potential differences in pharmacokinetics (metabolism) and pharmacodynamics (sensitivity and mode of action) between the animal species and humans is considered in the estimation of human equivalent doses and in extrapolation from high doses typically used in the animal bioassays to low doses to which humans may be potentially exposed. Pharmacokinetic, toxicity, and mode of action data for TBA were reviewed and data selected for quantitative dose-response modeling.

The major findings of the review of pharmacokinetic and toxicity data that influenced the dose-response assessment included:

- Pharmacokinetic data indicate that TBA is poorly metabolized *in vivo* (Baker et al. 1982; Thurman et al. 1980) and slowly eliminated from the blood of rats and mice (McComb and Goldstein 1979), likely as a conjugate with glucuronide (Aarstad et al. 1985; Thurman et al. 1980). However, pharmacokinetic data were insufficient at this time to develop a pharmacokinetic model for TBA or to make a chemical-specific adjustment for pharmacokinetic differences between rats and humans.

- TBA was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 at doses up to 10,000 μg/plate, with or without induced rat or hamster liver S9 fractions (Zeiger et al. 1987). *Tertiary*-butyl acetate (TBAc), a metabolic precursor for TBA was also not mutagenic in the same strains, with or without microsomal induction at doses up to 5000 μg/plate (McGregor et al. 2005). TBA was positive in *S. typhimurium* strain TA102 (Williams-Hill et al. 1999); however, this result was not replicated in two other studies in which TBA was dissolved in either dimethylsulfoxide or water at doses up to 5000 μg/plate; TBAc was also non-mutagenic in studies in which both TA102 and *Escherichia coli* WP2uvrA/pKM101 were evaluated (McGregor et al. 2004; McGregor et al. 2005). Both TA102 and E. coli WPs strains are sensitive to oxidative damage to DNA. TBA undergoes limited metabolism and is a free radical scavenger rather than a generator of reactive oxygen species; therefore, production of oxidative damage to DNA is highly unlikely. TBA should be considered to be non-mutagenic.

- Studies that assessed the potential genotoxicity of TBA with and without metabolic activation were all negative. A L5178Y mouse lymphoma mutagenicity test was negative at TBA doses up to 5,000 μg/mL (Zeiger et al. 1987). Neither sister chromatid exchanges nor chromosomal aberrations were noted in Chinese Hamster Ovary (CHO) cell cytogenetics tests (NTP 1995). A mouse peripheral blood micronucleus test performed on blood samples from male and female $B6C3F_1$ mice following a 13-week exposure to TBA in drinking water at concentrations up to 40,000 μg/mL did not show an increase in the frequency of micronucleated normochromic erythrocytes (NCEs) or the percentage of polychromatic erythrocytes (PCEs) (NTP 1995, 1997). No increase in percentage of PCEs was noted in rats receiving intraperioneal injections of TBA at doses up to 625 mg/kg (NTP 1997). Evidence of unspecified DNA damage was noted in human leukemia HL-60 cells using a Comet Assay at concentrations up to 2224 μg/mL (Tang et al. 1997) [only the English abstract of this Chinese study was available at this time]. Based on the weight of the evidence, TBA should be considered non-genotoxic.

- Data support the hypothesis that renal tumors in male rats formed in response to a cascade of biological events associated with α2u-globulin nephropathy, a condition unique to male rats (USEPA 1991). Nephropathy leading to tumor development results when α2u-globulin accumulates in the proximal tubule resulting in the characteristic protein droplet and lysosome accumulation in the proximal tubule. This leads to cellular necrosis and sustained cellular proliferation and the subsequent promotion of initiated cells, resulting in the formation of neoplasms (McGregor and Hard 2001; USEPA 1991). The available toxicity and other mode of action data satisfy the criteria outlined by the International Agency for Research on Cancer (IARC) (1999) and USEPA (1991) needed to conclude that renal tumors in male rats occur by a α2u-globulin-mediated mode of action. Further, the pattern of observed kidney effects in male rats exposed to TBA is the same as 9 other chemicals classified by the USEPA (1991) as "chemicals inducing α2u -globulin accumulation" (CIGA). These findings provide convincing evidence that the renal tumors are α2u-globulin-mediated. In addition, there is *in vivo* evidence that no proteins in human kidney can function in a manner analogous to α2u-globulin (Meek et

al. 2003). Consequently, compounds that produce renal tumors in male rats via the α2u-globulin-mediated pathway are not relevant to humans and the use of these renal tumors in human health risk assessment is not appropriate (USEPA 1991).

- The pattern of thyroid follicular cell hyperplasia and follicular adenoma, viewed in light of benign tumors only and in only one sex and species and the negative mutagenicity data, strongly suggests an epigenetic mode of action for TBA in the production of thyroid tumors in female mice. USEPA (1998) guidelines for the evaluation of thyroid tumors in rodents recommend the data needed to provide the evidentiary basis for a non-linear, threshold approach. While not all of the recommended data were available at this time, the overall weight of evidence strongly suggests that TBA is acting by indirectly disrupting thyroid hormone balance through enzyme induction (possibly by increased glucuronidation and biliary elimination of thyroid hormones). The possible ways in which the thyroid-pituitary axis can be disturbed are all likely to be processes that have thresholds, that is, a certain amount of disruption would be required before feedback loops result in sustained thyroid stimulating hormone (TSH) stimulation of follicular cells which then results in the biological cascade of cell proliferation, hyperplasia, and neoplasia (USEPA 1998). While expected to be quantitatively different in humans, it can't be said with certainty at this time that these observations are not qualitatively relevant for human health risk assessment. Therefore, the thyroid tumors in male and female mice were evaluated quantitatively.

Dose-response analyses consistent with USEPA guidelines (2005) were conducted using the incidence of thyroid adenomas in female and male mice reported by NTP (1995). Human equivalent doses were derived by considering species differences in pharmacokinetics; however, pharmacokinetic data were insufficient at this time to develop a pharmacokinetic model for TBA or to make an adjustment for pharmacokinetic differences between rats and humans. Therefore, the default assumption of an animal-to-human scaling factor of body weight$^{3/4}$ was applied. While it is likely that humans are not only less sensitive to induction of thyroid tumors and may be refractory to that induction, these species differences in sensitivity have not yet been quantified because of the complex nature of these thyroid-pituitary interactions. Therefore, no quantitative adjustment for pharmacodynamic difference was made.

As per USEPA guidance, dose-response models were used to estimate a dose in the observable range, termed a point of departure (POD), defined as the lower bound on dose at a specified level of risk, usually 10% (LED_{10}). Because of uncertainty around the estimate of the LED_{10} or lower bounds for other risk levels, values for the maximum likely estimate (ED_{10}) and the upper bound (UED_{10}) are reported along with the lower bound. Because no survival

differences were found between control and treated females, dose-response modeling for focal follicular cell adenomas was conducted for the selected data sets using the Multistage model. For comparison, follicular cell adenomas in male mice were also evaluated using the Multistage model. Also, because survival in the male mice in the high dose groups was significantly reduced compared to the survival in the vehicle control group, a time-to-tumor model, MultWeib, was used. For comparison, data for the female follicular adenomas was also evaluated using MultWeib model, which considers the differences in survival in the animals and how that impacts the estimation of risk. In addition, the data for follicular cell hyperplasia in male and female mice was modeled using a variety of Benchmark models currently found in the USEPA's BMDS software. For chemicals with a nonlinear mode of action, such as is likely for TBA, a non-linear extrapolation to lower doses was conducted.

The POD selected was the smallest of the LED_{10}s on $BMDL_{10adj}$ and was 22 mg/kg/day derived from female follicular cell hyperplasia data and the Multistage model. Using the POD, a Reference Dose (RfD) was estimated according to the new USEPA guidelines (2005). The uncertainty factors considered included inter- and intra-species variability in kinetics and sensitivity, the nature of the response (use of precursor data) and other data base limitations. The resulting RfD was 220 μg/kg/day (22 mg/kg/day divided by 100).

1.0 Introduction

Tertiary Butyl Alcohol (TBA) has multiple industrial and chemical uses. TBA is used in the manufacture of perfumes and cosmetics, as an additive in gasoline to improve the oxygen content, and is a metabolite of the fuel oxygenate, methyl-tert-butylether (MTBE) (NTP 1995). The National Toxicology Program (1995) has conducted a 2-year drinking water bioassay with TBA in rats and mice. Results of this study showed increases in the incidence of renal tubule hyperplasia and renal adenomas in male rats; however, the incidence of renal tubule hyperplasia or adenoma was not significantly increased in female rats. In male and female mice, the incidence of thyroid follicular cell hyperplasia was increased, and in female mice, the incidence of thyroid follicular cell adenoma was also increased. Based on these findings, the NTP (1995) concluded that there was: 1) some evidence of carcinogenic activity in male rats and female mice; 2) equivocal evidence of carcinogenicity in male mice; and 3) no evidence of carcinogenicity in female rats. The results of genotoxicity tests (*Salmonella* reverse mutation assay, mouse lymphoma cell mutation test, sister chromatid exchange assay, chromosomal aberration assay in Chinese hamster ovary (CHO) cells and micronucleus assay) conducted by NTP (1995) with TBA were negative.

The California Office of Environmental Health Hazard Assessment (OEHHA) (CALEPA 1999) developed an interim drinking water level of 12 μg/L based on the incidence of kidney tumors reported in male rats in the NTP (1995) study. In this calculation, OEHHA estimated a cancer slope factor using linear extrapolation and body weight$^{3/4}$ scaling to extrapolate to the human equivalent concentration at a 1 x 10^{-6} extra lifetime cancer risk level. Default assumptions were used for body weight (70 kg) and water consumption (2 L/day). In the period since OEHHA developed their interim water level, additional data has been published that suggests the kidney tumors observed in male rats form as a result of TBA binding to α2u-globulin (Borghoff et al. 2001; Williams and Borghoff 2001), the protein that also plays a key role in the development of protein droplet (α2u) nephropathy in male rats. Protein droplet nephropathy occurs when α2u-globulin, a protein synthesized in the liver of male rats, accumulates in tubular cells in the kidney (CIIT 1996). This condition is unique to male rats and an analogous condition does not occur in humans (CIIT 1996). Therefore, kidney tumors that

form in male rats via a α2u-globulin-mediated pathway should not be used in human health risk assessment (USEPA 1991). After reviewing these data and while agreeing with the concept that there are chemical agents that produce renal tumors in some species of rats that are not relevant to humans and acknowledging that TBA does induce α2u-globulin in male rats, Budroe et al. (2004) maintained that the kidney tumors in male rats could be used for human cancer risk assessment[1].

The purpose of this investigation was to conduct a quantitative risk assessment according to USEPA guidelines (2005) in which data on the mode of action by which TBA induced renal tumors in rats and thyroid tumors in mice was considered. When data from animal studies, such as the TBA bioassays, are extrapolated to humans to provide estimates of lifetime cancer risks, then potential differences in pharmacokinetics (metabolism) and pharmacodynamics (sensitivity and mode of action) between animal species and humans are considered in: 1) the estimation of human equivalent doses, and 2) the extrapolation from high doses typically used in the animal bioassays and low doses to which humans may be potentially exposed. Pharmacokinetic, toxicity, and mode of action data for TBA were reviewed and data selected for quantitative dose-response modeling.

2.0 Hazard Narrative

2.1 Overview

This section provides an overview of the experimental data for TBA. TBA has been tested in a number of different studies to include a two-year oncogenicity study in which TBA was administered to rats and mice in drinking water. The purpose of this section is to evaluate the chronic bioassay and other key data, in particular data related to the mode of action of TBA in the rodent studies and key species differences in kinetics, to develop a weight-of-evidence analysis of the potential that TBA may pose a carcinogenic hazard in humans (USEPA 2005).

[1] The authors of this paper are on the staff of OHEEA, CalEPA; however, the disclaimer notes that the contents and opinions expressed in the manuscript are those of the authors and not the official position of CalEPA.

2.2 Carcinogenicity Studies

2.2.1 Human Data

No epidemiological data were found in the literature.

2.2.2 Animal Data

The potential for TBA to induce tumors or result in chronic toxicity in laboratory animals has been investigated in a 2-year oral (drinking water) study in rats and mice (Cirvello et al. 1995; NTP 1995). Male and female F344/N rats were administered TBA in drinking water at concentrations resulting in doses of approximately 0, 90, 200, or 420 mg/kg/day in males and 0, 180, 330, or 650 mg/kg/day in females. Male and female $B6C3F_1$ mice were administered TBA in drinking water at concentrations resulting in doses of approximately 0, 540, 1040, or 2070 mg/kg/day in males and 0, 510, 1020, or 2110 mg/kg/day in females. Rats and mice were observed twice daily and body weights and clinical observations were recorded weekly for the first 13 weeks and every 4 weeks thereafter. Water consumption was recorded every 2 weeks.

At 15 months, hematology and urinalysis evaluations were conducted on rats in all exposure groups. At the 15-month interim sacrifice, a complete necropsy and microscopic evaluation was performed and brain, right kidney, and liver weights were recorded. Interim sacrifices were not conducted on mice due to decreased survival in the male high-dose group. Following final sacrifice, a complete necropsy and microscopic examination were performed on all mice and rats.

Survival analysis showed a significant decrease in survival rates in male and female rats in the high-dose groups and in male mice in the high-dose group (NTP 1995). The final mean body weight in male and female rats in the high-dose groups were 24% and 21% less than controls, respectively. Mean body weights in the male rats at 90 and 200 mg/kg/day were similar to controls through week 65 of the study and then decreased through the remainder of the study. Mean body weights in the female rats at 90 and 200 mg/kg/day were similar to control values throughout the study. Mean body weight in the high dosed male mice were significantly lower than the controls from week 9 of the study until the final 10 weeks of the study. Body weights and body weight gains in all other treated male mice were similar to controls. The mean body weights of the female mice in the high-dose group were 10% to 15% less than controls beginning at week 13 of the study and continuing through the end of the study (statistical significance was

not reported). The mean body weights in female mice in the mid-dose group were approximately 6% lower than controls, and mean body weights in the low-dose groups were only slightly less than controls throughout the study (statistical significance was not reported) .

A dose-related increase in water consumption in male rats was observed during the second year of the study, while a dose-related decrease in water consumption was observed in female rats (NTP 1995). Water consumption was unaffected in male and female treated mice.

Behavioral and general health and appearance was unaffected by treatment in rats and mice of both sexes with the exception of an increased incidence of hyperactivity in the female rats in the high-dose group. Urinary and hematological observations were not considered to be treatment-related. A significant increase in urine specific gravity and a significant decrease in urine volume were noted in the female rats receiving 330 and 650 mg/kg/day, which was consistent with the decreased water consumption noted in the female rats.

At the 15-month interim sacrifice (10 rats per group), mean body weights were significantly decreased in male and female rats in the high-dose group (NTP 1995). In the male rats, relative brain, right kidney, and liver weights were increased in the high-dose group and relative right kidney weights were decreased in the mid-dose group. In the female high-dose group, relative brain and liver weights were significantly increased. Also, in the female rats, absolute and relative right kidney weights were significantly increased in all treated groups at the 15-month interim sacrifice. One renal tubule adenoma was observed in male rats at the 15-month interim sacrifice in the high-dose group. In addition, the incidence and severity of mineralization of the kidney was significantly increased in the high-dose male rats when compared to controls sacrificed at 15-months. Nephropathy was observed in all male and female rats, including controls, at the 15-month interim sacrifice, and the severity was slightly increased in treated male rats and the mid- and high-dose group females. Incidence data for male and female rats at the 15-month interim evaluation are presented in Tables 1 and 2. Due to decreased survival in mice, a 15-month interim evaluation was not performed.

At the end of the 2-year study, microscopic evaluations noted changes in the kidneys of male and female rats and in the thyroid gland of male and female mice. In rats, while the incidence of nephropathy was the same across all groups, including controls, the severity of the observed nephropathy increased in both the male and female rats in the high-dose groups (NTP

1995). In the female rats, there was a significant increase in kidney inflammation in the 330 and 650 mg/kg/day group, and a significant increase in the kidney transitional epithelium hyperplasia at 650 mg/kg/day (Table 3). No renal tumors were found in any of the treated females.

In the male rats there were significant increases in the incidences of renal tubule hyperplasia and mineralization in the 420 mg/kg/day dose group and transitional epithelial hyperplasia was significantly increased at 200 and 420 mg/kg/day in male rats (Table 4) (Cirvello et al. 1995; NTP 1995). The incidence of linear foci in the renal papilla, which is a component of renal mineralization and nephropathy, was significantly increased in all treated groups of male rats. These foci consisted of distinctive linear deposits along radiating medullary collecting ducts and have been described as a lesion specifically associated with nephropathy due to increased accumulations of α2u-globulin in male rats. The incidence of adenomas alone was not significantly increased in any of the treated groups when either the single section or the step-sections were combined. In contrast to the usual 8 step-sections, 16 to 17 sections were taken for males in this study. The incidence of the combination of renal tubule adenomas and carcinomas was only significantly increased in the mid-dose group and only with step-sectioning.

In male mice, significant increases in hyperplasia in follicular cells were noted in dose groups; however, the incidence of adenomas alone or in combination with carcinomas was not increased compared to controls (Table 5). Thyroid follicular cell hyperplasia was increased in the two highest dosed female groups and a significant increase in the incidence of thyroid follicular cell adenomas but not carcinomas was found in female mice at 2110 mg/kg/day (Cirvello et al. 1995; NTP 1995). The incidence of these tumors was increased only in the highest dose tested and was not significantly increased in treated male mice at any dose tested. However, survival was affected in the highest dose group in males and the question remains, if survival was taken into account, would the incidence in the high-dose males have been increased. The incidence of thyroid follicular cell hyperplasia was increased in male mice in all treated groups and in female mice in the two highest dose groups.

The incidence of urinary bladder chronic inflammation was significantly increased in the male and female mice high-dose groups, and the incidence of urinary bladder transitional hyperplasia was significantly increased in the male high-dose group (NTP 1995). In the liver of exposed male mice, the incidence of hepatocellular carcinoma was significantly ($p = 0.049$)

increased at 1040 mg/kg/day when compared to the controls, but the incidence decreased in the high-dose group when compared to controls. When the incidence of hepatocellular adenoma and carcinoma were combined in the male mice, the incidence increased at 540 and 1040 mg/kg/day (not significantly), and significantly decreased in the high-dose group when compared to the controls.

The authors concluded that there was "some evidence of carcinogenicity" in male rats based on the increased incidence of renal tubule adenomas and carcinomas combined (from the step-sectioning) but "no evidence of carcinogenic activity" in female rats. In addition, there was "equivocal evidence of carcinogenicity" in male mice based on the increased combined incidence of follicular cell adenomas and carcinomas of the thyroid gland (although the incidence was not statistically significant); and "some evidence of carcinogenicity" in female mice based on increased incidences of follicular cell adenoma of the thyroid.

2.3 Analysis of Other Key Data

2.3.1 Pharmacokinetics

Absorption of TBA from the gastrointestinal tract and distribution throughout the body occurred rapidly following administration via gavage (Faulkner and Hussain 1989; Poet et al. 1997). In female C57BL/6J mice given TBA at a concentration of 10.5 mmol/kg (777 mg/kg) via gavage every 12 hours for a total of 5 doses, TBA absorption and distribution was complete within 1.5 hours following the last dose (Faulkner and Hussain 1989). In male and female F344 rats given single intravenous dose of 0, 37.5, 75, 150, or 300 mg/kg, TBA underwent a rapid distribution phase followed by a slower elimination phase (Poet et al. 1997).

