Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Colecalciferol
Cholecalciferol
Vitamin D3

EC Number: 200-673-2
CAS Number: 67-97-0

CLH-O-0000001412-86-144/F

Adopted

9 December 2016

9 December 2016

CLH-O-0000001412-86-144/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: **colecalciferol; cholecalciferol; vitamin D3**

EC Number: **200-673-2**

CAS Number: **67-97-0**

The proposal was submitted by **Sweden** and received by RAC **13 January 2016.**

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

PROCESS FOR ADOPTION OF THE OPINION

Sweden 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 **22 January 2016**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **11 March 2016.**

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Peter Hammer Sørensen**

Co-Rapporteur, appointed by RAC: **Agnes Schulte**

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 **9 December 2016** by **consensus**.

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

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	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	603-180-00-4	colecalciferol; cholecalciferol; vitamin D3	200-673-2	67-97-0	Acute Tox. 3* Acute Tox. 3* Acute Tox. 2* STOT RE 1	H301 H311 H330 H372**	GHS06 GHS08 Dgr	H301 H311 H330 H372**			
Dossier submitters proposal	603-180-00-4	colecalciferol; cholecalciferol; vitamin D3	200-673-2	67-97-0	**Modify** Acute Tox. 2 Acute Tox. 2 Acute Tox. 2 STOT RE 1 **Add** Carc. 2 Muta. 2	**Modify** H330 H310 H300 H372 **Add** H351 H341	GHS06 GHS08 Dgr	**Modify** H330 H310 H300 H372 **Add** H351 H341		STOT RE 1; H372: C ≥ 0.6 % STOT RE 2; H373: 0.06 % ≤ C < 0.6 %	
RACs proposal	603-180-00-4	colecalciferol; cholecalciferol; vitamin D3	200-673-2	67-97-0	Acute Tox. 2 Acute Tox. 2 Acute Tox. 2 STOT RE 1	H330 H310 H300 H372	GHS06 GHS08 Dgr	H330 H310 H300 H372		inhalation: ATE = 0.05 mg/l (dusts or mists) dermal: ATE = 50 mg/kg bw oral: ATE = 35 mg/kg bw STOT RE 1; H372: C ≥ 3 % STOT RE 2; H373: 0,3 % ≤ C < 3 %	
Resulting Annex VI entry if agreed by COM	603-180-00-4	colecalciferol; cholecalciferol; vitamin D3	200-673-2	67-97-0	Acute Tox. 2 Acute Tox. 2 Acute Tox. 2 STOT RE 1	H330 H310 H300 H372	GHS06 GHS08 Dgr	H330 H310 H300 H372		inhalation: ATE = 0.05 mg/l (dusts or mists) dermal: ATE = 50 mg/kg bw oral: ATE = 35 mg/kg bw STOT RE 1; H372: C ≥ 3 % STOT RE 2; H373: 0.3 % ≤ C < 3 %	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Colecalciferol (also known as cholecalciferol and Vitamin D3) has an existing entry in Annex VI of the CLP Regulation. It is an active biocidal substance in the meaning of Regulation (EU) No 528/2012. Colecalciferol is currently being evaluated as a new biocidal active substance under Directive 98/8/EC for use as rodenticide (Product Type 14).

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier submitter's proposal

The existing colecalciferol classification in Annex VI to the CLP Regulation has the following minimum classifications: Aute Tox. 3* (H301), Acute Tox. 3* (H311), and Acute Tox. 2* (H330). The DS proposed to revise all acute toxicity categories as Acute Tox. 2.

Acute oral toxicity

Table: Summary of acute oral toxicity studies

Method / Reliability score	Species	Dose levels	LD_{50} (95% confidence interval, when available)	Remarks
Similar to OECD TG 401 / 1.	Rat. Sprague-Dawley. 6/sex/dose group.	146, 219, 329, 493, 739, 1109 mg/kg bw	Males: 352 mg/kg bw (268-484 mg/kg bw) Females: 619 mg/kg bw (495-782 mg/kg bw)	Key study. Dosing solutions were analysed for the actual colecalciferol content. Anonymous, 1983a
Similar to OECD TG 401 / 2	Rat. Wistar 5/sex/dose group.	0, 12, 25, 50, 100, 200 mg/kg bw	Males: 35 mg/kg bw (24-53 mg/kg bw) Females: 47 mg/kg bw (28-79 mg/kg bw)	Key study. Anonymous, 1982a BASF/Bayer task force.
Comparable to OECD TG 401 / 2	Rat. Sprague-Dawley. 10/sex	A single oral dose of 1.5 mg/kg bw	Males and females: No mortality. LD_{50} > 1.5 mg/kg bw.	Not acceptable. Anonymous, 1983b

In these rat studies clinical signs included diarrhoea, hypoactivity, emaciation, ataxia, oily yellow or brown stained anal region, red staining around eyes, nose and/or mouth, lacrimation, bradypnoea, dyspnoea, piloerection, tremors, and death. There was a dose-related weight loss in all dose groups.

Comments received during public consultation

One Member State Competent Authority (MSCA) commented, supporting the classification as Acute Tox. 2 for the oral route.

Assessment and comparison with the classification criteria

Study using Sprague-Dawley rats

In the key study, the LD_{50} for both males and female rats, 352 and 629 mg/kg bw fall within the range for category 4 (300 < ATE < 2000 mg/kg bw). The doses were estimated by analysis of the test substances in the dosing solutions, the purity was not given.

Study using Wistar rats

The LD_{50} of 35 mg/kg bw in males and 47 mg/kg bw in females fall within the range for category 2 (5 < ATE < 50 mg/kg bw). The purity of the test substance is unknown, the analytical concentration in the test solution was not provided, and the reliability of the study is thus considered to be lower than the first study. However, the LD_{50} values are supported by the results from rodenticide efficacy tests that were in the same range for mice and rats of the Wistar and Sprague-Dawley strains.

In conclusion, taking into consideration the acute toxicity data presented by the DS, RAC agrees with the DS that classification in **acute toxicity category 2, H300 (fatal if swallowed)** is warranted based on the LD_{50} of 35 mg/kg bw (Anonymous, 1982a).

The acute toxicity estimate (**ATE**) for the classification of colecalciferol in a mixture is **35 mg/kg bw**. ATE values are derived according to Table 3.1.2 of Annex I to CLP.

Acute dermal toxicity

Table: Summary of acute dermal toxicity studies

Method / Reliability score	Species	Dose levels	LD_{50} (95% confidence interval, when available)	Remarks
Comparable to OECD 402 / 2	Rat. Wistar. 5/sex/dose group	Males: 0, 37.5, 75, 150, 300, mg/kg bw. Females: 75, 100, 300, 600 mg/kg bw	Males: 61 mg/kg bw (39-95 mg/kg) Females:185 mg/kg bw (103-332 mg/kg bw)	Key study. Anonymous (1982b)
Comparable to OECD 402 / 4	Rabbit. New Zeeland White 5/sex	A single dose of 2000 mg/kg bw of a commercial product	Males and females: No mortality. LD_{50} > 2000 mg/kg bw	Not acceptable. Commercial product with unknown purity tested. Commercial product with unknown purity tested Anonymous, 1982c

The calculated acute dermal LD_{50} values were 61 mg/kg bw (with 95% confidence limits of 39 – 95 mg/kg bw) in male rats and 185 mg/kg bw (with 95% confidence limits of 103 – 332 mg/kg bw) in female rats. The purity was not known. Clinical signs were mainly indicative of decreased motor activity and motor coordination, respiration difficulties and muscle weakness. Weight loss

was reported and also heart calcification (characterised by hardening of the muscle and presence of white spots) in the deceased or euthanized animals. There are no dermal absorption studies in rats and it is therefore not known if rats have a much higher dermal absorption than humans of this substance.

Comments received during public consultation

One MSCA commented supporting the classification as Acute Tox. 2 for the dermal route.

Assessment and comparison with the classification criteria

The LD_{50} values in Wistar rats, i.e. 61 mg/kg bw in males and 185 mg/kg bw in females fall within the range for category 2 (50 < ATE ≤ 200 mg/kg bw). The lower 95% confidence interval limit for males (39 mg/kg bw) falls within category 1 but as the purity of the test substance is unknown, the exact LD_{50} values cannot be determined.

