OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

Air Toxics Hot Spots Program

Tertiary-Butyl Acetate

Inhalation Cancer Unit Risk Factor

Technical Support Document for Cancer Potency Factors
Appendix B

Public Review Draft
August 2015

Air, Community, and Environmental Research Branch

Office of Environmental Health Hazard Assessment

California Environmental Protection Agency

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Public Review Draft

August 2015

Introduction

This document summarizes the carcinogenicity and derivation of an inhalation cancer unit risk factor for *tertiary*-Butyl Acetate (TBAc). Cancer unit risk factors are used to estimate lifetime cancer risks associated with inhalation exposure to a carcinogen.

OEHHA is required to develop guidelines for conducting health risk assessments under the Air Toxics Hot Spots Program (Health and Safety Code Section 44360(b)(2). In implementing this requirement, OEHHA develops cancer unit risk factors (URF) for carcinogenic air pollutants listed under the Air Toxics Hot Spots program. The TBAc URF was developed using the most recent "Air Toxics Hot Spots Program Technical Support Document for Cancer Potency Factors", finalized by OEHHA in 2009.

TBAC is commonly used as a solvent in a variety of products including coatings, inks, adhesives, industrial cleaners and degreasers. TBAc is listed as a substance for which emissions must be quantified under the Air Toxics Hot Spots Program.

The National Toxicology Program (NTP) conducted a carcinogenicity bioassay of *tertiary*-butanol (TBA) (NTP, 1995) in rats and mice. The study provides evidence of carcinogenicity of TBA, which is a primary metabolite of TBAc (Groth and Freundt, 1994; Huntingdon Life Sciences Ltd., 2000e). Accordingly, OEHHA has developed a TBAc cancer unit risk factor for use in the Hot Spots program, based on the carcinogenicity evidence of TBA.

The TBAc cancer unit risk factor document has been made available for public review on the OEHHA website at http://www.oehha.ca.gov. The document will then be revised in response to public comments, if necessary, and will subsequently receive external peer review by the Scientific Review Panel (SRP). After SRP review, the document will be revised in response to SRP comments, if necessary, and then be adopted by the Director of OEHHA for use in the Air Toxics Hot Spots program. After adoption, the document will be included in Appendix B of the Air Toxics Hot Spots Program Technical Support Document for Cancer Potency Factors.

TERTIARY BUTYL ACETATE

CAS No: 540-88-5

I. PHYSICAL AND CHEMICAL PROPERTIES (From HSDB, 2014)

Molecular formula	$C_6H_{12}O_2$
Molecular weight	116.16
Description	colorless liquid
Density	0.8593 at 25°C /4°C
Boiling point	95.1°C
Vapor pressure	47 mm Hg @ 25°C
Solubility	Practically insoluble in water; miscible with common industrial organic solvents including ethanol, ethyl ether, acetic acid and chloroform.
Conversion factor	1 ppm = 4.74 mg/m^3
Uses	Solvent.

II. HEALTH ASSESSMENT VALUES

Unit Risk Factor:	1.9×10^{-6} (µg/m^3)$^{-1}$
Inhalation Slope Factor:	6.7×10^{-3} (mg/kg-day)$^{-1}$
Oral Slope Factor:	7.0×10^{-3} (mg/kg-day)$^{-1}$

The derivation of these values is based on the male F344 rat kidney tumor incidence data for the primary metabolite of TBAc, *tertiary*-butanol (TBA) (NTP, 1995). The TBA cancer risk estimate was calculated using benchmark dose methodology. The lower 95 percent confidence limit on the dose associated with a 5 percent tumor response ($BMDL_{05}$) was derived by the multistage cancer model in BMDS version 2.6, and used as the point of departure. Linear extrapolation from the $BMDL_{05}$ to the origin was used to determine the slope of the dose-response curve for low level exposure, which is the cancer slope factor. The oral slope factor for *tertiary*-butyl acetate (TBAc) was derived from this value assuming 71% metabolism of TBAc to TBA and a molar conversion factor of 0.64. The unit risk factor and inhalation slope factor assumes that 95% of an inhaled dose of TBAc is absorbed systemically.

III. Carcinogenicity

No carcinogenicity studies are available for TBAc. However, exposure to TBA, the primary metabolite of TBAc, has been demonstrated to cause tumors in rats and mice. There are no human carcinogenicity data for either compound.

Metabolism and Pharmacokinetics

Tertiary-Butyl Acetate

The metabolism of inhaled TBAc was studied in Sprague-Dawley rats by Groth and Freundt (1994). A continuous 5 hour exposure to approximately 440 ppm TBAc (via a tracheal canula) resulted in continuously increasing blood concentrations of TBAc and TBA. Blood concentrations of TBA (approximately 350 µM/L blood) were slightly higher than those of TBAc (approximately 300 µM/L blood) at the end of the exposure period. Similar blood level accumulation kinetics of TBAc and TBA were noted during a continuous exposure to about 900 ppm TBAc in air over a period of 4.25 hours. The TBAc blood concentration decreased by approximately 50% within 45 minutes of the cessation of exposure, but TBA blood concentrations remained unchanged.

Metabolism and pharmacokinetics studies were performed using male Sprague-Dawley rats (Cruzan and Kirkpatrick, 2006). Animals (6/group) were exposed to either 100 or 1000 ppm ^{14}C-*tertiary*-butyl acetate (^{14}C-TBAc) by inhalation for 6 hours. TBAc was radiolabeled in this case to facilitate the study of its metabolism and elimination. Two animals/group were killed immediately after exposure, and their carcasses analyzed for radioactivity. The remaining 4 animals/group were placed in metabolism cages for the purpose of collecting feces, urine and expired air. These animals were sacrificed 7 days postexposure and body tissues were analyzed for radioactivity.

Low-dose animals excreted most of the inhaled TBAc-derived radioactivity (89% of dose) in urine. The remaining TBAc-derived radioactivity was found in feces, expired air and tissues (2.7, 4.8 and 0.7% of dose, respectively). Radioactivity was rapidly eliminated, mostly within 24 hours.

The primary excretion route of TBAc-derived radioactivity for high-dose animals was also urine (69% of dose), but a larger proportion was found in expired air (27% of dose) compared to low-dose animals. The remaining TBAc-derived radioactivity was found in feces and tissues (0.97 and 0.22% of dose, respectively). As with the low-dose animals, most of the excreted radioactivity was recovered during the first 24 hours.

A lower proportion of available radioactivity was absorbed by animals at the high dose level. The authors stated that the reduction in the proportion of radioactivity absorbed at the high dose level, and the increased proportion in expired air may indicate some saturation of absorption and metabolism.

The animal carcass and tissues retained very little of the residual radioactivity in both dose groups. Mean TBAc-equivalent concentrations of 0.925, 0.328, 0.891, 0.131 and 0.246 µg/g were detected in the nasal tissues, larynx, trachea, lungs and fat, respectively, of the low-dose animals. Mean TBAc-equivalent concentrations ranging

from 0.084 to 0.170 µg/g were detected in the liver, kidneys, spleen and blood. TBAc-equivalent concentrations in nasal tissues, larynx, trachea, spleen and blood from the high-dose animals were below detection limits. Liver, kidney and lung tissues demonstrated mean concentrations of between 0.40 and 0.55 µg/g; fat concentrations were 1.17 µg/g.

The inability to detect radioactivity in nasal tissues, larynx, trachea, spleen and blood from the high-dose animals was probably due to the low specific activity of the ^{14}C-TBAc used to expose the high-dose animals rather than metabolic differences at the different doses, making these study results inconclusive.

Four major urinary metabolites (2-hydroxymethylisopropyl acetate (U1), 2-hydroxyisobutyric acid (U2), a TBA-glucuronide (U4), and a 2-hydroxymethylisopropyl acetate-glucuronide conjugate (U6)) were detected. During the first 48 hours postexposure, these accounted for 9.3, 45.5, 11.0 and 10.0% of dose respectively in low-dose animals, and for 7.9, 38.9, 15.2 and 2.6% of dose in high-dose animals. Three minor urinary metabolites U3, U7 and a *t*-butyl-2-hydroxyacetate-glucuronide conjugate (U8) accounted for 1 to 1.6% and 0.4 to 1.1 % of dose in the low-dose and high-dose animals, respectively.

Two major radioactive components, A1 and A2, were detected in expired air from high-dose animals 6 hours postexposure. The authors stated that component A2 was chromatographically identical to parent TBAc, while component A1 was unidentified.

The authors concluded that TBAc metabolism appears to follow two major routes. One involved hydroxylation of the TBA portion of TBAc to produce U1, which then formed U6, or was oxidized to U2 (the major urinary metabolite). The second route was by cleavage of the ester linkage to produce TBA, which formed U4, or was oxidized to U2. A minor route was the hydroxylation of the acetate portion of TBAc to produce *t*-butyl-2-hydroxyacetate, followed by glucuronidation to form the U8 metabolite. Free TBA was not detected. These proposed metabolic pathways are shown in Figure 1.

