

Lead registrant comments on the CLH report
Proposal for Harmonised Classification and Labelling
Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

Chemical name: 3,4-dimethyl-1H-pyrazole

EC Number: 429-130-1; CAS Number: 2820-37-3; Index Number: 613-248-00-5

The comments refer to the following endpoints in the CLH report:

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

For the decision on appropriate classification or non-classification with regards to specific target organ toxicity after repeated exposure, carcinogenicity and reproductive toxicity, the available data on toxicokinetics as well as mechanistic data concerning ADME (mainly distribution and excretion) are key for proper interpretation of the effects observed in studies with repeated dosing and should have been taken into account by the DS.

Summary of important findings from toxicokinetic studies:

Based on the oral metabolism studies with 3,4-DMP in the Wistar rat (2017/1005940 amended by 2020/2096870) and goat (2016/7009352), the following can be summarized: A number of the identified metabolites are formed by typical phase I conversions of the parent compound, such as hydroxylation at various positions and further oxidation of the former methyl groups to carboxylic acids. A second main route of biotransformation is direct N-conjugation with glucuronic acid (also in combination with hydroxylation of the 3-methyl group). Most phase I metabolites are O-conjugated either with glucuronic acid or sulfuric acid (phase II metabolism). In the goat, glycine conjugates and N-conjugation of the main metabolite are observed in addition, whereas in the rat also some glutathione conjugation occurred. The unchanged parent was detected in samples of feces, urine or bile in the rat but not in the goat. Overall, the differences in 3,4-DMP metabolism between rat and goat are minimal; the main metabolite, 3-methylpyrazole-4-carboxylic acid (MPCA), is found in the urine of both rats and goats.

Investigations on absorption, distribution and elimination of 3,4-DMP after oral dosing in Wistar rats (2016/1196943) showed, that 3,4-DMP was almost completely absorbed (88-93 %) and only 4-10 % of radioactivity was found in the feces depending on the dosing-regimes. Excretion occurs mainly via renal pathway as 85-93 % of radioactivity were found in the urine depending on the dosing-regimens. For the low dose (10 mg/kg bw), the time course of the amount of radioactivity found in urine and feces indicated a rapid excretion. The overall available data indicated an excretion that occurred predominantly within 2 days after dosing. Administration of 100 mg/kg bw after either single or repeated dosing for 15 days indicated comparable kinetics / metabolism after single and multiple dosing at this dose level.

For the low and high dose tissue distribution (single oral dose of 10 or 100 mg/kg bw) one and four hours after administration, highest concentrations were found in kidney and liver. The lowest concentrations were noted in adipose tissue, approximately 3 times lower compared with kidney and liver. The concentrations in blood and plasma were only slightly below the concentrations found in kidney and liver one and four hours after dosing.

Mean excretion via bile was also examined and was shown to be in the range of 5-8 % depending on the dosing-regimes.

In the goat (2016/7009352), approximately 106.9% of the total dose was recovered, the majority of which was present in the urine (94.85%) feces (7.54%) and GI tract (2.74%). Relatively low amounts were recovered in milk (0.09%). Tissues generally accounted for ≤ 0.1% of the administered dose except for liver (0.55%) and kidney (0.14%).

Overall, no striking differences in 3,4-DMP absorption, distribution and elimination seem to exist between rats and goats.

Further investigations on the plasma and blood cell kinetics as well as on tissue distribution (not discussed here, radioactivity was found in all tissues at the first timepoints investigated: 1h post-dosing at 10 mg/kg bw and 4h post-dosing at 100 mg/kg bw) were performed in male and female Wistar rats (2016/7006240).

Kinetics were investigated after single oral (4, 12, 40, 120 and 360 mg/kg bw, nominal dose) and intravenous (4 mg/kg bw) administration. Blood samples (~200 µL) were taken by jugular vein catheter 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 h after dosing.

The investigations showed that the internal dose (as indicated by the area under the curve (AUC)) was comparable for males and females at 4, 41 and 349-350 mg/kg bw, but was slightly higher for females at oral doses of 12 and 119-120 mg/kg bw. The AUC values were over-proportional with doses for both sexes: The factor between the target doses was constantly at around 3, whereas the ratio of the AUC values increased with the dose level for both males and females. These data of non-linear kinetics indicated a saturation of kinetics that was already present at a dose level of 12 mg/kg bw. This effect was more pronounced in female than in male rats.

The parameters derived from the plasma kinetics are presented in the following table (actual dose levels given). The kinetics in blood cells were similar.

Sex	Dose [mg/kg bw]	C _{max} [µg Eq/g]	T _{max} [h]	initial half-life [h]	terminal half-life [h]	AUC _{0→168} [µg Eq h/g]	AUC _{0→∞} [µg Eq h/g]
male	4, p.o.	3.5	1	2.0	60.2	19.6	20.2
	4, p.o. ^A	3.3	1	2.1	61.6	19.6	20.2
	12, p.o.	9.6	1	2.5	74.0	79.2	80.8
	41, p.o.	31.0	2	2.8	87.6	440.2	449.3
	120, p.o.	72.8	4	3.1	70.9	1225	1239
	350, p.o.	136.0	8	2.8	72.3	4683	4729
	4, i.v.	8.4	directly ¹⁾	1.6	158.7	22.5	23.9

$$\frac{(AUC/dose)_{4 \text{ mg/kg bw p.o.}}}{(AUC/dose)_{4 \text{ mg/kg bw i.v.}}} : \mathbf{85 \%}$$

¹⁾ means directly after administration

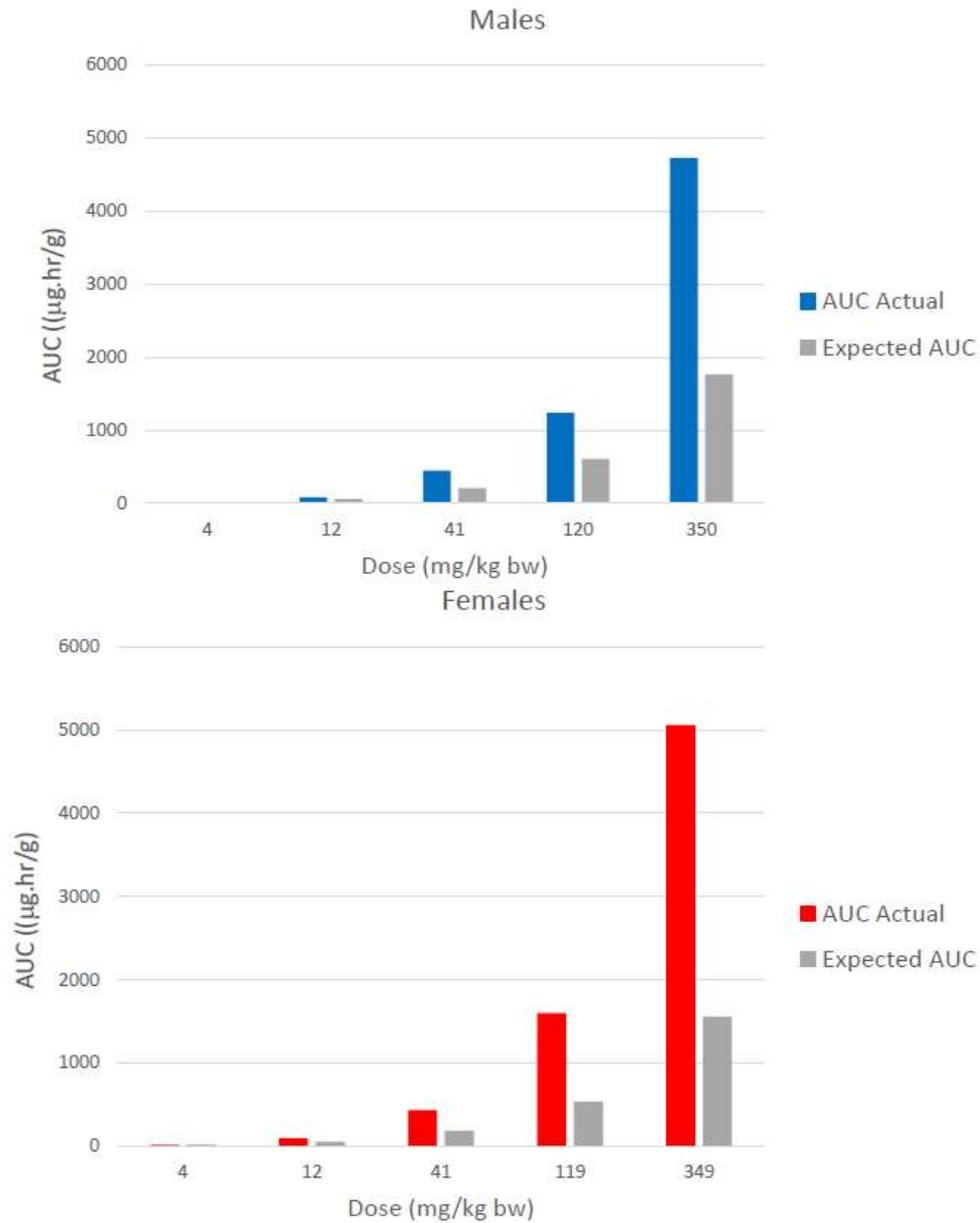
^{A)} Calculations used data from three animals excluding animal#4

Sex	Dose [mg/kg bw]	C _{max} [µg Eq/g]	T _{max} [h]	initial half-life [h]	terminal half-life [h]	AUC _{0→168} [µg Eq h/g]	AUC _{0→∞} [µg Eq h/g]
female	4, p.o.	3.0	1	1.7	72.9	17.3	17.8
	4, p.o. ^B	3.0	1	1.8	84.3	17.1	17.7
	12, p.o.	10.5	1	2.5	63.2	96.6	97.5
	41, p.o.	27.8	4	2.5	70.0	432.3	437.1
	119, p.o.	81.5	4	4.2	97.8	1579	1601
	349, p.o.	148.9	8	2.9	60.9	5032	5058
	4, i.v.	5.4	directly ¹⁾	2.6	140.6	24.8	25.6

$$\frac{(AUC/dose)_{4 \text{ mg/kg bw p.o.}}}{(AUC/dose)_{4 \text{ mg/kg bw i.v.}}} : \mathbf{70 \%}$$

¹⁾ means directly after administration

^{B)} Calculations used data from three animals excluding animal#8



Based on an observed AUC at the lowest dose of 4 mg/kg bw, the expected AUCs at the higher dose levels can be extrapolated. If excretion kinetics would follow a linear pattern, the AUCs should increase by roughly the same factor as the dose levels. As shown by the graphs above, this is neither the case in male nor in female rats.