The cause of the lack of toxicity at 2000 mg/kg bw in rabbits is unknown. The signs of dermal irritation (that could contribute to lower absorption) observed after single application remained for up to 14 days. As a product name is reported in the study summary, a commercial product was obviously tested on which the content of the active compound was not documented. No information was given on the purity of the test substance and it remains open whether the dose 2000 mg/kg was related to the test substance or the tested product. Therefore, the rabbit data are not considered for the classification on acute dermal toxicity.

RAC considers that the acute dermal toxicity of colecalciferol fulfils the criteria for classification in **acute toxicity 2, H310 (fatal in contact with skin)**. The acute toxicity point estimate (**ATE**) for use in the formulas for the classification of mixtures (Table 3.1.2 of CLP) is **50 mg/kg bw**.

Acute inhalation toxicity

Table: Summary of acute inhalation toxicity studies

Method / Reliability score	Species	Dose levels	LD_{50} (95% confidence interval, when available)	Remarks
Compliance to OECD 403.	Rat. Wistar. 5/sex/dose group	1.2, 1.7, 2.2, 4.6 mg/lmg/l.	0.13-0.4 mg/lmg/l (0.13-0.15 mg/lmg/l for males and 0.14 to 0.4 mg/lmg/l for females)	Key study. Anonymous, 1986

Under the conditions of the acute inhalation study available, the LC_{50} after a 4-hour exposure and a 35-day observation period was estimated to be in the range of 130 – 380 mg/m^3 (0.13 – 0.38 mg/l). Post-exposure observations showed clinical effects on the mood, motor activity and coordination, posture, muscle tone and the autonomic nervous system. However, since mortality occurred in some of the animals at the same dose levels, these symptoms are considered to reflect general toxicity rather than a specific effect on the nervous system. Macroscopic findings considered to be treatment related, for both male and female rats, included pale kidneys with roughened surface, white spots and areas of the myocardium, white area in the stomach and red spots on the lungs.

In females, there was an increase of both absolute and relative lung weights. The macro- and microscopic observations revealed calcification of the visceral organs and the lungs at all exposure levels. There were no signs of respiratory irritation. Body weights of surviving male and female rats were reduced in exposed groups up to 3 weeks after treatment. Thereafter the body

weights resumed to control values. Mortality occurred at all exposure levels, between 48 hours and 7 days after exposure. The mortality pattern in males did not show a clear dose-response but indicated a slightly higher sensitivity of males compared to females.

Comments received during public consultation

One MSCA commented supporting the classification as Acute Tox. 2 for the inhalation route.

Assessment and comparison with the classification criteria

The LC_{50} is within the range of 0.13 - 0.15 mg/l for males and 0.14 to 0.4 mg/l for females. These values fall within the range for classification in category 2 (0.05 < ATE ≤ 0.5 mg/l for dust and mist)).

Based on the results of the study in Wistar rats, RAC agrees with the DS's proposal to classify colecalciferol as **Acute Tox. 2, H330 (fatal if inhaled)**. The acute toxicity point estimate (**ATE**) for use in the formulas for the classification of mixtures (Table 3.1.2 of CLP) is **0.05 mg/l (dusts or mists)**.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

The DS proposed no classification for STOT SE. Macro- and microscopic observations revealed calcification of visceral organs and increased lung weights (relative and absolute) in female rats. These effects are consistent with the mode of action of colecalciferol and consist in a progressive hypercalcemia (mobilization of calcium from the bone matrix to plasma) with generalised tissue mineralisation that is mainly prominent during repeated exposure where hyperphosphataemia and mineralisation in the vasculature may also occur. Body weights were decreased during follow up period but regained control levels in both male and female rats. These effects were not considered severe enough by the DS to warrant a classification as STOT SE 1 or 2.

In addition, in the acute toxicity studies, there were various (neurological) clinical signs observed at doses slightly below or at lethal doses but they were not indicative of narcotic effects. These symptoms likely also reflect general toxicity rather than a specific effect on the nervous system. Finally, no sign of respiratory tract irritation (RTI) was observed. In summary, the clinical signs could be caused either by hypercalcemia, acute neurotoxicity or be indicative of a more generalized toxic response. The DS concluded that classification of colecalciferol as STOT SE 3 (RTI or narcotic effects) is not justified.

Comments received during public consultation

No comments were received for this endpoint.

Assessment and comparison with the classification criteria

RAC agrees with the DS that **the criteria for classification for STOT SE 1 and 2 or 3 are not fulfilled**. The toxicological and clinical effects that were observed in the acute toxicity studies are related to generalised toxicity.

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

Summary of the Dossier Submitter's proposal

Colecalciferol is currently classified as STOT RE 1; H372** in Annex VI of the CLP Regulation which is a classification translated from the previous legislation, i.e. Directive 67/548/EEC. The DS proposed to maintain the existing STOT RE 1 classification.

In the available 90-d oral rat study (Anonymous, 2013; cf. background document, Tables 10.9.c-d), the effects considered relevant for this category by the DS include progressive hypercalcemia with tissue mineralisation in several organs (i.e. aorta, heart, kidneys, stomach, trachea, femur and sternum) and in adrenal glands' medullar chromaffin cell, which were observed already at doses of 0.06 mg/kg bw/day and 0.3 mg/kg bw/day, respectively. Although associated microscopic findings were only seen in the kidneys (degeneration / regeneration, tubular dilation and proteinaceous cast), the DS hesitated to propose the kidneys as the target organ taking into consideration the possible influence of the study conditions and the absence of data in other species. Moreover, generalised tissue mineralisation is consistent with the mode of action of colecaciferol, i.e. mobilization of calcium from the bone matrix to plasma and death from hypercalcemia and mineralisation of major organs. There was no repeated dose toxicity data available for the dermal or inhalation route.

Overall, the effects observed in the 90-d rat study are consistent with the 26-week and 57-week rat studies (cf. background document, Table 10.9a) and indicated an impaired organ function at doses that are well within the guidance value set in the CLP regulation for a 90-d study in rats. Therefore, the DS proposed to maintain the existing STOT RE 1 classification. In addition, based on equations in the Guidance on the Application of the CLP Criteria (Version 4.0, Nov. 2015), the specific concentration limits (SCLs) assigned to the substance and applicable to mixtures containing the substance are proposed by the DS to be the following:

SCL *Cat*.1 = (ED/GV1)x100% or SCL *Cat.*1 = (0.06/10)x100 = 0.6% (for STOT RE 1) - Equation 3.9.2.6.a;

SCL *Cat*.2 = (ED/GV2)x100% or SCL *Cat*.2 = (0.06/100)x100 = 0.06% (for STOT RE 2) - Equation 3.9.2.6.b.

In both equations, the Effective Dose (ED) is set at doses of 0.06 mg/kg bw/day from the 90-day toxicity study in rats. The Guidance Values (GV) of 10 mg/kg (GV1) and 100 mg/kg (GV2) as STOT RE 1 and RE 2 (respectively) for a 90-day rat study are selected from the Guidance on the Application of the CLP Criteria (Version 4.0, Nov. 2015).

Comments received during public consultation

Two MSCA agreed with the classification as STOT RE 1. One of them agreed with the SCL proposal, the other proposed to use the dose of 0.3 mg/kg bw/d (from the 90-day rat study, oral) for the derivation of the SCL, since at 0.06 mg/kg bw/d no severe toxicity but rather minimal kidney

changes were observed and in only 2 males and 1 female of the 10 (per sex) rats examined (cf. Table 10.9.d of the back ground document).