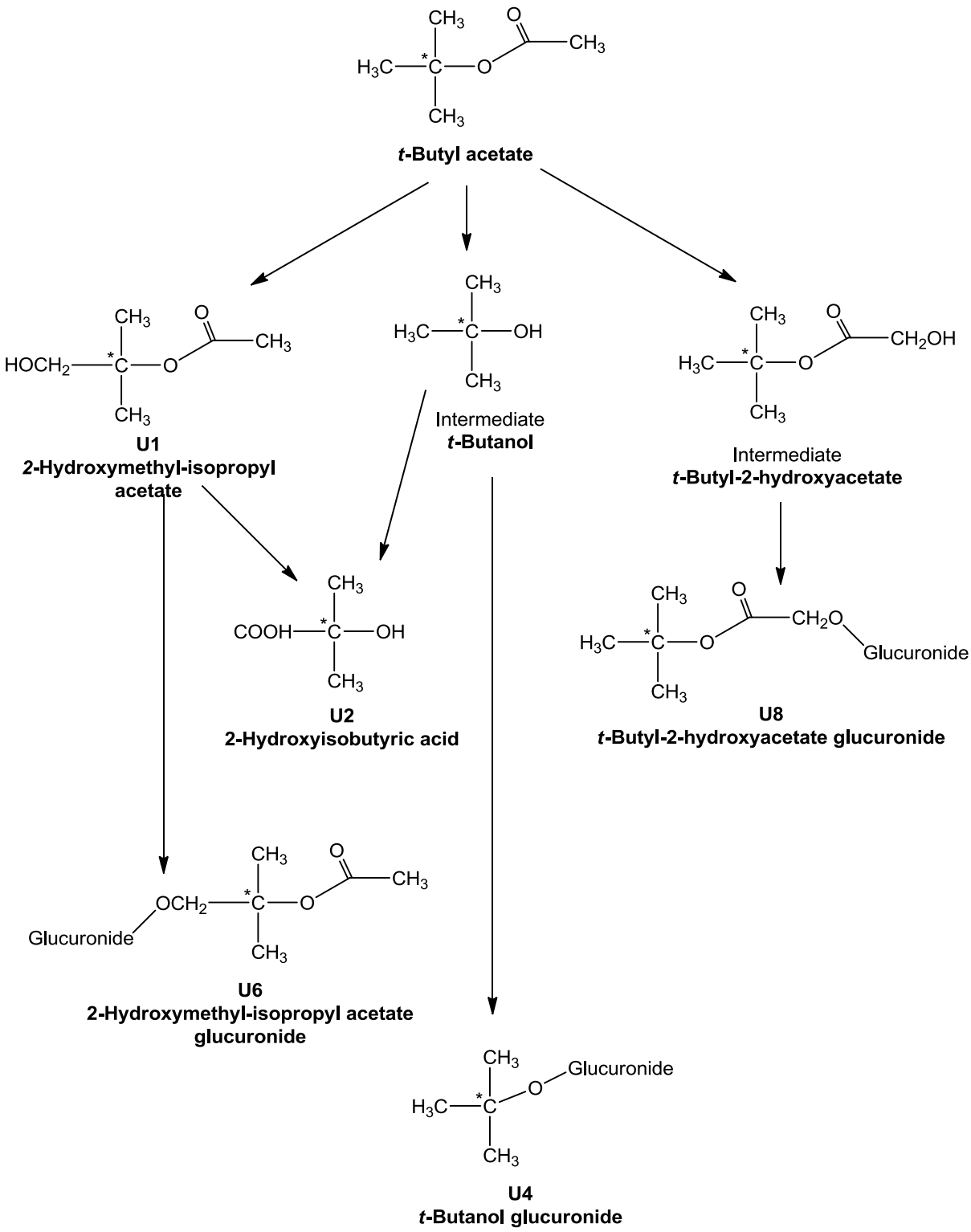

Figure 1. Proposed Metabolic Pathways for *tertiary*-Butyl Acetate in Rats (adapted from Cruzan and Kirkpatrick, 2006)

Cancer Bioassays

Tertiary-Butanol

The National Toxicology Program (NTP) conducted a two-year cancer bioassay using Fischer 344 (F344) rats and $B6C3F_1$ mice exposed to TBA in drinking water (NTP, 1995; Cirvello *et al*., 1995).

Groups of 60 F344 rats were administered daily doses of TBA via drinking water of 0, 1.25, 2.5 or 5 mg/mL (approximately 0, 90, 200, and 420 mg/kg-day) in males and 0, 2.5, 5 or 10 mg/mL (approximately 0, 180, 330, and 650 mg/kg-day) in females. Ten animals in each group were sacrificed at 15 months for evaluation; the remaining animals were exposed until the study was terminated at 103 weeks. The high dose groups of both sexes experienced decreased survival. A dose-related decrease in body weight gain was also observed. All treated groups of females showed a dose-related increase in kidney weight at the 15-month evaluation. Males exhibited increased kidney weight at the mid and high doses. Nephropathy was seen in all groups of treated females and caused early mortality in high exposure groups.

By the 24 month termination of the study, combined adenomas and carcinomas of the renal tubules had been found in 8/50, 13/50, 19/50, and 13/50 of the control, low, mid and high dose males, respectively (Table 1). The two-year survivals were 10/50, 6/50, 4/50, and 1/50, respectively. The increased incidence in the mid dose group was statistically significant (p = 0.012) by Fisher's exact test. The increased mortality in the high dose group may have reduced the observed incidence of renal tumors. Renal tubule hyperplasia was significantly increased in treatment groups. Although no renal (or other) tumors were observed in female rats, the incidence of renal hyperplasia was significantly elevated in the high dose group. Control male rat renal tubule adenoma incidence was 2/327, and carcinoma incidence was 0/327 in the six studies comprising the recent NTP historical control database for drinking water studies, indicating the rarity of these neoplasms in male F344 rats (http://ntp.niehs.nih.gov/ntp/research/database_searches/historical_controls/path/r_orlwr.txt). The renal tubule adenoma incidence in the concurrent control group of male rats in the NTP study was considerably greater than the corresponding historical control incidence. The pathogenesis of proliferative lesions of renal tubule epithelium is thought to proceed from hyperplasia to adenoma to carcinoma (Cirvello *et al*., 1995).

Groups of 60 $B6C3F_1$ mice of each sex were administered TBA in drinking water at doses of 0, 5, 10 or 20 mg/mL (approximately 0, 540, 1040, and 2070 mg/kg-day in males and 0, 510, 1020, and 2110 mg/kg-day in females). Reduced survival was observed in the high dose groups.

In the NTP mouse bioassay, incidence of thyroid follicular cell hyperplasia was significantly elevated in all treatment groups of males (5/60, 18/59, 15/59, 18/57) and in the mid and high dose groups of females (19/58, 28/60, 33/59, 47/59). A significant dose-dependent increase in follicular cell adenomas was observed in female mice (p = 0.007, Cochran-Armitage trend test), with adenoma incidence significantly increased compared to controls by pairwise comparison in high dose females (9/59, p = 0.028, Fisher exact test)(Table 1). NTP (1995) stated that "proliferation of thyroid gland follicular cells is generally considered to follow a progression from hyperplasia to adenoma and carcinoma".

Thyroid follicular cell adenoma incidence was also increased in the male mouse 10 mg/mL exposure group. This increased tumor incidence was not significant, but NTP noted that the 7% tumor incidence in this group exceeded the maximum rate of 2% observed in NTP historical drinking water controls, and that the incidence of 4% in the 20 mg/mL exposure group may have been related to reduced survival. For these reasons, NTP considered the increased thyroid follicular cell adenoma incidence in male mice to be equivocal evidence of carcinogenic activity. Chronic urinary bladder inflammation was seen in both sexes at the high dose, but no urinary bladder neoplasms were observed.

The increased incidence of renal tubule adenoma or carcinoma, combined, in male rats and of thyroid gland follicular cell adenoma in female mice is evidence of a carcinogenic response to TBA. Increased tumor incidences in the male rats and male and female mice in this study are listed in Table 1.

Table 1. Increased tumor incidences in Fischer 344 male rats and male and female B6C3F$_1$ mice exposed to *tertiary*-butanol in drinking water (NTP, 1995)

Sex, species	Tumor type	Administered dose (mg/mL)	Exposed dose (mg/kg-day)	Tumor incidence
Male rats	Renal tubule adenomas/carcinomas	0	0	8/50
		1.25	90	13/50
		2.5	200	19/50**
		5	420	13/50
Male mice	Thyroid follicular cell adenomas			
		0	0	1/60
		5	540	0/59
		10	1040	4/59
		20	2070	2/57
Female mice				
		0	0	2/58^{+}
		5	510	3/60
		10	1020	2/59
		20	2110	9/59*

Fisher exact test pairwise comparison with controls: * p = 0.028 ** p = 0.012

Cochran-Armitage trend test for dose response: $^{+}$ $p = 0.007$

IV. Supporting data

It has been established that TBAc is metabolized in rats to TBA (Groth and Freundt, 1994; Huntingdon Life Sciences Ltd., 2000e). Oral TBA exposure has been demonstrated to induce renal tumors in male rats and thyroid follicular cell tumors in female mice (NTP, 1995). The possibility has been raised that the male rat kidney tumors observed after oral TBA exposure may be related to a TBA-α2u interaction resulting in α2u nephropathy (Borghoff *et al.*, 2001; McGregor and Hard, 2001; Hard *et al.*, 2011). This mechanism would call into question the human relevance of the renal tumor data. We provide information in the following section relevant to this possible mode of action. Further, we provide some information on whether the thyroid tumors may be the result of hyperplasia due to thyroid hormone disruption. A discussion of the data supporting any particular mode of action is provided in Section V, Cancer Hazard Identification, below.

Biochemical Effects and Cell Proliferation

Tertiary-Butyl Acetate

Male and female CD(SD) rats and CD1(IGR) mice (10 animals/sex/exposure group for rats, 40 animals/sex/exposure group for mice) were exposed to TBAc by inhalation at concentrations of 0, 100, 400 or 1600 ppm for 6 hours/day, 7 days/week for 13 weeks by Faber *et al.* (2014). These concentrations were described as being approximately equal to 0, 135, 540 and 2160 mg/kg-day for rats, and 0, 237, 948 and 3792 mg/kg-day for mice.

Absolute rat kidney weights were significantly increased ($p < 0.05$) in the male 1600 ppm exposure group compared to controls. Relative rat kidney weights (relative to body weight) were significantly increased ($p < 0.05$) in all male exposure groups and in the 1600 ppm female exposure group compared to controls. The authors stated that renal α_2-microglobulin (α2u) accumulation was consistent throughout the male rat TBAc exposure groups as measured by both immunohistopathology and ELISA analysis, while tubular basophilia demonstrated a slightly higher incidence and severity in the 1600 ppm exposure group. However, statistical significance data were not provided for any of these endpoints. Additionally, a graphical depiction of male rat kidney α2u levels (Figure 2) appeared to show that there was probably no significant difference between the TBAc exposure groups, and possibly no significant difference between some of the TBAc exposure groups and the control group. The authors stated that TBAc exposure did not cause increases in rat kidney tubular cell proliferation as measured by proliferating cell nuclear antigen (PCNA) assessment.

