Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Melamine

EC Number: 203-615-4
CAS Number: 108-78-1

CLH-O-0000006932-69-01/F

Adopted

10 December 2020

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CLH-O-0000006932-69-01/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: **Melamine**

EC Number: **203-615-4**

CAS Number: **108-78-1**

The proposal was submitted by **Germany** and received by RAC on **14 November 2019.**

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

PROCESS FOR ADOPTION OF THE OPINION

Germany 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 **9 December 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **7 February 2020**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Nathalie Printemps**

Co-Rapporteur, appointed by RAC: **Normunds Kadiķis**

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 **10 December 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	TBD	Melamine	203-615-4	108-78-1	Carc. 2 STOT RE 1	H351 H372 (urinary tract)	GHS08 Danger	H351 H372 (urinary tract)			
RAC opinion	TBD	Melamine	203-615-4	108-78-1	Carc. 2 STOT RE 2	H351 H373 (urinary tract)	GHS08 Danger	H351 H373 (urinary tract)			
Resulting Annex VI entry if agreed by COM	TBD	Melamine	203-615-4	108-78-1	Carc. 2 STOT RE 2	H351 H373 (urinary tract)	GHS08 Danger	H351 H373 (urinary tract)			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Melamine has no existing entry to the CLP regulation. The proposal from the dossier submitter (DS) addressed the following endpoints: STOT RE, germ cell mutagenicity and carcinogenicity.

In the opinion, 'calculus' and 'stone' are used synonymously, likewise 'transitional cell epithelium' is used synonymously with 'urothelium'. Urolithiasis, nephrolithiasis and renal/kidney stones refer to the presence of calculi/stones in the urinary tract.

HUMAN HEALTH HAZARD EVALUATION

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

Summary of the Dossier Submitter's proposal

The DS identified the urinary tract system as the main target organ in rats, mice, monkeys and humans.

In rats, mice and monkeys, adverse toxic effects at dose levels relevant for classification STOT RE 2 (> 10 mg/kg and ≤ 100 mg/kg) were identified by the DS in two key reliable GLP studies:

- 90-day rat study (NTP, 1983): Calculus formation in the urinary bladder in males and calcareous deposits in the straight segments of the proximal tubules in females;
- 14-day rat study (Early *et al*., 2013): Renal crystals in female rats and renal injuries in male and female rats in a 14-day study.

In a weight-of-evidence assessment, the DS considered that other identified supportive studies showed consistent findings and justified classification as STOT RE 2. When exposure duration was different from 90-day, Haber's rule was used by the DS to derive an extrapolated effective dose.

The DS highlighted that due to improper experimental procedure, the occurrence of melamine crystals in experimental studies may have been underestimated (the use of formalin during tissue fixation could dissolved melamine crystals).

According to the DS, human urinary tract findings were in line with rats, mice and monkeys. Extensive literature was available in the dossier on the adverse health effects in children following consumption of melamine-tainted infant formula (deliberate adulteration scandal in China). The main limitation noted by the DS is the uncertainty related to individual exposure. Indeed, melamine exposure duration and daily intake was based on retrospective estimation of batch concentration analysis of infant formula performed in the study or, based on official numbers from the Chinese Ministry of Health. Variability in the concentration of melamine in infant formula has been observed. For example, higher concentrations were found in mainland china than in the Hong-Kong area. Nevertheless, a causal relationship between melamine exposure and significant adverse effects in humans has been established. The main effect was the occurrence of melamine-caused stones in the urinary tract of the affected children.

The DS summarised several published observational studies and noted that:

- Stones were most commonly found in the kidney, but to a minor extent also in the urinary bladder and ureter;

- A correlation between the prevalence of urolithiasis and exposure to the substance were observed. Nevertheless, the exact prevalence according to a specific quantity of melamine intake cannot be derived due to uncertainties in exposure assessment (a summary of the prevalence is provided in Table 22 of the CLH report);
- Several risk factors have been identified: male gender, prematurity, duration of consumption of contaminated products;
- Calculi from melamine exposure were distinguished from common calcium-oxalate calculi. The induced stones were mainly composed of uric acid and melamine. Other triazines (e.g. cyanuric acid) were not found relevant for stone formation;
- Humans and most particularly children may be more sensitive to melamine-induced calculi as uricase is not present in humans compared to other mammals (e.g. rats);
- Also, some studies suggested adversity at low dose (impaired renal function, contribution to common calcium urolithiasis), but effect in humans at low-dose exposure remain to be elucidated as major limitations were noted in these studies (e.g. study methodology).

Nephrotoxic effects observed in children accidentally exposed to melamine include renal injuries/lesions and renal inflammation. Macroscopic haematuria was described and may be the result of stone-related urothelial irritation/abrasion. Progression to acute obstructive renal failure and death was seen in some cases. Only in a few subjects was persistent nephrolithiasis reported in follow-up studies. According to the DS, paediatric patients with acute renal failure, may have an elevated risk to develop cardiovascular events and an increased mortality risk. The DS also highlighted that in a current meta-analysis, history of kidney stones is associated with an increased risk of chronic kidney disease and urinary tract carcinogenicity.

Based on adverse renal abnormalities observed in reliable and good quality human observational studies, a classification of melamine as STOT RE 1 was proposed by the DS.

No SCL was proposed.

Comments received during consultation

One member state (MS) agreed to classify melamine as STOT RE 1 for urinary tract system. Nevertheless, the they questioned if calculus formation could be considered as a significant/severe adverse effect.

One NGO supported the proposal to classify melamine as category 1.

Ten comments were received from mainly industrial organisations. All were in favour of no classification. Some comments provided on the mode of action (MoA) of melamine urinary tract toxicity were similar to the comments provided for the carcinogenicity hazard class and are addressed in the corresponding section of the opinion.
The main comments provided by industry were:

- The Early *et al*., (2013) study is of low reliability, with excessive dosage, no statistical analysis and too short an exposure duration;
- Other relevant 90-day key studies were available in rats and monkeys and did not support STOT RE classification (e.g. NTP, 1983).
- Melamine deposits will only occur if the solubility limit of melamine is exceeded in urine. The use of time extrapolation (Haber's rule) is questionable in this context as the threshold-based MoA is mainly dependant to concentration and mostly independent from exposure duration.
- The NOAEL/LOAEL set by the DS using the NTP 13-week study in rats for calculi formation is not supported in the absence of statistical significance at the LOAEL.

- Criminal adulteration is not relevant for classification as it is not a reasonably expected use.
- There is a major bias in the human data as the stones formed from melamine exposure and those of other origin (e.g. calcium stones) were not differentiated in the published human studies. Industry representatives considered that it is necessary to consider the potential confounding influence of stones not formed from melamine exposure when interpreting the data, notably the persistence of stones and effects at low levels of melamine.

An industry representative pointed out that the proposal for STOT RE classification is based on the primary effects of the MoA of tumour formation in male rats (formation of urinary bladder stones and its sequels). Consequently, the proposed STOT RE classification would serve as a double classification.

Assessment and comparison with the classification criteria

RAC considered the same studies as the DS for STOT RE hazard assessment. In addition, results of a recent EOGRTS study were also taken into account (Study report, 2020, results as summarised on the ECHA dissemination website).

In experimental animals, the urinary tract system was identified as the target organ in all the repeated-dose toxicity studies (including carcinogenicity and reproductive toxicity) in rats, mice and monkeys.

Urinary tract findings induced by melamine in mice and monkeys were observed only above the guidance value (GV) which would potentially justify classification in category 2.

In rats, at dose levels below the GV that may trigger STOT RE 2 classification, the following findings were noted (See also in-depth analysis by RAC of the studies in the BD):

Urinary tract	**Findings**	**Study duration (days)**	**Effective dose (mg/kg)***
Urinary bladder	Stone formation	90	LOAEL: 72 BMD10: 41.7
Kidney	Crystal deposits	14	LOAEL: 140 BMD10: 21.1
	Nephropathy: Tubular dilatation, tubular basophilia, infiltration of mononuclear inflammatory cells, degeneration/necrosis/ regeneration of the tubular epithelium, fibrosis	14	NOAEL: 700 BMD10: 292

* As calculated by the DS

With regards to the formation of **calculi in the urinary bladder**, as described in more detail in the carcinogenicity section of this opinion, the MoA of transitional cell epithelium changes induced by melamine is commonly accepted to be related to the precipitation of melamine above a certain threshold in urine, the formation of calculi and subsequent inflammation, and epithelium hyperplasia/proliferation leading to potential carcinogenesis. The first key-event of the MoA is threshold-based and involves stone formation. This is considered as an early event of urinary tract toxicity. Subsequent hyperplasia seen in the urothelium is considered to be an adaptive effect with no adverse consequences on its own upon cessation of exposure unless neoplasia develops, which is addressed under the carcinogenicity hazard class. Moreover, RAC notes that Haber's rule should be used with care in the case of stone formation as the first key events of

the MoA is mainly concentration-related. Exposure duration may also be involved in stone formation as seen in several studies in humans (e.g. Wang *et al*., 2009, Gao *et al*., 2011), but might be more correlated to the size of stones.

