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CHOLECALCIFEROL
COMMENTS ON APPARENT GENOTOXICITY

Summary

Experimental data available from *in vitro* and *in vivo* GLP studies, epidemiological reports and public scientific literature show that Cholecalciferol poses no concerns with respect to genotoxicity.

Notwithstanding this weight of evidence, the CLH report considers that borderline results in the *in vitro* Ames and *in vivo* comet assay might suggest some concern for genotoxicity and therefore proposes to classify Cholecalciferol as a Mutagen Category 2.

The Applicant considers that the weak positive response observed in the *in vitro* Ames test at high doses and in presence of precipitate is not relevant based on clear negative responses reported in *in vitro* higher tier mammalian test and two other Ames tests from the public literature.

The Applicant disagrees with CLH report's interpretation of the comet test data which is discordant from the Study Director's conclusion. The Applicant considers that the weak DNA migration observed in the liver of highly intoxicated animals is irrelevant with respect to genotoxicity for the following reasons:

- The effects are slight (<2-fold increase),
- The effects are neither dose-related nor associated with a concurrent significant increase in Tail Moment although this parameter is the product of the Tail Intensity and the comet length,
- The effects remained within the range of laboratory's historical control data,
- The effects are only observed in the presence of excessive systemic toxicity in the liver of highly intoxicated animals undergoing cholecalciferol toxicosis with severe hypercalcaemia.

This position is further supported by negative QSAR analysis for mutagenicity structural alerts, extensive literature which suggests a protective role of Cholecalciferol against cellular processes leading to cancer.

The Applicant considers that Cholecalciferol does not meet any criteria for Mutagenicity classification as defined in the Regulation (EC) No 1272/2008 taking into account the extensive animal data and human experience.

Cholecalciferol is an essential vitamin in humans for its role in calcium homeostasis and skeletal health. Cholecalciferol has a long history of beneficial use in fortified foodstuffs, dietary supplements and human medicinal products. Tolerable upper intake levels have been defined by the European Food Safety Authority (EFSA, 2012) and national supplementation and fortification policies have been adopted across Europe to prevent Vitamin D deficiency disorders, e.g. impaired bone mineralization and bone-softening diseases (EFSA, 2012 and Spiro, 2014). Classification of Cholecalciferol as a mutagen is not justified and is likely to impair community health by discouraging adequate intake of Vitamin D_3.

Introduction

Cholecalciferol was tested in a full battery of GLP and guideline-compliant tests to investigate its genotoxic potential. Results are summarised in Table 1.

Table 1: Overall summary of genotoxicity testing

In vitro	**Reference/Guideline**	**Results**		
Bacterial assay for gene mutation	[redacted] (2013a) OECD 471 () OPPTS 870.5100	**Weak** and **inconsistent** positive response at high and precipitating dose levels		
		Strain	**-S9**	**+ S9**
		TA98	-	-
		TA100	-	✓
		TA1535	✓	-
		TA1537	-	-
		WP2*uvrA*	-	-
Mammalian assay for gene mutation	[redacted] (2013b) OECD 476 (1997)	**Negative**		
Test for clastogenicity in mammalian cells	[redacted] (2013c) OECD 473 (1997)	**Negative**		
In vivo	**Reference/Guideline**	**Results**		
Rat Combined **Micronucleus** and Comet **test** via the oral route	[redacted] (2014) Recommendations from Expert WG (2000 & 2003)	**Negative**		
Rat Combined Micronucleus and **Comet test** via the oral route	[redacted] (2014) OECD Guideline 474, 1997 (OECD, 1997)	**Negative** [Considered positive by Swedish Authorities]		

Cholecalciferol was shown to be negative in two out the three tests performed *in vitro* and in two out of the two tests performed *in vivo*. The weak positive response observed in bacteria (Ames test) at high dose levels primarily in the presence of precipitate, was considered to be of limited relevance in view of the negative results observed in the two *in vitro* tests run in mammalian cells and the two *in vivo* tests performed in rat and liver bone marrow.