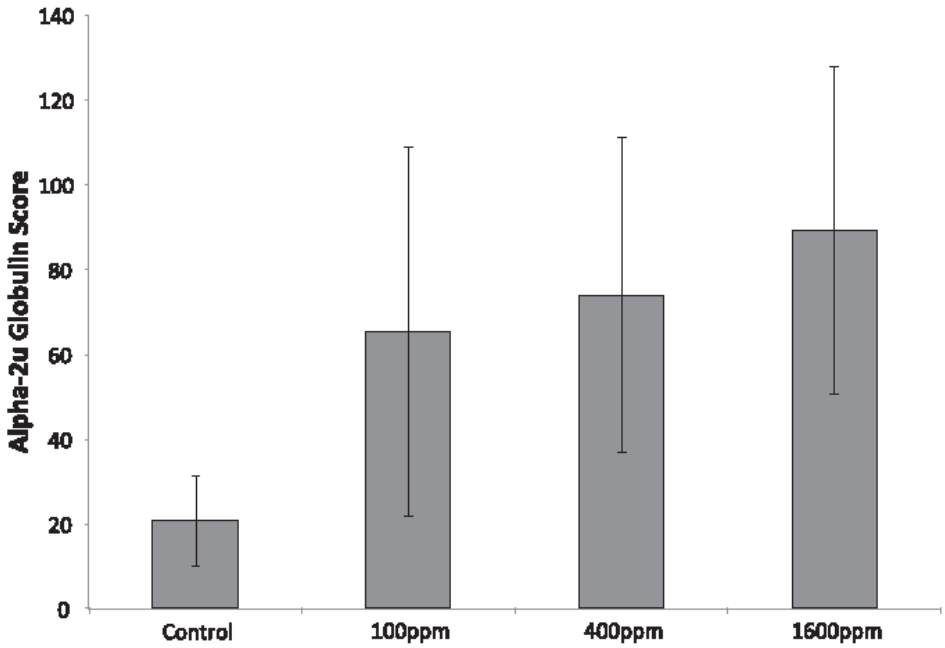

Figure 2. The effect of TBAC exposure on α-2u-globulin (ng/mg of total protein) in male rats during a 13-week inhalation study. (Figure 2. from Faber *et al.* (2014), reproduced unchanged by permission)

The authors listed absolute and relative liver weights, absolute thyroid/parathyroid gland weights and thyroid-stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) blood levels in mice. The data table legend (Table 6 in Faber et al, 2014) indicated that the mice had been exposed to TBAc for 20 weeks. However, the test description for these data indicated that the TSH, T_3 and T_4 data were collected at study week 4, and the thyroid/parathyroid gland weights were obtained at study week 13. No significant changes in TSH or T_3 levels were observed in TBAc-exposed male or female mice compared to controls. Significantly decreased T_4 levels ($p < 0.05$) were only noted in the male 1600 ppm mouse exposure group. The authors did not observe significant thyroid gland histopathological changes or alterations in thyroid/parathyroid gland weights in the TBAc-exposed mice compared to controls. Absolute liver weights were significantly increased in the 1600 ppm female mouse exposure group (23% increase compared to controls; $p < 0.05$). Relative liver weights were significantly increased in the male and female 1600 ppm exposure groups (11.6% and 20.4% increase compared to controls, respectively; $p < 0.05$).

Tertiary-Butanol

The NTP (1995) conducted a 13-week TBA drinking water exposure study in male and female F344 rats and $B6C3F_1$ mice. TBA drinking water exposure levels were 0, 2.5, 5, 10, 20 or 40 mg/mL.

Exposed doses were 0, 230, 490, 840, 1520 and 3610 mg/kg-day for male rats and 0, 290, 590, 850, 1560 and 3620 mg/kg-day for female rats. Exposed doses for male mice were 0, 350, 640, 1590, 3940 and 8210 mg/kg-day and 0, 500, 820, 1660, 6430 and 11,620 mg/kg-day for female mice.

All male rats in the 40 mg/mL exposure group died prior to study termination. Both absolute and relative (to body weight) kidney weights were significantly increased at all exposure levels for both male and female rats.

No increases in absolute liver weights were noted in either male or female TBA-exposed mice. Increases in relative liver weights were noted in the 20 and 40 mg/mL exposure group in male mice, and in the 40 mg/mL exposure group in female mice.

Takahashi *et al.* (1993) presented an analysis of male rat renal cortical sections from the NTP (1995) 13-week TBA drinking water exposure study. Statistically significant increases in hyaline droplet deposition were reported at all TBA exposure levels ($p < 0.05$). NTP (1995) reported similar increases in hyaline droplet deposition, but did not report statistical analysis data. Takahashi *et al.* (1993) also reported a significant increase in the median number of renal proximal tubular cell nuclei in S-phase as measured by PCNA incorporation in the 20 mg/mL exposure group. NTP (1995) did not report these data.

Borghoff *et al.* (2001) studied the potential induction of α2u nephropathy and enhanced renal cell proliferation by TBA and TBA renal dosimetry in male and female F344 rats. Male and female F344 rats were exposed to 0, 250, 450, or 1750 ppm TBA vapors 6 h/day for 10 consecutive days to assess α2u nephropathy (5 animals/sex/concentration) and renal cell proliferation (5 animals/sex/concentration). TBA dosimetry was assessed using male and female F344 rats following single or repeated (8-day) exposures to target concentrations of 250, 450, or 1750 ppm TBA (3 animals/sex/exposure concentration/time point) for 6 hours/day. Rats were killed at various time points following exposure (2, 4, 6, 8, and 16 hours) to measure levels of TBA in the liver, kidney, and blood and for 1 and 8 days to evaluate the dosimetry of TBA following either single or repeated exposures. Animals evaluated for cell proliferation were subcutaneously implanted with an osmotic pump for delivery of 5-bromo-2-deoxyuridine (BrdU). Incorporation of BrdU into renal cell DNA was used as a measure of cell proliferation.

Relative kidney weights (percentage of body weight) were increased in male rats exposed to 1750 ppm and in female rats exposed to 450 and 1750 ppm TBA compared to controls ($p < 0.05$). A statistically significant, concentration-dependent positive trend ($p < 0.05$) for the accumulation of protein droplets in the renal proximal tubule was noted in male but not female rats exposed to TBA. This increase was statistically significant in the male 1750 ppm exposure group ($p < 0.01$) compared to controls. Immunohistochemical staining of protein droplets for α2u in control and TBA-exposed male rat kidney sections within renal proximal tubules indicated α2u incorporation. α2u staining was slightly greater in male rats exposed to TBA as

compared to control male rats. However, the authors stated that no TBA exposure-related increase in α2u staining intensity in male rats was noted. Kidneys from control or TBA-exposed female rats did not stain positive for α2u.

Renal cytosol α2u concentrations in male rats were measured using an enzyme-linked immunosorbent assay (ELISA) assay. A significant increase in α2u concentration was noted in the 1750 ppm group ($p < 0.05$) compared to controls, in contrast to the α2u staining evaluation, where no TBA exposure-related increase staining was noted. However, very little increase α2u concentration if any was noted in the 250 and 450 ppm groups. The renal α2u concentration in the 1750 ppm group compared to controls also appears to be substantially less than that observed for the α2u inducer TMP (Prescott-Matthews *et al.*, 1997).

A statistically significant increase in cell proliferation in the renal proximal tubule epithelial cells was observed in all groups of TBA-exposed male rats as compared to control males. No similar significant differences in cell proliferation between TBA-exposed and control female rats were noted. The authors stated that renal α2u concentration was positively correlated with cell proliferation in male rat kidney (linear regression analysis, $r^2 = 0.70$). However, the authors also stated that "the concentration of α2u in the kidneys of male rats exposed to 250 or 450 ppm TBA was not significantly increased compared to control rats." These TBA concentrations were noted by the authors to significantly increase male rat renal tubule cell proliferation (see Figure 3), suggesting that TBA can induce male rat renal tubule cell proliferation at concentrations that do not increase renal α2u concentrations.

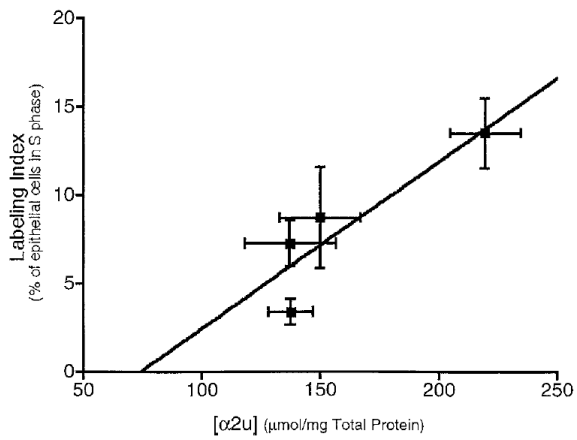

FIG. 6. Positive, exposure-related correlation between the labeling index (LI) and renal α2u concentration for each exposure group. The symbols represent the individual values for each animal at each exposure group (0, 250, 450, and 1750 ppm TBA) ($r^2 = 0.70$).

Figure 3. Showing α2u protein in the kidneys does not appear to be significantly different between control male rats and those exposed to 250 and 450 ppm TBA while proliferation as measured by epithelial cells in S phase does increase. (Figure 6. from Borghoff *et al.* (2001), reproduced unchanged by permission)

The TBA kidney-blood concentration ratios at 2, 4, and 6 hours post-exposure were similar (close to a 1:1 ratio) in female rats following either 1 or 8 days exposure. TBA kidney-blood concentration ratios were not provided for female rats at 16 hours post-exposure for all concentrations and exposure durations, or for male rats exposed to 250 and 450 ppm but not 1750 ppm TBA for both exposure durations. TBA liver-blood ratios tended to be higher post-exposure in male rats exposed for 8 days compared to those exposed for 1 day. Male rat TBA kidney-blood ratios tended to be higher at all concentrations and sampling points after 1 and 8 days of exposure than those of female rats. The exception to this appeared to be the 1 day 1750 ppm exposure groups, where male and female TBA kidney-blood ratios appeared to be similar at 2, 4 and 6 but not 16 hours post-exposure. Female rat TBA kidney: blood ratios tended to remain the same or decrease from 2 to 6 hours post-exposure after either 1 or 8 days of exposure. In contrast, male rat TBA kidney-blood ratios tended to increase from 2 to 6 hours post-exposure after either 1 or 8 days of exposure. TBA liver-blood ratios were approximately the same for both male and female rats at all post-exposure sampling time points after 8 days of TBA exposure; liver-blood ratio values were not provided for the 16 hours post-exposure timepoint.

