

**Response to: CLH Report, Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2 International Chemical Identification: 2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol (TBNPA), 14 June 2019**

**I. . Summary**

The brominated flame retardant, 2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol (TBNPA, TR-513, Trinol, CAS #36483-57-5) was assessed by the Norwegian Environment Agency for germ cell mutagenicity, carcinogenicity, and reproduction. Based on a read-across approach with the structural analog 2,2-bis(bromomethyl)propane-1,3-diol (BMP, FR-522, Dinol, CAS #3296-90-0) the CLH classification was proposed for mutagenicity of Muta 1B, H340 and for carcinogenicity of Carc 1B, H350. Classifications for developmental and reproductive toxicity were not determined as the results from the prenatal developmental toxicity study did not warrant classification. ICL believes that the proposed classification for mutagenicity and carcinogenicity is unjustifiably severe and the reproduction classification is unclear. Accordingly, ICL is providing comments on the proposed mutagenicity, carcinogenicity and reproduction classifications as proposed in the 14 June 2019 CLH report.

ICL believes that the proposed hazard category for germ cell mutagens 1B is too severe based on the TBNPA database. TBNPA and BMP share similarities in the *in vitro* mutagenicity assays but divergent results in the *in vivo* tests. BMP is positive *in vivo*. All experimental data indicates that TBNPA is not an *in vivo* genotoxin. This is a significant difference. It has immediate impact on the weight of the evidence approach that is needed in the assessment of the germ cell mutation hazard and in the subsequent classification. For the purpose of establishing hazard assessments for genotoxicity, the battery conducted for TBNPA is sufficient without the necessity of using a (Q)SAR approach which assumes a paucity of available data. The actual TBNPA genetic toxicity data negates the reliance on the (Q)SAR model prediction. Although a (Q)SAR approach comparing BMP and TBNPA appears reasonable, the specific genotoxicity data does not support its use in the classification of TBNPA germ cell mutagenicity.

ICL strongly believes that the classification has to be removed based on the lack of *in vivo* genetic damage shown experimentally, the differences between TBNPA and BMP with respect to *in vivo* genotoxicity and a weight of the evidence approach to the TBNPA genotoxicity battery.

ICL believes that the proposed hazard category for carcinogenicity 1B is also too severe. Although BMP and TBNPA were found to belong to the same (Q)SAR-based clusters for genotoxicity and carcinogenicity, the genotoxicity of TBNPA is only observed *in vitro* and the lack of *in vivo* gene mutation eliminates genotoxicity as a mode of action for potential carcinogenicity for TBNPA. In addition, the physicochemical properties differ in significant ways. Neither BMP nor TBNPA have direct data suggesting they are a known or presumed human carcinogen. In addition to the multiple species, multiple tumor sites, the genotoxicity of BMP (*in vitro* and *in vivo*) added the strength of evidence justifying the classification of category

1B. Genotoxic carcinogens tend to cross species lines and represent a potential human hazard. However, this is not the case with TBNPA, therefore, the classification of TBNPA should be no greater than as Category 2.

Moreover, a 13-week repeated dose oral toxicity study for TBNPA, was initiated per ECHA's decision # CCH-D-2114381478-36-01/F and is currently ongoing. Previously, it was found, that the kidney pathology in the 28-day toxicity study of TBNPA was different than the kidney pathology in the BMP 90-day toxicity study. A direct comparison is confounded by the different durations of the two studies

It will be clear if TBNPA and BMP are similar after this study is done, and hence we ask to postpone the classification for carcinogenicity until the study is completed within 7 months' time.

ICL agrees with the 14 June 2019 CLH report that there are no effects on developmental or germ cells that warrant classification for reproductive toxicity. However, several inconsistencies in data reporting and conclusion in the 14 June 2019 CLH report are present. A toxicity study evaluated many germ cell parameters and found no effect of TBNPA on these parameters. A prenatal developmental study that evaluated fertility, number of implantations, resorptions, live young and percentages of sex ratio and pre- and post- implantation loss found no effects. These studies were identified and described in separate sections of the CLH report but clarifications are needed in the final document on classification. Conclusions in some sections state the data is considered 'inconclusive' and in others 'not considered sufficiently severe to meet the criteria for classification'. ICL kindly request the references to the classification of reproductive toxicity to be uniformly expressed as 'not considered sufficiently severe to meet the criteria for classification'.

In summary, ICL recommends the proposed classifications be amended to Carcinogenicity Category 2 at a minimum, removal of the germ cell mutagen designation and clarification of the classification of reproductive toxicity.

## **II. GERM CELL MUTAGENICITY**

### **A Section 10.1 – 10.1.2 Germ Cell Mutagenicity (pages 7 – 10: Genetic Toxicology Data-base for TBNPA**

A number of both *in vitro* and *in vivo* genetic toxicity assays have been performed with TBNPA and this battery of tests is sufficient to define the mutagenicity of TBNPA.

These tests are briefly summarized individually and discussed below.

#### ***In vitro* Assays**

##### **1. Bacterial reverse gene mutation assay (Ames)**

#### **a. Ames test results**

The CLH report summarizes the study as follows: *“In the in vitro assays, one Ames test was included. In the presence of hamster S-9 mix however, there were clear evidence of mutagenic activity between 500 and 15 µg/plate with strains TA1535 and TA100. The test showed no evidence of mutagenic activity in the absence or presence of rat S-9 mix (Study report unnamed, 1996)”* Section 10.1.1 page 10

In this study (J Kitching. FR-513 Bacterial Mutation Assay. Huntingdon Life Sciences, Report DSB 94A/950638. 24 January 1996. Unpublished), FR-513 was assessed with and without metabolic activation in strains TA1595, TA1537, TA98 and TA100. Two independent mutation tests were performed in a pre-incubation assay. Three different metabolic activation systems were used. The traditional activation system, Aroclor-induced rat liver homogenate (S9) at 10% (v/v) in the S9 mix, an uninduced hamster liver S9 at 10% (v/v) and 30% (v/v) in the S9 mix.

No mutation was observed under the non-activated conditions in all strains. Likewise, no mutation was observed when the activation system was an Aroclor-induced rat liver homogenate, the traditional activation system used in this assay system.

The laboratory concluded that FR-513 was positive between 15 and 500 µg/plate with strains TA1535 and TA100 in the presence of hamster S9 with greater mutation frequency observed with the 30% S9 homogenate.

At the time, HLS used an evaluation criteria that judged a positive response when reproducible increases in revertants were at least 1.5 times the concurrent solvent controls. Since that time, the current evaluation criteria has changed. For TA1535 and TA1537 a positive response requires a 3-fold increase in revertants and for TA98 and TA100 a positive response requires demonstration of a 2-fold induction of revertants. Based on these criteria, the response in strain TA100 would be assessed as weakly positive in the 30% hamster S9 activation system and negative with the 10% hamster S9 activation system.

#### **i. Comment:**

S9 source: Rat liver has a high level of P450 enzymes that will either activate or inactivate promutagens. Aroclor induction increases the concentration of these types of metabolic enzymes in the rat liver. In contrast, hamster liver S9 has a different set of metabolic enzymes. The modification using hamster S9 was originally designed to increase the sensitivity of the Ames test to azo dyes and aromatic amines which have traditionally been negative in the Ames test. The hamster S9 enhances the reduction of azo-bonds leading to DNA reactive metabolites. Overall, the rat P450 system enhances oxidation. It must also be remembered that the enzyme systems that activate chemicals can also inactivate them, in particular by glucuronidation more prevalent in the rat liver activation system. Most alkyl halides are metabolically activated by P450 systems.

