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Re: *Tertiary*-Butyl Acetate Inhalation Cancer Unit Risk Factor
Technical Support Document for Cancer Potency Factors, Appendix B
Prepared by Office of Environmental Health Hazard Assessment (OEHHA)
Public Review Draft, August 2015

Dear Dr. Budroe:

Lyondell Chemical Company, a LyondellBasell Company, appreciates the opportunity to provide comments on California's Office of Environmental Health Hazard Assessment August 2015 draft Technical Support Document (TSD) for *tertiary*-butyl acetate (TBAc). LyondellBasell, as a producer of TBAc, has a significant interest in any review of TBAc's risks by an authoritative body. We are committed to accurate and up-to-date scientific information and assessments of our products and hence we have reviewed and provide comments on the draft TBAc TSD assessment to assure that the best assessment possible is achieved.

The present draft TBAc TSD assessment, in our opinion, draws erroneous conclusions regarding TBAc's potential for human health hazards and risks. Our comments focus primarily on why OEHHA reliance on *tertiary*-butanol (TBA)-induced male rat kidney tumors for the URF derivation is not justified on the basis of mode of action (MoA) considerations.

The draft Technical Support Document for *tertiary*-butyl acetate has proposed an inhalation cancer Unit Risk Factor (URF) of 1.9×10^{-6} $(\mu g/m^3)^{-1}$ based on the observation of kidney tumors in male Fischer 344 rats treated with *tertiary*-butanol. The potential carcinogenicity of TBAc has not been examined in rodent bioassays, and thus OEHHA's consideration of rat and mouse carcinogenicity findings of TBA is appropriate given the well demonstrated rapid and extensive metabolism of TBAc

to TBA. However, OEHHA's selection of and reliance on TBA-induced male rat kidney tumors as the primary basis for derivation of the URF for TBAc is not justified given robust MoA data demonstrating that this tumor response is mediated through the non-human-relevant $\alpha 2_u$-globulin MoA that is further augmented through the likewise non-human-relevant MoA of chemically-induced accentuation of rat chronic progressive nephropathy.

Although not selected by OEHHA as the basis for the URF derivation, potential use of TBA-induced mouse thyroid tumors also would not be justified based on MoA information suggesting a quantitative and/or qualitative lack of human relevance for this tumor response. Equally, or even more importantly, it is highly likely that dose-constraining inhalation toxicity of TBAc in mice would limit formation of TBA to a systemic dose that is below that identified as thyroid-tumorigenic in mice. Thus, if TBAc were to be tested at a Maximum Tolerated Dose (MTD) in a chronic mouse bioassay, it is highly unlikely it would be identified as a thyroid tumorigen.

Furthermore, an overall weight-of-evidence evaluation of the genotoxicity toxicity of TBAc and its metabolic surrogates conclusively indicate TBAc and TBA are not genotoxic. Expert judgment calls for primary reliance on well-conducted and reported tests of apical genotoxic endpoints. OEHHA TSD guidance calls for a weight-of-evidence evaluation of the "number and quality" and "methodological issues" as these are considered in the evaluation of cancer MoAs. It is clear that apical endpoints of genotoxicity (mutations and chromosomal damage) are completely negative in high-quality test systems. Positive results reported from some non-apical tests for DNA damage (adducts; Comet assays) suffer from major methodological deficiencies that significantly limit their interpretability and utility in demonstrating TBAc or TBA genotoxicity.

Because TBAc and TBA are not genotoxic and TBA-induced male rat kidney tumors are not a scientifically justified basis for derivation of a TBAc inhalation URF, an alternative approach to TBAc chronic risk assessment has been proposed based on non-cancer neurotoxicity findings. This alternative approach yielded acute and chronic TBAc reference concentrations (RfCs) of 1.5 and 0.3 ppm, respectively.

Therefore consideration of TBA's rodent tumor findings and MOA and lack of genotoxicity, the TSD assessment for TBAc should find:

1) TBAc is not a cancer concern for humans,

2) TBAc is a low concern to humans for chronic toxicity by inhalation exposure

Our conclusions are supported in our attached comments. These comments were also offered in summary form in an oral presentation to OEHHA at a Public Workshop for discussion of the draft TBAc TSD (September 14, 2015, Sacramento, CA; LyondellBasell Comments, 2015; Appendix A).

LyondellBasell appreciates your consideration of our comments. If you have any questions on our comments, please contact me to discuss.

Sincerely,

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Attachment

***Tertiary*-Butyl Acetate Inhalation Cancer Unit Risk Factor**
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Prepared by Office of Environmental Health Hazard Assessment (OEHHA)
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October 12, 2015

Summary

The draft Technical Support Document (TSD; OEHHA, 2015) for *tertiary*-butyl acetate (TBAc) has proposed an inhalation cancer Unit Risk Factor (URF) of 1.9 x 10^{-6} $(\mu g/m^3)^{-1}$ based on the observation of kidney tumors in male Fischer 344 rats treated with *tertiary*-butanol (TBA; NTP, 1995, Cirvello et al., 1995). The potential carcinogenicity of TBAc has not been examined in rodent bioassays, and thus OEHHA's consideration of rat and mouse carcinogenicity findings of TBA is appropriate given the well demonstrated rapid and extensive metabolism of TBAc to TBA (reviewed in Bus et al., 2015). However, OEHHA's selection of and reliance on TBA-induced male rat kidney tumors as the primary basis for derivation of the URF for TBAc is not justified given robust mode of action (MoA) data demonstrating that this tumor response is mediated through the non-human-relevant $\alpha 2_u$-globulin MoA (McGregor, 2010; Bus et al., 2015) that is further augmented through the likewise non-human-relevant MoA of chemically-induced accentuation of rat chronic progressive nephropathy (Hard et al., 2011).