Williams and Borghoff (2001) investigated the binding characteristics of TBA to α2u in male and female F344 rats. Animals used for α2u binding determinations (4/sex/group) were dosed once with 500 mg/kg TBA, 500 mg/kg ^{14}C-TBA, or vehicle (corn oil) by gavage. Animals used for TBA dosimetry determinations (3/sex/group) were dosed once with 500 mg/kg ^{14}C-TBA, or corn oil (vehicle) by gavage.

Kidney TBA levels were increased in male animals compared to female animals ($p \leq 0.06$), and liver, blood and urine TBA levels were significantly increased in male animals compared to female animals. However, the kidney-blood ratios were not substantially different between male and female animals (1.86 and 2.03, respectively).

Renal cytosolic α2u concentrations were significantly increased ($p = 0.0003$) in TBA-treated male rats compared to controls (311.3 mg and 180.4 mg α2u/mg total protein, respectively). Gel filtration analysis of kidney cytosol indicated that an α2u protein standard coeluted with the low-molecular-weight protein fraction (LMWPF). Anion-exchange chromatography demonstrated that ^{14}C-TBA-derived radioactivity coeluted with α2u from male kidney cytosol. Incubation of *d*-limonene oxide (a known high affinity α2u ligand) with LMWPF isolated from ^{14}C-TBA-treated male rat kidneys displaced ^{14}C-TBA-derived radioactivity. Gas chromatography-mass spectrometry analysis confirmed that TBA was present in this LMWPF. Dialysis with sodium dodecyl sulfate (SDS) caused loss of ^{14}C-TBA-derived radioactivity from the LMWPF fraction; this did not occur after dialysis without SDS, suggesting that TBA binding to α2u is reversible.

Blanck *et al.* (2010) exposed groups of 15 female B6C3F$_1$ mice to concentrations of 0, 2 or 20 mg/mL TBA in drinking water for either 3 or 14 days. The approximate exposure levels for the 2 and 20 mg/mL exposure groups were 344 and 818 mg/kg-day, respectively, for animals exposed for 3 days and 418 and 1616 mg/kg-day, respectively for animals exposed for 14 days. A positive control group (for liver enzyme induction and decreased circulating thyroid levels) received 10 mL/kg body weight (80 mg/kg-day) phenobarbital (PB) by oral gavage daily.

TSH, T_3 and T_4 levels were determined by radioimmunoassay for all animals at sacrifice. The thyroids and livers of 5 animals/group received histopathological examination. Hepatic gene transcription levels were determined for 1) Cyp1a1, 2) the constitutive androstane receptor (CAR)-responsive elements Cyp2b9, Cyp2b10, UGT1a1, UGT2b5 and Sult 2a2, 3) the pregnane-X-receptor mediated Cyp3a11, and 4) the sulfotransferase Sult1a1 by quantitative polymerase chain reaction (qPCR).

Cyp1A-dependent 7-ethoxyresorufin-O-deethylase (EROD), Cyp3A-dependent 7-benzoxyresorufin-O-debenzylase (BROD), Cyp2B-dependent 7-pentoxyresorufin-O-dealkylase (PROD), lauric acid hydroxylation (LAH) and uridine diphosphate glucuronyl transferase (UDPGT) activities were assayed in liver microsomes prepared from a pool of 3 mice/exposure group.

TBA did not induce treatment-related clinical signs or body weight changes in either exposure group. The authors reported visibly enlarged livers in 3 of 15 animals for both TBA exposure groups after 14 days but not 3 days of exposure. However, the authors also reported no effects on either absolute or relative liver weight in any TBA-exposed animals.

TBA was not observed to induce mouse thyroid histopathological changes at the exposure levels used. A diffuse centrilobular hepatocellular hypertrophy similar to that observed with PB was observed in the 20 mg/mL exposure group but not the 2 mg/mL exposure group, which had a lower incidence and severity than was observed in the PB-treated animals. The hepatocellular hypertrophy data is listed in Table 2.

Table 2. Centrilobular hepatocellular hypertrophy incidence and severity data from B6C3F$_1$ mice treated with *tertiary*-butanol (TBA) or phenobarbital (PB)

	TBA (mg/mL)			PB (mg/kg-day)
	0	2	20	80
# animals	6	5	5	6
Diffuse centrilobular hepatocellular hypertrophy				
Minimal (grade 1)	0	0	1	4
Slight (grade 2)	0	0	1	1
Total	0	0	2	5

PB significantly increased gene transcription of all the genes surveyed except Cyp2b9 and Ugt2b5 at both 3 and 14 days of exposure. TBA did not increase transcription of any of the genes surveyed after 3 days of exposure. After 14 days of TBA exposure, significant gene transcription increases were noted for Cyp2b10 and Sult1a1 in both exposure groups, and for Cyp2b9 in the 20 mg/mL exposure group.

PB significantly increased cytochrome P-450 content and EROD, PROD and BROD activity after both 3 and 14 days of exposure. TBA significantly increased BROD activity in both exposure groups, and significantly increased P-450 content, PROD and EROD activity in the 20 mg/mL exposure group but not the 2 mg/mL exposure group after 14 days of treatment.

TBA (both exposure groups) and PB did not produce a statistically significant increase in TSH levels compared to controls after either 3 or 14 days exposure. T_3 levels were not significantly reduced compared to controls by either TBA (both exposure groups) or PB after 3 days of exposure. TBA caused a small but statistically significant decrease in T_3 levels after 14 days of exposure (12% and 13% decrease in the 2 and 20 mg/mL exposure groups, respectively; $p < 0.01$ for both groups). PB-induced reductions in T_3 levels after 14 days of exposure were greater than those observed for TBA (21%, $p < 0.01$). T_4 levels were also not significantly reduced compared to controls by TBA (both exposure groups) after 3 days of exposure. A moderate statistically significant reduction in T_4 levels was noted after

14 days of TBA exposure in both the 2 and 20 mg/mL exposure groups (15% and 22% reduction, respectively; $p < 0.01$). In contrast, PB significantly reduced T_4 levels in the animals after both 3 and 14 days of exposure (34% and 48% reduction, respectively; $p < 0.01$), and produced a greater depression of T_4 levels than TBA in this study.

Genotoxicity

Both TBAc and TBA have been tested in several genotoxicity assays. A tabular summary of the results of that testing is listed in Table 3.

Table 3. Genotoxicity testing summary for *tertiary*-butyl acetate and *tertiary*-butanol

Assay	Cell type or species/strain	Metabolic Activation		Reference
		without	with	
tertiary-butyl acetate				
Bacterial gene mutation	*Salmonella* typhimurium TA 98, TA100, TA1535, TA1537, TA102	-	-	Huntingdon Life Sciences Ltd. (2000)
	Escherichia coli, strain WP2uvrA/pKM 101 (CM891)	-	-	Huntingdon Life Sciences Ltd. (2000)
Chromosomal aberrations	*in vitro* human lymphocytes	-	-	Cruzan and Kirkpatrick (2006)
Rat micronucleus assay	Male/female Sprague-Dawley rats	NA	-	Cruzan and Kirkpatrick (2006)
tertiary-butanol				
DNA damage, Comet assay	human HL-60 leukemia cells	+	NA	Tang *et al*. (1997)
	Rat-1 diploid rat fibroblast cells	+	NT	Sgambato *et al.* (2009)
8-OHdG DNA adducts (oxidative DNA damage)	Rat-1 diploid rat fibroblast cells	+	NT	Sgambato *et al.* (2009)
DNA adducts	Male Kunming (outbred) mice	NA	+	Yuan *et al.* (2007)
Bacterial gene mutation	*S*. typhimurium TA98, TA100, TA1537, TA1538	-	-	NTP (1995)

Assay	Cell type or species/strain	Metabolic Activation		Reference
		without	with	
Bacterial gene mutation	*S*. typhimurium TA102	NT	+	Williams-Hill *et al*. (1999)
	S. typhimurium TA102	-	-	Huntingdon Life Sciences Ltd. (2000)
Bacterial gene mutation	*S*. typhimurium TA102	-	-	McGregor *et al*. (2005)
Mammalian gene mutation	L5178Y mouse lymphoma cells	+/-	-	NTP (1995)
Chromosomal aberrations	Chinese hamster ovary (CHO) cells	-	-	NTP (1995)
Micronucleus induction	Male/female B6C3F$_1$ mice	NA	-	NTP (1995)

+/-: equivocal NA: not applicable NT: not tested

***Tertiary*-Butyl Acetate**

Bacterial mutation tests

Huntingdon Life Sciences Ltd. (2000) evaluated the genotoxicity of TBAc in bacteria, using the *Salmonella* typhimurium strains TA1535, TA1537, TA98, TA100 and TA 102, and a tryptophan dependent mutant of *Escherichia* coli, strain WP2uvrA/pKM 101 (CM891). Test chemicals were dissolved in dimethyl sulphoxide (DMSO), which was also used as a negative control. Appropriate positive controls were included in the assay procedure. Duplicate assays were performed in the presence and absence of Aroclor 1254-induced rat liver S9. The test strains were preincubated with TBAc in gas-tight glass vials (to minimize loss of volatile test substance) prior to plating out. Test concentrations of TBAc used were 5, 15, 50, 150, 500, 1500 and 5000 µg/plate in an initial range finding assay, and 50, 150, 500, 1500 and 5000 µg/plate in the final assay.

Test strain toxicity was observed after TBAc exposure at 5000 µg/plate. TBAc was not mutagenic in any bacterial test strain in the presence or absence of rat liver S9.

