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RE: Comments on the *NTP TECHNICAL REPORT ON THE TOXICOLOGY STUDIES OF TETRABROMOBISPHENOL A (CAS NO. 79-94-7) IN F344/NTac RATS AND B6C3F1/N MICE AND TOXICOLOGY AND CARCINOGENESIS STUDIES OF TETRABROMOBISPHENOL A IN WISTAR HAN [Crl:WI(Han)] RATS AND B6C3F1/N MICE (NTP TR587)*

ToxStrategies scientists have reviewed the *NTP Technical Report on the Toxicology Studies of Tetrabromobisphenol A (cas no. 79-94-7) in F344/NTac Rats and B6C3F1/n Mice and Toxicology and Carcinogenesis Studies of Tetrabromobisphenol A in Wistar Han [Crl:WI(Han)] Rats and B6C3F1/n Mice (NTP TR587)* recently released for public comment. We offer comments in the following three areas for your consideration:

1) Shortcomings in the comparison of the uterine tumor incidence to historical controls: In the interest of being fully transparent, statements related to comparisons to historical control incidence should be clarified to note that there are no comparable (same route of administration) NTP historical control data for this strain of rat. Additionally, there are no historical control data for the longitudinal review used to further identify uterine tumors in this specific study. For the traditional transverse section there are only 3 studies with historical control data and, as such, it is not as robust as are typical comparisons to historical controls.

2) Limitations in the evaluation and interpretation of the *Tp53* mutation data: There are a number of shortcomings with the methods and data handling that need to be addressed before the data can in fact be used to support the conclusions purported for the mutation analysis.

3) Limited relevance to humans of NTP study dose levels: Even the lowest doses tested in this animal study are substantially higher than current human exposures. Therefore, it is difficult to accurately extrapolate the findings in this study to humans—especially considering the uncertainties as to how the high doses, where effects were observed, may have perturbed normal physiological functions and protective mechanisms in the animals in the study.

Each of these key comments is discussed in more detail in Attachment A. These comments are being submitted on behalf of the American Chemistry Council's North American Flame Retardant Alliance (NAFRA). We thank the members of the NTP Technical Report Peer Review Panel in advance for their consideration of these comments.

Sincerely,

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Attachment A

ToxStrategies Comments on the
NTP TECHNICAL REPORT ON THE TOXICOLOGY STUDIES OF TETRABROMOBISPHENOL A (CAS NO. 79-94-7) IN F344/NTac RATS AND B6C3F1/N MICE AND TOXICOLOGY AND CARCINOGENESIS STUDIES OF TETRABROMOBISPHENOL A IN WISTAR HAN [Crl:WI(Han)] RATS AND B6C3F1/N MICE (NTP TR 587)

1. Shortcoming in the comparison of uterine tumor incidence to historical controls

Data presented in the NTP report indicate that chronic administration of TBBPA to female Wistar Han rats resulted in an increased incidence of uterine adenocarcinomas. The increase was reported to be statistically significant when residual longitudinal sections of the uterus were evaluated. The NTP report included a clear explanation for the need to go back to the residual tissue to perform this evaluation based on the initial results. However, questions always arise when a new approach, for which there are little to no historical control data, is used to evaluate non-neoplastic and neoplastic lesions. ToxStrategies has identified several areas where additional clarification/qualification is needed in the report.

Comment 1: In the interest of being fully transparent, statements related to comparisons to historical control incidence should be clarified to note that there are no comparable (same route of administration) NTP historical control data for this strain of rat. Additionally, there are no historical control data for the longitudinal review used to further identify uterine tumors in this specific study. For the traditional transverse section there are only 3 studies with historical control data and, as such, it is not as robust as are typical comparisons to historical controls.