Although not selected by OEHHA as the basis for the URF derivation, potential use of TBA-induced mouse thyroid tumors also would not be justified based on MoA information suggesting a quantitative and/or qualitative lack of human relevance for this tumor response. Equally, or even more importantly, it is highly likely that dose-constraining inhalation toxicity of TBAc in mice would limit formation of TBA to a systemic dose that is below that identified as thyroid-tumorigenic in mice (Bus et al., 2015). Thus, if TBAc were to be tested at a Maximum Tolerated Dose (MTD) in a chronic mouse bioassay, it is highly unlikely it would be identified as a thyroid tumorigen.

Because TBA-induced male rat kidney tumors are not a scientifically justified basis for derivation of a TBAc inhalation URF, an alternative approach to TBAc chronic risk assessment

has been proposed based on non-cancer neurotoxicity findings (Bus et al., 2015). This alternative approach yielded acute and chronic TBAc reference concentrations (RfCs) of 1.5 and 0.3 ppm, respectively.

The comments provided in this submission were offered in summary form in an oral presentation to OEHHA at a Public Workshop for discussion of the draft TBAc TSD (September 14, 2015, Sacramento, CA; LyondellBasell Comments, 2015; Appendix A). This submission focuses primarily on why OEHHA reliance on TBA-induced male rat kidney tumors for the URF derivation is not justified on the basis of MoA considerations.

TBA MoA data do not support selection of male rat kidney tumors as the basis for derivation of the inhalation cancer URF.

The TBAc TSD directs significant attention to an evaluation of whether the observation of TBA-induced male rat kidney tumors, used as the primary basis of the derivation of the TBAc URF, are mediated through the non-human-relevant $\alpha 2_u$-globulin MoA. The TSD concludes that the available TBA MoA data do not fulfill all of the seven criteria outlined by IARC to classify a chemical as operating by this MoA (IARC, 1999), and thus the OEHHA TBAc cancer risk assessment is defaulted to the assumption of a non-threshold genotoxic MoA.

The TBAc TSD sets out its position on the TBA cancer hazard evaluation by considering the toxicity and MoA data in the context of the International Agency for Research on Cancer (IARC) seven criteria for kidney tumor induction through an $\alpha 2_u$-globulin nephropathy MoA (IARC, 1999; Table 1). The TSD concludes TBA fits only 3 of these 7 criteria: criterion 3, induction of the characteristic sequence of histopathological changes with hyaline droplet formation is obligatory; criterion 4, immunohistochemical identification of the accumulating droplets as $\alpha 2_u$-globulin; and criterion 5, reversible binding of TBA to $\alpha 2_u$-globulin. LyondellBasell concurs with the TSD conclusions on these criteria, and thus no comments are offered on data addressing these criteria.

The TBAc TSD concludes that TBA does *not* fit two of the 7 criteria, namely: criterion 1, lack of genotoxic activity, and criterion 7, dose response relationships between end-points and tumor-associated doses. It further concludes that TBA does *not completely* fit criterion 2, male rat specificity for the TBA-induced nephropathy; and criterion 6, induction of sustained increased cell proliferation in the renal cortex. Each of these points will be considered in turn, and contrasted to the LyondellBasell position that a weight-of-evidence examination of the overall data support the conclusion that essentially all of IARC-identified criteria are indeed fulfilled (Table 1).

Table 1: Does TBA meet IARC criteria for an $\alpha 2_u$-globulin MoA?

	OEHHA	LyondellBasell View
Essential Criteria		
1. Non-genotoxicity	No	Yes
2. Male rat specificity	Not Completely	Yes
3. Characteristic histopath	Yes	Yes
4. $\alpha 2_u$-globulin identified	Yes	Yes
Additional Supporting Evidence		
5. Reversible binding to $\alpha 2_u$-globulin	Yes	Yes
6. Sustained, increased cell proliferation	Not completely	Probably
7. Dose response similarities between MoA and tumors	No	Yes

Although IARC (1999) stated that all seven criteria must be fulfilled in order to conclude that a chemical is operating by an $\alpha 2_u$-globulin MoA, the IARC Working Group paper used as the basis for these criteria differentiated the seven criteria into "essential" and "additional supporting evidence" (Swenberg and Lehman-McKeeman, 1999).

IARC Criterion # 1 – Non-genotoxicity

OEHHA guidance for evaluation of data used in derivation of cancer potency factors specifically notes that a "weight-of-evidence" approach should be employed for evaluating the body of evidence supporting a conclusion of whether a MoA is consistent with a cancer outcome (OEHHA, 2009). The TSD guidance specifically states that the "number and quality" and "methodological issues" are concerns to be addressed in toxicological studies used "in the interpretation of animal bioassay data and mechanistic studies." This TSD guidance is particularly critical to the OEHHA evaluation of TBAc genotoxicity in that a conclusion of non-genotoxicity would substantially alter the risk assessment approach.

A weight-of-evidence evaluation of the genotoxicity data summarized in the TBAc TSD results in the clear conclusion that both TBAc and its metabolic surrogate TBA are non-genotoxic. TBAc was negative in high-quality regulatory guideline and GLP-compliant studies examining bacterial (*Salmonella* typhimurium and *Escherichia* coli) reverse mutation, *in vitro* human lymphocyte chromosomal aberration, and *in vivo* rat bone marrow micronucleus assays (Table 3, OEHHA, 2015). TBA also was negative in high-quality bacterial reverse mutation, mammalian cell (L5178Y mouse lymphoma) mutation, mammalian cell (CHO) chromosomal aberration, and *in vivo* mouse micronucleus assays. The *in vivo* micronucleus tests are regarded as reliable indicators for both clastogenicity and aneugenicity.

