REGISTRATION REPORT

Part B

Section 6

Mammalian Toxicology

Detailed summary of the risk assessment

Product code: GWN-10616

Chemical active substances:

Zoxamide, 60 g/L

Potassium phosphonates, 755 g/L

Phosphonic acid equivalents, 500 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

Applicant: XXXX

Submission date: 31/10/2023 (update October 2024)

Evaluation date: 07/2024

MS Finalisation date: 11/2024

Version history

When	What
July 2024	Version submitted by the applicant and evaluated by zRMS PL
September 2024	Update based on zRMS request of July 2024: Chapter 6.1, 6.3, A 2.2
October 2024	Update based on zRMS request of October 2024: Chapter 6.1, 6.6.1, 6.6.2.1, 6.6.5.1, Appendix 1, A 2.5.1, A 2.6.1, A 2.6.2, A 2.10.1, A 2.10.2, A 3.1.1, A 3.1.2, A 3.2.1, A 3.2.2, A 3.3.1, A 3.3.2, A 3.4
November 2024	Version evaluated by zRMS taking into account comments received from cMSs and the applicant

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6 Mammalian Toxicology (KCP 7)

6.1 Summary

Table 6.1-1: Information on GWN-10616 *

Product name and code	GWN-10616
Formulation type	Suspension concentrate (SC)
Active substance(s) (incl. content)	Zoxamide, 60 g/L Potassium phosphonates, 755 g/L (Phosphonic acid equivalents: 500 g/L)
Function	Fungicide
Product already evaluated as the 'representative formulation' during the approval of the active substance(s)	No
Product previously evaluated in another MS according to Uniform Principles	No

* Information on the detailed composition of GWN-10616 can be found in the confidential dRR Part C.

Justified proposals for classification and labelling

According to the criteria given in Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008, the following classification and labelling with regard to toxicological data is proposed for the preparation:

Table 6.1-2: Justified proposals for classification and labelling for GWN-10616 according to Regulation (EC) No 1272/2008

Hazard class(es), categories	~~Acute Tox 4,~~ Skin Sens. 1
Hazard pictograms or Code(s) for hazard pictogram(s)	GHS07
Signal word	Warning
Hazard statement(s)	~~H302,~~ H317
Precautionary statement(s)	P261, P264, P270, P272, P273, P280, ~~P301+P312, P330,~~ P302+P352, P362+P364, P333+P313, ~~P321~~, P391, P501
Additional labelling phrases	To avoid risks to man and the environment, comply with the instructions for use. [EUH401]
	1,2-benzisothiazol-3(2H)-one

Table 6.1-3: Summary of risk assessment for operators, workers, residents and bystanders for GWN-10616

	Result	PPE / Risk mitigation measures
Operators	Acceptable	Normal work wear (arms, body and legs covered) according to the EFSA model 2022 during mixing/loading and application, and additionally gloves during mixing and loading, due to the skin sensitising potential of the product.

	Result	PPE / Risk mitigation measures
Workers	Acceptable	Work wear (arms, body and legs covered) according to the EFSA model 2022 and additionally gloves when handling treated crops, due to the skin sensitising potential of the product.
Residents	Acceptable	None
Bystanders	Acceptable	None

No unacceptable risk for operators, workers, residents and bystanders was identified when the product is used as intended. No specific PPE is necessary.

Further provisions are required to protect operators and workers from the skin sensitising potential of the concentrate and spray dilution.

A summary of the critical uses and the overall conclusion regarding exposure for operators, workers and residents/bystanders is presented in the following table.

Table 6.1-4 Critical uses and overall conclusion of exposure assessment

1	2	3	4	5	6	7	8	9	10			
Use-No.*	**Crops and situation (e.g. growth stage of crop)**	**F, Fn, Fpn G, Gn, Gpn or I ****	**Application**		**Application rate**		**PHI (d)**	**Remarks:** **(e.g. safener/synergist (L/ha))** **critical gap for operator, worker, resident or bystander exposure based on [Exposure model]**	**Acceptability of exposure assessment**			
			Method / Kind **(incl. application technique *****	**Max. number (min. interval between applications)** **a) per use** **b) per crop/ season**	**Max. application rate kg as/ha** **a) a.s. 1** **b) a.s. 2**	**Water L/ha** **min / max**			**Operator**	**Worker**	**Residents**	**Bystander**
1	Grapevine (table and wine) (BBCH 14-79)	F	Broadcast foliar spray, HCTM, HCHH	a) 3 (8-10 days) b) 3	a) 0.180 (Zoxamide) b) 2.265 (Potassium phosphonates)#	200 - 1000	28	Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products; EFSA Journal 2022;20(1):7032	A	A	A	A
3	Pome fruit (BBCH 51-69)	F	Broadcast foliar spray, HCTM	a) 2 (6-8 days) b) 2	a) 0.180 (Zoxamide) b) 2.265 (Potassium phosphonates) #	200 - 1000	nr		A	A	A	A
5	Potato (BBCH 21-89)	F	Broadcast foliar spray, LCTM	a) 3 (7-8 days) b) 3	a) 0.150 (Zoxamide) b) 1.8875 (Potassium phosphonates) ##	200 - 500	7		A	A	A	A

* Use number(s) in accordance with the list of all intended GAPs in Part B, Section 0 should be given in column 1
** F: professional field use, Fn: non-professional field use, Fpn: professional and non-professional field use, G: professional greenhouse use, Gn: non-professional greenhouse use, Gpn: professional and non-professional greenhouse use, I: indoor application
*** e.g. LC: low crops, HC: high crop, HH: hand held, TM: tractor-mounted
# Phosphonic acid equivalents: 1.5 kg a.s./ha
Phosphonic acid equivalents: 1.25 kg a.s./ha
nr: not relevant

Explanation for column 10 “Acceptability of exposure assessment”

A	Exposure acceptable without PPE / risk mitigation measures
R	Further refinement and/or risk mitigation measures required
N	Exposure not acceptable/ Evaluation not possible

Data gaps

- No data gaps.

6.2 Toxicological Information on Active Substance(s)

Information regarding classification of the active substances and on EU endpoints and critical areas of concern identified during the EU review are given in Table 6.2-1.