With regards to **kidney nephropathy** (distinguishable from age-related chronic progressive nephropathy), Hard *et al*., 2009 considered that melamine precipitation in the lower urinary tract could create pressure effects through transient obstruction leading to the observed renal changes (termed retrograde nephropathy by the authors). It may also be hypothesised that crystals deposits in the tubular lumen of the kidney may produce irritation and inflammation, resulting in tubule degeneration and/or necrosis and subsequent acute to chronic inflammation or obstructive nephropathy. In monkeys nephrotoxicity seen following 90-day exposure (Early *et al*., 2013), at 700 mg/kg was not fully reversible. RAC considered retrograde nephropathy relevant for classification of melamine as STOT RE. With regards to dose levels, classification for STOT RE 2 based on nephropathy would only be triggered by one 14-day key study in rats, supported by some short-term studies identified by the DS with study durations of 14 days or less (Early *et al*., 2013 five-day study, Stine *et al*., 2014). In the available 28-day supporting study, also renal crystals were seen in rats but no nephrotoxicity was noted up to 240 mg/kg (Research triangle institute). Moreover, nephrotoxicity observed in 90-day short-term studies or EOGRTS study (77-day exposure in males) was only observed at dose above GV for classification. For example, according to the reanalysis of Hard *et al*., 2009, nephrotoxicity was not seen below 100 mg/kg bw/day and only 1/9 males had nephropathy at around 500 mg/kg bw/day (NTP, 1983). Therefore, due to uncertainties on time extrapolation with Haber's rule and as nephrotoxicity was seen above the GV of 100 mg/kg in studies of more than 15-day duration and only following Haber's rule extrapolation in studies ≤ 14- day duration, the criteria for STOT RE 2 classification, based on animal data (rats, mice monkeys), are not fulfilled.

In humans, in line with the available animal data, the primary effect of consuming high doses of melamine was **calculi formation**. Numerous studies have established that the consumption of melamine at high dose results in urolithiasis in children. According to the available human observational studies (table 20 of the CLH report), stones were reported to be mainly located in kidney renal pelvis or calyx. In some studies, stones were also reported to be located, in few children, in ureter and to a lesser extent in bladder. Abnormalities at urinalysis (Haematuria, proteinuria and leukocyturia) were consistently reported in some children exposed to melamine. Obstruction features such as hydronephrosis were also noted (hydroureter was reported only in one study). Based on urinary microprotein profile (ex: microalbumine, acetyl-beta-D-glycosidase), tubular injuries and renal glomerulus injuries were also described. According to the 2-year longitudinal study from Zou *et al*., 2013, renal tubular and glomerular damage were resolved by 6-month after diagnosis. Obstruction features, haematuria and leukocyturia were still noted in few patients at 24-month follow-up. Persistent urolithiasis was noted in 8.85% of the patients at 24-month.

No renal function impairment (creatinine, blood urea nitrogen) was reported except in case of **acute obstructive renal failure** in children. In these patients, stones were found either in kidneys or ureters (Sun *et al.,* 2010c). Acute renal failure was reported in several studies (Lam *et al*., 2009; Yang *et al*., 2010b and Sun *et al*., 2010b and c, Wang *et al*., 2011). In a case report, Sun *et al*., 2010b reported the following histopathological findings from a biopsy of a paediatric patient with acute renal failure: lymphocytic infiltration in the glomeruli, sclerotic glomeruli, proliferation of fibrous tissue in the glomeruli and Bowman's capsule, swollen tubular cells, lymphocytic infiltration and fibrosis within the renal interstitium, and crystals within the lumen. According to the available follow-up studies (Sun *et al*., 2010b, 2010c; Yang *et al*., 2010b), following treatment, the renal function was fully recovered in patients with acute renal failure. During the consultation, industry highlighted that background levels of non-melamine stones

may bias these published results. RAC acknowledges that it could be a potential bias in the human data and contributes to the uncertainties on melamine effects at low exposure levels. Nevertheless, it does not affect the conclusion that at high exposure, kidney injuries were seen following melamine human exposure.

Although an association between stone formation and melamine exposure levels was found, the estimated daily exposures in the studies were uncertain (high variability from low to very high concentration in the analysed melamine-tainted infant formula batches).

Calculi formation is a key event for renal acute or chronic diseases in human. Other acute renal findings observed in humans, abnormalities at urinalysis (e.g. haematuria), hydronephrosis, glomerular and tubular injuries may be assumed to be cause by uroliths. Although these effects are expected to be reversible following stone treatment or removal, they may be considered as relevant for classification. In all the available studies, kidney renal function (creatinine, Serum blood urea nitrogen levels) was normal except in patients with acute obstruction renal failure. According to official numbers, in 2008, over the 22,384,000 infants examined following suspected melamine exposure, 294 000 infants had been diagnosed with urinary tract abnormalities, 51,900 infants were hospitalised, and 6 deaths were confirmed. Sun *et al*., 2010 also reported that 25 cases were diagnosed with complications of acute obstructive renal failure. According to WHO, the infants consumed the infant formula for 15 days to 13 months (median 8 months). The estimated daily intake depending on the age of children was around 10 to 110 mg/kg considering mean/maximum melamine levels in some batch of infant formula (WHO, 2009). RAC considers acute renal obstruction failure induced by melamine to be a severe adverse complication of concern and relevant for classification. Death has also been seen in 6 patients. Nevertheless, no diagnostic investigation was performed in these six children but the reason is thought to be the lack of, or delay of treatment (WHO, 2009). RAC notes that, in the case of melamine-tainted products, the deficiency in protein in the children's diet may additionally favour kidney failure. Moreover, the very low number of exposed subjects with complications decrease the concern. RAC also notes uncertainties on exposure-effect levels and particularly at low dose exposure (uncertainties on individual daily intake, background incidence of urolithiasis).

With regards to potential chronic renal disease, following calculi or crystal formation, lesions as observed in rats (retrograde nephropathy) and monkeys are expected to occur in humans. Supporting evidence are available from the human biopsy showing that fibrosis, tubular diseases and inflammation may occur. Moreover, chronic nephropathy seen in monkey following 90-day exposure that was fully reversible, increasing the concern.

Overall, based on its potential to cause human acute and chronic renal diseases, classification as STOT RE 1 would be required according to the CLP criteria. Nevertheless, RAC considers that a downgrading to category 2 is appropriate, mainly in view of the uncertainties on the effects of melamine at low doses. RAC also notes that animal data, showing similar effect as in humans, are supportive of the classification under STOT RE.

RAC considers a **classification of melamine as STOT RE 2 (urinary tract system) warranted**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification for germ cell mutagenicity.

Melamine was negative in gene mutation tests in bacteria (Haworth *et al*., 1983; Litton Bionetics Inc., 1977; Raltech Scientific Services, 1981a and Zhang *et al*., 2011). All the tests, performed similarly to OECD TG with some limitations, were negative with and without metabolic activation. The DS considered the studies reliable for their respective outcome. In addition, three negative gene mutation assay in bacteria were available but disregarded due to missing information on controls and test concentrations.

Melamine did not induce *in vitro* gene mutations in mammalian cells with and without metabolic activation in two studies, performed similarly to OECD TG 476 or 490 (Raltech Scientific Services, 1981b, McGregor et al., 1988).

Based on the negative results observed in Galloway *et al*., 1987, melamine was not found to induce clastogenic effects with and without metabolic activation *in vitro* in mammalian cells. One additional negative *in vitro* chromosomal aberration tests in mammalian cells (Zhang *et al*., 2011) was not considered reliable by the DS due to major deviations from the recommended OECD TG 473 (sampling time after exposure too short).

