VIA Email

August 8, 2017

Document Control Office
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, N.W.
Washington, DC 20460


Dear Sir or Madam:

The American Petroleum Institute (API) respectfully submits comments for consideration by the U.S. Environmental Protection Agency (EPA) Science Advisory Board Chemical Assessment Advisory Committee in their review of the EPA Integrated Risk Information System (IRIS) Toxicological Review of tert-butyl alcohol (tert-butanol or TBA). API commends the Agency for providing opportunities for public comment on this important assessment, and for convening the June 2016 Public Science Meeting on this topic. API participated in the 2016 Public Science Meeting and provided detailed comments prepared by Dr. Kenneth Bogen on the Agency’s previous Public Comment Draft of this assessment. API believes that a number of key aspects of the TBA assessment can be further improved, as discussed in the attached comments.
American Petroleum Institute Comments
U.S. Environmental Protection Agency
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I. Introduction and Summary

The American Petroleum Institute (API) respectfully submits these comments for consideration by the U.S. Environmental Protection Agency (EPA) Science Advisory Board Chemical Assessment Advisory Committee in their review of the EPA Integrated Risk Information System (IRIS) Toxicological Review of tert-butyl alcohol (tert-butanol or TBA). API is a national trade association representing all facets of the oil and natural gas industry, which supports 9.8 million U.S. jobs and 8 percent of the U.S. economy, and provides most of the nation’s energy. API’s more than 625 members include large integrated companies, as well as exploration and production, refining, marketing, pipeline, and marine businesses, and service and supply firms.

API’s members are involved in all major points of the chemical supply chain—from natural gas and crude oil production, to refinery production of fuels and other products, to service companies using chemicals. API and its members are dedicated to continuous efforts to meet chemical management responsibilities while economically developing energy resources and supplying high quality products and services. API recognizes the responsibility to work with other industries, the public, government, and nongovernmental organizations to achieve sound management of chemicals.

API commends the Agency for providing opportunities for public comment on this important assessment, and for convening the June 2016 Public Science Meeting on this topic. API participated in the 2016 Public Science Meeting and provided detailed comments prepared by Dr. Kenneth Bogen on the Agency’s previous Public Comment Draft of this assessment.

API believes that a number of key aspects of the TBA assessment can be further improved. Chief among these are the cancer modes of action and classification, the cancer risk quantification, and the critical endpoint chosen for noncancer risk assessment. Attention by the Advisory Committee to these aspects of the review is requested in finalizing the TBA toxicological review.

API’s major comments, which are further discussed in the remainder of this document, are as follows.
• A putative increase in thyroid follicular cell adenomas in TBA-exposed female B6C3F1 mice in the NTP (1995) bioassay is not relevant to humans.
  o The TBA-associated elevation observed in high-dose females is not statistically significant if an appropriate 2-tail test is applied; consequently, any conclusion of thyroid tumorigenicity must be deemed ambiguous.
  o A nongenotoxic, CAR-activation-mediated hormone-disruption mode of action that is irrelevant to humans can explain a putative TBA-associated elevation in female mouse thyroid follicular cell tumors.
• Renal tubule cell adenomas elevated in TBA-exposed male rats in the NTP (1995) bioassay are not relevant to humans.
  o TBA-associated male-rat kidney tumors were due at least in part to a recognized alpha2u-globulin mode of action specific to male rats.
  o TBA-associated male-rat kidney tumors were exacerbated by a Chronic Progressive Nephropathy (CPN) mode of action that is specific to aging rats.
• TBA is not genotoxic, based on a critical examination of relevant studies.
• Consequently, TBA-associated tumors observed in rodents are not relevant to humans.

II. Noncancer Kidney Toxicity

Charge Question 3a: “The draft assessment identifies kidney effects as a potential human hazard of tert-butanol. EPA evaluated the evidence, including the role of α2u-globulin and chronic progressive nephropathy, in accordance with EPA guidance (U.S. EPA, 1991). Please comment on whether this conclusion is scientifically supported and clearly described.”

Comment

API believes the kidney changes observed in rats following repeated exposure to TBA are associated with modes of action (α2u-globulin nephropathy and CPN) that have been characterized as rodent specific and are considered not relevant to humans. Therefore, these effects are inappropriate for characterizing potential human risk and should not be used as a basis for EPA’s noncancer risk assessment of TBA. Characterization of CPN and its relevance to humans has been discussed extensively, predominantly in publications by Hard et al. (2004; 2005; 2009; 2012; 2013). The weight of evidence supports an absence of a renal counterpart in humans. EPA acknowledges that there is no known counterpart to rat CPN in aging humans,
and that alpha-2u globulin nephropathy in male rats could exacerbate CPN and would not be considered relevant to human risk. The kidney effects of TBA identified in the Draft Review as critical effects are directly linked to these rodent specific effects. Thus, the rodent kidney effects of TBA exposure are considered inappropriate for characterizing potential human risk and should not be used as a basis for EPA’s noncancer risk assessment of TBA.

API commends the Agency for identifying kidney effects reported in rats following exposure to ETBE and its metabolite tertiary-butyl alcohol (TBA) as a key science topic for discussion during the June 2016 IRIS Public Science Meeting. API specifically endorses the written comments provided and source materials identified on this topic by Dr. Samuel Cohen following the June 2016 public meeting, which can be found in the TBA docket. These materials support the view that rat kidney effects of TBA exposure are inappropriate for characterizing potential human risk.

The relevance of kidney effects appearing in concert with rat kidney CPN is thus a key issue for consideration by the peer reviewers. In the absence of pathology distinct from that associated with CPN in the rat, API believes exacerbation of a disease process acknowledged as not occurring in humans is an inappropriate basis for human risk assessment. Acknowledging that rat CPN does not occur in humans, but then differentiating exacerbation of CPN as relevant to human health remains unsupported in the absence of specific pathology differentiating the underlying disease processes.

It is also noteworthy that the Agency states their evaluation of the rat kidney effects, including the role of α2u-globulin and chronic progressive nephropathy, was conducted in accordance with EPA guidance (U.S. EPA, 1991). The effort that has been focused on this topic in the intervening 26 year period since the 1991 EPA guidance document begs the question of whether an evaluation structured in accordance with the 1991 guidance adequately reflects the current state of the science on this topic. Attention by the peer reviewers to this fundamental question is requested.

III. Oral Reference Dose for Noncancer Outcomes

Charge Question 3c: “Section 2.1 presents an oral reference dose of 4x10⁻¹ mg/kg–day, based on increases in severity of nephropathy in female rats via drinking water (NTP, 1995). Please comment on whether this value is scientifically supported and its derivation clearly described. If an alternative data set or approach would be more appropriate, please outline how such data might be used or how the approach might be developed.”

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1 Comment submitted by Samuel M. Cohen, Department of Pathology & Microbiology, University of Nebraska Medical Center. Available at: https://www.regulations.gov/document?D=EPA-HQ-ORD-2013-0111-0039
Comment

Consistent with the previous comments that the rodent kidney effects of TBA exposure are inappropriate for characterizing potential human risk and should not be used as a basis for EPA’s noncancer risk assessment of TBA, dose-response assessment conducted for the purposes of extrapolating risks from these findings to humans is inappropriate.