Strain specificity: Both TA1535 and TA100 carry the same defective histidine gene, *hisG46*. They both also contain the mutation in the *uvrB* gene making the strains deficient in DNA repair processes. In order to increase the sensitivity of the tester

strains, a plasmid, pKM101, has been inserted in TA1535 to create TA100. This plasmid codes for an error-prone repair process which results in increased sensitivity to mutagens. The presence of the error-prone repair system seemed to mitigate the mutagenicity of FR-513 rather than enhance it.

The *hisG46* gene is reverted through a change in base-pairing. It requires a mutation at the A/T site that results in an AT to GC conversion to restore histidine function. Henderson et al (2001) in a study of eosinophil peroxidase indicated that bromination of DNA can be taken up as 5-bromodeoxyuridine. This thymidine analog can mispair with guanine. This is a possible MOA for the gene mutation observed in the Ames tester strain TA1535. The error-prone repair process, also known as translesion synthesis, allows DNA replication machinery to replicate past damaged DNA. This involved the use of specialized translesion DNA polymerases that can insert bases at the site of damage. Some mechanisms of translesion synthesis introduce mutations. For example Pol  $\eta$  mediates error-free bypass of lesions induced by UV irradiation, whereas Pol  $\zeta$  introduces mutations at these sites. The lesion is most likely repaired in TA100, which would explain the positive response in TA1535 and the negative result in TA100.

## **ii. Comparison of TBNPA with BMP bacterial mutation results**

### **BMP results:**

In the two *Salmonella* assays reported in the NTP report (TR452), 2,2-bis(bromomethyl)-1,3-propanediol (BMP) gave a positive response in TA100 with metabolic activation from 30% Aroclor 1254-induced male Syrian hamster liver S9. These test conditions were not evaluated in TA1535. No mutation was observed in non-activated trials or in the presence of either 10% hamster S9 and 10% and 30% S9 from Aroclor-induced rats.

### **Comparison:**

It should be noted that in the TBNPA study, both 30 and 10% v/v hamster S9 was from uninduced hamster liver. The BMP studies used Aroclor induced hamster liver. Both sets of studies used a pre-incubation assay. In both studies, the high dose represented an experimentally derived dose based on toxicity. The difference in concentrations tested reflect the increased solubility of BMP (19.4 g/L) compared to TBNPA (1.93 g/L)

Because the NTP study only evaluated the 30% hamster S9 with TA100, only TA100 data is presented in Table 1. The results of BMP and TBNPA show the range of activity with the different fractions and sources of S9 activation systems. Positive results with either BMP or TBNPA are only observed with the 30% fraction of hamster S9. Neither of the responses are highly mutagenic in TA100. The results are remarkably similar. Under normal conditions for the conduct of a *Salmonella typhimurium* gene mutation assay, both BMP and TBNPA are negative. Positive results are only obtained when a high volume of hamster liver S9 is used. This set of conditions optimizes for azo or aromatic amines.



## 2. *In vitro* Mammalian Gene Mutation

### a. L5178Y Mouse lymphoma TK+/- assay

The CLH report summarizes the study as follows: “*The OECD TG 476 Mammalian cell gene mutation assay was positive. TBNPA was mutagenic in the test system with incubations in the presence of metabolic activation. The presence of S9-mix in both tests resulted an increase in mutation frequencies more than threefold and outside the labs historical data (no more detailed information about historical data is available in the registration). The increases were considered biologically relevant and TBNPA is considered mutagenic in vitro (Study report unnamed, 2004).*” Section 10.1.1 page 10

In this study (A.M. Verspeek-Rip. Evaluation of the mutagenic Activity of FR-513 in an *in vitro* Mammalian Cell Gene Mutation Test with L5178Y Mouse Lymphoma Cells (with independent repeat). NOTOX B.V. Report number 419311. 17 November 2004. Unpublished), FR-513 was evaluated in concentrations ranging from 10 to 500 µg/mL and 100 to 350 µg/mL for non-activated treatments and from 50 to 500 and 100 to 535 µg/mL for Aroclor-induced rat liver activation treatments in two independent trials based on concurrently derived cytotoxicity evaluations. The method of Amacher was used and mutant colonies were scored using microtiter plates. Both small and large colonies were scored.

In the absence of S9 activation, FR-513 did not induce a significant increase in the mutant frequency in the first experiment using 3 hour treatment times. This result was confirmed in a repeat experiment with 24 hour treatment times.

In the presence of S9 rat liver activation, FR-513 induced a 6.6-fold increase in the mutant frequency at the TK locus. FR-513 showed up to an 8-fold increase in small colonies and a 5.4-fold increase in large colonies. The second experiment confirmed the positive result for both small and large colonies.

#### i. Comment

The mouse lymphoma assay (MLA) is capable of detecting both gene mutation and chromosomal aberrations. The TFT-resistant colonies are divided into small and large colonies. Mutant cells that have extensive genetic damage tend to have prolonged doubling times and thus form small colonies. These small colonies have been associated with the induction of chromosomal mutations. In contrast, the large colonies have been shown to result from mutants with single gene mutations. FR-513 induced both large and small colonies indicating that in this assay system, both gene mutation and chromosomal aberrations were most likely induced.

In the MLA, the use of a global evaluation factor (GEF) has become common practice. For the MLA using scoring with the microwell plates, this factor is 126 plus the spontaneous background (Moore et al, 2006). Overall, an exposure of 100 – 200 µg/mL resulted in a positive response.

## ii. Comparison of TBNPA with BMP mammalian mutation results

BMP has not been evaluated in an *in vitro* mammalian gene mutation assay. Chromosomal damage will be discussed below.

### 3. *In vitro* Mammalian Chromosomal Aberration

#### a. *In vitro* Chromosome Aberration in Cultured Human Lymphocytes

The CLH report summarizes the study as follows: *“In the OECD TG 473 In vitro cytogenicity/ chromosome aberration study in mammalian cells TBNPA was found to be clastogenic in the presence of metabolic activation and at the highest test substance concentration (1000 microgram/ml) in the absence of metabolic activation. TBNPA has the potential to disturb mitotic processes and cell cycle progression (Study report unnamed, 2004).”* Section 10.1.1 page 10

In this study (C.A.F. Buskens. Evaluation of the Ability of FR-513 to Induce Chromosome Aberrations in Cultured Peripheral Human Lymphocytes (With Repeat Experiment). NOTOX Report Number 419322. 14 December 2004. Unpublished) FR-513 was evaluated for the potential to induce chromosomal damage in cultured primary peripheral human lymphocytes. Concentrations evaluated for chromosome damage ranged 333 to 1000 ug/mL without metabolic activation (two trials using a 3 hour exposure with 24 hour fixation) and from 33 to 200 ug/mL in a 24 hour exposure and 24 hour fixation and 10 to 66 ug/mL in a 48 hour exposure with 48 hour fixation. Activated trials were scored at concentrations of 100 to 1020 ug/mL in the first and 666 to 1150 ug/mL in the second trial using a 3 hour exposure time with 24 and 48 hours fixation, respectively. The activation was a 1.8% (in culture media) S9 fraction of Aroclor-induced rat liver homogenates. Mitotic indices were used to assess cytotoxicity.