The Swedish Authorities came to a different conclusion in that they considered the comet assay was positive, and prepared a CLH report which proposed to classify Cholecalciferol as a Mutagen Category 2.

The Applicant strongly disagrees with this proposal. This position paper presents a thorough review of all the available genotoxicity data to demonstrate that Cholecalciferol does not meet any criteria for mutagenicity classification as defined in Regulation (EC) No 1272/2008. It shows that the proposed classification is highly questionable and lacks relevance in the light of the conclusions that can be drawn from animal data and more importantly from human experience.

Review of Cholecalciferol experimental data

1. *In vitro* data

Cholecalciferol was tested in three *in vitro* GLP and guideline-compliant mutagenicity tests to investigate its potential to induce gene mutation and chromosome effects in bacteria and mammalian cells.

- **Bacterial assay**

When tested in the bacterial Ames test, Cholecalciferol was clearly negative in TA98, TA1537 and *E.coli* WP2uvrA but induced a slight positive response in TA100 with metabolic activation at high dose levels in the presence of precipitate (2.6-fold increase over control) and in TA1535 without metabolic activation at high dose levels and again in the presence of precipitate (3.3-fold increase over control) ([redacted], 2013a).

Table 2: Summary of Ames test ([redacted] 2013a)

Strain	1st assay		2nd assay		Confirmatory assay	
	-S9	+S9	-S9	+S9	-S9	+S9
TA98	negative	negative	negative	negative	negative	negative
TA100	negative	+ (2.6), P	negative	+ (2.3), P	not tested	not tested
TA1535	+ (3.3), P	negative	not valid	not valid	+ (4.8), P	not tested
TA1537	negative	negative	negative	negative	negative	negative
E. coli WP2uvrA	negative	negative	negative	negative	negative	negative

P: presence of precipitate; in bracket fold-increase over control; n/a: test not valid; n/t: not tested

Knowing that precipitation can impact the interpretation and the reliability of the test (the presence of precipitate indicates the limit of solubility has been reached and results at concentrations where precipitate occurs are considered less reliable), the responses observed in TA100 and TA1535, which slightly exceeded the laboratory criteria for positivity (2-fold control value for TA100 and 3-fold control value for TA1535), were interpreted as weak effects. This position was supported by the fact that one the two "positives" was reported with metabolic activation, the other only observed without metabolic activation.

This conclusion is also supported by the results of two studies from the literature (Anonymous, 1977 and Mortelmans K., 1986) which showed that Cholecalciferol was negative in several bacterial strains including TA 100 and TA 1535 when tested at dose levels higher than those tested in the GLP Ames test.

Table 3: summary of studies published data in the open literature – TA100 and TA1535

Anonymous (1977) Mutagenicity evaluation of FDA 75-81 Vitamin D3				
Strain	TA100		TA1535	
Dose	-S9	+S9	-S9	+S9
50000 µg/mL	negative	negative	negative	negative
25000 µg/mL	negative	negative	negative	negative
12500 µg/mL	negative	negative	negative	negative
Mortelmans K. (1986) Salmonella mutagenicity test, Mutagenesis 8, sup. 7: 1-119.				
Strain	TA100		TA1535	
Dose	-S9	+S9	-S9	+S9
10000 µg/mL	negative	negative	negative	negative
3333 µg/mL	negative	negative	negative	negative
1000 an < µg/mL	negative	negative	negative	negative

- **Higher tier test in mammalian cells**

When tested in higher tier tests which use mammalian cells, Cholecalciferol was clearly negative in a Chromosome aberration test and a Mouse Lymphoma Assay [redacted] 2013b and c).

These results and more particularly the negative response reported in the Mouse Lymphoma Assay showed that the slight induction of gene mutation observed in bacteria was not reproducible in mammalian cells and further supported the conclusion that the weak positive response observed in the Ames test was due to the non-appropriateness of the experimental conditions for cholecalciferol (e.g. precipitation) and has to be considered to be of limited relevance.