Tertiary alcohols, such as TBA, are considered to be minimally metabolized via oxidative pathways (Baker et al. 1982; Thurman et al. 1980). Small amounts of acetone (ranging from 0.5% to 9.5% of the administered dose) were found in the urine and expired air of rats injected with TBA at doses ranging from 750 to 2000 mg/kg; however, the amount recovered was highly variable and did not change in a dose-related manner (Baker et al. 1982). In male rats treated with 250 mg/kg TBA via gavage, TBA was metabolized by conjugation to the TBA glucoronide and the TBA sulfate (main metabolite) and by oxidation to, 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate, and small amounts of acetone (Bernauer et al. 1998).

TBA did not form aldehydes or ketones by dehydrogenation and was not a substrate for alcohol dehydrogenase (Arslanian et al. 1971; Videla et al. 1982) as it lacks the carbonyl hydrogen required for alcohol dehydrogenase activity (Baker et al. 1982). Further oxidation of 2-methyl-1,2-propanediol can result in the formation of 2-hydroxyisobutyrate, which can also be further metabolized to form acetone. In both the Bernauner et al. (1998) and the Baker et al. (1982) studies, the doses of TBA were very high (250 to 2000 mg/kg) and the resulting production of acetone in urine was very small. No differences in blood acetone levels were seen in TBA-treated male or female rats (300 mg/kg) compared to controls (Poet et al. 1997). Baker et al. (1982) could not conclude that TBA was the sole source of acetone in rat urine.

In *in vitro* studies, TBA served as a substrate for rat mixed function oxidases (MFOs) and was demethylated to yield small amounts of formaldehyde apparently involving the interaction of TBA with hydroxy radicals generated from hydrogen peroxide (Cederbaum and Cohen 1980; 1983). Formaldehyde was not found in the urine of rats or humans following administration of TBA (Bernauer et al. 1998).

Human data regarding the movement of TBA in the body are limited to results obtained from a single volunteer who ingested a gelatin capsule resulting in a dose of 5 mg TBA/kg (Bernauer et al. 1998). The major TBA metabolite in the urine of that individual was 2-hydroxyisobutyrate, with smaller amounts 2-methyl-1,2-propanediol and TBA glucuronide**.** In contrast to rats**,** only trace amounts of TBA sulfate were recovered likely due to species differences in sulfotransferase(s) (Bernauer et al. 1998). No mention was made of the presence of acetone in the urine.

Elimination half-lives ranged from 3.8 hours at doses less than 300 mg/kg and were increased to 4.3 and 5 hours in the high-dose female and male rats, respectively. Poet et al. (1997) stated that the elimination of TBA appeared to saturate at higher doses. Other studies have estimated the elimination half-life to be 8 to 9 hours following oral (Thurman et al. 1980) or intraperitoneal injection (Baker et al. 1982). The doses were much higher in these studies than the doses administered by Poet et al. (1997). According to Poet et al., these doses were likely greater than metabolism/elimination saturation levels. Saturable metabolism/elimination has been reported in mice administered TBA by intraperitoneal injection at doses ranging from 5 to 20 mmoles/kg (approximately 360 to 1550 mg/kg) (Faulkner and Hussain 1989).

2.3.2 Animal Toxicity Studies

2.3.2.1 Acute and Subacute

The acute toxicity of TBA is low. An oral LD_{50} of 3,500 mg/kg in rats (NTP 1995, 1997) and an oral LD_{50} of 3,600 mg/kg in rabbits (NTP 1995, 1997) have been reported. Following an intraperitoneal injection, a LD_{50} of 441 mg/kg was reported in mice (NTP 1995, 1997).

The accumulation of triacylglycerols (TAGs) in the liver of rats was evaluated to determine if the effects of certain alcohols on hepatic liver metabolism in the rat were related to oxidative metabolism by alcohol dehydrogenase (ADH). Ethanol, n-propanol, and isobutanol are all metabolized through the ADH pathway and induced a fatty liver. TBA, which is not metabolized by ADH, was used to determine if the fatty liver effects were due to oxidation of these alcohols by ADH. Female Wistar rats were administered a single dose of TBA at 25 mmol/kg (1850 mg/kg) via a gastric tube (Beauge et al. 1981). At 2, 5, and 20 hours following exposure, there were significant increases in blood glucose, blood free fatty acids (FFA), liver TAGs, and liver wet weight. Significant decreases were noted in blood TAGs, blood phospholipids and liver phospholipids. The results indicated that TBA administration induced fatty liver without impairing hepatic fatty acid oxidation.

Male Sprague Dawley rats were exposed to TBA at concentrations of 2000 ppm for 3 days or 500 ppm for 5 days (6 hour/day exposures) via inhalation in order to evaluate the changes in the cytochrome P-450 enzyme system in the liver, kidney, and lung (Aarstad et al. 1985). Cytochrome P-450 levels were significantly increased in the kidney but not the liver following inhalation of 500 ppm TBA for 5 days; or in the liver but not the kidney following inhalation of 2000 ppm for 3 days. No significant changes were noted in the cytochrome P-450 levels in the lung following either exposure duration.

2.3.2.2 Subchronic

Oral

The potential toxic effects of repeated dosing of TBA in drinking water were evaluated in male and female F344/N rats and $B6C3F_1$ mice to determine the appropriate doses to be used in 2-year carcinogenicity studies (0, 5, 10, or 20 mg/mL for male and female mice; 0, 2.5, 5, or 10 for female rats; and 0, 1.25, 2.5, or 5 for male rats) (Lindamood et al. 1992; NTP 1995). Rats

and mice (10/sex/group) were given access to drinking water with TBA concentrations of 0, 2.5, 5, 10, 20, or 40 mg/mL for 92 to 94 (rats) or 94 to 95 (mice) consecutive days. In rats, the average daily doses were approximately 0, 230, 840, 1,520, or 3,610 mg/kg/day for males and 0, 290, 590, 850, 1,560, or 3,620 mg/kg/day for females based on water consumption. For mice, the average daily doses based on water consumption were 0, 350, 640, 1,590, 3,940, or 8,210 mg/kg/day for males and 0, 500, 820, 1,660, 6,430, or 11,620 for females. All male rats and 6 of the 10 female rats in the 40 mg/mL dose groups died during the study. Mean body weights and body weight gains were significantly decreased in male rats at 5, 10, and 20 mg/mL (all male rats died prior to study termination) and in the 40 mg/mL female mice. Treatment-related clinical findings in male and female rats included ataxia, hypoactivity and emaciation, indicative of alcohol intoxication.

Hematology results from day 15 of the study showed significant increases in mean cell volume and mean cell hemoglobin in male rats at 5 mg/mL, and a significant decrease in platelets in the male and female rats at doses of 40 mg/mL (Lindamood et al. 1992; NTP 1995). In the male rats examined at the end of the study, hemoglobin levels and erythrocytes counts were significantly decreased in the 10 and 20 mg/mL groups; there were significant increases in mean cell volume and platelet counts at 10 and 20 mg/mL and mean cell hemoglobin levels at 20 mg/mL. No significant hematological findings were noted in female rats in any dose group. Corresponding to the decreased water consumption by male and female rats, there were significant increases in urine specific gravity and decreases in urine volume at dose levels of 10, 20, and 40 mg/mL in both males and females when compared to controls. Clinical chemistry results showed significant decreases in serum alkaline phosphatase activity at days 15 and 20 in the 20 and 40 mg/mL males and 5 and 20 mg/mL females. Significant increases occurred in alanine aminotransferase activity in 40 mg/mL females at week 13. Sorbitol dehydrogenase was significantly increased in males at 10 and 20 mg/mL at week 13.

All of the treated male and female rats had statistically significantly increased absolute and relative kidney weights at week 13 (Lindamood et al. 1992; NTP 1995). Relative and absolute liver weights were significantly increased in all treated females, and relative liver weights were significantly increased in the 5, 10, and 20 mg/mL males. Other significant organ weight changes were considered secondary to body weight changes.

Upon gross and microscopic evaluation, the urinary bladder of the male rats in the 20 and 40 mg/mL dose groups contained grossly visible calculi, microscopic inflammation of the lamina propria, and hyperplasia of the transitional epithelium (Lindamood et al. 1992; NTP 1995). In the females, inflammation and hyperplasia were noted in the urinary bladder at 40 mg/mL. Examination of the kidney showed significantly greater severity of nephropathy in treated males compared to controls. However, while there were significant increases in the incidence of nephropathy in females in the 10, 20, and 40 mg/mL groups, the severity of the lesions was not increased in the treated animals when compared to controls

Preliminary results from the 2-year carcinogenicity study (NTP 1995) suggested the possibility of increased renal tubular neoplasms in the male rats given 2.5 mg/mL TBA. These results prompted a reexamination of the kidney slides from this 90-day study (Takahashi et al. 1993). Results showed a treatment-related increase in the incidence of hyaline droplet accumulation in male rats at all doses with the exception of the animals in the 40 mg/mL group (all died prior to study termination). In addition, there was a significant increase in the incidence of mineralization of the kidney in male rats at 10, 20, and 40 mg/mL and in all treated females (NTP 1995). Takahashi (1993) reported increased nephropathy in all treated groups of male rats, with the exception of the high-dose group, when compared to the controls. In addition, there was a direct relationship between the severity of nephropathy, hyaline droplet accumulation, and deposition of abnormal crystals.

Two male mice and one female mouse in the high-dose group died due to TBA exposure (Lindamood et al. 1992; NTP 1995). At necropsy, mean body weights were significantly decreased at 20 and 40 mg/mL in the male mice and at 40 mg/mL in the female mice when compared to controls. Dose-related clinical findings in mice following exposure to 40 mg/mL TBA included emaciation, ataxia, and hypoactivity in male mice and emaciation in female mice. Hematological results showed a significant increase in the segmented neutrophil count in the 40 mg/mL male mice when compared to controls; however, the results were highly variable. In male mice, the hematocrit, hemoglobin and erythrocyte counts were significantly increased at 40 mg/mL, and hemoglobin was significantly increased at 20 mg/mL. In the females, hemoglobin and erythrocyte counts were significantly increased at 20 mg/mL and erythrocytes counts only were significantly increased at 40 mg/mL. The relative and absolute kidneys weights of female

mice in the 40 mg/mL dose group were significantly increased when compared to controls. Other significant organ weight changes were considered secondary to body weight changes.

Histopathological results indicated a statistically significant increase in the incidence of urinary bladder inflammation and transitional epithelial hyperplasia in the 20 and 40 mg/mL groups of male mice when compared to controls (Lindamood et al. 1992; NTP 1995). In the female mice, there was a significant increase in the incidence of urinary bladder inflammation in the 40 mg/mL dose group when compared to controls. No other significant histopathological changes were reported in male or female mice.

Inhalation

Male and female F344/N Rats and B6C3F$_1$ mice (5/sex/group) were exposed to target air concentrations of 0, 450, 900, 1750, 3500, or 7000 ppm TBA for 6 hours plus a T_{90} of 10 minutes per day, 5 days per week, for 12 exposure days in an 18-day period (NTP 1997). The T_{90} was identified as the time required for TBA concentration to reach 90% of the final stable concentration following the beginning of exposure. All male and female rats in the 7000 ppm group died on day 2 of the study. Mean body weights and body weight gains in the 3500 ppm groups of male and female rats were significantly lower than controls at the end of the study. Clinical finding in all male and female rats exposed to 900 ppm and higher included ataxia, hyperactivity, and hypoactivity, indicative of alcohol intoxication. Thymus weights were significantly decreased in male and female rats in the 3500 ppm group when compared to controls. No treatment related gross or microscopic findings were reported in the treated male and female rats.

All male and female mice in the 7000 ppm group died on day 2 of the study and one male in the 3500 ppm groups died on day 3 of the study (NTP 1997). Mean body weights and body weight gains in the treated mice did not differ significantly from the control groups. Clinical signs in males and females in the 3500 ppm group included hypoactivity, ataxia, and rapid respiration, while at 1750 ppm clinical findings included hypoactivity, ataxia, and urogenital wetness. The relative liver weight of the male mice in the 3500 ppm group and the absolute and relative liver weights of the females in the 3500 ppm group were significantly greater than the controls. The absolute and relative thymus weights of female mice in the 3500 ppm group were significantly lower than the controls. No treatment related gross or microscopic findings were reported in the treated male and female rats.

NTP (1997) also performed 13-week inhalation studies in F344/N rats and $B6C3F_1$ mice. Rats and mice (10/sex/group) were exposed to TBA air concentrations of 0, 136, 270, 540, 1080 or 2100 ppm for 6 hours plus T_{90} of 10 minutes per day, 5 days per week, for 13 weeks, with at least 2 consecutive exposure days before sacrifice. Results in the treated male and female rats showed no significant changes in survival or body weights when compared to control animals. Clinical signs of toxicity in the 2100 ppm group female rats included emaciation and hypoactivity (at one observation period only). No clinical findings of toxicity were noted in the treated rats.

Significant decreases in hematocrit were noted in male rats in the 270, 1080, and 2100 ppm groups at week 13 when compared to controls (NTP 1997). Hemoglobin was significantly decreased in all treated groups of male rats at week 13 and erythrocyte counts were significantly decreased in the 1080 and 2100 ppm groups of male rats when compared to controls. Alkaline phosphatase was significantly decreased in male rats in the 540, 1080, and 2100 ppm groups at day 22 and at 1080 and 2100 ppm at week 13 when compared to controls. Urinalysis results showed a significant decrease in urine pH in male rats in the 540, 1080, and 2100 ppm groups at day 22 and in the 2100 ppm group at week 13. No significant hematology or clinical chemistry findings were reported for the treated female rats; however, urine pH was significantly increased in the 1080 and 2100 ppm groups at day 21 and week 13. Significant increases in relative and absolute right kidney weights occurred in the male rats at 1080 and 2100 ppm. In addition, there were significant increases in relative right kidney weights in female rats at 2100 ppm, and relative liver weights at 1080 and 2100 ppm. No treatment-related gross necropsy findings were reported in male or female rats, and no treatment-related microscopic findings were reported in female rats. A treatment-related increase in the severity of chronic nephropathy was noted in all exposed males relative to controls. Kidney sections from male rats in the 0, 1080, and 2100 ppm groups were stained and analyzed for the presence of tubular hyaline droplet accumulation; however, no differences in control and treat animals were noted.

One death in the high-dose male mice group was attributed to TBA treatment; no other treatment-related deaths were reported in either male or female mice (NTP 1997). Mean body weight gains were significantly lower than control in the female mice in the 1080 and the 2100 ppm groups. No treatment-related clinical findings were reported for male or female mice.

Hematology results showed a significantly increased segmented neutrophil count in the 2100 ppm group males when compared to controls; however, no other treatment-related changes in hematology were noted. The only significant organ weight change was an increase in relative liver weights in the 1080 and 2100 ppm groups of female mice when compared to controls. No significant gross or microscopic changes were noted in the treated male and female mice.

2.3.2.3 Reproductive and Developmental Studies

Faulkner et al. (1989) exposed pregnant female CBA/J and C57BL/6J mice to TBA via gavage at a concentration of 10.5 mmol/kg (777 mg/kg/day) every 12 hours during days 6 through 18 of gestation. A significant increase in the number of resorptions per litter and a significant decrease in the number of live fetuses per litter were reported in the treated groups of both strains of mice when compared to controls. Morphological examination of the surviving offspring showed no teratological effects in either strain of mice at blood levels equivalent to teratogenic ethanol treatment.

Daniel and Evans (1982) fed pregnant Swiss Webster mice liquid diets containing TBA at concentrations of 0, 0.5%, 0.75%, or 1.0% (w/v) (0, 3243, 4520, 6250 mg/kg/day) from day 6 to day 20 of gestation. According to preliminary studies by the same author, the highest concentration tested corresponded to the maximal tolerated concentration during a 15 day administration. Results showed decreases in the number of litters, litter size, and birth weight and an increase in the number of stillborn pups in the offspring of all exposed mice. In addition, there were significant delays in postnatal physiological and psychomotor performance in the offspring of exposed dams; however, maternal sedation and decreased food intake were noted at the higher exposure concentrations, which could contribute to the developmental effects observed in the offspring. In a similar study in Sprague Dawley rats exposed to concentrations of 6000 or 12,000 mg/m^3 TBA via inhalation from gestation day 1 through 19 (Nelson et al. 1989), TBA did not produce an increase in the number of birth defects relative to the frequency observed in the controls.

As part of the 13-week oral study, vaginal cytology and sperm morphology evaluations were also performed in F344/N rats and B6C3F$_1$ mice (10/sex/group) exposed to TBA drinking water concentrations of 0, 2.5, 5, 10, 20, or 40 mg TBA/mL for 92 to 94 (rats) or 94 to 95 (mice) consecutive days (NTP 1995). Parameters evaluated were the same as those in NTP (1997).

Results showed no significant differences in the reproductive endpoints of male or female rats or male mice evaluated or in estrous cycle length or percentage of time spent in various stages.

NTP (1997) performed vaginal cytology and sperm morphology evaluations in F344/N rats and $B6C3F_1$ mice (10/sex/group) exposed to TBA air concentrations of 0, 540, 1080 or 2100 ppm for 6 hours plus T_{90} per day, 5 days per week, for 13 weeks, with at least 2 consecutive exposure days prior to sacrifice. Parameters evaluated in the males included testis, epididymal, and caudal weight, sperm motility, sperm count, and sperm morphology. Females were evaluated for estrous cycle length or the percentage of time spent in the various estrous stages. The evaluations were performed at the end of the exposure and results showed no significant differences in the reproductive endpoints of male or female rats or mice.

2.3.3 Mutagenicity and Genotoxicity Studies

A battery of genetic toxicity studies were performed including the *Salmonella typhimurium* mutagenicity test, the mouse lymphoma mutagenicity test, the Chinese hamster ovary (CHO) cell cytogenetics test, the rat bone marrow micronucleus test, and the mouse peripheral blood micronucleus test (McGregor et al. 1988; NTP 1995, 1997; Zeiger et al. 1987). In the *S. typhimurium* mutagenicity tests, TBA was incubated with the *S. typhimurium* tester strains TA98, TA100, TA1535, and TA1537 both with and without metabolic activation with rat and hamster S9 mix for 20 minutes at 37ºC at doses of 0, 100, 333, 1000, 3333, or 10,000 µg TBA/plate (NTP 1995, 1997; Zeiger et al. 1987). TBA did not induce mutations (no increase in the number of histidine-independent reverants) in any of these *S. typhimurium* strains tested with or without activation following incubation at 37ºC for 2 days. The tester strains commonly used in the *S. typhimurium* mutagenicity test, TA98, TA100, TA1535, and TA1537, have G-C base pairs at the site of mutation (Levin et al. 1982).