In the absence of exogenous metabolic activation, FR-513 induced a statistically significant increase in the number of cells with chromosomal aberrations at the highest cytotoxic concentration (1000 ug/mL) when exposure was 3 hours whether gaps were included or excluded. FR-513 did not induce chromosomal aberrations the highest cytotoxic concentration when gaps were included or excluded after 24 hour exposure. It is general practice to exclude gaps. Polyploidy was observed at the highest concentration following the 3 hour exposure but not after the 24 hour exposure period. FR-513 did not induce any chromosomal damage following 48 hours of exposure.

In the presence of metabolic activation, FR-513 induced a statistically significant increase in the number of cells with chromosome aberrations at the lowest and highest (cytotoxic) tested concentrations both when gaps were included and excluded with a 24 hour fixation period. In the second trial with a fixation time of 48 hours, FR-513 induced a statistically significant increase in the number of cells with chromosome aberrations at an intermediate tested concentration when gaps were included only. Since the number of cells with chromosome aberrations was well within the laboratory's historical control data range, this increase was considered not to be biologically relevant.

It was noted that FR-513 increased the number of polyploid cells in the absence of activation (3 hour exposure) and in the presence of S9-mix. This may indicate that FR-513 has the potential to disturb mitotic processes and cell cycle progression, thereby inducing numerical chromosome aberrations. No increase in polyploidy was observed in the 24 hour treatment without metabolic activation.

Types of Damage: In the absence of metabolic activation utilizing a 3 hour exposure and a 24 hour fixation time excluding gaps, the primary type of damage at the high dose (1000 ug/mL and mitotic index of 40 or 46%) was chromatid breaks with a minor component showing chromosome breaks. Some exchanges were noted (6 and 5/200 in the two trials. Polyploidy was also observed (8 or 7/200 cells examined). No damage was noted when the cells were exposed for 24 hours with a fixation time of 24 hours or exposed for 48 hours with a fixation time of 48 hours.

In the activated trial (3 hour, 24 hour fixation) excluding gaps, the primary type of damage was chromatid and chromosome (break in both chromatids) breaks. Some minutes (single, usually circular, part of a chromatid lacking a centromere) were present. Two exchange figures were observed, as was polyploidy (5 per 200 metaphases scored). No significant damage was observed following a 48 hour fixation period.

#### **i. Comment**

In all experiments, either activated or non-activated, the types of damage observed were primarily chromatid or chromosomal breaks. More complex signs of damage were not present. These would include dicentric chromosomes, rings, exchange figures, interchanges or multiple aberrations per cell or endoreduplication. Polyploidy was observed which represents a chromosome number that is a multiple of the normal diploid number.

It would also appear that the longer the exposure and fixation time, the cells either have the time to repair these lesions or will die. Lesions were only observed following a 3 hour exposure and 24 hour fixation.

The finding of chromatid and chromosomal damage is consistent with the induction of the small colonies in the *in vitro* MLA which can detect chromosomal damage.

#### **ii. Comparison of TBNPA with BMP mammalian chromosomal aberration results**

##### BMP results

In an *in vitro* chromosomal aberration study in Chinese hamster ovary cells, 2,2-bis(bromomethyl)-1,3-propanediol (BMP), a dose-related increase in aberrations was observed in CHO cells treated in the presence of induced rat liver at a dose that induced marked cytotoxicity. A majority of the breaks which were observed in the aberration assay were located in the heterochromatic region of the long arm of the X chromosome. Only 100 metaphases were scored per dose.

SCEs were not induced. SCE is the reciprocal exchange of chromatin between two identical sister chromatids. This assay examines the ability of a test chemical to increase the exchange of DNA in duplicating chromosomes between two sister chromatids. In this assay, homologous recombination uses the nascent sister chromatid to repair potentially lethal DNA lesions. Homologous repair pathways are involved in the repair of double-strand breaks in DNA (those observed in chromatid and chromosome breaks). In the NTP report TR452, it states: “the type of damage pattern seen with 2,2-bis(bromomethyl)-1,3-propanediol (induction of chromosomal aberrations but not sister chromatid exchanges) is unusual. Most chemicals which induce aberrations also induce SCEs.”

Exposure for both studies was for 2 hours with and without S9. Harvest time for CA was 10-12 hours and for SCE was 25.5 hours. These studies were conducted in 1987 as part of a 108 coded chemical assessment of chromosomal aberration and sister chromatid exchange (SCE) in the Chinese hamster ovary cell.

#### Comparison:

Although the detailed data was not present for these studies, the dose range of both BMP and TBNPA were comparable in the induction of chromatid/chromosome breaks. The finding of an induction of small colonies in the MLA assay of TBNPA is also consistent with the *in vitro* findings of chromatid/chromosome breaks.

#### ***In vivo* Assays**

#### **4. Cytogenetics *in vivo***

##### **a. Mouse Bone Marrow Micronucleus Assay**

The CLH report summarizes the study as follows: “*In the in vivo mammalian somatic cell study TBNPA did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the mouse. Therefore, TBNPA can be considered to be non-mutagenic in this test (Study report, unnamed, 2007b).*” Section 10.1.1 page 10

In this study (N. Honarvar. Micronucleus Assay in Bone Marrow Cells of the Mouse with FR-513. RCC Report 1002501. 11 April 2007. Unpublished), FR-513 was evaluated for the potential to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the mouse. A single dose of 75, 150 or 300 mg/kg bw was administered by gavage (in corn oil) and bone marrow was harvested 24 hours post-dose. Bone marrow from one group, administered 300 mg/kg bw, was harvested 48 hours post-dose. Each group consisted of 5 males and 5 females. Doses were selected based on acute toxicity testing showing doses at and above 500 mg/kg bw resulted in the death of some animals after 48 hours. There were 2000 polychromatic erythrocytes (PCE) scored per animal.

The analysis of the blood plasma of animals treated with 300 mg test item per kg b.w. showed, that 1 h after treatment quantifiable amounts of the test item could be detected. The plasma of the animals contained between 38.7 and 65.6 ng test item per mL plasma. The samples from the 4 h

interval did not have any detectable levels of the test item. FR-513 did not have any cytotoxic effect on the bone marrow as assessed by PCE/NCE ratios.

Compared to the corresponding vehicle controls, there was no statistically significant or biologically relevant enhancement in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item. The mean values of micronuclei observed after treatment with FR-513 were generally below the values of the vehicle control group.

**Table 2. Frequency of micronucleated polychromatic erythrocytes in mouse bone marrow following treatment with TBNPA by gavage.\***

Dose (ppm)/hour	Micronucleated PCEs/1000 Cells		
	Male	Female	Combined**
0/24	1.4	1.6	1.5
75/24	1.0	0.7	0.85
150/24	0.9	1.3	1.1
300/24	1.5	1.0	1.25
300/48	1.1	1.2	1.15

\* 2000 PCEs scored per animal

\*\* as expressed in the report

### **i. Comment**

This assay will detect chromosomal damage. Micronuclei arise from acentric chromosomal fragments or whole chromosomes that remain in the PCE after the extrusion of the nucleus during maturation of the PCE from the immature erythroblast.