- **Overall Conclusion from *in vitro* studies**

In view of all the experimental data available from GLP studies and open literature the Applicant concludes that Cholecalciferol is devoid of *in vitro* genotoxic potential.

This position differed from the proposal of the CLH report which considered the weak positive response observed in the Ames test in isolation and without taking into account the fact that these weak effects were:

- observed at dose levels which were associated with precipitate,
- above the laboratory criteria for positivity only very slightly,
- biologically inconsistent with respect to metabolic activation,
- inconsistent with negative results reported in studies from the literature testing much higher dose levels,
- not reproducible in mammalian cells as evidenced by negative response in higher tier tests.

In the CLH report no explanations and justifications are provided why an overriding weight is attributed to a single marginal Ames test result, against 2 studies with negative results; and why a fully reliable higher tier negative mammalian cell gene mutation assay, is disregarded in favour of the apparent single marginal Ames positive.

The applicant considers that there is a strong weight of evidence to conclude that the overall biological significance of the weak positive response observed in bacteria is limited and irrelevant with respect to genotoxicity.

2. *In vivo* data

Cholecalciferol was tested in two *in vivo* GLP and guideline-compliant mutagenicity tests to investigate its potential to micronucleus and DNA damage in rat bone marrow and liver, respectively ([redacted] 2014).

- **Micronucleus test**

When tested in a micronucleus test Cholecalciferol was clearly negative and did not induce any increase in micronuclei following oral administration at up to 15 mg/kg/day.

- **Comet assay**

Cholecalciferol was tested in a GLP Comet assay which followed the recommendations of the Comet Workshop (2000 and 2003) as the OECD guideline 489 was not yet published ([redacted] 2014).

Animals (6 male Han Wistar rats/dose level) were dosed at 3.75, 7.5 and 15 mg/kg/day by oral gavage over 3 consecutive days and were sacrificed 3 hours after the last dosing. When tested under these conditions Cholecalciferol was clearly negative at 3.75 mg/kg/day but induced slight statistically

significant increases in Tail intensity (TI) in the liver of rats treated at 7.5 and 15 mg/kg/day (1.9 and 1.8- fold increase, respectively). However the changes observed at 7.5 and 15 mg/kg/day were slight (<2-fold increase), not dose-related and not associated with a concurrent significant increase in Tail Moment (TM) although this parameter is the product of the TI and the comet length; and values of TI and TM remained within the range of laboratory's historical control data (66 studies using the same vehicle and performed between July 2010 and August 2014).

Table 4: summary of liver comet data

Dose levels	**Cell scored**	**Tail intensity**		**Tail Moment**	
		Mean	HCD	Mean	HCD
Negative control	750	5.10 ± 0.63	0.01-12.64	0.55 ±0.07	0.00-1.70
3.75 mg/kg/day	750	6.16 ± 0.41 NS		0.65 ±0.06	
7.5 mg/kg/day	900	9.65 ± 0.70 **		1.05 ±0.08	
15 mg/kg/day	900	9.18 ±1.40 **		1.06 ±0.15	
Positive control	900	51.54 ±3.76 ***, S+R	5.02-84.19	10.25 ±1.08	0.57-39.88

NS: not significant, S+ two-sample t test; R Rank-transformation data – To be completed
HCD: historical control data from 66 studies using the same negative control and performed between July 2010 and August 2014

Knowing that excessive toxicity can play a key role in the interpretation and the reliability of the results of the comet assay (OECD Guideline 489, 2014), DNA migration reported at 7.5 and 15 mg/kg/day was analyzed in light of all the parameters reported in the course of the study to monitor the health status of the treated animals.

In-life parameters and post-mortem observations, which are summarised in Table 5, showed that a dose level of 3.75 mg/kg/day induced adequate and tolerable systemic toxicity whereas dose levels of 7.5 and 15 mg/kg/day produced excessive systemic toxicity.