Table 6.2-1: Information on active substances

	Active substance 1	**Active substance 2**
Common Name	Zoxamide	Potassium phosphonates
CAS-No.	156052-68-5	13977-65-6 for potassium hydrogen phosphonate 13492-26-7 for dipotassium phosphonate
Classification and proposed labelling		
With regard to toxicological endpoints (according to the criteria in Reg. 1272/2008, as amended)	Skin Sens. 1, H317	--
Additional C&L proposal	--	--
Agreed EU endpoints		
AOEL systemic	0.3 mg/kg bw/day based on a 90-day dog study (60 % oral absorption) and a safety factor of 100	5 mg/kg bw/day (bridging data from Fosetyl-Al)
Reference	EFSA Peer Review Conclusion (2017)	EFSA Peer Review Conclusion (2012)
Acute Acceptable Operator Exposure Level (AAOEL)	Not allocated, not necessary	Not allocated, not necessary
Reference	EFSA Peer Review Conlcusion (2017) EFSA Journal 2017;15(9):4980	EFSA Peer Review Conclusion (2012) EFSA Journal 2012;10(12):2963
Conditions to take into account/critical areas of concern with regard to toxicology		
	~~None~~ According to COMMISSION IMPLEMENTING REGULATION (EU) No 540/2011 "In this overall assessment Member States shall pay particular attention to: — the protection of groundwater from metabolite RH-141455. It is also noted that according to Peer review of the pesticide risk assessment of the active substance zoxamide (EFSA Journal 2017;15(9):4980) the human health and environmental risk assessments consequent to potential changes in the isomeric ratio could not be finalised.	None

6.3 Toxicological Evaluation of Plant Protection Product

A summary of the toxicological evaluation for GWN-10616 is given in the following tables. No specific studies related to acute oral toxicity, acute dermal toxicity, acute inhalation toxicity and skin sensitisation are available. The acute toxicity potential related to these endpoints was evaluated using the CLP calculation method according to Regulation (EC) 1272/2008 (classification based on ingredients of the mixture; additivity formula). Full summaries of studies on the product related to the endpoints skin corrosion/irritation and eye irritation that have not been previously considered within an EU peer review process are described in detail in Appendix 2.

Table 6.3-1: Summary of evaluation of the studies on acute toxicity including irritancy and skin sensitisation for GWN-10616

Type of test, species, model system (Guideline)	Result	Acceptability	Classification (acc. to the criteria in Reg. 1272/2008)	Reference
LD_{50} oral, rat Calculation method was applied*	~~1468 mg/kg bw~~ > 5000 mg/kg bw	YES	~~Acute Tox 4, H302~~ None	Not applicable
LD_{50} dermal, rat Calculation method was applied*	Not calculated since none of the components was classified	YES	None	Not applicable
LC_{50} inhalation, rat Calculation method was applied*	Not calculated since none of the components was classified	YES	None	Not applicable
Skin corrosion *in vitro*, EPISKIN model (OECD 431)	Non-corrosive	YES	None	Buda, 2022
Skin irritation *in vitro*, EPISKIN model (OECD 439)	Non-irritant	YES	None	Buda, 2022
Eye irritation *in vitro*, isolated chicken eyes (OECD 438)	No prediction could be made	YES	None	XXXX, 2022
Eye irritation *in vitro*, EpiOcular model (OECD 492)	Non-irritant	YES	None	Buda, 2022
Skin sensitisation Calculation method was applied*	Sensitising	YES	Skin sensitiser 1, H317	Not applicable
Supplementary studies for combinations of plant protection products	No data – not required			

* CLP calculation method according to Regulation (EC) 1272/2008 (classification based on ingredients of the mixture; additivity formula)

Table 6.3-2: Additional toxicological information relevant for classification/labelling of GWN-10616

	Substance (concentration in product, % w/w)*	Classification of the substance (acc. to the criteria in Reg. 1272/2008)	Reference	Classification of product (acc. to the criteria in Reg. 1272/2008)
Toxicological properties of active substance(s) (relevant for classification of product)	Zoxamide (> 2.5- ≤ 10 % (w/w))	Skin Sens. 1, H317	MSDS** Regulation 1272/2008	~~Acute Tox 4, H302~~ Skin Sens. 1, H317
	~~Phosphonic acid (≈34 %)~~	~~Acute Tox. 4 * H302 Skin Corr. 1A, H314~~	~~Regulation 1272/2008~~	-
	~~Potassium hydroxide (≈26%)~~	~~Acute Tox. 4 * H302 Skin Corr. 1A, H314~~	~~Regulation 1272/2008~~	-
Toxicological properties of non-active substance(s) (relevant for classification of product)	Information can be found in the confidential dRR Part C.			
Further toxicological information	No data – not required			

* Concentration range or concentration limit as provided in MSDS
** Material safety data sheet by the applicant

The toxicological evaluation of the plant protection product is not relevant for this submission / no additional data.
However, additional data are submitted for metabolites of Zoxamide, which appear in raw agricultural commodities (metabolite RH-141452) and processed commodities (metabolite RH-150721). The toxicological data were evaluated during active ingredient renewal (AIR) on EU level (please refer to RAR, 2017 and EFSA Peer Review Conclusion, 2017) and additional studies have been provided to RMS Latvia. Full summaries of studies on the metabolites submitted to Latvia are additionally described in detail in Appendix 2 (A 2.11 Other/Special Studies) for the sake of completeness.

RH-141452

RH-141452 is not genotoxic and it showed an acute oral toxicity of LC50 > 5000 mg/kg bw/day (see EFSA, 2017), which is above 2000 mg/kg bw/day, the dose for classification. Repeated dose toxicity data for RH-141452 were provided, *i.e.* a 14-day and 90-day repeated dose toxicity study in rats, as a basis to set a chronic reference value (ADI) for consumer risk assessment. Summary is presented below.