The table below shows the mean plasma concentration of radioactivity after single oral administration of ¹⁴C-3,4-DMP at nominal dose levels of 4, 12, 40, 120 and 360 mg/kg bw to male and female rats:

Plasma concentrations expressed in µg/g plasma

Time	Oral Dose									
	4 mg/kg bw		12 mg/kg bw		40 mg/kg bw		120 mg/kg bw		360 mg/kg bw	
	male	female	male	female	male	female	male	female	male	female
0										
0.5	3.143	2.896	9.010	9.674	27.355	23.536	46.970	62.619	102.346	69.480
1	3.463	3.032	9.621	10.508	30.845	25.752	56.176	64.792	93.697	77.132
2	2.817	2.731	8.626	10.106	31.305	26.884	68.979	77.820	82.342	95.893
4	1.688	1.742	7.087	8.450	29.474	27.760	72.815	81.508	99.288	120.843
8	0.381	0.267	2.584	3.830	23.868	25.403	71.148	75.874	136.005	148.917
24	0.034	0.025	0.102	0.085	0.531	0.518	4.399	17.050	132.705	142.102
48	0.026	0.014	0.055	0.041	0.214	0.163	0.468	0.506	1.751	1.603
72	0.013	0.007	0.045	0.032	0.157	0.113	0.331	0.309	1.142	0.921
96	0.010	0.008	0.040	0.026	0.131	0.111	0.293	0.266	0.866	0.635
120	0.009	0.008	0.053	0.020	0.158	0.078	0.260	0.249	0.687	0.531
144	0.007	0.006	0.026	0.017	0.099	0.061	0.185	0.198	0.562	0.392
168	0.007	0.005	0.015	0.010	0.072	0.048	0.129	0.153	0.449	0.299

In both male and female rats, the excretion after a single oral dose of 120 or 360 mg/kg bw/d is not completed within 24 hours and high levels of radioactivity remain in the plasma. Additional or repeated dosing within 24 hours would thus result in an increase of the internal dose. This effect is already marginally visible at 40 mg/kg bw/d.

Plasma kinetics were also investigated in male and female mice after receiving a single oral dose of 10, 30, 100, 250 and 600 mg/kg bw, respectively (02B0233/15B007). Animals of the highest dose level of 600 mg/kg bw were sacrificed 24 hours post administration in a moribund state. Blood samples were taken at defined time points after test-substance administration. Blood was separated into plasma and blood cells and radioactivity was measured in each sample.

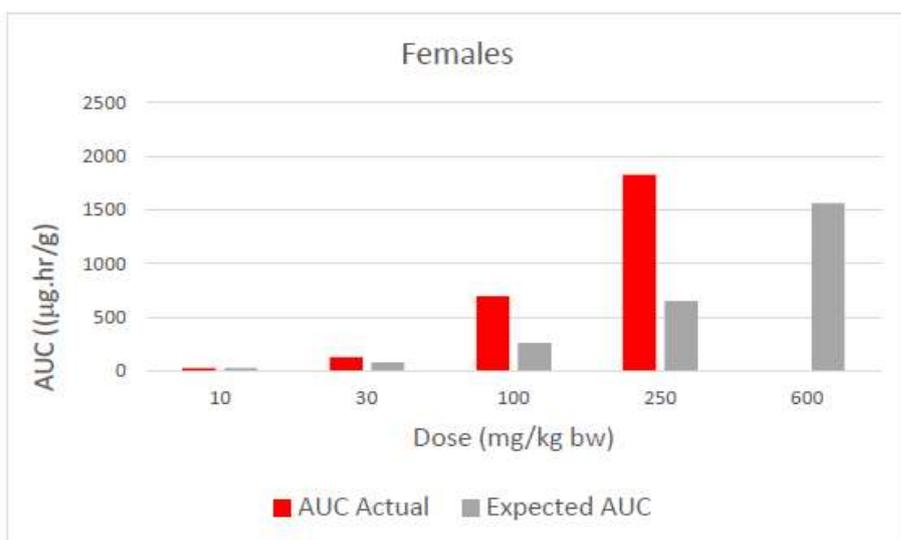
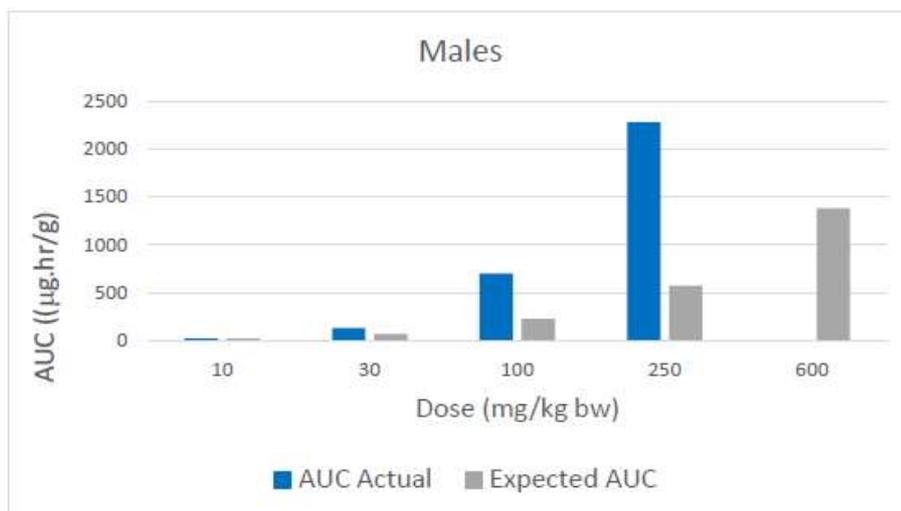
The pharmacokinetic parameters derived from time-dependent mean plasma concentrations of each dose group are presented below:

gender	target dose	C _{max}	T _{max}	terminal half life	AUC ₀₋₁₆₈	AUC _{0-∞}
	[mg/kg bw]	[µg Eq/g]	[hour]	[hour]	[µg Eq.xh/g]	[µg Eq.xh/g]
male	10	8.93	1	54.4	23	23
	30	25.37	1	39.0	132	133
	100	67.89	1	41.8	717	719
	250	97.75	8	33.6	2234	2256
	600	- ¹	- ¹	- ¹	- ¹	- ¹
female	10	8.90	1	60.5	25	26
	30	25.64	1	48.0	136	137
	100	67.67	1	46.1	681	693
	250	90.67	4	81.8	1790	1829
	600	- ¹	- ¹	- ¹	- ¹	- ¹

¹ parameter not determined

After single oral administration the test material was rapidly absorbed from the gastrointestinal tract. No marked gender differences in the time-course of absorption and elimination in plasma samples were observed. The internal dose, as indicated by the area under the curve (AUC), increased over-proportionally to the dose levels administered. This effect was already present starting at the target dose of 10 mg/kg bw when compared to the target dose of 30 mg/kg bw. These results clearly demonstrate non-linear kinetics of 3,4-DMP after oral dosing in mice.

Based on an observed AUC at the lowest dose of 10 mg/kg bw, the expected AUCs at the higher dose levels can be extrapolated. If excretion kinetics would follow a linear pattern, the AUCs should increase by roughly the same factor as the dose levels. As shown by the graphs below, this is neither the case in male nor in female mice.



The mean plasma concentrations after single oral administration of ¹⁴C-3,4-DMP at target dose levels of 10; 30; 100; 250 and 600 mg/kg bw to male and female mice is shown in the table below.

Plasma concentrations expressed in µg Eq/g plasma

Time [h]	10 mg/kg bw males	30 mg/kg bw males	100 mg/kg bw males	250 mg/kg bw males	600 mg/kg bw males
1	8.93	25.37	67.89	82.45	110.07
2	5.07	21.22	63.18	83.89	132.47
4	0.72	15.79	50.82	94.89	159.55
8	0.12	1.75	37.97	97.75	224.51
24	0.04	0.18	0.41	35.52	248.24
48	0.02	0.08	0.26	0.52	-
72	0.03	0.05	0.12	0.46	-
96	0.02	0.06	0.08	0.17	-
120	0.01	0.02	0.07	0.14	-
144	0.01	0.02	0.06	0.69	-
168	0.01	0.02	0.03	0.45	-

Time [h]	10 mg/kg bw females	30 mg/kg bw females	100 mg/kg bw females	250 mg/kg bw females	600 mg/kg bw females
1	8.90	25.64	67.67	84.46	133.72
2	5.64	22.67	61.65	88.83	136.03
4	0.79	12.98	49.64	90.67	144.68
8	0.14	2.84	33.57	82.45	215.54
24	0.06	0.16	0.31	20.93	234.79
48	0.03	0.08	0.39	0.53	-
72	0.03	0.05	0.43	0.47	-
96	0.02	0.04	0.10	0.27	-
120	0.02	0.03	0.19	0.32	-
144	0.01	0.03	0.10	0.63	-
168	0.01	0.02	0.06	0.33	-

In both male and female mice, the excretion after a single oral dose of 250 or 600 mg/kg bw/d is not completed within 24 hours (the mice dosed with 600 mg/kg bw/d were sacrificed moribund after 24 h) and high levels of radioactivity remain in the plasma. Additional or repeated dosing within 24 hours would thus result in an increase of the internal dose.