Fragments formed by chromosomal ‘breaks’ or ‘minutes’ as observed in the *in vitro* chromosomal aberration assay are readily detectable. The time frame, 24 to 48 hours post-dose) of the analysis is also consistent with the detection of the damage induced *in vitro* by FR-513. This study provides evidence that the event observed *in vitro* is not present in the whole animal. Acute toxicity and FR-513 plasma concentrations are evidence of bioavailability.

### **ii. Comparison of TBNPA with BMP in vivo micronuclei results**

#### **BMP results**

In a series of mouse micronucleus studies as reported in the NTP report TR452, 2,2-bis(bromomethyl)-1,3-propanediol (BMP) was also shown to be genotoxic *in vivo*. Significant increases in micronucleated normochromatic erythrocytes were observed in peripheral blood samples obtained from male and female mice exposed for 13 weeks to 2,2-bis(bromomethyl)-1,3-propanediol in feed. Bone marrow smears were immediately prepared at the end of the 13-week toxicity study. These increases were observed in the two highest dose groups of male mice (5,000 and 10,000 ppm, equivalent to 1,300 and 3,000 mg/kg bw/day) and the three highest dose groups of female mice (2,500 to 10,000 ppm, equivalent to 1,200 and 2900 mg/kg bw/day).

**Table 3. Frequency of micronucleated normochromatic erythrocytes in mouse peripheral blood following treatment with BMP in feed for 13 weeks.\***

Dose (ppm)	Micronucleated NCEs/1000 Cells	
	Male	Female
<b>0</b>	2.36	1.46
<b>625</b>	2.28	1.86
<b>1250</b>	2.55	1.86
<b>2500</b>	2.98	2.72*
<b>500</b>	3.80*	4.26*
<b>10000</b>	9.30*	11.81*

\* 10,000 NCEs scored per animal

In the first of two mouse bone marrow micronucleus tests performed to confirm the positive results seen in the 13-week feed study, inconsistent results were obtained between two trials, which used the same dose range of 100 to 400 mg/kg 2,2-bis(bromomethyl)-1,3-propanediol, administered by gavage three times at 24-hour intervals. Results of the first trial were negative (note only 2 of 5 males survived at the high dose); however, in the second trial, 2,2-bis(bromomethyl)-1,3-propanediol produced a clear, dose-related increase in micronucleated PCEs (note only 2 of 5 males survived at the high dose). Because the positive response was not reproduced, the results were concluded to be equivocal.

In an attempt to clarify the results obtained in the first bone marrow micronucleus test, a second investigation was performed using both male and female mice. 2,2-Bis(bromomethyl)-1,3-propanediol was administered as a single intraperitoneal injection (150 to 600 mg/kg) and bone marrow samples were taken 48 hours after dosing. The results of this experiment provides evidence of the ability of 2,2-bis(bromomethyl)-1,3-propanediol to induce micronuclei in bone marrow cells of female mice. Although male mice in all three dose groups showed a two-fold increase in the frequency of micronucleated PCEs (control 1.5 and 3.0 MN/1000 PCEs at 0 and 600 mg/kg respectively), the trend test was not significant due to the similarity in the responses, and pairwise analyses were also insignificant (an increase in 2-fold is biologically significant). The response in female mice was somewhat stronger (2.5-fold increase over background, at the highest dose, 2.0 and 5.2 MN/1000 PCEs PCEs at 0 and 600 mg/kg respectively) and was directly related to increasing doses of 2,2- bis(bromomethyl)-1,3-propanediol. These results were consistent with the stronger response observed in female mice (peripheral blood) in the 13-week feed study.

**iii. Comparison:** BMP was clearly positive in the mouse micronucleus assay by the oral and i.p. routes of administration and equivocal to positive in gavage studies. In contrast, TBNPA was negative. The TBNPA MN assay was an OECD guideline, GLP compliant study. The potential of TBNPA to induce chromosomal aberrations was not observed *in vivo*. Thus, what damage was detected *in vitro* was not induced *in vivo*. This is contrasted with the results of BMP which showed *in vivo* positive results for chromosomal damage.

## **b. *In vivo/in vitro* Unscheduled DNA Synthesis Assay (TBNPA)**

The CLH report summarizes the study as follows: “*In the OECD TG 486 Unscheduled DNA Synthesis (UDS) test with rat liver cells (liver hepatocytes) in vivo TBNPA did not induce any marked or toxicologically significant increases in the incidence of cells undergoing unscheduled DNA synthesis in isolated rat hepatocytes following in vivo exposure for 2 or 16 hr. Therefore, the test material was considered to be non-genotoxic under the conditions of the study (Study report, 2007a).*” Section 10.1.1 page 10

In this study (R. Durward. FR-513: *in vivo* Liver Unscheduled DNA Synthesis (UDS) Assay. Safefarm Laboratories report 0466/0269. 31 July 2007. Unpublished) FR-513 was evaluated for the potential to induce DNA repair in isolated rat hepatocytes following *in vivo* administration of 670 and 2000 mg/kg bw by gavage in arachis oil. Perfusion began either for 2 or 16 hours post-dosing. Animals were perfused for approximately 16 hours, livers excised and disassociated into single cell populations. Cultures were prepared, and cells were treated with methyl-<sup>3</sup>H-thymidine, washed and then autoradiographed to determine if DNA repair took place.

The mean nuclear grain counts, cytoplasmic grain counts and net nuclear grain counts/cell/slide for each animal was determined and the net nuclear grain count and percentage of cells in repair per slide for each animal reflected the induction of the DNA repair function.

FR-513 did not induce any marked increase in the incidence of cells in repair at either dose level or in either experiment. It was concluded that FR-513 did not induce unscheduled DNA synthesis in isolated rat hepatocytes following either 2 or 16 hours exposure.

### **i. Comment**

Where the micronucleus assay will detect chromosome breaks, the UDS will identify chromatid breaks. Unscheduled DNA synthesis measures the excision repair process by the incorporation of tritiated thymidine. This occurs after DNA is damaged, which may happen due to chemical damage to DNA at the site of covalent binding. It will also repair, via medium to long patch repair processes, small gaps in the DNA caused by certain chemical or physiological damage. Thus, this assay is capable to determining if the DNA damage observed *in vitro* is happening *in vivo*. The DNA damage obtained *in vitro* was not observed *in vivo*.

Based on the strain specificity observed in the *Salmonella typhimurium* assay, the UDS assay should have detected this type of gene mutation if it was occurring *in vivo*.

It should be noted that rat liver S9 was capable of activating TBNPA in the mammalian *in vitro* assays.

## **c. *In vivo* Comet Assay (BMP)**

The CLH report summarizes the study as follows: “*BMP induced oxidative stress and induced DNA damage in the urothelial cell line of Urotsa cells in two tests (Kong et al., 2011; 2013). No DNA damage was seen in an in vitro comet assay with primary hepatocytes (non-target) isolated from male SD rats (Kong et al., 2013). BMP increased DNA damage in urine bladder, but not in*

*liver in SD rats (Wada et al., 2014). In this study, BMP was administered orally over two days and bladders were sampled 3 hours after the second administration.”*

**i. Comment**

The comet assay evaluates, via a process of alkaline denaturation and gel electrophoresis, strand breaks in DNA in single cells. If there are single strand (or small double strand) breaks in the DNA, small fragments will be detected in this assay.