Williams-Hill et al. (1999) found that in the presence of S9 activation, TBA produced mutations in tester strain TA102; however, the proportional increase was modest and occurred at very high doses (less than a 1.5-fold at approximately 750 µg/plate; 1.9-fold at approximately 1500 µg/plate; 1.9-fold at approximately 2250 µg/plate; 1.4-fold at 5000 µg/plate). These results were not confirmed in another laboratory that tested TBAc (*tertiary*-butyl acetate from which TBA is generated) in *S. typhimurium* strains TA100, TA98, TA 1535, TA 1537 and TA102 or in *Escherichia coli* WP2uvrA/pKM101 and in two other laboratories that tested TBA

in *S. typhimurium* strain TA102 at doses ranging from 5 to 5000 μg/plate with or without metabolic activation (McGregor et al. 2005). The tester strain, TA102, has a mutation of the ochre *hisG248* gene with an A-T base pair at the site of the mutation that is sensitive to detection of oxidative damage to DNA (Levin et al. 1982). The *E. coli* strains WP2 are also used to detect oxidizing mutagens (Wilcox et al. 1990). TBA undergoes limited metabolism and is a free radical scavenger rather than a generator of reactive oxygen species; therefore, as indicated by the negative results in both TA102 and *E. coli* WPs strains that are sensitive to oxidative damage to DNA, production of oxidative damage to DNA is highly unlikely. TBA should be considered to be non-mutagenic.

Other genotoxicity studies have been uniformly negative. The L5178 mouse lymphoma test was negative both with and without metabolic activation with S9 and consisted of a vehicle control (4 cultures); a positive control (2 cultures); and TBA at concentrations of 1000, 2000, 3000, 4000, or 5000 μg/mL (2 cultures per concentrations) (McGregor et al. 1988; NTP 1995, 1997). All treatment levels were replicated. Following a 4-hour incubation with TBA, the medium and TBA were removed and the cells were incubated in fresh medium for an additional 3 days to express the mutant phenotype. The cells were then plated with trifluorothymidine (TFT) for selection of TFT-resistant cells. Plates were incubated at 37ºC for 10 to 12 days. Results of the mouse lymphoma test showed a small increase in mutant colonies in a single trial at the highest concentration tested in the absence of S9 activation. An additional trial was conducted without S9 activation and the results indicated no increase in mutant colonies. The trials conducted with S9 activations were negative. Based on these results, the authors concluded the mouse lymphoma test was negative (McGregor et al. 1988; NTP 1995, 1997).

TBA was tested in cultured CHO cells for the induction of sister chromatid exchanges and chromosomal aberrations (Galloway et al. 1987; NTP 1995, 1997). Tests were performed both with and without activation with Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix at TBA concentrations of 0, 160, 500, 1600, or 5000 μg/mL (Trail 1) or 0, 2000, 3000, 4000, or 5000 μg/mL (Trail 2). In the sister chromatid exchange test without activation, the CHO cells were incubated with TBA for 26 hours, then the medium containing TBA was removed and replaced with fresh medium plus bromodeoxyuridine (brdU) and colcemid and incubated for an additional 2 hours. In the test with S9 activation, the cells were

incubated with TBA and S9 for 2 hours, then the medium was removed and replaced with medium containing serum and brdU and no TBA and incubated for an additional 26 hours. A weakly positive response, defined as an increase of 20% or greater at a single dose, was noted in the first trial in which TBA was administered without the S9 fraction (at the highest dose, 5000 μg/mL, the increase was 20.32%). This result was not repeated in the second trial and the results of both trials with metabolic activation did not show sister chromatid exchanges at any of the concentrations tested.

In the chromosomal aberrations test, TBA was tested at concentrations of 0, 160, 500, 1600, or 5000 μg/mL (Trail 1) or 0, 1600, 3000, 4000, or 5000 μg/mL (Trail 2), with and without activation (Galloway et al. 1987; NTP 1995, 1997). Without S9 activation, CHO cells were incubated with TBA for 9 to 9.5 hours; colcemid was then added and incubation continued for 2 additional hours. In tests with S9 activation, CHO cells were incubated with TBA for 2 hours; the treatment medium was then removed and the cells were reincubated for 9.5 to 10 hours in fresh medium with colcemid present for the last 2 hours. Results of the tests, with and without activation, showed no chromosomal aberrations at any of the concentrations tested.

A mouse peripheral blood micronucleus test was performed on blood samples from male and female $B6C3F_1$ mice following a 13-week exposure to drinking water containing TBA at concentrations of 0, 3000, 5000, 10,000, 20,000, or 40,000 μg/mL (NTP 1995, 1997). The frequency of micronuclei in 10,000 normochromatic erythrocytes (NECs) in up to 10 animals per dose group was determined. There was no increase in the percentage of polychromatic micronucleated cells (PCEs) in the total erythrocyte population in male and female mice at any of the concentrations tested. TBA did not increase the percentage of PCEs in bone marrow cells in male rats administered TBA by intraperitoneal injection at doses of approximately 39, 78, 156, 312, or 625 mg/kg indicating that TBA was not toxic to bone marrow cells (NTP 1997).
Tang et al. (1997) reported that TBA *could* (emphasis added) cause DNA damage in human leukemia (HL-60) cells using single-cell gel electrophoresis (Comet assay) when incubated with these cells at concentrations ranging from 74, 371, 741, or 2224 μg/mL). Only the abstract was available in English at this time and details as to specifics of the experimental protocol, including the type Comet Assay, the type of DNA damage noted, the presence of DNA repair enzymes, the presence of a metabolic activation, or dose-response information was not provided. Budroe et al.

(2004) stated that the results of Tang et al. (1997) demonstrated that both the number of cells with DNA damage and the severity of that damage increased in a dose-related manner; however, this interpretation could not be verified at this time.

2.4 Potential Modes of Action

2.4.1 Mode of Action for Kidney Tumors in Rats

The association between exposure to TBA and the formation of renal tumors in male rats has been shown in a 2-year bioassay (Cirvello et al. 1995; NTP 1995). Agents that produce kidney tumors have been classified into two broad categories: 1) classic renal carcinogens, such as dimethylnitrosamine, that act by a mode(s) of action that is likely to have a linear, non-threshold dose response and may be relevant to humans; and 2) those producing kidney tumors by a series of biological events involving α2u-globulin-induced nephropathy that are likely to have a non-linear, threshold dose-response and are not considered relevant to humans (USEPA 1991). Following exposure to classical renal carcinogens, a high incidence of kidney tumors is generally produced in a both male and female rats and mice that occur with a shortened latency and that demonstrate a clear dose-response (USEPA 1991). While a number of putative modes of action have been reported, in general, classic renal carcinogens or their active metabolites are electrophilic species that bind covalently to marcromolecules including DNA (USEPA 1991). TBA does not fit the pattern of a classical renal carcinogen but rather, based on the observations in the bioassays and additional mode of action studies, TBA falls into the category the USEPA (1991) has defined for chemicals, such as isophorone and d-limonene, as "chemicals inducing α2u-globulin accumulation" (CIGA) that act by a mode of action not relevant to humans. The data for TBA demonstrate that renal tumors in male rats exposed to TBA form as part of the cascade of events associated with α2u-globulin nephropathy (Borghoff et al. 2001; Williams and Borghoff 2001), a condition unique to male rats (USEPA 1991).

2.4.1.1 Overview of α2u-globulin-induced renal tumors

α2u-globulin is a low molecular weight protein synthesized, under androgenic control, in the liver of male rats (McGregor and Hard 2001; USEPA 1991). Because of its small size, α2u-globulin is filtered by the glomerulus and approximately 60% is reabsorbed in the proximal tubule and catabolized with large proteins and about 40% is re-excreted in the urine (McGregor

and Hard 2001). Chemicals that bind to α2u-globulin in blood result in the formation of a protein-ligand complex. When unbound α2u-globulin is reabsorbed, it is taken up by lysosomes within the proximal tubule and digested by lysosomal enzymes to peptides and amino acids (Borghoff et al. 1996). Nephropathy results when the complex is reabsorbed in the P2 segment of the proximal tubules. This complex is more resistant to proteolysis and accumulates in phagolysosomes of the tubule cells resulting in histologically distinct hyaline droplets, which lead to lysosomal overload, shedding of cells of the lumen, and sustained tubule cell regeneration (McGregor and Hard 2001). Consequently, α2u-globulin accumulates in the proximal tubule resulting in the characteristic protein droplet and lysosome accumulation in the proximal tubule that leads to cellular necrosis and sustained cellular proliferation and the subsequent promotion of initiated cells resulting in the formation of neoplasms (McGregor and Hard 2001; USEPA 1991).

2.4.1.2 Evidence that TBA is a CIGA chemical

Because humans appear to be less like male rats, with regard to renal physiology, particularly in how the kidney handles low molecular weight proteins, the United States Environmental Protection Agency (USEPA 1991) has provided guidelines for discerning α2u-globulin associated renal carcinogenesis in the male rat and the use of these data in human health risk assessment. According to the International Agency for Research on Cancer (IARC 1999) and USEPA (1991), certain criteria must be demonstrated to conclude that renal tumors in male rats occur by a α2u-globulin-mediated mode of action. The following is a composite of these criteria. In light of the criteria recommended by USEPA (1991), mode of action studies have been conducted with TBA to provide data to satisfy these criteria. These results are discussed in this section. Also, the data for TBA were compared with data for corresponding data for 9 chemicals classified by the USEPA as CIGA chemicals (Tables 7 and 8). These criteria and studies are briefly discussed below.

Demonstrates species, strain, and sex specificity.

In the 2-year bioassay conducted by NTP (1995), TBA produced renal tubular tumors only in male rats and not in female rats or either sex in mice. These renal tumors were not statistically significant in any male dose group at the 15-month interim sacrifice (Table 1) and

were not significantly increased in the 2-year results when the standard single (a single sagittal and a single transverse) section was considered (Table 4). Statistical significance of renal tumors in male rats was only achieved after step sectioning of male rats in the mid-dose group. The incidence of renal tubule adenomas and carcinomas from the step sectioning evaluation was 7/50 in the control and 10/50, 18/50, and 11/50 in the low, mid, and high-dose groups. Significant differences in survival of the high-dose group is likely responsible for the lower incidence in the high-dose group compared to the mid-dose group. In all animals found with a renal tumor, these were benign; the incidence of renal carcinomas was not significantly increased (only 1 carcinoma was found in a treated animal that had a benign tumor as well). With the step sections, the incidence in the next-to-the-highest dose group was significantly different from control and statistical significance was achieved at the highest dose group only when a tumor found at an interim (15 month) sacrifice was included in the total. These findings were judged by NTP to provide "some evidence of carcinogenic activity" (NTP 1995). This condition is considered sex- and species-specific (e.g., unique to the male rat) and is not observed in female rats or other laboratory species (e.g., mice, rabbits or guinea pigs) (USEPA 1991). It is also not seen in male rats, such as the NCI Black Reiter, that do not synthesize α2u-globulin in the liver and are not susceptible to renal tubular cell proliferation or tumor formation resulting from chemicals that cause renal tubular tumors in male rats of strains that do synthesize α2u-globulin (Dietrich and Swenberg 1991). Also, transgenic mice modified to synthesize α2u-globulin develop renal tumors by chemicals that do not produce these tumors in conventional mice that do not synthesize α2u-globulin (Lehman-McKeeman and Caudill 1994).

Budroe et al. (2004) questioned the sex-specificity of TBA, stating that adverse renal effects occurred in female rats. According to NTP (1995), nephropathy, termed chronic progressive nephropathy (CPN), is an age-related, spontaneously occurring background lesion in F344 rats. In the 90-day study with TBA, signs of nephropathy were seen in both male and female rats at the end of the study (NTP 1995). A dose-related increase in the incidence of nephropathy was seen in females; however, no increase in the severity was seen in females. In males in this 90-day study, both the incidence and severity of the nephropathy increased with dose. Neither renal inflammation nor renal hyperplasia were found in either males or females after 90 days of treatment. In the 2-year study with TBA, the incidence of nephropathy in

females did not increase, but the severity score increased with dose, when evaluated using step sectioning (NTP 1995). According to the NTP (1995), the inflammation noted in the mid- and high-dose group females was regarded as part of the nephropathy associated with CPN. The incidence of inflammation and transitional epithelial hyperplasia occurs commonly with nephropathy and can occur more frequently with more severe nephropathy, as indicated in the 2-year study, where significant increases in transitional epithelial hyperplasia and inflammation were noted in the high-dose group only and in the two highest dose groups, respectively (NTP 1995). According to NTP (1995) this hyperplasia did not progress to renal tumors in the renal pelvis.

Further, renal tubular hyperplasia was comparable to controls in treated females (0/50, 0/50, 0/50 and 1/50 for controls and the 3 treatment groups) but was significantly increased in treated males. Renal tubular hyperplasia was absent in control females but was present in control males (14/50). The data in male rats support the connection between renal tubular hyperplasia and renal tubule tumors, that is, the progression with time from hyperplasia to adenomas in these cells. Renal tubular hyperplasia was significantly increased in the high-dose males but not in the other dose groups, in particular the mid-dose group. Conversely, the incidence of renal tubular adenomas was statistically significantly increased in only the mid-dose group. A possible explanation for this observation is the significantly decreased survival in the high-dose males, thereby, decreasing the time for progression from hyperplasia to adenomas in animals dying early.

A number of CIGA compounds, such as hexachloroethane, dimethlyphosphonate, and 1,4-dichlorobenzene, caused an increase in nephropathy, characteristic of age-related increases in CPN, in male and female rats (USEPA 1991) (Table 7). None of these chemicals caused an increase in the number renal tumors in either female rats or male and female mice (mice data not shown). Administration of a number of CIGA chemicals has been reported to exacerbate CPN; however, there is no clear evidence that CPN leads to renal tumor formation in rats or humans (USEPA 1991). Moreover, for 34 compounds identified as CIGA agents, none of these resulted in renal tumors in female rats or male and female mice despite signs of enhanced CPN for some of these compounds in female rats and male and female mice (USEPA 1991).

Mineralization, a component of CPN and as part of α2u-globulin-mediated tumors in treated rats, was seen in rats in both control and treated groups in the 2-year study. In treated males, this mineralization included linear foci in the renal papilla, a lesion specifically attributed to increased accumulations of α2u-globulin (NTP 1995). In contrast, the mineral deposits in control males and in all females occurred at the junction of inner and outer stripes of the cortico-medullary junction, which is common in male and female rats and is unrelated to α2u-globulin-induced nephropathy (NTP 1995). Similarly, in the 90-day study, the incidence of mineralization was significantly increased in male rats at the higher doses; all female rats (both control and treated groups) had evidence of mineralization. Mineralization in males and females consisted of focal mineral deposits at the cortico-medullary junction, consistent with that seen in CPN.

A pattern emerges for these renal effects that is both time and dose dependent and that reveals sex-specific differences. Two parallel events that are apparently occurring: 1) age-related nephropathy unrelated to tumor formation; and 2) biological events leading to tumor formation. Nephropathy as part of CPN occurs earlier and is more pronounced in males than in females after 90 days of treatment. Consequently, the conclusion can be drawn that some CIGA agents, including TBA, can have other effects on the rat kidney that are unrelated to, and do not progress to the benign renal tumors found in male rats (i.e., these are not considered precursor lesions for the benign renal tumors in male rats). The presence of nephropathy in female rats is unrelated to renal tumor formation and does not negate the mode of action for renal tumors in male rats.

Demonstrate the presence of induction of characteristic sequence of histopathological changes (cytoplasmic protein droplet accumulation, protein droplets are α2u-globulin, and the chemical binds to α2u-globulin).

Protein Droplet Accumulation

In a 90-day drinking water study, male and female rats and mice were given TBA in drinking water at concentrations of 0, 2.5, 5, 10, 20, or 40 mg/mL. In rats, a dose-related increased incidence of hyaline droplets and angular crystalline structures within renal tubule epithelium and tubule lumina was noted in treated males but was absent in control males and in

all females. A dose-response trend was evident with minimal increased droplet accumulation in the 2.5 mg/mL group and was more prominent in the 5, 10, and 20 mg/mL groups (all rats died prior to the end of the study in the 40 mg/mL group). Takahashi et al. (1993) re-evaluated renal tissue of male rats from a 90-day toxicity study conducted by the NTP (1995). Takahashi et al. (1993) resectioned the kidneys to reevaluate these kidney tissues. Treatment-related increases in the quantity of hyaline droplets and the number of abnormal intracytoplasmic deposits were noted only in the proximal tubules. Treatment-related nephropathy was also increased in all male rats in all dose groups (note the animals in the high dose group died by week 12 and were excluded). The authors found a direct correlation between protein hyaline droplet accumulation and deposition of abnormal crystals described as angular, crystalline structures typically associated with hyaline droplets.

Borghoff et al. (2001) exposed male and female F344 rats to TBA by the inhalation route at concentrations of 0, 250, 450, or 1750 ppm for 6 hours per day for 10 days. The highest exposure provided a dose that approximated the dose received by the 20 mg/mL dose group in the 90-day drinking water study. Significant and concentration-dependent increases in protein droplets were found in treated males, with no increases noted in male controls or females. In male rats, there was increased accumulation of protein droplets within the proximal tubule, resulting in the formation of large coalescing globules of protein and crystalloid protein structures. There was a dose-related increase in the protein droplet score (product of severity and percentage of tubules affected) that was statistically significantly increased in the highest exposure category. A statistically significant, concentration-dependent positive trend for protein droplet accumulation was determined for TBA-treated males.

Budroe et al. (2004) stated that the dose-response relationship between hyaline droplet formation and renal tumor incidence was not particularly strong. The statement was based on a comparison of the dose at which hyaline droplet formation was noted in the 10-day inhalation study (Borghoff et al. 2001) and the 90-day drinking water study (NTP 1995) with the dose at which renal tumors were found in the 2-year bioassay. In the 90-day study, hyaline droplet formation was “minimal” at a drinking water concentration of 2.5 mg/mL and not significantly increased following 10 days of exposure at TBA air concentrations that were assumed to result in a dose equivalent to that in the 2.5 mg/mL group in the 90-day drinking water study, In the 2-

year study, a significant increase in renal tumors was noted in male rats given drinking water at a TBA concentration of 2.5 mg/mL, which was not the highest concentration administered. Rather than being conflicting results, these illustrate a basic toxicological principal, in that the response is related to (dose) x (time). With many endpoints the incidence and severity of the observed effects will increase with increasing dose or with increasing duration at a constant dose. For example, survival of rats administered 5 mg/mL for 13 weeks was unaffected; however, survival was significantly reduced in the rats receiving the same amount in the 2-year study. It is not advisable to use different dosing durations and routes of exposure to reach conclusions as to the strength of the TBA-induced hyaline droplet/renal tumor relationship.

Identification that the accumulated protein is α2u-globulin.