As shown by the available *in vivo* studies, 3,4-DMP and its metabolites are mainly excreted via urine. It was thus examined both *in vitro* and *in vivo* whether kidney organic anion transporters facilitate the transport of 3,4-DMP from blood to urine.

In vitro studies with the main metabolite MPCA showed, that both human (h) and rat (r) OAT1 and OAT2 were transporters of this metabolite (99V0237/16X490 and 99V0237/16X549).

Furthermore, the K_m (given in µM) and V_{max} (given in pmol/mg/min) values were calculated in transporter-overexpressing HEK cells (99V0237/16X549): 262 ± 46 µM and 1215 ± 75 pmol/mg/min for hOAT1, 1210 ± 133 µM and 5990 ± 403 pmol/mg/min for hOAT2, 330 ± 19 µM and 1380 ± 30 pmol/mg/min for rOAT1 and 512 ± 47 µM and 11403 ± 461 pmol/mg/min for rOAT2, respectively.

With regard to the substrate affinity towards the investigated organic anion transporters, they can be ranked as follows:

hOAT1 > rOAT1 > rOAT2 >> hOAT2

As hOAT1 and hOAT2 are both localized basolaterally in humans whereas in rats only rOAT1 is localized basolaterally, the transport out of the blood could be slightly higher in humans compared to rats (1). In addition, since rOAT2 is mainly expressed at the luminal side of proximal tubule cells (1), rOAT2 could also be involved in the reabsorption from the primary urine. Hence, rOAT2 might contribute to less renal clearance and lower elimination from the blood circulation for a certain period in rats compared to humans. These investigations suggest that the transport out of the blood into the urine could be slightly more efficient in humans compared to rats, making humans less prone to the toxic effects of 3,4-DMP.

In addition, *in vivo* investigations on plasma kinetics were conducted in Wistar rats (02B0440/01B008) with phosphorylated 3,4-DMP and the main metabolite MPCA under the influence of Probenecid as a competitive inhibitor (1) of organic anion transporters (OATs). The study was designed to investigate, whether excretion of the dosed radioactivity occurs actively mediated by OATs *in vivo*. The investigations of the major metabolite MPCA in the rat are of special interest, because this metabolite is a carboxylic acid derivative and based on its structure and the results of *in vitro* investigations (see above), a substrate for rOAT1 and rOAT2 in the kidney. To focus on excretion, this metabolite is tested in a scenario of 100 % systemic bioavailability, e.g., in plasma kinetics after intravenous administration. Two groups of female Wistar rats received a single oral target dose of 10 mg/kg bw phosphorylated 3,4-DMP by gavage either with or without intraperitoneal cotreatment with Probenecid at a target dose of 150 mg/kg bw. Two further groups of female rats received a single intravenous target dose of 10 mg/kg bw of the main metabolite MPCA either with or without intraperitoneal cotreatment with Probenecid at a target dose of 150 mg/kg bw. Blood was sampled at various time points up to 72-hours post dosing and the plasma concentrations of radioactivity were determined. Although mean half-lives decreased with co-administration of Probenecid for both test substances, the internal dose increased when Probenecid was co-administered to the radiolabeled test substances compared to groups without coadministration of the OAT-inhibitor. In plasma kinetics of the main metabolite MPCA after intravenous dosing, the mean maximum plasma concentration was clearly increased for the dose group with Probenecid coadministration versus the group without Probenecid cotreatment. Taken together, these findings demonstrate that renal excretion of both parent and metabolite is inhibited by the OAT-inhibitor Probenecid. Consequently, it can be concluded that both test substances are excreted from the organism actively by OATs.

Oral repeated dose toxicity studies identified the olfactory epithelium as target tissue in the rat and the mouse. Since the organic anion transporter 6 (OAT6), shows strong expression in nasal epithelia (2) in rodents, its potential to transport the main metabolite MPCA, a carboxylic acid, has been examined *in vitro* (99V0237/16X548). OAT6 has been described in rats and mice, while in humans only a pseudogene (SLC22A20P) has been identified thus far, which is not protein coding (3). Since the available literature information for the rat OAT6 is limited, mouse OAT6 (mOAT6) was used in this investigation.

For mOAT6, the main metabolite MPCA showed clear substrate characteristics as displayed by a mOAT6-mediated uptake ratio of 8.1-fold compared to vector-cells at 100 μ M. Furthermore, its substrate character was confirmed by the successful uptake inhibition of the test item by ibuprofen: Percentages of inhibition were 56% and 83% at 100 μ M and 300 μ M ibuprofen, respectively. Additionally, 300 μ M of the probe substrate estrone sulfate also inhibited the mOAT6-mediated uptake of the main metabolite MPCA (43% inhibition). This *in vitro* experiment shows that OAT6-mediated transport of the main metabolite MPCA into the olfactory epithelium is possible.

Overall summary of toxicokinetics:

Overall, the differences in absorption, distribution, metabolism and elimination of 3,4-DMP between rats and goats are minimal. MPCA, the main carboxylic acid metabolite of 3,4-DMP is found in the urine of both rats and goats and mainly quantitative differences between the different metabolites are observed.

The available data show that excretion of the test item and/or its metabolites occurs mainly via urine and involves an active transport via organic anion transporters in the kidney. The excretion via the kidneys can be saturated as confirmed by *in vivo* studies: The kinetics demonstrate non-linearity at dose levels of 12 mg/kg bw/d and higher in rats (2016/7006240). Therefore, a dose level in between 12-30 mg/kg bw/d is considered as kinetic MTD for repeated oral administration in the rat based on all available data. Similarly, also in mice, non-linearity was observed at dose levels of 30 mg/kg bw/d and higher (02B0233/15B007). In mice, a dose level in between 30-64 mg/kg bw/d is considered as kinetic MTD for repeated oral administration based on all available data.

Saturation of excretion is a crucial point that must be taken into account when examining toxicity after repeated dosing because excretion is a key detoxification process.

This issue is *inter alia* addressed in the OECD 443 guideline (extended one generation study):

“If TK data are available which indicate dose-dependent saturation of TK processes, care should be taken to avoid high dose levels which clearly exhibit saturation, (...). In such cases, the highest dose level should be at, or just slightly above the inflection point for transition to nonlinear TK behavior.”

Similarly, the ECHA Guidance Document on Information Requirements and Chemical Safety Assessment R7a (2017) states the following:

“When conducting repeated dose toxicity studies, it is necessary to ensure that the observed treatment-related toxicity is not associated with the administration of excessive high doses causing saturation of absorption and detoxification mechanisms. The results obtained from studies using excessive doses causing saturation of metabolism are often of limited value in defining the risk posed at more relevant and realistic exposure levels where a substance can be readily metabolized and cleared from the body.”

Non-linearity between external and internal doses directly impacts the analysis of dose-response effects. Since derived no effect levels (DNELs) for exposure assessment were calculated from doses where no saturation of kinetics occurred (and where no adverse effects were seen), toxicity at kinetically saturated doses is not relevant to real life human exposure scenarios.

References:

- (1) Burckhardt G: Drug transport by organic anion transporters (OATs). *Pharmacol Ther.* 2012;136(1):106-30.
- (2) Nigam SK et al: The organic anion transporter (OAT) family: a systems biology perspective. *Physiol Rev.* 2015 Jan;95(1):83-123.
- (3) <https://www.ncbi.nlm.nih.gov/gene/440044>

10 EVALUATION OF HEALTH HAZARDS

10.1 Acute toxicity - oral route

10.1.3 Conclusion on classification and labelling for acute oral toxicity

The lead registrant agrees with the proposed classification as **Acute Tox. Cat. 4, H302 (Harmful if swallowed)** and the proposed **ATE_(oral) of 500 mg/kg bw.**

10.2 Acute toxicity - dermal route

10.2.3 Conclusion on classification and labelling for acute dermal toxicity

The lead registrant agrees with the proposed classification as **Acute Tox. Cat. 4, H312 (Harmful in contact with skin)** and the proposed **ATE_(dermal) of 1100 mg/kg bw.**

10.3 Acute toxicity - inhalation route

10.3.3 Conclusion on classification and labelling for acute inhalation toxicity

The lead registrant agrees with the proposed classification as **Acute Tox. Cat. 4, H332 (Harmful if inhaled)** and the proposed **ATE_(inhalation-dust/mist) of 2.1 mg/L.**

10.9 Carcinogenicity

The lead registrant would like to point out, that the DS did not include the results of a carcinogenicity study in C57BL/6 J Rj mice (87C0429/02C033, 2018) according to OECD 451 guideline and GLP:

In this study, 3,4-DMP was administered via the diet to groups consisting of 50 male and 50 female C57BL/6 J Rj mice at nominal dose levels of 0 (test group 0), 2 (test group 1), 20 (test group 2) and 80 mg/kg bw/d (test group 3) over a period of 18 months. Food consumption and body weight were determined once a week during the first 13 weeks and thereafter at 4-week intervals at the end of the study. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Blood smears were prepared after 12 months and at the end of the administration period. All animals were assessed by gross pathology, followed by histopathological examinations.

The following test substance-related, adverse findings were noted:

Test group 3: Actual dose in males: 85.5 mg/kg bw/d and in females: 98.6 mg/kg bw/d
Clinical parameters, and clinical pathology

• No treatment-related, adverse findings were observed

Pathology

• Respiratory metaplasia and hyperplasia of the olfactory epithelium in all male and female animals in level III of the nasal cavity and in 49/50 male and female animals in level II of the nasal cavity

Test group 2: Actual dose in males: 20.8 mg/kg bw/d and in females: 24.4 mg/kg bw/d
Clinical parameters, clinical pathology and pathology

• No treatment-related adverse findings.

