Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol

EC Number: 253-057-0
CAS Number: 36483-57-5; 1522-92-5

CLH-O-0000006818-61-01/F

Adopted

11 June 2020

11 June 2020

CLH-O-0000006818-61-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol**

EC Number: **253-057-0**

CAS Number: **36483-57-5; 1522-92-5**

The proposal was submitted by **Norway** and received by RAC on **14 June 2019.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Norway has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **24 July 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **24 September 2019**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Christina Tsitsimpikou**

Co-Rapporteur, appointed by RAC: **Nikolaos Spetseris**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **11 June 2020** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	603-RST-VW-Y	2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol	253-057-0	36483-57-5; 1522-92-5	Muta. 1B Carc. 1B	H340 H350	GHS08 Dgr	H340 H350			
RAC opinion	603-RST-VW-Y	2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol	253-057-0	36483-57-5; 1522-92-5	Muta. 2 Carc. 1B	H341 H350	GHS08 Dgr	H341 H350			
Resulting Annex VI entry if agreed by COM	603-RST-VW-Y	2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol	253-057-0	36483-57-5; 1522-92-5	Muta. 2 Carc. 1B	H341 H350	GHS08 Dgr	H341 H350			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol (TBNPA) is a small brominated alkyl alcohol. TBNPA is a REACH registered substance and according to the information published on the ECHA dissemination site, this substance is used in the manufacture of polymers, plastic products and chemicals, as well as an intermediate. TBNPA is a reactive flame retardant in polymer synthesis (100-1000 tonnes per year, tpa) for the manufacture of plastic products and chemicals. It is used in industrial, professional and consumer settings in formulation and use of commercial mixture(s). The substance currently does not have an Annex VI entry according to the CLP regulation. The dossier submitter (DS) evaluated Germ Cell Mutagenicity, Carcinogenicity, Reproductive Toxicity, Specific Target Organ Toxicity after Repeated Exposure and proposed classification as Carc. 1B and Muta. 1B.

In the CLH dossier, extensive use is made of read-across of data from another brominated alkyl alcohol, 2,2-bis(bromomethyl)propane-1,3-diol (BMP), recently evaluated by RAC (CLH-O-0000001412-86-212/F, adopted June 2018). The read-across of data is mainly based on a document by the Danish Environmental Protection Agency, which grouped several brominated flame retardants. RAC agrees with the proposed read-across and also extends it to include 2,3-dibromo-1-propanol (2,3-DBPA), which is a structurally similar brominated flame retardant with a harmonised classification and labelling. The reasoning for the use of read-across of data is explained and justified as follows.

Read-across

The toxicological data presented in the CLH dossier and/or available in the open literature for TBNPA are very limited and the available studies are the following (see more details in the table below):

1. a 28-day oral repeated dose toxicity (RDT) study in rats with a 14 day recovery period where some relevant reproduction parameters were investigated (Anonymous, 2015; REACH registration dossier)
2. an OECD TG 414 pre-natal developmental toxicity study on female Sprague-Dawley (SD) rats (20 /dose) requested by ECHA on 2015 (TPE-D-21 I43LO292-65-OUF), (Anonymous, 2016)
3. a 14-day repeated dose oral toxicity by gavage in rats used as supporting study in the evaluation of STOT RE (Anonymous, 2011)
4. a non-guideline, 30-day feeding study in rats used as supporting study in the evaluation of STOT RE (Anonymous, 1973)
5. Several *in vitro* mutagenicity studies in bacteria and mammalian cells and 2 *in vivo* mutagenicity studies

In addition, a sub-chronic toxicity study (90-day) by oral route (EU B.26./OECD TG 408) in rats was also requested by ECHA with a deadline of submission 25th March 2020 (CCH-D-21 L43BI47B-36-0UF). Industry submitted the study, which became available to RAC on 23rd March 2020.

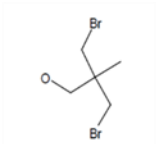
Therefore, the need for identifying substances with similar chemical structure and toxicological properties has arisen in order to evaluate the human health hazards discussed in the CLH dossier.

As mentioned above, the DS proposed read-across from BMP. RAC, following suggestion from a commenting Member State Competent Authorities (MSCA) during the general consultation, has

also identified another similar substance, 2,3-DBPA, which has a harmonised classification and labelling (Annex VI, Index number 602-088-00-1, CLP 00) and could also be used for read-across for the classification of TBNPA.

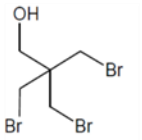
2,3-DBPA is mentioned in the CLH dossier as member of the Small Brominated linear and branched Alkyl Alcohols (SBAA) group described by the Danish Environment Protection Agency (DEPA) in its respective report entitled "Category approach for selected brominated flame retardants - preliminary structural grouping of brominated flame retardants" (Wedebye *et al.,* 2016). This SBAA group was originally predicted by a number of (Q)SAR models including the OECD QSAR Toolbox. The members of the SBAA group (61 identified in the DEPA report) had *a priori* very similar chemical structures with 3-5 carbons, 2-3 bromine atoms and 1-2 alcohol groups (see the following Figure).

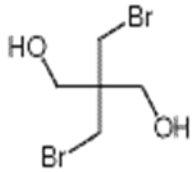
Figure*:* Chemical structures and identifiers of TBNPA, BMP and 2,3-DBPA



The most prominent members of the SBAA group

Dominant chemical structure of the SBAA group





2,2-dimethylpropan-1-ol, tribromo derivative; 3-bromo-2,2-bis(bromomethyl)propan-1-ol (TBNPA)
EC 253-057-0
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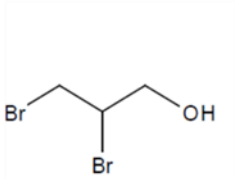

2,2- bis(bromomethyl)propane-1,3-diol (BMP)
CAS 3296-90-0

2,3-Dibromo-1-propanol (2,3-DBPA)
CAS 96-13-9

Regarding chemical similarity between TBNPA and BMP the following can be noted:

- In TBNPA, one OH group is replaced by one Br, making TBNPA less symmetric, more polarized and more reactive compared to BMP
- In both substances all the Br and OH groups are attached to primary carbons (labile C-Br bond, reactive hydroxyl groups)
- Both substances share a common 5-carbon backbone

Regarding chemical reactivity of TBNPA and BMP the following can be noted:

- Both substances share similar electrophilic properties of the base molecule
- For both substances, nucleophilic substitution of the Br (more labile) can take place and/or of the OH group, when enzymatically activated
- For both substance, radical activation is possible, which constitutes also an alert for a genotoxic mechanism
- For both substances, the aliphatic halogen is a structural alert both for carcinogenicity and mutagenicity

Regarding chemical similarity between TBNPA and 2,3-DBPA the following can be noted:

- Br and OH groups attached to primary carbons
- Both substances share a common 3-carbon backbone

Nevertheless, the chemical structure of 2,3-DBPA, which comprises 2 carbon atoms less than both TBNPA and BMP, has a Br group on a secondary carbon, vicinal both to a primary carbon

Br and primary carbon OH group, which renders 2,3-DBPA more reactive compared to both TBNPA and BMP and through different mechanisms, which probably do not operate in the other 2 SBAAs. More specifically, dehydrohalogenation of 2,3-DBPA has been experimentally proven by the detection of 2-bromoacrylic acid as a metabolite. In addition, oxidation to form epoxides, which can enter different metabolic pathways, can also take place. Despite this, all the mechanisms and the structural alerts described above for TBNPA and BMP cannot be excluded for 2,3-DBPA

In the DEPA report, it is explained that all members of the SBAA group have specific structural alerts for mutagenicity and carcinogenicity, for example the "aliphatic halogen" (alert for *in vitro* and *in vivo* mutagenicity and carcinogenicity in the OECD QSAR Toolbox. This alert identified 34% false positives among the mutagenicity training set chemicals (Kazius *et al.,* 2005). According to Benigni *et al.* (2008 and 2010), this alert has a positive predictivity for carcinogenicity of 74%. Nevertheless, as there are multiple chemical reactions possible in a biological system, it does not seem that there is one single mechanistic interpretation to explain this alert in relation to mutagenicity and cancer. Some alerts were identified in all the SBAA group members and/or their metabolites pointing to possible common mechanism(s) of action (e.g. metabolic activation to reactive carbonyl compounds and aldehyde Schiff-base formation of DNA adducts and cross-links). In addition, the same nuclear substitution (S_N2) reaction mechanism, which has been proposed as the primary method of DNA alkylation, is expected to be shared due to the presence of the bromide group (Sobol *et al.,* 2007). In the DEPA report, BMP and TBNPA were found to belong to the same (Q)SAR-based clusters identified for genotoxicity and carcinogenicity, while 2,3-DBPA (which has harmonised classification as Carc. 1B and Repr. 2) did not result in the same clusters . For reproductive toxicity the three substances are in separate clusters (positive predicted indications in several reproductive toxicity models). All three substances have similar profiles for endocrine activity and skin sensitization (positive predicted indications for airway allergy).