The BMP positive result in UROtsa cells *in vitro* were confirmed to be present in the urinary bladder cells *in vivo*. The analysis of the liver was performed *in vitro* on primary hepatocyte cultures and did not allow for a comparison of the *in vitro* to *in vivo* response.

**d. Comparison of TBNPA with BMP *in vivo* Responses**

TBNPA was positive for gene mutation and chromosomal damage *in vitro*. Additional testing showed that TBNPA did not induce genetic damage *in vivo*. In contrast, BMP was positive *in vitro* and additional *in vivo* testing showed that BMP induces genetic damage in the whole animal. The mouse MN test in both peripheral blood and in bone marrow indicated that chromosomal damage was present. Studies utilizing the Comet assay demonstrated that DNA damage observed *in vitro* was also observed in the whole animal.

This is a stark difference between BMP and TBNPA, although they share many structural similarities, do not have the same genotoxic effects *in vivo*. A ‘read-across’ approach for genotoxicity should not be used.

**B. Overall Weight of the Evidence for Genotoxicity of TBNPA**

TBNPA was positive in a bacterial mutation assay (Ames test) optimized for activation of azo or aromatic amines in *Salmonella* tester strains that detect base-pair gene mutation. TBNPA was negative without activation and was also negative when the standard S9-activation system was used.

TBNPA was positive with metabolic activation in the *in vitro* mouse lymphoma TK<sup>+/-</sup> assay and induced both large and small colonies, indicating that TBNPA was both mutagenic and clastogenic. This assay was negative without metabolic activation.

In *in vitro* human lymphocyte cultures, TBNPA was positive for clastogenicity with and without metabolic activation when exposed for 3 hours and with 24 hour fixation times. Exposures of longer duration or with longer fixation times, showed either no or very limited induction of chromosomal aberrations. The primary damage observed were chromatid and chromosome breaks. Thus, although TBNPA was positive in these two assays, the level and magnitude of damage was limited.

TBNPA was evaluated *in vivo* in the mouse micronucleus and unscheduled DNA synthesis (UDS) assays. TBNPA was negative. Thus, *in vivo*, TBNPA was a non-genotoxic. These two *in vivo* assays would have detected the types of chromosomal damage observed *in vitro*. It is

clear from these studies, that TBNPA although it has a potential to induce gene and chromosomal mutation, does not have genotoxic activity *in vivo*.

Based on this weight of the evidence approach, TBNPA would not be considered a somatic cell genotoxin. Since TBNPA does not have the intrinsic ability to mutate or damage chromatin *in vivo*, it is not likely that it has the potential to cause heritable effects in germ cells.

### **C. Section 10.1.2 – 3 Other relevant Information, Comparison with the CLP criteria for mutagenicity (page 10 - 11):**

#### **Read-across for mutagenicity**

CLH classified TBNPA as Germ Cell Mutagenicity Cat. 1B based on the structural activity relationship with BMP (2,2- bis(bromomethyl)propane-1,3-diol).

BMP was positive in a bacterial mutation assay (Ames test) optimized for activation of azo or aromatic amines in *Salmonella* tester strains that detect base-pair gene mutation. BMP was negative without activation and was also negative when the standard S9-activation system was used.

BMP was not evaluated in an *in vitro* mammalian gene mutation assay.

BMP was positive in an *in vitro* chromosomal aberration assay in Chinese hamster ovary cells. BMP induced a dose-related increase in chromosomal aberrations treated in the presence of induced rat liver at a dose that induced marked cytotoxicity. A majority of the breaks which were observed in the aberration assay were located in the heterochromatic region of the long arm of the X chromosome. In the same assay, SCEs were not induced.

BMP was evaluated in *in vivo* somatic cell chromosomal aberration assays. BMP was clearly positive in the mouse micronucleus assay by the oral and i.p. routes of administration.

BMP increased DNA damage *in vivo* in urine bladder, but not in liver in SD rats (Wada et al., 2014) using the comet assay. In this study, BMP was administered orally over two days and bladders were sampled 3 hours after the second administration. Thus, BMP was capable of inducing DNA damage *in vivo* in the bladder.

**Comparison of TBNPA and BMP:** Although there are structural relationships between TBNPA and BMP, the results of the genotoxicity studies are different. The *in vitro* assays, both bacterial and mammalian, show TBNPA and BMP have the potential of inducing gene mutation and structural chromosomal damage when metabolically activated. However, *in vivo* results diverge. None of the *in vivo* studies with TBNPA showed any induction of DNA or chromosomal damage. In contrast, BMP was positive in the mouse micronucleus assay as well as the comet assay following *in vivo* exposures.

The comparisons of the structural relationships and the use of the (Q)SAR approach is presented in the Carcinogenicity section of the CLH report, 10.2.1.1. In addition to the differences in the genetic toxicity data, there are also differences in the physiochemical properties of BMP and TBNPA. This is discussed in detail in Section III Carcinogenicity of this document.

**Table 4. Comparison of Genotoxic Endpoints of TBNPA and BMP**

Assay	TBNPA	BMP
<b>In vitro</b>		
<i>Salmonella typhimurium</i>	Negative: without activation Negative: with rat S9	Negative: without activation Negative: with rat S9
	Positive: with 30% hamster S9	Positive: with 30% hamster S9
<i>In vitro</i> mouse lymphoma – gene mutation, chromosomal aberration	Negative: without activation	
	Positive: with activation Gene mutation and Chromosomal aberration	
<i>In vitro</i> Chinese hamster ovary – chromosomal aberration		Negative: without activation Negative: SCE
		Positive: with activation
<b>In vivo</b>		
Mouse micronucleus	<b>Negative:</b> (gavage, multiple doses)	<b>Positive:</b> (feeding, i.p. bone marrow and peripheral blood)
Comet assay		Negative in liver <b>Positive</b> in bladder
UDS	<b>Negative:</b> liver	

As can be seen, TBNPA and BMP share similarities in the *in vitro* assays but divergent results in the *in vivo* tests. TBNPA is negative *in vivo*. BMP is positive *in vivo*.

The CLH concluded that TBNPA should be classified as Muta. 1B, H340. However, in the rationale it is clearly stated:

*TBNPA was clastogenic in human lymphocytes in vitro in the presence of metabolic activation and at the highest test concentration without metabolic activation, and mutagenic in mouse lymphoma cells in vitro in the presence of metabolic activation. In bacterial reverse mutation assays, mutagenicity was seen. Two in vivo tests with TBNPA were negative: a) in rat hepatocytes (UDS test) and b) micronucleus test in femur bone marrow cells of the mouse. We have no reproductive toxicity studies that indicate that TBNPA reaches the germ cells. The database is limited to a single prenatal developmental toxicity study. (10.1.3 page 11)*

[Note: The data is not limited to just the prenatal developmental study. In Section 10.3.1 Adverse effects on sexual function and fertility, the CLH report clearly states in reference to a 28-day toxicity study, “No treatment related changes in sperm count and motility were observed. Vaginal lavages which were taken early morning during the 3 week period from all females, prior to termination of the animals showed no treatment related changes in the oestrus cycle. In

*addition, there were no dose related changes in organ weight of ovaries, seminal vesicles, testis, ureter, uterus, vagina in comparison to control animals.” No measurements of gestation parameters in the developmental study sowed TBNPA to case any effect on implantation loss, litter size or weights.]*

The CLH report goes on to state regarding mutation:

*However, as described above, we propose to read across from the source substance BMP to the target substance TBNPA, see section 10.2.1 and table 10 (Data matrix for studies relevant for assessing germ cell mutagenicity, Analogue Approach). RAC states in the recent RAC-opinion for BMP that “there is positive evidence of somatic cell mutagenicity for BMP from in vitro/in vivo studies and evidence from the reproductive toxicity studies and that this supports that BMP reaches the (female) germ cells”. According to RAC “both facts in combination are sufficient to give ‘some’ evidence that the substance has the potential to cause mutations to germ cells”. RAC agreed that BMP should be classified as a germ cell mutagen, Cat. 1B; H340. We propose the same classification for TBNPA. (10.1.3 page 11)*

The CLH report considered the genotoxicity of TBNPA and BMP essentially the same.