Human lymphocyte chromosomal damage *in vitro*

Cruzan and Kirkpatrick (2006) examined the ability of TBAc to cause chromosomal aberrations in human lymphocytes exposed *in vitro*. Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of rat liver S9 mix. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using colcemid and the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of TBAc to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

First test:
Without and with S9 mix -
3 hours treatment, 18 hours recovery: 290, 580 and 1160 µg/mL.

Second test:
Without S9 mix - 21 hours continuous treatment: 290, 580 and 1160 µg/mL.
With S9 mix - 3 hours treatment, 18 hours recovery: 290, 580 and 1160 µg/mL.

The maximum test concentration, 1160 µg/mL, corresponded to a 10 mM solution of TBAc.

TBAc caused no significant increase in the proportion of metaphase figures containing chromosomal aberrations (excluding gap damage) at any dose level when compared with the solvent control, in either test in both the absence and presence of S9 mix. A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No statistically significant increases in the proportion of polyploid cells were seen. The authors concluded that TBAc did not induce chromosomal aberration in human lymphocytes exposed *in vitro*.

Rat micronucleus assay

The ability of TBAc to induce micronuclei (indicative of chromosomal damage) in the bone marrow cells of rat exposed to TBAc by inhalation was studied by Cruzan and Kirkpatrick (2006). Male and female Sprague-Dawley rats were subjected to a single 6 hour nose-only inhalation exposure to TBAc at exposure levels of 100, 400 and 1600 ppm (5 animals/sex/100 and 400 ppm groups; 10 animals/sex/1600 ppm group). A negative control group received clean air only (10 animals/sex/group). A positive control group was dosed by gavage with cyclophosphamide at 20 mg/kg bodyweight.

Bone marrow smears were obtained from five male and five female animals in the negative control group, each of the test substance groups and the positive control group 24 hours after treatment. In addition, bone marrow smears were obtained from five male and five female animals in the negative control and high dose treatment groups 48 hours after treatment. One smear from each animal was examined for the presence of micronuclei in 2000 immature erythrocytes. The proportion of immature erythrocytes was assessed by examination of at least 1000 erythrocytes from each animal. The incidence of micronucleated mature erythrocytes was also evaluated.

No significant increases in the frequency of micronucleated immature erythrocytes and no substantial decrease in the proportion of immature erythrocytes were observed in rats treated with TBAc and killed 24 or 48 hours later, compared to vehicle control values ($p > 0.01$ in each case).

The authors concluded that TBAc did not show any evidence of causing chromosome damage or bone marrow cell toxicity in male or female rats exposed to TBAc by nose-only inhalation exposure.

***Tertiary*-Butanol**

DNA damage

Tang *et al*. (1997) examined the ability of TBA to induce DNA damage in human leukemia HL-60 cells using a Comet assay (single cell gel electrophoresis). TBA was not significantly cytotoxic at any of the concentrations tested (1, 5, 10, and 30 mmol/L; 74, 371, 741, and 2224 µg/mL) as determined by lactate dehydrogenase (LDH) release. However, both the number of cells demonstrating DNA damage and the severity of damage increased significantly in a dose–responsive manner at all concentrations tested.

Yuan *et al.* (2007) used an accelerator mass spectrometry assay to study DNA damage induced in mice by ^{14}C-radiolabeled TBA. Male Kunming (outbred) mice (6/group) were exposed to 0, 0.099, 0.99, 10, 101 or 997 µg ^{14}C-TBA/kg body weight dissolved in saline, using a dosing volume of 8 mL/kg body weight. Animals were sacrificed at six hours post-treatment and the liver, kidneys and lungs were collected for DNA isolation. Extracted DNA samples from the organs listed above were then analyzed for DNA adducts using an accelerator mass spectrometer. A dose-dependent increase in ^{14}C-TBA-DNA adducts was demonstrated for liver, kidney and lung.

The effects of TBA on a diploid rat fibroblast cell line (Rat-1) were studied by Sgambato *et al.* (2009). Exposure of Rat-1 cells to 0.44 mM (33 µg/mL) TBA for 4 hours caused a significant increase (225%; $p < 0.01$) in 8-hydroxydeoxyguanosine (8-OHdG) DNA adducts as measured by an immunohistochemistry assay using an

anti-8-OHdG antibody. 8-OH-dG is generally considered to be a marker of DNA oxidative damage.

TBA was also evaluated for induction of DNA damage in Rat-1 cells in the comet assay. Rat-1 cells were exposed to 0.44 mM (33 µg/mL) TBA for 4 hours, and the cells were assayed for DNA damage at 30 minutes, 4 hours and 12 hours. DNA damage peaked at 30 minutes, as demonstrated by increases in tail length, percent migration and tail moment, and then declined at 4 and 12 hours.

Bacterial mutation assays

NTP (1995) found TBA to be negative in the *Salmonella*/mammalian microsomal mutation assay in the presence and absence of Aroclor 1254-induced rat or hamster liver S9 in test strains TA98, TA100, TA1537 and TA1538 at concentrations of 100, 333, 1000, 3333 and 10,000 µg/plate.

The genotoxicity of TBA was assayed using the standard *Salmonella*/mammalian microsomal mutation assay with TA102, a strain that detects oxidative DNA damage and contains a functional *uvr*B gene, rendering it excision repair proficient (Williams-Hill *et al*., 1999). The assay was performed in the presence of Aroclor-induced rat liver S-9, using concentrations ranging from approximately 750 to 3750 µg/plate. TBA caused a maximum of 800 revertants/plate (approximately 2-fold greater than control) at a concentration of approximately 2500 µg/plate. The authors concluded that TBA induces a mutagenic pathway involving oxidation of DNA bases and an intact repair system.

Huntingdon Life Sciences Ltd. (2000) evaluated the genotoxicity of TBA concurrently with TBAc in *Salmonella* strain TA102 only, employing the same methodology as used for TBAc. Duplicate assays were performed in the presence and absence of Aroclor 1254-induced rat liver S9. The test strains were preincubated with TBA in gas-tight glass vials (to minimize loss of volatile test substance) prior to plating out. Test concentrations of TBA used were 5, 15, 50, 150, 500, 1500 and 5000 µg/plate in an initial range finding assay, and 50, 150, 500, 1500 and 5000 µg/plate in the final assay. No toxicity was observed after TBA exposure. TBA was not genotoxic to *Salmonella* strain TA102 in the presence or absence of rat liver S9.

McGregor *et al*. (2005) tested TBA for mutagenicity in S. typhimurium (test strain TA102) in the presence and absence of Aroclor 1254 induced rat liver S9 in two separate experiments. TBA was dissolved in either DMSO or water at concentrations of 0, 100, 200, 500, 1000, 2500 and 5000 µg/plate. TBA dissolved in water in the absence of rat liver S9 caused a small increase in mutagenicity in the two experiments (maximum response 1.5-fold and 1.7-fold greater than control in experiments 1 and 2, respectively), but the increase was less than the 2-fold increase that is usually considered significant.

Mammalian gene mutation assays

TBA did not induce mutations in the presence of Aroclor 1254-induced rat liver S9 in the L5178Y mouse lymphoma mutation assay in two trials at concentrations of 1000, 2000, 3000, 4000 and 5000 µg/mL (NTP, 1995). TBA did cause a significant increase in mutations in the absence of S9 in one trial at the 5000 µg/mL concentration. The number of mutations induced increased with dose, but NTP did not present trend test data. A second TBA trial in the absence of S9 was negative, but NTP did not present the results from that trial because it did not meet quality control standards for that assay. NTP concluded that TBA did not induce mutations in the L5178Y mouse lymphoma mutation assay.

Mammalian chromosomal damage assays

TBA did not induce chromosomal aberrations in vitro in Chinese hamster ovary (CHO) cells in the presence and absence of Aroclor 1254-induced rat liver S9 at concentrations ranging from 160 to 5000 µg/mL (NTP, 1995). TBA also did not induce sister chromatid exchanges (SCEs) in the presence of Aroclor 1254-induced rat liver S9 in CHO cells. TBA did cause a significant increase in SCEs in the absence of S9 in one trial at the 5000 µg/mL concentration. The number of SCEs induced increased with dose, but NTP did not present trend test data. A second TBA trial in the absence of S9 was negative. NTP concluded that TBA did not induce SCEs in CHO cells.

Additionally, TBA did not induce micronuclei (indicative of chromosomal damage) in normochromatic erythrocytes (NCEs) obtained from male and female $B6C3F_1$ mice exposed to 0, 3000, 5000, 10,000, 20,000 or 40,000 ppm TBA in drinking water for 13 weeks (NTP, 1995).

V. Cancer Hazard Evaluation

Male rat kidney tumor data

It has been established that TBAc is metabolized in rats to TBA (Groth and Freundt, 1994; Huntingdon Life Sciences Ltd., 2000e). Oral TBA exposure has been demonstrated to induce renal tumors in male rats and thyroid follicular cell tumors in female mice (NTP, 1995). The possibility that the male rat kidney tumors observed after oral TBA exposure may be related to a TBA-α2u interaction resulting in α2u nephropathy has been raised by Borghoff *et al.* (2001), McGregor and Hard (2001) and Hard *et al.* (2011). The defining feature of α2u nephropathy is the rapid accumulation of protein (or hyaline) droplets in the renal proximal tubule cells of many strains of male rats following chemical treatment. Female rats do not appear to produce α2u. α2u is usually degraded in the lysosomal compartment of the renal proximal tubule cells. It is believed that binding of α2u to chemicals results in an impairment of the lysosomal degradation of this protein, leading to an accumulation of protein droplets containing α2u. Protein droplet accumulation occurs rapidly, and

leads to progressive renal injury, characterized by single cell degeneration and necrosis in the renal proximal tubule. Renal proximal tubule necrosis causes compensatory cell proliferation in the renal cortex. Cessation of cell proliferation occurs shortly after stopping acute exposures to α2u-inducers, and a restoration of normal renal architecture can occur. Chronic exposure to α2u-inducers leads to linear papillary mineralization, which is typical of the chronic progressive nephropathy often seen in older male rats. Atypical hyperplastic cellular foci may eventually be observed in the proximal tubules, and these foci may eventually progress to renal adenomas and carcinomas (Swenberg and Lehman-McKeeman, 1999).