As outlined on page 46 of the draft NTP report, "*For meaningful comparisons, the conditions for studies in the historical control database must be generally similar*." Clearly, one factor that would affect the incidence of lesions reported in historical controls rats would be the approach used for sectioning and evaluating the tissue. Although the NTP provided a clear and reasonable explanation for the need to go back to the residual uterine tissue for further evaluation, it needs to be emphasized when these data are presented (pages 60-61; Table 6) that there are no historical control incidence data of uterine adenomas, adenocarcinomas, or malignant mixed Mullerian tumors with a longitudinal review of the uterus, or when transverse and longitudinal reviews are

combined. Given that no such historical data are available, it is difficult to understand the basis for the statement in the Discussion and Conclusion, (page 84) of the draft NTP report, "*In addition, the incidences of the malignant uterine epithelial tumors exceeded the historical control ranges in all treatment groups*." Further, this statement should be clarified to indicate if it is based on the transverse and longitudinal reviews combined, or if it relies on comparison of the transverse review only. If the above statement is based on the transverse review only, then it should be noted that historical control data are limited to just 3 studies and, as such, are not as robust as are typical comparisons to historical controls. Given the general lack of relevant historical control data available for this strain of rat and review for evaluating uterine histopathology, any use of historical control data in the evaluation of carcinogenicity should be carried out cautiously.

The NTP report also discusses that the historical database for a given study typically includes studies using the same route of administration (p. 46). However, because the current study was the only study conducted to date in Wistar Han rats using corn oil as a gavage vehicle, this technical report included historical control tumor incidence from all previous studies in Wistar Hans rats no matter the route of exposure. It would also be useful if the NTP could address how these historical control data are "*generally similar*," (as noted on p. 46), as well as discuss potential limitations and shortcomings associated with making comparisons to historical control database that consists of data from different studies using different routes of exposure.

Finally, the specific data used to characterize the incidence rates in the historical control groups that served as comparisons should be provided in the NTP report. Without this additional information, it is difficult to fully interpret any comparisons to historical control data.

2. Limitations in the evaluation of *Tp53* mutation data

Based on the uterine tumor type and the appearance of a similarity to human tumors, NTP conducted an investigation of the *Tp*53 mutations in the uterine adenocarcinomas. The objective was to evaluate spontaneous adenocarcinomas and adenocarcinomas from TBBPA dosed rats for alterations in the frequencies of *Tp*53 mutations to determine if the incidences of these mutations in treated rats differed from spontaneous tumors. In the draft report, there is significant focus on the analysis and interpretation of the *Tp*53 mutations based solely on a very limited number of spontaneous tumors from animals in the control groups of various 2-year cancer studies and tumors from animals administered TBBPA. Based on review of these data, as well as the interpretation of the findings, a number of concerns were identified and are discussed below. When considered collectively, it appears that the presentation of these results may be premature or at a minimum should be clarified as being from a pilot study, particularly considering that the quality of data analysis, reporting, and review was significantly less than the main components of the standard bioassay.

Comment 2A: The information regarding the methods used to conduct the *Tp53* mutation analysis is not complete.

When reviewing the methodology as provided in Appendix M, ToxStrategies scientists identified

a number uncertainties in the text provided. First, it was not clear that the PCR product was sequenced more than once to confirm the mutation. At a minimum there should be 2x coverage, which is standard practice. If the sequence confirmation was in fact performed, it is requested that this information be addressed in the draft report. Without confirmation that such a standard procedure was followed, it is difficult to have confidence in the results reported.

It would be useful to provide a thorough description of the uterine carcinomas collected, the fixation procedures, and the length of time stored in FFPE blocks prior to mutation analysis. Considerations for these parameters are especially important for the spontaneous uterine carcinomas collected from control animals from other chronic studies. With respect to the selection of samples, it would also be useful to provide a thorough description of the statistical methods used to select the uterine tumors from the NTP archive (e.g., was this process conducted using software for randomized sample selection). It is requested that the NTP provide information regarding the preparation and storage of all the samples used in this analysis, and in doing so, discuss any similarities and differences with sample storage and preparation conditions (including length of storage time).

Additionally, it is also not clear that the methods and data generated in the analysis of *Tp*53 mutations were as thoroughly reviewed as the histopathology of the tumors, or if the data were conducted according to GLP as was the case with the pathology.