The physico-chemical and structural properties for TBNPA, BMP and 2,3-DBPA that are of interest in the present opinion are summarised in the following table. The physico-chemical properties between these 3 SBAAs present similarities and differences, with some properties of 2,3-DBPA lying between the values reported for TBNPA and BMP (relative density, LogP, ALogP). TBNPA is considerably less soluble compared to BMP and 2,3-DBPA. Nevertheless, availability of TBNPA in biological fluids, where the temperature is higher (around 36°C compared to 20°C), is expected to be enough for the substance to exert similar toxicological effects as BMP and 2,3-DBPA. These toxicological effects are due to the similar chemical structure/functional groups and comparable physicochemical properties of these three SBAAs.

***Table**: Summary of physico-chemical properties and structural features for TBNPA, BMP and 2,3-DBPA*

Property	TBNPA [1,2]	BMP [1,2,3]	2,3-DBPA [2,4]
Physical state at 20°C and 101.3 kPa	Solid, white to off-white flakes	Off white crystalline powder, odourless	Clear colourless to slightly yellow viscous liquid
Melting point	Melting / freezing point at 101 kPa: 68.96 °C	Melting / freezing point at 101 kPa: 109 °C	-
Flash point	-	-	> 235 °F (113 °C)
Boiling point	-	270 °C at 101 kPa	426 °F (219 °C) at 760 mm Hg (101 kPa)
Relative density	2.286 at 20°C	1.2 at 20°C	2.120 at 20 °C/4 °C
Vapour pressure	0±0.21 kPa at 25°C	0.85 kPa at 25 °C	1 mm Hg (0.13 kPa) at 134.6 °F (57 °C)
Polar surface area	20.23	40.46	20.23
Water solubility	1.93 g/L at 20 °C	19.4 g/L at 20 °C	50 to 100 g/L at 68° F (at 20 °C)
Partition coefficient n-octanol/water Log Kow (Log Pow)	2.6 at 22.5°C (2.47)	0.85 (1.06)	- (1.13)
ALogP[5]	2.15	0.75	1.14
Hydrogen bond acceptors	1	2	1
Hydrogen bond donors	1	2	1
Rotable bonds	4	4	2
Lipinski score[6]	0	0	0
Molecular weight	324.8	261.9	217.9
Parent atom counts	9	9	6

[1] ECHA dissemination site
[2] Danish Environment Protection Agency (DEPA) report entitled "Category approach for selected brominated flame retardants - preliminary structural grouping of brominated flame retardants" (Wedebye et al., 2016)
[3] US EPA; Estimation Program Interface (EPI) Suite. Ver. 4.0. Jan, 2009. Available from, as of Oct 25, 2010: http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm
[4]National Toxicology Program, Institute of Environmental Health Sciences, National Institutes of Health (NTP). 1992. National Toxicology Program Chemical Repository Database. Research Triangle Park, North Carolina
[5] Atom based method of measuring distribution coefficients using atomic contributions usually in pharmaceutical industry. The most common elements contained in chemical substances (hydrogen, carbon, oxygen, sulfur, nitrogen, and halogens) are divided into several different atom types depending on the environment of the atom within the molecule. While this method is generally the least accurate, the advantage is that it is the most general, being able to provide at least a rough estimate for a wide variety of molecules
[6] Determines if a chemical compound with a certain pharmacological or biological activity has chemical properties and physical properties that would make it a likely orally active drug in humans

None of the members are predicted to be persistent or bioconcentrating.

Regarding toxicokinetics and metabolism, there are no data available for TBNPA and the data on BMP are rather limited. More specifically, glucuronidation is the sole established route of metabolism of BMP in liver microsomes or primary liver cells of rodents, Rhesus monkeys and humans. The rate of BMP glucuronidation in rodent cells was 150-fold higher than in human hepatocytes. It is assumed that this is a detoxification route and this is expected to be the same for TBNPA and 2,3-DBPA. In addition, BMP has been detected in the gonads (Hoehle *et al.*, 2009). In the testis of rats only 0.01% BMP was recovered after up to 10 days of exposure. No female rats were used in this specific study. There were no other toxicokinetic data examining whether BMP reaches the ovaries of mammals. No data were available on the distribution of the metabolite(s) before and after internal reabsorption, or whether BMP or its glucuronide metabolite is the active compound. On the other hand, similar to many halogenated aliphatic alcohols, 2,3-DBPA is oxidized and dehalogenated. After conjugation to glutathione, the intermediate epoxide is metabolized further to mercapturic acid. As a result of hydrolysis of the

epoxide and successive oxidation, bromoacetic acid and oxalic acid may also form. The glutathione conjugate can also be metabolized to a highly reactive episulphonium ion, as a result of which there is the possibility of adduct formation at N-7 of the guanine (NTP, 1993). Apart from these experimental findings, all three SBAAs can undergo a variety of different reactions, either through a xenobiotic metabolic pathway (i.e. cytochrome P450 oxidases, UDP-glucuronosyltransferases, glutathione S-transferases) or by interacting with DNA via multiple mode of actions.

A summary of the available experimental data for TBNPA, BMP and 2,3-DBPA (data sources: CLH report for TBNPA; RAC opinion for BMP; Treinen *et al*., 1989; Lamb *et al*., 1997; Lamb *et al*., 1997; NTP, 1993; ECB, 1998) is contained in the BD.

Based on the available studies, the toxicological properties for the three SBAAs can be summarised as follow.

- The kidney is recognized as the common target organ for all three SBAAs and as the target organ where the most prominent and severe effects were observed in various species (TBNPA – rats, BMP – rats & mice, 2,3-DBPA – male rats). Among the other organs affected by the three SBAAs, the liver was shown to be a common target organ for both TBNPA (rats), with mild effects (reduced serum glutamic-pyruvic transaminase [SGPT] activity, increased liver weight and minimal centrilobular hypertrophy) and for 2,3-DBPA (rats & mice), with the severity of the effects for the latter being equally prominent as those observed in the kidney. Urinary bladder was the common main target organ for TBNPA and BMP. Lung was only targeted by 2,3-DBPA.

- With regard to fertility, data exist only for BMP and 2,3-DBPA, and the effects observed reveals some obvious differences as well as some similarities. 2,3-DBPA mainly exerts its action on male reproductive organs, while BMP exerts fertility impairment on F0 and F1 animals, mainly, if not exclusively, to females (litters/pair, fertility index, no live pups/litter). Nevertheless, examination of the available data for BMP, provided by an NTP study using the protocol for Reproductive Assessment by Continuous Breeding (RACB), showed some common effects between BMP and 2,3-DBPA on male fertility parameters: in the F1, absolute testis weight was significantly decreased (16%) at the highest dose, along with significantly decreased epididymal sperm density (14%) after BMP administration. These latter findings are comparable with 2,3-DBPA effects on fertility.

- Regarding mutagenicity, a full dataset is available for TBNPA. TBNPA and 2,3-DBPA have the same *in vitro* tests positive (Ames test, Mouse Lymphoma Assay, Thymidine Kinase mutation test), but 2,3-DBPA is active both with and without metabolic activation, while TBNPA only with metabolic activation. TBNPA and BMP were positive in the same *in vitro* tests (Ames test, chromosome aberration test) both with metabolic activation, but again differences in reactivity are noted. TBNPA is also active without metabolic activation at the highest dose in the chromosome aberration test, while the Ames test for BMP is reported negative in 10% of metabolic S9 activation mixture and positive only with 30% S9. TBNPA and 2,3-DBPA were negative in the same *in vivo* test (erythrocyte micronucleus test) but with limitations, while BMP was positive in two different *in vivo* erythrocyte micronucleus tests (at higher doses than TBNPA and 2,3-DBPA) as well as in an *in vivo* comet assay in urinary bladder, but was negative in a comet assay in liver cells. It can be concluded that the mechanistic pathways operating for TBNPA and BMP are possibly similar, with TBNPA being slightly more reactive, while 2,3-DBPA shares some common mechanisms but also exhibits extra reactivity compared to the other 2 SBAAs.