IV. Inhalation Reference Concentration for Noncancer Outcomes

**Charge Question 3d:** “Section 2.2 presents an inhalation reference concentration of 5x100 mg/m3, based on increases in severity of nephropathy in female rats via drinking water (NTP, 1995), converted for inhalation exposure using a toxicokinetic model (Borghoff et al., 2016). Please comment on whether this value is scientifically supported and its derivation clearly described. If an alternative data set or approach would be more appropriate, please outline how such data might be used or the approach might be developed.”

Comment

Consistent with the comments above that the rodent kidney effects of TBA exposure are inappropriate for characterizing potential human risk and should not be used as a basis for EPA’s noncancer risk assessment of TBA, dose-response assessment conducted for the purposes of extrapolating risks from these findings to humans is inappropriate.

V. Cancer Modes-of-Action in Male Rat Kidney and Mouse Thyroid

**Charge Question 4a:**

“(i) Cancer modes-of-action in the kidney. As described in section 1.2.1, kidney tumors were observed in male rats following tert-butanol exposure, and a mode-of-action involving α2u-globulin and/or chronic progressive nephropathy was evaluated. The analysis, conducted in accordance with EPA’s guidance on renal toxicity and neoplasia in the male rat (U.S. EPA, 1991), considered the kidney tumors in male rats to be relevant to human hazard identification. Please comment on whether this conclusion is scientifically supported.”

Comments

Renal tubule cell adenomas elevated in TBA-exposed male rats in the NTP (1995) bioassay are not relevant to humans.

*TBA-Associated Male-Rat Kidney Tumors Were Due At least in Part to a Recognized*
American Petroleum Institute

Comments on Draft Toxicological Review of TBA

alpha2u-globulin Mode of Action Specific to Male Rats.

In the NTP (1995) study, male and female F344 rats and B6C3F1 mice were administered TBA via drinking water for 103 weeks. Survival was significantly decreased in male and females rats at the highest dose levels. The final mean body weight of high dose males and females was significantly lower than controls. The body weights of other treated groups were similar to controls. Hyperactivity in high dose female rats was the clinical sign reported. A few minor or sporadic changes were observed in hematology and urinalysis; however, these were not considered to be treatment related. Renal tubule hyperplasia and mineralization was observed with dose-dependence in treated males although not all observations reached significance. Nephropathy was observed with significantly increased severity in all dosed females and in males at the highest dose. Significant neoplastic observations were limited to increased incidence of renal tubule cell adenomas in male rats, and also to significantly decreased incidence in female mammary gland tumors and in male pancreatic islet cell tumors. Significantly increased renal tumors were not observed in the treated female rats.

NTP (1995) concluded that based on the histopathology of the kidney, alpha2u-globulin accumulation was likely a factor contributing to development of TBA-associated renal tubule cell hyperplasia and tumorigenesis in male rats. Because both male and female rats also displayed a dose-related increase in CPN, NTP (1995) concluded that alpha2u-globulin accumulation could not account entirely for the observed TBA-induced exacerbation of CPN. NTP thus concluded that TBA adversely affects kidney tissue of rats exposed experimentally for prolonged periods, and does so with greater potency in male rats involving some forms of pathology observed to occur exclusively in male rats.

A male-rat-specific alpha2u-globulin-mediated carcinogenic MOA for certain xenobiotic chemicals (including nongenotoxic hydrocarbons such as d-limonene, unleaded gasoline, and 2,4,4-trimethylpentane) has long been well recognized to involve chemically induced renal neoplasms in male rats resulting from chemical binding to alpha2u-globulin (one of the most abundant low molecular-weight proteins synthesized in male rat liver), lysosomal accumulation of this bound protein (forming “hyaline droplets” that exhibit considerably enhanced visualization after Mallory-Heidenhain staining), consequent nephropathy and cell death, and compensatory cell proliferation as components of a carcinogenic MOA widely recognized as not being relevant to predicting cancer risk to humans (U.S. EPA 1991; Swenberg 1993; Hard et al. 1999; IARC 1999; Doi et al. 2007).

Histopathology evidence supports the current scientific consensus that this male-rat-specific MOA is at least the predominant cause of renal tubule tumors in male rats.
exposed chronically to TBA, which is nongenotoxic (as discussed later in this section), and which selectively binds to alpha2u-globulin and induces kidney proximal tubule tumors only in male rats by the recognized alpha2u-globulin MOA (NTP 1995; Borghoff et al. 2001; McGregor and Hard 2001; Williams and Borghoff 2001; NSF 2003; API 2005; McGregor 2010; Hard et al. 2011).

Specifically, in male but not female rats exposed to TBA, protein droplets accumulate in kidney tubules (Borghoff et al. 2001). TBA exposure is also associated with accumulation of angular protein-droplet precursors of outer-medulla granular casts and linear papillary mineralization that are typical of alpha2u-globulin nephropathy in treated male rats (Hard et al. 2011). Among male F344 rats exposed subchronically to 0, 0.25, 0.5, 1, 2, or 4% (w/v) TBA administered in drinking water for 13 weeks, there were large reductions in urine volume in the three highest dose groups with crystaluria occurring in the two highest dose groups. Nearly all exposed rats had increased bile acids, and PCNA-stained S-phase nuclei (indicative of cell replication) in renal sections were significantly increased in male rats in the 1% and 2% TBA exposure groups (Lindamood III et al. 1992). In another study in which the same concentrations of TBA in drinking water were administered to male F344 rats for 90 days, hyaline droplets accumulated in renal proximal tubules with crystalline, rectangular, and rhomboid forms of the protein evident (Takahashi et al. 1993). In that study, associated nephropathy increased in a dose-related pattern, except (evidently due to increased mortality) in the 4% dose group. Replicative DNA synthesis also increased in proximal tubules of rats dosed with 2% TBA as measured by immunohistochemical staining for proliferating cell nuclear antigen.

In vivo interaction of relatively nonvolatile TBA with alpha2u-globulin was observed in experiments in which male and female F344 rats were given a single gavage 14C-radiolabeled TBA dose of 500 mg/kg (Williams and Borghoff 2001). Concentrations of radiolabel found in renal cytosol 12 hours after dosing were higher in males than in females, and both gel filtration and ion-exchange chromatography showed that radiolabel detected in male rat renal cytosol co-eluted with alpha2u-globulin (Williams and Borghoff 2001). The radiolabel in renal cytosol of rats dosed with radiolabeled TBA could be displaced from the low-molecular-weight protein fraction of male, but not female, rat kidney by d-limonene oxide (Williams and Borghoff 2001), which has a particularly high affinity to alpha2u-globulin (Lehman-McKeeman et al. 1989).