Assessment and comparison with the classification criteria

The key events resulting from repeated exposure to high doses of colecalciferol are hypercalcaemia and hyperphosphataemia that promote mineralisation in the vasculature and in several tissues (e.g. myocardium, kidney, bone). Vascular calcification is a known risk factor for cardiovascular diseases. RAC notes that mineralisation should not be interpreted as an increased deposition of calcium only, as it can be expected in dystrophic tissues. Colecalciferol treatment induces osteoblastic differentiation in animal and human vascular smooth muscle cell cultures and promotes calcium uptake into aortic smooth muscle cells while calciferol did not (see review of Hsu *et al.*, 2008). In human atherosclerotic plaque, the calcium deposits develop complete bone architecture. As calcium deposits may occur at any site of the cardiovascular system, RAC agrees with the DS proposal that the kidney should not be indicated as the only target organ.

RAC notes that there are differences in the transport and internalisation of endogenously produced vitamin D3 and vitamin D3 from the diet. In their review, Hsu *et al.* (2008) stated that vitamin D3 generated by ultraviolet (sun) exposure is primarily carried in the bloodstream by the vitamin D3 binding protein, while dietary colecalciferol may be carried into the bloodstream from the intestinal villi inside chylomicrons and transferred to Low-density lipoprotein (LDL) particles in the liver. These chylomicrons accumulate in artery walls producing atherosclerotic plaque and bring vitamin D3 with them. Overall, binding to the vitamin D3 binding protein is considered to reduce vitamin D3 bioavailability to target tissues and reduce uptake by the liver (Haddad *et al.*, 1993). These differences are thought to be relevant for the interpretation of endogenously produced vitamin D3 and vitamin D3 uptake from external sources.

RAC therefore agrees with the DS that STOT RE 1 is warranted since the observed adverse effects occur well within the CLP guidance value for classification as STOT RE category 1 (C ≤ 10 mg/kg bw/d) after a 90-day repeated-dose study (oral, rat).

Regarding the DS proposal for a SCLs of 0.6% for STOT RE 1, RAC considers the proposal of one MSCA that commented during the public consultation, i.e. to use 0.3 mg/kg bw/d instead of 0.06 mg/kg bw/d, as appropriate. As indicated in table 10.9.d of the background document, at 0.06 mg/kg bw/d, mineralisation in the kidney and the trachea was only reported for 1/10 rats, and not in other organs of male and female rats. Adverse effects in the aorta, heart, kidney and bones were present in the majority of animals at 0.3 mg/kg bw/d.

Then the SCLs assigned to the substance and applicable to mixtures containing colecalciferol are the following:

SCL *Cat*.1 = (ED/GV1) x 100% or SCL *Cat.*1 = (0.3/10) x 100 = 3% - Equation 3.9.2.6.a;

SCL *Cat*.2 = (ED/GV2) x 100% or SCL *Cat*.2 = (0.3/100) x 100 = 0.3% - Equation 3.9.2.6.b.

Correspondingly, a **SCL of 3%** will be the concentration limit at/above which the mixture warrants classification as **STOT RE 1**. Accordingly the **SCL for STOT RE 2 is 0.3%**.

Overall, RAC agrees with the DS to classify colicalciferol as **STOT RE 1 (H372)** but proposing a lower SCL for mixtures.

RAC evaluation of mutagenicity

Summary of the Dossier Submitter's proposal

The genotoxic potential of colecalciferol was investigated in three standard *in vitro* test systems (Ames test, mammalian cell gene mutation test, mammalian chromosome aberration test) and in an *in vivo* combined Comet and micronucleus assay.

In vitro studies

Method / Reliability	Dose levels / test substance	Relevant information	Results and observations	Reference
OECD TG 471 / Reliability: 2-3	5.0, 0.5, 0.05, 0.005, 0.0005 % w/v Purity > 97%	*Saccharomyces cerevisiae* Strain D4	**Negative**	Anonymous, 1977
OECD TG 471 / Reliability: 2-3	5.0, 0.5, 0.05, 0.005, 0.0005 % w/v Purity > 97%	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	**Negative**	Anonymous, 1977
OECD TG 471 / Reliability: 1	D0074 Purity = 100.1%	*S. typhimurium* TA98, TA100, TA1535, TA1537 *E.coli WP2 uvrA* 78 – 5000 µg/plate +/- S9 all strains	**Positive** Mutagenic activity was noted in two of the bacterium tester strains (*S. typhimurium* TA100 with S9 mix at > 1250 µg/plate, and TA1535 with S9 mix at > 2500 µg/plate (x2 response and x3 response respectively). Reproducible dose dependent mutagenicity was observed in TA100 (and TA1535, initial –S9 and +S9 confirmatory test). Precipitate / slight precipitate was observed in Initial Mutation Test and Complementary Mutation Test in all examined bacterial strains at 5000 and 3750 µg/plate with/without metabolic activation and in Confirmatory Mutation Test in all examined bacterial strains at 5000 µg/plate with metabolic activation. Slight inhibitory, cytotoxic effect of the test item (reduced / slightly reduced background lawn development) was observed in the Initial Mutation Test and Complementary Mutation Test in all examined bacterial strains at 5000 µg/plate with and without metabolic activation, and in the Confirmatory Mutation Test in *S. typhimurium* TA98 at 5000 µg/plate without metabolic activation.	Hargitai, 2013a
No guideline but the standardised protocol adopted by the National Toxicology Program (NTP) was	Not stated	Deficiencies in reporting (study accepted as supplementary data only) *S. typhimurium*	**Negative**	Mortelmans *et al.,* 1983

used. / Reliability: 3		TA98, TA100, TA1535,TA1537 33-10000 µg/plate		
OECD TG 473 / Reliability: 1	D0074 Purity = 100.1%	5-400 µg/mL Chinese hamster V79 cells +/- S9	**Negative** No induction of chromosome aberrations (Chinese hamster V79 cells) Colecalciferol was tested up to cytotoxic concentrations. Cytotoxicity was observed at 300 and 250 µg/mL without metabolic activation (relative survival 18 and 37%, respectively) and at 400 µg/mL with metabolic activation (relative survival 41%).	Hargitai, 2013c
OECD TG 476 / Reliability: 1	D0074 Purity = 100.1%	Mouse Lymphoma L5178 TK cells 10 – 325 µg/mL Concentrations tested (range): 5 to 325 µg/mL, test concentrations were based on an initial cytotoxicity test and on test substance solubility. +/- S9	**Negative** No biologically relevant or statistically significant increase in the mutation frequency was observed. No significant dose response to the treatment was indicated by the linear trend analysis. Assay 1, 3-hour treatment with metabolic activation: 325; 300; 275; 250; 225; 200, 175; 150; 125; 100; 75 and 50 µg/mL. Excessive toxicity > 225 µg/mL: relative survival value of 17% at 225 µg/mL. Assay 1, 3-hour treatment without metabolic activation: 325; 300; 275; 250; 225; 200, 175; 150; 125; 100; 75 and 50 µg/mL. Relative survival value of 11% at 200 µg/mL Assay 2, 3-hour treatment with metabolic activation: 325; 300; 275; 250; 225; 200, 175; 150; 125; 100; 75 and 50 µg/mL. Relative survival value of 28% at 225 µg/mL. Assay 2, 24-hour treatment without metabolic activation: 80; 70; 65; 60; 55; 50; 45; 40; 35; 30; 25; 20; 10 and 5 µg/mL. Relative survival value of 7% at 35 µg/mL.	Hargitai, 2013b

According to the DS, the criteria for a positive mutagenic response were fulfilled in the study by Hargitai (2013a), since mutagenic effects were identified for two of the five tested strains (TA100 and TA1535). The two other Ames test available were negative but both studies were considered of low reliability.

The potential for mutagenicity was also tested *in vitro* in a mammalian cell gene mutation test and in a mammalian chromosome aberration test where negative responses were obtained in both tests. The studies were well-conducted and performed in accordance with GLP and OECD guidelines.