Borghoff et al. (2001) demonstrated by immunohistochemical staining specific for α2u-globulin that the protein droplets in the renal proximal tubules in male rats administered TBA by inhalation (see above) was α2u-globulin. No positive staining was observed in male controls or female rats. The authors noted that there did not appear to be a dose-related increase in the intensity of this staining. However, the concentration of renal α2u-globulin per gram of tissues measured using an Enzyme-linked Immunoabsorbent Assay (ELISA) increased with treatment in a dose-related manner and was statistically significant in the high concentration group. The lack of a linear or dose-related increase in α2u-globulin staining is not unexpected. According to Borghoff et al. (2001) a similar pattern was seen with MTBE-exposed rats in both 10-day and 13-week inhalation studies. In the MTBE studies, immunohistochemical staining did not reveal a dose-related increase in intensity of the stain; however, as with TBA, the concentration of α2u-globulin in kidney cytosol increased in a dose-related manner when measured using ELISA. These results are not contradictory. MTBE and TBA may be very weak inducers of α2u-globulin in comparison to a strong inducer, such as TMP. According to Borghoff et al. (2001), the small increases in α2u-globulin may be more readily detected using the ELISA as it is a more sensitive method than immunohistochemical staining.

Renal concentrations of α2u-globulin were also measured by Williams and Borghoff (2001). Male and female F344 rats were given a single gavage dose of 500 mg TBA/kg. The concentration of α2u-globulin in kidney cytosol was significantly increased over concentrations

in control. The dose selected was based on the drinking water concentration (5 mg/mL; 420 mg/kg/day) administered for 13 weeks in the NTP study in which protein droplets were found in male but not female rats.

Reversible binding of the chemical to α2u-globulin.

In a study conducted by Williams and Borghoff (2001), male and female F344 rats were administered a single gavage dose of 500 mg [^{14}C]-TBA/kg or corn oil vehicle. A statistically significant higher percentage of the radiolabeled TBA was detected in male kidney, liver and blood than in these organs in females. Radiolabled TBA coeluted with α2u-globulin from male, but not female, kidney cytosol. Protein dialysis demonstrated that the interaction between radiolabeled TBA and α2u-globulin was reversible. Further, d-limonene oxide (a chemical with a high affinity for α2u-globulin) displaced radiolabeled TBA from the Low Molecular Weight Protein Fraction (LMWPF) from the kidney. Equilibrium dialysis with sodium dodecyl sulfate (SDS) with the LMWPF from treated male rats also resulted in the release of TBA. SDS is a detergent that denatures protein, resulting in the release of bound chemical if that binding is reversible. The study demonstrated the reversible binding of TBA to α2u-globulin.

Induction of sustained cell proliferation in the renal cortex.

Both Takahashi et al. (1993) and Borghoff et al. (2001) demonstrated dose-dependent increases in cell proliferation. In the Borghoff et al. (2001) study, the labeling index (LI) on brdU immunohistochemically stained renal cortex section increased in a concentration-dependent manner and was statistically significant in all treated males compared to control males. No significant differences were noted in control versus treated females. A significant positive correlation was found between α2u-globulin concentration and LI in the male kidney.

Treatment-related increases in PCNA-stained S-phase nuclei from proximal renal tubule epithelial cells were seen and were statistically significant in male rats receiving 20 mg/mL TBA in drinking water for 90 days (Takahashi et al. 1993). An increase in cell proliferation (as measured by PCNA staining) was also noted in the 10 mg/mL group but was not statistically significantly increase. According to the authors, these data support the concept that the accumulation of excess hyaline droplets is a key factor in enhanced cell proliferation.

Budroe et al. (2004) stated that the cell proliferation, hyaline droplet accumulation, and α2u-globulin concentration data in the Borghoff et al. (2001) study were contradictory. A significant increase in cell proliferation was noted in rats exposed at 250 ppm TBA, while both hyaline droplet accumulation and α2u-globulin concentrations were statistically significantly increased (pair-wise) at the highest exposure concentration of 1750 ppm. While not significant by a pair-wise comparison, a significant positive trend was found for both endpoints, as well as a significant positive correlation between α2u-globulin accumulation and LI in that study. Borghoff et al. (2001) noted and discussed a number of reasons for the difference in concentration at which these 3 responses occurred. First, the LI was measured in the kidney using a 3-day brdU pump, which may be a more sensitive method than evaluating protein droplet or α2u-globulin accumulation that was measured at one time point following exposure, and, therefore, could detect changes at lower exposure concentrations. Second, α2u-globulin concentrations were measured 24 hours after exposure, which for strong inducers results in the maximum difference between treated groups and controls; however, the time course for a weak inducer, such as TBA, may indeed be different. Third, cell proliferation, as measured by brdU, may, in short-term exposures, represent an adaptive response to TBA present in the kidney. In addition, not all of the increase in cell proliferation may have been caused by α2u-globulin accumulation. A close correspondence between the dose at which hyaline droplet accumulation, deposition of abnormal crystals, and cell proliferation (measured by PCNA) were increased was noted by Takahashi et al. (1993) in the kidneys of male rats in the 90-day drinking water study conducted by the NTP (1995). According to Borghoff et al. (2001) and Takahasi et al. (1993), there are no data that suggest a mechanism other than that mediated by α2u-globulin.

Lack of genotoxicity activity based on a weight-of-evidence evaluation of in vitro and in vivo data.

TBA did not induce mutations (no increase in the number of histidine-independent reverants) in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 with and without metabolic activation with rat and hamster S9 mix (NTP 1995, 1997; Zeiger et al. 1987). Williams-Hill et al. (1999) found that in the presence of S-9 activation, TBA did produce mutations in tester strain TA102; however, the proportional increase was modest and occurred at

very high doses (less than a 1.5-fold at approximately 750 μg/plate; 1.9-fold at approximately 1500 μg/plate; 2-fold at approximately 2250 μg/plate; 1.4-fold at 3750/plate μg/plate). The decline in revertants per plate was steep at doses above 2250 μg/plate; it was not reported if TBA was or was not toxic at those levels. These results were not confirmed in another laboratories that tested TBAc (*tertiary*-butyl acetate from which TBA is generated as a metabolite) in *S. typhimurium* strains TA100, TA98, TA 1535, TA 1537 and TA102 and *E. coli* strain WP2uvrA/pKM101 at doses ranging from 5 to 5000 μg/plate with or without metabolic activation (McGregor et al. 2004; 2005). The results of two laboratories that conducted independent tests of TBA in TA102 at doses up to 5000 μg/plate, with or without metabolic activation were also negative (McGregor et al. 2005). The tester strains commonly used in the *S. typhimurium* mutagenicity test, TA98, TA100, TA1535, and TA1537, have G-C base pairs at the site of mutation (Levin et al. 1982). While the tester strain, TA102, has a mutation of the ochre *hisG248* gene with an A-T base pair at the site of the mutation that is sensitive to detection of oxidative damage to DNA (Levin et al. 1982). The *E. coli* strains WP2 are also used to detect oxidizing mutagens (Wilcox et al. 1990).

It has been suggested by Budroe et al. (2004) that TBA is mutagenic by way of oxidative damage and cites Williams-Hill et al. (1999) as stating that MTBE and its metabolites required an intact excision repair system to produce oxidative damage to DNA. If so, then it is expected that TBA would be negative in tester strains not responsive to oxidative damage and that have *uvr*B excision repair deletions. The conclusion that TBA would require an intact repair system can not be drawn from the published data in Williams-Hill et al. (1999). Such a claim was made for MTBE based on the positive results in TA102 (with an intact repair system) and negative results in TA104 (a tester strain also sensitive to oxidative damage but lacking an intact repair system). TBA was only tested in the TA102 strain and not the TA104 and, therefore, a statement regarding excision repair can not be supported. Further, formaldehyde, another MTBE metabolite tested was positive in TA100, TA102, TA104, indicating that an intact excision repair system was not required, The results of Williams-Hill et al. (1999) were not confirmed in other laboratories which tested TBA in TA102 and TBAc in TA102 and an *E. coli* WP2 strain also sensitive to oxidative damage (McGregor et al. 2004; McGregor et al. 2005).

The likely antecedent of oxidative damage to DNA is the production of free radicals. A number of agents, including x-rays, UV light, hydrogen peroxide, bleomycin, mitomycin C, a variety of aldehydes, such as formaldehyde, and others are positive in TA102 (Levin et al. 1982). The mutagenicity of hydrogen peroxide in TA102, which is due to the production of free radicals with resulting oxidative damage, was blocked by cashew apple juice, an antioxidant that scavenges free radicals (Melo Cavalcante et al. 2003). TBA is also thought to be a free-radical scavenger (Cederbaum and Cohen 1980), which is thought to be the mechanism by which TBA protected DNA in mouse leukemia cells (Roots and Okada 1972) or in bacteriophage (Lafleur and Loman 1982) from the effects of radiation. Consequently, the positive response in TA102 noted by Williams-Hill et al. is puzzling.

If TBA is indeed positive in the TA102 assay, its potency is minimal to negligible compared to known oxidizing agents. At a dose of approximately 750 µg TBA/plate, only about 100 revertants above background were noted (Williams-Hill et al. 1999) compared to almost 3,000 revertants at 0.5 µg/plate of mitomycin C or over 700 revertants at 100 µg/plate of hydrogen peroxide (Levin et al. 1982). A possible, though unlikely, explanation for the result reported by Williams-Hill et al. may be formaldehyde-mediated. In *in vitro* studies, TBA was demethylated by rat Mixed Function Oxidases (MFOs) to yield small amounts of formaldehyde, apparently involving the interaction of TBA with hydroxyl radicals generated by hydrogen peroxide (Cederbaum and Cohen 1980; Cederbaum et al. 1983). However, *in vivo*, TBA is biologically stable and not readily metabolized and does not form aldehydes or ketones by dehydrogenation and is apparently not a substrate for alcohol dehydrogenase (Arslanian et al. 1971; Videla et al. 1982). *In vivo,* production of oxidative damage either directly by TBA or indirectly by the formation of formaldehyde is highly unlikely, and, consequently, genotoxicity by way of oxidative damage to DNA when TBA is administered to whole animals is unlikely as well.

As importantly, TBA was negative in the mouse lymphoma mutagenicity test (McGregor et al. 1988), the Chinese Hamster Ovary Cell cytogenetics test (NTP 1995), the rat bone marrow micronucleus test (NTP 1997), and the mouse peripheral blood micronucleus test (NTP 1995). If oxidative damage to DNA were operative, some effects in these mammalian systems, in particular in the *in vitro* tests, should have been noted given the high doses administered.

On the basis of the Williams-Hill et al. and Tang et al. studies only, Budroe et al. (2004) state that TBA does not meet the IARC criteria for "Negative for genotoxicity in a battery of tests". This statement is inconsistent with the application of the weight-of-evidence approach used by USEPA (1991) in their comparative evaluation of 9 CIGA chemicals. Several of these chemicals were positive in one or more of the standard tests for genotoxicity (Table 8). Dimethyl methyl phosphonate, isophorone, pentachloroethane, and hexachloroethane were positive in sister chromatid exchange tests in CHO cells, while positive results in the L5178Y micronucleus test were found for dimethyl methyl phosphonate, and pentachloroethane.

Moreover, USEPA guidelines for human cancer risk assessment (USEPA 2003) acknowledge that a non-linear dose-response may be operable even when there are positive genetic toxicity data. Genotoxicity tests provide information about the presence or absence of specific effects that may or may not be related to the adverse health outcomes measured for the animal (and by extrapolation, humans) as a whole and it is not clear how these relate to effects seen in animals in other organs. Genotoxicity test results are generally classified as "positive/negative", which ignores the fundamental toxicological concept of dose-response. The weight of evidence for TBA indicates that it is not genotoxic nor would it be expected to be at the estimated environmental exposures.

In summary, these data provide convincing evidence that the mode of action for male rat renal tumors is the α2u-globulin-mediated cascade of biological events. There is *in vivo* evidence that there are no proteins in human kidney that could function in a manner analogous to α2u-globulin (Meek et al. 2003). Consequently, compounds that produce renal tumors in male rats are not relevant to humans.

2.4.2 Mode of Action for Thyroid Tumors in Mice

Several chemicals have been shown to interfere with thyroid homeostasis. Although these chemicals interfere at different points in the biochemical feedback control of thyroid hormone secretion, the end result is that in the rodent, each chemical induces an increase in TSH levels. When these levels are sustained, the thyroid gland follicular cells are continuously stimulated, resulting in hyperplasia that can progress to neoplasia.

In the rodent, chemicals that interfere with thyroid hormone secretion or that increase thyroid hormone metabolism induce a compensatory increase in TSH. Consequently, the thyroid gland is continuously stimulated as long as the chemical interference is sustained. Some of the mechanisms by which chemicals can interfere with thyroid hormone synthesis, release and metabolism are:

- blocking iodide uptake,
- inhibition of iodine organification (conversion of I^- to I_2) and coupling with tyrosine,
- inhibition of thyroid hormone secretion and release,
- inhibition of 5'-deiodinase,
- induction of hepatic microsomal enzymes that metabolize thyroid hormone, or
- accumulation of pigment within the follicular cells or within the colloid resulting in decreased ability to synthesize hormone.

Chemicals that interfere with thyroid hormone homeostasis are structurally diverse and act by different mechanisms (see Table 9). However, the end result is that in rodents following the administration of these chemicals, thyroid hormone levels (T_3 and T_4) were decreased, resulting in a compensatory increase in TSH in order to maintain T_3 and T_4 levels at physiological levels (Capen et al. 1991; Capen 1996). With continued chemical interference, the elevated levels of TSH would be sustained. Due to the lack of binding globulins and subsequent rapid metabolism and degradation of thyroid hormones, the rodent thyroid gland is at near maximum stimulation in order to maintain physiological levels of thyroid hormone. The addition of a chemical that results in decreased peripheral levels of T_3 and T_4 with a compensatory increase in TSH would further stress a system that was near capacity. If the TSH stimulation was sustained, hyperplasia, and possibly progression to neoplasia, could result (Capen et al. 1991; Capen 1996).

The possible ways in which the thyroid-pituitary axis can be disturbed mentioned above are all likely to be processes that have thresholds, that is, a certain amount of disruption would be required before feedback loops result in sustained TSH stimulation of follicular cells resulting in the biological cascade of cell proliferation, hyperplasia, and neoplasia (USEPA 1998). USEPA (1998) guidelines for the evaluation of thyroid tumors in rodents recommend the data needed to

provide the evidentiary basis for a non-linear, threshold approach. The data for TBA has been evaluated here using that approach.

Mutagenicity

As discussed in Section 2.3.2.4 above, TBA was not mutagenic or genotoxic in any of the tests conducted in bacterial or mammalian tests systems (*in vivo or in vitro*). Studies that assessed the potential for TBA to damage genes were all negative (McGregor et al. 1988; Zeiger et al. 1987). NTP (1995; 1997) performed a battery of genetic toxicity studies including the S. *typhimurium* mutagenicity test (strains TA98, TA100, TA1535, and TA1537), the mouse lymphoma mutagenicity test, the Chinese Hamster Ovary Cell cytogenetics test, the rat bone marrow micronucleus test, and the mouse peripheral blood micronucleus test. The results of all genetic test performed by NTP (1997) were negative.

Thyroid Growth

Chemicals that act by way of disrupting thyroid-pituitary functioning stimulate thyroid enlargement (USEPA 1998). Absolute and relative (to body weight) thyroid weight were significantly increased in male mice. The incidence of follicular cell hyperplasia was increased in all dose groups in male mice and in the two highest dose groups (1,015 and 2,105 mg/kg/day) in female mice. The incidence of hyperplasia in male mice in the control and in all treated groups was less than that in females for corresponding dose groups. The background rate in males was 8%, while that in females was 33%. The incidence in the high-dose males was 32%, while that in females was 82%. These hyperplastic lesions were histologically similar to spontaneously occurring follicular cell hyperplasia indicating that TBA is likely acting in a similar manner to that producing hyperplasia in untreated animals.

Hormone Changes

No studies were conducted that measured thyroid hormone levels following TBA treatment; therefore, there is no direct evidence that thyroid hormone levels changed with TBA administration. There is indirect evidence that TBA could affect circulating hormone levels that would trigger feed back loops with resulting disruption in the thyroid-pituitary axis. Alcohols,

such as ethanol and 2-propanol, induce mixed function oxidases (MFOs), particularly CYP2E1 in rats (Donato et al. 2003; Miguez et al. 1994) and mice (Gong et al. 2003). TBA also induced MFOs (Aarstad et al. 1985; Bechtel and Cornish 1972). At a high dose (1000 mg/kg), TBA resulted in a 3-fold induction of MFO activity after a single oral dose (Bechtel and Cornish 1972). Enzyme induction, specifically of CYP2E1, may be occurring in mice in the 2-year bioassay; however, no signs, such as liver hypertrophy and other signs of enzyme induction, were noted. One reason for this is that activation of CYP2E1 is associated with alcoholic cirrhosis through the formation of reactive oxygen radicals and acetaldehyde (Bruckner and Warren 2001). It is hypothesized that TBA is a scavenger of free radicals (Lafleur and Loman 1982; Roots and Okada 1972). In one study, TBA induced MFO activity without resulting in increases in liver weight; however, this was a single dose study (Bechtel and Cornish 1972). While this activation may be ongoing, a tie to alterations in thyroid hormone homeostatis is unknown. An alternative possibility is by induction of UDP-glucuronyltransferase (UDP-GT) (NTP 1995). A number of MFO inducers also induce UDP-GT (Parkinson 2001). Ethanol has been shown to induce UDP-GT in rabbits to a greater extent than 3-methylcholanthene (Yost and Finley 1983). Glucuronidation forming glucuronide conjugates of endogenous substrates, such as bilirubin and thyroid hormones, is catalyzed by UDP-GT (Parkinson 2001). These are polar, water-soluble conjugates that are eliminated in the urine and bile. A number of classic MFO inducers produced a significant increase in biliary excretion of T_4 as T_4-glucuronide with a corresponding reduction in serum T_4 levels (Hosokawa et al. 1993; Vansell and Klassen 2001). It has been proposed that increased glucuronidation and biliary elimination of T_4 results in disruption of the thyroid-pituitary axis and underlies the production of thyroid tumors (Curran and DeGroot 1991; Kolaja et al. 1999; McClain et al. 1989; Parkinson 2001; Vansell and Klassen 2001). TBA is conjugated by glucuronidation and TBA-glucuronide is one of the urinary TBA metabolites (NTP 1995). TBA could be acting by way of increasing UDP-GT activity and, consequently and indirectly, increasing the glucuronidation of T_4 with enhanced biliary excretion of T_4. A mode of action involving enhanced biliary excretion of T_4 by way of induction of UDP-GL is also operative in the rat and thought to be the basis of phenobarbital-induced thyroid tumors (Curran and DeGroot 1991). Thyroid tumors were not seen in the NTP

study in rats. However, there are species differences in the ease of induction of UDP-GT and rats are less inducible than mice (Litterst et al. 1975; Vessey and Zakim 1972).

Site of Action

A possible mode of action for TBA is indirectly through enzyme induction in the liver. Relative but not absolute liver weights were increased in the two highest dose groups in the 13-week study but so were body weight decreases. Organ-weight data were not presented in the two-year bioassay. No other indications of enzyme induction, such as enlarged livers or hypertrophy were noted in the two-year study but clinical chemistry parameters were not conducted in mice in the 2-year study. UDP-GT is located in the liver but also in other tissues, such as the kidney, intestine, skin, brain, and spleen (Parkinson 2001). While these are suggestive, the site of action remains uncertain.