- Regarding carcinogenicity, there are no data for TBNPA, while the carcinogenic profile of BMP and 2,3-DBPA seems rather similar, with many common tumours in both sexes of rats and mice as shown in the tables below:

Table: *Common tumours in rats for BMP and DBPA*

Site	BMP	DPBA	BMP	DPBA
	Rats		Rats	
	Male	Male	Female	Female
Skin	+	+		
Zymbal's gland	+	+		
Mammary gland			+	+
Oral cavity - Oral Mucosa	+	+	+	+
Oesophagus	+	+	+	+
Forestomach	+	+		
Small intestine	+	+		
Large intestine	+	+		
Kidney	?	+		

Table: *Common tumours in mice for BMP and DBPA*

Site	BMP	DBPA	BMP	DBPA
	Mice		Mice	
	Male	Male	Female	Female
Forestomach	+	+	?	+
Lung	+	+	+	?

+: positive results
?: equivocal results

RAC has applied the Read-Across Assessment Framework (RAAF) (2017) developed by ECHA for the two possible source substances, BMP and 2,3-DBPA, and the results are contained in the Background Document.

It is evident that RAAF indicates 'high to medium' confidence for reading across from BMP to TBNPA, while confidence for reading across from 2,3-DBPA to TBNPA is only 'sufficient'. This is in line with the partial similarity of 2,3-DBPA with TBNPA, substantiated above, with regards to chemical structure and reactivity, physico-chemical and toxicological properties.

In conclusion, for classification purposes RAC makes use of the available experimental data on TBNPA and, when this is not available, insufficient or inadequate due to, for example, deficiencies in the testing methods, data is read-across from BMP (but not 2,3-DBPA) for the reasons stated above.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The toxicity of TBNPA following repeated exposure has been evaluated by the DS based on three studies, two oral and one feeding study all in rats.

Study report (Anonymous, 2015)

In a 28-day oral by gavage study (OECD TG 407, GLP) with TBNPA, liver was the target organ of Sprague-Dawley rats as indicated by increased organ weight and slight minimal centrilobular hypertrophy. Moreover, slight changes were observed in the kidney weight as well as in salt blood concentrations. The effects in liver and kidney were fully reversible with the exception of kidney weights, which remained slightly high at the end of the recovery period for males in the top dose group.

Study report (Anonymous, 2011)

In a 14-day oral by gavage study (no guideline study) with TBNPA in Crl:CD(SD) rats urine staining occurred in males and females in the top dose group (1000 mg/kg bw/d). Males receiving the top dose were killed early on day 4 of treatment for animal welfare reasons.

Study report (Anonymous, 1973)

In a 30-day oral feeding study (no GLP/guideline study) with TBNPA in Sprague-Dawley rats, the effects in male rats from 100 mg/kg bw/d included kidney damage and urine bladder hyperplasia. Additional treatment-related effects were: increase in serum urea nitrogen content in male rats receiving 300 mg/kg bw/d TBNPA in their diet. No changes were noted in any of the female rats in this study.

According to the DS, based on the three available studies, the target organs are the liver, the kidneys and the urine bladder after exposure to TBNPA. The effects were mild and reversible, except for one of the studies (Anonymous, 2011), where the high dose on day 4 lead to high acute toxicity making it necessary to kill animals for animal welfare reasons. No significant toxic effects were observed and the DS proposed no classification for the STOT RE hazard endpoint.

Comments received during consultation

There were two comments received from MSCAs. The first MSCA supported the DS' proposal and stated that although there were effects seen on liver (increased organ weight, minimal centrilobular hypertrophy), kidney (increased organ weight), and urine bladder (hyperplasia of the mucosal lining of urinary bladders), these effects showed full recovery. Thus, no significant toxic effects were observed in the three available studies and therefore no classification is warranted.

The second MSCA noted that based on the available data no significant toxic effects were observed in the animals at low or moderate exposure concentrations of TBNPA and thus no classification is warranted for TBNPA regarding STOT-RE.

Assessment and comparison with the classification criteria

The evaluation of the STOT RE endpoint for the target substance TBNPA is presented below.

***Table**: Repeated dose toxicity studies for TBNPA*

A/A	Species/ Reference	Method/ Test Substance	Results
1	**Study report Anonymous, 2020** Sprague-Dawley rats 10/sex/dose 5/sex/dose recovery groups	*OECD TG 408 Repeated Dose 90-Day Oral Toxicity in Rodents* *Reliability 1 given by the registrant* TBNPA 97% Oral/gavage Doses (mg/kg bw/d): 0, 50, 150, 450 90 days daily dosing 28-d recovery/observation period NOAEL = 50 mg/kg bw/d for m NOAEL = 150 mg/kg bw/d for f	Body weight: No effects observed Organ weights: No effects observed Food/water consumption: No effects observed Clinical signs: The clinical sign of perineum wet with urine was observed in both males and females at 150 and 450 mg/kg bw/d. By the end of the treatment period, this clinical sign was observed in 9/10 males and 7/10 female rats of the main group and 4/5 male and 3/5 female rats of the recovery group. The finding was fully reversible at the end of the recovery period. The rest of the observations of all tested groups were comparable to the vehicle control group. No behavioural changes observed. Haematological/Coagulation effects: There were no TBNPA exposure related changes in haematology, prothrombin time and activated partial thromboplastin time in males and females. Urinalysis: No treatment related findings in urine parameters Clinical biochemistry: An increase in blood urea nitrogen at 450 mg/kg bw/d and in creatinine at ≥ 150 mg/kg bw/d in males was considered as test item related. This increase was associated with microscopic findings of increased eosinophilic droplets in the tubular epithelium consisting tubular casts in kidneys. These findings reversed at the end of recovery period. Gross pathology: No effects seen Histopathology: Test item related microscopic changes were noted in kidneys and urinary bladder in males. In kidneys, increased eosinophilic droplets were noted in the tubular epithelium in the cortex of 6 males treated at 150 mg/kg bw/d and all males treated at 450 mg/kg bw/d but not in females. A single incidence of papillary necrosis was also observed at 450 mg/kg bw/d males and considered as test item related. Diffuse epithelial hyperplasia was noted in urinary bladder of 6 males at 150 mg/kg bw/d and all males and one female at 450 mg/kg bw/d. Both these changes reversed at the end of recovery period. No neoplastic findings were observed. Mortality: None observed