In vivo study

Method / Reliability	Dose levels / test substance	Results and observations	Remarks	Reference
Combined bone marrow micronucleus test (OECD TG 474) and Comet assay Reliability: 1	Rat, Wistar 3/sex/group (dose range-finder study) 6 M/group (main study) 3 applications (dosed at Day 1, 2 and 3) 0, 3.75, 7.5, 15 mg/kg bw Sampling times: Day 3 (3 hrs after last dose) Batch D0074 Purity = 100.1%	Bone marrow micronucleus test: **Negative** No cytotoxicity was seen in bone marrow. Comet assay: **Positive** Increased DNA migration in liver cells was observed in the 7.5 and 15 mg/kg bw/d doses. No increased migration in duodenum. Comet analysis of liver resulted in increases in % tail intensity and tail moment following dosing at 7.5 and 15 mg/kg bw/d. The increases in % tail intensity were statistically significant when compared to the concurrent vehicle control group and also demonstrated a significant linear trend, although the degree of DNA damage did not increase when the dose level was increased from 7.5 to 15 mg/kg bw/d. The mean % tail intensity observed in groups dosed at 7.5 and 15 mg/kg bw/d (9.65 and 9.18, respectively) was outside the historical control range for the vehicle and laboratory in question. The maximum tolerated dose (MTD) was set at 15 mg/kg bw/d based on mortality at higher doses. <u>3.75 mg/kg bw/d:</u> Calcium ↑24% Phosphor ↑70% Urea 16% Glucose ↓18% <u>7.5 mg/kg bw/d:</u> ↓ Bw 3.6% Calcium ↑37 % Phosphor ↑70% Urea 126% Glucose ↓31% ↑AST 81% ↓ALT 27% Unclear histopathological effects in liver ↓ Glycogen vacuolation Myositis in duodenum in 2/6 males (grade minimal to slight) <u>15 mg/kg bw/d:</u> ↓ Bw loss 7.8% ↑AST 119% Calcium ↑31% Phosphor ↑55% Urea 314%	↑ Alizarin red staining in one animal at 7.5 mg/kg bw/d (mucosa of duodenum) and in in one animal at 7.5 mg/kg bw/d and another at 15 mg/kg bw/d (muscularis of pylorus) There was no dose-related increase in % clouds or % cells with halos in the liver or duodenum following treatment with colecalciferol, thus demonstrating that colecalciferol did not cause excessive DNA damage (which can interfere with Comet analysis) in either the liver or the duodenum following oral administration. A complete depletion of glycogen was noted in the liver of all animals of mid and high dose groups. Complete depletion of glycogen was also noted in the liver in one control animal, and the rest of the controls (5/6) showed *minimal* glycogen vacuolation. Thus, the effect could be a consequence of fasting.	Beevers (BASF 2014)

		Glucose ↓23% ↓ALT 49%No adverse histopathology effects in liver but> ↓ Glycogen vacuolation ↓ Inflammatory cell foci of liver ↑ Myositis in duodenum in 6/6 males (grade minimal to slight)		

As presented in the Table above, Colecalciferol was recently tested in a combined *in vivo* Comet and micronucleus assay (Beevers, 2014). The study was performed in accordance with GLP and the OECD TG (TG 489 for Comet assay was adopted on 29 July 2016 i.e. after the test was conducted).

The DS considered the result of the micronucleus assay as negative. However, the micronucleus assay is not considered to be the adequate *in vivo* follow up of a positive *in vitro* result in the Ames test since the micronucleus test detects structural and/or numerical damage to chromosomes rather than point mutations. In contrast, the result of the Comet assay was concluded as positive by the DS. Colecalciferol induced an increase in DNA migration in the liver. The mean percent (%) tail intensity (TI) observed in groups administered 7.5 and 15 mg/kg bw/d was 9.65 and 9.18, respectively. The TI were not outside of the historical control range for the vehicle and in the testing laboratory. The TI historical control range generated from 66 studies between July 2010 and August 2014 showed an upper limit of 5.51% and, the historical control range generated from 6 studies between May 2008 and March 2013 showed an upper limit of 4.81%.

The DS concludes that the MTD was considered to be reached at 15 mg/kg bw/d based on the following findings from a 28-day dose-range finding study (Bayer CropScience, 2014):

- Severe body weight losses occurred over a short period of 3 days in young rats which are supposed to grow rapidly at this stage of development.
- Severe alteration of several clinical chemistry parameters (i.e. increases in Ca and P, urea and liver enzymes) reflecting renal and muscular failure.
- Decreases in liver glycogen vacuolation considered to be hallmarks of colecalciferol toxicosis, e.g. loss of appetite and the use of endogeneous glycogen.

The DS recognised that excessive toxicity may give false positive results due to induction of necrosis followed by hyperplasia. However, although the effects occurred at doses above the MTD, there was no increased frequency of inflammation observed in the livers of animals treated with 7.5 mg/kg bw/d and 15 mg/kg bw/d (the frequency was in fact decreased). In addition, complete glycogen depletion was noted in the liver at 7.5 and 15 mg/kg bw/d. However, complete glycogen depletion was also noted in the liver in one control animal, and the rest of controls (5/6) showed minimal glycogen vacuolation. This effect could be a consequence of fasting and there is thus no apparent reason to disregard the observed DNA effects based on the liver toxicity observed.

Comments received during public consultation

Industry considered that the weak positive response observed in the *in vitro* Ames test at high doses and in the presence of precipitate is not relevant for classification based on clear negative responses reported in *in vitro* higher-tier mammalian tests and in two other Ames tests from the

public literature. Industry (in line with other comments from third parties) also disagreed with DS's interpretation of the positivity of the *in vivo* comet assay. They considered that the weak DNA migration observed in the liver of highly intoxicated animals (severe hypercalcaemia) is irrelevant with respect to genotoxicity. A second comment submitted by Industry concluded that vitamin D3 should not be considered a germ cell mutagen (category 2) because it does not match the criteria laid down in CLP (2015). In addition, the increased DNA migration seen in the *in vivo* Comet assay in the liver after administration of doses close to the LD_{50} was considered to be a secondary response to the hypercalcemia and/or liver damage.

Under the biocide review program, the applicant provided a position paper stating that the loss of glycogen in the livers of animals receiving 7.5 and 15 mg/kg bw/d was a consequence of pronounced weight loss indicating severe systemic toxicity (Bayer CropScience, 2014). However, the DS does not support this position.

Similarly to industry, the European Medicinal Agency (EMA) disagreed with the classification proposed by the DS based on weak/borderline effects in the Ames test considered as positive by the DS. Specifically, the weak increase at the highest concentrations (3750 and 5000 µg/plate) was seen in only one experiment (TA1535 without S9) but not confirmed in the two other experiments with the same strain (TA1535 without S9) and thus EMA considered these findings as not reproducible in the same tested strain. EMA took into consideration the negative results in the mouse lymphoma TK gene mutation assay that were reported for cholecalciferol (CLH Report), 1-hydroxy-colecalciferol and calcipotriol and the consistently negative (non-clastogenic) results from *in vitro* and *in vivo* studies with colecalciferol and two derivatives (24,25-dihydroxycholcalciferol and calcipotriol) for testing chromosomal aberrations/ micronuclei. EMA considered the positive *in vivo* comet assay in the liver result as likely due to a secondary effect either related to the pharmacology (e.g., Ca-activated nucleases that may have caused DNA strand-breaks) or to the toxicity (comet effects occurred at doses above the MTD). Based on the weight-of-evidence assessment of the available genotoxicity data, EMA concluded that colecalciferol has no relevant mutagenic and/or genotoxic potential, therefore they did not support the DS proposal to classify colecalciferol as a germ cell mutagen.

Two MSCAs agreed and one disagreed on mutagenicity classification proposal.

Assessment and comparison with the classification criteria

In vitro

The OECD test guideline-compliant Ames assay by Hargitai (2013a), was regarded as an overall positive result by the DS. However, RAC considers the weak effects observed in the TA 100 and TA 1535 E. Coli strains as borderline positive cases. Two mammalian cell tests (mouse lymphoma assay and chromosomal aberration test) are negative. In summary, RAC concluded that the results from *in vitro* testing indicate a possible weak positive indication for point mutations. No indications were observed for structural aberrations.