A criteria list for determining whether chemicals that induce male rat kidney tumors and may cause increased renal α2u production should be considered to be potential human carcinogens has been defined by the International Agency for Research on Cancer (IARC).

The criteria for an agent causing kidney tumors through an α2u-globulin-associated response in male rats (IARC,1999) are as follows:

1. Lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of in-vitro and in-vivo data

2. Male rat specificity for nephropathy and renal tumorigenicity

3. Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory

4. Identification of the protein accumulating in tubule cells as α2u-globulin

5. Reversible binding of the chemical or metabolite to α2u-globulin

6. Induction of sustained increased cell proliferation in the renal cortex

7. Similarities in dose-response relationship of the tumor outcome with the histopathological end-points (protein droplets, α2u-globulin accumulation, cell proliferation).

<u>TBA fits some of these criteria:</u>

Criterion 3:

Acute and subchronic TBA exposure has been shown to exacerbate hyaline droplet formation in male rats (Takahashi *et al.*, 1993; NTP, 1995; Borghoff *et al.*, 2001), and chronic histopathological changes including granular cast formation and linear papillary mineralization have been observed in TBA-exposed male rats (NTP, 1995).

Criterion 4:

Immunohistochemical staining of protein droplets for α2u in control and TBA-exposed male rat kidney sections within renal proximal tubules indicated α2u incorporation (Borghoff *et al.*, 2001). Additionally, Faber *et al*. (2014) noted consistent renal α2u-globulin accumulation in male rat 13-week TBAc exposure groups as measured by both immunohistopathology and ELISA analysis.

Criterion 5:

Reversible binding of TBA to α2u has been demonstrated (Criterion 5) (Williams and Borghoff, 2001), although a binding affinity was not determined.

<u>TBA does not completely fit several of the other criteria listed above:</u>

Criterion 2:

The NTP TBA 2-year study (1995) demonstrated renal tumors only in male rats.

However, adverse renal effects associated with TBA exposure have been observed in female rats. The severity of nephropathy was significantly increased in all TBA-exposed female rats in the NTP (1995) study, and this increased severity exhibited a dose-response. Renal inflammation was significantly increased in the 5 and 10 mg/mL female rat exposure groups, and significantly increased transitional epithelial hyperplasia was noted in the 10 mg/mL female rat exposure group. These data do not fit Criterion 2. Melnick *et al*. (1997) noted that this nephrotoxic response in the female rat suggests the possibility of other processes leading to or influencing the kidney tumor response.

Criterion 6:

The data on the sustained induction of renal tubular epithelial cell proliferation in male rats by TBA is mixed. A dose-dependent increase in cell proliferation in the renal tubular epithelial cells of male rats exposed to TBA by inhalation was observed by Borghoff *et al.* (2001). The animals in this study were only exposed for 10 days, so this study did not provide information on whether cell proliferation was sustained chronically.

Takahashi *et al.* (1993) presented an analysis of renal cortical sections from the NTP (1995) 13-week TBA drinking water exposure study which included an evaluation of male rat renal tubular epithelial cell proliferation as measured by proliferating cell nuclear antigen (PCNA)-incorporation.

A statistically significant ($p < 0.05$) increase in median (not mean) cell proliferation was only noted in the second highest exposure group at 20 mg/mL.

More recent data by Faber *et al.* (2014) indicated that TBAc exposure did not cause increases in rat kidney tubular cell proliferation after 13 weeks of TBAc exposure by inhalation as measured by PCNA assessment.

Finally, TBA does not fit the following criteria:

Criterion 1:

Positive genotoxicity data exist for TBA. TBA has been reported to cause DNA damage in human leukemia HL-60 cells using a Comet assay (Tang *et al*., 1997), induce ^{14}C-TBA-DNA adducts in mouse liver, kidney and lung (Yuan *et al*., 2007) and induce 8-OHdG DNA adducts and DNA damage measured via the Comet assay in Rat-1 cells (Sgambato *et al*., 2009). Additionally, TBA has been demonstrated to induce mutations in a *Salmonella* strain known to be sensitive to oxidative DNA damage (TA102) in the presence of rat liver S9 (Williams-Hill *et al.*, 1999). These data indicate that TBA should not be determined to be non-genotoxic.

Criterion 7:

While α2u staining was slightly greater in male rats exposed to TBA as compared to control male rats (Criterion 3) (Borghoff *et al.*, 2001), the authors stated that no TBA exposure-related increase in α2u staining intensity in male rats was noted. A significant increase in renal cytosol α2u concentrations as determined by an ELISA assay was noted in the male rat 1750 ppm group ($p < 0.05$) compared to controls, in contrast to the α2u staining evaluation, where no TBA exposure-related α2u increase was noted. However, very little increase, if any, in α2u concentrations as determined by an ELISA assay was noted in the 250 and 450 ppm groups. The renal α2u concentration in the 1750 ppm group compared to controls also appears to be substantially less than that observed for the α2u inducer TMP (Prescott-Matthews *et al.*, 1997).

Additionally, Faber *et al.* (2014) stated that renal α2u-globulin accumulation was consistent throughout male rat 13-week TBAc exposure groups as measured by both immunohistopathology and ELISA analysis. The authors did not present a statistical analysis of these data. However, a graphical depiction of male rat kidney α2u-globulin levels appeared to show that there was probably no significant difference between the TBAc exposure groups, and possibly no significant difference between some of the TBAc exposure groups and the control group, suggesting a weak dose-response relationship between TBAc exposure and renal α2u-globulin accumulation in this study. Since TBAc is quickly and extensively metabolized to TBA, this study is relevant to a TBA-induced male rat kidney tumor mode of action consideration.

The dose response relationship between hyaline droplet severity and renal tumor incidence was not particularly strong in the NTP 1995 study. Male rats exposed to TBA for 13 weeks developed increased hyaline droplet accumulation within renal tubule epithelium and lumens at all doses of TBA, but the droplet accumulation was minimal at the dose demonstrating a significantly increased tumor incidence (2.5 mg/mL). Also, male rats in the TBA inhalation study by Borghoff *et al.* (2001) did not demonstrate significant increases in either hyaline droplet accumulation or renal α2u

concentrations at estimated doses approximating those resulting in increased renal tumors in the 1995 NTP study.

Takahashi *et al.* (1993) presented an analysis of renal cortical sections from the NTP (1995) 13-week TBA drinking water exposure study which included an evaluation of male rat renal tubular epithelial cell proliferation as measured by PCNA-incorporation.

A statistically significant ($p < 0.05$) increase in median (not mean) cell proliferation was only noted in the 20 mg/mL exposure group. This is well above the exposure level (2.5 mg/mL) which induced male rat renal tubule tumors in the NTP (1995) 2-year TBA drinking water exposure study.

The hypothetical progression of events leading from α2u nephropathy to renal tumors is that an accumulation of hyaline droplets containing α2u in the renal proximal tubules results in cell death, then compensatory cell proliferation, which leads to increased renal tumors. Swenberg and Lehman-McKeeman (1999) note that "Dose related and male rat specific increases in cell proliferation have been demonstrated with all of the (α2u-inducing) chemicals evaluated, and the dose response relationships for cell proliferation parallel those for hyaline droplet formation and the induction of renal tumours." However, in the case of TBA, male rat kidney tumor induction takes place at exposure levels where cell proliferation and hyaline droplet formation are not substantially increased.

The above data do not rule out the possibility of increased renal α2u concentrations playing a role in the increased renal tumor incidence seen in TBA-exposed male rats, either directly through the induction of nephropathy, or indirectly through increasing the TBA concentration in the kidney.

As described above, 1) the dose response relationship between hyaline droplet severity, cell proliferation and renal tumor incidence in TBA-exposed rats is weak; 2) TBA exposure has been demonstrated to cause adverse renal effects (nephropathy, inflammation, transitional epithelial hyperplasia) in female rats; 3) positive TBA genotoxicity data exists. These data indicate that it would not be appropriate to determine that the increased renal tumors observed in TBA-exposed male rats are solely due to α2u-induced nephropathy. Thus, the increased renal tumor incidences observed by NTP (1995) in TBA-exposed male rats should be considered to be suitable for use in human cancer risk assessment.

Female mouse thyroid follicular cell tumor data

McClain (2001) prepared an expert opinion for Lyondell Chemical Co. regarding the increased thyroid follicular cell tumor incidence observed by NTP (1995) in female TBA-exposed mice, which stated that "The thyroid gland hyperplasia noted in male and female mice and the increased follicular cell tumor incidence in female mice are consistent with an epigenetic mode of action. It is postulated that the thyroid gland hyperplasia and neoplasia are the consequence of microsomal enzyme induction of

thyroid hormone metabolism. This results in a compensatory increase in pituitary TSH which, with long term, low level stimulation of the thyroid gland results in follicular cell hypertrophy, hyperplasia and ultimately neoplasia in rodents."

It has been suggested that thyroid hormone hepatic metabolism and excretion may lead to compensation by the thyroid gland for this increased excretion, potentially resulting in stimulation of the hypothalamic-pituitary-thyroid axis and possibly secondary hyperplastic or neoplastic changes in the thyroid (Curran and de Groot, 1991).

This opinion did not state that the female mouse thyroid tumor data was not applicable to human cancer risk assessment. However, some have taken the position that "the available evidence indicates that the thyroid tumors observed in the high dose female mice are due to a threshold mechanism - one to which humans are much less susceptible than rodents. Since the high dose of TBA was 2110 mg/kg/day, and yet higher doses of TBAc would be required to achieve such TBA levels (see next section), there is very little likelihood that humans could experience exposures sufficient to cause such tumors (Lyondell Chemical Co., 2001)."

However, the available mouse data does not support a threshold mechanism for TBA carcinogenicity in that species.

Liver weights in male and female mice exposed orally to TBA for 13 weeks (NTP, 1995) were only slightly increased in the 20 and 40 mg/mL male and 40 mg/mL female groups (115% and 107% of control for the male 20 and 40 mg/mL groups, respectively; 113% of control for the female 40 mg/mL group). Hepatic enzyme induction is often associated with increases in liver weight (Maronpot *et al.*, 2010).