2	**Study report Anonymous, 2015** Sprague-Dawley rats 5/sex/dose CLH report	*OECD TG 407 Repeated Dose 28-Day Oral Toxicity in Rodents, GLP compliant.* *Reliability 1 given by the registrant* TBNPA 97% Oral/gavage Doses (mg/kg bw/d): 0, 30, 150, 500 28 days daily dosing 14-d recovery/observation period NOAEL = 500 mg/kg bw/d both for m and f	Body weight: no effects observed Organ weights: Increased liver weight (predominantly at ≥ 150 mg/kg bw/d), and a correlative microscopic finding of slight minimal centrilobular hypertrophy were reported. Full or partial recovery were seen at the end of the study. Slightly higher kidney weights were observed in females in the low dose group and in males in the medium dose group. All findings showed full recovery, with the exception of kidney weights which remained slightly high at the end of the recovery period for males in the top dose group. Food/water consumption: no effects observed Clinical signs: (500 mg/kg bw/d) Chin rubbing and/or salivation in all females and most males was reported. Females also displayed unsteady gait. All signs occurred following dosing and disappeared 1-2 hours after. Haematological effects: No clear treatment related findings Urinalysis: After exposure to TBNPA (≥ 30 mg/kg/ bw/d) a dose-related increase in urinary volume and slightly high total protein and glucose output in males was seen. All of these findings showed full recovery at the end of the 2-week recovery Clinical biochemistry: (500 mg/kg bw/d) Transient slightly low sodium concentration (0.98X control) and slightly high potassium concentration (1.18X control). Gross pathology: No effects Histopathology: No effects Mortality: None observed
3	**Study report Anonymous, 2011** Crj: CD(SD) rats 5/sex/dose CLH report	*No Guideline* *Repeated Dose 14-Day Oral Toxicity in Rodents), No GLP* *Reliability 2 given by the registrant* TBNPA 98.4% Oral/gavage Doses (mg/kg bw/d): 0, 100, 300 and 1000 14 days daily dosing Day 15 sacrifice No post exposure observation period	Body weight: no major changes except for changes in males receiving the top dose, where some of them lost weight. Organ weights: Liver, kidneys and spleen were examined with no effects observed. Food/water consumption: no effects observed Clinical signs: Post dose salivation and chin rubbing was observed on occasion in the majority of animals at all dose levels. Haematological effects: Not examined Clinical biochemistry: Not examined Gross pathology: Enlargement of the liver with associated dark areas was seen in one female at 1000 mg/kg bw/d. Histopathology: Not examined Mortality: Males receiving 1000 mg/kg bw/d (top dose) were killed on day 4 of treatment for animal welfare reasons. Last in-life signs included, abnormal gait, unresponsive, underactive, flat

		NOAEL (m) = 300 mg/kg bw/d both for m and f Due to mortality at the top dose NOAEL (f) = 1000 mg/kg bw/d	posture, prostrate posture and high levels of urine staining in all males at this dosage. Macroscopic examination revealed abnormal contents and pallor of the jejunum in three of the five animals, but there were no other consistent macroscopic observations recorded for these animals.
4	**Study report Anonymous, 1973** Sprague-Dawley rats 5/sex/dose CLH report	*No Guideline* *Repeated Dose 30-Day Oral Toxicity in Rodents), No GLP* *Reliability 2 given by the registrant* TBNPA 98% Oral/feeding Doses (mg/kg bw/d): 0, 10, 30, 100, 300 30 days daily dosing No post exposure observation period NOAEL (m) = 30 mg/kg/ bw/d for based on the kidney and urinary bladder effects NOAEL (f) = 300 mg/kg/ bw/d	Body weight: No effects observed Organ weights: Liver, kidneys and spleen were examined with no effects observed. Food/water consumption: No effects observed Clinical signs: Not examined Haematological effects: No effects observed Clinical biochemistry: Decrease in SGPT (300 and 100 mg/kg bw/d) and increase in blood urea nitrogen in males (300 mg/kg bw/d) Gross pathology: Effects in male rats from 100 mg/kg bw/d included kidney damage and urine bladder hyperplasia. No effects in females. Hyperplasia of transitional epithelium of the urinary bladder (100 and 300 mg/kg bw/d; males); eosinophilic clumping of the cytoplasm and darkening of nuclei in cortical tubular epithelial cells. Regenerative changes in the large epithelial tubular cells. Histopathology: Changes in kidneys and urinary bladders (male at 300 and 100 mg/kg bw/d). Mortality: No mortality occurred

The recently submitted sub-chronic 90-day toxicity study (Anonymous, 2020) () by the oral route (EU B.26./OECD TG 408) in rats, as requested by ECHA had the goal to assess the systemic toxicity potential of TBNPA and to compare its toxicological profile and more specifically the kidney pathology of TBNPA with that of BMP.

TBNPA was administered by gavage for 90 days in SD rats, with a 28 day recovery period in order to access the reversibility of any effects observed. The doses of 50, 150 and 450 mg/kg bw/d resulted in no mortalities, no changes in haematology, coagulation parameters, thyroid hormone levels and urine parameters. There were no gross pathological changes observed at any of the doses tested. There were clinical signs of perineum wet with urine observed in both males and females which were fully reversed at the end of the recovery period.

The main effects observed were in the kidneys and in the urinary bladder. These effects were correlated with blood urea and creatinine changes and with the clinical sign of perineum wet with urine. More specifically, at 150 mg/kg bw/d in males, an increase in creatinine correlated with increased eosinophilic droplets in tubular epithelium in kidneys and urinary bladder epithelial hyperplasia were considered. These were considered as test item related changes. At 450 mg/kg bw/d in males, a minimal increase in blood urea nitrogen and creatinine was correlated with morphological changes in the kidneys and the urinary bladder. In kidneys, increased eosinophilic

droplets were noted in the tubular epithelium in the cortex of 6 males treated at 150 mg/kg bw/d and all males treated at 450 mg/kg bw/d but not in females. A single incidence of papillary necrosis was also observed at 450 mg/kg bw/d males and considered as test item related. Diffuse epithelial hyperplasia was noted in urinary bladder of 6 males at 150 mg/kg bw/d and all males and one female at 450 mg/kg bw/d. Both these changes reversed at the end of recovery period. Based on the above findings, treatment did not cause any adverse effects at 50 mg/kg bw/d in males and at 150 mg/kg bw/d in females during the 90 days treatment period. It is worth noting that at the highest dose (450 mg/kg bw/d) no signs of systemic toxicity were observed. It could be argued that a higher dosing schedule should have been applied in order to observe a full toxicological spectrum of TBNPA.

In the key experimental study (Anonymous, 2015), the oral gavage administration of TBNPA to Sprague-Dawley rats at doses of 30, 150 or 500 mg/kg bw/d for four weeks was well-tolerated and did not cause any adverse change. A substance related response was evident in the liver (predominantly at ≥ 150 mg/kg bw/d) as indicated by increased organ weight and a correlative microscopic finding of slight minimal centrilobular hypertrophy. Some changes in blood chemistry (low sodium and high potassium concentrations in males at 500 mg/kg bw/d) or urine composition/output (increased urinary volume and total protein and glucose output in males at 500 mg/kg bw/d) occurred and a slight increase in kidney weight was evident in both sexes (predominantly at ≥ 150 mg/kg bw/d). None of these changes were considered adverse in nature, however, and the majority showed full or at least partial recovery.

In the supporting 14-day repeated dose oral (gavage) toxicity study in rats (non-guideline, no GLP, Anonymous, 2011), administration of TBNPA to CD rats at doses up to 1000 mg/kg bw/d in females and 300 mg/kg bw/d in males was well tolerated. However, doses of 1000 mg/kg bw/d in males necessitated premature sacrifice of these animals on day 4 and was considered to exceed the maximum tolerated dose.

In the supporting 30-day repeated dose oral (feeding) toxicity study in rats (non-guideline, no GLP, Anonymous, 1973), the ingestion of up to 30 mg/kg bw/d of TBNPA in the diet of Sprague-Dawley rats for 30 days did not cause changes in the toxicological parameters evaluated. At levels of 100 and 300 mg/kg bw/d, histologic changes in kidneys and urinary bladder were noted in male rats. No changes were noted in any of the female rats.

Based on the available studies, it is apparent that repeated exposure to TBNPA targets primarily the kidneys (increased organ weight, increased eosinophilic droplets, papillary necrosis) and the urine bladder (hyperplasia of the mucosal lining of urinary bladders) and secondarily the liver (increased organ weight, minimal centrilobular hypertrophy). The effects in the kidneys and the urinary bladder are more of concern since they were also observed in the supporting study (Anonymous, 1973) and are accompanied by clinical findings and altered biochemistry. However, the findings in all studies were mild, reversible and observed at doses above the guidance values for classification (STOT RE 2, ≤ 100 mg/kg bw/d for 90-day study).

In conclusion, the available RDT data for TBNPA is adequate for evaluation and RAC bases the evaluation of the STOT RE endpoint on the TBNPA RDT studies, which provide a complete database for classification. In these studies, mild, reversible effects in the kidneys and urinary bladder were observed at doses above the guidance values for classification. Therefore, RAC considers that despite the fact that clinical signs (perineum wet with urine) and biochemistry (minimal increase in blood urea nitrogen and creatinine) support the histopathological observations, **no classification for STOT RE is warranted**, in agreement with the DS.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS based the evaluation of mutagenic properties of TBNPA on the following studies:

1. *In vitro studies:*

i) 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 µg/mL) in the absence of metabolic activation. TBNPA has the potential to disturb mitotic processes and cell cycle progression (Anonymous, 2004a).

ii) 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 in 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* (Anonymous, 2004b).

iii) In the OECD TG 471 Ames test, in the presence of hamster S9-mix, there was clear evidence of mutagenic activity between 500 and 15 µg/plate with strains TA1535 and TA 100. The test showed no evidence of mutagenic activity in the absence or presence of rat S9-mix (Anonymous, 1996).