TBA was clearly associated with dose-related, statistically significant increases in BrdU measures of mitotic DNA-synthesis labeling index in epithelial cells of the renal cortex of male but not female rats exposed to TBA in air for 10 days at concentrations of 250–1,750 ppm (Borghoff et al. 2001).
**TBA-Associated Male-Rat Kidney Tumors Were Exacerbated by a Chronic Progressive Nephropathy (CPN) Mode of Action that is Specific to Rats.**

In the two-year NTP drinking-water study of TBA, renal hyperplasia was observed in 14/50, 20/50, 17/50, and 25/50 male rats of the 0, 1.25, 2.5, and 5.0 mg/mL drinking-water dose groups, respectively (p < 0.01), while renal tubule hyperplasia developed in only a single rat in the highest dose group among females administered up to 10 mg/mL of TBA in water (Cirvello et al. 1995; NTP 1995). As a subsidiary contributing nongenotoxic MOA, chronic TBA exposure may also act to elevate renal tubule tumor incidence observed in male rats by exacerbating CPN. CPN is a spontaneous age-related disease that occurs with high incidence (particularly among males) in strains of rat commonly used for subchronic and chronic toxicity studies; like the male rat alpha2u-globulin MOA, CPN has no human counterpart and is not considered relevant for purposes of characterizing human risk (McGregor 2010; Hard et al. 2011, 2013). Both alpha2u-globulin nephropathy (involving cell loss through lysosomal overload) and CPN (involving a spontaneous disease process with a high rate of cell turnover) represent secondary events and not direct forms of cytotoxicity (Hard et al. 2011). Other than these two processes that have no human counterpart, there is no evidence of TBA-induced renal cytotoxicity in either male or female rats (Hard et al. 2011). Moreover, increased rates of cytotoxicity-mediated cell turnover/proliferation *per se* are well recognized to generate an expectation of increased tumor likelihood due solely to nongenotoxic amplification of premalignant cells generated spontaneously by background rates of premalignant conversion (NRC 1994). This well-recognized nongenotoxic tumorigenic MOA cannot be ignored in a scientifically based cancer risk assessment of a nongenotoxic chemical. The argument that cytotoxicity-induced cell proliferation must have a virtually linear low-dose dose-response in view of intercellular or population variability in cytotoxic thresholds (NRC 2009) has been disproved mathematically and was shown to conflict with directly relevant experimental data (Bogen 2016).

CPN has enhanced proximal-tubule apoptosis and regenerative hyperplasia as a characteristic in common with the male-rat-specific pattern of alpha2u-globulin nephropathy that is induced together with low renal-tumor incidence by chronic exposure to TBA and certain light hydrocarbons (Hard et al. 1999). MTBE generates TBA as its major, persistent metabolite, and like TBA, MTBE induces kidney tubule tumors in exposed male but not female rats (McGregor 2006; Leavens and Borghoff 2009). Like TBA, MTBE binds to alpha2u-globulin in the kidney of male F344 rats as measured by headspace concentrations of MTBE in kidney samples from rats exposed orally to MTBE (Prescott-Mathews et al. 1999), either with or without the addition of d-limonene-1,2-oxide, a metabolite of d-limonene that is also known to bind alpha2u-globulin (Lehman-
McKeeman et al. 1989). There was also an increase in the measured alpha2u-globulin concentration in the kidneys of male F344 rats that was significant at the highest exposure concentration, 1750 ppm, and that also correlated with increased renal cell proliferation in the latter study. The measured binding affinity of MTBE to alpha2u-globulin (Poet and Borghoff 1997) is much lower than that of other known binders such as 2,4,4-trimethyl-2-pentanol (Borghoff and Lagarde 1993) and d-limonene oxide (Lehman- McKeeman et al. 1989), but is similar to that of 1,4-dichlorobenzene, a chemical that also binds alpha2u-globulin and induces associated nephropathy (Charbonneau et al. 1989). The BrdU labeling index, indicating increased rates of cell division, was increased in the renal cortex of all MTBE-treated male groups (lowest dose 400 ppm) and in the outer part of the medulla in the two highest male exposure groups of F344 rats exposed to MTBE for 10 days (Prescott- Mathews et al. 1997). After male and female F344 rats were exposed to MTBE vapors of 0, 413, 1516, or 3013 ppm for 6 hours/day for 10 consecutive days, there were significant proximal tubule necrosis and protein-droplet accumulation in the two highest exposed groups of male rats, a significantly increased amount of kidney alpha2u-globulin in male rats exposed to 3013 ppm, significantly greater cell-proliferation labeling indices in all the exposed groups of male rats, and a strong positive correlation ($r = 0.994$) between the observed exposure-related levels of enhanced kidney proximal tubular-cell proliferation and alpha2u-globulin concentration; no similar responses were observed in female rats (Prescott-Mathews et al. 1997). After exposure of F344 rats to 0, 400, 3,000, or 8,000 ppm MTBE for 5 or 28 days, increased protein accumulation was observed in Mallory-Heidenhain-stained kidney tubular epithelial cells of male rats in the two highest exposure groups, and those cells in the male (but not female) rats exhibited significantly enhanced rates of cell-proliferation labeling in the two lower exposure groups by day 5, and in all exposure groups by day 31 (Bird et al. 1997). In their corresponding two-year cancer bioassay of F344 rats exposed to MTBE concentrations up to 8,000 ppm, kidney tumors were elevated only in male rats that were exposed to 3,000 ppm MTBE in air.

McGregor (2006, 2010) concluded that evidence for carcinogenicity of TBA in rodents is unconvincing, the strongest being for an increased low-level incidence of renal tubule-cell adenoma increase by a mechanism that is specific to male rats and has no human relevance. Cruzan (2007) and Bogen and Heilman (2015) reached the same conclusion.

Melnick et al. (2012) challenged the hypothesis that enhanced CPN is an MOA for chemically induced kidney cancers with an analysis of NTP bioassay tumor data indicating that chemically exacerbated CPN is not associated with, and by extension not the cause of, renal tubular tumor induction in rats. While this study showed that the mean CPN severity did not correlate with renal tubule tumorigenicity, the Melnick et al. (2012) analysis of CPN severity and renal tubule tumors only compared aggregated
tumor data, and was not based on analysis of data pertaining to tumors in individual rats. In contrast, when data pertaining to tumors in individual rats were analyzed, CPN of advanced severity was shown to be strongly and positively associated with renal tumor development (Hard et al. 2013). A re-evaluation of archived kidney sections obtained from NTP studies in which CPN was graded from animals treated with one of 20 compounds reported that advanced stages of CPN represent a risk factor for the development of renal tumors (Hard et al. 2012). The difference in CPN severity between males and females was also observed to correlate well with tumor outcomes. Thus, severity of CPN—an endpoint relevant to rats but not humans—that was observed in studies of MTBE and TBA contributed to exposure-related elevations of renal-tumor incidence in those studies.

The Review Draft (pg. 1-36) discusses the arguments related to whether CPN-related renal tumors in rats are relevant to humans and concludes that this issue is unresolved. API encourages the peer reviewers to pay special attention to this aspect of the draft document and the information presented above with the goal of providing better clarity on this topic.

Finally, it is also noteworthy that the Agency states their evaluation of the rat kidney effects, including the role of α2u-globulin and chronic progressive nephropathy, was conducted in accordance with EPA guidance (U.S. EPA, 1991). The effort that has been focused on this topic in the intervening 26 year period since the 1991 EPA guidance document begs the question of whether an evaluation structured in accordance with the 1991 guidance adequately reflects the current state of the science on this topic. Attention by the peer reviewers to this fundamental question is requested.