Table 6.3-3: Summary of the results of toxicity studies for RH-141452

Type of test, species (Guideline)	Result	Acceptability	Reference
Ames (OECD 471)	non-genotoxic	YES	Sames, J.L., Ciaccio, P.J. (1998)*; Report no. 98R-050
In vitro mammalian cell gene mutation test (HPRT-Locus) in Chinese Hamster V79 cells (OECD 476)	non-genotoxic	YES	Voges, 2020**; Report no. 188620

Type of test, species (Guideline)	Result	Acceptability	Reference
In vitro mammalian micronucleus assay in Chinese Hamster V79 cells (OECD 487)	non-genotoxic	YES	Donath, 2019**; Report no. 188616
Acute oral mouse (OECD 401)	LD_{50} > 5000	YES	XXXX, 1998*; Report no. 98R-049
14 d dietary toxicity in rats (OECD 407)	NOAEL > 400 mg/kg bw/day[#]	YES	XXXX, 2021**; Report no. U-20188
90 d dietary toxicity in rats (OECD 408) incl. 28 d recovery and plasma TK	NOAEL > 538 mg/kg bw/day[##]	YES	XXXX, 2022**; Report no. U-20281

* indicates that a study was reviewed at EU level
** indicates that a study was reviewed on national level (RMS Latvia)
[#] NOAEL of 5000 ppm, which is equivalent to 415 mg/kg bw/day in males and 432 mg/kg bw/day in females.
[##] NOAEL of 9000 ppm, hich is equivalent to 538 in males and 625 mg/kg body weight/day in females.

Based on the available results, an ADI for RH-141452 can be calculated. The NOAEL of the 90-day study in rats of 538 mg/kg bw/day can be used as a point of departure. An additional UF of 10 (overall UF 1000) is proposed to take into account for the missing data (including the lack of dog studies, the most sensitive species with the parent compound).
Therefore, the ADI of RH-141452 would be 0.54 mg/kg bw/day, which is similar to the ADI of the parent of 0.5 mg/kg bw/day, and it can be concluded that the ADI of the parent Zoxamide of 0.5 mg/kg bw/day covers the toxicity profile of the metabolite RH-141452.

RH-150721

A full genotoxicity package was provided for RH-150721, showing that the metabolite is not genotoxic. Further toxicological data was generated, *i.e.* a 14-day and a 90-day repeated dose toxicity study in rats, as a basis to set a chronic reference value (ADI) for consumer risk assessment. Summary is presented below.

Table 6.3-4: Summary of the results of toxicity studies for RH-150721

Type of test, species (Guideline)	Result	Acceptability	Reference*
Ames (OECD 471)	non-genotoxic	YES	Sokolowski, 2013*; Report no. 1549300
In vitro mammalian cell gene mutation assay in mouse lymphoma L5178Y cells (OECD 490)	non-genotoxic	YES	Schreib, 2017**; Report no. 171360
In vitro mammalian micronucleus assay in Chinese hamster V79 cells (OECD 487)	non-genotoxic	YES	Donath, 2017**; Report no. 171361
14 d dietary toxicity in rats (OECD 407)	LOAEL: 66 mg/kg bw/day[#]	YES	XXXX, 2020***; Report no. U-19189
90 d dietary toxicity in rats (OECD 408) incl. 28 d recovery and plasma TK	NOAEL: 44 mg/kg bw/day[##]	YES	XXXX, 2020***; Report no. U-19235

* indicates that a study was reviewed at EU level
** submitted to EFSA in 2017, but not yet reviewed
*** indicates that a study was reviewed on national level (RMS Latvia)
[#] LOAEL of 1000 ppm, which is equivalent to 66 mg/kg bw/day for males 77 mg/kg bw/day for females.
[##] NOAEL of 670 ppm, which is equivalent to 44 mg/kg bw/day for females. NOAEL of 2000 ppm (111 mg/kg bw/day) for males.

ADI and ARfD for RH-150721

The following reference values were proposed by EFSA following EU expert meeting conclusions on April 2023 and agreed by Latvia as RMS.

An ADI of 0.04 mg/kg bw/day can be set for the metabolite RH-150721, based on the NOAEL of 44 mg/kg bw/day in the 90-day toxicity study in female rats and applying an UF of 1000 (an additional UF of 10 is used to take into account for the missing data (including the lack of dog studies, the most sensitive species with the parent compound).
An effect on body weight gains were noted from day 1-2 of the 14-day study (>10 % in males and females at all dose levels), therefore, an ARfD could be set, based on the LOAEL of 66 mg/kg bw/day. Considering only acute effects, an UF of 300 could be applied to the LOAEL, resulting in an ARfD of 0.22 mg/kg bw for the metabolite RH-150721.

6.4 Toxicological Evaluation of Groundwater Metabolites

The following data on Zoxamide metabolites with the potential to reach the groundwater in concentrations above 0.1 µg/L and requiring relevance assessment were submitted. Note that the relevance assessment of the metabolites is reported in Part B.10.

The toxicological studies were evaluated during active ingredient renewal (AIR) on EU level (please refer to RAR, 2017 and EFSA Peer Review Conclusion, 2017) and additional studies have been provided to RMS Latvia. The zoxamide metabolite RH-141455 (3,5-dichloro-4-carboxybenzoic acid) can appear > 0.1 µg/L but < 0.75 µg/L.

6.4.1 RH-141455

An overview on the results of the accepted toxicological studies for the groundwater metabolite RH-141455 is given in the following table. Full summaries of studies on the metabolite submitted to Latvia are additionally described in detail in Appendix 2 (A 2.11 Other/Special Studies) for the sake of completeness.

Table 6.4-1: Summary of the results of toxicity studies for RH-141455

Type of test, species (Guideline)	Result	Acceptability	Reference
Ames (OECD 471)	non-genotoxic	YES	Sames, J.L., Ciaccio, P.J., 1998*; Report no. 98R-048
In vitro mutation test using mouse lymphoma L5178Y cells (OECD 476)	non-genotoxic	YES	Woods, 2014*; Report no. FRK0049
In vitro micronucleus test in human lymphocytes (OECD 487)	non-genotoxic	YES	Woods, 2014*; Report no. FRK0050
Acute oral mouse (OECD 401)	LD_{50} > 5000 mg/kg bw	YES	XXXX, 1998*; Report no. 98R-047
14 d dietary toxicity in rats (OECD 407)	NOAEL: 368 mg/kg bw/day[#]	YES	XXXX, 2020**; Report no. U-19071
90 d dietary toxicity in rats (OECD 408) Limit test incl. 28 d recovery and plasma TK	LOAEL: 924 mg/kg bw/day[##]	YES	XXXX, 2020**; Report no. U-19102

* indicates that a study was reviewed at EU level
** indicates that a study was reviewed on national level (RMS Latvia)
[#] NOAEL of 5000 ppm, which is equivalent to 368 mg/kg bw/day for males and NOAEL of 15000 ppm (1069 mg/kg bw/day) for females.
[##] LOAEL of 16000 ppm, which is equivalent to 924 mg/kg bw/d in males and NOAEL of 16000 ppm (1119 mg/kg bw/day) for males and females.