Dose Correlations

There is a dose correlation among the two endpoints of interest: follicular cell hyperplasia and focal follicular cell adenomas and carcinomas in males and more strongly in females (Figures 1 and 2). In addition, when the correspondence between hyperplasia and adenomas in females (Figure 2) was adjusted by that background rate for follicular hyperplasia (33%), there was a even closer correlation between both endpoints.

Reversibility

No studies were located that evaluated reversibility.

Other Studies

TBA was not identified as a carcinogen in an initiation/promotion study following initiation with 4-nitroquinoline (Hoshino et al. 1970). It should be noted that details of the protocol were not provided.

In summary, the pattern of follicular cell hyperplasia and follicular adenomas, viewed in light of benign tumors only and in only one sex and the negative mutagenicity data, strongly suggests an epigenetic mode of action for TBA in the production of these tumors in mice. There

is suggestive evidence as to the putative mode of action by way of enzyme induction and the potential effects on circulating hormones.

2.5 Weight of Evidence

As discussed in detail above, TBA was administered in drinking water for two years to groups of male and female rats at concentrations resulting in estimates of intake of 85, 195, or 420 mg/kg/day for males and 175, 330, or 650 mg/kg/day for females (Cirvello et al. 1995; NTP 1995). The only tumor response noted was in male rats and was an increase in renal tubular adenomas and only after combining the incidence found in the initial standard histopathological (single section) evaluation and extended histopathological evaluation (step sections) were combined. The incidence of renal tubular carcinomas alone was not significant either in the standard or extended evaluations and did not increase the incidence in any dose group when evaluated in combination with renal tubular adenomas. In the published results of the NTP investigation, the authors concluded that the proliferative renal lesions seen with TBA administration are similar to those reported for a number of chemicals associated with the accumulation of α2u -globulin (Cirvello et al. 1995). Cirvello et al. (1995) concluded that the increase in hyaline droplets in the 13-week study and the mineralization in the two-year study suggest that α2u-globulin accumulation may be the mode of action for TBA. As discussed in detail above, a series of mechanistic studies support α2u-globulin accumulation as the mode of action for TBA in male rat kidneys. The evidence can be summarized as follows:

- demonstrated species and sex specificity – found only in male rats;
- increased protein/hyaline droplet accumulation and angular crystalline structures within renal tubule epithelium;
- evidence that the accumulated protein is α2u-globulin;
- demonstrated that TBA binding to α2u-globulin is reversible;
- sustained cell proliferation in the renal cortex has been demonstrated; and
- weight-of-evidence evaluation supports the conclusion that TBA is not mutagenic or genotoxic.

Renal tumors in male rats that are caused by a α2u-globulin mode of action are not considered relevant to humans, and are not used in dose-response modeling or risk assessments (Meek et al. 2003; USEPA 1991).

A companion study in mice was conducted in which male and female mice received TBA in drinking water for 2 years resulting in doses of 540, 1040, or 2070 mg/kg/day in males and 510, 1020, or 2110 mg/kg/day in females. The only tumor type significantly increased was focal follicular cell adenomas in female mice. The incidences of focal follicular cell adenomas and carcinomas were not significantly increased in male mice. However, follicular cell hyperplasia was significantly increased in all treated males. Survival was significantly reduced in the high-dose group in males compared to control values and may have resulted in intercurrent mortality (deaths due to causes other than the tumor in question) that affected the tumor incidence, that is, some male mice may not have lived long enough to have developed this tumor. The incidence of focal follicular cell adenomas was significantly increased in the high-dose females and follicular hyperplasia was significantly increased in the two highest dose groups.

The mode of action for the formation of thyroid follicular cell adenomas in mice was evaluated according to USEPA guidelines, as discussed above. According to the USEPA (1998), chemicals that disrupt the thyroid-pituitary axis resulting in changes in circulating hormone levels with the resulting cascade of hyperplasia and neoplasia do so by a mode of action that is not relevant to people. No studies were conducted that measured changes in thyroid hormone levels that provide direct evidence for an interruption of the thyroid-pituitary axis; however, as discussed above, indirect evidence indicates that mode of action. These include:

- evidence of thyroid growth (increase in absolute and relative thyroid weights and increases in hyperplasia);
- possible connection between induction of UDP-GT and enhanced elimination in the bile of T_4;
- correlation between hyperplasia and adenomas (when corrected for background in the females)
- lack of promoter effect in initiation/promotion studies; and
- weight-of-evidence evaluation supports the conclusion that TBA is not mutagenic or genotoxic.

The pattern of follicular cell hyperplasia and follicular cell adenomas, viewed in light of benign tumors only and in only one sex and species along with the lack of mutagenicity or genotoxicity, strongly suggests a non-linear, epigenetic mode of action that has a threshold. Because the mode of action (direct evidence for hormone changes and for the site of action) is uncertain, the relevance of these finding to human health is also uncertain. However, given the weight-of-the-evidence, TBA is *Not Likely to be a Human Carcinogen.*

3.0 Dose-Response Assessment

When a chemical is classified as a carcinogen, quantitative dose-response analyses are typically conducted using animal bioassay or human epidemiological data to derive estimates of daily intake that would result in exposures that are below acceptable risk-based levels. Historically, excess lifetime human cancer risk has been estimated using a linear extrapolation in the low-dose region of the dose-response curve (USEPA 1986). Use of the linear approach assumed that the underlying mode of action for a chemical was a non-threshold process and that the probability of response was proportional to dose, i.e., a linear relationship between dose and response. It has been recognized in the scientific community that many chemicals may exert their effects in animal models by a nonlinear mode of action, produce an effect by a mode of action that has a threshold, or be a carcinogen in rodents by a mode of action that is not relevant to humans (Alison et al. 1994). The most recent USEPA guidance on human health cancer risk assessment recommends using data from experimental or epidemiological studies to estimate a dose at the bottom end of the observable range, termed a point of departure (POD), which is usually defined as the lower bound on dose at a 10% risk (LED_{10}) (USEPA 2005). Then, depending on the weight-of-evidence, either a linear (estimate a slope factor and risk-specific doses) or non-linear extrapolation to low doses is used to derived Reference Dose/Reference Concentration (RfD/RfC)] in accordance with USEPA guidelines.

3.1 Selection of Data for Dose-Response Modeling

The data selected for dose-response modeling was the incidence of adenomas in male and female mice and the incidence of hyperplasia in both sexes. Both quantal and time-to-tumor data were available for the incidence of adenomas; however, only quantal data were available for the hyperplasia. Use of precursor data is intended to extend the shape of the dose-response curve to doses that may not have been tested in the chronic bioassay; however, follicular cell hyperplasia was not seen in the 13-week study at any dose tested (2.5 mg/mL to 40 mg/mL).

3.2 Estimation of the Human Equivalent Dose

When data from animal studies, such as the TBA bioassays, are extrapolated to humans to provide estimates of lifetime cancer risks, potential differences in pharmacokinetics

(metabolism) and pharmacodynamics (sensitivity) between the animal species and humans should be considered in the estimation of human equivalent doses. Toxicokinetic data indicate that TBA is poorly metabolized *in vivo* (Baker et al. 1982; Thurman et al. 1980) and slowly eliminated from the blood of rats and mice (McComb and Goldstein 1979), likely as a conjugate with glucuronide (Aarstad et al. 1985; Thurman et al. 1980). However, pharmacokinetic data were insufficient at this time to develop a pharmacokinetic model for TBA or to make an adjustment for pharmacokinetic differences between rats and humans. Therefore, the default assumption of an animal-to-human scaling factor of body weight$^{3/4}$ was applied. This is the equivalent of multiplying the administered dose in the mouse study by (BW Mouse/BW Human)$^{1/4}$ (human body weight assumed to be 70 kg; default body weights of 0.03 kg for male mice and female mice). This correction assumes that clearance of TBA in the human is slower than in the animal model, which may not be the case for TBA. Humans have a higher capacity for glucuronidation and may, therefore, clear TBA faster than the rodent. Consequently, the default scaling factor for animal-to-human kinetic differences will likely result in an under-estimate of the appropriate dose-metric and an over-estimate of human risk.

Pharmacodynamic differences must also be considered when extrapolating from the animal species to humans. When the mode of action for a chemical is well characterized, first a qualitative assessment is made to determine if that mode of action is operative in humans. If assumed relevant, an adjustment to provide the human equivalent dose metric is made and used in dose-response modeling to quantitatively account for the differences in species sensitivity. For example, an international multidisciplinary workshop on Leydig cell tumorigenesis has recommended such an adjustment to the input doses used in dose-response analyses to account for quantitative differences in response to increases in Luteinizing Hormone (LH) levels, the trigger for Leydig proliferation (Clegg et al. 1997).

Qualitatively, it is not known if humans are susceptible to disruption of the thyroid-pituitary axis and develop thyroid tumors (USEPA 1998). If so, humans are expected for numerous reasons to be less sensitive to these carcinogenic effects than rodents (USEPA 1998). Several candidates to quantify species differences could be postulated, such as the difference in serum proteins that lend humans much less sensitive to small changes in circulating thyroid hormone levels or a dampened response to enzyme induction with less, if any, effect on thyroid

hormone metabolism and elimination. However, because of the complex nature of these thyroid-pituitary interactions, these species differences in sensitivity have not yet been quantified. Therefore, no quantitative adjustment for pharmacodynamic difference was made.

3.3 Estimation of Point of Departure

Because environmental exposures are often outside the range of experimental observations, the USEPA (1999) uses dose-response models to estimate a dose in the observable range that serves as the point of departure (POD) for extrapolation to lower doses. The POD is defined as the lower bound on dose at a specified level of risk, usually 10% (LED_{10}). The level of risk may be less than 10% (e.g., 1% or 5%), depending on the slope of the dose-response curve. Because of uncertainty around the estimate of the LED_{10} or lower bounds for other risk levels, values for the maximum likely estimate (ED_{10}) and the upper bound (UED_{10}) are reported along with the lower bound. However, the LED_{10} is used as the point of departure (POD) for extrapolation to lower doses.

Because there were no survival differences among control and treated females, dose-response modeling for focal follicular cell adenomas was conducted for the selected data sets using the multistage model. For comparison, follicular cell adenomas in male mice were also evaluated using the multistage model, which has the form:

$$p(d) = 1 - e^{-(q_0 + q_1 \times d + q_2 \times d^2 \cdots + q_k \times d^k)} \qquad \text{Equation 1}$$

for dose (d). For each data set, the ED_{10} and the upper and lower bounds on the ED_{10} were estimated (Appendix A). The multistage dose-response model results were estimated using TOX_RISK Version 5.3 (ICF Consulting 2001).

Because survival in the male mice in the high-dose groups was significantly reduced compared to the survival in the vehicle control group, a time-to-tumor model, MultWeib, was used. For comparison, data for the female follicular adenomas was also evaluated using this model. This model considers the differences in survival in the animals and how that impacts the

estimation of risk. This model is Multistage in dose and Weibull in time and essentially assesses the probability that a tumor would have been identified at time t. The MultWeib has the form:

$$p(d,t) = 1 - e^{-(q_0 + q_1 \times d + q_2 \times d^2 \cdots + q_k \times d^k) \times (t - t_0)^c} \quad \text{Equation 2}$$

The ED_{10} and upper and lower bounds were estimated using the MultWeib model in the program QRisk (ENVIRON International Corporation 2003). The results of the dose-response modeling are given in Appendix A.

The LED_{10}s based on the incidence of follicular cell adenomas and carcinomas in males and adenomas in females were 301 and 206 mg/kg/day for males and females, respectively, based on the Multistage model (Table 10) (Appendix A). The LED_{10} for females using the time-to-tumor model was virtually the same, 200 mg/kg/day, as that estimate using the Multistage model, as would be expected because of the lack of survival problems in female mice. Use of the Multweib for the male mouse data resulted in an LED_{10} of 207 mg/kg/day, which is considerably lower than the results from the Multistage model. This would be expected because of the reduced survival in the high-dose males. The fit of the Multistage model to the data was evaluated using the Chi-Square Goodness of Fit test for the female data. The fit of the model to the data was very good for the data set in females and adequate for the data set in males. With time-to-tumor data sets, the fit of the model is evaluated using the Log-likelihood method and is estimated using the dose-response curves (Appendix B). With the log-likelihood method, the value closest to zero provides the best fit; however, the fit of the model to both data sets was comparable.

The hyperplasia data were evaluated using a number of Benchmark models available from the USEPA. These include the Multistage, logistic, gamma, quantal-linear, weibull, quan-quadratic, and the probit (Table 11). The results are reported as the Benchmark Dose (BMD), the lower bound on the BMD at a Benchmark risk of 10% ($BMDL_{10}$), and the $BMDL_{10}$ after adjusting the dose by body weight$^{3/4}$ ($BMDL_{10adj}$). Use of the 10% risk and the body weight conversion was to provide results comparable to those for the follicular adenomas analyses. For females, the multistage model provided an excellent fit (p=.86); however, none of the models provided a very good fit to the male hyperplasia data. This is not unexpected as there were

survival problems in the males that were unable to be accounted for because of the lack of time to incidence data. The BMDLs and $BMDLs_{adj}$ were 267.4 mg/kg/day and 22.2 mg/kg/day, respectively, for females, and 702.1 mg/kg/day and 61.7 mg/kg/day for males. As might be expected for the obligatory precursor event, an equivalent change, i.e., a 10% change in response over background, is estimated to occur at a lower dose than for the neoplastic lesion.

The smallest of the LED_{10}s or $BMDL_{10adj}$, 22 mg/kg/day, was selected as the POD.

3.4 Evaluation of the Approach for Low-dose Extrapolation

Administration of TBA resulted in a significant increase in benign follicular cell adenomas in female mice and only in the highest dose group. In male mice, the incidence of follicular cell adenomas alone or in combination with carcinomas were not statistically significantly increased above control values even when survival differences were also considered. The incidence of follicular cell hyperplasia was increased in all dose groups in males; however, the response was not dose-related and did not apparently progress to adenomas. Hyperplasia was significantly increased in the two highest dose groups in females with the incidence of adenomas following the same pattern as that seen with the hyperplasia. The hyperplasia seen in treated females and males was histopathologically identical to that seen in controls (NTP 1995).

The lack of a response in other tissues suggests a focused effect on some part of the thyroid-pituitary axis. The possible ways in which the thyroid-pituitary axis can be disturbed (as mentioned above) are likely processes that have thresholds, that is, a certain amount of disruption would be required before feedback loops result in sustained TSH stimulation of follicular cells resulting in the biological cascade of cell proliferation, hyperplasia, and neoplasia (USEPA 1998). The pattern of follicular cell hyperplasia and follicular adenomas, viewed in light of the negative mutagenicity data, suggests an epigenetic mode of action for TBA in the production of these tumors in mice. As discussed above, a possible mode of action for TBA would be through induction of UDP-glucuronyl transferase and the subsequent increased conjugation of T_4, increased elimination in the bile, and decreased serum levels. The decrease in serum levels could then trigger the thyroid-pituitary feedback with resulting increases in TSH and the biological cascade to hyperplasia and adenoma formation. However, while there is suggestive evidence as

to the putative mode of action, clear evidence is lacking at this time, but it is highly likely to be non-linear.

3.5 Extrapolation to Low Doses

The latest USEPA Guidelines (2005) indicate that for chemicals with a nonlinear mode of action, such as is likely for TBA, the POD is used to derive an RfD/RfC according to USEPA guidelines. Uncertainty factors associated with extrapolation across and within species and other types of uncertainty can be placed in a quantitative context. Then the POD is divided by the numerical value of those factors in the manner used in the derivation of a RfD.

An uncertainty factor for interspecies extrapolation (UHs) was considered. Extrapolation across species considers pharmacokinetic and pharmacodynamic differences and a default value of 10 (3 for kinetics and 3 for dynamics) is typically applied. In the absence of data on kinetic differences and a model or other means to quantify those differences, kinetic differences across species can be approximated by scaling by body weight$^{3/4}$, which was done in the derivation of the TBA human equivalent dose. Therefore, no further adjustment for species difference in kinetics was applied. It is unlikely that humans would be as sensitive to the effects of TBA as was the mouse for a number of reasons. However, in the absence of chemical-specific data, USEPA science policy is to assume humans are equally sensitive to the induction of thyroid cancer as rodents; therefore, an uncertainty factor of 1 was applied to account for species differences in pharmacodynamics. This is a conservative approach when tumors arise by way thyroid-pituitary disruption, as rodents are much more sensitive to this effect than humans (USEPA 1998).

An uncertainty factor for intraspecies extrapolation (UHh) was considered. This factor takes into account the variability in both kinetics and sensitivity within human populations. In the unlikely event that these mouse thyroid tumors are relevant to people and a mode of action involving thyroid-pituitary disruption is also operative in people, the default uncertainty factor of 10 was applied.

An uncertainty factor of 1 was used for UFsub, study duration. When chronic study data is not available and subchronic study data are used instead, an UFsub of 10 is applied. The POD was developed from a 2-year chronic study.

An uncertainty factor for Data Base limitations (UFdb) was considered. While it is assumed that the thyroid tumors result from a progression from hyperplasia to adenomas, the mode of action is not known with certainty. This could be viewed as a data base limitation; however, it is expected that hyperplasia is the key precursor event and because it produced the lowest POD, an additional uncertainty factor of 10 was not applied. An uncertainty factor of 10 for data base limitations was deemed reasonable because there is a lack of a two-generation reproductive study. This is reasonable because essentially the RfD is based on non-cancer, precursor data. It is uncertain if TBA would also be a reproductive toxicant, as are other alcohols, at doses that could result in a lower RfD.

The resulting RfD is 220 µg/kg/day (22 mg/kg/day/100).

4.0 Discussions and Conclusions

This analysis was intended to provide quantitative estimates of an acceptable human intake of TBA. The pharmacokinetic and toxicity data were reviewed to select the relevant data for quantitative assessment and to inform both the approach to species extrapolation and extrapolation to low doses relevant to human exposure. The potential modes of action of the only tumor types noted in a 2-year chronic drinking water bioassay, renal tubular tumors in male rats and follicular cell tumors in female mice were critically reviewed. Convincing evidence has been published that establishes the mode of action of TBA in the induction of renal tubular neoplasms in male rats as a male rat-specific response that is mediated by the accumulation of TBA- α2u-globulin complex in the renal proximal tubules resulting in nephropathy, cell proliferation, and tumor formation. Chemicals acting by that mechanism, which is not operative in humans who do not have a protein comparable in behavior to α2u-globulin, are unlikely to produce renal tumors in humans. Consequently, the use of renal tumors in male rats for human health risk assessment is not appropriate (USEPA 1991).

A definitive mode of action for TBA-induced thyroid tumors in humans has not been demonstrated experimentally. Moreover, while expected to be quantitatively different in humans, it cannot be said with certainty that these observations are not qualitatively relevant for human health risk assessment. Therefore, the thyroid tumors in male and female mice were evaluated quantitatively. An argument can be made based on the lack of mutagenicity data, the limited tumor array (only thyroid tumors in female mice), and the potential for induction of MFOs and UDP-GT for a mode of action acting by way of a threshold. This would involve increasing UDP-GT activity and, consequently and indirectly, increasing the glucuronidation of T_4 with enhanced biliary excretion of T_4 is plausible. A mode of action involving enhanced biliary excretion of T_4 by way of induction of UDP-GT is also operative in the rat and thought to be the basis of Phenobarbital-induced thyroid tumors (Curran and DeGroot 1991). Consequently, non-linear extrapolation to low doses was applied.