Blanck *et al*. (2010) did not observe increases in either absolute or relative (to body weight) liver weights in groups of 15 female $B6C3F_1$ mice exposed to concentrations of 0, 2 or 20 mg/mL TBA in drinking water for either 3 or 14 days. TBA significantly increased BROD activity in both exposure groups, and significantly increased P-450 content and PROD activity in the 20 mg/mL exposure group but not the 2 mg/mL exposure group after 14 days of treatment. The magnitude of increase observed after 14 days of exposure to 20 mg/mL TBA (156% , 210% and 1231% of controls, for P-450, PROD and BROD activity, respectively) was substantially less than the increase induced by the liver enzyme inducer PB (220% , 813% and 7213% of controls, for P-450, PROD and BROD activity, respectively).However, Blanck *et al*. (2010) also found that TBA did not induce mouse thyroid histopathological changes, and did not produce a statistically significant increase in TSH levels compared to controls after either 3 or 14 days exposure. TBA caused a small decrease in T_3 levels and a moderate decrease in T_4 levels after 14 days of exposure. PB-induced decreases in T_3 and T_4 levels in this study were substantially greater than those produced by TBA.

Faber *et al.* (2014) did not observe significant thyroid gland histopathological changes or alterations in thyroid/parathyroid gland weights in TBAc-exposed mice compared to controls after 13 weeks of exposure. Additionally, no significant increases in TSH levels or decreases in T_3 levels were observed in TBAc-exposed male or female mice compared to controls. Moderately decreased T_4 levels (32% reduction compared to controls) were only noted in the male 1600 ppm mouse exposure group. TBAc-exposed female mice demonstrated no change in T_4 levels compared to controls.

The above data on the effects of TBA in mice indicate that TBA: 1) causes little or no increases in absolute or relative liver weights; 2) does not induce cytochrome P450 activity to the same degree as seen with PB; 3) does not cause large decreases in T_3 or T_4 levels; 4) does not increase TSH levels; 5) does not cause acute abnormal mouse thyroid histopathological changes. Additionally, TBAc has not been observed to cause significant thyroid gland histopathological changes or alterations in thyroid/parathyroid gland weights in mice. TBAc has been observed to induce moderate decreases in T_4 levels at relatively high dose exposures in male but not female mice, but did not cause increases in TSH levels or decreases in T_3 levels in male or female mice. This, plus the fact that positive genotoxicity data for TBA has been published, indicates that TBA should not be considered to be strictly a hormonally-mediated threshold carcinogen for the thyroid, and that the female mouse thyroid follicular cell tumor data reported by NTP is relevant for use in determining the potential human cancer risk from TBA exposure.

VI. Quantitative Cancer Risk Assessment

OEHHA has previously calculated a cancer slope factor (CSF) for TBA from the Cirvello (1995) TBA male rat kidney tumor data using TOX_RISK version 3.5 software (OEHHA, 1999). In this case, the CSF is a cancer potency derived from the lower 95 percent confidence limit on the 10 percent tumor dose (LED_{10}), where CSF = 10 percent/LED_{10}. The potency estimate was converted to human equivalents [in $(mg/kg\text{-}day)^{-1}$] using body weight $(BW)^{3/4}$ scaling. The TBA CSF value was calculated using data from the most sensitive species and sex; in this case, 3×10^{-3} $(mg/kg\text{-}day)^{-1}$ from the NTP (1995) male F344 rat kidney tumor data.

A CSF of 2×10^{-3} $(mg/kg\text{-}day)^{-1}$ was then derived for TBAc, assuming 100% metabolism of TBAc to TBA and a molar conversion factor of 0.64 (TBA molecular weight 74.12 / TBAc molecular weight 116.16). An inhalation unit risk factor (UR) for TBAc of 4×10^{-7} $(\mu g/m^3)^{-1}$ was derived from the CSF value for TBAc by assuming a human breathing rate of 20 m^3/day, 70% fractional absorption, and an average human body weight of 70 kg. These CSF and UR values for TBAc were included in a 2006 ARB report (ARB, 2006).

The final NTP report for TBA (NTP, 1995) listed exposed dose values that were slightly different from the values published in Cirvello *et al.* (1995). Lifetime average daily dose estimates from the NTP TBA drinking water exposure study (1995) were

reported in Cirvello *et al.* (1995) as 0, 85, 195 or 420 mg/kg-day for male rats, and 0, 510, 1015 or 2105 mg/kg-day for female mice, and were reported in NTP (1995) as 0, 90, 200 or 420 mg/kg-day for male rats, and 0, 510, 1020 or 2110 mg/kg-day for female mice. The tumor incidence data was the same for both Cirvello (1995) and NTP (1995).

TBA CSFs can be calculated from the NTP (1995) TBA drinking water exposure study male rat kidney and female mouse thyroid follicular cell tumor data sets using the Multistage Cancer Model function of Benchmark Dose Software (BMDS) version 2.6 (U.S. EPA, 2015). The administered and exposed doses and tumor incidence data uncorrected for mortality for both tumor types are listed in Table 1, and are from NTP (1995).

A poly-3 survival-adjusted lifetime tumor incidence correction was applied to the data listed in Table 1 by OEHHA. The poly-3 correction was performed using the methods described in Bailer and Portier (1988) and Portier and Bailer (1989). The poly-3 correction required time-on-study and tumor diagnosis data for individual animals. The time-on-study and tumor diagnosis data for the female mice were obtained from the NTP (1995) study report. The time-on-study and step section kidney tumor diagnosis data were obtained for the male rats in the NTP study from two pathology reports provided to OEHHA by NTP (NTP 1992a, b).

Table 4 lists the administered and exposed doses and the poly-3 survival-adjusted lifetime tumor incidence for male rat kidney and female mouse thyroid follicular cell tumors from the 1995 NTP TBA study.

Table 4. Increased poly-3 survival-adjusted tumor incidences in Fischer 344 male rats and male and female B6C3F$_1$ mice exposed to *tertiary*-butanol in drinking water (NTP, 1995)

Sex, species	Tumor type	Administered dose (mg/mL)	Exposed dose (mg/kg-day)	Poly-3 corrected tumor incidence
Male rats	Renal tubule adenomas/carcinomas	0	0	8/35
		1.25	90	13/34
		2.5	200	19/34**
		5	420	13/33
Male mice	Thyroid follicular cell adenomas			
		0	0	1/60
		5	540	0/59
		10	1040	4/59
		20	2070	2/57
Female mice				
		0	0	2/58$^+$
		5	510	3/60
		10	1020	2/59
		20	2110	9/59*

Fisher exact test pairwise comparison with controls: * p = 0.028 ** p = 0.005
Cochran-Armitage trend test for dose response: $^+$ p = 0.007

A CSF_{animal} could not be derived from the poly-3 corrected NTP male rat kidney tumor data set due to lack of model convergence. Therefore, a CSF_{animal} of 4.3 x 10^{-3} (mg/kg-day)$^{-1}$ was calculated from the NTP male rat kidney tumor data set with the high dose (420 mg/kg-day) eliminated, using a 1st degree polynomial (a goodness-of-fit p value could not be determined for a 2nd degree polynomial).

The NTP female mouse thyroid tumor data set was evaluated using both 2nd degree and 3rd degree polynomial multistage cancer models. The 3rd degree polynomial multistage cancer model provided a better fit of that dataset (based on lower AIC values and a higher p-value), and was used to calculate a CSF_{animal} of 7 x 10^{-5} (mg/kg-day)$^{-1}$ from the female mouse thyroid tumor data set. All CSF_{animal} values were derived using a Benchmark Response Value (BMR) of 5% (5% extra risk) to calculate the Benchmark Dose (BMD).

A summary of the BMDS output is listed in Table 5. The associated graphic representation of the model results for the NTP male F344 rat renal tubule adenomas and carcinomas data set is provided in Figure 3.

Table 5. BMDS output from an analysis of the NTP (1995) *tertiary*-butanol tumor data

Sex/strain/species/tumor site	BMD mg/kg-day	BMDL mg/kg-day	Goodness of fit *p*-value	AIC
Male F344 rat renal tubule adenomas/carcinomas; high dose dropped	18.76	11.55	0.86	133.55
Female $B6C3F_1$ mouse follicular cell adenomas	1523.69	647.06	0.74	109.31

AIC: Akaike Information Criterion
BMD: Benchmark Dose
BMDL: lower 95% confidence limit on the BMD

The TBAc CSF value can then be calculated using the TBA CSF from the most sensitive species and sex; in this case, 4.3×10^{-3} $(mg/kg\text{-}day)^{-1}$ from the poly-3 adjusted NTP (1995) male F344 rat kidney tumor data. The potency estimate for TBA was converted to human equivalents [in $(mg/kg\text{-}day)^{-1}$] using body weight $(BW)^{3/4}$ scaling. A time-weighted average body weight for the control rats (0.431 kg) was calculated from information presented by NTP (1995) for control animals during the study, and a default human body weight of 70 kg was used. The resulting TBA CSF_{human} value was 1.5×10^{-2} $(mg/kg\text{-}day)^{-1}$.

The CSF and UR values for TBAc included in the 2006 ARB report (ARB, 2006) assumed a 70% fractional absorption for TBAc, and 100% metabolism of TBAc to TBA.

However, the absorption and metabolism to TBA for a 6-hr dose at 100 ppm of TBAc can be derived from the data presented in Cruzan and Kirkpatrick (2006). These data indicate that 50.7 mg/kg of the radiolabeled TBAc was inhaled over 6 hours, and within 7 days after exposure ended, 92.6% of the radioactivity was recovered in urine, feces and tissues, and 4.8% of the radioactivity was recovered in exhaled air. Within the first 12 hours after exposure, 4.5% of the radioactivity (95% of the total amount exhaled) was exhaled suggesting that this minor route of excretion could occur before or after absorption through lung tissue. Therefore it is reasonable to assume that at least 95% of TBAc inhaled over 6 hours is absorbed to blood.