The following reference values were proposed by EFSA following EU expert meeting conclusions on April 2023 and agredd by Latvia as RMS.

An ADI of 0.3 mg/kg bw/day can be set for the metabolite RH-141455.

As a result, the metabolite RH-141455 can be regarded as toxicologically not relevant in groundwater. Further details are provided in dRR Part B.10.

6.5 Dermal Absorption (KCP 7.3)

A summary of the dermal absorption rates for the active substances in GWN-10616 are presented in the following table.

Table 6.5-1: Dermal absorption rates for active substances in GWN-10616

	Zoxamide		Potassium phosphonates	
	Value	**Reference**	**Value**	**Reference**
Concentrate	0.55 %	New study reported in Appendix 2	0.071 %	New study reported in Appendix 2
Dilution (dilution factor)	14 % (1:400)	New study reported in Appendix 2	1.7 % (1:500)	New study reported in Appendix 2

6.5.1 Justification for proposed values - Zoxamide

Proposed dermal absorption rates for Zoxamide are based on dermal absorption studies on a formulation identical to GWN-10616. The study results are summarised in the following table. Full summaries of studies on the dermal absorption of Zoxamide that have not previously been evaluated within an EU peer review process are described in detail in Appendix 2.

Table 6.5-2: Summary of the results of submitted dermal absorption studies for Zoxamide

Test	Concentrate	Spray dilution (dilution factor)	Formulation in study	Acceptability of study	Justification provided on representativity of study formulation for current product	Acceptability of justification	Reference
In vitro (human)	0.55 %	14 % (1:400)	GWN-10616	YES	Not required	YES	Finlayson, Z., 2022

6.5.2 Justification for proposed values – Potassium phosphonates

Proposed dermal absorption rates for Potassium phosphonates are based on dermal absorption studies on a formulation identical to GWN-10616. The study results are summarised in the following table. Full summaries of studies on the dermal absorption of Potassium phosphonates that have not previously been evaluated within an EU peer review process are described in detail in Appendix 2.

Table 6.5-3: Summary of the results of submitted dermal absorption studies for Potassium phosphonates

Test	Concentrate	Spray dilution (dilution factor)	Formulation in study	Acceptability of study	Justification provided on representativity of study formulation for current product	Acceptability of justification	Reference
In vitro (human)	0.071 %	1.7 % (1:500)	GWN-10616	YES	Not required	YES	Spa, S., 2022

6.6 Exposure Assessment of Plant Protection Product (KCP 7.2)

Table 6.6-1: Product information and toxicological reference values used for exposure assessment

Product name and code	GWN-10616	
Formulation type	SC	
Category	Fungicide	
Active substance(s) (incl. content)	**Zoxamide** (60 g/L)	**Potassium phosphonates** (755 g/L) Phosphonic acid equivalents: 500 g/L
AOEL systemic	0.3 mg/kg bw/day	5 mg/kg bw/day
Inhalation absorption	100 %	100 %
Oral absorption	60 %	100 %
Dermal absorption	Concentrate: 0.55 % Dilution: 14 % (0.15 g/L) (Based on product (formulation))	Concentrate: 0.071 % Dilution: 1.7 % (1.51 g/L) Phosphonic acid equivalents: 1 g/L (Based on product (formulation))

6.6.1 Selection of critical use(s) and justification

The critical GAP used for the exposure assessment of the plant protection product is shown in Table 6.1-4. A list of all intended uses within the central zone is given in Part B, Section 0.

Justification

Operator exposures from applying GWN-10616 on grapevine (max. rate 3 L GWN-10616/ha corresponding to 0.18 kg Zoxamide/ha and 2.265 kg Potassium phosphonates/ha sprayed 3 times with vehicle mounted applications and with minimum 8 days between applications) are the critical uses for upward spraying, covering the use of pome fruit and also the use on low crops (potato, downward spraying). Based upon the request by zRMS, operator exposures from applying GWN-10616 on grapevine with manual-hand held and manual-knapsack applications is presented herewith.

Grapevine is also the critical use for worker and residents/bystanders, covering the use of pome fruit and potato.

6.6.2 Operator exposure (KCP 7.2.1)

6.6.2.1 Estimation of operator exposure

A summary of the exposure models used for estimation of operator exposure to the active substances during application of GWN-10616 according to the critical use is presented in Table 6.6-2. The outcome of the estimation is presented in **Błąd! Nie można odnaleźć źródła odwołania.**3 (longer term exposure). Detailed calculations are in Appendix 3.

Table 6.6-2: Exposure models for intended uses

Critical use(s)	Grapevine (max. 3 L product/ha)
Model(s)	Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products; EFSA Journal

	2022;20(1):7032 Online calculator - OPEX version: 1.0.1

Table 6.6-3: Estimated operator exposure (short term exposure)

		Zoxamide		Potassium phosphonates	
Model data	**Level of PPE**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
Tractor mounted spray application outdoors to high crops					
Application rate		3 x 0.180 kg a.s./ha		3 x 2.265 kg a.s./ha	
Spray application (AOEM; 75th percentile) Body weight: 60 kg	Work wear (arms, body and legs covered) M/L and A	0.02	6	0.03	0.5
Manual-hand held spray application outdoors to high crops					
Application rate		3 x 0.180 kg a.s./ha		3 x 2.265 kg a.s./ha	
Spray application (AOEM; 75th percentile) Body weight: 60 kg	Work wear (arms, body and legs covered) M/L and A	0.009	3.1	0.01	0.3
Manual-knapsack spray application outdoors to high crops					
Application rate		3 x 0.180 kg a.s./ha		3 x 2.265 kg a.s./ha	
Spray application (AOEM; 75th percentile) Body weight: 60 kg	Work wear (arms, body and legs covered) M/L and A	0.006	2	0.006	0.1