Excessive systemic toxicity at 7.5 and 15 mg/kg/day consisted in severe effects on body weight (no body weight gain and body weight loss of 8.8g at 7.5 and 15 mg/kg/day, respectively, after 3 days while control animals gained 19.5g over the same period of time), marked increases in blood calcium/phosphorous (+30%/+70%) and urea (2.2 and 4.2-fold, respectively), alteration of enzymatic activities (AST, ALP and ALT) and liver histopathological changes as evidenced by the absence of glycogen vacuolation in all animals. All these effects bear the hallmarks of cholecaciferol toxicosis as a rodenticide and are very characteristic of excessive intakes of cholecalciferol:

- Severe effects on body weight (no body weight gain and body weight loss of 8.8g at 7.5 and 15 mg/kg/day, respectively, after 3 days when control animals gained 19.5g over the same period of time) resulting from appetite loss,
- Marked hypercalcemia/hyperphosphatemia resulting from the disturbance of calcium/phosphorous homeostasis,
- Marked increase in blood urea resulting from the calcification of kidneys and likely renal failure,
- Absence of glycogen vacuolation in the liver resulting from the use of endogenous glycogen to compensate appetite loss and absence of food consumption for 3 days.

Table 5: Summary of in-life and post-mortem data (6 male rats)

Dose levels	**Negative control**	**3.75 mg/kg/day**	**7.5 mg/kg/day**	**15 mg/kg/day**
Pre-dose bodyweight (g)	206.9 ± 9.70	216.8 ± 14.71	209.8 ± 8.31	209.4 ± 8.70
Final Body weight (g)	226.4 ± 12.76	227.0 ± 20.67	209.0 ± 11.17	200.6 ± 8.32

Body weight change (g) day 1-3	+ 19.5	+10.2	+0.8	-8.8
Calcium (mmol/l)	2.65 ± 0.077	3.29 ± 0.092	3.62 ± 0.353	3.47 ± 0.156
Phosphorous (mmol/l)	2.0 ± 0.35	3.4 ± 0.36	3.4 ± 0.16	3.1 ± 0.25
Urea (mmol/l)	4.2 ± 0.36	5.0 ± 1.24	9.5 ± 7.31	17.4 ± 7.86
Aspartate aminotransferase (IU/l)	77 ± 9.9	86 ± 12.8	139 ± 32.3	169 ± 40.8
Alanine aminotransferase (IU/l)	74 ± 15.1	67 ± 15.6	54 ± 15.6	38 ± 9.1
Alkaline phosphatase (IU/l)	149 ± 17.5	159 ± 23.6	178 ± 42.5	186 ± 12.2
Liver: glycogen vacuolation	5/6	4/6	0/6	0/6

In addition, all rats at the top dose and two at the mid-dose showed fluid (2-5 ml) in the thoracic cavity at necropsy. This fluid will have interfered with lung function, reducing systemic oxygenation; and is typically a consequence of heart failure, inferring impaired delivery of blood to organs (including liver). The toxic effects noted at 7.5 and 15 mg/kg/day were also entirely consistent with those reported in a 28-day range-finding study ([redacted], 2013) in which ten rats treated at 10 mg/kg/day died within one week with 1st death being reported only 3 days after the onset of the treatment by oral gavage. These findings strongly suggest that mortality would have been reported in the comet assay if animals would have been observed on a slightly longer period and not sacrificed shortly after the 3rd day of dosing.

Taken together all these information showed that the dose levels of 7.5 and 15 mg/kg/day, at which weak DNA migration was observed, induced an excessive systemic toxicity and that comet liver samples were likely taken from lethally–intoxicated animals undergoing cholecalciferol toxicosis with severe hypercalcemia.

Therefore the fact that DNA migration noted at 7.5 and 15 mg/kg/day was slight (<2-fold increase), was not dose-related, was not associated with a concurrent significant increase in TM, and that values of TI and TM remained in the range of laboratory's historical control data, questioned the biological relevance of these findings. This doubt is further supported by the fact that these slight effects were only observed at dose levels which were shown to induce excessive systemic toxicity, expressed by severe effects on body weight and hypercalcemia. The Study Director concluded on that basis that the DNA damage observed in the liver of highly intoxicated animals was irrelevant with respect to genotoxicity.