Although not presented in the TSD, the non-genotoxicity of TBA also is consistent with the non-genotoxic profile of two other metabolic surrogates of TBA, methyl *tertiary*-butyl ether (MTBE) and ethyl *tertiary*-butyl ether (ETBE). MTBE and ETBE are extensively metabolized to TBA, and thus represent metabolic surrogates of TBAc through their common metabolism to TBA (reviewed in McGregor 2006, 2007). The negative genotoxicity profiles of both of these informative chemicals, which include the same spectrum of high-quality *in vitro* and *in vivo* regulatory guideline and GLP-compliant tests, should be included in the overall weight-of-evidence evaluation supporting a conclusion of non-genotoxicity in the TBAc TSD.

The conclusion that TBAc and TBA are not genotoxic also is consistent with several recent reviews of the data described in the TBAc TSD.

> Dr. James S. Felton, in a letter dated May 20, 2011, to George Alexeeff (Acting Director of OEHHA), stated:
>
> "…the genetic toxicology data does not support a positive overall genetic toxicology assessment for [TBAC and TBA]; …the bulk of the evaluation comes down to three studies, all of which have *major discrepancies* [emphasis added]; …This negative genetox assessment when used with the rat kidney tumor data fits an alpha-2u globulin production mechanism for cancer induction not relevant to the human."
>
> The European Food Safety Authority (2012) concluded:
>
> "The available data on *tert*-butyl acetate and on acetate and *tert*-butanol, its major metabolites, do not give rise to concerns regarding systemic toxicity, developmental toxicity or genotoxicity. Any carcinogenic risk would likely be from a non-genotoxic mode of action."
>
> and, the Toxicology Excellence in Risk Assessment (TERA, 2009) Peer Consultation review of TBAc also concluded:
>
> "…the overall weight of evidence indicates that TBAC is not likely to be genotoxic..."

The TBAc TSD weight-of-evidence conclusion that genotoxicity is a MoA underpinning potential TBAc and TBA carcinogenicity rests largely on a small number of methodologically flawed studies evaluating *in vitro* or *in vivo* effects of TBA only. Substantive concerns exist for each of the cited positive studies and are described below (discussed in the order of presentation in Table 3 of the draft TBAc TSD).

Increased DNA damage in a Comet assay performed in HL-60 cells (Tang et al., 1997) is not informative as evidence of DNA-damaging activity of TBA in that a non-standard method was used in which only the qualitative appearance or lack of appearance of a Comet tail was evaluated (reviewed in Felton, 2011). In addition, McGregor (2010) has noted that Tang et al. reported that MTBE, TBA and the MTBE/TBAc/TBA metabolite α-hydroxyisobutyric acid all resulted in the approximately the same DNA damage, even though the structures of these chemicals share little commonality as to possible reactive groups. Although McGregor

suggested that the equivalent responses might have been due to metabolic convergence to the common terminal metabolite of α-hydroxyisobutyric acid, this possibility was questionable given that the limited metabolic capacity of HL-60 cells would likely not be able to drive a quantitative metabolic conversion of MTBE or TBA to α-hydroxyisobutyric acid. In addition, the study also did not provide important study details impacting interpretation, e.g., although the effects were apparently collected from three experiments, no indication of cross-experiment variation was reported.

A second Comet assay reported in a Rat-1 cell line employed a single IC50 test concentration (Sgambato et al., 2009). This test concentration exceeded the upper limit concentration (IC30) recommended for such tests to avoid potential confounding of increased cytotoxicity (Tice et. al., 2000). The investigators themselves cautioned that interpretation of the Comet response might have been confounded by increased cell death, and that "…further studies with time- and dose-dependent curves are needed to investigate these issues." This conclusion is further reinforced by the report that only a single indicator of cytotoxicity (MTT test) was used in this assay; a combination of multiple methods are recommended to effectively assess cytotoxicity in the Comet assay (Storer et al., 1996; Speit et al., 2015).

A report of increased DNA adduct formation in various tissues of male Kumming mice treated with TBA (Yuan et al., 2007) is substantially confounded by use of an accelerated mass spectroscopy method that is prone to false positive results due to metabolic *incorporation* of radiolabel as compared to true adduction, and/or possible cross-contamination from protein adducts and other metabolites (Phillips et al., 2000; reviewed in Felton, 2015). Such incorporation cannot be ruled out given the demonstrated formation of acetone as a TBA metabolite (Baker et al., 1982; Cederbaum et al., 1983). Interpretation of Yuan et al. (2007) is further complicated in that no synthetic standards of adducted DNA bases were used, as is necessary, to confirm the hypothesized DNA adduct formation (Himmelstein et al., 2009).

Finally, observation of a mutagenic response in a non-GLP *in vitro S.* typhimurium TA102 assay (Williams-Hill et al., 1999) is not a reliable result (reviewed in McGregor, 2010; Felton, 2015). TBA induced only a very weak response in that the requirement for a positive response of a 2-fold increase in mutation incidence for this assay was barely, if at all, fulfilled. In addition, the weak effect was noted in a tester strain with high and variable background incidence of revertants. Perhaps most importantly, these findings were not replicated in two independent and GLP-compliant tests in the TA102 tester strain (McGregor, 2005). These replications further confirmed that the negative findings were not attributable to use of DMSO, an oxidative stress inhibitor, in that test results were also negative with use an aqueous vehicle. It is also important to note that the TA102 strain is specifically designed to be highly sensitive to oxidative stress (McGregor, 2005, 2010; Felton, 2015), and thus the negative findings in these studies do not support the findings of Sgambato et al. (2009) reporting increased Comet assay damage and formation of 8-OHdG DNA oxidative damage observed under highly cell toxicity treatment conditions.