The calculations show that there is no undue health risk to the operator when normal work wear is worn during mixing/loading and application. With respect to the classification of the product for skin sensitisation (calculation method), the wear of gloves during mixing/loading is recommended.

zRMS:
The exposure of operator not wearing PPE, but wearing a work clothing (long sleeved shirt, long trousers) and applying formulation GWN-10616 on high crops at dose of 3.0 L/ha, using tractor-mounted/trailed sprayer (upward spraying , calculated with the EFSA AOEM 2022 and expressed as the sum of percentage of respective AOELs for all two active substances of the products (Zoxamide – 6.0 % of AOEL and Potassium phosphonates 0.5 % of AOEL = 6.5%) is below of 100%, therefore it is concluded that operator is not at risk if applying GWN-10616 according to its intended use.
The exposure of operator not wearing PPE, but wearing a work clothing (long sleeved shirt, long trousers) and applying formulation GWN-10616 on high crops (grapes) at dose of 3.0 L/ha, using manual-hand held spray application outdoors, calculated with the EFSA AOEM 2022 and expressed as the sum of percentage of respective AOELs for all two active substances of the products (Zoxamide – 3.1 % of AOEL and Potassium phosphonates 0.3 % of AOEL = 3.4 %) is below of 100%, therefore it is concluded that operator is not at risk if applying GWN-10616 according to its intended use.

Given the toxicological properties and classification of the formulation GWN-10616 according to Regulation 1272/2008/EC) as Skin Sens. 1 wearing protective gloves is recommended when handling the concentrate.

6.6.2.2 Measurement of operator exposure

Since the operator exposure estimations carried out indicated that the acceptable operator exposure level (AOEL) will not be exceeded under conditions of intended uses and consideration of the above mentioned personal protective equipment (PPE), a study to provide measurements of operator exposure was not necessary and was therefore not performed.

6.6.3 Worker exposure (KCP 7.2.3)

6.6.3.1 Estimation of worker exposure

Table 6.6-4 shows the exposure model(s) used for estimation of worker exposure after entry into a previously treated area or handling a crop treated with GWN-10616 according to the critical use(s). Outcome of the estimation is presented in Table 6.6-5 (longer term exposure). Detailed calculations are in Appendix 3.

Table 6.6-4: Exposure models for intended uses

Critical use(s)	Grapevine (max. 3 L product/ha)
Model	Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products; EFSA Journal 2022;20(1):7032 Online calculator - OPEX version: 1.0.1

Table 6.6-5: Estimated worker exposure (longer term exposure)

		Zoxamide			Potassium phosphonates		
Model data	**Level of PPE**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Re-entry restriction (days)**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Re-entry restriction (days)**
Grapevine, Hand harvesting, Outdoor, Work rate: 8 hours/day, DT_{50}: 30 days, DFR: 3 µg/cm²/kg a.s./ha, Interval between treatments: 8 days							
Number of applications and application rate		3 x 0.18 kg a.s./ha			3 x 2.265 kg a.s./ha		
Body weight: 60 kg	Potential TC: 30000 cm²/person/h	0.8	254	41	1.2	23.3	0
	Work wear (arms, body and legs covered) TC: 10100 cm²/person/h	0.3	85.5	0	0.4	7.8	0

The calculations show that there is no undue health risk to the worker when normal work wear is worn for worker activities. With respect to the classification of the product, further provisions are required to pro-

tect workers from the skin sensitising potential of the product (gloves) when handling treated crops.

zRMS:
The potential systemic exposure estimation of worker entering for 8 hours for hand harvesting a vineyard sprayed with GWN-10616 to its two active substances (Zoxamide and Potassium phosphonates) using worst-case scenario and acceptable model (EFSA 2022 AOEM model) demonstrates that such exposures are well above respective AOELs set in EU for Zoxamide (254 % of AOEL) and below of AOEL of Potassium phosphonates (23.3 %). A sum of exposures to both active substance expressed as percentage of their AOELs (277.3 %) is also above 100% indicating that worker not wearing normal workwear has an unacceptable risk due to exposure to Zoxamide and due to combined exposures to these two substances simultaneously.
In case a worker entering for 8 hours for hand harvesting a vineyard sprayed with GWN-10616 is wearing work wear (arms, body and legs covered) exposure is reduced and is below respective AOELs (85.5% of AOEL of Zoxamide and 7.8 % of AOEL of Potassium phosphonates), and a sum of exposure of worker wearing work wear (arms, body and legs covered) to both active substances expressed as percentage of their AOELs (93.3 %) is also below 100%. It is concluded that the risk of worker wearing work wear (arms, body and legs covered) and entering for 8 hours for hand harvesting a vineyard sprayed with GWN-10616 is acceptable. The wear of gloves is recommended for worker activities since the product is classified for skin sensitization.

6.6.3.2 Refinement of generic DFR value (KCP 7.2)

Not required as risk is acceptable for workers.

6.6.3.3 Measurement of worker exposure

Since the worker exposure estimations carried out indicated that the acceptable operator exposure level (AOEL) will not be exceeded under conditions of intended uses and considering above mention PPE, a study to provide measurements of worker exposure was not necessary and was therefore not performed.

6.6.4 Resident and bystander exposure (KCP 7.2.2)

6.6.4.1 Estimation of resident and bystander exposure

The acute exposure assessment for bystanders covers the exposure that a resident could reasonably be expected to incur in a single day. Therefore, there is no need for a separate acute risk assessment for residents.

No bystander risk assessment is required for PPPs that do not have significant acute toxicity or the potential to exert toxic effects after a single exposure. Exposure in this case will be determined by average exposure over a longer duration, and higher exposures on one day will tend to be offset by lower exposures on other days. Therefore, exposure assessment for residents also covers bystander exposure.

Table 6.6-6 shows the exposure model used for estimation of resident and bystander exposure to Zoxamide and Potassium phosphonates. The outcome of the estimation is presented in Table 6.6-7 (longer term resident exposure). Detailed calculations are in Appendix 3.