- **Overall Conclusion from *in vivo* studies**

In view of all the experimental data available from GLP tests the Applicant concluded that Cholecalciferol is devoid of *in vivo* genotoxic potential.

This position differed from the proposal of the CLH report which considered the DNA migration observed at 7.5 and 15 mg/kg/day in isolation and without taking into account the fact that these effects:

- were slight (<2-fold increase),
- were neither dose-related nor associated with a concurrent significant increase in TM though this parameter is the product of the Tail Intensity and the comet length,
- remained within the range of laboratory's historical control data,
- were only observed in the presence of excessive systemic toxicity in highly intoxicated animals undergoing cholecalciferol toxicosis with severe hypercalcemia.

The reason why the Swedish Authorities considered that the comet assay is positive though they wrote in the CLH report that "it could be argued that the positive result are irrelevant and should not be considered for classification since the dose where DNA migration occurred were about the Maximal

Tolerated Dose" remains unclear. Moreover, the reason why the CLH report attributes an overriding weight to a weak, inconsistent and non-dose related response observed in the presence of excessive systemic toxicity and changed both the conclusion of the Study Director and the position of the applicant, is poorly justified.

In contrast, data are available that indicate the comet assay not to be relevant to human. Fedirko *et al* (2010) reported a small randomised clinical trial, examining DNA damage in biopsies of colorectal tissue of patients receiving cholecalciferol. No increase in DNA damage was seen in the group of patients receiving cholecalciferol; indeed, a slight protective effect might be inferred. Fedirko *et al* (2014) also reported a nested case-control study, examining the incidence of hepatocellular carcinoma among subjects from a cohort with known pre-diagnostic blood levels of cholecalciferol; finding a statistically-significant protective effect of cholecalciferol against hepatocellular carcinoma. The substantial cancer epidemiology of cholecalciferol is described in a companion paper (Exponent 1408446.uk0-7619), but the results of Fedirko (2010, 2014) support the broad conclusion of epidemiological data: cholecalciferol is not associated with an increase in cancer. Cholecalciferol might indeed be protective against some forms of cancer. The studies of Fedirko however directly contradict the relevance of the data used in the CLH report to propose mutagenesis: cholecalciferol is seen not to be mutagenic, *in vivo*, in human; and cholecalciferol is particularly seen not to cause hepatocellular (liver) cancer in human, exactly the same target tissue as the rat comet assay.

The Applicant considers that there is a strong weight of evidence to conclude that the overall biological significance of the weak DNA migration observed in the liver of highly intoxicated animals is irrelevant with respect to genotoxicity.

3. Overall conclusion

Cholecalciferol was tested in a full battery of GLP and guideline-compliant tests to investigate its genotoxic potential.

In view of all the experimental data available *in vitro* from GLP studies and public scientific literature the Applicant concludes that cholecalciferol is devoid of *in vitro* genotoxic potential. This position differed from the proposal of the CLH report which considered the weak positive response of the Ames test in isolation without taking into account the fact that these weak effects were:

- observed at dose levels which were associated with precipitate,
- exceeding the laboratory criteria for positivity only very slightly,
- biologically inconsistent with respect to metabolic activation,
- inconsistent with negative results reported in studies from the literature testing much higher dose levels,
- not reproducible in mammalian cells as evidenced by negative response in higher tier mammalian tests.

The applicant disagrees with the Swedish Authority's position and considers that the overall biological significance of the weak positive response observed in the Ames test is limited and irrelevant with respect to genotoxicity.

In view of all the experimental data available *in vivo* from GLP tests the Applicant concludes that cholecalciferol is devoid of *in vivo* genotoxic potential. This position differed from the proposal of the CLH report which considered the DNA migration observed in the comet assay in isolation without taking into account the fact that these effects were:

- slight (<2-fold increase),

- neither dose-related nor associated with a concurrent significant increase in TM though this parameter is the product of the TI and the comet length,
- remained within the range of laboratory's historical control data,
- only observed in the presence of excessive systemic toxicity in highly intoxicated animals undergoing cholecalciferol toxicosis with severe hypercalcemia.