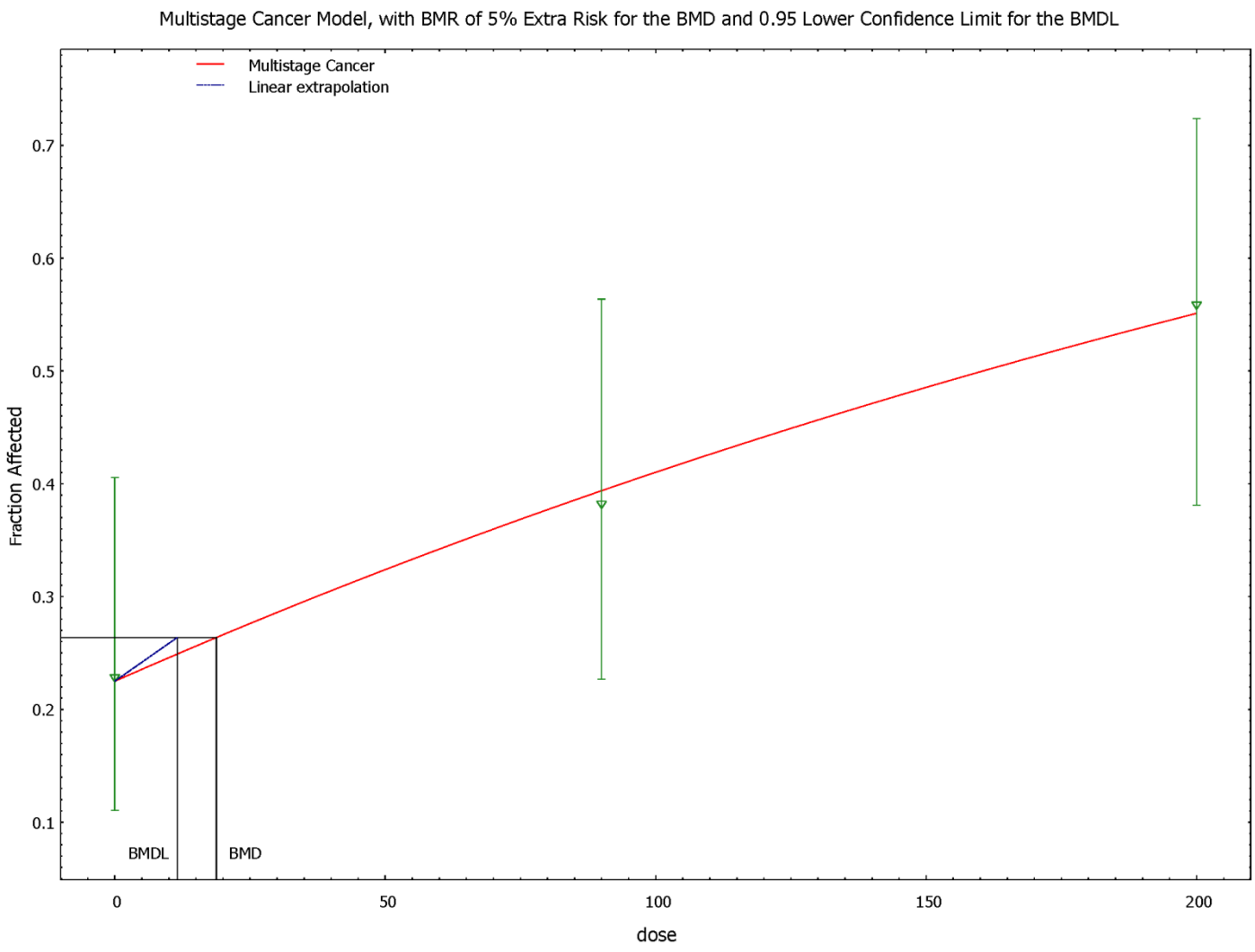

Figure 3. BMDS Multistage Cancer Model data for NTP (1995) male rat kidney adenomas/carcinomas

Additionally, Table V and Figure 1 in Cruzan and Kirkpatrick (2006) reports that 3.5% plus 1.3% of TBAc that is excreted through the urinary pathway converts to *t*-butyl-2-hydroxyacetate or unidentified metabolites and 24.2% converts to 2-hydroxymethyl-isopropyl acetate. This leaves 71% of urinary products of metabolism that could originate from TBA. In the absence of fecal and tissue TBAc quantitative metabolite data, it can be reasonably assumed that the percentage of metabolism products that could originate from TBA in those compartments is similar to the percentage found in the urinary compartment. It should be noted that the TBA cancer bioassay was performed using F344 rats, while the TBA metabolism studies were done in Sprague-Dawley rats. Possible differences in TBA metabolism between the rat strains are a potential source of uncertainty in the TBAc oral CSF calculation presented below.

An oral CSF of 7.0×10^{-3} (mg/kg-day)$^{-1}$ can be derived for TBAc from the TBA CSF_{human} value of 1.5×10^{-2} (mg/kg-day)$^{-1}$, assuming a metabolic conversion factor of 0.71 (TBAc MC) for conversion of TBAc to TBA and a molar conversion factor (MCF) of 0.64 (TBA molecular weight 74.12 / TBAc molecular weight 116.16), using the following relationship:

$$\text{TBAc CSF} = \text{TBA CSF}_{human} \times \text{TBAc MC} \times \text{MCF}$$

An inhalation TBAc CSF of 6.7×10^{-3} (mg/kg-day)$^{-1}$ can be calculated from the oral TBAc CSF using the following relationship, where FA = 95% fractional absorption:

$$\text{inhalation TBAc CSF} = \text{oral TBAc CSF} \times \text{FA}$$

A unit risk factor (UR) for TBAc of 1.9×10^{-6} (µg/m^3)$^{-1}$ can then be derived from the inhalation CSF value for TBAc by assuming a human breathing rate (BR) of 20 m^3/day, an average human body weight (BW) of 70 kg, and a mg to µg conversion (CV) of 1000 using the following relationship:

$$UR = \left(\frac{CSF \times BR}{BW \times CV}\right)$$

VII. Conclusions

No carcinogenicity data are available for TBAc. However, TBAc has been demonstrated to be substantially metabolized to *tertiary*-butanol (TBA) in rats (Groth and Freundt, 1994). TBA genotoxicity data are mixed, but positive DNA damage (Tang *et al*., 1997; Yuan *et al.*, 2007; Sgambato *et al.*, 2009) and bacterial gene mutation studies for TBA (Williams-Hill *et al*., 1999) suggest that TBA may cause oxidative DNA damage resulting in the induction of gene mutations. Further, a DNA adduction study observed a dose-dependent increase in ^{14}C-TBA-DNA adducts for liver, kidney and lung. TBA has been shown to induce tumors in both rats and mice (NTP, 1995; Cirvello *et al*., 1995), and OEHHA (1999) has previously calculated a cancer slope factor for TBA of 3×10^{-3} (mg/kg-day)$^{-1}$, corresponding to an air unit risk

factor of 9×10^{-7} $(\mu g/m^3)^{-1}$. (This was part of an expedited evaluation supporting a drinking water Action Level for TBA developed by OEHHA (OEHHA, 1999) and adopted by the Department of Health Services.) This raises a concern that exposure to TBAc may result in a cancer risk because of the metabolic conversion to TBA. It has been proposed that the NTP (1995) TBA carcinogenicity data may not be relevant to human cancer risk assessment because 1) the male rat kidney tumors are the result of TBA-induced α2u nephropathy, a pathological effect specific for male rats, and 2) the female mouse thyroid follicular cell tumors may be due to an effect on thyroid hormone levels, which would have a threshold above the level of expected human exposures. However, the data pertaining to both these possibilities are insufficient to allow the determination that the NTP (1995) TBA carcinogenicity data are not relevant to human cancer risk assessment. Therefore, TBAc should be considered to pose a potential cancer risk to humans.

A CSF of 0.002 $(mg/kg\text{-}day)^{-1}$ was previously derived for TBAc by OEHHA, assuming 100% metabolism of TBAc to TBA and a molar conversion factor of 0.64 (TBA molecular weight 74.12 / TBAc molecular weight 116.16) (ARB, 2006). An inhalation unit risk factor for TBAc of 4×10^{-7} $(\mu g/m^3)^{-1}$ was derived from the CSF value for TBAc by assuming a human breathing rate of 20 m^3/day, 70% fractional absorption, and an average human body weight of 70 kg.

In this document, OEHHA has calculated TBA CSFs from the NTP (1995) TBA drinking water exposure study's male rat kidney and female mouse thyroid follicular cell tumor data sets using BMDS version 2.6 (U.S. EPA, 2015). CSF_{animal} values of 4.3×10^{-3} $(mg/kg\text{-}day)^{-1}$ and 7.7×10^{-5} $(mg/kg\text{-}day)^{-1}$ were derived from the male F344 rat kidney tumor data and the female mouse thyroid tumor data, respectively. The TBAc CSF value was then calculated using the TBA CSF from the most sensitive species and sex; in this case, 4.3×10^{-3} $(mg/kg\text{-}day)^{-1}$ from the NTP (1995) male F344 rat kidney tumor data. This potency estimate was converted to human equivalents [in $(mg/kg\text{-}day)^{-1}$] using body weight $(BW)^{3/4}$ scaling. The resulting TBA CSF_{human} value was 1.5×10^{-2} $(mg/kg\text{-}day)^{-1}$.

An oral CSF of 7.0×10^{-3} $(mg/kg\text{-}day)^{-1}$ was calculated for TBAc, assuming 71% metabolism of TBAc to TBA and a molar conversion factor of 0.64 (TBA molecular weight 74.12 / TBAc molecular weight 116.16). An inhalation TBAc CSF of 6.7×10^{-3} $(mg/kg\text{-}day)^{-1}$ was calculated from the oral TBAc CSF assuming 95% fractional absorption. A unit risk factor for TBAc of 1.9×10^{-6} $(\mu g/m^3)^{-1}$ was then derived from the inhalation CSF value for TBAc by assuming a human breathing rate of 20 m^3/day, and an average human body weight of 70 kg.

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