Table 6.6-6: Exposure models for intended uses

Critical use(s)	Grapevine (max. 3 L product/ha)
Model	Guidance on the assessment of exposure of operators, workers, residents and

	bystanders in risk assessment for plant protection products; EFSA Journal 2022;20(1):7032 Online calculator - OPEX version: 1.0.1

Table 6.6-7: Estimated resident exposure (longer term exposure)

		Zoxamide		Potassium phosphonates	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
Grapevine, Outdoor, Vehicle mounted spray application, Upward spraying Buffer zone: 5 (m), Drift reduction technology: no (0%) DT_{50}: 30 days, DFR: 3 µg/cm^2/kg a.s./ha, Interval between treatments: 8 days					
Number of applications and application rate		3 x 0.18 kg a.s./ha		3 x 2.265 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.02	5.8	0.03	0.6
	Vapour (75th perc.)	0.0008	0.3	0.0008	0.02
	Deposits (75th perc.)	0.0006	0.2	0.003	0.07
	Re-entry (75th perc.)	0.01	3.6	0.02	0.3
	Sum (mean)	0.02	7.1	0.03	0.7
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.01	3.2	0.02	0.3
	Vapour (75th perc.)	0.0003	0.09	0.0003	0.005
	Deposits (75th perc.)	0.0002	0.08	0.0004	0.007
	Re-entry (75th perc.)	0.006	2	0.009	0.2
	Sum (mean)	0.01	3.9	0.02	0.4

The calculations show that there is no undue health risk to the resident/bystander under conditions of the intended uses.

zRMS:
The exposure estimation of resident (adult and child) to each of the two active substances of GWN-10616: (Zoxamide and Potassium phosphonates) using appropriate assumptions and model EFSA AOEM 2022 demonstrates that such a exposure is well below respective AOELs set in EU for these active substances and a sum of exposures to both active substance expressed as percentage of their AOELs is also below 100%, therefore the application of product GWN-10616, does not pose an unacceptable risk to the health of residents (adult and child) for its
No bystander acute exposure estimation for Zoxamide and Potassium phosphonates is required since no acute acceptable operator exposure value (AAOEL) has be set for these active substance. Therefore, as indicated in the EU guidance (SANTE-10832-2015 rev. 1.7; 24 January 2017), no unacceptable risk is expected for bystanders due to short-term single exposure to Zoxamide and Potassium phosphonates as a result of application of a product GWN-10616with accordance with intended use within good agricultural practice.
These estimates lead to conclusion that application of a product GWN-10616 in line with GAP on high crops at maximal dose of 3.0 L product/ha, using tractor-mounted/trailed sprayer does not pose an unacceptable health risk for residents and bystanders.

6.6.4.2 Measurement of resident and/or bystander exposure

Since the resident and/or bystander exposure estimations carried out indicated that the acceptable operator exposure level (AOEL) for Zoxamide and Potassium phosphonates will not be exceeded under conditions of intended uses and considering above mentioned risk mitigation measures, a study to provide measurements of resident/bystander exposure was not necessary and was therefore not performed.

6.6.5 Combined exposure

The product is a mixture of two active substances.

6.6.5.1 Exposure assessment of Zoxamide and Potassium phosphonates in GWN-10616

Note: The combined toxicological effect of these active substances has not been investigated with regard to repeated dose toxicity.

At the first tier, combined exposure is calculated as the sum of the component exposures without regard to the mode of action or mechanism/target of toxicity. Initially, the individual Hazard Quotients (HQ) are calculated for all active substances in the PPP by assessing the exposure according to appropriate models and dividing the individual exposure levels by the respective systemic AOEL. This is equivalent to the predicted exposure as % of systemic AOEL is converted to decimal. The Hazard Index (HI) is the sum of the individual HQs.

Table 6.6-8: Risk assessment from combined exposure (short term exposure) for the critical use

Application scenario	Active ingredient	% AOEL (HQ)
Operators – vehicle-mounted application, grapevine	Zoxamide	6.0 (0.06)
	Potassium phosphonates	0.5 (0.005)
	Cumulative risk operators (HI)	**0.06**
Operators – manual-hand held application, grapevine	Zoxamide	3.1 (0.031)
	Potassium phosphonates	0.3 (0.003)
	Cumulative risk operators (HI)	**0.03**
Operators – manual-knapsack application, grapevine	Zoxamide	2.0 (0.02)
	Potassium phosphonates	0.1 (0.001)
	Cumulative risk operators (HI)	**0.02**
Workers – Hand harvesting (workwear), grapevine	Zoxamide	85.5 (0.855)
	Potassium phosphonates	7.8 (0.078)
	Cumulative risk workers (HI)	**0.9**
Resident - child (grapevine)	Zoxamide	
	Drift	5.8 (0.058)
	Vapour	0.3 (0.003)
	Deposits	0.2 (0.002)
	Re-entry	3.6 (0.036)
	Sum of all pathways	**7.1 (0.071)**
	Potassium phosphonates	
	Drift	0.6 (0.006)
	Vapour	0.02 (0.0002)
	Deposits	0.07 (0.0007)
	Re-entry	0.3 (0.003)
	Sum of all pathways	**0.7 (0.007)**
	Cumulative risk resident – child (HI)	
	Drift	0.06
	Vapour	0.003
	Deposits	0.003
	Re-entry	0.04
	Sum of all pathways	**0.08**
Resident – adult (grapevine)	Zoxamide	
	Drift	3.2 (0.032)
	Vapour	0.09 (0.0009)
	Deposits	0.08 (0.0008)
	Re-entry	2.0 (0.02)

Application scenario	Active ingredient	% AOEL (HQ)
	Sum of all pathways	3.9 (0.039)
	Potassium phosphonates	
	Drift	0.3 (0.003)
	Vapour	0.005 (0.00005)
	Deposits	0.007 (0.00007)
	Re-entry	0.2 (0.002)
	Sum of all pathways	0.4 (0.004)
	Cumulative risk resident – adult (HI)	
	Drift	0.04
	Vapour	0.001
	Deposits	0.0009
	Re-entry	0.02
	Sum of all pathways	**0.04**

HQ Hazard Quotient
HI Hazard Index

The Hazard Index is < 1. Thus, combined exposure to all active substances in GWN-10616 is not expected to present a risk for operators, workers, residents and bystanders. No further refinement of the assessment is required.