Test group 1: Actual dose in males: 2.2 mg/kg bw/d and in females: 2.6 mg/kg bw/d
Clinical parameters, clinical pathology and pathology
• No treatment-related, adverse findings were observed

The oral administration of 3,4-DMP via the diet to C57BL/6 J Rj mice over a period of 18 months did not demonstrate a carcinogenic potential up to the highest dose tested: 85.5 mg/kg bw/d in males and 98.6 mg/kg bw/d in females.

There were no significant increases in neoplastic lesions, and life span was unaffected.

The NOAEL for systemic toxicity was the mid dose level of 20.8 mg/kg bw/d in males and 24.4 mg/kg bw/d in females based on alteration of the olfactory epithelium in the nasal cavity.

The NOAEL for carcinogenicity was the highest dose level of 85.5 mg/kg bw/d in males and 98.6 mg/kg bw/d in females.

Discussion of chronic/carcinogenicity studies with regards to carcinogenicity classification or non-classification

3,4-DMP was not mutagenic in the Salmonella typhimurium/Escherichia coli reverse mutation assay (40M0429/02M011) in the absence and the presence of metabolic activation. When tested in a mouse lymphoma assay using L5178Y TK+/- cells (52M0429/02M014), no forward mutations or structural chromosome aberrations were induced in the absence and the presence of metabolic activation. Finally, neither a chromosome-damaging (clastogenic) effect nor impairment of chromosome distribution in the course of mitosis (aneugenic activity) was observed in bone marrow cells of NMRI mice in vivo (26M0429/02M013) at dose levels up to 500 mg/kg bw.

In the oral carcinogenicity study with 3,4-DMP in C57BL/6 J Rj mice (87C0429/02C033), respiratory metaplasia and hyperplasia of the olfactory epithelium in all male and female animals in level III of the nasal cavity and in 49/50 male and female animals in level II of the nasal cavity were observed at the highest dose level tested (males: 85.5 mg/kg bw/d and in females: 98.6 mg/kg bw/d). These findings are most likely progressions of the degeneration/regeneration of the olfactory epithelium in the nasal cavity observed in the sub-chronic and sub-acute studies. The oral administration via the diet over a period of 18 months did not demonstrate a carcinogenic potential up to the highest dose tested. There were no significant increases in neoplastic lesions, and life span was unaffected. The NOAEL for systemic toxicity was the mid dose level of 20.8 mg/kg bw/d in males and 24.4 mg/kg bw/d in females based on alteration of the olfactory epithelium in the nasal cavity at the highest dose level.

In the combined chronic toxicity/carcinogenicity study in Wistar rats (80C0429/02S024), a **carcinogenic potential** of 3,4-DMP was observed in **male animals**.

After two years of exposure, neuroepithelial carcinomas were observed in the nasal cavity (level III) of seven of 50 males. In addition, an adenocarcinoma was seen in the nasal cavity (level IV) of one male. These tumors occurred at the highest dose level of 60 mg/kg bw/d only. The neuroepithelial carcinomas arose from the olfactory epithelium (sustentacular cells, basal cells, immature sensory cells, and possibly ductal cells of Bowman's glands) and the adenocarcinoma was located unilateral in the lumen between ethmoid turbinates in level IV of the nasal cavity.

Additional findings in the nasal cavity were minimal to massive (multi)focal hyperplasia of the olfactory epithelium in the posterior part of the nasal cavity (level III and/or IV) in six male and two female animals, minimal to severe degeneration/regeneration of the olfactory epithelium in the dorsal meatus of the nasal cavity in 47 males and in 47 females and a significantly higher incidence of minimal to severe inflammation and/or inflammatory cells in the lumen in the nasal cavity, especially in levels III and/or IV in both males and females.

The occurrence of neuroepithelial carcinomas in seven males (nasal cavity, level III, statistically significantly increased) and the adenocarcinoma in the nasal cavity (level IV) in one male of the high dose group were regarded to be treatment-related and adverse and are thus potentially relevant for classification with regards to carcinogenicity. Female animals did not show tumors in the nasal cavity.

In addition, an increased incidence of malignant lymphomas in six males (12%), i.e., four T-cell, one B-cell, and one unclassifiable, versus one control male (unclassifiable) was observed at 60 mg/kg bw/d without statistical significance but above the range of historical control data (8%). The relationship to treatment is not as clear because lymphomas were not of the same cellular origin. In females, the incidence of malignant lymphomas was comparable between control and high dose animals.

Nevertheless, a treatment-related effect cannot be completely ruled out based on the laboratories historical control data. If, however taking a publication by Walsh and Poteracki (4) into account where the authors reported spontaneous neoplasms in control Wistar rats with incidences for lymphomas in male control Wistar rats in the range of 2-12 %, a relation to treatment is more than questionable as this publication shows that the higher incidence of lymphomas observed in this study is still within the normal biological range for male Wistar rats. **The increased incidence of malignant lymphomas is therefore considered to be not relevant for classification with regards to carcinogenicity.**

It should be noted that no lymphomas or any other kind of tumors were observed in male and female mice at even higher dose levels.

As all three genotoxicity studies conducted with 3,4-DMP were negative, a non-mutagenic mode of action for tumor formation in the nasal cavity must be assumed.

Generally, toxic effects of 3,4-DMP were observed in the combined chronic toxicity/carcinogenicity study in Wistar rats at dose levels of 30 and 60 mg/kg bw/d. For discussion of carcinogenicity, especially the observations made in male rats are key:

At 30 mg/kg bw/d, the following test substance-related, relevant findings were observed in male rats at the end of the study:

- Significantly decreased body weight change values.
- Minimal or slight (multi)focal hyperplasia of the olfactory epithelium in the nasal cavity (level III) in two male animals.
- Minimal to **moderate** degeneration/ regeneration of the olfactory epithelium in the dorsal meatus of the nasal cavity in 48 males.
- Minimal to **severe** diffuse atrophy in the mandibular glands in 35 males.
- Increased number of males with (peri-)vasculitis in different organs (mainly testes)

At 60 mg/kg bw/d, the following test substance-related, relevant findings were observed in male rats at the end of the study (tumors not discussed here):

- Increased mortality rate in male animals, i.e., 44% (34 % if excluding the five decedents that died of a tumor in the nasal cavity) vs. 22% in controls.
- Significantly decreased body weights with a maximum of -12% in males.
- Significantly decreased body weight change values with a maximum of -16% in males.
- Significantly decreased terminal body weight in males (-13%).
- Minimal to **massive** (multi)focal hyperplasia of the olfactory epithelium in the posterior part of the nasal cavity (level III and/or IV) in six males.
- Minimal to **severe** degeneration/ regeneration of the olfactory epithelium in the dorsal meatus of the nasal cavity in 47 males.
- Significantly higher incidence of minimal to **severe** inflammation and/or inflammatory cells in the lumen in the nasal cavity, especially in levels III and/or IV.
- Minimal to **severe** diffuse atrophy in the mandibular glands in 48 males.
- Increased number of males with (peri-)vasculitis in different organs (mainly testes and pancreas)
- Higher incidence of a mostly minimal (multi)focal degeneration of the skeletal muscle.

OECD 453 guideline states that "...the highest dose level should be chosen to identify the principal target organs and toxic effects while avoiding suffering, **severe toxicity**, morbidity, or death. The highest dose level should be normally chosen to elicit evidence of toxicity, as evidenced by, for example, **depression of body weight gain (approximately 10%)**. However, dependent on the objectives of the study (see paragraph 6), a top dose lower than the dose providing evidence of toxicity may be chosen, e.g., if a dose elicits an adverse effect of concern, which nonetheless has little impact on lifespan or body weight."

OECD guidance document 35 (2002) states that "The highest dose to be used in a chronic toxicity or carcinogenicity study needs to be carefully considered and the reasons for the final choice clearly defined" and "the largest administered dose should not compromise the biological interpretability of the observed responses. For example, it is generally considered that it should not:

(a) in a chronic study, **exceed the maximum tolerated dose (or MTD)** defined as the highest dose to produce toxic effects without causing death and **to decrease body weight by no more than 10% relative to controls**".

OECD guidance document 116 (2012) further states that "If the main objective of the study is to identify a cancer hazard, there is broad acceptance that **the top dose should ideally provide some signs of toxicity such as slight depression of body weight gain (not more than 10%), without causing e.g., tissue necrosis or metabolic saturation** and without substantially altering normal life span due to effects other than tumors".

ECHA guidance on the application of the CLP criteria (5.0, 2017) states that "In lifetime bioassays compounds are routinely tested using at least three dose levels to enable hazard identification and hazard characterization as part of risk assessment. Of these doses, the **highest dose needs to induce minimal toxicity, such as characterized by an approximately 10% reduction in body weight gain (maximal tolerated dose, MTD dose)**" and "excessive toxicity, for instance toxicity at doses exceeding the MTD, can affect the carcinogenic responses in bioassays. Such toxicity can cause effects such as cell death (necrosis) with associated regenerative hyperplasia, which can lead to tumor development as a secondary consequence unrelated to the intrinsic potential of the substance itself to cause tumors at lower less toxic doses" and "tumors occurring only at excessive doses associated with severe toxicity generally have a more doubtful potential for carcinogenicity in humans. In addition, tumors occurring only at sites of contact and/or only at excessive doses need to be carefully evaluated for human relevance for carcinogenic hazard." and "If a test compound is only found to be carcinogenic at the highest dose(s) used in a lifetime bioassay, and the characteristics associated with doses exceeding the MTD as outlined above are present, this could be an indication of a confounding effect of excessive toxicity. This may support a classification of the test compound in Category 2 or no classification".