Comment 2B: The findings from *Tp*53 mutation assay are not statistically significant when the dose groups are evaluated separately

To evaluate *Tp*53 mutations, the NTP combined all of the tumors from all the TBBPA treated animals into one single group. In doing so, there was a significant increase in the incidence of mutations in the uterine tumors in all TBBPA-dosed animals combined compared to tumors from the control animals. However, if the tumors from the individual dose groups are evaluated separately, as was done by the NTP when evaluating the significance of other endpoints evaluated in the two-year bioassay, only the incidence of mutations from tumors resulting from exposures to the low dose group is statistically different from the spontaneous tumors reported in Appendix M as seen in the table below:

Group	*Tp53* mutations	FET (p-value)*
Controls	2/10	NA
250 mg/kg	3/3	0.035
500 mg/kg	3/7	0.31
1000 mg/kg	4/6	0.092
All treated	10/16	0.042

* one-sided Fisher's exact test

The NTP should provide a rationale for why it is appropriate to combine the mutation incidences from all treated groups into a single group, as well as discuss the lack of significance when the data are evaluated in individual dose groups. This is particularly important given that the tumor outcome differed between some of the groups.

Another approach may be to combine tumors from the treatment groups with statistically significant elevations in uterine tumors (i.e., 500 and 1000 mg/kg groups). The 250 mg/kg group is clearly not a control group, nor were uterine tumors significantly elevated relative to control (P=0.168; Table 6, pg 61). When taking this approach in evaluating the mutation incidence data from only the carcinogenic groups, the incidence of mutations do not differ significantly from the control as indicated in the table below:

Group	*Tp53* mutations	FET (p-value)*
Spontaneous	2/10	NA
250 mg/kg TBBPA (no statistically significant neoplastic lesions)	3/3	0.035
500-1000 mg/kg TBBPA (statistically significant neoplastic lesions)	7/13	0.11

* one-sided Fisher's exact test

Comment 2C: An independent *post hoc* analysis that accounted for Type 1 errors (the NTP analysis did not) indicated that the mutation incidence was not statistically significant.

Because the NTP combined treated groups in a *post hoc* analysis, it must be recognized that the treated groups could be combined in four ways (250/500, 250/1000, 500/1000, 250/500/1000). In conducting *post hoc* tests, it is necessary to correct for the inflated risk of Type I error that arises from the fact that the analyst has conducted (after looking at the pattern of data) just one test from among all the possible tests. As such, the obtained p values should be Bonferroni-corrected by multiplying the p-values by 4. This correction increases the original NTP p-value of 0.042 to 0.168, which is greater than their target significance level of 0.05:

Group	*Tp53* mutations	FET (p-value)*	Bonferroni-corrected
Spontaneous	2/10	NA	NA
All treated	10/16	0.042	0.168

* one-sided Fisher's exact test

Comment 2D: Additional discussion is needed to support the appropriateness of utilizing historical control data from studies associated with different routes of exposure when interpreting mutation incidence rates for TBBPA

Five of the spontaneous tumors were from corn oil gavage studies, and five were from inhalation studies. If only the tumor incidences from corn oil gavage studies are considered, the spontaneous incidence changes from 2/10 to 1/5. Although both represent a 20% incidence, the difference in sample sizes (2/10 and 1/5) can influence statistical power and the ability to detect differences among groups. However, there is a biological rationale for considering only spontaneous tumors from corn oil gavage studies because corn oil gavage was the vehicle used in the TBBPA study. It is conceivable that the mutation rate might differ in rats on a high fat diet. This is consistent with the discussion on page 46 of the NTP report regarding the importance of similar study designs when comparing to historical control animals, "*A second potential source of variability is route of administration.*" As such, it is requested that NTP provide discussion on how pooling these tumors from studies conducted by different routes of administration impacts the findings, and specifically, how such pooling can impact the quantitative variability of the findings.