ZRMS:
The combined exposure to both active substances of GWN-10616 does not pose an unacceptable risk for operators, workers, bystanders and residents.

Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 7.1.4/01	Buda, I.	2022	*In Vitro* Skin Corrosion Test with GWN-10616 in the EPISKIN Model Gowan Crop Protection Ltd, UK TOXI-COOP ZRT., Hungary, Study No. 912-431-6556, Doc. No. 565-001, EFSA Study Identification: EF-SA-2021-00006664 GLP Not published	N	XXXX
KCP 7.1.4/02	Buda, I.	2022	*In vitro* skin irritation test with GWN-10616 in the EPISKIN Model Gowan Crop Protection Ltd, UK TOXI-COOP ZRT., Hungary, Study No. 912-439-6625, Doc. No. 565-002, EFSA Study Identification: EF-SA-2022-00007879 GLP Not published	N	XXXX
KCP 7.1.4/03	Buda, I.	2019	*In Vitro* Skin Corrosion Test with Proficiency Chemicals in the EPISKIN Model TOXI-COOP ZRT., Hungary, Study No. 392-431-4224, Doc. No. 565-003 GLP Not published	N	XXXX
KCP 7.1.5/01	XXXX	2022	GWN-10616: *In vitro* Eye Irritation Test in Isolated Chicken Eyes XXXX, Study No. 912-438-6756, Doc. No. 566-001, EFSA Study Identification: EF-SA-2022-00009174 GLP Not published	N	XXXX
KCP 7.1.5/02	Buda, I.	2022	*In vitro* Eye Irritation Test with GWN-10616 in the EpiOcularTM Model Gowan Crop Protection Ltd, UK TOXI-COOP ZRT., Hungary, Study No. 912-492-6757, Doc. No. 566-002, EFSA Study ID: EFSA-2022-00009175	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP Not published		
KCP 7.1.5/03	XXXX	2012	REFERENCE SUBSTANCES *IN VITRO* EYE CORROSIVES IN ISOLATED CHICKEN EYES XXXX, Study No. 392.549.3229, Doc. No. 566-003 GLP Not published	N	XXXX
KCP 7.1.5/04	Buda, I.	2016	*In vitro* Eye Irritation Test with GWN-10616 in the EpiOcularTM Model TOXI-COOP ZRT., Hungary, Study No.302-492-1722, Doc. No. 566-004 GLP Not published	N	XXXX
KCP 7.3/01	Finlayson, Z.	2022	The *In Vitro* Percutaneous Absorption of Radiolabelled GWN-8030 in a Concentrate Formulation and One In-Use Dilution Through Human Split-Thickness Skin Gowan Crop Protection Ltd, UK Charles River Laboratories Edinburgh Ltd, UK, Study No. 787018, Doc. No. 511-001 GLP Not published	N	XXXX
KCP 7.3/02	Spa, S.	2022	The *In Vitro* Percutaneous Absorption of Phosphonate from a Concentrate Formulation (GWN-10616) and One In-Use Dilution through Human Split-Thickness Skin Gowan Crop Protection Ltd, UK Charles River Laboratories Den Bosch BV, The Netherlands, Study No. 20352081, Doc. No. 511-002, EFSA Study Identification: EFSA-2022-00012083 GLP Not published	N	XXXX
KCP 7.3/03	Anonymous	2023	Calculation in vitro dermal absorption Zoxamide EFSA calculator V.3 2018 Doc. No. 511-003 Scientific Consulting Company, Bad Kreuznach, Germany Non-GLP Not published	N	XXXX
KCP 7.3/04	Anonymous	2023	Calculation in vitro dermal absorption Phosphonate EFSA calculator V.3 2018 Doc. No. 511-004	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Scientific Consulting Company, Bad Kreuznach, Germany Non-GLP Not published		

List of data submitted or referred to by the applicant and relied on, but already evaluated at EU peer review