In vivo

RAC questioned the positive *in vivo* Comet assay due to the following reasons:

- a weak effect was observed at the two highest doses tested of 7.5 and 15 mg/kg bw/d (1.9 and 1.8 fold increase in mean % TI, respectively)
- no dose-dependency was observed. It was uncertain whether a plateau was already reached at 7.5 mg/kg bw/d, since no additional increase was observed at 15 mg/kg bw/d
- positive effect were observed only in the liver; negative result are reported in the duodenum

- it cannot be excluded that the weakly increased DNA migration is induced by a liver specific secondary mechanism

Similarly, the study director of the combined micronucleus/comet assay concluded in the final study report (available to RAC) that colecalciferol did induce an increase in DNA migration in the liver, but this only occurred at dose levels where severe toxicity was observed and therefore concluded that the DNA damage observed in the liver was biologically irrelevant with respect to genotoxicity.

Overall, RAC recognises a positive result reported in a bacterial gene mutation assay but it is considered irrelevant due to a lack of reproducibility in other point mutation tests. In addition, the occurrence of precipitation at the relevant test concentrations were observed. Two mammalian cell tests (mouse lymphoma assay and chromosomal aberration test) were considered negative. The positive result of the *in vivo* Comet assay (indicator test) was questioned by RAC as only a weak increase in % Tail Intensity (TI) was observed in the liver of rats at maximum tolerated doses (MTD) and without a dose-dependency. No such increase in TI was seen in the duodenum. RAC notes that it cannot be excluded that the weakly increased TI was induced by a liver specific secondary mechanism. Overall, the results from *in vivo* testing confirm no indications for structural aberrations.

In view of the above, RAC proposes not to classify colecalciferol as a germ cell mutagen. More specifically, RAC considers that cholecalciferol, taking the weight of the evidence into account, does not meet the criteria for classification as defined in the CLP regulation. The condition '*other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays*' is not fulfilled with the weakly positive Comet assay alone. This view was shared by RAC and **no classification was agreed for germ cell mutagenicity**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS summarised the available relevant information but recognised that the carcinogenicity database for colecalciferol is very limited. The Table below presents a summary of the available information.

Table: Summary of long term or carcinogenicity studies

Method, guideline, deviations if any / reliability	**Species, strain, sex, no/group**	**Dose levels duration of exposure**	**Results**	**Reference**
26 week study, limited comparability to an OECD TG 408 guideline study and is not performed in accordance with GLP Reliability: 2-3	Rat, Charles River-Crl:CD BR rats, 30/group	0.125, 0.25, 0.5 mg/kg bw/d Test substance: cat. No. C9756, purity and batch not stated	***At 0.125 mg/kg bw/d:*** 1/12 rat with proliferative adrenal lesion (hot spot). Stat. sign. ↑ in BrdU labelled chromaffin cells at/during week 4, 8, 12 but not at/during week 26. Stat. sign. increased serum calcium and phosphorous and urinary calcium/creatinine ratio weeks 4 to 26. No effect on body weight gain, no effect on adrenal weight. ***At 0.25 mg/kg bw/d:***	Tischler *et al.*, 1999

Method, guideline, deviations if any / reliability	Species, strain, sex, no/group	Dose levels duration of exposure	Results	Reference
			↑ Absolute (weeks 12, 26) and relative adrenal weight (week 12) Adrenal proliferative lesions (hotspots) in 8/10 rats (none in controls). Hyperplastic nodules 5/10 rats, pheochromocytoma 1/10 rat ↓ body weight gain approximately 10% lower than mean control body weight at end of study. ↓ weight gain (growth curves followed the control and low dose group until approx. day 77 for 0.25 mg/kg group) Stat. sign. ↑ serum calcium and phosphorous and urinary calcium/creatinine ratio week 4-26. Hypercalcaemia (based on serum calcium and urinary calcium excretion), mild nephrocalcinosis without scarring. ***At 0.5 mg/kg bw/d:*** ↑ Abs. (week 12) and ↑ rel adrenal weight week 12/26: i.e. 22% vs 12% in controls resp. 22% vs 10% in controls. Adrenal proliferative lesions in 9/9 anrats (hotspots: 8/9 rats, hyperplastic nodules: 7/9 rats, pheochromocytoma: 1/9 rat) ↓ body weight gain, 30-35% lower than mean control body weight at end of study. Hypercalcinosis based on mild to moderate nephrocalcinosis with scarring. Stat. sign. increased serum calcium and phosphorous and urinary calcium/creatinine ratio between week 4 and 26 Figures represent differences (in %) to controls	
57 week study in rats Limited comparability to an OECD 452 guideline study, and is not performed in accordance with GLP	Rat, Wistar males 20/group	0 and 5 ppm in the diet Test substance: 24R,25-dihydroxycolecalciferol (24,25-$(OH)_2D3$), an active form of vitamin D3. Unknown purity.	↑ Serum phosphorus ($p < 0.05$) no significant differences to controls in the other serum parameters including calcium. ↑ Urinary calcium levels at all investigated time points ($p<0.05$ or 0.01). Max. observed at 22 weeks. ↑ Urinary phosphorous at 22 weeks only ($p<0.05$). ↑ Absolute adrenal weight approximately 20% (right, $p<0.01$) and 19% (left, $p<0.05$) and relative by 19% (right, $p<0.01$) and 16% (left $p<0.01$). ↑ Absolute and relative femur weight by 17% ($p<0.01$) and 19% ($p<0.01$) respectively. ↑ Absolute and relative kidney weight (1.5 to 4%) but only statistically significant for the 4% relative increase ($p<0.05$) observed for left kidney. ↑ Medullary adrenal hyperplasia (6/20) ↑ pheochromocytoma in 1/20 rats ↑ PCNA labelling index in the normal area of the adrenal compared to control animals (1.82% vs 0.87%) with increasing indices in the area of medullary hyperplasia (5.88%) and in the pheochromocytoma (16%). The cortical bone of femur was also thicker in treated rats. It was specifically stated in the paper that no apparent toxic changes including calcifications was histopathologically recognised in the lungs, heart, liver,	Ikezaki *et al.* (1999)

Method, guideline, deviations if any / reliability	Species, strain, sex, no/group	Dose levels duration of exposure	Results	Reference
			kidneys, thyroid, parathyroid, spleen, stomach and intestines.	

Experimental data

The DS summarised a 26-week study in rats obtained from the scientific literature performed with a special focus on adrenal investigations. The study was not performed in accordance with GLP and the comparability to an OECD TG 408 study is low since only selected parameters were investigated. The authors concluded that Vitamin D3 is a powerful mitogen for adrenal medullary chromaffin cells in male rats and causes focal proliferative adrenal lesions including hot spots and hyperplastic nodules.

The DS noted that pheochromocytoma, a neuroendocrine tumour secreting high amount of cathecolamine, was observed in 1/10 animals at 0.25 mg/kg bw/d and 1/9 animals at 0.5 mg/kg bw/d. The serum calcium and phosphorus levels as well as the calcium creatinine ratio were statistically significantly increased from week 4. Calcification, graded "none to minimal", was observed by week 4 in the kidneys of animals administered 0.125 and 0.25 mg/kg bw/d. The calcinosis was mild at 0.25 mg/kg bw/d by week 26 with minimal or no scarring. Animals in the low dose group (0.125 mg/kg bw/d) had normal or minimal calcifications. The kidney calcification was also minimal by week 4 in 3/6 animals of the high dose group (0.5 mg/kg bw/d) but had progressed by week 26 to "mild to moderate" nephrocalcinosis with patchy tubular atrophy and scarring. The body weights were only reduced at week 4 in animals administered 0.25 mg/kg bw/d. The body weights deviated from the control- and low-dose weight curves around week 11 and were continuously reduced until week 26. The adrenal weights were increased from week 12 in the 0.25 and 0.5 mg/kg bw/d groups. Loss of animals due to premature deaths or euthanasia occurred during the study i.e. 3 animals at 0.5 mg/kg bw/d, 2 animals at 0.250 mg/kg bw/d and one control but the cause and timing was not reported. Nevertheless, it was noted that there were no detectable abnormalities on adrenal glands in these animals.