Comment 2E: An independent analysis in which all mutation types were accounted for (NOTE- NTP did not account for all mutation types) resulted in an increased incidence of background mutations.

NTP opted not to include silent mutations in their analysis, a decision that lacks biological rationale. Silent (synonymous) mutations in *TP*53 have been shown to affect *TP*53 mRNA translation rates and protein levels via disruption of interactions with MDM2, an E3 ubiquitin-protein ligase (Whibley et al., 2009, Nature Reviews Cancer). NTP should further justify this exclusion, and consider their results with and without silent mutations, and the implications of their findings. For example, the incidence of mutations in tumors from control animals increases from 2/10 to 3/10 if silent mutations are included. Furthermore, if one considers spontaneous mutations only in corn oil gavage studies, the incidence increases from 1/5 to 2/5 if silent mutations are included. Thus, it is requested that NTP include discussion on the scientific rationale for the selection of mutations used in the analysis presented in the draft report.

Comment 2F: Insufficient data are provided to support the conclusions purported on the *Tp*53 mutation analysis.

First, it is requested that NTP correct the factually incorrect and misleading statement in Appendix M, page M-3, "*Mutant Tp53 is nonfunctional and results in loss of cell cycle checkpoint control, and uncontrolled cell growth and proliferation, leading to carcinogenesis*." In human cells, it is widely known that many *TP*53 mutations actually cause gain of functionality (Oren and Rotter, CSH Perspectives, 2012). Furthermore, the above quote also suggests that if *Tp53* is mutated, carcinogenesis will occur. Clearly the mutation of *Tp53* is critical and can be a key event in the formation and progression of many tumors, but the statement as written does not convey the complexities of the carcinogenic process. Indeed, *TP53* is mutated in at least half of all human tumors, but the other half of tumors contain wild-type copies of *TP53* at both alleles, indicating that cancer can form without direct mutation of *TP53* (Freed-Pastor and Prives, Genes

& Dev., 2012). Mutation of *TP53* is also typically one part of multiple mutational events that drive carcinogenesis (Vogelstein et al., Science. 2013). Given the lengthy speculative discussion on this topic in the discussion section of the report, it is requested that these concepts be included in the report.

Second, it is requested that discussion be added to the draft report, as well as Appendix M, regarding the study design and inherent limitations in using the data to interpret potential impacts on functionality. The method used to analyze the *Tp*53 mutations in the uterine carcinomas was DNA- and not mRNA-based. The DNA based methodology used in this study cannot confirm whether the mutations identified affect one (heterozygous) or both alleles (homozygous). This information is useful in interpreting these data since the gene may still be partially functional if only one allele is mutated. Furthermore since this was a DNA based assay, it is also uncertain if the mRNA encoding the mutant p53 protein was actually being expressed in the tumors evaluated. It is critical to understand and identify the limitations in the methodology used in this analysis and whether these mutations are expressed in the tumor that lead to an altered p53 function and avoid over interpreting the data presented.

Third, on page 62 of the report, the following statement is made "*An increase incidence of point mutations in the rat Tp53 gene was observed in uterine adenocarcinomas from TBBPA exposed animals (10/16; 63%) compared to spontaneous uterine adenocarcinomas in control animals (2/10; 20%).*" TBBPA was found to be negative in the Ames assay, an assay that would pick up chemicals that cause point mutations. It is requested that NTP provide comment on the potential discrepancies in findings, as well as provide rationale as to which types of mechanisms may be involved with the *Tp53* mutations given the lack of direct mutagenicity observed in the Ames assay.