“(ii) Cancer modes-of-action in the thyroid. As described in section 1.2.2, thyroid tumors were observed in male and female mice following tert-butanol exposure, and an anti-thyroid mode-of-action was evaluated. The analysis, conducted in accordance with EPA’s guidance on thyroid follicular cell tumors in rodents (U.S. EPA, 1998), found the information inadequate to determine whether an anti-thyroid mode-of-action was operating and considered the thyroid follicular cell tumors in male and female mice to be relevant to humans. Please comment on whether this conclusion is scientifically supported.”

Comments

*The Mode of Action of Putative TBA-Associated Thyroid Tumor Elevation in Female Mice is Not Applicable to Humans.*

A nongenotoxic MOA for statistically significantly increased thyroid tumors in female mice is supported strongly by a substantial body of evidence indicating that TBA is not mutagenic and does not break chromosomal DNA in vitro or in vivo (as discussed later in this section). A
nongenotoxic MOA for these tumors is also supported directly by evidence that TBA simulates thyroid follicular cell proliferation, and is thereby expected to amplify spontaneous thyroid follicular cell tumors when administered at any dose that is patently mitogenic. The incidences of thyroid follicular cell hyperplasia were significantly increased in all groups of TBA-exposed males, and in the two highest groups of TBA-exposed females (NTP 1995).

Among the nine high-dose-group female mice that exhibited thyroid follicular cell adenoma, six also exhibited thyroid follicular cell hyperplasia (McGregor 2010). Hyperplastic follicular cell lesions that occurred in the TBA-treated mice were similar in appearance to those that occurred in untreated mice (NTP 1995).

The U.S. EPA has long recognized that antithyroid activity is a common mode of thyroid carcinogenic action in rodents and that most chemicals with this activity appear to be inducers of hepatic microsomal enzymes (Hurley et al. 1998). Experiments conducted by Blanck et al. (2010) showed that 14 (but not three) days of exposure to either TBA at 20 mg/mL in drinking water (the same TBA concentrations as that used for the highest TBA exposure group of NTP bioassay B6C3F1 mice), or to an 80-mg/kg dose of phenobarbital by oral gavage, but not to TBA at 2 mg/mL in drinking water, can stimulate B6C3F1 mouse thyroid cell proliferation indirectly by significantly reducing circulating levels of triiodothyronine (T3) and thyroxine (T4) hormones that tightly control thyroid growth, and this occurs by TBA induction of P450 liver-enzyme activities known to affect circulating thyroid hormone levels in rodents. Notably, results of these experiments very clearly show that exposure to 20 mg/mL (but not 2 mg/mL) of TBA in drinking water for 14 days significantly increased total hepatic P450 by ~1.5-fold, and expression of hepatic CYP2B10 in particular (which is a CYP2B enzyme) by ~17-fold (Blanck et al. 2010).

Nongenotoxic elicitation of rodent thyroid hormone disruption can be caused when a xenobiotic chemical activates the xenobiotic-sensing constitutive androstane (CAR) receptor involving increased hepatic enzyme activities, which indirectly stimulates thyroid cell proliferation and thyroid tumors in a mechanistically based rodent tumorigenic mode of action (MOA) that is adequately documented (e.g., Wilson et al. 1996 [in view of evidence that alachlor activates the CAR receptor (Baldwin and Roling 2009)]; Qatanani et al. 2005; Barbier 2009; Maronpot et al. 2010; Rouquié et al. 2014). Among other endpoints, the CAR receptor regulates many phase I and II P450 liver (including CYP2B) enzymes that can affect the metabolism of numerous endogenous signaling molecules and hormones, including estrogen, progesterone, and thyroid hormones (Tien and Negishi 2006). As explained below, this MOA has no human relevance in view of well recognized differences in thyroid hormone physiology between rats and humans (Capen et al. 2002).

Phenobarbital is a well-studied CAR activator (Tien and Negishi 2006). CAR induction, as illustrated by phenobarbital, has been proposed as a nongenotoxic mode of action of
chemically induced rodent liver tumors (Elcombe et al. 2014). Barbiturates and chemicals that induce CYP2B are understood first to interact with the CAR receptor (Maronpot et al. 2010), induction of hepatic CYP2B gene expression and enzymes has been used as a surrogate indicator of CAR activation (Elcombe et al. 2014), and such induction is the best identified predictor of CAR activation (Oshida et al. 2015). It follows that induction of CYP2B observed by Blanck et al. (2010) in female B6C3F1 mice fed 20 mg/mL of TBA in drinking water for 14 days is virtually certain to have been associated with CAR activation. Phenobarbital also induces hepatic metabolism and excretion of thyroxine, resulting in compensatory increased TSH secretion by the pituitary, which is expected to promote thyroid tumorigenesis (McClain 1989).

Oxazepam, which also induces CYPB2 (as noted above) and the CAR receptor (Oshida et al. 2016), is another chemical carcinogen in B6C3F1 mice that induces hepatic P450 (particularly CYP2B) enzymes as well as hepatocellular and thyroid follicular cell proliferation and adenomas in those tissues (NTP 1993; Griffin et al. 1996; Parkinson et al. 2006). NTP (1993) concluded that, in addition to inducing liver tumors in B6C3F1 mice, “Increased incidences of hyperplasia of thyroid gland follicular cells in male and female B6C3F1 mice and of follicular cell adenomas in female B6C3Fl mice were also related to oxazepam exposure.”

The synthetic CAR agonist 1,4-bis(2-(3,5-dichloropyridyloxy))benzene (TCPOBOP) is a potent and highly selective mouse CYPB2 and CAR activator (Elcombe et al. 2014) and induces liver and thyroid tumors in rats (Diwan et al. 1996). Wild-type mice treated with TCPOBOP exhibited decreased serum T4 concentration, whereas treatment of Car(-/-) mice with TCPOBOP failed to elicit these changes (Maglich et al. 2004).

Doxylamine succinate (the first-generation ethanolamine-class antihistamine, Unisom) was observed to induce (by up to 2.6-fold) hepatic microsomal P450 activity as well as markedly increase (by 7- to 38-fold) CYP2B enzyme activity when fed to male and female B6C3F1 mice, and to exhibit a hepatic-enzyme-induction pattern in those mice that could not be distinguished from that of phenobarbital in a study that compared the effect of administering each compound (Bookstaff et al. 1996). IARC (2001) described doxylamine succinate as a nongenotoxic, potent phenobarbital-like inducer of CYP2B enzymes and phenobarbital-like inducer of thyroxine glucuronosyltransferase activity in B6C3F1 mice, which increased both hepatocellular and thyroid follicular cell adenomas in both sexes of these mice (though not in a dose-related manner for male thyroid tumors) after chronic lifetime dietary exposure to this chemical. IARC (2001, p. 156) concluded that “Doxylamine induces thyroxine glucuronidation in mice, with concomitant decreases in serum thyroxine and increases in serum thyroid stimulating hormone concentrations” and that “this is the probable mechanism of action for the induction of thyroid tumours in animals.”

The shift in homeostasis caused in rodents by chronic reduction in thyroid hormones from induced hepatic P450 metabolism does not occur in humans, due to a longer (5- to 10-fold
greater) circulating half-life of T4 in humans and primates mediated by binding to thyroid binding globulin that is absent in rodents (Capen et al. 2002).