Based on the severity of effects observed at 60 mg/kg bw/d (severe histopathology findings, body weight decreased $\geq 10\%$, and body weight gain decreased $>10\%$) the high dose level exceeded the MTD provision for dose selection set forth in the OECD 453 guideline as well as OECD and ECHA guidance documents.

In addition, OECD 453 guideline also states that toxicokinetic data available for the test substance are also key for dose setting.

Points to be considered in dose selection include:

"Known or suspected nonlinearities or inflection points in the dose–response;"

"Toxicokinetics, and dose ranges where metabolic induction, saturation, or nonlinearity between external and internal doses does or does not occur;"

As outlined above it is also emphasized in OECD guidance document 116 (2012) that metabolic saturation should be avoided: "Toxicokinetic non-linearity should also be considered in the selection of the top dose to be used. Although top dose selection based on identification of inflection points in toxicokinetic nonlinearity may result in study designs that fail to identify traditional target organ or body weight effects, it must be appreciated that metabolic saturation in fact represents an equivalent indicator of biological stress. In this case, the stress is evidenced by

appearance of non-linear toxicokinetics rather than appearance of histological damage, adverse changes in clinical chemistry, hematology parameters or decrease in body weight gain”.

The available toxicokinetic data show, that excretion of the test item and/or its metabolites occurs mainly via urine and involves an active transport via organic anion transporters in the kidney. The excretion via the kidneys can be saturated as confirmed by *in vivo* studies: Non-linear kinetics indicated a saturation of kinetics that was already present at a dose level of 12 mg/kg bw in rats (2016/7006240).

Based on all available data a dose level between 12-30 mg/kg bw/d can be considered as kinetic MTD for repeated oral administration in the rat.

The dose level of 30 mg/kg bw/d could possibly already satisfy the guideline requirements as an appropriate high dose level as severe effects were at least observed in the mandibular gland together with moderate effects in the olfactory epithelium and some depression of body weight gain which occurred as well.

Based on the toxicokinetic considerations together with the severe toxicity observed at the high dose level of 60 mg/kg, the MTD, as recommended in various guidelines, was clearly exceeded.

A dose level between 30-60 mg/kg bw/d would have been more appropriate.

Concerning the tumors in nasal cavity of rats, there is additional evidence that these are likely not relevant to humans.

The postulated mechanism is transport of the main metabolite MPCA, which is a carboxylic acid, by rodent specific organic anion transporter 6 (OAT6) into the olfactory epithelium (5, 6). This transport and the potential subsequent accumulation led to cytotoxicity, necrosis and inflammation (seen in some high dose animals) in the long-term. Clearly, cell proliferation is also involved in the process. These processes are very likely the cause for the observed degeneration and regeneration of the olfactory epithelium and subsequent tumor formation in male rats. Transport of the main metabolite MPCA by OAT6 has been confirmed *in vitro* (99V0237/16X548). As OAT6 has not been identified in humans (to date only a pseudogene was identified, which is not protein coding (7)) and there were no treatment related effects observed in the olfactory epithelium in dogs, a rodent specific mechanism is plausible based on the available data.

In vitro experiments with both rat and human OAT1 and OAT2 (99V0237/16X549) with regards to potential excretion kinetics in the kidneys suggest that the transport out of the blood into the urine could be slightly higher in humans compared to rats making humans less prone to the toxic effects of 3,4-DMP.

Although not necessarily relevant for the discussion on the appropriate cancer classification (or non-classification), the effect of non-linear kinetics resulting in a saturation of excretion was more pronounced in female rats compared to males. Nevertheless, no tumor formation was observed in the nasal cavity of female rats.

The explanation for this phenomenon is likely related to the differences in OAT-expression dependent on age and sex (5, 6).

Rat OAT1 and OAT2 are transporters of the main metabolite (MPCA) of 3,4-DMP (99V0237/16X549).

In male rats, the OAT1 mRNA levels in the kidneys were higher compared to females whereas OAT2 mRNA levels in the kidneys were higher in female rats compared to males (8, 9).

In rats, rOAT1 is localized basolaterally (5) whereas rOAT2 is mainly expressed at the luminal side of proximal tubule cells (5). Thus, rOAT2 could also be involved in the reabsorption from the primary urine.

The substrate affinity of the main metabolite (MPCA) of 3,4-DMP was higher towards rOAT1 than rOAT2 (99V0237/16X549). This might explain together with the sex differences in rOAT1 and rOAT2 levels why saturation of kinetics was more pronounced in female than in male rats

(2016/7006240). Based on the observations summarized above, lower clearance via rOAT1 and higher potential re-uptake via rOAT2 would be expected in female rats compared to males. When the experiment was conducted, the rats were approximately 10 weeks old, a time where rOAT1 and rOAT2 transporter mRNA-levels in the kidneys are at its peak (8) in both sexes. Studies in male rats show however, that protein expression levels of OAT1 in the kidneys increased over time, peaking at 60 days but were slightly decreasing with age (10). At the next investigated time points in this publication of 180, 540 and 850 days of age, the OAT1 protein expression levels in the kidneys were lower compared to 60 days (10). Lower OAT1 kidney levels in ageing male rats could potentially lead to higher internal exposures if excretion in the kidney decreases and might thus be another potential explanation why tumors were ultimately only seen in male rats.

In rats and mice, sex and species differences have been observed in the expression levels of mRNAs and/or proteins (for example OAT1, OAT2, OAT3, OAT5) of several organic anion transporters (5, 6, 11). This could be one explanation as to why 3,4-DMP did not demonstrate a carcinogenic potential up to the highest dose tested in mice. Unfortunately, there are no investigations published dealing with sex (or species) differences with regards to OAT6 expression. Most research published to date was conducted in mice and only very limited information on rat OAT6 is available in the literature.

Nevertheless, in analogy to observed sex differences in mRNA or protein expression levels with other OATs it is plausible, that OAT6 levels in the olfactory epithelium of male rats could be higher compared to female rats offering a logic explanation for tumor formation in the nasal cavity in male rats only (higher uptake of the main metabolite MPCA in males compared to females). Reduced excretion (lower OAT protein levels) via the kidneys in ageing male rats could intensify this effect.

This assumption is substantiated by observations made in a two-generation study in Wistar rats (76R0429/02R044) where minimal degeneration/regeneration of the olfactory epithelium was observed at a dose level of 25 mg/kg bw/d in the F0 parental animals in 23/25 male but only in 4/25 female animals. This observation contradicts investigations (2016/7006240) showing that the internal dose was slightly higher for females at oral doses above 4 and below 41 mg/kg bw compared to males, which should result in findings in the olfactory epithelium in females of at least similar magnitude. However, this was not the case. A higher transport rate of the main metabolite MPCA into the nasal epithelium in males facilitated by a higher amount of OAT6 can serve as possible explanation for the observed higher number of male rats with degeneration/regeneration of the olfactory epithelium in this study.

Summary of Carcinogenicity:

In mice, the available carcinogenicity study did not demonstrate a carcinogenic potential of 3,4-DMP.

In the combined chronic toxicity/carcinogenicity study in rats, tumor formation with clear relation to treatment was observed in the nasal cavity of male animals only and only at a dose exceeding the maximum tolerated dose (the highest dose level tested 60 mg/kg bw/d). For reason given above (no statistically significant increase, lymphoma incidence within published values for male Wistar rats), the observed increased number of lymphomas is not considered relevant for classification and labeling.

As all three genotoxicity studies conducted with 3,4-DMP were negative, a non-mutagenic mode of action for tumor formation in the nasal cavity can be assumed.

The renal excretion can be saturated as confirmed by *in vivo* studies: disproportionate increases for internal exposure, indicating detoxification has been compromised by saturation of excretion processes, are already present at a dose level of 12 mg/kg bw in rats. Based on the severity of effects observed at 60 mg/kg bw/d (severe histopathology findings and depression of body weight

gain above 10 %) together with the toxicokinetic considerations, the high dose level of 60 mg/kg bw/d was too high considering the provision for dose selection set forth in the OECD 453 guideline, and thus of doubtful relevance to hazard assessment.

Tumor formation in the nasal cavity (mainly level III) was accompanied by minimal to massive (multi)focal hyperplasia of the olfactory epithelium in the posterior part of the nasal cavity (level III and/or IV) in some animals, minimal to severe degeneration/regeneration of the olfactory epithelium in the dorsal meatus of the nasal cavity in almost all animals and a significantly higher incidence of minimal to severe inflammation and/or inflammatory cells in the lumen in the nasal cavity, especially in levels III and/or IV.

Inflammation can promote the occurrence and development of cancer by e.g., promoting blood vessel growth, cancer cell proliferation, tumor invasiveness and by negatively regulating immune response (12, 13).

The postulated mechanism for the observed tumor formation in the nasal epithelium in male rats is a consequence of the transport of the main metabolite MPCA, a carboxylic acid, by rodent specific organic anion transporter 6 (OAT6) into the olfactory epithelium (5, 6). This transport and the potential subsequent accumulation led to cytotoxicity, necrosis and inflammation in the long-term. Clearly, cell proliferation is also involved in the process. These processes are very likely the cause for the observed tumor formation in the nasal cavity. Transport of the main metabolite MPCA by OAT6 has been confirmed *in vitro* (99V0237/16X548). As OAT6 has not been identified in humans (to date only a pseudogene was identified, which is not protein coding (7)) and as no treatment related effects were observed in the olfactory epithelium in dogs, a rodent specific mechanism is plausible based on the currently available data. The reason for male-specific tumor formation in rats could potentially be linked to sex-specific differences in OAT6 expression levels (higher OAT6 levels in males) as sex-specific differences have been observed for protein expression levels of several other OATs (5, 6, 11). This assumption is supported by *in vivo* data showing that the olfactory epithelium of male rats is affected in higher numbers at the lowest LOAEL of 25 mg/kg bw compared to females.