*TBNPA and BMP have almost identical structure, similar physicochemical properties (table 11) and almost similar genotoxicity test results. We assume that the target substance TBNPA and the source substance BMP share the same toxic mode of action for genotoxicity. BMP and other brominated chemicals have been shown to be genotoxic in a spectrum of tests. It is hypothesized that the carcinogenic activity of brominated chemicals is due to genotoxic mechanisms (NTP, 1996). (10.2.1.1 page 15).*

This is not the case. **All experimental data indicates that TBNPA is not an *in vivo* genotoxin. This is not an insignificant difference. It has immediate impact on the weight of the evidence approach that is needed in the assessment of the germ cell mutation hazard and in the subsequent classification. (It also raises the question of the assumption of a similar mode of action for carcinogenicity.)** There are also significant differences in the structure and physicochemical properties as discussed in Section III Carcinogenicity, A Read across.

ICL recognizes the value of the (Q)SAR based approach when there is a paucity of data. Just because TBNPA and BMP are in the same clusters does not mean that the actual activity is the same. In the September 9-10, 2005 meeting of the Expert Working Group on Hazard Identification and Risk Assessment in Relation to *in vitro* Testing, they write:

*Chemicals with structural alerts for mutagenicity but with negative results in an initial regulatory battery would usually not require additional testing, provided that the initial battery is sensitive to the type of effect indicated by the alert. The Working Group agreed that a structural alert can raise a concern, but study data are usually the final arbiter of hazard. However, if a chemical is in a structural class known to give positive results in*

*specific genotoxicity tests or under specific experimental conditions that were not employed, then additional testing that includes these specific tests or conditions should be conducted. (Thybaud et al, 2007)*

ICL agrees with this conclusion. TBNPA has been sufficiently evaluated in a series of GLP, OECD guideline genotoxicity studies. This battery consisted of *in vitro* bacterial and mammalian tests, using both gene mutation and chromosomal endpoints. *In vivo* rodent assays were employed that would have detected the damage observed *in vitro* on both a gene mutation and chromosomal basis. The results obtained did not warrant further germ cell testing. **For the purpose of establishing hazard assessments for genotoxicity, the battery conducted for TBNPA is sufficient without the necessity of using a (Q)SAR approach which assumes a paucity of available data.**

#### **D. Section 10.1.3 Comparison with the CLP criteria for mutagenicity (page 10 - 11):**

##### **Mutation Hazard Class and Category Code**

The Guidelines for the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) stipulates that to arrive at a classification, 1) test results are considered from experiments determining mutagenic and/or genotoxic effects in germ and/or somatic cells of exposed animals...*in vitro* tests may also be considered, 2) the system is hazard based, classifying substances on the basis of their intrinsic ability to induce mutations in germ cells, and 3) the classification for heritable effects in human germ cells is made on the basis of well conducted, sufficiently validated tests. Evaluation of the test results should be done using expert judgement and all the available evidence should be weighed for classification.

These studies have been performed, individually assessed and described above and a weight of the evidence approach was used to assess the hazard. TBNPA does not meet this criteria.

Based on the ‘Note: Substances which are positive in *in vitro* mammalian mutagenicity assays, and which also show structure activity relationship to known germ cell mutagens, should be considered for classification as Category 2 mutagens.’

BMP was assessed a germ cell mutagen as stated above “*there is positive evidence of somatic cell mutagenicity for BMP from in vitro/in vivo studies and evidence from the reproductive toxicity studies and that this supports that BMP reaches the (female) germ cells*” (10.1.3 page 11). This classification was not based on the results of a positive *in vivo* heritable germ cell assay in mammals, or a metabolite capable of interacting with germ cell genetic material, or positive results showing effects in humans. BMP caused reduced fertility in a 2-generation reproduction study. This study was evidence that the BMP reached the ovary but did not show that there as any interaction of the genetic material in the ovary. Decreases in fertility and altered histopathology of the ovary are indications of reproductive toxicity but not germ cell mutation. The etiology of fertility effects is diverse and can include biochemical, enzymatic and hormonal modes of action. It is not clear as to the scientific judgement that resulted in the conclusion that genetic material from germ cells was damaged. The classification of 1B for BMP appears to be overly cautious.

Thus, the (Q)SAR approach resulting in the ‘read-across’ to BMP based on the fertility effect being of genotoxic nature does not apply to TBNPA. In addition, the *in vivo* gene mutation potential of BMP and TBNPA are very different. Therefore, it is not warranted to classify TBNPA as Category 1B to match the classification of BMP.

Based on a prenatal SD rat study, TBNPA was determined by CHL to not warrant classification for reproductive toxicity. Germ cells were evaluated in the toxicity study and no fertility effects were observed in the rat developmental study. Thus, for TBNPA, there is evidence that the germ cell was not affected by exposure in the rat. This is discussed further in Section IV. Reproduction.

**Using the available experimental data from the OECD guideline, GLP studies, ICL asserts that TBNPA should not be categorized as a potential human germ cell mutagen.** It shows neither *in vivo* genotoxicity nor effects in rodent germ cells. Category 2 requires that positive evidence be obtained from experiments in mammals and/or in some cases from *in vitro* experiments. There is clearly no evidence that TBNPA causes somatic cell mutagenicity *in vivo* in mammals or in other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays. TBNPA induces genetic damage only in *in vitro* endpoints. It should not be classified as Category 2 based on any experimentally derived TBNPA data.

#### **E. Section 10.1.4 Conclusion on classification and labelling for mutagenicity**

As stated in the 14 June 2019 CHL report, “TBNPA should be classified as Muta. 1B, H340”

ICL concludes that this proposal should be removed. TBNPA does not warrant a classification of Mut 1B. Although under a precautionary principle approach, TBNPA could be classified as Category 2 based on a read-across approach with BMP. However, since TBNPA shows neither *in vivo* mutagenicity nor fertility effects, the factors raising the classification for BMP do not exist for TBNPA. **Therefore, TBNPA does not warrant a Germ Cell Mutagenicity classification.**

### **III CARCINOGENICITY**

#### **A. Section 10.2.1 Read across for mutagenicity and carcinogenicity**

1. ICL has no comments with respect to the use of the read-across approach for animal carcinogenesis. No oncogenicity studies have been performed for TBNPA and therefore, it is reasonable to extrapolate the results from other structurally related molecules to predict potential animal carcinogenicity. ICL does not believe that it is scientifically valid to extrapolate a genotoxic mode of action from BMP as only *in vitro* genetic damage was observed in TBNPA. Additionally, the other TBNPA studies demonstrate that germ cells are not a target of toxicity as evidenced by normal morphology and function. These two factors, genotoxicity and germ cell

damage, used to increase the level of concern for BMP are not experimentally present for TBNPA. A weight of the evidence approach for TBNPA must be taken when determining carcinogenesis classification.