API encourages the peer reviewers to carefully consider that substantial amount of experimental evidence, summarized above, that clearly is relevant to and that directly and specifically supports a hormone-disruption-related MOA for elevated thyroid tumors observed in female B6C3F1 mice exposed chronically to 20 mg/mL TBA in drinking water. Such evidence includes, for example, experimental results indicating that the CAR receptor was virtually certainly activated in female B6C3F1 mice that chronically ingest 20 mg/mL TBA, but not in those exposed to a drinking water concentration of ~2 mg/mL TBA. The Draft Review fails to acknowledge how CYP2B-induced reduction of T3 and T4 is very clearly expected to induce thyroid cell proliferation in rodents and thus (in a nongenotoxic way) promote or amplify the spontaneous incidence of rodent thyroid tumors. Nor does it acknowledge how this hormone-disruption-related MOA for rodent thyroid tumors is not expected to be relevant to humans, due to well understood species differences in thyroid hormone physiology.

**TBA Genotoxicity is not a Plausible Basis for Human Relevance of Mouse Thyroid Follicular Cell or Rat Kidney Tumors.**

In its evaluation of TBA genotoxicity, the Draft Review indicates that DNA adducts in male Kunming mice (Yuan et al. 2007) and DNA damage in human HL-60 leukemia cells (Tang et al. 1997) have been observed, and that, overall, the mode(s) of carcinogenic action for tert-butanol in the kidney and the thyroid are not known, and these tumor data are considered relevant to humans. In another study by Sgambato et al. (2009), an initial increase in DNA damage was observed as measured by nuclear fragmentation, but the damage declined drastically following 4 hours of exposure and disappeared entirely after 12 hours of exposure to tert-butanol.

Genotoxicity and mutagenicity studies for TBA were also summarized by McGregor (2006, 2010), Cruzan (2007), and Bogen and Heilman (2015), all of whom concluded that, overall, an adequate set of available studies supports the conclusion that TBA is consistently nongenotoxic. As summarized below and in Table 1 appended to these comments, results of those assays that were positive either were not confirmed in independent studies or possessed flaws that preclude meaningful interpretation of the assays (McGregor 2010; Bogen and Heilman 2015).

In an assay of reverse mutation in the Salmonella typhimurium strain TA102, TBA treatment resulted in a positive response (Williams-Hill et al. 1999); however, in two additional studies conducted under GLP standards, no significant increases in mutation frequency were observed using either DMSO or water as vehicles in the same strain (McGregor et al. 2005).

Additional assays of reverse mutation in other strains of Salmonella typhimurium are also negative (Zeiger et al. 1987). EG&G Mason (1981a) observed a positive result with and without
metabolic activation in TA1535; however, Zeiger et al. (1987) failed to confirm this, and further studies with the TA1535-derived and more sensitive strain TA100 were also negative (Zeiger et al. 1987; EG&G 1981b).

A single finding of increased mutation in mouse lymphoma L5178Y/TK cells both with and without exogenous metabolic activation was also reported (McGregor et al. 1988). In the mouse lymphoma test system, the results indicated weak mutagenicity, with a small but significant increase relative to solvent control at the highest concentrations observed both with and without metabolic activation. Based on this, the authors concluded that TBA demonstrated no evidence of mutagenicity. In agreement with the finding of no mutagenic potential for TBA, EG&G (1981c) reported, in a similar test system with and without metabolic activation, that TBA was negative for mutagenicity. A sister chromatid exchange assay of TBA conducted by NTP (1995) was reported as being weakly positive, but was not corroborated in a replicate NTP assay.

Sgambato et al. (2009) reported that DNA fragmentation was increased as measured by the single-cell gel electrophoresis (or “comet”) assay following TBA treatment of cultured (normal diploid, contact-inhibitable) Rat-1 fibroblast cells; however, neither viability nor positive-control data were included in the publication for comparison, so the authors’ conclusion that a purely genotoxic mechanism of damage occurred cannot be verified or adequately assessed. Sgambato et al. (2009) also measured DNA-adduct formation and cell growth inhibition. Evidence of TBA-induced DNA fragmentation was studied by incubating Rat-1 fibroblasts for 0.5, 4, or 12 hours in 0.84 mM (~74 ppm) MTBE or in 0.44 mM (~33 ppm) TBA, each concentration corresponding to the concentration in medium containing these respective chemicals observed in a separate study (involving exposures to concentrations of 0–100 mM) to inhibit growth by 50% inhibition during 48 hours of incubation as measured using the colorimetric MTT assay of cell viability. Similar reductions in cell viability were also observed after only 24 hours of incubation in 44 mM TBA (Sgambato et al. 2009, Figure 1). TBA- exposure associated DNA adducts (measured as an 8-OHdG marker of oxidative DNA damage) were observed to be elevated significantly after 4 hours of exposure to 0.44 mM TBA. A related study (Iavicoli et al. 2002) done by the same (latter) group of investigators determined that MTBE concentrations of 0.084 and 0.84 mM during exponential growth reduced cell survival to ~75% and ~40%, respectively, of that in control cells. At these MTBE concentrations, the corresponding percent of cells undergoing apoptosis was increased ~1.8- and 11-fold above control levels, respectively (Iavicoli et al. 2002, Table 1). Likewise, Sgambato et al. (2009) showed that incubation in 0.44 mM TBA induced not only reduced cell viability, but also measurable programmed (apoptotic) cell death, noting that “we observed a comparable progressive increase in the subdiploid peak indicative of apoptotic cells, which increased from approximately 1.3% to approximately 18.7 and 12.2% for MTBE and TBA, respectively, after 48 h of treatment.”
The report by Tang et al. (1997) on DNA fragmentation in (neoplastic, chromosomally abnormal) human acute myelogenous leukemia (HL-60) cells exposed to TBA, MTBE, or hydroxisobutyric acid (HIBA) detected using the alkaline comet (single-cell gel electrophoresis) assay is a Chinese-language paper that was published in English only in abstract form. The original paper indicates that exposure to these chemicals at concentrations of 1, 5, 10, and 30 mmol/L (i.e., mM) for one hour induced DNA fragmentation in a dose-dependent fashion, and the corresponding English abstract “suggested” that detected DNA damage occurred but that “no cytotoxic effect by MTBE, TBA and HIBA was observed.” Thus, while Sgambato et al. (2009) reported that incubation in 0.44 mM TBA for 48 hours corresponded to 50% cell inhibition in Rat-1 fibroblasts with accompanying apoptosis, Tang et al. (1997) claimed that incubation for only 1 hour in TBA concentrations of up to 30 mM were not cytotoxic to HL-60 cells. That is, while Tang et al. (1997) reported they observed no cytotoxicity after they incubated their cells at maximum time-integrated TBA concentration of 30 mM, Sgambato et al. (2009) detected cytotoxicity and apoptosis after they incubated their cells at maximum time-integrated TBA concentration of only 0.44 mM, which represents a ~2.8-fold lower integrated TBA exposure and a ~68-fold lower maximum TBA concentration than those applied by Tang et al. (1997). This discrepancy concerning reported TBA-induced cytotoxicity is very difficult to reconcile. The absence of TBA-associated cytotoxicity claimed by Tang et al. (1997) would appear implausible, particularly in view of the similarity in growth kinetics and DNA-fragmentation response of HL-60 and Rat-1 cells (Wolf et al. 2005), and the similar (generally within 5-fold) concentration-wise sensitivity of HL-60 and normal human diploid TIG-3 cells to cytotoxicity induced by exposure to cisplatin and to each of 12 different sesquiterpene compounds (Matsuo et al. 2014). It is possible that the 1-hour exposure duration applied by Tang et al. (1997) was insufficient in duration for TBA-induced cytotoxicity to be exhibited in HL-60 or in other cell types had some others also been tested. The original Tang et al. (1997) Chinese publication also indicates that study applied a lactate dehydrogenase (LDH) assay to measure induced cytotoxicity, which differs from the MTT viability assay used by Iavicoli et al. (2002) and Sgambato et al. (2009). It has become recognized that in certain conditions the LDH assay can fail to detect cytotoxicity that can be detected by the MTT assay, dose-dependent growth inhibition can act as a confounding variable that causes the traditional LDH cytotoxicity assay to underestimate dead cells substantially, and that, ideally, assay combinations should be applied to obtain unbiased measures of induced cell death (Fotakis and Timbrell 2006; Galluzzi et al. 2009; Smith et al. 2011).