In an assessment of the relevance of thyroid follicular cell tumors observed in rodents and their relevance to human health, the USEPA (1998) noted that despite qualitative similarities in the control of thyroid hormone synthesis and secretion in rodents and humans, humans were

likely not as sensitive quantitatively to the development of thyroid tumors as a result of disruption of the pituitary-thyroid axis. The USEPA further noted that the presence of the high-affinity binding protein in humans was likely the reason for this quantitative difference. Although rats have binding proteins, these are low-affinity proteins, which allow the protein-bound thyroid hormone to be removed from the blood, metabolized and excreted more readily (USEPA 1998). As a result, the half-life of T_4 is much shorter in the mouse (1 day) than in the human (5 to 9 days). Consequently, the mouse thyroid gland is chronically stimulated by TSH in order to compensate for the rapid turnover of thyroid hormone, and as a result, it is likely that increases in TSH would be more likely to induce growth and potentially neoplastic changes in the mouse thyroid gland than in the human thyroid gland (USEPA 1998). Moreover, thyroid follicular cell tumors induced by the chemical perturbation of the pituitary-thyroid axis, which would result in increases in TSH, were considered secondary to the effects on thyroid gland function, and represented nonlinear or threshold events (USEPA 1998). Therefore, if in the rodent TBA is acting by interfering with thyroid hormone synthesis via induction of TSH, thereby, increasing the metabolism of thyroid hormone, it is questionable that these events would result in thyroid cancer in humans.

The RfD estimated using the non-linear approaches to low dose extrapolation was 220 µg/kg/day. The resulting drinking water concentration could be calculated as follows:

$$Drinking\ water\ concentration = \frac{220\ \mu g / kg / day\ x\ 0.001\ \mu g / mg\ x\ 70\ kg}{2\ L}$$

Assuming an average body weight of 70 kg and an average daily water consumption of 2 liters, the average drinking water concentration for TBA associated with the RfD would be approximately 8 mg/L.

Table 1
Incidences of Neoplastic and Nonneoplastic Lesions in Male Rats at 15-Month Interim Sacrifice
(NTP 1995)

Endpoint	Dose (mg/kg/day)			
	0	**90**	**200**	**420**
Kidney - mineralization	1/10	2/10	5/10	9/10**
Kidney - nephropathy	10/10	10/10	10/10	10/10
Kidney – renal tubule hyperplasia	0/10	0/10	2/10	0/10
Kidney – renal tubule adenoma	0/10	0/10	0/10	1/10

**Significantly different ($p \leq 0.01$) from control group by the logistic regression test.

Table 2
Incidences of Neoplastic and Nonneoplastic Lesions in Female Rats at 15-Month Interim Sacrifice (NTP 1995)

Endpoint	Dose (mg/kg/day)			
	0	180	330	650
Kidney - mineralization	10/10	10/10	10/10	10/10
Kidney - nephropathy	10/10	10/10	10/10	10/10

** Significantly different ($p \leq 0.01$) from control group by the logistic regression test.

Table 3
Incidences of Neoplastic and Nonneoplastic Lesions in Female Rats at Final Sacrifice
(NTP 1995)

Endpoint	Dose (mg/kg/day)			
	0	**180**	**330**	**650**
Kidney – inflammation, suppurative	2/50	3/50	13/50**	17/50**
Kidney – mineralization	49/50	50/50	50/50	50/50
Kidney – nephropathy	48/50	47/50	48/50	50/50
Kidney – renal tubule hyperplasia	0/50	0/50	0/50	1/50
Kidney – transitional epithelium, hyperplasia	0/50	0/50	3/50	17/50**

**Significantly different ($p \leq 0.01$) from control group by the logistic regression test.

Table 4
Incidences of Neoplastic and Nonneoplastic Lesions in Male Rats at Final Sacrifice
(NTP 1995)

Endpoint	Dose (mg/kg/day)			
	0	**90**	**200**	**420**
Kidney – nephropathy	49/50	49/50	50/50	50/50
Kidney – transitional epithelium, hyperplasia	25/50	32/50	36/50**	40/50**
Kidney – mineralization	26/50	28/50	35/50	48/50**
Kidney – mineralization, linear foci in the renal papilla	0/50	5/50*	24/50**	46/50**
Kidney - renal tubular hyperplasia	14/50	20/50	17/50	25/50**
Kidney - renal tubule adenoma (single section evaluation)	1/50	3/50	4/50	2/50
Kidney - renal tubule adenoma (step section evaluation)	6/50	4/50	9/50	9/50
Kidney - renal tubule adenoma (includes single section and step section evaluation)	7/50	7/50	10/50	10/50
Kidney – renal tubule carcinoma (single section evaluation)	0/50	0/50	0/50	1/50
Kidney – renal tubule carcinoma (step section evaluation)	0/50	2/50	1/50	0/50
Kidney – renal tubule carcinoma (includes single section and step section evaluation)	0/50	2/50	1/50	1/50
Kidney - renal tubule adenoma or carcinoma (single section evaluation)	1/50	3/50	4/50	3/50
Kidney - renal tubule adenoma or carcinoma (step section evaluation)	7/50	10/50	18/50**	11/50
Kidney - renal tubule adenoma or carcinoma (includes single section and step section evaluation)	8/50	13/50	19/50**	13/50

*Significantly different ($p \leq 0.05$) from control group by the logistic regression test.
**Significantly different ($p \leq 0.01$) from control group by the logistic regression test.

Table 5
Incidences of Neoplastic and Nonneoplastic Lesions in Male Mice at Final Sacrifice
(NTP 1995)

Endpoint	Dose (mg/kg/day)			
	0	**540**	**1040**	**2070**
Thyroid follicular cell hyperplasia	5/60	18/59**	15/59*	18/57**
Thyroid follicular cell adenoma	1/60	0/59	4/59	1/57
Thyroid follicular cell adenoma or carcinoma	1/60	0/59	4/59	2/57
Urinary bladder chronic inflammation	0/59	3/59	1/58	37/59**
Urinary bladder transitional epithelial cell hyperplasia	1/59	3/59	1/58	17/59**

*Significantly different ($p \leq 0.05$) from control group by the logistic regression test.
**Significantly different ($p \leq 0.01$) from control group by the logistic regression test.

Table 6
Incidences of Neoplastic and Nonneoplastic Lesions in Female Mice at Final Sacrifice
(NTP 1995)

Endpoint	Dose (mg/kg/day)			
	0	**510**	**1020**	**2110**
Thyroid follicular cell hyperplasia	19/58	28/60	33/59*	47/59**
Thyroid follicular adenoma	2/58	3/60	2/59	9/59*
Urinary bladder chronic inflammation	0/59	0/60	0/59	4/57**
Urinary bladder transitional epithelial cell hyperplasia	0/59	0/60	0/59	3/57

*Significantly different ($p \leq 0.05$) from control group by the logistic regression test.
**Significantly different ($p \leq 0.01$) from control group by the logistic regression test.

Table 7
Comparison of Nephropathy and Carcinogenicity in Male and Female Rats exposed to Chemicals Inducing α2u -Globulin Accumulation (CIGA) and TBA[a] (USEPA 1991)

Chemical	Hylaine Droplet Formation	Increased renal α2u -globulin levels	Cast Formation	Mineralization	Hyperplasia	Nephropathy	Carcinogenicity		
							Adenoma	Adenocarcinoma	Carcinoma
MALE RATS									
Decalin (decahydronaphthalene)	+	+	+	+	+	NR	+	-	+
Dimethyl methyl phosphonate	+	NR	+	+	+	+	-	+	-
Isophorone	+	+	+	NR	+	+	+[b]	+[b]	-
d-Limonene	+	+	+	+	+	+	+/[b]	+[b]	-
Pentachloroethane	+	+	+	+	+	+	+	-	-
Unleaded gasoline	+	+	+	+	+	NR	+	-	+
1,4-Dichlorobenzene	+	+	+	+	+	+	+	+[b]	-
Hexachoroethane	+	NR	+	+	+	+	+[c]	-	+[c]
Tetrachloroethylene	+	+	+	NR	+	+	-	-	-
TBA	+	NR	+	+	+	+	+	-	-

Table 7 (continued)

Chemical	Hylaine Droplet Formation	Increased renal α2u -globulin levels	Cast Formation	Mineralization	Hyperplasia	Nephropathy	Carcinogenicity		
							Adenoma	Adenocarcinoma	Carcinoma
FEMALE RATS									
Decalin (decahydronaphthalene)	-	-	-	-	-	**NR**	-	-	-
Dimethyl methyl phosphonate	-	**NR**	-	-	-	+	-	-	-
Isophorone	-	**NR**	-	-	-	+	-	-	-
d-Limonene	-	-	-	-	-	+	-	-	-
Pentachloroethane	-	**NR**	-	-	-	-	-	-	-
Unleaded gasoline	-	**NR**	-	-	+	**NR**	-	-	-
1,4-Dichlorobenzene	-	**NR**	-	+	-	+	-	-	-
Hexachoroethane	-	**NR**	+	+	-	+	-	-	-
Tetrachloroethylene	-	-	+	-	+	+	-	-	-
TBA	-	**NR**	+	+	-	+	-	-	-

NR - Not reported

a - TBA data presented in section 2.2 added to table adapted from USEPA (1991).

b - Combined incidence of tubular cell adenoma or adenocarcinoma

c - Combined incidence of renal tubule adenoma and renal tubule carcinoma

Results presented as positive (+) or negative (-)

Table 8
Genotoxicity of Chemicals Inducing α2u-Globulin Accumulation (CIGA) and TBA[a] (USEPA 1991)

Chemical	Genotoxcity			
	Salmonella	**Chromosome Aberrations in CHO cells**	**Sister Chromatid Exchange in CHO cells**	**Thymidine-kinase-gene mutation assay in L5178Y Cells**
Decalin (decahydronaphthalene)	**NR**	**NR**	**NR**	**NR**
Dimethyl methyl phosphonate	-	**E**	+	+
Isophorone	-	-	+	+/-
d-Limonene	-	-	-	-
Methyl isobutyl ketone	**NR**	**NR**	**NR**	**NR**
Pentachloroethane	-	-	+	+
Unleaded gasoline	-	-		+/-
1,4-Dichlorobenzene	-	-	-	**E**
Hexachoroethane	-	-	+	**NR**
Tetrachloroethylene	-	-	-	-
tertiary-Butyl Alcohol	-[b]	-	-	-

NR - Not Reported

a - TBA data presented in section 2.3.3 added to table adapted from USEPA (1991)

b - Negative predominately in al genotoxicity tests.

Results presented as positive (+), negative (-), questionable (+/-), or equivocal or weakly positive (E)

Table 9
Chemicals That Interfere with Thyroid Homeostatis

Mechanism	Chemicals	Effect on T_3 and T_4	Effect on TSH
Blockage of iodine uptake	perchlorate and thiocyanate	↓	↑
Inhibition of peroxidase and coupling of iodine with tyrosine	thiourea, thiouracil, propylthiourcil, methimazole, carbimazole, goitrin, sulfonamides, p-aminobenzoic acid, p-aminosalicylic acid, amphenone, resorcinol, phloroglucinol, 2,4-dihydroxybenzoic acid, aminotriazole, tricyanoaminopropene, antipyrine and iodopyrine.	↓	↑
Blockage of thyroid hormone release	iodide and lithium	↓	↑
Inhibition of 5'-deiodinase	FD&C Red No. 3, amiodarone and iopanoic acid	↓T_3 ↑T_4	↑
Induction of hepatic microsomal enzymes	phenobarbital, benzodiazepines, nicardipine, bepridil, steroids, chlordane, DDT, TCDD, PCB, and PBB	↓	↑
Accumulation of pigment	minocycline	↓	↑

Source: (Capen et al. 1991)

Table 10:
Mouse Thyroid Tumor Dose-Response Modeling Results

			Using BW ¾ Animal to Human Conversion		
		Model	**LED_{10}**	**ED_{10}**	**UED_{10}**
Female Mice					
	Follicular Cell Adenoma	Multistage	206.01	287.32	545.71
	Follicular Cell Adenoma	Multistage Weibull	199.78	281.49	502.90
Male Mice					
	Follicular Cell Adenoma and Carcinoma	Multistage	301.40	836.63	NC
	Follicular Cell Adenoma and Carcinoma	Multistage Weibull	206.59	373.79	NC

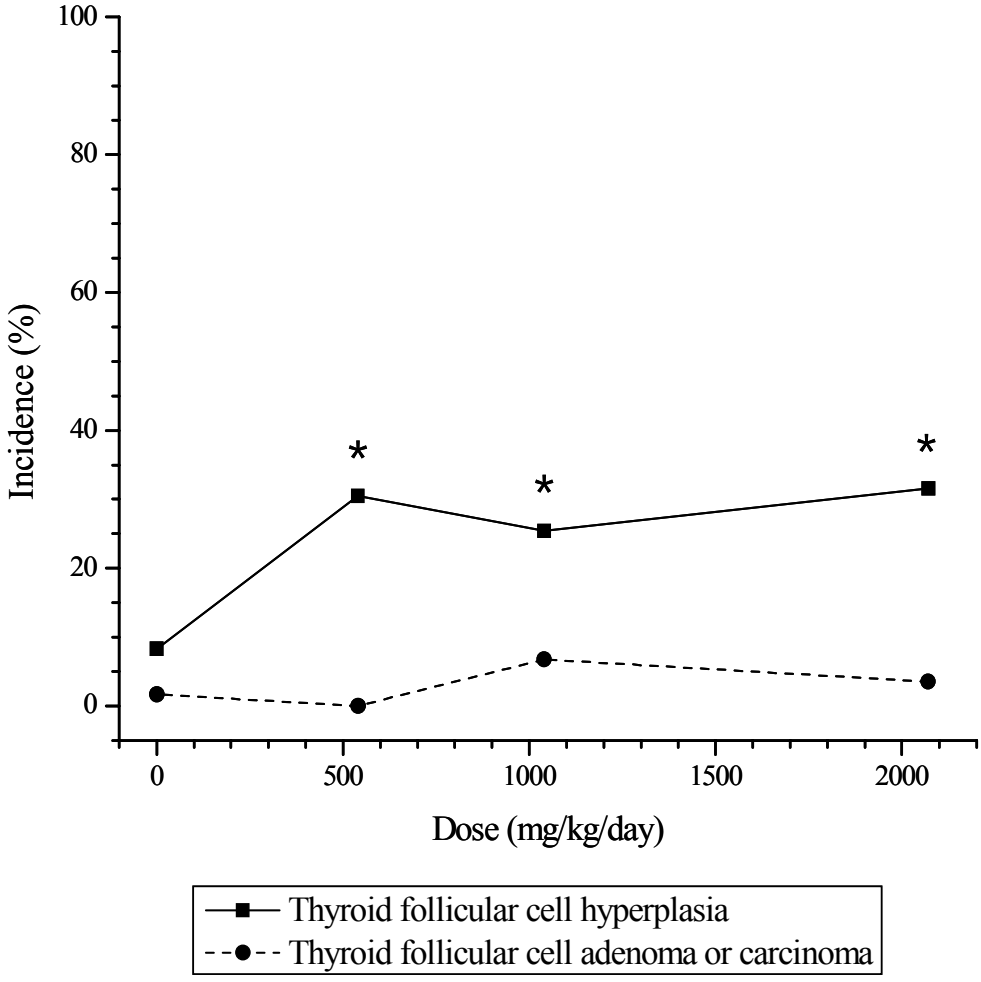
NC = Not calculated. It was not possible to calculate an upper bound for these data.
ED_{10}= Effective Dose at 10% extra lifetime risk
LED_{10}= Lower bound on ED_{10}
UED_{10}= Upper bound on ED_{10}

Table 11:
Results of the Benchmark Modeling for Follicular Cell Hyperplasia in Male and Female Mice in the 2-year Chronic Bioassay (in mg/kg/day).

Sex/Model	BMD_{10}[a]	$BMDL_{10}$[b]	$BMDL_{10adj}$[c]
Females			
Quantal-linear	202.1	150.1	21.6
Quantal-quadratic	626.6	525.7	75.7
Multistage	267.4	154.5	22.2
Weibull	319.3	153.5	22.1
Gamma	326.3	153.3	22
Logistic	373.3	115.3	16.6
Probit	386.8	275.9	39.7
Males			
Quantal-linear	702.1	428.8	61.7
Quantal-quadratic	1491.7	1053.4	152
Multistage	702.1	428.8	61.7
Weibull	702.1	428.8	61.7
Gamma	702.1	428.8	61.7
Logistic	585.4	338.9	48.8
Probit	1321.4	810.3	117

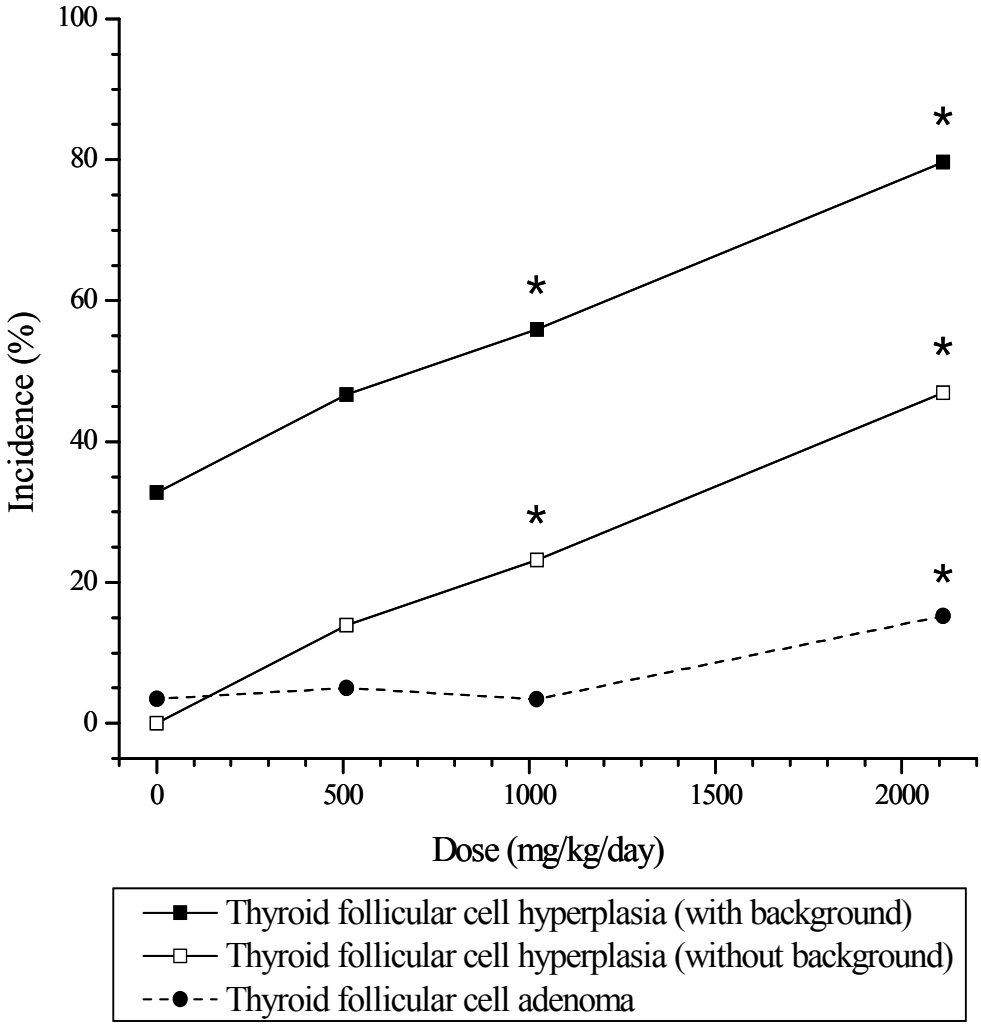
a - Benchmark Dose at a 10% extra risk
b - Lower bound on the BMD
c - Lower bound on the BMD after adjusting bioassay doses by body weight 3/4

Figure 1.
Incidence of Thyroid Neoplastic and Non-Neoplastic Lesions versus Dose in Male Mice
(NTP, 1995)



* Statistically significantly different from controls.