2. *In vivo studies:*

i) In the *in vivo* mammalian somatic cell study (similar to OECD TG 474, no major deviations), 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 (Anonymous, 2007b).

ii) In the OECD TG 486 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 16h. Therefore, the test material was considered to be non-genotoxic under the conditions of the study (Anonymous, 2007a).

The DS concluded that 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 also 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. The DS added that there were no suitable studies to indicate that TBNPA reaches the germ cells. The database available for TBNPA is limited to a single prenatal developmental toxicity study.

The DS, recognizing the limited database on TBNPA available for reaching a conclusion, proposed to read-across from the substance BMP, which was evaluated by RAC (adopted RAC opinion CLH-O-0000001412-86-212/F, 8 June 2018) and classified as a germ cell mutagen 1B. Originally, the DS in the CLH dossier proposed the same classification (germ cell mutagen 1B; H340) for TBNPA. After receiving comments both from Industry and from MSCAs during the consultation (for more details see section below), the DS recognized that:

- blood plasma results provided by Industry showed bioavailability for TBNPA
- the sensitivity of different bacterial strains used for the *in vitro* testing and whether the two-fold rule may be too insensitive for *Salmonella* strains with relatively high reversion frequencies, such as TA100, TA97, and TA102, and too sensitive for chemicals with low reversion frequencies, such as TA1535 and TA1537 (Mortelmans & Zeiger, 2000). Nevertheless, the DS still considered the results of the Ames test to be positive in both mutation tests, but only in the highest concentration for TA100.
- the results on the germ cells in the 28-day toxicity study show that no treatment related changes in sperm count and motility were observed.
- the shortcomings in the *in vivo* data, such as the dose selection in the *in vivo* micronucleus test or the general validity of the UDS test.

The DS, after reconsidering the available information listed above, agreed that the note to table 3.5.1 in CLP section 3.5.2.2 can be considered relevant to TBNPA based on the QSAR clustering for genotoxicity (BMP and TBNPA in same cluster; Wedebye *et al*., 2016), and thus revised the original conclusion and proposed TBNPA to be classified as a germ cell mutagen, Category 2.

Comments received during consultation

There were 4 comments received during the consultation, 1 from Industry and 3 from MSCAs.

Industry argued that the proposed hazard category for germ cell mutagen 1B is too severe based on the TBNPA database. While Industry recognized that TBNPA and BMP share similarities in the *in vitro* mutagenicity assays, the results in the *in vivo* tests are substantially different. Since the available experimental data for TBNPA are sufficiently robust and indicates that the substance is not an *in vivo* genotoxin, Industry considered that there is no need to read-across from BMP. Therefore, they suggested classification by the DS should be removed based on the lack of *in vivo* genetic damage shown experimentally.

The 3 MSCAs did not in general reject the category approach established by the Danish Environmental Protection Agency and applied by the DS within the current proposal, but expressed concerns regarding the category proposed by the DS for classification for germ cell mutagenicity. All 3 rather favoured classification in category 2, rather than 1B. The MSCAs supported the read-across from BMP, supporting a classification in Category 2. Two of the 3 commenting MSCAs provided similar reasoning and arguments with regards to shortcomings of the *in vivo* data set for TBNPA, which were accepted by the DS and led to review of the classification proposed for TBNPA to germ cell mutagen Cat. 2).

Assessment and comparison with the classification criteria

The following tables present a summary of the mutagenicity/genotoxicity tests *in vitro* and *in vivo* for TBNPA:

***Table**: Summary of mutagenicity/genotoxicity tests in vitro*

Study/Method/ Guideline/deviations if any	**Test substance**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**
Study report Anonymous, 2004a *In vitro* cytogenicity/chromosome aberration study in mammalian cells (lymphocytes: peripheral human lymphocytes) OECD TG 473, GLP	TBNPA (commercial preparation FR-513) Purity 97%	Based on range finding study, the doses in the main studies ranged from 100 to 2000 µg/mL with and without metabolic activation (S9-mix**). ✓ Positive control was Mitomycin C (MMC), a clastogen active without metabolic activation, and cyclophosphamide (CP), a clastogen requiring metabolic activation ✓ Negative control (solvent only) was DMSO 3h exposure, 24 h fixation. Vehicle choice according to TG 473	TBNPA **was found to be clastogenic** in the presence of metabolic activation (Aroclor-1254 induced rat liver S9-mix, according to the Industry the activation was a 1.8% in culture media S9-mix fraction of Aroclor induced rat liver homogenates), and at the highest test substance concentration (1000 µg/mL) in the absence of metabolic activation. Cytotoxicity seen as low as at 100 µg/mL, with metabolic activation. TBNPA has the **potential to disturb mitotic processes and cell cycle progression (chromatid and chromosome breaks, some minutes, single usually circular, part of a chromatid lacking a centromere, polyploidy).**
Study report Anonymous, 2004b Mammalian cell gene mutation assay (mouse lymphoma L5178Y cells, gene mutation) following OECD TG 476, GLP compliant	TBNPA (commercial preparation FR-513) Purity 97%	Dose range finding test (without/with metabolic activation): Solvent control, 33, 100, 333, 1000, 3250 µg/mL Metabolic activation: rat liver microsomal enzymes, S9-fraction** Experiment 1 (without metabolic activation): first solvent control, second solvent control, 10, 50, 100, 200, 300, 400, 500,	Mutant frequencies: In the absence of S9-mix TBNPA did not induce a significant increase in mutant frequencies in the first experiment. This result was confirmed in a repeat experiment with modifications in the duration of the time treatment from 3 to 24 hours.

Study/Method/ Guideline/deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations
		positive control (MMC) Experiment 1 (with metabolic activation 8%): first solvent control, second solvent control, 50, 100, 200, 300, 375, 450, 500, positive control (CP) Experiment 2 (without metabolic activation): first solvent control, second solvent control, 10, 50, 100, 225, 250, 300, 325, 350, positive control (MMC) Experiment 2 (with metabolic activation 12%): first solvent control, second solvent control, 100, 200, 300, 350, 400, 470, 500, 535, positive control (CP) Positive control methyl methane sulfonate/cyclophosphamide Vehicle DMSO	FR-513 was mutagenic in the test system with incubations in the presence of metabolic activation. The presence of S9-mix in both tests resulted in an increase in mutation frequencies colonies more than threefold and outside the laboratoty historical data (exact data not available). Industry claimed a 6.6-fold increase in the mutant frequency at the TK locus and 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. The increases were considered biologically relevant and FR-513 is considered mutagenic *in vitro*. Cytotoxicity was seen at concentration of 333 µg/plate and above with and without metabolic activation.
Study report Anonymous, 1996 Bacterial reverse mutation assay: *in vitro* gene mutation study in bacteria (Ames test) OECD TG 471	TBNPA (commercial preparation FR-513) Purity 98%	*S. typhimurium* TA1535, TA1537, TA 98 and TA 100[#] (with and without metabolic activation from hamster S9-mix treated with Aroclor-1254**). Strains TA98 and TA1537 are capable of detecting frameshift mutagens, strains TA100 and TA1535 are capable of detecting base-pair substitution mutagens. Test concentrations: 0, 5, 50, 500, 5000 µg/plate in the preliminary toxicity determination (with and without metabolic activation), and 0, 15, 50, 150, 500, 1500 µg/plate in	5000 µg/plate in the preliminary toxicity determination was toxic so the highest concentration was set to 1500 µg/plate in the main test. Toxicity was observed in the preliminary test at the concentration of 5000 µg/plate. Large, dose-related increases* in revertant colony numbers were observed in both mutation tests with

Study/Method/ Guideline/deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations
		the main test (with and without metabolic activation).	strains TA1535 and TA100, at concentrations between 15 and 500 µg/plate, but this was only observed with metabolic activation. DMSO used as negative control. Positive control was Congo red (CAS no. 573-58-0) which demonstrated the sensitivity of the assay and the metabolizing activity of the liver preparations. **Mutagenicity** was reported in both mutation tests with strains TA1535 and TA100 at concentrations between 15 and 500 µg/plate, only with metabolic activation.