Apoptosis involves DNA fragmentation that also is detected by the alkaline comet assay, and apoptotic or necrotic fragmentation of cellular DNA is difficult to distinguish definitively from DNA fragmentation that may be induced directly by chemical exposure, unless careful measures are taken at multiple time points to make such distinctions unambiguously (Brink 2006, 2007). Careful measures of cell viability have been recommended to safeguard against
unresolved issues concerning cytotoxicity-related comet-assay misinterpretation (EFSA 2012). Only detailed measures of viability at multiple time points can effectively distinguish DNA fragmentation due to cell death from the direct, chemically mediated DNA damage that the comet assay detects (Brink 2006, 2007). Such detailed measures of cell death have only rarely been included as part of any comet assays done to date, and none were included in any reported studies of comet assays applied to TBA, so no such TBA studies to date can be interpreted as having demonstrated TBA-induced DNA damage independent of cytotoxicity.

Yuan et al. (2007) reported the formation of TBA-induced DNA adducts concurrent with their report of MTBE adducts, but their method of adduct detection could not differentiate between true adducts and carbon metabolically incorporated through natural processes into the DNA structure. Yuan et al. (2007) reported positive findings for adduction of MTBE and (in parallel experiments) of TBA to DNA using accelerator mass spectrometry (AMS) methods. Another study reporting very similar positive results from the same research group, Du et al. (2005), also suffers from this same flaw in that it failed to verify that the formation of AMS-detected radioisotope was the result of direct covalent binding of an MTBE metabolite to DNA.

TBA can be metabolized in part to acetone (Bogen and Heilman 2015), which in turn in rodents and other mammals can be metabolized further into lactate and pyruvate participants of gluconeogenesis and glycolysis that can generate and incorporate formate (Coleman 1980; Kalapos 2003). Consequently, because carbon atoms derived from TBA metabolism are expected to be incorporated via the 1-carbon (including formate) pool into nucleotide and DNA synthesis, AMS-detection of increased 14C in DNA after rodent exposure to [14C]TBA does not necessarily imply direct, potentially mutagenic DNA-adduction of [14C]TBA or any of its reactive metabolites. That is, the AMS technique used and its corresponding method of application were and remain incapable of determining whether radiolabeled DNA detected is the result of adduct formation, or simply the result of endogenous carbon metabolism and recycling resulting in the incorporation of labeled carbon into nucleotide pools used to synthesize and/or repair DNA via processes that are unrelated to adduct formation (Wang et al. 2004; McGregor 2010; Zhu et al. 2010). Only isolation and chemical characterization of TBA-DNA adducts can unambiguously demonstrate that TBA-exposure-induced TBA adducts of DNA have been generated, and no such demonstration was provided by Yuan et al. (2007).

Finally, it is also noteworthy that the Agency states their evaluation of the mouse thyroid tumor effects was conducted in accordance with guidance on thyroid follicular cell tumors in rodents (U.S. EPA, 1998). The effort that has been focused on this topic in the intervening 19 year period since the 1998 EPA guidance document begs the question of whether an evaluation structured in accordance with the 1998 guidance adequately reflects the current state of the science on this topic. Attention by the peer reviewers to this fundamental question is requested.
VI. Cancer Characterization

**Charge Question 4b:** “As described in sections 1.2.1, 1.2.2, and 1.3.2, and in accordance with EPA’s cancer guidelines (U.S. EPA, 2005), the draft assessment concludes that there is suggestive evidence of carcinogenic potential for tert-butanol, based on thyroid follicular cell tumors in male and female B6C3F1 mice via drinking water and on renal tubule tumors in male F344 rats via drinking water. Please comment on whether this cancer descriptor is scientifically supported. If another cancer descriptor should be selected, please outline how it might be supported.”

**Comments**

An observed marginal elevation in female thyroid follicular cell tumors in TBA-exposed mice is of questionable statistical significance (see discussion below), and to the extent that elevation is real it is clearly consistent with an established nongenotoxic, CAR-activation MOA involving indirectly (metabolically) mediated thyroid effects that are well recognized to be irrelevant to humans and that do not contribute to human thyroid follicular cell cancer (see response to Charge Question 4a above). The only other tumor type known to be significantly elevated after chronic exposure to TBA (male rat proximal renal tubule tumors associated with enhanced CPN and alpha2u-globulin accumulation) involves two MOAs that are recognized as irrelevant to humans (see response to Charge Question 4a above). Consequently, the existing set of cancer bioassay data for TBA do not provide evidence suggestive of potential human carcinogenicity.

*The TBA-associated elevation observed in high-dose females is not statistically significant if an appropriate 2-tail test is applied; consequently, any conclusion of thyroid tumorigenicity must be deemed ambiguous.*

Data concerning the chronic toxicity or carcinogenicity of TBA are available primarily from a 2-year drinking water study conducted by the NTP (1995) also published as Cirvello et al. (1995). In this study, combined thyroid follicular cell adenoma and carcinoma were marginally increased in male mice in the middle dose group only, which was considered to indicate “equivocal evidence of carcinogenic activity in male mice” (NTP 1995, p. 57). The incidence rate of follicular cell adenomas of the thyroid was 2/58, 3/60, 2/59, and 9/59 in female mice exposed to 0, 5, 10, or 20 mg/mL, respectively, corresponding to approximate lifetime time-weighted average doses of 0, 510, 1,020, or 2,110 mg/kg/day (where the response for the high-dose group includes one bilateral tumor). The latter set of tumor-response data were reported as being significantly increased in the three exposed groups compared to that in controls by a “logistic” trend test \( p = 0.011 \), and significantly increased in the highest exposure group compared to that in the controls and by an unspecified “pairwise comparison” \( p = 0.039 \) (NTP 1995, Table 14, p. 53). As reported by NTP (1995), “In 20 mg/mL females, the incidence of follicular cell adenoma was significantly greater than that of the controls and occurred
bilaterally in one female in this exposure group” and “incidences of follicular cell hyperplasia were significantly increased in all groups of exposed males and in 10 and 20 mg/mL females.” Cirvello et al. (1995) also indicated that they had used Fisher exact tests to assess tumor incidence data, and that “incidence of follicular cell adenoma in 20-mg/mL female mice was statistically significant [p < 0.05] and the rate of 15% exceeds the maximum rate of 5% observed in controls groups from previous NTP drinking water studies.”