In vitro experiments with both rat and human OAT1 and OAT2 (99V0237/16X549) with regards to potential excretion kinetics in the kidneys suggest that the transport out of the blood into the urine could be slightly higher in humans compared to rats making humans less prone to the toxic effects of 3,4-DMP.

Conclusion on classification or non-classification with regards to carcinogenicity:

According to the CLP (REGULATION (EC) No 1272/2008), classification of a substance as a carcinogen is a process that involves two interrelated determinations: evaluations of strength of evidence and consideration of all other relevant information to place substances with human cancer potential into hazard categories (3.6.2.2.2.).

3.6.2.2.3. (b): Limited evidence of carcinogenicity: the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) **the evidence of carcinogenicity is restricted to a single experiment**; (b) **there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies** (e.g., human relevance of results at doses exceeding the maximum tolerated dose) (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

ECHA guidance on the application of the CLP criteria (5.0, 2017) states that “In lifetime bioassays compounds are routinely tested using at least three dose levels to enable hazard identification and hazard characterization as part of risk assessment. Of these doses, the highest dose needs to induce minimal toxicity, such as characterized by an approximately 10% reduction in body weight gain (maximal tolerated dose, MTD dose)” and “excessive toxicity, for instance toxicity at doses exceeding the MTD, can affect the carcinogenic responses in bioassays. Such toxicity can

cause effects such as cell death (necrosis) with associated regenerative hyperplasia, which can lead to tumor development as a secondary consequence unrelated to the intrinsic potential of the substance itself to cause tumors at lower less toxic doses” and “tumors occurring only at excessive doses associated with severe toxicity generally have a more doubtful potential for carcinogenicity in humans. In addition, **tumors occurring only at sites of contact and/or only at excessive doses need to be carefully evaluated for human relevance for carcinogenic hazard.**“ and **“If a test compound is only found to be carcinogenic at the highest dose(s) used in a lifetime bioassay, and the characteristics associated with doses exceeding the MTD as outlined above are present, this could be an indication of a confounding effect of excessive toxicity. This may support a classification of the test compound in Category 2 or no classification”.**

As tumor formation with clear relation to treatment was only seen in a single experiment in a single sex and species limited to the nasal cavity (lymphomas in male rats at the highest dose level not considered relevant due to the lack of a statistically significant increase and due to lymphoma incidence being within published values for male Wistar rats) at a dose level where non-linear kinetics indicate saturation of kinetics and where severe histopathology findings and depression of body weight gain above 10 % were observed (exceedance of provisions for high dose selection set forth in the OECD 453 guideline), it has to be questioned whether a classification is warranted at all especially in light of the postulated rodent-specific mode of action via OAT6 transport of the main metabolite MPCA into the nasal epithelium which is not relevant to humans.

The lead registrant therefore thinks that there is enough evidence to not classify for carcinogenicity, a Cancer Cat. 2 classification should only be seen as a precautionary approach and worst case scenario.

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10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

As a general principal, all OECD guidelines for toxicity testing after repeated dosing emphasize the importance of proper dose setting taking all available data including toxicokinetics studies addressing absorption, distribution, metabolism and excretion of the test substance into account.

The current OECD 416 guideline (adopted in 2001) states: “Dose levels should be selected taking into account any existing toxicity data, especially results from repeated dose studies. Any available information on metabolism and kinetics of the test compound or related materials should also be considered. In addition, this information will also assist in demonstrating the adequacy of the dosing regimen.”

The current OECD 414 guideline (adopted in 2018) contains a similar paragraph: “Dose levels should be selected taking into account any existing toxicity data as well as additional information on metabolism and toxicokinetics of the test chemical or related materials. This information will also assist in demonstrating the adequacy of the dosing regimen.”

The current OECD 443 guideline (adopted in 2018) addressing the same endpoint as the OECD 416 guideline is most specific regarding the relevance of toxicokinetics studies: “Although not required, TK data from previously conducted dose range-finding or other studies are extremely useful in the planning of the study design, selection of dose levels and interpretation of results. Of particular utility are data which: 1) verify exposure of developing fetuses and pups to the test compound (or relevant metabolites), 2) provide an estimate of internal dosimetry, and 3) evaluate for potential dose-dependent saturation of kinetic processes.”

“When selecting appropriate dose levels, the investigator should consider all available information, including the dosing information from previous studies, TK data from pregnant or non-pregnant animals, the extent of lactational transfer, and estimates of human exposure. If TK data are available which indicate dose-dependent saturation of TK processes, care should be taken to avoid high dose levels which clearly exhibit saturation, provided of course, that human exposures are expected to be well below the point of saturation. In such cases, the highest dose level should be at, or just slightly above the inflection point for transition to nonlinear TK behaviour.”

Similarly, ECHA Guidance Document on Information Requirements and Chemical Safety Assessment R7a (2017) states as follows:

“When conducting repeated dose toxicity studies, it is necessary to ensure that the observed treatment-related toxicity is not associated with the administration of excessive high doses causing saturation of absorption and detoxification mechanisms. The results obtained from studies using excessive doses causing saturation of metabolism are often of limited value in defining the risk posed at more relevant and realistic exposure levels where a substance can be readily metabolized and cleared from the body.

As summarized in paragraph 9 (toxicokinetics), the available data show that excretion of the test item and/or its metabolites occurs mainly via urine and involves an active transport via organic anion transporters in the kidney. The excretion via the kidneys can be saturated as confirmed by *in vivo* studies: The kinetics demonstrate non-linearity at dose levels of 12 mg/kg bw/d and higher

in rats (2016/7006240). Therefore, a dose level in between 12-30 mg/kg bw/d is considered as kinetic MTD for repeated oral administration in the rat based on all available data. In both male and female rats, the excretion after a single oral dose of 120 or 360 mg/kg bw/d is not completed within 24 hours and high levels of radioactivity remain in the plasma. Additional or repeated dosing within 24 hours would thus result in an increase of the internal dose. The resulting internal exposure become irrelevant to hazard assessment because effects occur only under experimental conditions where detoxification by excretion is compromised, circumstances not present in human exposure. In essence, it is possible to create an adverse effect in rodents, but only under exposure conditions where detoxification is impaired, an event that does not occur at lower exposures.

Similarly, also in mice, non-linearity was observed at dose levels of 30 mg/kg bw/d and higher (02B0233/15B007). In mice, a dose level in between 30-64 mg/kg bw/d is considered as kinetic MTD for repeated oral administration based on all available data. In both male and female mice, the excretion after a single oral dose of 250 or 600 mg/kg bw/d is not completed within 24 hours and high levels of radioactivity remain in the plasma. Additional or repeated dosing within 24 hours would thus result in an increase of the internal dose. Similar to rats, this indicates detoxification is impaired due to saturation of excretion, and therefore effects observed at these doses are disproportionate and do not reflect human hazard potential.

Two-generation reproduction toxicity study according to OECD 416 guideline

The dose levels for the available two-generation reproduction toxicity study (76R0429/02R044) according to OECD 416 guideline and GLP in rats were carefully chosen with respect to the above cited provisions.

The lead registrant therefore disagrees with the DS that the doses used in this study were relatively low.

In this study, the **NOAEL** (no observed adverse effect level) for **general, systemic toxicity** is 6 mg/kg bw/d for the F0 and F1 parental rats, based on minimal to moderate regeneration/regeneration of the olfactory epithelium in most male and female animals, at doses as low as 25 mg/kg bw/d.

The **NOAEL** for **fertility and reproductive performance** for the F0 and F1 parental rats is 100 mg/kg bw/d, the highest dose tested. A LOAEL was not established.

The **NOAEL** for **developmental (offspring) toxicity** in the F1 and F2 progeny is 100 mg/kg bw/d, the highest dose tested. A LOAEL was not established.

Nevertheless, the DS claims that some effects are observed in this two-generation reproduction toxicity study which could be relevant for classification regarding sexual function/fertility:

Male fertility

The DS explains “Regarding male reproductive organ weight, absolute prostate weight was significantly and dose-dependent lower at the highest dose in the F0 generation, while relative prostate weight was lower at this highest dose, but the decrease was not significant. At the F1 generation, prostate was more affected, absolute as well as relative weights were significantly and dose-dependent decreased. Furthermore, at the F1 generation, absolute and relative seminal vesicle weights were significantly, and dose-dependent decreased at 25 and 100 mg/kg bw/d.”

In the study report, the effects on the prostate weight in F0 male animals in the high dose is discussed as follows:

“The minimally decreased absolute weights of prostate glands in high dose male animals were regarded as incidental, since only the absolute weights were affected and since

there were no correlating histopathologic findings in prostate glands. Furthermore, all weight parameters lay within the range of historical controls.”

In the study report, the effects on the prostate weight in F1 male animals in the high dose and the effects on seminal vesicles in the mid and high dose are discussed as follows:

“The significantly decreased absolute and relative weights of seminal vesicles in the mid and high dose male animals were regarded incidental, since all weight parameters lay within the range of historical control values and no treatment-related histopathologic changes of seminal vesicles were seen.”

“The significantly decreased absolute and relative weights of prostate glands in the high dose male animals were regarded as incidental and not treatment-related, since they lay within the range of historical control values, whereas the absolute and relative prostate gland weights in control animals were high and lay above the range of historical controls. Furthermore, no treatment-related histopathologic changes were noted.”

Female fertility

The DS indicates “...in the F0 generation, fertility index tended to decrease at the highest dose, even if the change was not dose related. At this highest dose, absolute and relative ovaries weight were significantly lower in the F1 parental generation.”