***** *According to Industry comment, at the time of the test, the laboratory used 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 have 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. TA1535 had positive responses. Nevertheless, it has been shown that the 2-fold rule may be too insensitive for Salmonella strains with relatively high reversion frequencies, such as TA100, TA97, and TA102, and too sensitive for chemicals with low reversion frequencies, such as TA1535 and TA1537 (Mortelmans & Zeiger, 2000).*

***** *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. 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 (Industry comment).*

*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. Some lesions could be most likely repaired in TA100, which would explain the different intensity of positive responses in TA1535 and in TA100.*

***Table**: Summary of mutagenicity/genotoxicity tests in mammalian somatic or germ cells in vivo*

Study/Method/ Guideline/deviations if any	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**
Study report Anonymous, 2007 UD) test with rat liver cells *in vivo.* GLP and OECD TG 486	TBNPA (commercial preparation FR-513) Purity not given	Sprague-Dawley rats (CD (Ctr;CD(SD) IGS BR) strain) No. of animals per sex per dose: 2 range finder studies: (1 male; 1 female) and (2 males 0 females); 2 main tests: study 1: 4 per dose (males); study 2: 4 per dose (males) All animals were dosed once. In the range finding tests the dose was 2000 mg/kg bw. In the main studies the dose was 670 and 2000 mg/kg bw. Administration: oral by gavage. Treatment: 16h (experiment 1); 2h (experiment 2). Negative control: animals not treated Positive control 2-acetamididofluorene at 50 mg/kg bw, and sym-dimethylhydrazine dihydrochloride at 40 mg/kg bw.	Results: Negative The test material did not induce any marked or toxicologically significant increases (actual data not available) in the incidence of cells undergoing unscheduled DNA synthesis in isolated rat hepatocytes following *in vivo* exposure for 2 or 16h. Therefore, **the test material was considered to be non-genotoxic under the conditions of the study.** Concurrent positive control data: Both positive and negative controls produced marked increases in the incidence of cells in repair and the vehicle control groups gave **acceptable values for net nuclear grain counts.** Administration of the test substance in the range finding study produced **toxicity in the dosed animals manifested as ataxia, lethargy, red coloured urine** (no deaths). Lethargy and ataxia were also seen in the main studies.
Study report Anonymous, 2007 *In vivo* mammalian somatic cell study: cytogenicity/ erythrocyte micronucleus OECD TG 474 is relevant, but the study was done prior to the guideline. No major deviations from the guideline.	TBNPA (commercial preparation FR-513) Purity: 98.1%	NMRI mice male and female No. of animals per sex per dose: Ten animals (5 males, 5 females) per dose. Preliminary test: 2000, 1500, 1000, 500, 400, 300 (mg/kg bw/d) main test: 300, 150, 75 (mg/kg bw. On the day of the experiment, the test item was formulated in DMSO+corn oil (30%-	**TBNPA did not induce micronuclei as determined by this micronucleus test** with femur bone marrow cells of the mouse. The % micronuclei was 0.085, 0.110 and 0.125 at dose 75, 150, 300 mg/kg bw 24 hours post-treatment.

Study/Method/ Guideline/deviations if any	Test substance	Relevant information about the study (as applicable)	Observations
		70%). The vehicle was chosen to its relative non-toxicity for the animals. All animals received a single standard volume of 10 mL/kg bw orally. Negative controls: "valid" (no more information available) Positive control substance(s): Cyclophosphamide (CPA > 98%); Dosing: 40 mg/kg bw; volume administration: 10 mL/kg bw	

Based on the data presented above and the whole data set available for TBNPA the following findings can be summarised:

1. TBNPA was mutagenic in mouse lymphoma cells *in vitro* in the presence of metabolic activation.
2. TBNPA was clastogenic in human lymphocytes *in vitro* in the presence of metabolic activation and at the highest test concentration without metabolic activation is consistent with the mouse lymphoma test findings.
3. In bacterial reverse mutation assays, mutagenicity was seen. Nevertheless, the intensity of the positive results were debated by Industry during the consultation (cf. public attachment to the RCOM).
4. 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.
5. Regarding the micronucleus test in femur bone marrow cells, in order to conclude that a substance is clearly negative, it has to be demonstrated that the bone marrow had been exposed (adequate evidence of target tissue exposure). To this end, concentrations of the test item can be determined in the blood plasma. Industry provided data on the analysis of the blood plasma of animals treated with 300 mg/kg bw (maximum dose tested): 1h after treatment the plasma of the animals contained between 38.7 and 65.6 ng test item per mL plasma. The samples from the 4h interval did not have any detectable levels of the test item. In addition, Industry stated that TBNPA did not induce any cytotoxic effects, as determined by the ratio between polychromatic and normochromatic erythrocytes, without providing actual data. A sound conclusion as to whether these values indicate systemic exposure including the bone marrow is questioned. Furthermore, acute toxicity testing at and above 500 mg/kg bw resulted in the death of some animals after 48 hours. In RAC's opinion, there are several limitations with the micronucleus test in femur bone marrow cells. Since 4h after exposure there is no TBNPA present in blood plasma, administration of the test substance could have been done differently. One variation according to OECD TG 474 is to administrate the test chemical as a split dose, i.e., two or more treatments on the same day separated by no more than 2-3 hours. A second

variation is to administrate the test item in more than one daily treatment for a more efficient exposure. Both of these variations could have improved the quality of the study. In addition, Industry stated that acute toxicity testing at and above 500 mg/kg bw resulted in the death of some animals after 48 hours. However, this result is equivocal since in the 90-day repeated toxicity study the top dose of 450 mg/kg bw/d was well tolerated with no acute, systemic or mortality effects observed. Moreover, the LD_{50} is > 2000 mg/kg bw for TBNPA so the dose selection for the specific study can definitely be disputed.

As assessed and explained in the RAC GENERAL COMMENT SECTION, RAC believes that the read-across among the three SBAA is justified. However, RAC disagrees with Industry's conclusion that the mutagenic profiles of TBNPA and BMP are different based on the comparison of the available *in vivo* micronucleus studies for TBNPA and BMP. The reasoning behind this argument is based on the substantially different parameters of the available studies.

In the mouse bone marrow micronucleus tests with TBNPA, the test substance was administrated in a single dose (top dose of 300 mg/kg bw/d) orally although it is not clear whether the route of exposure was gavage or feed (Industry/gavage, CLH report/orally, CSR/feed). In the key positive study with BMP, significant increases in micronucleated normochromatic erythrocytes were observed in peripheral blood samples obtained from male and female mice exposed for 13 weeks to BMP in feed. These increases were seen in the two highest dose groups of male mice (1300 and 3000 mg/kg bw/d) and the three highest dose groups of female mice (600, 1200 and 2900 mg/kg bw/d). It is apparent that the exposure is much higher in the BMP experiment because of longer duration and higher dosing. In the first of two mouse bone marrow micronucleus tests performed with BMP, a three dose scheme was used with a top dose of 400 mg/kg bw/d with equivocal results as the first trial was negative and the second was positive. In this experiment, the dosing is once more different that the one with TBNPA (3X400 vs 1X300 mg/kg bw/d). In the second mouse bone marrow micronucleus test, BMP was administered as a single intraperitoneal injection (150 to 600 mg/kg/d) and was positive with a significant dose related increase in micronucleated PCEs in females. In this study both the route of exposure (i.p. vs feed) and the dosing is different (600 vs 300 mg/kg bw/d). In conclusion, RAC notes that the micronucleus test in femur bone marrow cells with TBNPA has serious limitations and the comparison of the *in vivo* mutagenicity properties between TBNPA and BMP is not justified as the study parameters are not consistent across the studies.