### **B. Section 10.2.1.1. Hypothesis for the analogue approach (page 11)**

2. The CHL report states, “*TBNPA and BMP have almost identical structure, similar physicochemical properties (Table 11) and almost similar genotoxicity test results. We assume that the target substance TBNPA and the source substance BMP share the same toxic mode of action for genotoxicity. BMP and other brominated chemicals have been shown to be genotoxic in a spectrum of tests. It is hypothesized that the carcinogenic activity of brominated chemicals is due to genotoxic mechanisms (NTP, 1996). This corresponds to the RAAF scenario 2.*” (10.2.1.1, page 15)

Table 10: Data Matrix, Analogue Approach (10.2.1.1, page 13) clearly shows, as does Table 4 of this document, the difference between BMP and TBNPA with respect to *in vivo* genotoxicity. Although they may appear almost similar, BMP is positive in all *in vivo* tests and TBNPA is negative in the two *in vivo* tests (one defining gene mutation and the other chromosomal damage). This is a significant difference. This is discussed in greater detail in Section C of this document.

Likewise, there are significant differences in the structure and physicochemical properties as listed in Table 11 of the CHL report (10.2.1.1, page 16). The additional hydroxyl group and the fewer bromide groups can make a large difference in biological reactivity. It should be noted that the melting freezing point is 68.96 compared to 109°C for TBNPA and BMP respectively. The relative density of TBNPA is nearly twice that of BMP. Water solubility is nearly 10-fold greater for BMP and the Log Kow (partition coefficient) for TBNPA is twice that of BMP. Because the partition coefficient is expressed as a log scale, this difference represents over an order of magnitude difference. The CLH report, therefore, incorrectly asserts that the physicochemical properties are similar. As shown, only the physical state at 20°C and the vapour pressure are analogous, all others differ significantly. The differences in solubility and partition coefficient can also have a significant impact on biological activity.

ICL does not agree with the ‘Score’ assigned to each of the analogue assessments as shown in Tables 12 and 13 of the CLH report. In Table 12, the AE A.3 Source study is provided as the NTP study and assigned a score of 5. Studies done by NTP at this time had significant problems with purity of the test samples. In the case of BMP (Table 13), the purity was only 78.6%. The presence of TBNPA in the sample does not mitigate the low purity of BMP. The lack of sufficient purity of the BMP should result in a much lower score, yet the score assigned was 5, the highest score.

Also presented in Table 13 was a score of 4 for the A.2.2 Underlying mechanism, qualitative aspects. This score was assessed based on BMP and TBNPA sharing the same genotoxic properties and the assumption that they also shared the same toxic mode of action. As discussed above in this document, TBNPA does not share the same mutagenic properties. This score is far too high considering that TBNPA does not have genotoxic activity in *in vivo*.

Likewise, the assessment in AE 2.3 Underlying mechanism, quantitative aspects is equally high as assessed at 4. If there are poor qualitative aspects, the quantitative aspects cannot be established. Based on actual data, the genotoxic responses are not similar. BMP is an *in vivo* mutagen but TBNPA is not mutagenic *in vivo*. This is a significant difference and does not warrant a score of 4.

For AE 2.4 Other compounds, a score of 3 is too high for an assessment of the comparison to metabolic pathways. The speculation that the activation/detoxification route of TBNPA is similar to BMP is based solely on speculation and not supported by data. Nearly all xenobiotic chemicals are detoxified through some form of glucuronidation. It is the activation pathway that is important in determining similarity of underlying modes of action. No experimental evidence exists for BMP showing activation of BMP to a presumptive mutagen or carcinogen.

For A.2.5 Other effects it states that the ‘mechanism of carcinogenicity for the source substance is not described beyond genotoxicity. ICL has clearly documented that the genotoxicity of TBNPA and BMP are not the same. A.2.5 goes on to state that the data for BMP demonstrates an induction of oxidative DNA damage. This is speculation (discussed below) and does not warrant a score of 4.

The CHL proposed assessment assumed that the target substance TBNPA and the source substance, BMP, share the same toxic mode of action for genotoxicity. This has not been experimentally established. There are hypotheses that the mode of action is the induction of oxidative DNA damage, but this is speculation. Based on the lack of *in vivo* genotoxicity, it is clear that there are substantive differences in the mode of action or the metabolism and distribution of TBNPA. The NTP report (TR452) was finalized in 1996. NTP speculated that the carcinogenic activity of BMP could be due to oxidative damage to DNA or the C-Br bond is broken leaving the carbon containing electrophilic group to form DNA adducts with subsequent damage. Additional work by NTP to isolate and identify any DNA lesion was not undertaken. In the last 20-plus years since the drafting of the NTP report, many modes of action leading to tumor formation have been elucidated. At the time of the writing of the NTP report, they could only speculate on the mode of genotoxic action and assumed that the BMP was resulting in tumors due to a genotoxic mode of action. It is still speculation.

Again, the lack of *in vivo* genotoxicity of TBNPA strongly suggests that TBNPA will not act via a genotoxic mode of action.

### **C. 10.2.2 – 10.2.3 Comparison with the CLP criteria for carcinogenicity (page 17), Conclusion (page 18)**

There is sufficient evidence of the carcinogenicity of BMP in the rat and mouse as reported in the NTP TR452 report. No information exists with respect to the activity of BMP in humans. The RAC agreed to classify BMP as Carc. 1B; H350.

A read across approach was taken from BMP. No carcinogenicity study was available for TBNPA. However, the lack of *in vivo* genotoxicity of TBNPA discounts the direct relevance of the animal tumors observed with BMP and the potential TBNPA carcinogenicity in humans. The read across approach is only as valid as the (Q)SAR model and the data inputs used. If all

the carcinogenicity data used was from either BMP or DBP, then any short chain alkyl molecule with hydroxyl groups and bromides would be an alert. Because of this uncertainty, TBNPA would be more logically classified as Carcinogen Category 2.

**Conclusion:** ICL believes that the proposed hazard category for carcinogenicity 1B is also too severe. Although BMP and TBNPA were found to belong to the same (Q)SAR-based clusters for genotoxicity and carcinogenicity, the genotoxicity of TBNPA is only observed *in vitro* and the lack of *in vivo* gene mutation eliminates genotoxicity as a mode of action for potential carcinogenicity for TBNPA. Neither BMP nor TBNPA have data suggesting they are a known or presumed human carcinogen. The genotoxicity of BMP (*in vitro* and *in vivo*) added the strength of evidence for classification of Carc. 1B. Genotoxic carcinogens tend to cross species lines and represent a potential human hazard. This is not the case with TBNPA, therefore **TBNPA should be classified as Category 2**

Moreover, a 13-week repeated dose oral toxicity study for TBNPA, was initiated per ECHA's decision # CCH-D-2114381478-36-01/F, and is currently ongoing. Previously, it was found, that the kidney pathology in the 28-day toxicity study of TBNPA is different than the kidney pathology in the BMP 90-day toxicity study. BMP shows renal papillary degeneration and urinary hyperplasia whereas TBNPA showed an increase in minimal tubular basophilia a typical background finding in the rat, considered to be non-adverse. TBNPA showed no effects in the bladder, however, the BMP 90-day study observed urinary bladder hyperplasia in 9 of 10 males. It will be clear if TBNPA and BMP are similar after this study is done and the data assessed. Hence, we ask to postpone the classification decision until the study is completed within 7 months' time.