Human data

The DS reported some information on human exposure to colecalciferol from the scientific opinion prepared by EFSA's panel on Dietetic Products, Nutrition and Allergies (EFSA, 2012), and from the 5th edition of the Nordic Nutrition Recommendations (NNR5 project; Lamberg-Allardt *et al*., 2013). Available studies do not suggest an association between exposure and cancer at doses comparable with or slightly above the recommended supplement range. The dose range investigated in these studies were 10 to 27.5 µg/person/day taken for 4 to 7 years. There were also no clear association between exposure to 25-hydroxycolecalciferol, i.e. an active form of vitamin D3, and cancer. The data included in this report were inconsistent with respect to any cancer association between low- (deficiency) and high 25-hydroxycolecalciferol levels.

EFSA's panel concluded on no increased cancer risk in controlled clinical studies from exposure in the dose range 10 to 27.5 µg/person/day for 4 to 7 years. However, breast and colon cancer were the secondary outcomes in these trials. In addition, a meta-analysis of observational studies published up to 2011 found no association between 25-hydroxycolecalciferol concentration and breast cancer (5 studies) or prostate cancer (11 studies). Inverse correlation was found with colorectal cancer (9 studies). The EFSA's panel also reported an inconsistency between the reported associations for 25-hydroxycolecalciferol and all-cause mortality or cancer.

EFSA's panel also reviewed data from one observational study and one cohort study. In the observational study there was a significant increase in total cancer mortality in elderly Swedish men with baseline serum 25-hydroxycolecalciferol at > 98 nmol/L (but not > 93 nmol/L). In the cohort study there was a significant increase in total cancer mortality in US men (but not women) with baseline 25-hydroxycolecalciferol in the highest two categories (80 to < 100 nmol/L and ≥ 100 nmol/L) compared to men with 25-hydroxycolecalciferol concentrations < 37.5 nmol/L (overall trend was not significant). Four other cohort studies did not report any association. Inconsistent associations of vitamin D3 and 25-hydroxycolecalciferol concentrations with pancreatic cancer were also reported by EFSA.

The NNR5 project (Lamberg-Allardt *et al*., 2013) assessed four systematic literature reviews on vitamin D and cancer that met their inclusion criteria. These systematic literature reviews included cohort studies and randomised clinical trials. The authors found no consistent evidence of a direct association between vitamin D3 status and total cancer. They found some observational evidence of an inverse association between vitamin D3 status and risk of colorectal cancer but there was no evidence for a causal relationship. The evidence for an inverse association between vitamin D3 status and breast cancer risk was also considered weak in the NNR5 project due to lack of good quality studies and heterogeneity between studies. There is, according to the study authors, little or no evidence for a protective effect of vitamin D on prostate cancer.

***DS's considerations on the relevance to humans of* pheochromocytomas**

Background frequency

There is a high background frequency of adrenal pheochromocytomas in male rats compared to humans which is a very rare tumour. Pheochromocytoma occur spontaneously, mostly in older rats with an age of > 80 weeks (Greim *et al*., 2009). According to this publication, the frequency varies depending on species, strain, sex and age, it is generally higher in males than females and, it is reported to be 9-20% in males and 2-6.2% in females for Sprague-Dawley and approximately 10% and 2% in Wistar males and females after 24 months. However, the published historical control data (HCD) in old rats is less relevant for this rat study since the tumours were observed already after 26 weeks. The lack of relevant HCD complicates the assessment of the relevance of these findings. Moreover, female rats were not included in the 26 week study but increased proliferation in adrenals was observed in female rats in the 90-day study (background document, table 10.9.d).

Mechanism of tumour formation

Tischler *et al*., (1996) found that colecalciferol is a mitogen *in vivo* which may be related to the *in vivo* mode of action of colecalciferol on calcium. The adrenal tumours occurred at the same doses as increased serum calcium and nephrocalcinosis. In another study, adrenal cell proliferation was stimulated by hypocalcemia in rats when induced by intravenous infusion of calcium gluconate (Isobe *et al.*, 2012). Pheochromocytoma was also observed in a 57-week study in Wistar rats with a vitamin D3 metabolite (24R,25$(OH)_2$D3) that did not increase the serum calcium (Ikezaki *et al,* 1999). In this study medullary hyperplasia and pheochromocytoma occurred in adrenals in 1/20 animals at 57 weeks, when 24R,25$(OH)_2$D3 was administrated. The male rats had increased urinary calcium whereas the serum calcium, measured only at study termination, was not increased. The femur and adrenal weight was increased. The study was not performed according to GLP or following OECD test guidelines and the results are not considered to bring further clarity to the mechanism for pheochromocytoma formation.

Relevance to humans

Overall, it is not considered possible to conclude on whether there is an association between hypercalcemia and pheochromocytoma in humans. In addition, information is lacking with

respect to any association of high dose (i.e. doses outside the supplement range) exposure to vitamin D3 in humans and cancer. The human data described above only support lack of association with cancer in the dose range 10 to 27.5 µg/day. The data on high plasma concentration of 25-hydroxycolecalciferol were inconsistent and cannot be used to clarify the question.

Greim *et al*. (2009) investigated the relevance to humans and mechanisms of chemically induced pheochromocytomas for human risk assessment. They reviewed the occurrence of this tumour type in animal studies directly or indirectly induced by chemicals and found that pheochromocytomas are often found in test animals in addition to other tumours (especially in male rats). They listed conditions such as hypoxia, i.e. impaired respiration or pulmonary toxicity, uncoupling of oxidative phosphorylation, disturbances in calcium homeostasis, e.g. kidney damage that lead to disturbed calcium secretion via urine, and, disturbance of the hypothalamic endocrine axis which appears to be involved in the tumours formation. In addition, they suggested that interference with biochemical mechanisms lead to pheochromocytoma especially via interference with enzymes in catecholamine synthesis, receptor tyrosine kinase, hypoxia inducible factor and fumarate hydratase. Although there is no evidence that the mechanism of action suggested in rats is not relevant for humans, the authors did not find evidence to support that substances producing pheochromocytoma in animals also induced corresponding tumours in humans.

The DS reported that with respect to classification, the CLP guidance section 3.6.2.3.2 only lists pheochromocytoma arising in male rats exposed to particulates through inhalation (secondary to hypoxemia) as a mechanism that is not relevant to humans.

Comments received during public consultation

During public consultation, Industry disagreed with the proposed classification of colecalciferol as a Carcinogen. They stated that the classification proposal is based on the results of two published investigative experiments in rats (one with colecalciferol itself and the other with a colecalciferol metabolite). According to Industry, the proposal ignored the relevant epidemiological data available on vitamin D3. Incorporation of the epidemiological and experimental data into a weight of evidence approach leads to the conclusion that no classification for carcinogenicity is appropriate.

In their comment, EMA indicated that colecalciferol and four of its analogues are likely to induce adrenal pheochromocytomas in rats after long-term administration most likely due to their effects on Ca^{2+} metabolism. EMA reported that chronic endocrine hyperactivity may lead to compensatory hyperplasia and neoplasia and is induced by agents like lactose, sugar alcohols, and Ca^{2+}. As such EMA concluded that pheochromocytomas through this type of interference are most likely not relevant for humans (Greim et al, 2009).

Three MSCA agreed with the DS proposal to classify colecalciferol as Carcinogen 2; H351.

An individual from the United States (identified himself as the principal investigator on the paper Tischler *et al.*, 1999) suggested that the paper offers no evidence that vitamin D3 is a carcinogen. He found that a large volume of literature demonstrates that rats as a species are uniquely susceptible to induction of pheochromocytomas by dietary factors, drugs and other agents that do not cause tumours in humans or mice.

An individual from Israel commented on the relationship between pheochromocytoma and chronic progressive nephropathy and between pheochromocytoma and lung lesions causing hypoxemia.

Assessment and comparison with the classification criteria

The data available to assess the carcinogenic potential of colecalciferol are limited. The human data reported are restricted to doses in the range used for vitamin D supplementation while the animal data are limited to studies performed with a duration only representing approximately 25% of the lifespan of a rat. Nevertheless, pheochromocytomas were observed already after 26 weeks and may be considered providing evidence from animal studies that high doses of colecalciferol could be carcinogenic in human and thus classification as a carcinogen in category 2 should be considered.