It should be noted that the fertility index was not decreased in the F1 generation and both the fertility indices in the F0 and F1 generation were within the normal range of biological variation inherent in the strain of rats used for this study.

The lower ovaries weight effects are discussed in the study report as follows:

“The significantly decreased absolute and relative weights of ovaries in high dose F1 female animals were regarded as incidental, since they lay within the range of historical controls and since no histopathologic changes were seen.”

The DS also noted slightly reduced uterus weights in high dose females. However, the reduction was not statistically significantly changed and within the normal range of biological variation inherent in the strain of rats used for this study.

Overall conclusion on male and female fertility based on results of the two-generation reproduction toxicity study according to OECD 416 guideline:

As all weight effects on sex organ in male and female rats were considered as being incidental with no relation to treatment due to being within the range of historical controls and due to lack of histopathologic changes, these should not be considered relevant for classification towards fertility especially as adversity was not established.

Studies with repeated dosing

Male fertility

28-day repeated dose toxicity study performed in rats according to OECD 407 guideline and GLP (30C0429/02C019):

Effects on prostate, seminal vesicles, coagulating glands and epididymides in male rats at 526 mg/kg bw/d and 235 mg/kg bw/d are considered to be treatment-related and adverse but only occur in conjunction with other severe toxic effects and at dose levels where detoxification via excretion mechanisms are saturated based on available toxicokinetic data (discussed in detail above). This data is thus of limited relevance for classification purposes concerning fertility.

Importantly, the severity of effects on sex organs observed in males at 235 mg/kg bw/d in this 28-day study was not replicated in the subsequent 90-day study at a much higher dose level of 374 mg/kg bw/d (see below).

90-day repeated dose toxicity study performed in rats according to OECD 408 guideline and GLP (50C0429/02S021):

Statistically significantly decreased absolute weights in epididymides, prostate and seminal vesicles were only seen at the highest dose levels tested (374 mg/kg bw/d) and thus again at a dose level where detoxification via excretion mechanisms are saturated.

Relative organ weight of seminal vesicles was only statistically decreased in dose group 3 (129 mg/kg bw/d) but not in dose group 4 (374 mg/kg bw/d). Relative testes weights were statistically significantly increased in the highest dose level (374 mg/kg bw/d) tested at a dose level where detoxification via excretion mechanisms is saturated.

These effects were discussed in the study report as follows:

“The significantly decreased terminal body weight in males of test group 4 (6000 ppm) resulted in a decreased absolute weight of the epididymides and in an increased relative testes weight. In these organs, treatment-related histopathological findings could not be observed.

The mean absolute prostate (0.729 g) and seminal vesicle (0.952 g) weights were significantly decreased in males of test group 4 (374 mg/kg bw/d). The relative prostate (0.22%) and seminal vesicle (0.289%) weights were not significantly different from controls and were within or only minimally below the historical control range (prostate 0.872 - 1.076 g / 0.225 - 0.283%; seminal vesicle: 0.955 - 1.321 g / 0.259 - 0.375%). Therefore, these weight changes were related to the body weight reduction in this test group.

The decreased relative weight of the seminal vesicle (0.283%) in test group 3 males was within the historical control range (0.259 - 0.375%) and was regarded to be incidental.”

Thus, the effects on male reproductive organ weight effects are not relevant for classification as they have to be seen in conjunction with the reduced body weight in males, lack of histopathologic changes and the available historical controls. Again, adversity of the effects could not be established.

Combined chronic toxicity/carcinogenicity study performed in rats according to OECD 453 guideline and GLP (80C0429/02S024)

There were no effects on male sex organ weights after 12 months of exposure up to the highest dose level tested (60 mg/kg bw/d).

After 24 months of exposure, there were no effects on absolute sex organs weights in male rats up to the highest dose level tested (60 mg/kg bw/d).

Relative organ weights of epididymides were increased at 30 and 60 mg/kg bw/d but have to be seen in conjunction with the reduced body weights in both test groups and lack of relevant histopathological findings and thus should not be considered relevant for classification with regards to fertility especially as adversity was not established.

An increased incidence of vascular lesions ((peri-)vasculitis, synonyms: vasculitis, arteritis, perivascular inflammation, periarteritis, polyarteritis nodosa) was seen after 24 months of exposure but not after 12 months of exposure. At 30 mg/kg bw/d it occurred mainly in male rats in the testes (12 % vs. 2 % in controls) and was not statistically significantly increased vs. controls.

At 60 mg/kg bw/d, the increased number of males with (peri-)vasculitis in testes (34 % vs. 2 % in controls) and pancreas (18 % vs. 2 % in controls) was statistically significantly increased in both organs.

Polyarteritis is frequently seen in aging rats. The prevalence is higher in males. Arterial lesions most frequently occur in medium-sized arteries of the mesentery, pancreas, kidney, pancreaticoduodenal artery, testes, and most other organs, except the lung (14). The testes are a particularly common site for this change (15). The disease also most frequently occurs in rats with late-stage chronic nephropathy (14). In mice and hamsters polyarteritis is also commonly observed (16) in aging animals.

Thus the (peri-)vasculitis in the testes should not be considered relevant for classification with regards to fertility as it is a common finding in the ageing rat especially in the testes.

28-day (OECD 407), 90-day (OECD 408) and carcinogenicity (OECD 451) studies in mice (31C0429/02S032; 51C0429/02S022; 87C0429/02C033)

In the 28-day study in mice according to OECD 407 guideline and GLP (87C0429/02C033) no relevant or statistically significant effects on male sex/reproductive organs were seen up to the highest dose of 885 mg/kg bw/d.

In the 90-day study in mice according to OECD 408 guideline and GLP (51C0429/02S022) no relevant or statistically significant effects on male sex/reproductive organs were seen up to the highest dose of 944 mg/kg bw/d except for a significant absolute decrease in epididymides weight at the highest dose tested which has to be seen in relation to the significantly decrease body weight at this dose level. No treatment-related histopathological finding was made. In addition, relative testes weights were also significantly increased at the highest dose level. Histopathology was unremarkable and the weight changes were also attributed to body weight changes. Again, adversity of the effects could not be established, and the effects only occurred at a dose level where detoxification and excretion mechanisms are saturated.

In the carcinogenicity study in mice according to OECD 451 guideline and GLP (87C0429/02C033) no relevant or statistically significant effects on male sex/reproductive organs were seen up to the highest dose of 85.5 mg/kg bw/d except for a small relative weight increase of epididymides at the highest dose level which was regarded as incidental.

28-day (OECD 409) and 90-day (OECD 409) studies in dogs (30D0429/02D023; 31D0429/02D039)

In both the 28- and 90-day study in dogs according to OECD 409 guideline and GLP (30D0429/02D023; 31D0429/02D039) no relevant or statistically significant effects on male sex/reproductive organs were seen up to the highest dose of 90 mg/kg bw/d.

Female fertility

28-day repeated dose toxicity study performed in rats according to OECD 407 guideline and GLP (30C0429/02C019):

Weight effects on ovaries (at 480 and 244 mg/kg bw/d) and uterus (at 480 mg/kg bw/d only) together with some pathology findings in the ovaries and uterus are considered to be treatment-related and adverse but only occur in conjunction with other severe toxic effects and at dose levels where detoxification via excretion mechanisms is saturated based on available toxicokinetic data (discussed in detail above). This data is thus of limited relevance for classification purposes concerning fertility.

Importantly, the severity of effects on sex organs observed in females at 244 mg/kg bw/d in this 28-day study could not be replicated in the subsequent 90-day study at a much higher dose level of 375 mg/kg bw/d (see below).

90-day repeated dose toxicity study performed in rats according to OECD 408 guideline and GLP (50C0429/02S021):

Weights of ovaries and uterus were slightly reduced at the highest dose level (375 mg/kg bw/d) but without statistical significance.

When compared to the controls, interstitial glands in the ovaries showed a minimal to moderate dose-related increased vacuolation in 4/10 females of the test group 3 (143 mg/kg bw/d) and in all females of test group 4 (375 mg/kg bw/d). The increased vacuolation of ovarian interstitial glands was regarded to be treatment-related but non-adverse, as no other histopathological findings such as cellular degeneration were associated with it.

Two females of test group 4 (375 mg/kg bw/d) showed atrophy of the uterus, the cervix, and the vagina. This finding is considered treatment-related and adverse but only occurred at a dose level where detoxification via excretion mechanisms is saturated.

The slight reduction of weights of ovaries and uterus at the highest dose must be seen in conjunction with the significantly lower body weights (-19 %) and significantly reduced body weight changes (-44 %) in female rats.

The vacuolation of ovarian interstitial glands is regarded as being non-adverse and the only clear treatment-related and adverse effect on sex organs was the atrophy which occurred only at a dose level where detoxification via excretion mechanisms is saturated based on available toxicokinetic data (discussed in detail above). This data is thus of limited relevance for classification purposes concerning fertility.

Combined chronic toxicity/carcinogenicity study performed in rats according to OECD 453 guideline and GLP (80C0429/02S024)

There were no statistically significant effects on female sex organ weights after 12 and 24 months of exposure up to the highest dose level tested (60 mg/kg bw/d).

The extremely increased mean ovary weight in females at a dose level of 30 mg/kg bw/d after 12 months of exposure was caused by a single female showing a mass (diameter of 15 mm) in one ovary which correlated histopathologically with a benign thecoma.

28-day (OECD 407), 90-day (OECD 408) and carcinogenicity (OECD 451) studies in mice (31C0429/02S032; 51C0429/02S022; 87C0429/02C033)

In the 28-day study in mice according to OECD 407 guideline and GLP (87C0429/02C033) no relevant or statistically significant effects on female sex/reproductive organs were seen up to the highest dose of 846 mg/kg bw/d.