According to ECHA guidance on Information Requirements and Chemical Safety Assessment – Chapter R.7a: Endpoint specific guidance (IR CSA R.7a), the use of the *in vivo* UDS indicator test should always be justified on a case-by-case basis and may only be sufficient under certain circumstances (considering target organ and substance specific factors). Only if it can be reasonably assumed that the liver is a target organ, the UDS may be an adequate test. No available data indicate the liver to be the target organ. TBNPA is not expected to be highly metabolized in the liver, where glucuronidation activity is high. Furthermore, the guidance on IR CSA R.7a states that a negative result in a liver UDS test alone cannot be considered proof of absence of gene mutation inducing properties of the substance, despite the fact that 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*.

6. There is no indication that TBNPA reaches the germ cells, albeit the available database is limited.

Therefore, since positive results with TBNPA have been obtained from *in vitro* studies addressing both gene mutations and chromosome aberrations a relevant *in vivo* follow-up test is necessary

to find out whether the *in vitro* results are also relevant *in vivo*. Negative results provided by the *in vivo* micronucleus test may indicate that TBNPA does not induce chromosome aberrations. However, uncertainties arise regarding dose scheme selection and the availability of the test substance at such doses. In addition, the fact that liver has not been proven to be a target organ renders the results from the UDS test questionable. Hence, as it cannot be ruled out that TBNPA has the potential to generate gene mutations *in vivo*, RAC recognises a data gap for the induction of gene mutations *in vivo* and read-across from BMP is applied. Mutagenicity data on BMP are briefly presented and not assessed, as these data come from an adopted RAC opinion for germ cell mutagenicity 1B.

Data on BMP

The database for BMP comprised of three *in vitro* Ames tests, two giving clear concentration related positive results in the presence of 30% Syrian hamster liver S9-mix (National Toxicology, 1996; Zeiger *et al*., 1992) and one with negative result where the S9-mix concentrations were limited to 10% (Mortelmans *et al*., 1986). In summary, positive findings were obtained when using high concentrations of hamster S9-mix (30%), while no mutagenic activity was detected when using rat liver S9-mix, or low concentrations of hamster S9-mix for metabolic activation. Moreover the OECD TG 473 Chinese hamster ovary chromosome aberration test was positive in the presence of low concentrations of rat liver S9-mix and scoring of only 100 cells per sample (Galloway *et al*., 1987a). The same authors also conducted a Sister chromatid exchange assay that was negative (Galloway *et al*., 1987b). Several *in vitro* Comet studies measuring DNA damage were identified using BMP of high purity (98%). Positive results were obtained in urothelial cells (Kong *et al*., 2011; Kong *et al*., 2013), but no genotoxic effect was evident in the hepatocytes (Kong *et al*., 2013). Regarding *in vivo* studies, 2 OECD TG 474 mouse peripheral blood micronucleus tests were reported positive at 400 mg/kg bw/d and 1300 mg/kg bw/d for males and at 600 mg/kg bw/d for females. A positive *in vivo* comet assay (Wada *et al*., 2014) in urinary bladder at of 600 mg/kg bw/d was also identified, with no signs of toxicity. In the same study, negative results were obtained *in vivo* for the liver, similar to observations from the *in vitro* comet study by Kong *et al*. (2013), due to detoxification by glucuronidation in the hepatocytes. No germ cell mutagenicity studies are included. Availability to germ cells: Treinen *et al.* (1989) revealed that BMP leads to reduced fertility, specific effect on female reproductive capacity. BMP is not a selective reproductive toxicant, because these findings are concomitant with general toxicity. However, Bolon *et al*. (1997) showed significantly and dose-response related reduction in follicle numbers in both F0 and F1 mice from the same experiment. Moreover the reduction in follicle numbers occurs also at the mid dose in F1 mice not mediating clear reproductive effects or overt body weight decrease. This indicates that BMP reaches the germ cells.

Based on the above, RAC agreed that BMP should be classified as a germ cell mutagen, Cat. 1B; H340.

In conclusion, according to the CLP Regulation (Annex I: 3.5.2.2., note to table 3.5.1), classification as category 2 mutagens may be justified for substances "which are positive in *in vitro* mammalian mutagenicity assays", which is the case for TBNPA, and "which also show chemical structure activity relationship to known germ cell mutagens". This scenario can be considered to be relevant to TBNPA, where the results from the *in vivo* studies are not conclusive and read-across from a structurally related and known category-1B mutagen (BMP) is applicable.

Therefore, RAC considers that **classification of TBNPA as a germ cell mutagen Category 2, H341: Suspected of causing genetic defects – is warranted.**

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

There is no carcinogenicity data for the target compound TBNPA. The DS evaluated the carcinogenicity hazard endpoint based on read-across from the source substance BMP. The latter substance has a recent RAC opinion that considers BMP to be a multi-site carcinogen in two species with tumours of human relevance. Therefore, RAC classified BMP as Carc. 1B; H350 (CLH-O-0000001412-86-212/F). The DS proposed the same classification for TBNPA.

Comments received during consultation

Regarding carcinogenicity, three comments were received, one from Industry and two from MSCAs.

Industry

Industry disagreed with the proposed classification for TBNPA as Carc. 1B, based on read-across from the source substance BMP. The reasoning is mainly founded on the following points:

- The two substances have different mutagenic profiles.
- There are significant differences in the structure and physico-chemical properties between the two read across substances.
- The additional hydroxyl group and the fewer bromide groups can make a large difference in biological reactivity.
- The quality of the NTP study, the purity of the BMP used and the underlying mechanism were questioned and, consequently, the score in the RAAF scenario 2, as evaluated by the DS, was incorrectly assigned.
- Previously, it was found, that the kidney pathology in the 28-day toxicity study of TBNPA is different compared to the kidney pathology in the BMP 90-day toxicity study. BMP showed renal papillary degeneration and urinary bladder hyperplasia, whereas TBNPA showed an increase in minimal tubular basophilia, a typical background finding in the rat, considered to be non-adverse. TBNPA had no effects in the bladder, however, the BMP 90-day study observed urinary bladder hyperplasia in 9 out of 10 males. Industry stated that a direct comparison of the results of the 90-day RDT study of BMP with the results of the recently submitted 90-day of TBNPA (Anonymous, 2020) would provide insight about the similarity of the toxicological profile of the two substances.

Furthermore, Industry recognised that, although BMP and TBNPA were found to belong to the same (Q)SAR-based clusters for genotoxicity and carcinogenicity (Wedebye *et al*., 2016), 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. The genotoxicity of BMP (*in vitro* and *in vivo*) added the strength of evidence for classification of BMP as Carc. 1B. Genotoxic carcinogens tend to cross species lines and represent a potential human hazard. This is not the case with TBNPA. In addition, Industry noted that neither for BMP nor for TBNPA human data exist suggesting these SBAA substances are known or presumed human carcinogens. In conclusion, Industry believed that the proposed hazard category for carcinogenicity 1B is too severe and that TBNPA should be classified as Category 2.

MSCA

The first MSCA stated that as the classification for carcinogenicity in the CLH report is assumed to rely on mutagenic activity, the uncertainties associated with the *in vivo* genotoxicity data contradict the classification of TBNPA as a genotoxic carcinogen, Category 1B. Furthermore, additional uncertainties regarding the robustness of the read-across (lack of toxicokinetic data for the target substance, differences in physico-chemical properties, lack of comparable data regarding reproductive toxicity), which were not discussed in the proposal, raise doubts about the validity of the approach for classification of TBNPA.

The second MSCA noted that, based on the read-across from the source substance BMP classified as Carc. 1B, the same classification for TBNPA is warranted.

Assessment and comparison with the classification criteria

Experimental data addressing the carcinogenicity of TBNPA were not available. However, RAC accepts that data from studies with BMP can be read across for this endpoint.

BMP was recently evaluated by RAC and was classified as Carc. 1B based on multi-site tumours in two species, rats and mice, with human relevance, in the presence of limited general toxicity. The opinion was adopted by consensus on June 8, 2018.

Briefly, from the BMP RAC Opinion, "*BMP induced dose-dependent multi-site tumours in two species, rats and mice, in a well conducted OECD TG 453 oral study carried out by the NTP under GLP conditions and with limited general toxicity. Both benign and malignant tumours were observed in the respective tissues, showing the ability of the tumours to progress to malignancy. The stop-exposure group in male rats showed that only 3 months of exposure induced tumours at most sites where tumours were observed in the 2-year continuous-exposure groups. The incidences of neoplasms were greater at some sites (lungs, small and large intestine, thyroid). Adenoma and carcinoma of the seminal vesicle were also found, which did not occur in the other groups, and which are extremely rare in rats. Based on the findings from this group, genetic damage appears to occur within the first few months of exposure and that can develop into tumours, also in the absence of a toxic response in these tissues. Some of the tumours observed fit into the pattern of genotoxic chemicals (NTP, 1996).*"

In the table below a summary of the tumours observed in rats and mice in the source substance, BMP, is shown.