RAC notes that the lack of information on whether pheochromocytomas are to expect after prolonged vitamin D3 supplementation and on the prevalence of hypercalcaemia in humans could be related to the low dose levels of recommended amounts for acceptable intake (AI). Vitamin D deficiency leads to impaired mineralisation of bone due to an inefficient absorption of dietary calcium and phosphorus, and is associated with an increase in PTH serum concentration. Clinical symptoms of vitamin D deficiency manifest as rickets in children, and osteomalacia in adults. EFSA's panel set an AI for vitamin D at 15 µg/day for adults and children and at 10 µg/day for infants aged 7– 11 months (EFSA, 2016).

Rats which developed pheochromocytomas received at least 0.25 mg/kg bw/day (250 µg/kg bw/d). theoretically, if a 10 year old child weights 30 kg and receive 15 µg/day, the daily dose would be 0. 5 µg/kg bw/day i.e. 500 fold below the doses received by rats.

RAC notes that the rat data cannot be ignored based on the possibly huge differences in dosing. Excessive ingestion of vitamin D3 may also occur in humans due to continuous overdosing of supplementational intake or intoxication. A recent incident in Denmark describes poisoning of infants due to incorrect levels of vitamin D3 in drops given to newborns. An error in the manufacturing process increased the content of vitamin D3 to 75 times higher than the recommended therapeutic dose of 10 µg/day. The infants received 750 µg/day over a period from April to July 2016, i.e during 4 months. This daily dose is equivalent to 214 µg/kg bw/day for an infant of 3.5 kg. The majority of the children had non-specific symptoms - symptoms which are not really otherwise lead to hospitalisation. However, one child was hospitalised due to more severe symptoms upon admission and diagnosed with an hypercalcaemic crisis.

In rats, malignant and benign pheochromocytomas are well known to occur spontaneously as tumors in older animals (age > 80 weeks) (Greim *et al.*, 2009) with a relatively high spontaneous incidences. Their occurrence depends on species, strain, sex, and age. RAC notes that high spontaneous tumour incidences and uncertainties regarding the interpretation of chemical-induced increased tumour rates in comparison to high background rates generally decrease the level of concern for these tumours (see CLP guidance section 3.6.2.3.2). However, in this specific case, published data on spontaneous incidences from old rats were found to be less informative. The significance of the HCD from various sources (reflecting the historical incidences in aged rats and used as argument by some commenters that pheochromocytomas in male rats are a spontaneous phenomenon and thus are not relevant for humans) seems to be limited regarding the facts that none of the control animals in the study had a proliferative lesion (hot spot/hyperplastic nodule/pheochromocytoma). RAC recognises that the study duration was only 26 weeks and that the tested rats were rather young in comparison to 18-40 months, where pheochromocytomas were assessed in the literature. In addition the incidences of the (internal) laboratory control animals of the same age and from the same genetic background (Sprague-Dawley and Wistar rats of the same laboratory) were not available; the supplying breeder Charles River published incidences of 1.69-5% for the aged male Sprague-Dawley rats in 1999.

Cellular proliferation in the adrenal medulla is also observed with hypercalcaemia from e.g. increased calcium absorption, excessive dietary intake of calcium and phosphate and accompanied by increased production of norepinephrine and a decrease in epinephrine/norepinephrine ratio (Standberg, 1996). Hypercalcaemia is known to increase catecholamine synthesis. This is in agreement with the increase of epinephrine-positive and norepinephrine-positive cells at week 26 of the Tischler study (Tischler *et al.,* 1999).

In humans, pheochromocytomas are mainly benign progressing in 10-15% of the cases to malignancy. They are observed as sporadic tumours or as part of a familial cancer syndrome (multiple endocrine neoplasia type 2, von Hippel-Lindau syndrome, neurofibromatosis type 1 and the hereditary paraganglioma syndromes) for which germline heritable mutations were identified. According to Molatore *et al.* (2010), pheochromocytoma in rats could develop concomitantly with tumours in other endocrine organs (endocrine pancreas, C-cell tumours, pituitary glands). The rat (like the human) is suspected to develop multiple endocrine neoplasia syndromes. However, because the Tischler *et al.* (1999) study only examined histopathological changes in the adrenals, the kidneys and a section of the duodenum, no information on the occurrence of endocrine tumours at other sites is available. No information on tumour development (including preneoplastic lesions) on the parathyroid was reported in the 57-week study on 24,25-dihydroxycolecalciferol (other relevant organs such as pancreas and pituitary were not examined). Based on the available 26-/57-week studies, it therefore remains open whether pheochromocytoma was the only tumour site.

The principal investigator of the publications of Nyska *et al.* (1999) and Ozaki (2001) concluded that "*pheochromocytoma in rats are caused by dietary factors, drugs and other agents that do not cause tumors in humans or mice*". Based on the observed similarities on the altered gene expression leading to both rat and human pheochromocytomas, Elkahloun *et al.* (2006) concluded that the rat pheochromocytomas is relevant for human.

RAC concludes that there is limited evidence from rat chronic/carcinogenicity studies that colecalciferol induces tumours in the adrenals. Compared to the standard design of a 2-year carcinogenicity study in rats, the treatment duration was rather short (26 and 57 weeks instead of 104 weeks), only one sex was examined and, the number of animals dosed per group was low (30 instead of 50). It may thus be hypothesised that the low number of benign pheochromocytomas was a consequence of these shortcomings. As proliferative changes were seen in a high number of animals in the mid- and high-dose groups and no lesions were seen in the control group animals, this is unlikely to be a spontaneous lesion. Hot spots of increased cell proliferation and (multiple) hyperplasia are considered as preneoplastic effects. It cannot be ruled out that a mitogenic mode of action, which is currently not further characterised, may be operative in rats and potentially relevant to humans.

Overall, based on the short and poorly designed available chronic carcinogenicity studies where a single tumour in two dosed groups of male rats was observed, RAC concludes that the currently available data may be indicative of a carcinogenic potential, but the strength of evidence is not enough to classify vitamin D3 as a carcinogen in category 2. Overall, RAC considers that vitamin D3 should not be classified for carcinogenicity.

RAC evaluation of reproductive toxicity

Classification of the active metabolite calcitriol (1α,25-dihydroxycholecalciferol) for reproductive effects has been considered previously by the Commission Working Group on Classification and Labelling of Dangerous Substances and by the Specialised Experts (see below: extract from the

document denoted ECBI/76/00 - Rev. 3, 30.08.2001; ECBI/59/00 – Add. 1 - Rev. 2, 11.04.2001) as there was no standard OECD guideline reproductive toxicity study available with colecalciferol. The experts decided that ergocalciferol (vitamin D2) and colecalciferol (vitamin D3) should not be classified for developmental toxicity or fertility effects.

Regarding effects on development, this document informs: "*The Specialised Experts discussed ergocalciferol and colecalciferol in parallel. The Specialised Experts noted that there were few studies that had been adequately designed, conducted and reported that could be considered for purposes of classification of effects on the development. A majority of the Specialised Experts recommended no classification of colecalciferol for effects upon the development. The arguments were that there is no evidence suggesting that the substance is teratogenic in humans, even at high doses. In animal studies, effects on development but not malformations occurred at dose levels, for which hypercalcemia had been demonstrated or must have been present. Other lesions in foetuses were considered non-specific and secondary to maternal toxicity. A large minority of Experts in favour of Category 3 argued that external, visceral and skeletal abnormalities observed in rabbit foetuses after administration of high doses of the active metabolite 1α,25-dihydroxyvitamin D3 (calcitriol) are specific (McClain et al., 1980) and should lead to classification"* (citation from ECBI/ECBI/59/00 - Rev. 2).

With respect to fertility effects, the document informs: "*The Specialised Experts discussed ergocalciferol and colecalciferol in parallel. The Specialised Experts noted that there were few studies that had been adequately designed, conducted and reported that could be considered for purposes of classification. The Specialised Experts unanimously agreed that classification of ergocalciferol and colecalciferol for effects upon fertility is not warranted, as no valid and conclusive evidence is available from investigations on humans and animals."* (citation from ECBI/ECBI/59/00 - Rev. 2).