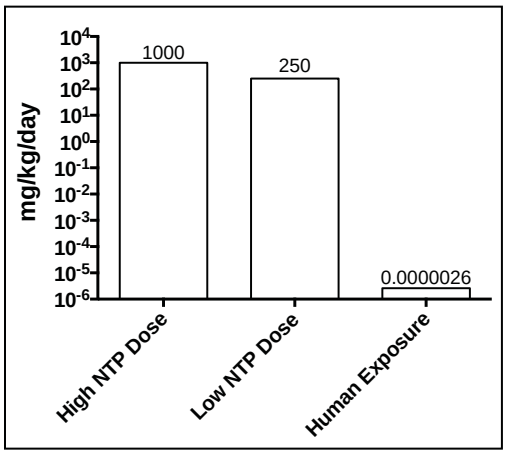
And lastly, the draft report does not currently discuss the role of time to tumor formation with respect to the *Tp53* mutation analysis. The draft report currently states, "*Tp53 mutations in human endometrial cancer are associated with advanced disease*" and that *TP53* mutations "*occur at a high rate in high grade tumors (80% to 90%)…. and are thought to possibly occur as a late event in the development of aggressive endometrial cancer.*" (pg. 87). Since the first incidence of uterine adenocarcinoma in TBBPA-dosed female rats occurs anywhere from 100-400 days prior to the first incidence of the spontaneous uterine adenocarcinoma, (Table 6), the *Tp53* mutational status may or may not be a function of tumor age/stage of progression and the amount of time available for mutational accumulation. This concept of mutational accumulation in human tumors is elegantly described in work by Bozic et al. (Bozic et al., PNAS. 2010). NTP should comment on this and also, if available, include data regarding the size/weight of the tumors used for *Tp53* mutational analysis (i.e., provide animal ID numbers).

3. Limited relevance to humans of the NTP study dose levels

As NTP studies are designed to maximize the potential for induction of adverse effects in laboratory animals (i.e., focused on hazard identification and not true risk to humans), the dose levels tested are often not relevant to humans. This poses unique challenges when attempting to extrapolate findings from these studies to humans. This is a point that should be clearly noted in

the NTP report. The importance of this point with respect to TBBPA in particular is elaborated on in the comment that follows.

Comment 3: Even the lowest doses tested were substantially higher than is human exposure and therefore it is difficult to accurately extrapolate the findings in this study to humans, particularly considering the uncertainties as to how the high doses where adverse effects were observed in the NTP study may have perturbed normal physiological functions and protective mechanisms.



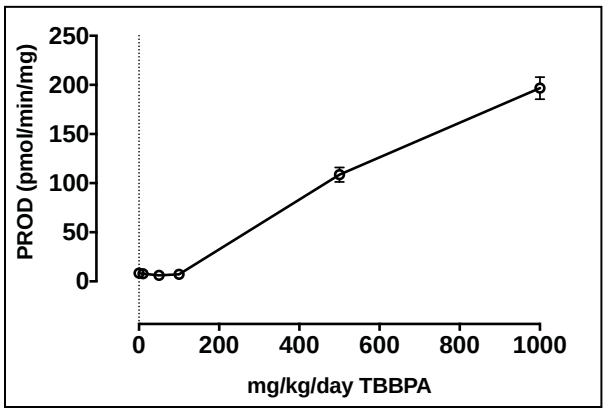
TBBPA, despite its high level of global production and use, is associated with very low levels of exposure in human populations (EFSA 2011, Health Canada 2012)[1]. For perspective, exposure estimates from European and Canadian agencies are orders of magnitude lower than the dose levels selected for both the 90-day (10-1,000 mg/kg-day) and 2-year studies (250-1,000 mg/kg-day; inset and footnote). Given the wide disparity between environmentally relevant doses and the doses used in the 2-yr study (see inset; adult human exposure dose as cited in the NTP report, p. 19), it is difficult to extrapolate the findings of this study with respect to evaluating current and future human exposures. This is particularly notable given that the draft report includes a number of rather speculative comments in the discussion section comparing findings between rodents and humans.