An overall weight-of-evidence evaluation of the genotoxicity toxicity of TBAc and its metabolic surrogates TBA, MTBE and ETBE conclusively indicates TBAc and TBA are not genotoxic. Expert judgement calls for primary reliance on well-conducted and reported tests of apical

genotoxic endpoints. As noted earlier, such an approach is fully consistent with OEHHA TSD guidance (OEHHA, 2009) calling for a weight-of-evidence evaluation of the "number and quality" and "methodological issues" as these are considered in the evaluation of cancer MoAs. As reviewed above, it is clear that apical endpoints of genotoxicity (mutations and chromosomal damage) are completely negative in high-quality test systems. Positive results reported from some non-apical tests for DNA damage (adducts; Comet assays) suffer from major methodological deficiencies that significantly limit their interpretability and utility in demonstrating TBAc or TBA genotoxicity.

Thus, an overall weight-of-evidence review of TBAc and its metabolic surrogates TBA, MTBE and ETBE conclusively demonstrate that Criterion #1 is fulfilled without qualification.

IARC Criterion # 2 – Male rat specificity of renal toxicity and tumorigenicity

Before reviewing the details of data supporting male rat-specificity, it must be noted that TBA is, at most, a very weak kidney tumorigen. A positive finding of tumorigenicity was not identified in the original NTP bioassay using standard histopathological sectioning of the kidney (NTP, 1995, Cirvello et al., 1995). The combined incidence of adenoma and carcinoma of the kidney was 1/50, 3/50, 4/50 and 3/50 for the control and 1.25, 2.5 and 5.0 mg/ml drinking water doses, and statistical significance of the response was only achieved in the mid-dose when subsequent step-sectioning of the kidney was conducted; NTP declared the TBA kidney finding only as *some evidence of carcinogenicity* in male rats.

As required of an $\alpha 2_u$-globulin MoA, no kidney tumors were observed in female rats or either sex of mice. TBA-induced male kidney tumor responses are relatively modest compared to other agents identified as operating via an $\alpha 2_u$-globulin MoA (Swenberg and McKeeman, 1999), and particularly so when compared by standard sectioning evaluations. Finally, it is also important to note that subsequent re-evaluations of the NTP bioassay male and female kidneys revealed TBA accentuation of rat chronic progressive nephropathy (CPN) in males and females (Hard et al., 2011). The CPN MoA, which also is not regarded as having a human clinical correlate, thus likely contributes to the overall expression of kidney tumorigenicity in male and toxicity in female rats.

The TBAc TSD (2015) lack of fulfillment of criterion # 2 on male rat specificity is largely based on kidney changes described in the female rat, namely exacerbation of CPN, increases in renal inflammation, and renal pelvis transitional cell hyperplasia (NTP, 1995). In the 2-year study, females were affected with a dose-related exacerbation of CPN. Although an adverse effect, this is not a nephrotoxic effect (Hard et al, 2009). It is an enhancement of the development of a spontaneous disease process that is very common in the F344 rat (Hard and Khan, 2004). In its advanced stages, CPN represents a wide spectrum of renal parenchyma alterations in both sexes, including inflammatory cell clusters and a characteristic form of transitional cell hyperplasia of the renal papilla lining (Hard et al, 2011; Frazier et al, 2012). It should be noted that CPN is not associated with mineralization in the renal papilla, in contrast to the statement on page 20, paragraph 1, lines 6-7 of the OEHHA document. Although Cirvello et al (1995) in their description of the NTP study linked both of these changes (inflammation and pelvic transitional cell hyperplasia) to CPN (nephropathy in their paper) this was ignored in the TBAc TSD (2015).

However, a Pathology Working Group (PWG) re-evaluating the renal histopathology of the 13-week and 2-year studies of TBA, recognized, and described the papilla lining alteration in both sexes as typical of advanced CPN (Hard et al, 2011). The arguments presented in the TBAc TSD (2015) are inaccurate and there were no TBA-induced nephrotoxic changes in the female kidney.

TBA therefore fulfills IARC criterion # 2 without qualification.

IARC Criterion # 6 – Sustained cell proliferation

The TBAc TSD (2015) used two studies to assess whether the criterion of sustained, increased tubule cell proliferation was supported. Borghoff et al (2001), using 5-bromo-2-deoxyuridine (BrdU) immunostaining, showed that TBA caused a dose-dependent increase in renal tubule cell proliferation, but as pointed out in the TSD, this was demonstrated at only one time point of 10 days. To examine this key event at a later time, the TSD cites the work of Takahashi et al (1993), which had applied PCNA staining to recuts of kidney tissue from the NTP (1995) 13-week drinking water study, as evidence questioning the presence of sustained cell proliferation. Takahashi et al. reported an increase in the *median* cell proliferation only in the second highest exposure group receiving 20 mg/ml of TBA, a dose much higher than the high dose (5 mg/ml) used in the NTP carcinogenicity study. The TSD then cites the negative cell proliferation response observed in a 13 week study of TBAc after 13 weeks of exposure by Faber et al (2014). This comparison was not strictly appropriate as the tumor finding applied to TBA, not TBAc. The TSD (2015) did not refer to the work of Lindamood et al. (1992), who demonstrated, in a 13-week drinking water study, a statistically significant increase in renal tubule S-phase nuclei with anti-PCNA staining at doses of 1 and 2% of TBA in male rats, matching the occurrence of the hyaline droplet response in their study. At the high dose of 4% in male rats there were no hyaline droplets or any cell proliferation response (Lindamood et al, 1992). Inhibition of hyaline droplet formation is seen with other chemicals at very high doses, for example, with β-myrcene (NTP, 2010). The shortcoming of the Lindamood paper is that the negative data for female rats were not presented and the increases at 0.25 and 0.5% were not statistically significant.