Table: *Tumours observed in the available studies for BMP*

	BMP (NTP study; oral); (study conducted by industry; oral)			
Site	**Rats**		**Mice**	
	Male	**Female**	**Male**	**Female**
Skin	+			
Subcutaneous tissue	+			?
Nose				
Mammary gland	+	+		?
Zymbal's gland	+			
Oral cavity - Oral Mucosa	+	+		
Oesophagus	+	+		
Forestomach	+		+	?
Small intestine	+			
Large intestine	+			

Mesothelium - Peritoneum	+			
Liver				
Kidney	±		+	
Urinary bladder	+			
Lung	+		+	+
Spleen				
Thyroid gland	+	+		
Seminal vesicle	+	NA		NA
Tunica Vaginalis		NA		NA
Clitoral Gland	NA		NA	
Haematopoietic system	+			
Pancreas	?			
Harderian gland			+	+
Circulatory system				?

+: positive

?: equivocal results

NA: not applicable

It is apparent that the source substance, BMP, is a multi-site, multi-species carcinogen, as tumours were observed in both sexes of rats and to a lesser extent in mice.

Conclusion

Since there are no epidemiological studies available for either the target or the source substance, classification as Carc. 1A is not justified. There are also no animal data on TBNPA. In the animal studies, it is apparent that the source compound, BMP, is a multi-site and multi-species carcinogen. BMP has an adopted RAC opinion as Carc. 1B. Based on the detailed read-across analysis presented above, RAC supports the **classification of TBNPA as Carc 1B, H350: May cause cancer** in agreement with the DS's proposal. Regarding the route of exposure, RAC noted that it should not be specified.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Adverse effects on sexual function and fertility

Effects on fertility have not been assessed as no relevant studies are available, except for a 28-day RDT study where no relevant effects were identified.

The required study (OECD TG 421 or 422) was waived by the registrant based on a 28-day oral RDT study in rats with a 14 day recovery period, where some relevant reproduction parameters were investigated (Anonymous, 2015). The results showed no systemic toxicity effects and the No Observed Adverse Effect level (NOAEL) was determined as > 500 mg/kg bw/d (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.

Adverse effects on development

In a prenatal developmental toxicity study in SD rats (see table below), the highest dose was reduced from 1000 to 500 mg/kg bw/d due to post dosing toxicity. Two animals were killed due to animal welfare reasons. No clear findings of developmental toxicity were observed. Minor effects on ossification in the medium and high dose groups were within the historical control data. More details in the studies have not been available to the DS and it was considered not necessary to request the full study report from the registrant.

Overall, the DS considered the data on reproductive toxicity as inconclusive. The results from the RDT studies do not warrant classification. However, the 90-day study is insufficient to fully assess fertility effects, while the results from the prenatal developmental toxicity study do not warrant classification.

Comments received during consultation

Industry agreed with the DS that there are no effects on sexual function or fertility and developmental indices that warrant classification for reproductive toxicity. In addition, Industry stated that the results observed in the 28-day RDT study "are not considered sufficiently severe to meet the criteria for classification".

All 3 MSCAs commenting agreed that the data on TBNPA both for fertility and developmental toxicity are not sufficient for classification. Nevertheless, two MSCAs suggested whether based on structural similarity, read-across from BMP and 2,3-DBPA for reproductive toxicity could be explored.

Assessment and comparison with the classification criteria

Adverse Effects on Sexual Function & Fertility/Development

Table*: pre-natal developmental toxicity study for TBNPA*

Study/Method/ guideline/deviations if any/ species/groups	**Test substance/ dose levels duration of exposure**	**Results**
Study report Anonymous, 2016 Oral, by gavage, OECD TG 414 SD rats (ca 70 days old, 231 to 292 g), 20 females/dose	TBNPA (commercial preparation FR-513) Purity 97.6% Administration to pregnant females via gavage on gestation days 6-19 Doses: 0, 100, 300, 500/(1000) mg/kg bw/d The highest dose was	<u>General toxicity</u> Transient effects on body weight were seen in the high dose group. At the high dose (1000 mg/Kg bw/d), *mean body weight loss (2%)* was observed during days 6-7 of gestation (after the first dose) and lower body weight gain later in the study (500 mg/Kg bw/d) compared to controls, even when maternal body weight was adjusted for the weight of the uterus. On days 6-9, dams in the high dose group *had significantly lower food consumption* (4 g/day lower). No effects seen on maternal body weight in the low and medium dose groups. <u>Organs weight:</u> Gravid uterine weight in the dosed animals was lower. <u>Mean number of live pups (litter size)</u>: Embryo-

<table>
<tr><th>Study/Method/ guideline/deviations if any/ species/groups</th><th>Test substance/ dose levels duration of exposure</th><th>Results</th></tr>
<tr><td></td><td>reduced from 1000 to 500 mg/kg bw/d due to post-dosing toxicity after 2-3 doses.
Two animals were killed due to animal welfare reasons.</td><td>foetal survival was considered to have been at all doses, 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 litter or pup weight by sex and with sexes combined: Mean placental, male, female and overall foetal weights/ litter weight, at all doses, were similar to controls and unaffected by treatment.

External, soft tissue and skeletal malformations and other relevant alterations: No dose-related major foetal abnormalities were found.

Incidences of delayed / incomplete ossification / unossified pelvic bones:
<table><tr><td>300 mg/kg bw/d</td><td>11 foetuses from 7 litters</td></tr><tr><td>500 mg/kg bw/d</td><td>12 foetuses from 8 litters</td></tr><tr><td>Concurrent control</td><td>4 foetuses from 3 litters</td></tr><tr><td>Historical control data</td><td>15 foetuses from 12 litters</td></tr></table></td></tr>
</table>

In the 28-day RDT study, no treatment related changes in sperm count and motility were observed. Vaginal lavages, which were taken early morning during the 3rd week period from all females prior to termination of the animals, showed no treatment related changes in the oestrus cycle. In addition, no treatment related changes in the weight of seminal vesicles, ovaries, testes, ureter, uterus, vagina were observed.

In the oral 90-day RDT study in rats (EU B.26./OECD TG 408, Anonymous, 2020), TBNPA was administered by oral gavage to Sprague Dawley rats at 50, 150 and 450 mg/kg bw/d. No mortalities were observed up to the highest dose over the 90-day dosing period and the 28-day recovery period. The body weights, body weight gains and food consumption were not altered by the treatment at any of the doses tested in either sex.

Very few fertility parameters were evaluated in this study, like the oestrous cycle in females. More specifically, the stage of oestrous cycle was recorded prior to necropsy in the treated groups only to facilitate interpretation of ovary and uterus organ weight (no test item related changes in the organ weights reported) and histopathology. No intergroup differences were observed in either parameter. In the males, no significant intergroup differences in sperm motility, sperm morphology and sperm counts were observed.

An isolated incidence of dilated uterus one each in 50 and 450 mg/kg bw/d dose group female was observed and considered as incidental finding and not related to test item administration.

No hormone analysis related to reproduction was reported.

It is evident that the dataset for any possible fertility and developmental effects for TBNPA is limited. Although no actual findings were reported that could raise concern for either cluster of effects, for fertility the findings are not conclusive to decide for classification or no-classification. For developmental effects, the findings reported from the OECD TG 414 study in rats with TBNPA are not sufficient to trigger classification.

In addition, since in the OECD TG 414 study with TBNPA the exposure started on gestation day 6, no conclusions could be drawn on female fertility.

Hence, taking into consideration the scattered data on fertility from the 28-days and 90-days RDT studies with TBNPA, RAC considers that classification of TBNPA for **sexual function and fertility is not warranted <u>due to lack of data</u>**.

In addition, based on OECD TG 414 prenatal developmental toxicity study for TBNPA, RAC considers that classification of TBNPA for **developmental effects is not warranted**.

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ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).