The application of a one-tailed test makes sense only under the condition that variation is logically or physically constrained to apply only in a single direction, rather than in both directions, about an estimated effect. Otherwise, according to Fleiss (1981), if a scientific investigator “intends to report the results to professional colleagues, he is ethically bound to perform a two-tailed test.” In their comprehensive assessment of this subject, Lombardi and Hurlbert (2009) concluded, “One-tailed tests rarely should be used for basic or applied research in ecology, animal behaviour or any other science.” The U.S. Environmental Protection Agency (EPA) recently recommended that “Two-sided intervals are commonly encountered in general scientific use and are appropriate when the overall uncertainty of an estimate needs to be characterized” (U.S. EPA 2012).

The p-value of 0.011 for trend for the pattern of thyroid adenoma incidence reported by NTP (1995) and that of p < 0.05 implied by Cirvello et al. (1995) are similar to the p-value of 0.0101 that is obtained for positive linear trend using a 2-tail exact Cochran-Armitage trend test, e.g., implemented using Mathematica 10.4 (Armitage 1955; Wolfram Research 2016).

However, an extended Fisher exact test of tumor-incidence contingency-table homogeneity (Baglivo et al. 1988) indicates the set of four observed response rates for female thyroid follicular cell adenomas are statistically homogeneous (p = 0.0600), and a 2-tail exact Cochran-Armitage trend test performed after excluding the high-dose group of female mice yields a non-significant negative trend in relation to administered concentration (p = 0.816). Consequently, except for the fact that incidence in the high-dose group in this study exceeded that of historical control mice for this endpoint, any interpretation that a significant positive linear trend occurred for this endpoint depends entirely on the assignment of relative exposure levels used for trend analysis. The administered water concentrations of TBA may not realistically reflect the corresponding biologically relevant internal exposure levels achieved in each exposure group (e.g., maximum blood and tissue concentrations of TBA that may best predict associated cytotoxicity), given the high likelihood that TBA has a nongenotoxic tumorigenic mode of action (MOA) as discussed in Section 3.

As noted by Bogen and Heilman (2015), the value “P = 0.028” reported in NTP (1995, Table D3) for a Fisher exact comparison of the incidence of follicular cell adenomas of the thyroid in control (2/58) vs. high-dose (9/59) female mice represents a 1-tail statistical test result (the more exact value is p = 0.0285), whereas the corresponding 2-tail chi-square and Fisher exact
tests comparing these proportions yields $p = 0.0614$ and $p = 0.0533$, respectively. Neither of the 2-tail p-values would normally be considered statistically significant. Health agencies might sometimes be assumed to analyze tumor data using a one-sided test because carcinogenicity studies are viewed as being designed to determine if an agent causes an increase in tumor incidence compared to controls. However, chemical carcinogens can significantly increase tumor incidence, or significantly decrease tumor incidence, or do both for different tumor types observed to be affected significantly in the same study. A rather stark illustration of this fact is provided in the case of male and female F344/N rats exposed to anthraquinone, which in each of three different anthraquinone-exposure groups studied per sex effectively eliminated the substantial background rates of mononuclear cell leukemia in non-exposed rats (Bogen 2011).

In the case of tumors examined in the NTP (1995) bioassay involving mice exposed chronically to TBA in drinking water (as listed in Tables D3 and D4), pairwise high-dose/control comparisons or trend tests conducted indicate negative trends for the following tumor types for female B6C3F1 mice (as listed in Table D3), each of which is statistically significant (as listed in Table D3 using a 1-tail p-value $\leq 0.025$ significance criterion, as a convenient approximation for corresponding 2-tail p-values as applicable):

- hepatocellular adenoma;
- hepatocellular adenoma or carcinoma; and
- all malignant neoplasms.

Applying the same approach identifies the following tumor types with significant negative trends identified for male B6C3F1 mice (as listed in Table D4):

- Hardarian gland adenoma;
- Hardarian gland adenoma or carcinoma;
- liver hepatocellular adenoma or carcinoma; and
- lung alveolar/bronchiolar adenoma.

Consequently, as for most if not all NTP cancer bioassays, there is no empirical basis for assuming that the NTP (1995) bioassay design was inherently limited in ability to identify only significant elevations in tumor incidence. Indeed, the NTP (1995) bioassay for mice that chronically ingested TBA in drinking water showed that TBA exposure reduced the incidence of at least as many tissue-specific tumor types as it elevated in female mice, and reduced the incidence of more tissue-specific tumor types than it elevated in male mice. Consequently, where applicable, only results of two-tailed tests of statistical significance are scientifically appropriate to use as a basis for determining the relevance (including potential human relevance) of tumor incidence associations with TBA exposure in the NTP (1995) study. Under this constraint, the evidence that TBA exposure induced thyroid follicular cell tumors in female mice in that study is marginal and ambiguous at best. Only the overall tumor incidence trend
was statistically significant, and neither the heterogeneity of response rates in all four test groups nor the response in any (including the highest) TBA-exposure group compared with that in the control female mice, notwithstanding the greater incidence of this tumor type in historical control female BC3F1 mice.

The same conclusion is reached if instead of raw thyroid follicular cell adenoma incidence data for the female mice, incidence data adjusted for intercurrent mortality are examined (i.e., 2/47, 3/47, 2/51, 9/53 for the control through highest dose groups, respectively, based on life table data listed in NTP 1995, Table D2, as using 646 days as the day of first tumor occurrence for this tumor type in female mice): p = 0.0808 for homogeneity by extended Fisher exact test, p = 0.0562 for high-dose vs. control response comparison by Fisher exact test, and p = 0.0175 by exact Cochran-Armitage trend test. It follows that the unqualified claim on page 1-27 of the Draft Review that there was “a statistically significant increase in follicular cell adenomas in the high-dose group” of female mice in the NTP (1995) study is misleading.

This unqualified claim is not based on applying an appropriate, scientifically objective criterion to define statistical significance in this context. If an appropriate pairwise 2-tail Fisher exact test is applied, the incidence of thyroid follicular cell adenomas in high-dose female mice in the NTP (1995) study must be interpreted as exhibiting no statistically significant difference from that which occurred in female control mice.

VII. Cancer Toxicity Values

Charge question 4c: “Section 3 of EPA’s cancer guidelines (2005) states:

When there is suggestive evidence, the Agency generally would not attempt a dose-response assessment, as the data generally would not support one, however, when the evidence includes a well-conducted study, quantitative analyses may be useful for some purposes, for example, providing a sense of the magnitude and uncertainty of potential risks, ranking potential hazards, or setting research priorities. In each case, the rationale for the quantitative analysis is explained, considering the uncertainty in the data and the suggestive nature of the weight of evidence.

Please comment on whether Section 2.3 of the draft assessment adequately explains the rationale for quantitative analysis, and whether the NTP (1995) study is suitable for this purpose.”