Nevertheless, the male rat data of Lindamood et al (1992) and Takahashi et al (1993) at 13-weeks were consistent and, coupled with the results of Borghoff et al (2001) at 10-days, provide some evidence that IARC criterion # 6 is fulfilled.

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes

The TBAc TSD (2015) uses immunohistochemical staining of rat kidney for $\alpha 2_u$-globulin as evidence of an absence of dose-response. This is a capricious staining technique and should not be relied upon to support regulatory decision-making. It should only be used for qualitatively identifying that accumulating hyaline droplets stain positively for the $\alpha 2_u$-globulin protein, i.e. Criterion # 4. Borghoff et al. (2001) clearly state that $\alpha 2_u$-globulin immunohistochemical staining is not as sensitive as an ELISA for measuring changes in the kidney accumulation of this protein. The ELISA is a quantitative measure of protein changes and the immunohistochemical staining is used to determine the localization of $\alpha 2_u$-globulin within the area of proximal tubule

where protein droplets are formed. As such, the $\alpha 2_u$-globulin staining should not be, and was not, used to assess an increase in staining with increasing TBA exposure concentrations.

In the second sentence of paragraph 2 on p.10 of the TSD it is stated "A significant increase in $\alpha 2_u$ concentration was noted in the 1750 ppm group ($p<0.05$) compared to controls, in contrast to the $\alpha 2_u$ staining evaluation, where no TBA exposure-related increase staining was noted." In fact this statement is not correct and does not reflect the data presented by Borghoff et al. (2001). As described by Borghoff et al. (2001), "In male rats exposed to TBA, there was an increased accumulation of protein droplets within the proximal tubule characterized by the formation of large coalescing globules of protein and rare crystalloid protein structures (Fig. 1). Grading on a scale of 0-16 demonstrated an increase in protein droplets at 1750 ppm TBA (Fig. 2). Linear regression analysis supported a statistically-significant, concentration-dependent, positive trend for the accumulation of protein droplets in male rats exposed to TBA." In this study the data provided evidence that there was a TBA exposure-related increase in protein droplet accumulation, that these droplets stained positive for $\alpha 2_u$-globulin, and that there was a significant increase in the concentration of $\alpha 2_u$-globulin at the highest exposure concentration in the male, but not female rat kidneys. To clarify, the authors of this study did not use $\alpha 2_u$-globulin staining of the protein droplets to evaluate a TBA exposure-related increase since it is not an appropriate method for evaluating a quantitative response.

The TSD suggests (p.10, paragraph 3) that TBA induces male rat renal tubule cell proliferation at concentrations that do not increase renal $\alpha 2_u$-globulin concentration. Although this may appear to be the case in the data presented in Borghoff et al. 2001, it is critical to consider that the sensitivity of these assays that measure renal cell proliferation and $\alpha 2_u$-globulin concentration are very different. The change in the concentration of $\alpha 2_u$-globulin is measured at a point in time post-exposure; $\alpha 2_u$-globulin concentration was measured approximately 18 hours following 10-days of exposure to TBA. Renal cell proliferation is evaluated with a 3-day osmotic mini-pump containing 5-bromo-2-deoxyuridine (BrdU) infused over a period of 3 days. The dynamic process of $\alpha 2_u$-globulin accumulation may peak at different times and the actual maximum concentration may not be captured. Chemical binding to $\alpha 2_u$-globulin is reversible, therefore it is possible that with a chemical that binds with low affinity, the time course for capturing the peak concentration of protein in the kidney may be different compared to a chemical such as 2,2,4-trimethylpentane, which was used as a positive control. As such, the correlation between renal $\alpha 2_u$-globulin concentration and renal cell proliferation was evaluated over the increased TBA exposure concentrations and not specific changes at each concentration.

The TSD also states (page 10, paragraph 3 – page 11, paragraph 1) "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 $\alpha 2_u$ concentrations." The concentrations that were referred to were 250 and 450 ppm TBA. There was an increase in renal cell proliferation measured at these lower concentrations where there was not a measureable change in the concentration of $\alpha 2_u$-globulin. However, these data need to be evaluated again keeping in mind that the renal cell proliferation is measured as an accumulation of a response over 3-days where the change in $\alpha 2_u$-globulin and in TBA concentration is at a fixed point in time. Also critical to note are the data presented in the Borghoff et al. (2001) publication (Figure 6) that clearly show

the concentrations of TBA in the kidneys of male and female rats are similar if not higher in the female rats with no measure of increased renal cell proliferation in exposed female rats.

Not discussed in the TSD (2015) is that a more reliable marker of renal tubule cell accumulation of $\alpha 2_u$-globulin is the typical polyangular or crystal-like shape of the droplets in $\alpha 2_u$-globulin nephropathy, as observed in Hard (2005) and illustrated in Hard (2008). Lindamood et al (1992) also noted that the shape of the $\alpha 2_u$-globulin-associated protein droplets was rhomboid and crystalline, and recorded them in male rats at doses from 0.25 to 2.0% in the drinking water. These typical droplets were observed in the NTP 13-week study at doses of 2.5 and 5.0 mg/ml, doses that were later associated with renal tubule tumors, by Hard (2005).