Figure 2.
Incidence of Thyroid Neoplastic and Non-Neoplastic Lesions versus Dose in Female Mice (NTP, 1995)



* Statistically significantly different from controls.

5.0 References

Aarstad, K., Zahlsen, K. and Nilsen, O. G. (1985). Inhalation of butanols: changes in the cytochrome P-450 enzyme system. *Archives of Toxicology Supplement* 8: 418-421.

Alison, R H, Capen, C C and Prentice, D E (1994). Neoplastic lesions of questionable significance to humans. *Toxicologic Pathology* 22: 179-186.

Arslanian, M. J., Pascoe, E. and Reinhold, J. G. (1971). Rat liver alcohol dehydrogenase. ***Biochem. J.*** 125: 1039-1047.

Baker, R. C., Sorensen, S. M. and Deitrich, R. A. (1982). The in vivo metabolism of tertiary butanol by adult rats. *Alcoholism: Clinical and Experimental Research* 6(2).

Beauge, F., Clement, M., Nordman, J. and Nordmann, R. (1981). Liver lipid disposal following t-butanol administration to rats. *Chem Biol Interactions* 38: 45-51.

Bechtel, D. H. and Cornish, H. H. (1972). Effect of the butyl alcohols on liver microsomal enzymes. *Abstracts: Eleventh Annual Meeting*: 298-299.

Bernauer, U., Amberg, A., Scheutzow, D. and Dekant, W. (1998). Biotransformation of 12C- and 2-13C-labeled methyl tert-butyl ether, ethyl tert-butyl ether, and tert-butyl alcohol in rats: identification of metabolites in urine by 13C nuclear magnetic resonance and gas chromatography/mass spectrometry. *Chem Res Toxicol* 11(6): 651-658.

Borghoff, S. J., Murphy, J. E. and Medinsky, M. A. (1996). Development of physiologically based pharmacokinetic model for methyl tertiary-butyl ether and tertiary-butanol in male Fisher-344 rats. *Fundam Appl Toxicol* 30(2): 264-275.

Borghoff, S. J., Prescott, J. S., Janszen, D. B., Wong, B. A. and Everitt, J. I. (2001). alpha 2u-Globulin nephropathy, renal cell proliferation, and dosimetry of inhaled tert-butyl alcohol in male and female F-344 rats. *Toxicol Sci* 61(1): 176-186.

Bruckner, J. V. and Warren, D. A. (2001). Toxic Effects of Solvents and Vapors. Casarett and Doull's Toxicology. The Basic Science of Poisons. C. D. Klassen, McGraw-Hill Medical Publishing Division**:** 869-916.

Budroe, J. D., Brown, J. P., Salsmon, A. G. and Marty, M. A. (2004). Acute toxicity and cancer risk assessment values for *tert* - butyl acetate. *Regulatory Toxicology and Pharmacology* 40: 168-176.

CALEPA (1999). Expedited Evaluation of Risk Assessment for Tertiary Butyl Alcohol in Drinking Water. http://www.oehha.org/water/pals/tba.html.

Capen, C. C., DeLellis, R. A. and Yarrington, J. T. (1991). Endocrine System. Handbook of Toxicologic Pathology. W. M. Haschek and C. G. Rousseaux. New York, Academic Press**:** 675-760.

Capen, C. C. (1996). Toxic Responses of the Endocrine System. Casarett and Doull's Toxicology: The Basic Science of Poisons. C. D. Klaassen. New York, McGraw-Hill**:** 617-640.

Cederbaum, A. I. and Cohen, G. (1980). Oxidated demethylation of t-butyl alcohol by rat liver microsomes. *Biochemical and Biophysical research communications* 97(2): 730-736.

Cederbaum, A. I., Qureshi, A. and Cohen, G. (1983). Production of formaldehyde and acetone by hydroxyl-radical generated systems during the metabolism of tertiary butyl alcohol. *Biochemical Pharmacology* 32(23): 3517-3524.

CIIT (1996). The mechanism of male rat kidney tumors induced by methyl tert-butyl ether and its relevance in assessing human risk. *CIIT Activities* 16(10).

Cirvello, J. D., Radovsky, A., Heath, J. E., Farnell, D. R. and Lindamood, C., 3rd (1995). Toxicity and carcinogenicity of t-butyl alcohol in rats and mice following chronic exposure in drinking water. *Toxicol Ind Health* 11(2): 151-165.

Clegg, E. D, Cook, J. C, Chapin, R. E, Foster, P. and Daston, G. P. (1997). Leydig cell hyperplasia and adenoma formation: mechanisms and relevance to humans. *Reproductive Toxicology* 11(1): 107-121.

Curran, P. and DeGroot, L. (1991). The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. *Endocrine Rev* 12: 135-150.

Daniel, M. A. and Evans, M. A. (1982). Quantitative comparison of maternal ethanol and maternal tertiary butanol diet on postnatal development. *J Pharmacol Exp Ther* 222(2): 294-300.

Dietrich, D. R. and Swenberg, J. A. (1991). NCI-Black-Reiter (NBR) male rats fail to develop renal disease following exposure to agents that induce alpha 2u-globulin (alpha 2u) nephropathy. *Fundamental And Applied Toxicology* 16: 749-762.

Donato, M. T., Klocke, R., Castell, J. V., Stenzel, K., Paul, D. and Gomez-Lechon, M. J. (2003). Constitutive and inducible expression of CYP enzymes in immortal hepatocytes derived from SV40 transgenic mice. *Xenobiotica* 33(5): 459-473.

ENVIRON International Corporation (2003). QRisk.

Faulkner, T. P., Wiechart, J. D., Hartman, D. M. and Hussain, A. S. (1989). The effects of prenatal tertiary butanol administration in CBA/J and C57BL/6J mice. *Life Sci* 45(21): 1989-1995.

Faulkner, T. P. and Hussain, A. S. (1989). The pharmacokinetics of tertiary butanol in C57BL/6J mice. *Research Communications in Chemical Pathology and Pharmacology* 64(1): 31-39.

Galloway, S. M., Armstrong, M. J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A. D., Nakamura, F., Ahmed, M., Duk, S. and al, et (1987). Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Env Mol Mutagen* 10(Suppl 10): 1-75.

Gong, P., Cederbaum, A. I. and Nieto, N. (2003). Increased expression of cytochrome P450 2E1 induces heme oxygenase-1 through ERK MAPK pathway. *J Biol Chem* 278(32): 29693-29700.

Hoshino, H., Chihara, G. and Fukuoka, F. (1970). Detection of potential weak carcinogens and procarcinogens. II. Carcinogenicity of tertiary butyl hydroperoxide. *Gann* 61: 121-124.

Hosokawa, S., Nakamura, J., Ito, S., Murakami, M., Ineyama, M., Yoshioka, K., Yamada, T., Seki, T., Matsuo, M. and Yamada, H. (1993). Hormonal disregulation mechanism in the rat thyroid tumor induced by diniconazole. *J Toxicol Sci* 18(1): 57-67.

IARC (1999). Consensus Report. In Species Differences in Thyroid, Kidney, and Urinary Bladder Carcinogenesis. C. C. Capen, E. Dybing, J. M. Rice and J. D. Wilbourn. Lyon, France, International Agency for Research on Cancer. IARC Sci. Pub. No. 147**:** 5-9.

ICF Consulting (2001). TOX_RISK. Version 5.3.

Kolaja, K. L., Hood, A. M. and Klassen, C. D. (1999). The UDP-glucuronultransferase inducers, phenobarbital and pregnenolone-16alpha-carbonitrile, enhance thyroid-folicular cell apoptosis: association with TGF-beta 1 expression. *Toxicol Lett* 106(2-3): 143-150.

Lafleur, M. V. and Loman, H (1982). Influence of anoxic sensitizers on the radiation damage in biologically active DNA in aqueous solution. *Int J Radiat Biol* 41: 295-302.

Lehman-McKeeman, L. D. and Caudill, D. (1994). d-Limonene induced hyaline droplet nephropathy in alpha 2u-globulin transgenic mice. *Fundam Appl Toxicol* 23: 562-568.

Levin, D. E., Hollstein, M., Christman, M. F., Schwiers, E. A. and Ames, B. N. (1982). A new Salmonella tester strain (TA102) with A-T base pairs at the site of mutation detects oxidative mutagens. *PNAS* 79: 7445-7449.

Lindamood, C., Farnell, D. R., Giles, H. D., Prejean, J. D., Collins, J. J., Takahashi, K. and Maronpot, R. R. (1992). Subchronic toxicity studies of t-butyl alcohol in rats and mice. *Fundam Appl Toxicol* 19(1): 91-100.

Litterst, C. L., Mimnaugh, E. G., Reagan, R. L. and Gram, T. E. (1975). Comparison of in vitro drug metabolism by lung, liver, and kidney of several common laboratory species. *Drug Metabolism and Disposition* 3(4): 259-265.

McClain, R., Levin, A., Posch, R. and Downing, J. (1989). The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. *Toxicol Aappl Pharmacol* 99: 216-228.

McComb, J. A. and Goldstein, D. B. (1979). Quantitative comparison of physical dependence on tertiary butanol and ethanol in mice: correlation with lipid solubility. *J Pharmacol Exp Ther* 208(1): 113-117.

McGregor, D., Brown, A., Cattanach, P., Edwards, I., McBride, D. and Caspary, W.J. (1988). Responses of the L5178Y tk+/tk- mouse lymphoma cell forward mutation assay. II: 18 coded chemicals. *Environ Mol Mutagen* 11(1): 91-118.

McGregor, D. and Hard, G. C. (2001). Renal tubule tumor induction by tertiary-butyl alcohol. *Toxicol Sci* 61(1): 1-3.

McGregor, D., Cruzan, G., Callander, R. D., May, K. and Banton, M. (2004). The mutagenicity of tertiary-butyl alcohol in Salmonella typhimurium TA102**:** 1-10.

McGregor, D., Cruzan, G., Callander, R., May, K. and Banton, M. (2005). The mutagenicity of tertiary-butyl alcohol, tertiary-butyl acetate and methyl tertiary-butyl ether in Salmonella typhimurium. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 565(2): 181-189.

Meek, M. E., Bucher, J. R., Cohen, S. M., Dellarco, V., Hill, R. N., Lehman-McKeeman, L. D., Longfellow, D. G., Pastoor, T., Seed, J. and Patton, D. E. (2003). A framework of human relevance analysis of information on carcinogenic modes of action. *Critical Reviews in Toxicology* 336(6): 591-653.

Melo Cavalcante, A. A., Rubensam, G., Picada, J., Gomes da Silva, E., Fonseca Moreira, J. and Henriques, J. (2003). Mutagenicity, antioxidant potential, and antimutagenic activity against hydrogen peroxide of cashew (Anacardium occidentale) apple juice and cajuina. *Environ Mol Mutagen* 41(5): 360-369.

Miguez, M. P., Anundi, I., Sainz-Pardo, L. A. and Lindros, K. O. (1994). Hepatoprotective mechanism of silymarin: no evidence for involvement of cytochrome P450 2E1. *Chem Biol Interact* 91(1): 51-63.

Nelson, B. K., Brightwell, W. S., Khan, A., Krieg Ef, J. R. and Massari, V. J. (1989). Behavioral teratology investigation of tertiary-butanol administered by inhalation to rats. *Teratology* 39(5).

NTP (1995). NTP Toxicology and Carcinogenesis Studies of t-Butyl Alcohol (CAS No. 75-65-0) in F344/N Rats and B6C3F1 Mice (Drinking Water Studies). *Natl Toxicol Program Tech Rep Ser* 436: 1-305.

NTP (1997). NTP Technical Report on Toxicity studies of t-Butyl Alcohol. Administered by inhalation to F344/n rats and B6C3F1 mice. *Natl Toxicol Program Tox Rep Ser* 53(56).

Parkinson, A. (2001). Biotransformation of Xenobiotics. Casarett and Doull's Toxicology. The Basic Science of Poisons. C. D. Klassen, McGraw-Hill Medical Publishing Division.

Poet, T. S., Valentine, J. L. and Borghoff, S. J. (1997). Pharmacokinetics of tertiary butyl alcohol in male and female Fischer 344 rats. *Toxicol Lett* 92(3): 179-186.

Roots, R. and Okada, S. (1972). Protection of DNA molecules of cultured mammalian cells from radiation-induced single-strand scissions by various alcohols and SH compound. *Int J Radiat Biol* 21: 329-342.

Takahashi, K., Lindamood, C. and Maronpot, R. R. (1993). Retrospective study of possible alpha-2 mu-globulin nephropathy and associated cell proliferation in male Fischer 344 rats dosed with t-butyl alcohol. *Environ Health Perspect* 101 (Suppl 5): 281-285.

Tang, G., Wang, J. and Zhuang, Z. (1997). [Cytotoxicity and genotoxicity of methyl tert-butyl ether and its metabolite to human leukemia cells]. *Zhonghua Yu Fang Yi Xue Za Zhi* 31(6): 334-337.

Thurman, R. G., Winn, K. and Urquhart, B. (1980). Rat brain cyclic AMP levels and withdrawal behavior following treatment with t-butanol. *Adv Exp Med Biol* 126: 271-281.

USEPA (1986). The Risk Assessment Guidelines of 1986. Office of Health and Environmental Assessment. EPA/600/8-87/045. Washington, DC. August 1987.

USEPA (1991). Alpha 2_u-globulin: Association with chemically induced renal toxicity and neoplasia in the male rat. Risk Assessment Forum. EPA/625/3-91/019F. Washington, D. C. September 1991.

USEPA (1998). Assessment of Thyroid Follicular Cell Tumors. U.S. Environmental Protection Agency. EPA/630/R-97-002. Washington, DC.

USEPA (1999). Guidelines for Carcinogen Risk Assessment. NCEA-F-0644. Review draft. July.

USEPA (2003). Draft Final Guidelines for Assessing Cancinogen Risk Assessment. EPA/630/P-03/001A. Risk assessment Forum. Washington, DC.

USEPA (2005). Guidelines for carcinogen risk assessment. EPA/630/P-03/001b.

Vansell, N. R. and Klassen, C. D. (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicol Appl Pharmacol* 176(3): 187-194.

Vessey, D. A. and Zakim, D. (1972). Regulation of microsomal enzymes by phospholipids. IV. Species differences in the properties of microsomal UDP-glucuronyltransferase. *Biochem Biophys Acta* 268(1): 61-69.

Videla, L. A., Fernandez, V. and de Marinis, A. (1982). Liver lipoperoxidative pressure and glutathione status following acetaldehyde and aliphatic alcohols pretreatments in the rat. *Biochemical and Biophysical Research Communications* 104(3): 965-970.

Wilcox, P., Naidoo, A., Wedd, D. J. and Gatehouse, D. G. (1990). Comparison of Salmonella typhimurium TA102 with Escherichia coli WP2 tester strains. *Mutagenesis* 5(3): 285-291.

Williams-Hill, D., Spears, C. P., Prakash, S., Olah, G. A., Shamma, T., Moin, T., Kim, L. Y. and Hill, C. K. (1999). Mutagenicity studies of methyl-tert-butyl ether using the Ames tester strain TA102. *Mutat Res* 446(1): 15-21.

Williams, T. M. and Borghoff, S. J. (2001). Characterization of tert-butyl alcohol binding to alpha2u-globulin in F-344 rats. *Toxicol Sci* 62(2): 228-235.

Yost, G. S. and Finley, B. L. (1983). Ethanol as an inducer of UDP-glucuronyltransferase: a comparison with phenobarbital and 3-methlcholanthrene induction in rabbit hepatic microsomes. *Biochem Biophys Res Commun* 111(1): 219-223.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., Mortelmans, K. and Speck, W. (1987). Salmonella mutagenicity tests. III Results from the testing of 255 chemicals. *Environ Mutagen* 9 (Supp 1): 1-110.