Other negative *in vitro* studies (sister chromatide exchange, unscheduled DNA synthesis, bioluminescence assay) were considered of lower weight by the DS. The only positive study was a microscreen assay insufficiently validated (Rossman *et al*., 1991).

Based on two reliable *in vivo* mammalian micronucleus assays in mice (NTP, 1989b; Pharmakon Research international, 1981), the DS concluded that there is no evidence of genotoxic effects *in vivo* in somatic cells.

In addition, four *in vivo* genotoxicity studies were disregarded in the dossier: a negative mammalian Comet assay in liver and bladder cells (Wada *et al*., 2014), a positive mammalian Comet assay in epididymides (Zhang *et al*., 2011), a negative micronucleus test (Zhang *et al*., 2011) and an ambiguous mammalian bone marrow chromosomal aberration test (NTP, 1989a). These studies were disregarded by the DS due to experimental shortcomings or missing information compared to respective OECD TG.

Overall, the DS concluded that the classification criteria for germ cell mutagens were not fulfilled for melamine.

Comments received during consultation

Ten industry or trade association representatives agreed with the DS's proposal. One member state (MS) also agreed that no classification for germ cell mutagenicity was warranted for melamine.

Assessment and comparison with the classification criteria

In vitro results

Seven negative studies for gene mutation in the Ames test were provided on melamine. Three negative studies were disregarded due to missing information on methods and results (Seiler, 1983; Ishiwata *et al*., 1991; Kubo *et al*., 2002). Considering the overall database, all strains recommended in OECD TG 471 were tested up to maximum recommended concentration (5000 µg/plate), including strain TA 102 in studies similar to OECD TG 471. Both the preincubation methods or direct plate incorporation were used. Overall, RAC agrees with the DS that melamine did not induce gene mutation in bacteria in presence or absence of metabolic activation.

Two negative studies for gene mutation in mammalian cells, performed similarly to OECD TG, were available. RAC agrees with the DS that melamine did not induce gene mutation in mammalian cells in presence or absence of metabolic activation.

Melamine was negative in an in vitro mammalian chromosome aberration test (Galloway *et al*., 1987). RAC notes that in this test, time exposure was insufficient in presence of metabolic activation (only 2 hours). The study from Zhang *et al*., 2011 was disregarded by the DS as sampling after exposure was too early.

RAC agrees with the DS that other *in vitro* studies available in the dossier were of lower weight.

Overall, melamine did not induce gene mutation *in vitro* with and without metabolic activation. In addition, melamine was not clastogenic *in vitro* with and without metabolic activation. Nevertheless, RAC notes that the study investigating clastogenic effects with metabolic activation had limitations.

In vivo results

The available data are summarised in the table below:

Test method		**Results**	**Reference**
Micronucleus test CD1 mice (m, f)	2 consecutive days gavage study 0, 1000 mg/kg bw/day	Negative	Pharmakon Research, 1981
Bone marrow CA test B6C3F1 mice (m)	Single ip injection 0, 150, 300, 600 mg/kg	Negative : 150, 600 mg/kg Positive: 300 mg/kg	NTP, 1989a
Micronucleus test B6C3F1 mice (m)	Three ip injections 0, 500, 1000, 2000 mg/kg bw/day	Negative	NTP, 1989b
Micronucleus test NIH mice (m) *Limit: inappropriate sampling time (6h instead of 18-24h)*	Two ip injection 0, 400, 800, 1600 mg/kg bw/day	Negative	Zhang *et al*., 2011
Comet assay: epididymides NIH mice (m) *Limits: no positive controls, no data on cytotoxicity, non-validated method*	Five ip injections 0, 400, 800, 1600 mg/kg bw/day	Positive	
Comet assay: liver and kidney SD rats *Limits: non-standard positive controls*	Oral, single gavage 0, 1000, 2000 mg/kg	Negative	Wada *et al*., 2014

m: males, f: females

In vivo, melamine did not induce damage at chromosomal levels based on the negative results in the micronucleus assays up to the limit dose of 2000 mg/kg (Pharmakon, Research, 1981, NTP, 1989b, Zhang *et al*. 2011). No information on bone marrow exposure were provided in the dossier. The positive result observed at only the mid dose in the chromosomal aberration test (NTP, 1989a) is considered of low weight compare to the consistent negative results obtained in the micronucleus assays in mice.

The available Comet assay in liver and kidney was negative. Nevertheless, RAC notes that positive control used in the study were not in line the ones recommended in OECD TG. According to industry comments during the consultation, 3 out of 4 Ames-test positive substances were positive in this Comet assay. A positive result was observed in the Comet assay performed in epididymides following melamine ip exposure. Although this study may indicate an intrinsic potential of melamine to induce DNA damage in epididymides, the study had limitations as no information on cytotoxicity and general health of animals was provided and as positive control results were not published. Negative historical control range of the laboratory would also have been useful to assess the positive results as high background variability may have occurred.

Moreover, RAC considers that the positive result in this Comet assay is of low weight as it was not supported by positive *in vitro* assays in mammalian cells.

Conclusion

Overall, based on negative results *in vitro* and mostly negative results *in vivo*, RAC agrees with the DS that **no classification for germ cell mutagenicity is warranted for melamine**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

For the purpose of classification, both experimental animal and human data were considered by the DS.

Four long-term studies in F344 rats were identified as key studies by the DS. Two oral feeding studies were performed similarly to OECD TG 451 (NTP, 1983; Hazleton, 1983). Two non-guideline long-term oral studies also investigated specifically the carcinogenicity potential of melamine on the urinary tract system (Okumura *et al*., 1992; Ogasawara *et al*., 1995). Although some deviations were noted in these two studies by the DS, they were considered relevant for classification purposes. In addition, two long-term oral studies (Cremonezzi *et al*., 2004 and Hazleton, 1953) were identified as supporting studies. The positive tumour initiation study (Mori *et al*., 2000) in rats was disregarded for classification purposes.

In mice, only one long-term study, similar to OECD TG 451 was identified as a key study (NTP, 1983). In addition, Cremonezzi *et al*., 2001 was used as a supporting study. The negative dermal initiation study in mice (Perrella and Boutwell, 1983) was conducted by applying one dose of melamine followed by treatment with a promotor. Due to this study design it was thus disregarded for classification purposes.

An NTP carcinogenicity study in rats and mice with uracil, acting through the same MoA, was also provided in the dossier as part of the discussion of the mode of action (MoA) of melamine in experimental animals.

Moreover, data were also derived from epidemiological studies in humans.

Based on the dose-related induction of urinary bladder tumours (transitional cell carcinoma and papilloma) in male rats in different studies conducted at different times and in different laboratories (NTP, 1983; Okumura *et al*., 1992; Ogasawara *et al.,* 1995), the DS considered that there was sufficient evidence of carcinogenicity in animals. Nevertheless, additional factors were considered to assess the overall level of concern and human relevance.

A critical factor concerning the evaluation of the carcinogenic potential of melamine is the MoA that has been established in experimental animals. Based on rats, mice and monkey data, the DS proposed the following MoA for experimental animals: *melamine-related precipitation originates in the kidneys and damages the epithelium along the urinary tract (including urinary bladder, ureter, and kidney), giving rise to transitional cell tumours in the bladder and precarcinogenic event (i.e. proliferative lesions of the transitional cell epithelium (bladder, ureter, renal pelvis/papilla) and renal tubular epithelium injuries and inflammation) that may be considered precursor lesions of neoplasms.*

ECHA guidance on the CLP criteria considers that tumours due to crystals in the urinary bladder are not relevant to humans, referring to IARC, 1999a. Based on the IARC, 1999b consensus report, the DS pointed out that, **only the formation of calcium phosphate-containing**

precipitates in the urine of rats were considered species-specific and not relevant to humans. For substances inducing the formation of microcrystals, amorphous precipitates and/or calculi (e.g. melamine), IARC did not exclude a carcinogenic response to chemical-mediated calculi in humans. It is stated in the IARC, 1999b report that "F*or chemicals producing bladder neoplasms in rats and mice as a result of calculus formation in the urinary bladder, the response cannot be considered to be species-specific; thus, the tumour response is relevant to an evaluation of carcinogenicity to humans. There are quantitative differences in response between species and sexes. Calculus formation is dependent on the attainment in the urine of critical concentrations of constituent chemicals which form the calculus; therefore, the biological effects are dependent on reaching threshold concentrations for calculus formation."*

The evidence of an association between melamine exposure and the formation of calculi and renal damage in humans came mainly from adverse health effects reported in children following the accidental consumption of melamine-tainted infant formula (China voluntary adulteration scandal).
The DS acknowledged that there are uncertainties as to whether exposure to melamine at low-doses may promote urolithiasis. Some reports related to infant urolythiasis suggested that the risk of calculi may even be increased at low dose (Chen *et al*., 2009; Lam *et al*., 2008, Li *et al*., 2010). Nevertheless, there are uncertainties regarding the actual level of accidental exposure to melamine in children. In some studies investigating potential kidney effects in human exposed to environmentally chronic low-doses of melamine, it was suggested that melamine may promote the development of calcium-related urinary stones including the formation of a nidus that subsequently promotes the growth of calcium uroliths, renal tubular injuries, or enhanced precipitation of calcium oxalate. Nevertheless, major study methodology deficiencies were identified in these studies. Thus, the DS concluded that whether the calculi could be induced at low dose levels remain to be elucidated.