Fertility

Summary of the Dossier Submitter's proposal

The DS based the assessment of fertility effects on the following information (see the background document, Tables 10.10.1a-c):

- A non-GLP fertility study performed with metabolite calcitriol in rats (McClain et al., 1980).
- A systematic literature review by Lerchbaum and Obermayer-Pietsch (2012).

Animal data

The reproductive and developmental toxicity of the metabolite 1α,25$(OH)_2$D3) in rats and rabbits was investigated in a teratogenicity study which is described in McClain *et al*. (1980). The study however lacks several analyses and parameters that are important for determining reproductive effects (cf. the background document for the study deviations). The McClain *et al*. study used Charles River CD rats (cf. the background document, Tables 10.10.1a for details), and tested a metabolite rather than colecalciferol. It is not clear if the doses used were close to the maximum tolerable dose in the rats. However, the results in the rat dams showed an increased serum calcium level at 0.3 µg/kg bw/d (high dose) whereas phosphate levels decreased at all doses tested. According to the study authors, there were no effects with respect to the percentage of pregnant animals, average litter size at day 13, number of implantations, percentage of resorptions, average number of corpora lutea per litter, average litter size at birth, gestation index, viability index at day 4 and lactation index at day 21. No structural effects on reproductive organs were observed in the 90-day oral rat study (Anonymous, 2013).

Human data

Neither EFSA's panel opinion nor the Nordic Nutrition Recommendations (NNR5 project) specifically addressed fertility effects. The DS has noted a recent review on human and animal data which includes a discussion on the involvement of vitamin D on fertility (Lerchbaum and Obermayer-Pietsch, 2012). This review found some evidence that vitamin D is involved in fertility but mostly in relation to deficiency. However, the study authors concluded that there is a lack of human studies.

Based on the fact that no robust information on colecalciferol was available, the DS proposed no classification on fertility.

Comments received during public consultation

Agreement for no classification on reproductive toxicity were submitted by Industry.

Assessment and comparison with the classification criteria

Based on the information available, RAC concludes that no effects on the fertility parameters or on sexual function was observed in the study performed with calcitriol (McClain *et al.*, 1980) or in the 90-day rat study (Anonymous, 2013). Therefore, RAC agrees with the DS that **no classification for effects on fertility is warranted**.

Development

Summary of the Dossier Submitter's proposal

The study performed with the vitamin D3 metabolite calcitriol (McClain *et al.*, 1980) in rats and rabbits is not a guideline study. A stated above, the study does not include all analyses and parameters required by a guideline study and it is deficient with respect to reporting. Moreover, exposure was restricted to the period of organogenesis and only one third of foetuses were examined for visceral alterations.

Neverthelsess, the results (cf. background document, Table 10.10a) showed no effects on development in rats up to a dose of 0.3 µg/kg bw/day. A more marked toxicity was observed in rabbits (cf. background document, Tables 10.10d-e) at 0.3 µg/kg bw/d, i.e. 18.8% mortality in the mothers and decreased 24-h viability index (76 vs. 98%) in the pups. Since no maternal body weights nor serum calcium data were presented for the mid- and low-dose groups, the impact of maternal toxicity could not be fully evaluated. The DS agreed that the study has limitations but considered it relevant for classification at doses causing no mortality.

The vitamin D3 metabolism in pregnant rabbits has also been investigated in a published study (Kubota *et al*., 1982), where a high dose of cholecaliferol of approximately 250 µg/kg bw/day was given for 3 consecutive days. The intra-uterine foetal mortality was significantly higher in the colecalciferol exposed foetuses compared to controls, i.e. 17.9% versus 2.8%. However, the decreased foetal viability should be interpreted with care in view of the low quality of the study. Although the calcium levels increased by ~20% compared to controls at GD-29, it is questionable if this had been the cause of the decreased foetal viability, since the calcium level is yet within the range observed in non-treated females and were comparable to untreated pregnant females at GD 27-30. Overall, the DS considered this study as supplementary information.

The DS alos cited the literature of medicinal products containing colecalciferol (400 to 10 000 IU per oral dose) contains preclinical data indicating teratogenicity in test animals at high doses (i.e.

greater than those used in clinical practice) (Commission Regulation No 37/2010). The effects were described in one summary of product characteristics (SmPC) as malformations in rats, mice and rabbits (skeletal defects, microcephaly, and cardiac malformation) but as the full reference is not available in the summary of product characteristics, the information could not properly be assessed.

Human data

Human data can be found in different reviews (cf. background document, Tables 10.10f-g) but this information is not considered sufficiently robust to support an accurate assessment whether or not the intrinsic properties of the substance fulfils criteria for classification. The information available is scarce and confounding factors cannot be excluded. Moreover, information is restricted to effects of doses in the supplement range and to an exposure duration covering only a part of the gestation period.

Comments received during public consultation

Agreement for no classification on reproductive toxicity were submitted from two Industry commenters.

Assessment and comparison with the classification criteria

There are no standard OECD developmental or teratogenicity studies on colecalciferol in animals available.

Based on the information available for the assessment of possible effects on development:

- Two non-GLP developmental toxicity studies with the metabolite **calcitriol** in rats and rabbits.
- Two separate reports of clinical trials in humans.
- EFSA scientific opinion reporting data from studies on women administered vitamin D3 as a supplement during a part of the pregnancy.
- A systematic literature review within the NNR5 project including also data from studies of women taking vitamin D3 supplements during a part of pregnancy.

In addition, the previous discussion on classification by the Commission's Specialised Experts (ECBI/76/00 - Rev. 3, 30.08.2001; ECBI/59/00 – Add. 1 - Rev. 2, 11.04.2001) was taken into account.

RAC recognises that due to the limitations of the animal studies (i.e. deficiencies with respect to methodology and reporting) and the use of doses in the lethal or high toxicity range, the data are not considered sufficiently robust to assess whether the intrinsic properties of colecalciferol fulfil the criteria for classification.

The effects by calcitriol in rabbits reported by McClain *et al.* (1980) include cleft palate, microphthalmia, open eyelids and other skeletal abnormalities in 9/109 animals from a single litter (cf. background document, Table 10.10e). They were observed at a dose without apparent severe toxicity to the mother. However due to deficiencies in the study design and reporting, it is not possible to analyse if there is any correlation between these effects in offspring and the toxicity in mothers. Therefore, it is not possible to conclude if effects could be considered non-specific and secondary to maternal toxicity or substance related. Microphthalmia was seen in 2/68 foetuses of the high dose group in which excessive toxicity also occurred (maternal death 19% and decreased foetal viability).

The excessive toxicity may however have masked effects at the high dose. The frequency of microphthalmia was 1/195 in controls. The malformation was observed in one litter of each control, mid dose and high dose animals. There is no historical control data from the performing laboratory. However, although historical control data can be found in the open literature and although these types of malformations are rare in rabbits, the fact that they were restricted to the same litter indicates that they represent chance findings rather than an effect of treatment.

The human clinical trials reported do not add any concern at the vitamin D3 dietary supplement dose levels used.

Therefore, in agreement with the DS's proposal, RAC concludes that **no classification for developmental toxicity is warranted**.

Lactation

Summary of the Dossier Submitter's proposal

High doses of colecalciferol/calcitriol administered to rats in gavage or diet (cf. Background document, Table 10.10j) resulted in significant increases in serum calcium in mothers and in rat pups. The content of calcium in milk was unaffected by treatment but the milk contained high levels of colecalciferol. Investigations of toxicity of pups were limited to analyses of serum calcium, body weight and kidney calcium content. Due to limited reporting of the study, it is not known wheather these increased levels in milk were associated with any toxic effect.

Overall conclusion for all reproductive toxicity endpoints

RAC concludes that due to limitations of data on reproductive toxicity regarding all parameters required for an accurate assessment of effects on fertility, development and toxicity via lactation, RAC considers the available data for not sufficient to assess if the instrinsic properties of colecalciferol fulfil the criteria for classification. In addition, the human clinical trials reported do not add any concern at suplemental levels. **Overall, RAC agrees with the DS that colecalciferol should not be classified as toxic to reproduction.**

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ANNEXES:

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

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