In the 90-day study in mice according to OECD 408 guideline and GLP (51C0429/02S022) no relevant or statistically significant effects on female sex/reproductive organs were seen up to the highest dose of 1279 mg/kg bw/d. Increased absolute and relative uterus weights in the lowest dose tested were judged as being incidental as they lacked a dose response relationship.

In the carcinogenicity study in mice according to OECD 451 guideline and GLP (87C0429/02C033) no relevant or statistically significant effects on female sex/reproductive organs were seen up to the highest dose of 98.6 mg/kg bw/d.

28-day (OECD 409) and 90-day (OECD 409) studies in dogs (30D0429/02D023; 31D0429/02D039)

In both the 28- and 90-day study in dogs according to OECD 409 guideline and GLP (30D0429/02D023; 31D0429/02D039) no relevant or statistically significant effects on female sex/reproductive organs were seen up to the highest dose of 90 mg/kg bw/d.

When comparing the above summarized study results with provisions of the CLP, in order to classify for **adverse effects** on sexual function and fertility, paragraph 3.7.2.2.1. states that “Classification is made on the basis of the appropriate criteria, outlined above, and an assessment of the total weight of evidence.”

Weight of evidence is further detailed in paragraph 3.7.2.3.1 to 3.7.2.3.5.

The ECHA guidance document “Guidance on the Application of the CLP Criteria” Version 5.0 – July 2017 states in paragraph 3.7.2.3.1. “Use of data from standard repeat dose tests”:

Fertility effects:

Toxicological effects, including marked effects, observed in a standard repeat dose study could be considered valid for the pre-mating phase for adult females and the pre- and post-mating phase for adult males. However, in case of contradictions between the standard repeat dose studies and reproductive studies, the result from the latter should be considered more relevant.

Thus, if applying the weight of evidence approach as outline in the CLP and respective guidance documents it should be concluded that no Repro Cat. 2 classification for fertility would be needed:

1. There were **no adverse** effects on sexual function and fertility in a **Two-generation reproduction toxicity study according to OECD 416 guideline**. Sex organs/the reproductive system was not adversely affected. Appropriate dose levels were chosen based on available data including toxicokinetic data.
2. There were no consistent effects on sex organs/ the reproductive system when looking at the available data from rats, mice, and dogs. Both dogs and mice did not show any adverse effects in sex organs/the reproductive system. In mice, the dose levels tested were much higher than in rats. In rats, effects on sex organs were ambiguous at most at the highest dose levels tested where saturation of excretion kinetics occurred.
3. The very few adverse effects on sex organs/ the reproductive system in rats which partially were not consistent between studies only occurred at dose levels where detoxification via excretion mechanisms are saturated based on available toxicokinetic data.
ECHA Guidance Document on Information Requirements and Chemical Safety Assessment R7a (2017) clearly states that adverse effects seen at these dose levels are of lesser relevance:
“When conducting repeated dose toxicity studies, it is necessary to ensure that the observed treatment-related toxicity is not associated with the administration of excessive high doses causing saturation of absorption and detoxification mechanisms. The results obtained from studies using excessive doses causing saturation of metabolism are often of limited value in defining the risk posed at more relevant and realistic exposure levels where a substance can be readily metabolized and cleared from the body.
4. Available mechanistic data suggests that detoxification via excretion by the kidneys is more efficient in humans compared to rats, making humans less prone to the toxic effects of the substance.

Conclusion on classification and labelling for sexual function and fertility

The lead registrant therefore proposes to remove the proposed Repro Cat. 2 classification for fertility based on the weight of evidence approach presented above and to not classify.

10.10.4 Adverse effects on development

The lead registrant agrees with the DS that no classification for development is needed.

10.10.7 Adverse effects on or via lactation

The lead registrant agrees with the DS that no classification for effects on or via lactation is needed.

10.10.10 Conclusion on classification and labelling for reproductive toxicity

As outlined above, the lead registrant does not agree with the DS that a classification for reproductive toxicity is warranted. The proposed classification should thus be removed.

References:

- (14) Barthold SW, Griffey SM and Percy DH (2016) Pathology of Laboratory Rodents and Rabbits, Fourth Edition (Wiley Blackwell), chapter 2, p 156
- (15) Creasy D et al: Proliferative and nonproliferative lesions of the rat and mouse male reproductive system. Toxicol Pathol. 2012 Aug;40(6 Suppl):40S-121S.
- (16) https://ntp.niehs.nih.gov/nnl/cardiovascular/blood_vessel/polart/index.htm
<https://ntp.niehs.nih.gov/atlas/nnl/cardiovascular-system/blood-vessel/PolyarteritisNodosa>

10.12 Specific target organ toxicity-repeated exposure

The lead registrant agrees, that no STOT RE classification for liver, blood and mandibular gland is warranted based on the available data and considering salivary gland species differences between rodents and humans.

In addition, the proposed STOT RE Cat. 2 H353 (nasal cavity) must be questioned:

According to the CLP (REGULATION (EC) No 1272/2008), 3.9.2.8.1. (e), substance-induced species-specific mechanisms of toxicity, i.e., demonstrated with reasonable certainty to be not relevant for human health, shall not justify classification.

Concerning the adverse effects observed in the olfactory epithelium in rats and mice, there is evidence that these are plausibly not relevant to humans (see also discussion of human relevance of nasal cavity tumors in male rats).

The postulated mechanism is transport of the main metabolite MPCA, which is a carboxylic acid, by rodent specific organic anion transporter 6 (OAT6) into the olfactory epithelium (17, 18). This transport and the potential subsequent accumulation led to cytotoxicity, necrosis and inflammation (seen in the chronic rat study) in the long-term. Clearly, cell proliferation is also involved in the process. These processes are very likely the cause for the observed degeneration and regeneration of the olfactory epithelium. Transport of the main metabolite MPCA, a carboxylic acid, by OAT6 has been confirmed *in vitro* (99V0237/16X548). As OAT6 has not been identified in humans (to date only a pseudogene was identified, which is not protein coding (19)) and there were no treatment related effects observed in the olfactory epithelium in dogs, a rodent specific mechanism is plausible based on the available data.

In vitro experiments with both rat and human OAT1 and OAT2 (99V0237/16X549) with regards to potential excretion kinetics in the kidneys suggest that the transport out of the blood into the urine could be slightly higher in humans compared to rats making humans less prone to the toxic effects of 3,4-DMP.

10.12.3 Conclusion on classification and labelling for STOT RE

The lead registrant therefore suggests to not classify concerning specific target organ toxicity after repeated exposure as the adverse effects observed in the nasal cavity are not relevant to humans and all other organ effects occurred either above the cut-off limit for classification or are also not considered relevant to humans due to species differences between rodents and humans (salivary gland) as discussed by the DS.

References:

- (17) Burckhardt G: Drug transport by organic anion transporters (OATs). *Pharmacol Ther.* 2012;136(1):106-30.
- (18) Nigam SK et al: The organic anion transporter (OAT) family: a systems biology perspective. *Physiol Rev.* 2015, Jan;95(1): 83-123.
- (19) <https://www.ncbi.nlm.nih.gov/gene/440044>

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Unfortunately, the DS did not evaluate the newly available data concerning environmental hazards.

Based on the overall available data, DMP should not be classified as acutely or chronically hazardous to the aquatic environment according to the CLP.

Rationale:

Chronic data are available for all three trophic levels; therefore, classification must be based on the available chronic toxicity data.

The following available data for chronic endpoints are the most sensitive and should be used:

Marine aquatic invertebrates (EPA OPPTS 850.1350, *Americamysis bahia*, 28 -d NOEC = 2 mg/L (mean measured concentrations), 986.6231, 2015) and Algae (OCSP 850.4550, *Anabaena flos-aquae*, 96-hour NOErC = 5.7 mg/L, 96-hour ErC50 = 25 mg/L (mean measured concentrations), 2015/7000486, 2015).

Additionally, for the chronic fish endpoint, data for phosphorylated 3,4-DMP is available: OECD 210, *Pimephales promelas*, 33 -days NOEC ≥ 10 mg/L (nominal, analytically verified), 51F0439/01510, 2002.

Furthermore, 3,4-DMP is not readily biodegradable, based on data available for phosphorylated 3,4-DMP: OECD 301A, < 10% DOC removal after 28 days, 96/0582/21/1, 1997.

These data show that all NOEC values lay above the threshold of 1 mg/L. Since the substance is non-rapidly biodegradable, the substance does not present a long-term hazard according to the categories outlined in Table 4.1.0(b) (i) (Commission Regulation (EU) No 286/2011 amending Regulation (EC) No 1272/2008) for non-rapidly degradable substances.

For non-rapidly biodegradable substances there are only classification categories 1 and 2 available with the respective thresholds of ≤ 0.1 mg/L and ≤1 mg/L (outlined in Table 4.1.0(b) (i) (Commission Regulation (EU) No 286/2011 amending Regulation (EC) No 1272/2008)). Therefore, based on the experimental chronic data, there is no existing classification category for DMP.

The classification as chronic 3 proposed by several applicants could be due to the lack of data on chronic ecotoxicity missing in other applications. Therefore, their C&L is based solely on acute data and the extrapolation on these data considering Table 4.1.0(b) (iii) (Commission Regulation (EU) No 286/2011 amending Regulation (EC) No 1272/2008). In these cases, C&L is based only on the acute data, hence a classification as aquatic chronic 3 would have been justified. But valid chronic experimental data are existing for DMP as explained above.

In conclusion, based on the acute and chronic data, the substance should therefore not be classified as chronically and acutely hazardous to the aquatic environment under the CLP Regulation, since the lowest chronic effect value is $> 1\text{mg/L}$ (Commission Regulation (EU) No 286/2011 amending Regulation (EC) No 1272/2008).