Another important factor to evaluate is whether melamine-induced calculi could persist in humans. Persistence in humans is an important factor to assess if melamine can produce chronic irritation followed by inflammation and proliferation and the development of neoplastic changes. The DS stressed that some human follow-up studies suggested that melamine-mediated calculi and kidney abnormalities could persist (Wang *et al*., 2014; Zou *et al*., 2013, Dai *et al*. 2012, Yang *et al*., 2013). A more recent 5-year follow-up study showed that although most of the children expelled their stones, asymptomatic intrarenal uroliths in kidney were seen in around 9% of children (Chang *et al*., 2017).
The DS considered that there are insufficient data and long-term follow-up studies on melamine exposure to conclude on a higher incidence of urinary tract cancer in humans. To date, no follow-up studies reported an increase cancer risk. Nevertheless, the DS pointed out that the history of urinary tract stones in humans is associated with carcinomas in urinary bladder and kidney.

Potential quantitative differences in response to calculi between experimental animals species, sexes and humans have been extensively discussed by the DS for each key event of the proposed MoA (e.g. horizontal body posture in rodents compared to humans, differences in localisation of stones between rodents and humans, higher sensitivity of human to uric acid).

Overall, the DS concluded that a consistent MoA can be established in experimental animals and humans. The data provides sufficient evidence that the MoA observed in experimental animals may be relevant to humans.

In conclusion, the DS proposed to classify melamine as Carc. 2, H351 on the following basis:

- Sufficient evidence of carcinogenicity only in urinary bladder of male rats;

- Non-genotoxic mode of action;
- Secondary mechanism of action with a threshold;
- Sufficient evidence indicating relevance to human.

Based on T25 calculation from transitional cell carcinoma observed in male rats in the available life-time exposure (NTP, 1983) study, the substance would fall into the low potency group. Nevertheless, based on the two additional key 36-week exposure studies (Ogasawara *et al*., 1995; Okumura *et al*, 1992) a T25 values supporting a medium potency of melamine was calculated. Therefore, the DS did not recommend SCL.

Comments received during consultation

Three MS agreed that a classification of melamine as a carcinogen is warranted. One MS agreed with Carc. Cat. 2, while another MS was in favour of at least a Cat. 2 classification and one expressed the view that both Cat. 2 and Cat. 1B could be justified. Two of these MSs agreed to use the general concentration limit but provided comments on the methodology used for specific concentration limit (SCL) derivation.

One non-governmental organisation (NGO) considered that classification as Carc. Cat. 1B is more appropriate than Cat. 2 due to extensive evidence in experimental animals supported by human data. They considered speculative to assume species differences in the urinary tract and indicated that it is too early to understand the long-term effects of melamine exposure in humans.

Thirty-six comments were received from industrial organisations or individuals, which were all in favour of no classification.

Industry questioned the selection of studies, the reliability and the weight of the studies used for classification purposes. In particular, they highlighted some recent relevant review papers that were dismissed in the dossier (Swaen *et al*., 2019, Cohen *et al*., 2018a and b). Moreover, an in-depth analysis of the quality of the carcinogenicity studies in mice and rats was provided.

As stated in the ECHA CLP guidance document, industry supported that urinary bladder tumours due to crystals observed in male rats are not relevant to humans and noted that the guidance document was not limited to specific type of crystals. Extensive commentary was provided on each of the key events of the proposed MoA of melamine-induced tumours.

The precipitation of uroliths in human urinary tract is the first step of carcinogenesis. Uroliths will only be formed if a threshold exposure level is exceeded. There are evidence that melamine exposure is associated with uroliths at high dose exposure in humans (deliberate adulteration scandal in China) but industry pointed out that they are no evidence that uroliths could be formed at low exposure. They highlighted the low reliability and the limitations of the studies, quoted by the DS, suggesting effects even at low exposure (Li, 2010; Liu, 2011, Wu, 2010, Lam, 2008 and Chen, 2009). Indeed, one of the major limitation is that the non-reversible stones observed in the follow-up studies of children accidentally exposed to melamine may be due to confounding factors such as the presence of non-reversible non-melamine stones not distinguished from melamine stones.

The first step is followed by chronic irritation and cytotoxicity through prolonged exposure. The uroliths must be persistent over a long period to become a decisive step in carcinogenesis. Industry strongly disagree that bladder stone may persist in humans**:**

- Early lesions such as bladder stones and hyperplasia are reversible through cessation of exposure;
- Humans will seek medical assistance in case a stone has formed (medical treatment, stone removal);

- Life-long conditions of criminal use of melamine is not plausible;
- There are variety of differences in anatomy between rats and humans that could explain that humans is not susceptible to the carcinogenic effects of urinary tract solids.

Other practical consideration on species-specificity were discussed by industry. Differences in locations of urinary tract stones in rats and humans did not allow a direct extrapolation between species. Indeed, based on the NTP study, proliferative lesions were only seen in the urinary bladder and not in the kidney. In addition, tumours did not developed in male mice in this study. There is differences in susceptibility to carcinogenesis from urinary solids that has not been completely explain for melamine. The rat is the most susceptible species.

Moreover, industry commented on other factors that need to be taken into account for classification. Tumours were only seen in presence of excessive primary toxicity (e.g. necrosis), associated with hyperplasia that lead to tumours as a secondary consequence. Therefore, according to the ECHA guidance, melamine tumours in rats should be considered as a secondary consequence of a very high dose that cause excessive local toxicity, pointing to a doubtful potential for carcinogenicity in humans. On this basis, melamine should not be classify.

Industry also commented the T25 approach used by the DS. They considered that the T25 should be derived from the NTP study as the presence of bladder stone is driven by melamine concentration to reach the solubility limit and not exposure duration. On this basis a high specific concentration limit of 5 % should be applied to melamine.

Specific comments received during the consultation have been considered by RAC and discussed below.

Assessment and comparison with the classification criteria

Assessment of key data

Animal data

Seven oral long-term studies were included in the CLH dossier to evaluate carcinogenicity in rats. In addition, a tumour initiation study was available in rat. Two oral carcinogenicity studies were available in mice. Initiating activity by skin application was also tested in one study in mice.

In the **NTP, 1983** study, performed similarly to OECD TG 451 in rats and mice of both sexes, a dose-related increase in transitional cell carcinoma was found with a statistically significant trend. The increase was statistically significant compare to control at 263 mg/kg bw/day in rats and was considered treatment-related and biologically relevant. Transitional cell carcinoma were not seen in the available historical control data (0/3351 through the bioassay program). Seven of the eight rats had bladder stones and an association was found between bladder stones and bladder tumours in rats. A transitional cell papilloma was seen in an additional high-dose male rat. The historical control data for papilloma were 4/3351 (0.1%). A correlation between bladder calculi and the occurrence of bladder transitional cell carcinoma was noted. Pre-neoplastic lesions, consisting of transitional cell hyperplasia, were also increased at 263 mg/kg bw/day. In female rats, an increase in chronic inflammation (different from chronic progressive nephropathy) was seen but no calculi or bladder tumours were induced except one transitional cell papilloma at each dose level. On the three male rats having bladder stones without carcinoma, one had papilloma and the two other had epithelial hyperplasia in the bladder. In the male rat having carcinoma without the presence of stone, the authors suggest that the stone may have passed before the post-mortem examination.