Comment

The Agency’s 2005 cancer guidelines (pg. 3-2) also states that such analyses, based on suggestive evidence, “generally would not be considered Agency consensus estimates” and that
“Dose-response assessments are generally not done when there is inadequate evidence” of carcinogenic potential. These qualifiers are appropriate in the case of TBA, and argue against the approach taken in the Draft Review.

Consistent with the previous arguments that TBA-associated tumors observed in the NTP (1995) study are not relevant to humans, dose-response assessments conducted for the purposes of extrapolating risks from these findings to humans is inappropriate.

VIII. Oral slope factor for cancer

Charge Question 4d: “Section 2.3 presents an oral slope factor of 5 x 10⁻⁴ per mg/kg–day, based on thyroid tumors in male or female mice via drinking water (NTP, 1995). Please comment on whether this value is scientifically supported and its derivation clearly described. If an alternative approach would be more appropriate, please outline how it might be developed.”

Comment

Consistent with the previous arguments that TBA-associated tumors observed in the NTP (1995) study are not relevant to humans, dose-response assessments conducted for the purposes of extrapolating risks from these findings to humans is inappropriate.

IX. Inhalation unit risk for cancer

Charge Question 4e: “Section 2.4 presents no inhalation unit risk. The lack of a toxicokinetic model for mice precluded the use of the oral thyroid tumor data, and the inability to determine the relative contribution of α₂u-globulin nephropathy and other processes precluded the use of the oral renal tumor data from male rats. If an alternative approach would yield an inhalation unit risk estimate, please outline how it might be developed.”

Comment

Consistent with the previous arguments that TBA-associated tumors observed in the NTP (1995) study are not relevant to humans, dose-response assessments conducted for the purposes of extrapolating risks from these findings to humans is inappropriate.
X. References


Borghoff SJ, Lagarde WH. Assessment of binding of 2,4,4-trimethyl-2-pentanol to low-molecular-weight proteins isolated from kidneys of male rats and humans. Toxicol


Borghoff, SJ; Ring, C; Banton, MJ; Leavens, TL. (2016). Physiologically based pharmacokinetic model for ethyl tertiary-butyl ether and tertiary-butyl alcohol in rats: Contribution of binding to α2u-globulin in male rats and high-exposure nonlinear kinetics to toxicity and cancer outcomes. J Appl Toxicol. 37(5): 621-640.


Diwan BA, Henneman JR, Rice JM, Nims RW. Enhancement of thyroid and hepatocarcinogenesis by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene in rats at doses that cause maximal induction of CYP2B. Carcinogenesis 1996; 17(1):37–43.


Fotakis G, Timbrell JA. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett
2006; 160:171–177.


Oshida K, Waxman DJ, Corton JC. Chemical and hormonal effects on STAT5b-dependent


Takahashi K, Lindamood C III, Maronpot RR. Retrospective study of possible alpha-2 mu-globulin nephropathy and associated cell proliferation in male Fischer 344 rats dosed with t-


### Table 1

#### Table 1. Genotoxicity studies of TBA

<table>
<thead>
<tr>
<th>Reference</th>
<th>Assay</th>
<th>Test system</th>
<th>With exogenous metabolic activation</th>
<th>Without exogenous metabolic activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG&amp;G Mason 1981a</td>
<td>Reverse mutation</td>
<td>S. typhimurium, TA98, TA100, TA1537 and TA1538</td>
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<td>–</td>
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<tr>
<td>EG&amp;G Mason 1981b</td>
<td>Reverse mutation</td>
<td>S. typhimurium, TA1535</td>
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<td>+/-</td>
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<tr>
<td>EG&amp;G Mason 1981c</td>
<td>Gene mutation</td>
<td>Mouse lymphoma L5178Y cells, tk locus in vitro</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>McGregor et al. 1988</td>
<td>Mutation</td>
<td>Rats F344/N metabolic activation, L5178Y tk⁺/tk⁻ lymphoma cell line</td>
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<tr>
<td>McGregor et al. 1988</td>
<td>Mutation</td>
<td>Rats F344/N metabolic activation, L5178Y tk⁺/tk⁻ lymphoma cell line</td>
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<td>–</td>
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<tr>
<td>McGregor et al. 1988</td>
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<td>Mouse lymphoma L5178Y cells, tk locus in vitro</td>
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<td>–</td>
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<td>McGregor et al. 2005</td>
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<td>S. typhimurium, TA102</td>
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<td>Williams-Hill et al. 1999</td>
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<td>Zeiger et al. 1987</td>
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#### DNA Adducts, Fragmentation, or Oxidation

<table>
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<tr>
<th>Reference</th>
<th>Assay</th>
<th>Test system</th>
<th>With exogenous metabolic activation</th>
<th>Without exogenous metabolic activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sgambato et al. 2009</td>
<td>Comet assay for DNA strand breaks; DNA oxidative damage</td>
<td>C3H/10/Tg/C1/8 cells</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>Tang et al. 1997</td>
<td>Comet assay for DNA strand breaks</td>
<td>Human HL-60 leukemia cells in vitro</td>
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<td>+</td>
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<tr>
<td>Yuan et al. 2007</td>
<td>AMS analysis of DNA radiolabel</td>
<td>Mice (Kunming, male); liver, lung, kidney tissues 6 h after 1⁴C-MTBE dose by gavage</td>
<td>ND</td>
<td>**+</td>
</tr>
<tr>
<td>Reference</td>
<td>Assay</td>
<td>Test system</td>
<td>Results with exogenous metabolic activation</td>
<td>Results without exogenous metabolic activation</td>
</tr>
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<tr>
<td>EG&amp;G Mason 1981d</td>
<td>Sister chromatid exchange</td>
<td>Hamster/Chinese ovary cells</td>
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<td>+/-</td>
</tr>
<tr>
<td>NTP 1995</td>
<td>Sister chromatid exchange</td>
<td>Chinese hamster ovary 95% MTBE</td>
<td>—</td>
<td>[+]</td>
</tr>
<tr>
<td>NTP 1995</td>
<td>Sister chromatid exchange</td>
<td>Chinese hamster ovary 95% MTBE</td>
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<td>NTP 1995</td>
<td>Micronucleus test</td>
<td>Peripheral blood of male and female mice following 13 wk treatment via drinking water</td>
<td>ND</td>
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</tr>
</tbody>
</table>

* Table A-3 reprinted from Bogen and Heilman (2015), Supplemental Online Materials, Appendices A and E, available at: http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1052367. Studies reported in NSF (2003) Oral Risk Assessment, updated with new studies via literature search through 2012, and from McGregor (2005, 2010). Primary publications were not obtained for all studies, only studies for which additional information was required to appropriately represent the study herein. Positive results in brackets indicate only negative results occurred in a repeat study. Comet assay = single cell gel electrophoresis (SCGE) assay; AMS = accelerator mass spectrometry without gel-electrophoresis confirmation of purported DNA adducts. Brackets indicate study results were all negative when repeated by different investigators (see text).

* A lower purity sample gave a significant response in the absence of metabolic activation.

** Exogenous metabolic system not specified so none was assumed, or metabolic system was not appropriate for the specific test system, or AMS analysis without corresponding verification of purported adduct(s).