The difficulty in interpreting the study findings is further compounded by the lack of discussion in the draft NTP report regarding the effects of the high doses used in the study on physiological function, and the potential for the high doses to saturate protective mechanisms. It is well accepted that there are dose-dependent changes in modes of action (MOA) at high doses:

> *"It is highly likely that critical, limiting steps in any given mechanistic pathway may become overwhelmed with increasing exposures, signaling the emergence of new modalities of toxic tissue injury at these higher doses. Therefore, dose-dependent transitions in principal mechanisms of toxicity may occur, and could have significant*

[1] For example, the European Food Safety Authority (EFSA) on Contaminants in the Food Chain conducted an assessment to characterize the exposure to TBBPA in the diet (EFSA 2011; note this assessment is also cited in the NTP report). All of the samples evaluated were in the food group "Fish and other seafood," though data from all 344 food samples were below the limit of quantification (LOQ). Based on these findings, EFSA estimated an upper bound dietary exposure estimate of 0.0000026 mg/kg-day based on the LOQ. EFSA (2011) also reported an intake in infants with a high consumption of breastmilk (0.000257 mg/kg-day), as well as a reasonable intake associated with dust (0.0000012 mg/kg-day). More recently, Health Canada evaluated intake of TBBPA and derived upper-bounding estimates of daily intake by Canadians (though data from China and other countries were used in the calculations) (Health Canada 2012). These estimates considered potential exposures from breastmilk, air (indoor and ambient), drinking water, food, and soil/dust. The total intake was highest for breastfed infants, with an estimated daily intake of 0.000195 mg/kg-bw.

impact on the interpretation of reference data sets for risk assessment." Slikker et al 2004



This aspect seems very important given the limited data included in the draft report. For example, cytochrome P450 activity in liver microsomes from TBBPA treated F344 rats in the 90-day study increased dramatically at the two highest exposure doses (see inset). Although these changes were not associated with liver lesions, they clearly represent a change in liver physiology, with a possible effect on TBBPA disposition as well as metabolism of other endogenous substrates (e.g. hormones). Such changes may also play a role in the overall response to TBBPA in Wistar Han rats of the 2-yr bioassay.

The draft report discusses a potential metabolic scheme for TBBPA that was constructed using a variety of studies in the peer-review literature (Figure 1, p. 22). As part of this discussion, the draft report stated that TBBPA was rapidly conjugated with glucuronic acid or sulfate in disposition and metabolism studies conducted in rats. Given this knowledge regarding metabolism, it is requested that the NTP comment and include discussion on why Phase 1 enzymatic activity was evaluated, but Phase 2 enzymatic activity was not. Additionally, it appears that NTP scientists have published kinetic data in the literature rather than include such in the report:

Knudsen, G.H., Sanders, J.M., Sadik, A.M., and Birnbaum, L.S. (2013). Disposition and kinetics of tetrabromobisphenol A (TBBPA) in female Wistar Han rats. Toxicology (in press).

Because this information is not yet available to the public, it is requested that these data be made available, as it is important to consider kinetics when interpreting the toxicological findings.

Moreover, it is notable that the MOA underlying adverse effects in the NTP study associated with exposures to 250-1000 mg/kg, are unlikely to occur at environmentally relevant concentrations. The vertical dotted line in the inset above represents upper bounds on human TBBPA daily exposure as reported by EFSA (2011). Clearly, there are dose-dependent changes induced by TBBPA that are unlikely to occur in rodents at typical human exposure levels.

Historically the NTP has chosen to avoid discussions related to risk assessment in their technical reports. However, in the case of TBBPA there is in fact quite a bit of speculation related to human risk. If such speculation remains in the report then all of the above points should in fact be addressed in the NTP report to ensure a balanced presentation of the science.

References

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Environmental Canada Health Canada. Draft Screening Assessment Report Phenol, 4,4'-(1-methylethylidene) bis[2,6-dibromo-

Chemical Abstracts Service Registry Number 79-94-7

Ethanol, 2,2'-[(1-methylethylidene)bis[(2,6-dibromo-4,1- phenylene)oxy]]bis Chemical Abstracts Service Registry Number 4162-45-2 Benzene, 1,1'-(1-methylethylidene)bis[3,5-dibromo-4-(2- propenyloxy)-Chemical Abstracts Service Registry Number 25327-89-3 November 2012.

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