Survival was decreased in male rats at the top dose (38%) but 60% survived 92-week of treatment. Mean body weight was decreased after 10 weeks in low and high dose groups (5-10%). No excessive general toxicity was noted.

	Males			Females		
Dose (mg/kg bw/day)	**Control**	**126**	**263**	**Control**	**262**	**542**
Urinary bladder						
Transitional cell carcinoma	0/45	0/50	8/49* (16%)	0/49	0/49	0/49
Transitional cell papilloma	0/45	0/50	1/49** (2%)	0/49	1/49 (2%)	1/47 (2%)
Transitional cell hyperplasia	0/45	1/50 (2%)	2/49 (4%)	0/49	0/49	0/49
Stones (calculi)	0/45	1/50 (2%)	10/49** (20%)	0/49	0/49	0/49
Kidney						
Chronic inflammation	2 /49 (4%)	3/50 (6%)	6 /49 (12%)	4/50 (8%)	17/50** (34%)	41/50** (82%)

*p≤ 0.05, **≤0.001;

In male mice, an increase in stones (4%, 85% and 93% in control, low and high dose, respectively), chronic inflammation and pre-neoplastic lesions (hyperplasia) were also observed in urinary bladder but no tumours were induced. Similar lesions were noted in female mice but only at the top dose of 1090 mg/kg bw/day and showing low incidences.

In **Okumura *et al*., 1992,** male rats were treated during 36 weeks through diet followed by a 4-week recovery period. Only kidney lesions were investigated. In line with the above results, the authors reported a dose-related increase in transitional cell carcinoma and papilloma in the urinary bladder of male rats. The increase was statistically significant at 1090 mg/kg bw/day and significantly correlated with calculi formation. In addition, a statistically significant increase in transitional cell hyperplasia was noted in the ureters and renal pelvis at the top dose group (only in the renal pelvis at the mid dose group). A carcinoma and 3 papilloma in the ureters were also reported at the top dose. A dose-related increase in urinary bladder weight was noted at mid and high dose groups. Haematuria and polyuria was noted in the top dose group.

With the exception of one male rats, the authors reported that all rats survived until the end of the study. Body weight gain was markedly affected at the top dose (no detailed data available). Terminal body weight was significantly less than control at the top dose following the 4-week recovery period (323 g vs 450g in controls). Body weight was not significantly affected at the low and mid dose groups.

Although a low number of animals per group was used and only kidney findings were analysed in male rats, RAC agrees with the DS that the published results of the study are sufficiently reliable and relevant to assess the carcinogenic potential of melamine. Based on body weight changes, RAC notes that the MTD may have been exceeded at the top dose. Although not statistically significant, an increase in carcinoma (5%) was already noted in absence of general toxicity at 330 mg/kg bw/day

Dose (mg/kg bw/day)		**Control**	**100**	**330**	**1090**
Urinary bladder					
Transitional cell carcinoma		0/20	0/20	1/20 (5%)	15/19* (79%)
Transitional cell papilloma		0/20	0/20	1/20 (5%)	12/19** (63%)
papillomatosis		0/20	0/20	5/20* (25%)	17/19** (89%)
Transitional cell hyperplasia		0/20	1/20	6/20* (30%)	12/19 ** (63%)
Calculi		0/20	4/20 (20%)	9/20* (45%)	8/19 ** (42%)

*p≤ 0.05, **≤0.001;

In a similar study design (36-week exposure followed by 4-week recovery), a statistically significant increase in transitional cell carcinoma and papilloma in the urinary bladder were

observed in male rats at ≥ 350 mg/kg bw/day in **Ogasawara *et al*., 1995**. In this study a correlation with uroliths and preneoplastic lesions (e.g. hyperplasia) was also found at this dose. An increase in transitional cell hyperplasia was also noted at 350 and 1030 mg/kg bw/day in kidney papilla. No effect on survival was noted in the study. The final body weight in the groups treated with melamine at 1030 mg/kg bw/day (top dose) was reported to be particularly very low. Food consumption was also decreased in this group. Urinary blood was observed in most of the rats at this dose. RAC notes that 1030 mg/kg bw/day may have been above MTD. Similarly to Okumura *et al*., 1992, the study was not performed according to OECD TG but for similar reason was considered acceptable for classification purposes. RAC acknowledges that the limitations raised by industry during the consultation on the inconsistency between the urinary volume and the water consumption and the low urine volumes in control raised doubt on the reporting in the study. Nevertheless, the study still provide useful information on the implication of bladder stones in the induction of tumours and the chemical composition of melamine-induced stones in rats. Indeed, in this study, male rats were also treated with melamine in the presence of different NaCl concentrations. Urinary bladder tumours were prevented in the presence of NaCl presumably through the facilitation of excretion of microcrystals. An analysis of four stones reported that the composition of the calculi was melamine and uric acid in an equimolar ratio.

Treatment (dose of NaPT expressed as mg/kg bw/day)	**No.**	**Calculi (%)**	**Papillomatous hyperplasia (%)**	**Papilloma (%)**	**Carcinoma (%)**
0	10	0	0	0	0
10% NaCl	10	0	0	0	0
350	19	37	9	42	21
350 + 5% NaCl	19	11	11	0	0
350 + 10% NaCl	19	5	0	0	0
1030	20	30	75	50	90
1030 + 5% NaCl	20	75	75	25	90
1030 + 10% NaCl	20	30	10**	15*	0

*p≤ 0.05, **≤0.001

In **Hazleton, 1983,** performed in rat similarly to OECD 451, no treatment-related neoplastic lesions in the urinary bladder were noted using lower doses (≤ 40 mg/kg bw/day in males and 80 mg/kg bw/day in females). Transitional cell hyperplasia in the urinary bladder was only noted at the top dose in males (6/37 vs 2/39 in controls). Calculi were seen in 1 male at 20 mg/kg bw/day and 2 males at 80 mg/kg bw/day in the urinary bladder.

RAC agrees with the DS that with respect to transitional cell carcinoma in the urinary bladder, a dose-response relationship can be established based on the four above studies (table 15 of the CLH report).

Two additional rat studies and one additional mice study were identified by the DS as supportive due to lower reliability. Major limitations (e.g. exposure time, number of animals, number of dose levels) were identified in these studies. In Hazleton, 1953, urinary bladder calculi, epithelial hyperplasia and benign papilloma were noted in the urinary bladder of rats at the top dose (350 mg/kg bw/day in males and 470 mg/kg bw/day in females). In this study small deposit of crystalline deposits in the kidney were also noted in 1 male and 2 females. Cremonezzi *et al*., 2004 reported an increase in proliferative lesions (metaplasia, hyperplasia and dysplasia) in the renal papilla and renal pelvis of rats (male or female not specified) at around 750 mg/kg bw/day. In mice, Cremonezzi *et al.,* 2001 published an increase combined incidence of dysplasia and/carcinoma in situ in the bladder, the ureter and the renal pelvis in mice (sex not specified) at around 1800 mg/kg bw/day. According to the DS there are uncertainties on the cancerous

potential of this type of lesion. In this study, increase transitional cell hyperplasia and calculus formation was also noted.

In addition, an oral tumour initiation study was available in WS/Shi rat (Mori *et al*., 2000). An increase in urinary bladder lesions and formation of calculi was observed in the studies following treatment with a known inducer of bladder cancer. In this study, 14/15 rats that displayed calculi developed tumours. In contrast, in tumours dermal initiation study in female mice (Perrella and Boutwell 1983), no tumours were observed. Nevertheless, very few details on study methods were available to RAC to assess the reliability of the study.

Human data

As described in detail in the STOT RE section of the CLH dossier (Table 20), in children accidentally exposed to melamine, stones were mostly found in the kidney and a few were also found in the ureters and in the bladder. They were mainly composed of acid uric and melamine. In most of the reported cases, the stones were successfully treated but some larger stones required surgical treatment. Persistent urolithiasis and kidney abnormalities (urinalysis, hydronephrosis) were also reported by several authors in children in longitudinal or follow-up studies as described in the STOT RE section of the opinion. There is no data showing an association between melamine exposure in humans and kidney tumours. Nevertheless, follow-up in melamine-exposure studies may have been to date insufficient (short follow-up).