The applicant disagrees with the Swedish Authority's position and considers that the overall biological significance of the weak DNA migration observed in the liver of highly intoxicated animals is irrelevant with respect to genotoxicity.

In conclusion the applicant considers that Cholecalciferol poses no concerns with respect to genotoxicity. This position is supported by negative QSAR analysis for mutagenicity structural alerts ([redacted] 2012), extensive literature which suggests a protective role of Cholecalciferol against cellular process leading to cancer and a long history of beneficial use.

The applicant considers that Cholecalciferol does not meet any criteria for Mutagenicity classification as defined in the Regulation (EC) No 1272/2008 and that the proposal made by the CLH report is highly questionable and lacks relevance in the light of the conclusions that can be drawn from the extensive animal data and human experience.

Cholecalciferol is an essential vitamin in humans for its role in calcium homeostasis and skeletal health. Cholecalciferol has a long history of safe use in fortified foodstuffs, dietary supplement and human medicinal products. Tolerable upper intake levels have been defined by the European Food Safety Authority (EFSA) and national supplementation and fortification policies have been adopted across Europe to prevent Vitamin D deficiency disorders, e.g. impaired bone mineralization and bone-softening diseases (Ref EFSA 2012 + Spiro 2014). Classification of Cholecalciferol as a mutagen is not justified and is likely to impair community health by discouraging adequate intake of Vitamin D.

References

Anonymous (1977) Mutagenicity Evaluation of FDA 75-81 Vitamin D_3 (Cholecalciferol) IIIA 6.1.1-02

[redacted] (2014) Cholecalciferol: Combined bone marrow micronucleus test and Comet assay in the liver and duodenum in treated rats. Confidential report. IIIA 6.6.4 & 6.6.5

[redacted] (2012) Cholecalciferol: Structural Activity Relationship Analysis using DEREK Nexus® Version 3.0.1. Exponent Confidential report 1006019.UK0 DERK 1212 0028 for BASF Agro BV and Bayer SAS

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on the Tolerable Upper Intake Level of vitamin D. EFSA Journal 2012;10(7):2813. [45 pp.] doi:10.2903/j.efsa.2012.2813. Available online: www.efsa.europa.eu/efsajournal

Fedirko V, Bostick RM , *et al.* (2010) Effects of Supplemental Vitamin D and Calcium on Oxidative DNA Damage Marker in Normal Colorectal Mucosa: A Randomised Clinical Trial Cancer Epidemiol. Biomarkers Prev **19(1)**: 280-291

Fedirko, V., Duarte-Salles, T., Bamia, C. *et al* (2014) Prediagnostic Circulating Vitamin D Levels and Risk of Hepatocellular Carcinoma in European Populations: A Nested Case-Control Study. Hepatology **60**: 1222-1230

[redacted] (2013a) Cholecalciferol - A Pilot Toxicity Study in Wistar Rats following Oral (Gavage) Administration. Confidential Report No. 11/363-101PE.

[redacted] (2013a) Cholecalciferol: *In vitro* Mammalian Cell Gene Mutation Test (Mouse Lymphoma Assay) Confidential report. IIIA 6.6.3.

[redacted] (2013b) Cholecalciferol: Bacterial Reverse Mutation Assay. Confidential report. IIIA 6.1.1-01

Mortelmans K., Haworth S., Lawlor T., Speck W., Tainer B. and Zelger E. (1986) *Salmonella* Mutagenicity Tests: II. Results From the Testing of 270 Chemicals. Environmental Mutagenesis, **8**, Supp. 7: 1-119. IIIA 6.1.1-03

OECD Testing Guideline 489 (2014) *In vivo* Mammalian Alkaline Comet Assay

Spiro A, Buttris J. (2014) Vitamin D: an overview of vitamin D status and intake in Europe, Nutrition Bulletin **39**, 322-350.