Other evidence of dose-response correlation is seen in presence of precursors of granular casts and mature granular casts observed at the junction of outer and inner stripes of outer medulla in male rats of the NTP 13-week study at relevant doses, low frequency at 2.5 mg/ml, and higher frequency at higher doses (Hard, 2005). Linear papillary mineralization, a characteristic chronic feature of $\alpha 2_u$-globulin nephropathy was observed in male rats, but not females, at doses correlating with renal tumor induction (Hard, 2005). Thus, linear papillary mineralization was scored as mild to moderate in the high-dose (5.0 mg/ml) male group where CPN compromised survival of the rats, and minimal to moderate in the mid-dose (2.5 mg/ml) male group, which demonstrated a significantly increased renal tumor incidence. Linear papillary mineralization was absent in the low dose and control male groups, but was present in the 15 month interim sacrifice males of the 5.0 and 2.5 mg/ml male dose groups. Cirvello et al. (1995) also quantified linear papillary mineralization showing major increases in incidence in male rats at doses of 2.5 and 5.0 mg/ml, but not in female rats. The difference in renal tumor incidence between the 2.5 mg/ml male group and the 5.0 mg/ml male group could be associated with a lower survival of male rats in the 5.0 mg/ml group, which would have reduced the time for more tumors to develop, but the comment made below on renal tubule hyperplasia is also relevant. For criterion # 7, allowance should be made to accommodate the confounding factors of CPN exacerbation and survival.

By placing all of the emphasis on the lone statistically-significant end-point of renal tumors in the mid-dose (2.5 mg/ml), the OEHHA assessment does not reveal all of the facts concerning this particular chemical. The TSD (2015) gives no consideration to data on renal tubule hyperplasia. Atypical tubule hyperplasia is the obligatory precursor of, and on a developmental continuum with, renal tubule adenomas, and subsequently, carcinomas (Hard, 1990; Dietrich and Swenberg, 1991; Nogueira et al, 1993). In the extended evaluation of the NTP 2-year study (NTP, 1995), the only statistically increased value among the renal tubule hyperplasia data was the number of foci in the high-dose male group (5.0 mg/ml) at 23. In this particular case, combining foci of hyperplasia with renal tumors provides a more valid indication of the factors involved in the rat kidney response to TBA. When this is done, there is a dose-related score of 19, 26, 32, and 34, for the 0, 1.25, 2.5, and 5.0 mg/ml, respectively. Although not every one of the hyperplastic lesions may have conformed to atypical hyperplastic foci, it can be deduced that the overall tumorigenic response is similar at both the mid-dose of 2.5 mg/ml and the high-dose of 5.0 mg/ml in the NTP 2-year carcinogenicity study of TBA.

The TSD (2015) concludes that $\alpha 2_u$-globulin cannot be the only operative MoA responsible for the increased renal tumors (page 23, paragraph 5. lines 6-7). It has defaulted to the opinion that genotoxicity is the reason, although as noted above, this conclusion is not justified by a weight-of-evidence evaluation of that dataset. However, the TSD also has given no consideration to a more likely alternative MoA. A feature of the TBA studies was exacerbation of spontaneous CPN, which was probably the cause of lower survival in the high-dose male rats. Advanced and end-stage CPN has been shown to be responsible for a low incidence of renal tubule tumors in control rats, and is therefore a risk factor for renal cancer development, particularly in male rats (Hard et al, 2012). A number of chemicals can exacerbate CPN to advanced stages of severity, and in so doing, marginally increase the incidence of renal tubule tumors (Hard et al 2013). A high rate of tubule cell turnover occurs throughout the developmental course of CPN in association with sustained tubule cell injury, and is likely to act as the underlying basis for the spontaneous renal tubule tumor formation associated with advanced to end-stage CPN.

TBA was associated with a dose-related increase in CPN in both the 13-week study and 2-year studies (NTP, 1995; Hard et al, 2011). In the 2-year study, CPN severity increased from 3.0 in control males to 3.3 in the high dose group (NTP, 1995). The mean CPN severity grade in male rats with renal tubule tumors in the TBA study was 3.6 compared to grade 2.8 in male rats without renal tumors (Hard et al, 2011). Furthermore, foci of hyperplasia due to exacerbated CPN are slow-growing and start to develop at a relatively late stage in carcinogenicity bioassays (Hard et al, 2013), as the NTP findings showed for the high-dose of TBA (NTP, 1995). There was also a dose-related increase in CPN severity in female rats, ranging from 1.6 in the control females to 2.9 in the high dose group (NTP, 1995). However, the degree of CPN severity was less in the females than in the males, which would explain the relative absence of renal tubule tumors in the dosed female rats. There is a well-known gender difference in the incidence and severity progression of CPN, with female rats being less predisposed than males (Hard and Khan, 2004). Because of this lower susceptibility to CPN, renal tubule tumors resulting from advanced CPN occur less frequently in females than in male rats, because fewer females reach end-stage CPN (Hard et al, 2013).