Mode of action

It is commonly accepted that melamine-induced carcinogenicity acts through the formation of calculi. The postulated MoA is that the urinary tumours in rats may be due to the formation of urinary crystals or calculi producing persistent irritation/inflammation and consequent transitional cell epithelium proliferation and urinary tract tumours.

Based on the available data, RAC considered that melamine is not genotoxic.

MoA in male rats

The following key events were described by the DS for tumour induction in the urinary bladder of male rats:

- Urinary concentration above the solubility limit adequate for precipitation and formation of calculi;
- Transitional cell epithelium irritation due to the persistence of calculi;
- Transitional cell epithelium proliferation;
- Transitional cell tumour formation.

Based on the four carcinogenicity key studies identified by the DS, all the events were found in male rats (presence of calculi, transitional cell hyperplasia and tumour formation). RAC agrees with the DS that there is supporting evidence that the presence of melamine-related stones in experimental male rats is linked to pre-neoplastic lesions along the urinary tract and tumour formation in the urinary bladder. RAC notes that the tumours observed at the highest dose in the NTP study in male rats were not seen in the presence of excessive general toxicity.

MoA in female rats and other species

In male and female mice and in female rats, formation of calculi and transitional cell hyperplasia was seen in the carcinogenicity studies (NTP, 1983). Nevertheless, no urinary tract tumours were seen up to 542 mg/kg bw/day in female rats and up to 688 and 1065 mg/kg bw/day in male and female mice, respectively. In the study of Cremonezzi *et al*., 2001, an increase in lesions in the urinary tract of unclear neoplastic potential were seen only at very high dose (c.a. 1800 mg/kg bw/day) in mice. Although the proposed MoA is plausible in female rats and mice, RAC notes a

clear difference between species and sex susceptibility to urinary calculi induced by melamine. This is further supported by the data available on uracil, acting via a similar MoA, for which transitional cell carcinoma were seen in both rats and mice in both sexes. As commented by industry during the consultation, species-specificity for tumour induction observed with melamine in the NTP study may disappear at higher dose levels maybe exceeding MTD.

In monkeys, following a 13-week exposure, the kidney was also identified as the primary target organ (Early *et al*., 2013). In the urinalysis, urine crystals were noted in weeks 9 and 13. Renal tubular degeneration/regeneration, mononuclear cell infiltration, tubular dilation and single cell necrosis were noted in the kidneys at 700 mg/kg bw/day. Due to the use of formalin in the study, the melamine induced crystals may have been underestimated. Kidney findings in monkeys were consistent with the findings observed in rats in 90-day studies (nephropathy) but no findings in the urinary bladder were found in monkeys. No longer-term studies were available in monkeys to investigate the potential carcinogenic potential of the observed proliferative kidney lesions.

In domestic pets (cats and dogs), crystals and kidney lesions (renal tubular necrosis and inflammation, crystalluria, haematuria), were also noted following accidental exposure though contaminated diet. Nevertheless, in these studies, animals were exposed to a mixture of triazine, notably cyanuric acid, which is a known synergist of melamine-induced renal damages.

MoA in humans

The relevance of the proposed MoA to humans needs to be carefully evaluated.

- *First step : urinary concentration adequate for precipitation and formation of calculi*

The first step of the presumed MoA may be relevant to humans if a threshold above a certain exposure is exceeded to form precipitation in the urinary tract.
Human infants were exposed to melamine following an adulteration incident in China in 2008. Kidney damage caused by stones in the urinary tract was found in exposed children. The predominant location of melamine induced calculi were the kidney, although fewer stones were found in the urinary bladder or the ureters.

RAC agrees with the DS that whether melamine induced calculi at low exposure remains to be elucidated (e.g. below the WHO Tolerable Daily Intake of 0.2 mg/kg bw) due to the limitations in the available human epidemiological studies suggesting an increased risk (See table below).

(Reference), study method	**Source of exposure**	**Prevalence of urolithiasis**	**Main limitations**
(Lam *et al*., 2008) Cross-sectional	Melamine-tainted formula	1/3170 urolithiasis + 7 suspected at 0.01-0.21 mg/kg bw/day	High uncertainties on exposure, study methodology
(Li *et al*., 2010) observational	Melamine-tainted formula	OR: 1.7, 95% CI: 1.3-2.4 at < 0.2 mg/kg bw/day	High uncertainties on exposure, enrolment bias
(Chen *et al*., 2009) observational	Melamine-tainted formula	63/3976 at 0.01-62.67 mg/kg bw/day (one kidney stone at 0.04 mg/kg bw/day) vs one case in the control group	High uncertainties on exposure

(Liu *et al*., 2011) Case-control	Unknown	Increased risk of calcium urolithiasis: RR= 7.64 (95% CI: 1.98-29.51) at > 3.11 mg/ml melamine in urine	Study methodology, potential confounders
(Wu *et al*., 2010) Cross-sectional	Environmental	Increased risk of human calcium and uric acid urolithiasis	High uncertainties on melamine measurements in urine, no analysis of melamine content in stones

During the consultation, industry also highlighted that background levels of non-melamine related stones may bias the results. Although the background incidence of stones in infants is low compare to adults, it was reported by Swaen *et al*., that in China, at the time of the accidental exposure, the prevalence rates were in the range of 2.5-3.61% in some part of the China. Lower prevalence was reported in Hong-Kong (0.03-0.6%). RAC notes that melamine can be distinguished from other stones such as calcium stones in humans. Nevertheless, RAC acknowledges that it contributes to the uncertainties on melamine effects at low exposure levels.

Overall, RAC considered the studies of low reliability and weight, due to the inherent limitation of the study design (case-control study do not allow to assess causality) and potential confounding factors (e.g. considerableuncertainties on exposure).

The DS also notes that humans may be more susceptible to stone formation than rats. When analysed, melamine-mediated uroliths in humans were mainly composed of melamine and uric acid naturally present in urine. It is thought that melamine interact with uric acid to form melamine-uric acid salts that precipitate to form microcalculi within the renal pelvis. Humans lack enzyme uricase compared to other mammals and higher level of uric acid could make them more susceptible. Moreover, uric acid concentration in neonates is higher compared to older children and adults that could also make them even more susceptible (WHO, 2009). Several studies investigated the composition of calculi induced by melamine in rats. Ogasawara *et al*., 1995 reported that the analysed stones induced by melamine were constituted of melamine and uric acid as seen in humans. Quantitative differences were noted as higher concentration of uric acid were reported in humans than in rats (2:1 ratio in humans vs 1:1 ratio in rats). Nevertheless, the presence of uric acid in melamine-induced calculi in rodents was not consistently found in the studies (e.g. Research triangle institute, 1982).

Overall, RAC considered the first step of the MoA is plausible in humans. The precise threshold of exposure in humans leading to precipitation of melamine is not possible to derive based on the available data. Humans may be more sensitive than rats to the formation of uric-acid calculi. Thus, there are no strong evidence that low exposure to melamine could not form uroliths in humans.

- *Second step: transitional cell epithelium irritation due to the persistence of calculi*

In order to induce prolonged irritation of the urinary tract, uroliths should persist in humans. Several studies suggested potential persistence of uroliths and kidney abnormalities following children exposure to melamine tainted formula.