#### IV. REPRODUCTION

##### A. 10.3.1 Adverse effects on sexual function and fertility (page 18)

When referring to TBNPA, the CHL report states, “*Effects on fertility have not been assessed as no relevant studies are available, except for a 28-day repeated dose toxicity study where no relevant effects were identified.*” (10.3.1 page 18)

The 28-day study showed:

*The results showed no systemic toxicity effects and the No Observed Adverse Effect level (NOAEL) was determined as >500 mg/kg/day (highest dose tested). No treatment related changes in sperm count and motility were observed. Vaginal lavages which were taken early morning during the 3 week period from all females, prior to termination of the animals showed no treatment related changes in the oestrus cycle. In addition, there were no dose related changes in organ weight of ovaries, seminal vesicles, testis, ureter, uterus, vagina in comparison to control animals.* (page 18)

ICL agrees with this assessment of the results of this study. However, the statement above implies that no other relevant studies are available.

In section 10.3.2 Adverse effects on development, the prenatal developmental toxicity study in SD rats is described. This study provides information on fertility and reproduction function. In the rubric in Table 14 it states:

*Mean number of live pups (litter size): Embryo-fetal survival was considered to have been unaffected by treatment at 100, 300 or 1000 / 500 mg/kg/day with mean numbers of implantations, resorptions, live young and percentages of sex ratio and pre- and post-implantation loss being similar to control values across all treated groups. (10.3.2, Table 14 page 19)*

The developmental study begins dosing, generally at gestation day 6 (this study in particular), assessment of mating and fertility indexes are not assessed. However, the viability of the implantation sites, resorptions of unviable conceptus and mean litter weights are all indications of reproductive function and can define loss of fertility. As such, aspects of fertility can be assessed from GD6 through delivery.

In this study (S. Renaut, FR-513: Study for Effects on Embryo-Fetal Development in Sprague-Dawley Rats by Oral Gavage Administration. Envigo CRS study number YK56MT. 15 December 2016),

All females reaching scheduled termination were pregnant with a live litter on Day 20 of gestation. The two females receiving 1000 mg/kg/day killed for reasons of animal welfare before the dose was lowered to 500 mg/kg/day were both confirmed as pregnant. The assessment of litter data is therefore based on a total of 20, 20, 20 and 18 females at scheduled termination on Day 20 of gestation at 100, 300 or 1000 / 500 mg/kg/day, respectively. Embryo-fetal survival was considered to have been unaffected by treatment at 100, 300 or 1000 / 500 mg/kg/day with mean numbers of implantations, resorptions, live young and percentages of sex ratio and pre and post-implantation loss being similar to Control values across all treated groups.

Mean placental, male, female and overall fetal weights at 100, 300 or 1000 / 500 mg/kg/day were similar to Controls and unaffected by treatment. At 300 mg/kg (mid-dose) there was a slightly increased incidence of delayed or incomplete ossification of pelvic bones compared to concurrent control animals but the incidence was within the laboratories historical control data range.

It needs to be noted that in the BMP 2-generation reproductive study, BMP exposure caused significantly decreased numbers of litters, pups born alive per litter and pup weights in mice (as reported in the NTP report TR452). These endpoints were not observed in the TBNPA developmental study in rats.

#### **B. 10.3.5 CONCLUSION ON CLASSIFICATION AND LABELLING FOR REPRODUCTIVE TOXICITY (page 19)**

The CHL report concluded “*The data for reproductive toxicity is inconclusive. The results from the repeated dose toxicity studies do not warrant classification. The results from the prenatal developmental toxicity study do not warrant classification.*” (10.3.5 page 20)

ICL agrees.

ICL would ask that in Table 4 (page 5) that the item for **Reproductive toxicity** read ‘**Does not warrant classification**’ rather than ‘Data inconclusive’.

## V. CONCLUSION:

ICL believes that the proposed hazard category for germ cell mutagens 1B is too severe based on the TBNPA database. TBNPA and BMP share similarities in the *in vitro* mutagenicity assays but divergent results in the *in vivo* tests. BMP is positive *in vivo*. All experimental data indicates that TBNPA is not an *in vivo* genotoxin. This is a significant difference. It has immediate impact on the weight of the evidence approach that is needed in the assessment of the germ cell mutation hazard and in the subsequent classification. For the purpose of establishing hazard assessments for genotoxicity, the battery conducted for TBNPA is sufficient without the necessity of using a (Q)SAR approach which assumes a paucity of available data. The actual TBNPA genetic toxicity data negates the reliance on the (Q)SAR model prediction. Although a (Q)SAR approach comparing BMP and TBNPA appears reasonable, the specific genotoxicity data does not support its use in the classification of TBNPA germ cell mutagenicity.

ICL strongly believes that the classification be removed based on the lack of *in vivo* genetic damage shown experimentally, the differences between TBNPA and BMP with respect to *in vivo* genotoxicity and a weight of the evidence approach to the TBNPA genotoxicity battery.

ICL believes that the proposed hazard category for carcinogenicity 1B is also too severe. Although BMP and TBNPA were found to belong to the same (Q)SAR-based clusters for genotoxicity and carcinogenicity, the genotoxicity of TBNPA is only observed *in vitro* and the lack of *in vivo* gene mutation eliminates genotoxicity as a mode of action for potential carcinogenicity for TBNPA. In addition, the physicochemical properties differ in significant ways. Neither BMP nor TBNPA have direct data suggesting they are a known or presumed human carcinogen. The genotoxicity of BMP (*in vitro* and *in vivo*) added to the strength of evidence in the classification of category 1B. Genotoxic carcinogens tend to cross species lines and represent a potential human hazard. This is not the case with TBNPA, therefore the classification of TBNPA should be no greater than as Category 2.

ICL agrees with the 14 June 2019 CLH report that there are no effects on developmental or germ cells that warrant classification for reproductive toxicity. However, several inconsistencies in data reporting and conclusion in the 14 June 2019 CLH report are present. A toxicity study evaluated many germ cell parameters and found no effect of TBNPA on these parameters. A prenatal developmental study that evaluated fertility, number of implantations, resorptions, live young and percentages of sex ratio and pre- and post- implantation loss found no effects. These studies were identified and described in separate sections of the CHL report but clarifications are needed in the final document on classification. Conclusions in some sections

state the data is considered 'inconclusive' and in others 'not considered sufficiently severe to meet the criteria for classification'. ICL would like the references to the classification of reproductive toxicity to be uniformly expressed as 'not considered sufficiently severe to meet the criteria for classification'.

In summary, ICL recommends the proposed classifications be amended to Carcinogenicity Category 2 at a minimum, removal of the germ cell mutagen designation and clarification of the classification of reproductive toxicity. ICL would also request that the proposed classification for carcinogenicity be postponed until the completion of the 90-day toxicity study.

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