Scientists from the US EPA and Texas A&M University have recently described the development of a new rat PBPK model for evaluating liver and kidney toxicity of ethyl *tert*-butyl ether and TBA based on internal dose metrics (Salazar et al, 2015). The internal dose metrics included the daily average of TBA blood concentration and the daily amount of TBA metabolized in the liver. The analyzed toxicity end-points included kidney tumors (males only), CPN (both sexes), and urothelial hyperplasia (males only). The results for kidney tumors indicated that the internal dose of TBA was inadequate to explain differences in tumor response, but CPN and urothelial hyperplasia were strongly correlated with TBA dose metrics. Salazar et al (2015) conclude that another factor besides the dose of TBA is involved in the kidney tumor induction, and their suggestion is that the NIH-07 diet used in the TBA study increased CPN and consequently the renal tumors. As discussed under Criterion # 2, urothelial hyperplasia is a component of CPN, and particularly a marker of advanced disease.

The procedure of step-sectioning of kidney tissue for obtaining increased renal tumor data for statistical analysis also selects for renal tubule tumors (usually late-occurring adenomas) and precursor foci of tubule hyperplasia associated with advanced CPN (Hard and Khan, 2004). In

first describing this procedure, Eustis et al (1994) stated that a notable feature of all of the studies associated with higher renal tumor incidences in male rats after step-sectioning (including the 2-year TBA study) was the presence of chemical-related, increased severity of CPN (Eustis et al, 1994). The only study in female rats in which step-sectioning increased the tumor incidence also showed a chemical-related increased severity of CPN (Eustis et al, 1994).

In the case of TBA it is very likely that the low renal tubule tumor incidence is due to the combination of an $\alpha 2_u$-globulin nephropathy MoA and the chemically associated exacerbation of CPN. This combined effect has been discussed by Lock and Hard (2010), who drew particular attention to the intimate association of $\alpha 2_u$-globulin nephropathy with exacerbating CPN throughout the course of $\alpha 2_u$-globulin nephropathy disease progression. Lock and Hard (2010) used TBA as an example of the likelihood of these two separate MoAs combining to increase the incidence of renal tubule tumors above the background level. There is no renal disease counterpart of CPN in humans (Hard et al, 2009) and therefore, as with $\alpha 2_u$-globulin nephropathy, a MoA involving exacerbation of CPN should have no relevance for extrapolation to humans in cancer risk assessment. Importantly, consideration of an overlay of a CPN MoA was also suggested by the IARC Working Group paper addressing the criteria for an $\alpha 2_u$-globulin MoA, in which it was concluded that "…minor exacerbations of common spontaneous renal disease" in female rats "should not pertain" to ruling out an $\alpha 2_u$-globulin MoA (Swenberg and McKeeman, 1999). This observation is entirely consistent with the conclusions of the Pathology Working Group review of TBA-induced rat kidney tumors (Hard et al., 2011), in which it was stated:

> "There was unanimous agreement among the members of this independent PWG that both a2u-g nephropathy and CPN exacerbation were the only causative factors in the development of renal tubule tumors observed in male rats exposed to TBA in drinking water. As neither of these modes of action have human counterparts, the PWG concluded that TBA-related renal changes in rats could not be extrapolated for human health risk assessment, and were unlikely to pose any risk for humans."

The above evaluation indicates that not only is Criterion # 7 fulfilled for a weakly active $\alpha 2_u$-globulin MoA, but also that CPN provides an alternative ancillary MoA further explaining the lack of human-relevance of TBA induced rat renal toxicity and tumors.

Use of TBA-induced mouse thyroid tumors are not justified as a basis for derivation of a TBAc inhalation URF given MoA information suggesting a lack of quantitative/qualitative human biological and exposure relevance of this tumor response

Similar to the male rat kidney response, TBA-induced female mouse thyroid tumors also are not a justifiable basis for derivation of an inhalation URF given both biological MoA and exposure considerations. Although MoA data investigating TBA thyroid tumors are more limited than that supporting the male rat kidney MoAs, it nonetheless suggests that the high-dose specific thyroid tumorigenicity of TBA results from a non-mutagenic MoA associated with enhanced catabolism of thyroid hormone mediated by TBA induction of liver oxidative metabolism and associated compensatory response of the hypothalamic-pituitary-thyroid regulatory axis (reviewed in McGregor, 2010; Bus et al., 2015). This MoA is common to multiple rodent thyroid

carcinogens, with phenobarbital as the prototypical agent of the class, and this tumor MoA is not regarded as quantitatively, or possibly qualitatively, relevant to humans (McClain, 1989; Dellarco et al, 2006).

The plausibility of TBAc as a potential mouse thyroid tumorigen is further tempered by the observation that intrinsic TBAc mouse toxicity would limit achieving of TBA tumorigenic doses. As reviewed in Bus et al. (2015), all mice exposed to 3000 ppm TBAc for 6 hours were prostrate during most of the exposure period. TBA-induced tumors were observed only in female mice at the top drinking water dose of 2110 mg/kg/day, which toxicokinetic assessments indicate are equivalent to 3300 ppm TBAc conservatively *assuming 100% metabolism of absorbed TBAc to TBA.* Given that toxicokinetic data indicate approximately 50% of systemically absorbed TBAc is metabolized to TBA, the 2110 mg/kg/day TBA mouse thyroid tumorigenic dose more realistically equates to 6657 ppm TBAc (Bus et al., 2015). Such a high chronic TBAc exposure would therefore almost certainly far exceed an MTD in mice, and thus it is very unlikely that tolerable chronic TBAc exposures would result in thyroid cancer.

Given that TBA rodent carcinogenicity and TBAc/TBA genotoxicity findings suggest TBAc is an unlikely rodent carcinogen, Bus et al. (2015) have recently developed an alternative TBAc risk assessment based on mouse neurotoxicity.