(Reference), study method	Results	Main limitations
(Chang *et al*., 2017) 5-year follow up study N=207 children	No renal damage observed in any children. Asymptomatic residual stones (< 4mm) in 17/198, proteinuria (10/198) and hematuria (6/198). No need to treat these residual stones clinically but further follow-up was suggested by the authors	Selection bias, limited examination, no controls. Stone analysis on 12 stones in the retrospective study (not in the follow-up study)
(Wang *et al*., 2014) 18-month follow-up N=73	5/73 (15%) persistent calculi	Selection bias
(Wang *et al*., 2013) Meta-analysis N= 26 studies	Persistent kidney abnormalities at 12-month follow-up: 7.7%	Possible selection bias, lack of sub-group analysis
(Yang *et al*., 2013) 48-month follow-up N=45	6/45 stones dissolved partially, 4/45 did not change and 1/ 45 increase in size	Selection bias
(Zou *et al*., 2013) 2-year longitudinal study N=240	At 24-month, 8.85% had persistent urolithiasis, obstruction features (hydronephrosis, hydroureter) were observed in 1.3% of patients, haematuria and leucocyturia were also still observed in a few cases	Selection bias, only 5 stones from melamine-exposed children were analysed, no measurement of melamine level in patients
(Dai *et al*., 2012) 12-month follow-up N=36	9/36: residual stones (6 with decreasing size and three stone with increasing size)	Selection bias
(Liu *et al*., 2010b) Population-based screening study	Remaining abnormalities (nephrolithiasis or hydronephrosis) in 5/48 (12%) patients 6 months after cessation of exposure	Selection bias, uncertainties on exposure in controls, lack of information on maternal feeding behavior
(Shang *et al*., 2012) 18-month follow-up N=38	5/38 (13%) showed residual renal stones	Selection bias

During the consultation, industry highlighted that uroliths of non-melamine origin may be a confounding factor in the available epidemiological studies. Thus, non-melamine stones may have been erroneously diagnosed as irreversible melamine stones. Moreover, industry pointed out that cyanuric acid may be present at low level in food and may play a role in infant kidney lithiasis. The DS was in the view that the background incidence of stones in children is low and that following accidental exposure of children, melamine induced stones were analysed and distinguishable from calcium stones. Moreover, cyanuric acid was only present at trace levels and was not identified to play a role in the aetiology of urolithiasis in Chinese children (WHO, 2009). According to the DS, there is no evidence that the stones were formed independently of melamine exposure.

Industry also commented that in case of stones in humans, depending on the size, calculi will be either spontaneously voided or lead to painful obstruction and be subject to surgical removal. Thus, stones will not persist beyond removal. In addition, early lesions that might occur before

treatment will be reversible. This was supported by data in mice, as following cessation of exposure, rapid dissolution and discharge of stones were seen in mice exposed to melamine (Sun *et al*., 2014 and Ren *et al*., 2012). According to the DS, persistent stones were seen in numerous follow-up studies. These stones (usually < 4mm) remained in the urinary tract system and did not obstruct the urinary tract as these would have cause severe symptoms. RAC agrees that in humans, higher size stones will lead to medical assistance and potentially stone removal. Nevertheless, it is plausible based on the available data that asymptomatic stones may persist. Indeed, kidney stones may not always cause symptoms. According to the systematic review and meta-analysis of Wang *et al*., 2013, 76.2% of the patients were asymptomatic.

In order to assess the relevance of this key events in humans, potential differences in the anatomical and physiological aspects of the bladders in rodents and humans need also to be carefully evaluated.
Species-specific anatomical and physiological factors in the urolithiasis-mediated induction of neoplastic lesions have also been discussed in the dossier and during the consultation. The retention time of calculi has been linked to the anatomy of the rodents. Calculi in rodents are sustained as they are normally horizontally positioned favouring the remaining of the calculi within the lumen of the urinary bladder, with less chance of elimination. The vertical deportment of humans may facilitate the elimination of stones. It has thus been a hypothesis that humans will be less susceptible to urolithiasis-mediated cancer compared to rodents. Nevertheless, as highlighted by the DS, following cessation of treatment in mice, rapid calculi discharge was found in mice (Ren *et al*., 2012, Sun *et al*., 2014) and thus elimination of stones was also possible in rodents.

Overall, RAC agrees that humans may be less susceptible to the persistence of stones due to anatomical and physiological aspects. Although the available studies have limitations, it is plausible that asymptomatic residual stones may persist in humans. Longer follow-up studies would be needed to exclude persistence of residual stones in patient with melamine-induced urolithiasis.

Therefore, although quantitative differences in humans and uncertainties have been identified (higher susceptibility of rats, limitation in the available studies), the data do not indicate that the persistence of stones in humans can be disregarded.

- *Last step: transitional cell epithelium proliferation and tumour development*

The last key events are the inflammation and proliferation of urothelium and lastly tumour induction. In humans, nephrotoxicity including renal inflammation and renal injuries/lesions have been seen. In a case report in paediatric patient having acute renal failure following accidental exposure to melamine, lymphocytic infiltration in the glomeruli, sclerotic glomeruli, proliferation of fibrous tissue in the glomeruli and Bowman's capsule, swollen tubular cells, lymphocytic infiltration and fibrosis within the renal interstitium, and crystals within the lumen were observed in a kidney biopsy (Sun *et al*., 2010b). In this case report, at follow-up, renal damage were fully resolved.

Differences in localisation of calculi in rats and humans have been noted. Indeed, rat carcinoma were seen in the urinary bladder only whereas humans developed stones mostly in the upper urinary tract (kidney). Differences were also noted in the 90-day studies in monkey and rats. Whereas rats showed kidney and urinary bladder toxicity, toxicity was limited to kidney in monkeys. According to the DS, preneoplastic lesions in rats and mice were not limited to the urinary bladder and were also seen in kidney and ureters. It has been suggested that renal cancer

in humans could occur at the same site as the site of calculi. RAC notes that as shown by several studies, stones in the ureters and bladder were also noted in few children. It may also be noted that with uracil, acting with a similar MoA as melamine, carcinoma were also noted in the renal pelvis in male and female rats.

RAC notes that preneoplastic lesions seen in the ureters and bladder in rats and mice were only seen at very high dose (possibly exceeding MTD) compared to urinary bladder preneoplastic changes and considered that chronic inflammation and regenerative lesions did not always lead to cancer as seen in mice and female rats at the tested dose levels. Nevertheless, also potential differences in location were seen between rats and humans, renal inflammation and proliferative lesions were observed.

There is no data showing an association between melamine exposure in
humans and kidney tumours. Nevertheless, follow-up in melamine-exposure studies may have been to date insufficient (small size cohorts, short follow-up).

More in general, according to IARC, 2019, there is epidemiological evidence that cancer of the urinary tract in humans is associated with a history of calculi in the bladder. Nevertheless, RAC notes that according to IARC, 1999, there are uncertainties on the association between micro-crystalluria (associated with irritation and cell proliferation) and bladder carcinomas.

Overall, although there are potential quantitative differences between rats and humans (e.g. localisation of tumours), the proposed MoA in humans cannot be disregarded.

<u>IARC assessment and CLP guidance document</u>

In the CLP guidance document (Version 5.0, 2017), urinary bladder tumours due to crystals in the bladder were considered not relevant to humans. The ECHA guidance document referred to the IARC, 1999 document.

IARC, 1999 published a consensus report on kidney tumours. Regards to urinary bladder neoplasms, melamine is quoted as a non-genotoxic chemical that have been shown to induce formation of microcrystals, amorphous precipitate and/or calculi in the urine of mice and rats. The report quoted also uric acid, calcium oxalate, uracil, thymine acting with a similar MoA. Inert materials such as glass beads and paraffin were also considered as potentially leading to calculus formation (following surgical implantation).

With regards to human relevance, IARC considered that *the risk in humans may not be as great as that in rodents because the calculi are usually voided spontaneously or removed by surgical procedures. Thus through quantitative differences in the carcinogenic response to calculi between species, the effect is not species-specific. However, calculus formation is dependent of the attainment in the urine of critically high concentrations of the constituent chemicals which form calculus. The carcinogenic effects are also dependent on reaching a threshold concentration for calculus formation.*

In contrast, regarding urinary bladder carcinogenesis produced by chemicals such as sodium salts (e.g. saccharine or ascorbate) causing calcium phosphate-containing precipitates in the urine of rats was considered as a species- and dose specific phenomenon that does not occur in humans. Urinary precipitation is based on the presence of high urinary concentrations of alpha-2u globulin and albumin. The interaction with these proteins with sodium salts is necessary to form precipitates.

As also pointed by the DS during the consultation, unlike substances such as sodium saccharin or sodium ascorbate, melamine induces urolithiasis in both experimental animals and humans. This is also one of the reasons why the DS considered the established MoA in animals relevant to humans.

RAC acknowledges that quantitative differences between species may exist but considered that the proposed MoA cannot be disregarded as potentially relevant in humans.

Conclusion on human relevance of the proposed MoA

Overall, RAC considered the evidence on urolithiasis in humans is sufficient to consider the proposed MoA plausible in humans. Nevertheless, the Committee notes that there are potential quantitative differences between rats and humans and some uncertainties in the assessment (low dose exposure, persistence of stones in humans, limitations in the available studies).

Comparison of the evidence for carcinogenicity with the classification criteria

In humans, a positive association has been observed between exposure to melamine and urolith formation which is the first step of the proposed MoA in experimental animals. Nevertheless, there is no evidence of carcinogenicity reported in the available epidemiological studies. Therefore, RAC agrees with the DS that classification in category 1A is not appropriate. However, the negative epidemiology data do not overrule the animal data.