Appendix A

TOX_RISK Output

Generating Model Fit Table ---

```
TITLE: Female Mice - Follicular Cell Hyperplasia

             Model: Multistage             Dataset: J:\API\TOX_RISK\FM_FCHyperplasia.TXD
Functional form: 1 - EXP( -Q0 - Q1 * D - Q2 * D^2 ... - Qk * D^k )
       Chi-square: 0.05                          P-value: 0.83
         Maximum Log-Likelihood =  -1.499997e+002

       Parameter Estimates :  k = 3
                              Q 0 = 4.020207E-001
                              Q 1 = 3.982399E-004
                              Q 2 = 0.000000E+000
                              Q 3 = 3.050651E-011
```

Experimental Doses (mg/kg/day)	#responses/ #subjects	Expected number of responders	90.0% Binomial Limits Lower	Upper
0	19 / 58	19.20	13.168	25.649
510	28 / 60	27.37	21.384	34.776
1015	33 / 59	33.48	26.246	39.471
2105	47 / 60	46.94	40.671	51.998

```
Generating ED10 Non-Linear Table ---
               Dataset:  J:\API\TOX_RISK\FM_FCHyperplasia.TXD

              TITLE: Female Mice - Follicular Cell Hyperplasia
```

Doses (mg/kg/day)	#responses/ #subjects
0	19/58
510	28/60
1015	33/59
2105	47/60

Body Weight	0.03	kg	70	kg
LifeSpan	103	weeks	70	years
Breathing Rate	0.0347	l/min	0.833	m^3/hr
Food Consumption	3.9	g/day	1400	g/day
Drinking Rate	6	ml/day	2	l/day
Route	WATER	(mg/kg/day)	N/A	
Dosing: Hrs/Day	24		N/A	
Days/Week	7		N/A	
Weeks	103		N/A	
Weeks of Study	103		N/A	
Averaging Factor	1		1	

```
       Model: Multistage                      Risk Type: Extra Risk
Molecular Wt.:     0.000                  Confidence limit: 95.000%

          Adjustment for Experiment Length:     1.000 (EPA METHOD)
       Animal to human conversion method: MG/KG  BODY WEIGHT(3/4)/DAY

    Constant Dose [mg/kg/day] Over Lifetime Computed for a Risk of 1 in 10
  Lower Bound = 2.2691E+001    MLE = 3.7865E+001    Upper Bound = 1.1873E+002
```

```
Generating Model Fit Table ---
TITLE: Female Mice - Follicular Cell Adenoma

             Model: Multistage             Dataset: J:\API\TOX_RISK\FM_FCAdenoma.TXD
Functional form: 1 - EXP( -Q0 - Q1 * D - Q2 * D^2 ... - Qk * D^k )
       Chi-square: 0.56                          P-value: 0.76
         Maximum Log-Likelihood =  -5.483039e+001

       Parameter Estimates :  k = 3
                              Q 0 = 3.679501E-002
                              Q 1 = 0.000000E+000
                              Q 2 = 0.000000E+000
                              Q 3 = 1.323109E-011

     Experimental                        Expected              90.0%
        Doses         #responses/       number of        Binomial Limits
     (mg/kg/day)       #subjects        responders      Lower      Upper
       0                 2 / 58            2.10         0.340      6.086
     510                 3 / 60            2.27         0.820      7.443
    1015                 2 / 59            2.91         0.346      6.072
    2105                 9 / 59            8.73         4.796     14.812



Generating ED10 Non-Linear Table ---
                  Dataset:  J:\API\TOX_RISK\FM_FCAdenoma.TXD

                 TITLE: Female Mice - Follicular Cell Adenoma


   Doses      #responses/
 (mg/kg/day)  #subjects
    0           2/58
  510           3/60
 1015           2/59
 2105           9/59



  Body Weight           0.03      kg                    70        kg
  LifeSpan              103       weeks                 70        years
  Breathing Rate        0.0347    l/min                 0.833     m^3/hr
  Food Consumption      3.9       g/day                 1400      g/day
  Drinking Rate         6         ml/day                2         l/day
  Route                 WATER  (mg/kg/day)              N/A
  Dosing: Hrs/Day       24                              N/A
          Days/Week     7                               N/A
          Weeks         103                             N/A
  Weeks of Study        103                             N/A
  Averaging Factor      1                               1

       Model: Multistage                        Risk Type: Extra Risk
Molecular Wt.:     0.000                    Confidence limit: 95.000%

           Adjustment for Experiment Length:     1.000 (EPA METHOD)
       Animal to human conversion method: MG/KG  BODY WEIGHT(3/4)/DAY

    Constant Dose [mg/kg/day] Over Lifetime Computed for a Risk of 1 in 10
  Lower Bound = 2.0601E+002    MLE = 2.8732E+002    Upper Bound = 5.4571E+002
```

Generating Model Fit Table ---
TITLE: Female Mice Follicular Cell Adenoma

Model: Multistage Weib Dataset: J:\API\TOX_RISK\FM_FCAdenoma.ttd
Functional form: 1 - EXP[(-Q0 - Q1 * D - Q2 * D^2 ... - Qk * D^k)
* (T - T0)^Z]
Maximum Log-Likelihood = -5.739578e+001

Parameter Estimates : k = 3
Q 0 = 3.012286E-022
Q 1 = 0.000000E+000
Q 2 = 0.000000E+000
Q 3 = 1.046985E-031
Z = 1.000000E+001
T0 = 0.000000E+000

Avg. Doses (mg/kg/day)	Number of animals	with fatal tumors	with incidental tumors
0	57	0	2
510	60	0	3
1015	59	0	2
2105	59	2	7

Generating ED10 Non-Linear Table ---
Dataset: J:\API\TOX_RISK\FM_FCAdenoma.ttd

TITLE: Female Mice Follicular Cell Adenoma

Avg Dose (mg/kg/day)	#fatal	#incidental	#animals
0	0	2	57
510	0	3	60
1015	0	2	59
2105	2	7	59

	MOUSE		HUMAN	
Body Weight	0.03	kg	70	kg
LifeSpan	103	weeks	70	years
Breathing Rate	0.0347	l/min	0.833	m^3/hr
Food Consumption	3.9	g/day	1400	g/day
Drinking Rate	6	ml/day	2	l/day
Route	WATER	(mg/kg/day)	N/A	
Dosing: Hrs/Day	24		N/A	
Days/Week	7		N/A	
Weeks	103		N/A	
Weeks of Study	103		N/A	
Averaging Factor	1		1	

Model: Multistage Weib Risk Type: Incid Extra Risk
Molecular Wt.: 0.000 Confidence limit: 95.000%

Animal to human conversion method: MG/KG BODY WEIGHT(3/4)/DAY

Constant Dose [mg/kg/day] Over Lifetime Computed for an Incidental Risk of 1 in 10
Lower Bound = 1.9978E+002 MLE = 2.8149E+002 Upper Bound = 5.0290E+002

Generating Model Fit Table ---
TITLE: Male Mice - Follicular Cell Hyperplasia

Model: Multistage Dataset: J:\API\TOX_RISK\MM_FCHyperplasia.TXD
Functional form: 1 - EXP(-Q0 - Q1 * D - Q2 * D^2 ... - Qk * D^k)
Chi-square: 5.94 P-value: 0.05
Maximum Log-Likelihood = -1.253519e+002

Parameter Estimates : k = 3
Q 0 = 1.430535E-001
Q 1 = 1.500735E-004
Q 2 = 0.000000E+000
Q 3 = 0.000000E+000

Experimental Doses (mg/kg/day)	#responses/ #subjects	Expected number of responders	90.0% Binomial Limits Lower	Upper
0	5 / 60	8.00	2.016	10.040
535	18 / 59	11.81	12.274	24.650
1035	15 / 59	15.22	9.686	21.473
2065	18 / 57	20.76	12.299	24.572

Generating ED10 Non-Linear Table ---
Dataset: J:\API\TOX_RISK\MM_FCHyperplasia.TXD

TITLE: Male Mice - Follicular Cell Hyperplasia

Doses (mg/kg/day)	#responses/ #subjects
0	5/60
535	18/59
1035	15/59
2065	18/57

Body Weight	0.03	kg	70	kg
LifeSpan	103	weeks	70	years
Breathing Rate	0.0347	l/min	0.833	m^3/hr
Food Consumption	3.9	g/day	1400	g/day
Drinking Rate	6	ml/day	2	l/day
Route	WATER	(mg/kg/day)	N/A	
Dosing: Hrs/Day	24		N/A	
Days/Week	7		N/A	
Weeks	103		N/A	
Weeks of Study	103		N/A	
Averaging Factor	1		1	

Model: Multistage Risk Type: Extra Risk
Molecular Wt.: 0.000 Confidence limit: 95.000%

Adjustment for Experiment Length: 1.000 (EPA METHOD)
Animal to human conversion method: MG/KG BODY WEIGHT(3/4)/DAY

Constant Dose [mg/kg/day] Over Lifetime Computed for a Risk of 1 in 10
Lower Bound = 6.1699E+001 MLE = 1.0101E+002 Upper Bound = 2.6239E+002

Generating Model Fit Table ---
TITLE: Male Mice Follicular Cell Adenoma and Carcinoma

Model: Multistage Dataset: J:\API\TOX_RISK\MM_FCAdenoma&Carcinoma2.TXD
Functional form: 1 - EXP(-Q0 - Q1 * D - Q2 * D^2 ... - Qk * D^k)
Chi-square: 4.10 P-value: 0.13
Maximum Log-Likelihood = -3.085602e+001

Parameter Estimates : k = 3
Q 0 = 1.410168E-002
Q 1 = 1.811969E-005
Q 2 = 0.000000E+000
Q 3 = 0.000000E+000

Experimental Doses (mg/kg/day)	#responses/ #subjects	Expected number of responders	90.0% Binomial Limits Lower	Upper
0	1 / 60	0.84	0.070	4.592
535	0 / 59	1.39	0.000	2.917
1035	4 / 59	1.91	1.383	8.771
2065	2 / 57	2.86	0.356	6.078

Generating ED10 Non-Linear Table ---
Dataset: J:\API\TOX_RISK\MM_FCAdenoma&Carcinoma2.TXD

TITLE: Male Mice Follicular Cell Adenoma and Carcinoma

Doses (mg/kg/day)	#responses/ #subjects
0	1/60
535	0/59
1035	4/59
2065	2/57

Body Weight	0.03	kg	70	kg
LifeSpan	103	weeks	70	years
Breathing Rate	0.0347	l/min	0.833	m^3/hr
Food Consumption	3.9	g/day	1400	g/day
Drinking Rate	6	ml/day	2	l/day
Route	WATER	(mg/kg/day)	N/A	
Dosing: Hrs/Day	24		N/A	
Days/Week	7		N/A	
Weeks	103		N/A	
Weeks of Study	103		N/A	
Averaging Factor	1		1	

Model: Multistage Risk Type: Extra Risk
Molecular Wt.: 0.000 Confidence limit: 95.000%

Adjustment for Experiment Length: 1.000 (EPA METHOD)
Animal to human conversion method: MG/KG BODY WEIGHT(3/4)/DAY

Constant Dose [mg/kg/day] Over Lifetime Computed for a Risk of 1 in 10
Lower Bound = 3.0140E+002 MLE = 8.3663E+002 Upper Bound = Can't Calc

```
Generating Model Fit Table ---
TITLE: Male Mice Follicular Cell Adenoma and Carcinoma

             Model: Multistage Weib        Dataset: J:\API\TOX_RISK\MM_FCAdenoma&Carcinoma.ttd
Functional form: 1 - EXP[( -Q0 - Q1 * D - Q2 * D^2 ... - Qk * D^k )
                       * (T - T0)^Z]
         Maximum Log-Likelihood =  -3.826239e+001

       Parameter Estimates :  k = 3
                              Q 0 = 2.856598E-022
                              Q 1 = 3.848817E-025
                              Q 2 = 1.243916E-028
                              Q 3 = 0.000000E+000
                              Z   = 9.815995E+000
                              T0  = 0.000000E+000
```

Avg. Doses (mg/kg/day)	Number of animals	Number with fatal tumors	Number with incidental tumors
0	60	0	1
535	59	0	0
1035	59	2	2
2065	57	1	1

```
Generating ED10 Non-Linear Table ---
            Dataset:  J:\API\TOX_RISK\MM_FCAdenoma&Carcinoma.ttd

           TITLE: Male Mice Follicular Cell Adenoma and Carcinoma
```

Avg Dose (mg/kg/day)	#fatal	#incidental	#animals
0	0	1	60
535	0	0	59
1035	2	2	59
2065	1	1	57

	MOUSE		HUMAN	
Body Weight	0.03	kg	70	kg
LifeSpan	103	weeks	70	years
Breathing Rate	0.0347	l/min	0.833	m^3/hr
Food Consumption	3.9	g/day	1400	g/day
Drinking Rate	6	ml/day	2	l/day
Route	WATER	(mg/kg/day)	N/A	
Dosing: Hrs/Day	24		N/A	
Days/Week	7		N/A	
Weeks	103		N/A	
Weeks of Study	103		N/A	
Averaging Factor	1		1	

```
       Model: Multistage Weib             Risk Type: Incid Extra Risk
Molecular Wt.:     0.000                   Confidence limit: 95.000%

       Animal to human conversion method: MG/KG  BODY WEIGHT(3/4)/DAY

 Constant Dose [mg/kg/day] Over Lifetime Computed for an Incidental Risk of 1 in 10
  Lower Bound = 2.0659E+002    MLE = 3.7379E+002    Upper Bound = Can't Calc
```

Appendix B

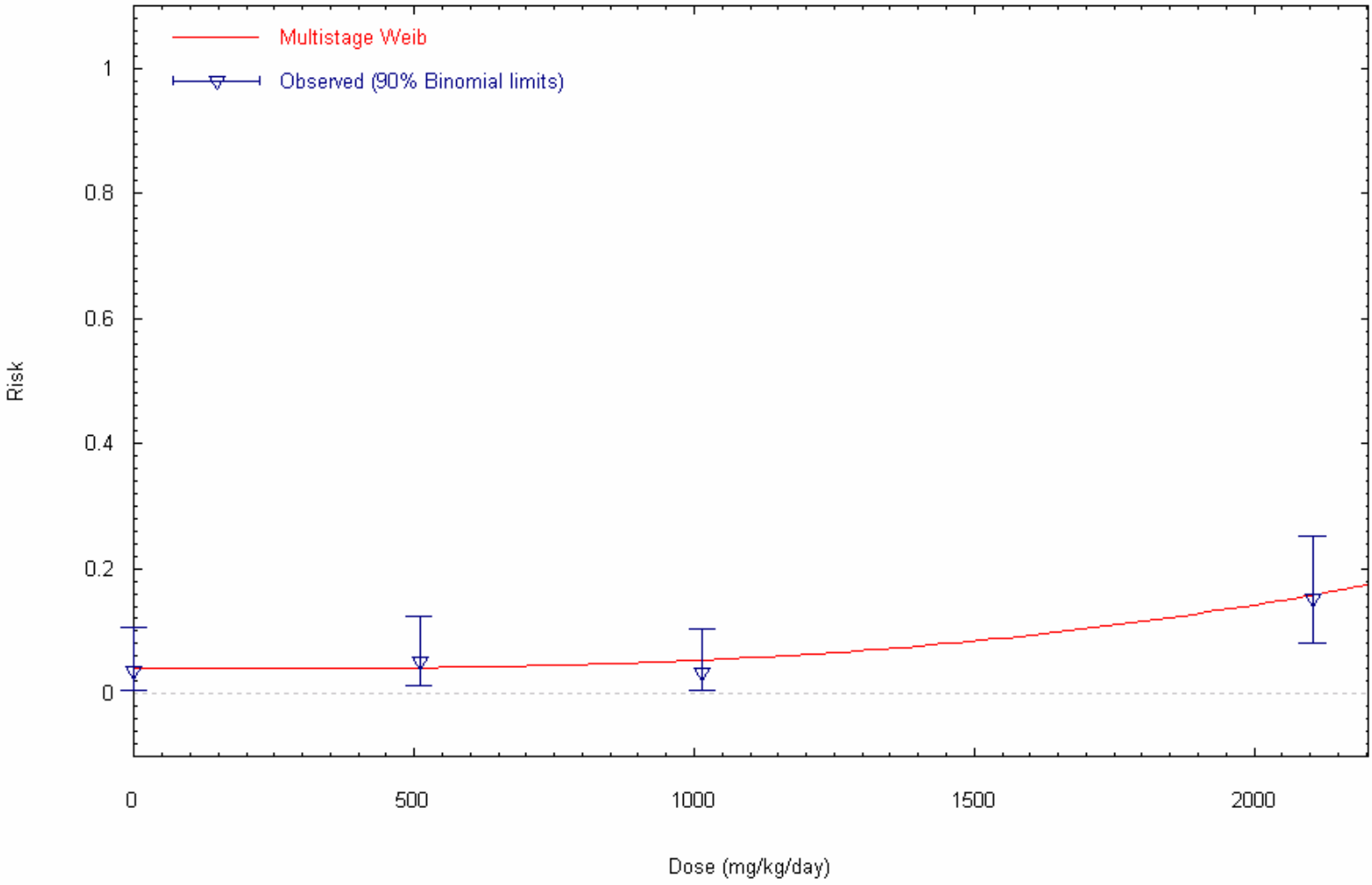
Graphs of Time-to-Tumor Models

11:42 04/28/2004

Incidence Graph at Week 103

FM_FCAdenoma&Carcinoma.ttd - Female Mice Follicular Cell Adenoma and Carcinoma

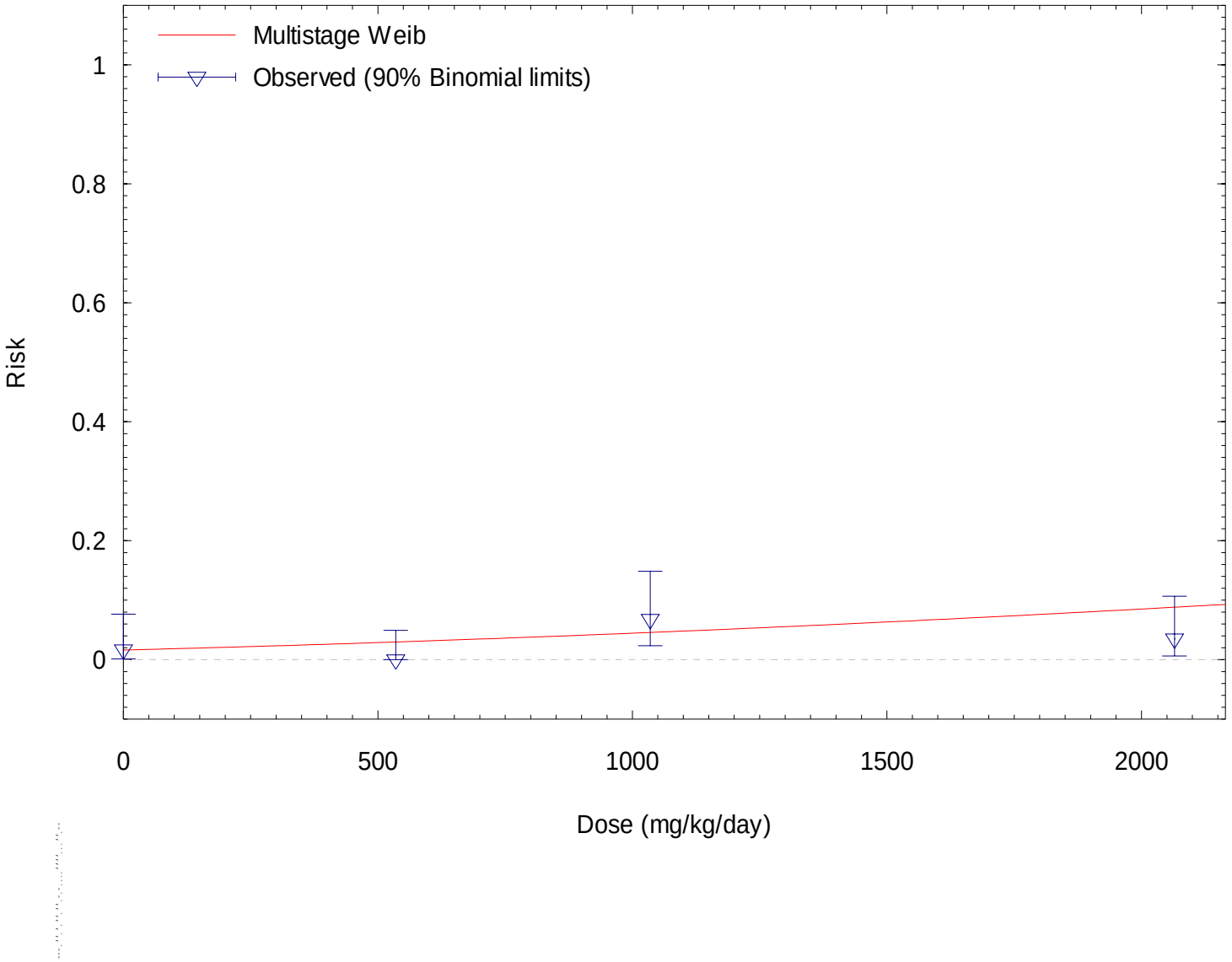
Model: Multistage Weib



Incidence Graph at Week 103

11:44 04/28/2004 MM_FCAdenoma&Carcinoma.ttd - Male Mice Follicular Cell Adenoma and Carcinoma

Model: Multistage Weib



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