Bus and coworkers (2015) have proposed a chronic TBAc RfC of 0.3 ppm conservatively based on neurotoxicity (hyperactivity) observed in mice immediately after termination of daily 6 hour exposures to TBAc. Although the neurotoxicity was observed immediately after a series of short-term 6 hour TBAc exposures, it was assumed that this response would continue over repeated exposures and thus represented a potential chronic effect. The TBAc inhalation Unit Risk of 1.9×10^{-6} $(\mu g/m^3)^{-1}$ described in the TBAC TSD (2015) translates to a TBAC 10^{-6} risk of 0.0001 ppm, or 3000-fold less than the non-cancer chronic RfC proposed by Bus et al. (2015).

Miscellaneous Observations

Male rat kidney evaluations

The statement is made that NTP observed granular casts in TBA-treated male rats (page 20, Criterion 3, bottom of page). That is not correct. In fact the statement in NTP (1995) and in Cirvello et al (1995) states the opposite, that is, there was "no morphologic evidence of extensive cell necrosis (granular cast formation)." Recognition of granular casts in TBA-exposed male rats was first recorded by Hard (2005) when examining slides from the NTP 13-week study of TBA that had been stained with Mallory Heidenhein and anti-PCNA stains (the latter from a cell proliferation investigation of the TBA-exposed rats of the TBA 13-week study). Subsequently precursors of granular casts were recorded in hematoxylin and eosin (H&E) stained kidney sections from the same NTP study by Hard et al (2011).

The derivation of the various health assessment values was based on the male F344 rat kidney tumor incidence data for TBA (NTP, 1995). Normally this would have used the tumor incidence values from the standard histopathological evaluation. However, this data was presumably unsuitable because the renal tumors were not statistically increased. Consequently, the data from

an extended histological evaluation of the kidney was used involving step-sectioning of the kidneys to produce 6 to 8 additional sections per kidney. This elevated the renal tubule tumor incidence in groups of 50 male rats from 1, 3, 4, 3 for the 0, 1.25, 2.5, and 5 mg/ml exposure levels, respectively, to 8, 13, 19, and 13, respectively (NTP, 1995). The 2.5 mg/ml dose group was statistically significant at 19 tumors. This departure from normal practice, though reasonable, should have been indicated and adequately explained in the TSD (2015). Eustis et al (1994), who introduced the multiple-sectioning procedure for kidney tumor evaluation, tabulated the combined adenoma/carcinoma incidence in the NTP 2-year study of TBA as 7, 10, 18 and 11 for control, low, middle, and high doses of TBA in male rats, respectively.

TBAc Inhalation Unit Risk Derivation

The TBAc TSD provides no rationale for selection of a 5% BMR response of the TBA male rat kidney response as the point of departure dose for risk derivation, despite OEHHA TSD guidance recommending selection of a 10% BMR as the standard default. In addition, no rationale is provided for elimination of the top dose in characterization of the male rat kidney BMR, resulting in a less than preferable two-point dose response curve as the basis for selection of the point of departure tumorigenic dose in rats.

The TSD also assumes that 95% of inhaled 100 ppm TBAc is absorbed into blood over the course of a 6 hour exposure (page 28, bottom paragraph). This estimate is very likely erroneous in that it was based on the assumption that the total amount of TBAc (radioactivity equivalents) determined in the bodies of rats at the termination of a 6 hour exposure (equivalent to 50.7 mg/kg TBAc) was essentally equivalent to the total amount of TBAc *inhaled* over the entire course of that 6 hour exposure. This projected inhaled dose is certainly a significant underestimate. Assuming an EPA default Minute Volume for rats of 0.174 L/min (0.000174 m^3/min), a total volume of 0.06264 m^3 of air (TBAc vapor) will be inhaled over a 6 hour exposure (360 min X 0.000174 m^3/min), which in turn equates to a total of 29.7 mg of inhaled TBAc for an individual rat (0.06264 m^3 X 474 mg/m^3 TBAc; 1 ppm TBAc = 4.74 mg/m^3). Assuming a rat body weight of 0.21 kg, this equates to a total potential inhaled dose of TBAc of 141 mg/kg TBAc over the course of a 6 hour total exposure. This inhaled dose is substantially higher than the dose estimated from collection of total radioactivity in animals at termination of a 6 hour exposure (50.7 mg/kg), and projects that the total systemically absorbed dose is substantially less than 95%, and could be as low as 35% (50.7 mg/kg divided by 141 mg/kg). Such an absorption percentage more closely aligns with approximate 50% respiratory retention values of inhaled organic compounds.

Page 5, paragraph 3 – description of historical control incidence of male rat kidney tumors.

It is important to note that NTP historical control incidence of male rat renal tubule adenomas (2/327) and carcinomas (0/327) in drinking water studies described here is derived from rats in which standard kidney sectioning, and not step-sectioning, was conducted. Thus, the inference stated here that such tumors are rare, although somewhat true, is misleading when these values are compared to the adenoma/carcinoma values reported in TBA treated rats in which step-sectioning was the basis of the tumor incidence determinations. The historical control incidence of kidney tumors in step-sectioned rats is not known.

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Appendix A: LyondellBasell Oral Public Comments (Presentation) on the Draft Inhalation Cancer Unit Risk Factor for *t*-Butyl Acetate. OEHHA Public Workshop, September 14, 2015, CAL/EPA Headquarters, Sacramento, CA.

(double-click on title below to view presentation)

LyondellBasell Comments on the Draft Inhalation Cancer Unit Risk Factor for *t*-Butyl Acetate

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September 14, 2015
Public Workshop
CAL/EPA Headquarters
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