In experimental animals, a statistically significant increase in transitional cell carcinoma was noted in male rats in three key studies (NTP, 1983; Ogasawara *et al*., 1995; Okumura *et al*., 1992). In the NTP study, tumours were seen in the absence of general toxicity. Moreover, RAC notes that the increase in malignant tumours was already observed following only 40-week exposure (36 week exposure followed by 4-week recovery) in male rats. On this basis, RAC agrees with the DS that there is sufficient evidence for carcinogenicity in experimental animals.

According to the CLP regulation (Annex I: 3.6.2.2.4), additional considerations like human relevance have to be taken into account for a classification for carcinogenicity. These are assessed in the following table:

Factor	Evidence with melamine	Conclusion
Tumour type and background incidence	- Transitional cell papilloma and carcinoma in male F344 rats - Rare tumours, above HCD - MoA may be relevant to humans (IARC, 1999)	Sufficient evidence in animals: category 1B
Multi-site responses	No. Tumours were only seen in the urinary tract system	Decreased concern
Progression of lesions to malignancy	Yes, transitional cell carcinoma are malignant lesions.	Increased concern
Reduced tumour latency	Yes. Reduced tumour latency was noted as following 36 weeks treatment in rats, tumours were already observed (Okumura *et al*., 1992, Ogasawara *et al*., 1995).	Increased concern
Whether responses are in single sex or both	Responses were only seen in male rats. Male predisposition to calculus was also seen in humans. Potential differences in anatomy, hormone and uric acid levels in humans has been suggested in the dossier.	-
Whether responses are in a single species or several	Single species	Decreased concern
Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	Other substances such as uracil, acting via a similar MoA, provide evidence of urinary tract carcinogenicity in both sexes and species in rodents	-
Routes of exposure	The oral route of exposure used in the long-term carcinogenicity studies is considered a relevant route in humans.	-
Comparison of ADME between test animals and humanss	No species specific differences identified in the available toxicokinetics studies.	-
The possibility of a confounding effect of excessive toxicity at test doses	No excessive general toxicity was not found in rats in the NTP, 1983 study at the high dose.	-
Mode of action and its relevance for humanss	Melamine is not genotoxic. Precipitation of melamine within the urine is responsible of calculi and subsequent tumour formation. The MoA is considered potentially relevant to humans also some unresolved question on potential quantitative differences have been noted. RAC notes the existence of a secondary mode of action, with the implication of a practical threshold above a certain dose level for calculi formation and chronic stimulation of cell proliferation.	downgrade to category 2

- … no influence on the concern (neither increase nor decrease)

During the consultation, industry noted that the bladder tumours are formed as a results of the physical presence of bladder stones and that such particle effects should be exempt from classification. The MoA is not considered by industry to be related to specific intrinsic properties of melamine, as only the dose leading to uroliths results in cancer. Moreover, they considered that calculi by themselves are not carcinogenic to the human urinary tract.

RAC considers nonetheless that the precipitation of crystals in the urinary tract is most likely to be responsible for tumour formation. The CLP regulation does not exclude a carcinogenicity classification due to the physico-chemical properties of a chemical.

In conclusion, RAC considers that in principle there is sufficient evidence of carcinogenicity in experimental animals (urinary bladder tumours in male rats) to justify classification in category 1B.

Regarding the relevance of the proposed MoA to humans, calculi have been associated with high melamine exposure. As calculi formation represents the first step of the proposed MoA of carcinogenesis, RAC agrees with the DS that there is sufficient evidence indicating that the MoA is of relevance to human carcinogenicity. Nevertheless, RAC notes potential quantitative differences in response to calculi between species. Notably, the following differences and uncertainties were noted:

- Humans may be more sensitive than rodents to calculi formation due to higher level of uric acid;
- Although calculi were seen in male and female mice, no tumours were induced below MTD
- humans may be less sensitive than rat to persistence of calculi in the urinary tract system due to anatomical differences;
- Differences in localisation of uroliths in animals (bladder) and humans (mostly kidneys) have been noticed. Nevertheless, RAC considers the MoA relevant within the urinary tract system and that stones were also noted in the ureters and bladder in few children.
-

Overall, also some quantitative differences were noted, the MoA cannot be disregarded as potentially relevant in humans.

The proposed mode of action is non-genotoxic and secondary to the formation of calculi that will only occur above a practical threshold. On this basis, according to the CLP guidance document, a downgrading of a category 1 classification to category 2 may be considered (Guidance to CLP version 5.9, 2017 3.6.2.3.2 (k); *In addition, the existence of a secondary mechanism of action with the implication of a practical threshold above a certain dose level (e.g., hormonal effects on target organs or on mechanisms of physiological regulation, chronic stimulation of cell proliferation) may lead to a downgrading of a Category 1 to Category 2 classification.*

Thus, the critical issue is the ability of melamine to reach a threshold concentration in human urine in order for calculi to form. Such a threshold cannot be established based on the available human data as there are too many uncertainties on actual exposure levels in the available studies. RAC notes that, to date, there is no strong evidence that calculi could occur following low exposure of melamine. Due to the uncertainties on potential effect of melamine at low dose exposure, **RAC agrees with the DS to classify melamine as Carc. 2 (H351).**

Specific concentration limit

In line with the EC (1999) guidance RAC, calculated the following T25 values based on the combined transitional cell carcinoma or papilloma in the urinary bladder of male rats observed following life-time dietary exposure (NTP, 1983). Start of treatment was 6 week of age and the duration of the study was 103 weeks. The lowest effective dose in male rats was 263 mg/kg bw/day (top dose in this study). At this dose, 9/49 male rats showed urinary tract tumours (18.4%). No background correction is needed as no tumours were seen in controls. The T25 is equal to 354 mg/kg bw/day (T25 = 103/104 x 25/18.4x 263 mg melamine/kg bw/day).

According to defaults situation, a T25 ≥ 100 mg/kg bw/day is considered a low potency carcinogen and an SCL of 1-5% could be assigned according to the CLP guidance document.

Nevertheless, other considerations should be considered for assigning a potency class:

Dose-response relationship: there is no data indicating a supralinear dose-response that would justify to move the substance into a high potency group.

Site/species/strain/gender activity: melamine induced tumours in rats in a single specific tissue in a single gender of a single species. This would be in favor of a low potency carcinogen.

Mechanism including genotoxicity: melamine is not considered as a genotoxicant. Melamine is a threshold carcinogen which is probably determined by the concentration at which melamine will form calculi.

As melamine is threshold-based, one of the MSs proposed to use the NOAEL of 126 mg/kg bw/day instead of the LOAEL in the NTP study to derive the T25 as recommended in EC, 1999. A T25 slightly above 100 mg/kg bw/day was obtained by the DS. As melamine might be more sensitive than rats due to higher uric acid levels relevant for the formation of melamine-uric acid calculi, the MS preferred not to derive an SCL.

RAC agrees that the use of a NOAEL instead of the LOAEL could be appropriate in the case of melamine. RAC notes that a T25 of 170 mg/kg bw/day would be obtained (T25=103/104 x 25/18.4 x 126) that would still support a low potency class.

Mechanistic relevance to humans

Mechanistic relevance to humans may also need to be taken into account. RAC considers that there is no reason to change the potency class from the starting assumption of low potency. Quantitative differences in rats and humans have been identified. Potential differences in susceptibility have been identified in both directions (high sensitivity of human formation of calculi but lower sensitivity of humans for persistence of calculi and differences in localisation).

Toxicokinetics

There is no data suggesting that the toxicokinetic behavior will be different in animals and humans.

Other elements

The short latency period observed in the studies increase the concern. Indeed, transitional cell tumours were already observed following 36-week exposure to melamine (Ogasawara *et al*., 1995; Okumura *et al*., 1992). Based on these studies, a T25 < 100 mg/kg bw/day is obtained as calculated by the DS. These data support a medium potency of melamine. Although it is recommended to use a 2-year study over the 36-week study in the EC guidance, the short latency period strongly increases the concern. RAC notes that due to the specific melamine MoA, the 36-week studies may be relevant for deriving a T25 value.

Overall, RAC considers that **the generic concentration limit is appropriate for melamine**.

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).