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 7.4/01	Voges, Y.	2020	*In vitro* mammalian cell gene mutation test (HPRT-Locus) in Chinese Hamster V79 Cells with RH-141452 Gowan Crop Protection Ltd, UK Eurofins BioPharma Product Testing Munich GmbH, Germany, Report No. 188620 GLP Not published	N	XXXX
KCP 7.4/02	Schreib, G.	2017	*In vitro* mammalian cell gene mutation assay (Thymidine Kinase Locus/$TK^{+/-}$) in mouse lymphoma L5178Y cells with RH-150721 Gowan Crop Protection Ltd, UK Eurofins BioPharma Product Testing Munich GmbH, Germany, Report No. 171360 GLP Not published	N	XXXX
KCP 7.4/03	Donath, C.	2019	*In vitro* mammalian micronucleus assay in Chinese Hamster V79 cells with RH-141452 Gowan Crop Protection Ltd, UK Eurofins BioPharma Product Testing Munich GmbH, Germany, Report No. 188616 GLP Not published	N	XXXX
KCP 7.4/04	Donath, C.	2017	*In vitro* mammalian micronucleus assay in Chinese Hamster V79 cells with RH-150721 Gowan Crop Protection Ltd, UK	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Eurofins BioPharma Product Testing Munich GmbH, Germany, Report No. 171361 GLP Not published		
KCP 7.4/05	XXXX	2019	RH-141455: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats XXXX, Report No. U-19044 No GLP Not published	Y	XXXX
KCP 7.4/06	XXXX	2020	RH-141455: 14-day oral dietary dose range finding study in Sprague Dawley rats XXXX., India, Report No. U-19071 No GLP Not published	Y	XXXX
KCP 7.4/07	XXXX	2020	RH-141455: 90-day oral dietary toxicity study with toxicokinetics and 28-day recovery period in Sprague Dawley rats XXXX, Report No. U-19102 GLP Not published	Y	XXXX
KCP 7.4/08	XXXX	2020	RH-150721: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats XXXX, Report No. U-19134 No GLP Not published	Y	XXXX
KCP 7.4/09	XXXX	2020	RH-150721: 14-day oral dietary dose range finding study in Sprague Dawley rats XXXX, Report No. U-19189 GLP Not published	Y	XXXX
KCP 7.4/10	XXXX	2020	RH-150721**:** 90-day oral dietary toxicity study and 28-day recovery period in Sprague Dawley rats XXXX, Report No. U-19235 GLP Not published	Y	XXXX
KCP	XXXX	2021	RH-141452: 14-day dietary study in Sprague Dawley rats	Y	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
7.4/11			XXXX Report No. U-20188, No GLP Not published		
KCP 7.4/12	XXXX	2022	RH-141452: 90-Day dietary toxicity study with 28-day recovery period in Sprague Dawley rats XXXX Report No. U-20281 GLP Not published	Y	XXXX
KCA 5.8.1	Sames, J.L., Ciaccio, P.J.	1998	RH-141,455: Salmonella typhimurium gene mutation assay (Ames test) Rohm and Haas Co., Report No. 98R-048, September 23, 1998, ER Ref No. 27.4 GLP Not published	N	XXXX
KCA 5.8.1	Woods, I.	2014	RH-141455: In vitro mutation test using mouse lymphoma L5178Y Huntingdon Life Sciences Eye Research Centre, Report No. FRK0049, July 8, 2014 GLP Not published	N	XXXX
KCA 5.8.1	Woods, I.	2014	RH-141455: In vitro micronucleus test in human lymphocytes Huntingdon Life Sciences Eye Research Centre, UK, Report No. FRK0050, July 8, 2014 GLP Not published	N	XXXX
KCA 5.8.1	Sokolowski, A.	2013	RH-150,721: *Salmonella typhimurium* reverse mutation assay Harlan CCR, Report No. 1549300, October 7, 2013 GLP Not published	N	XXXX
KCA 5.8.1	XXXX	1998	RH-141,452: Acute oral toxicity study in male and female mice XXXX Report No. 98R-049, September 24, 1998, ER Ref No. 25.2 GLP Not published	Y	XXXX
KCA 5.8.1	Sames, J.L., Ciaccio, P.J.	1998	RH-141,452: *Salmonella typhimurium* gene mutation assay (Ames test) Rohm and Haas Co., Report No. 98R-050, October 1, 1998, ER Ref No. 25.3	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP Not published		
KCA 5.8.1	XXXX	1998	RH-141,455: Acute oral toxicity study in male and female mice XXXX Report No. 98R-047, September 24, 1998, ER Ref No. 27.3 GLP Not published	Y	XXXX

List of data submitted by the applicant and not relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
-	-	-	-	-	-

List of data relied on not submitted by the applicant but necessary for evaluation

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
-	-	-	-	-	-

Appendix 2 Detailed evaluation of the studies relied upon

A 2.1 Statement on bridging possibilities

Comments of zRMS:	No bridging statement is necessary

Not relevant for this submission since no bridging was necessary. Detailed confidential information is addressed in Part C.

A 2.2 Acute oral toxicity (KCP 7.1.1)

Comments of zRMS:	The calculation provided in part C is appropriate. The product GWN-10616 warrants classification Acute Tox. 4; H302

Calculation formula for acute oral toxicity of mixtures and classification categories for the oral exposure route according to Regulation (EC) 1272/2008:

$$\frac{100}{ATEmix} = \sum_{n} \frac{Ci}{ATEi}$$

Ci concentration of ingredient i (% w/w or % v/v)
i the individual ingredient from 1 to n
n the number of ingredients
ATEi Acute Toxicity Estimate of ingredient i

Exposure route	Classification category	Converted acute toxicity point estimate
Oral [mg/kg bw]	0 < Cat 1 ≤ 5	0.5
	5 < Cat 2 ≤ 50	5
	50 < Cat 3 ≤ 300	100
	300 < Cat 4 ≤ 2000	500

ATE_{mix} = ~~1468~~ 800000 mg/kg bw
Classification of GWN-10616 for acute oral toxicity ~~Cat 4 (H302)~~ is not warranted.

A 2.3 Acute percutaneous (dermal) toxicity (KCP 7.1.2)

Comments of zRMS:	The assessment is appropriate. The product GWN-10616 does not warrants classification for acute dermal toxicity

Calculation formula for acute dermal toxicity of mixtures and classification categories for the dermal exposure route according to Regulation (EC) 1272/2008:

$$\frac{100}{ATEmix} = \sum_{n} \frac{Ci}{ATEi}$$

Ci concentration of ingredient i (% w/w or % v/v)
i the individual ingredient from 1 to n
n the number of ingredients
ATEi Acute Toxicity Estimate of ingredient i

Exposure route	Classification category	Converted acute toxicity point estimate
Dermal [mg/kg bw]	0 < Cat 1 ≤ 50	5
	50 < Cat 2 ≤ 200	50
	200 < Cat 3 ≤ 1000	300
	1000 < Cat 4 ≤ 2000	1100

None of the components in the formulation was classified for acute dermal toxicity resulting in no classification of GWN-10616 (no calculation).

A 2.4 Acute inhalation toxicity (KCP 7.1.3)

Comments of zRMS:	The assessment is appropriate. The product GWN-10616 does not warrants classification for acute inhalation toxicity

Calculation formula for acute inhalation toxicity of mixtures and classification categories for the inhalation exposure route according to Regulation (EC) 1272/2008:

$$\frac{100}{ATEmix} = \sum_{n} \frac{Ci}{ATEi}$$

Ci concentration of ingredient i (% w/w or % v/v)
i the individual ingredient from 1 to n
n the number of ingredients
ATEi Acute Toxicity Estimate of ingredient i

Exposure route	Classification category	Converted acute toxicity point estimate
Inhalation - vapours [mg/L]	0 < Cat 1 ≤ 0.5	0.05
	0.5 < Cat 2 ≤ 2.0	0.5
	2.0 < Cat 3 ≤ 10.0	3
	10.0 < Cat 4 ≤ 20.0	11

None of the components in the formulation was classified for acute inhalation toxicity resulting in no classification of GWN-10616 (no calculation).

A 2.5 Skin irritation (KCP 7.1.4)

Comments of zRMS:	The study KCP 7.1.4/01 performed according to internationally recognised guidelines and in laboratory with demonstration of proficiency and with GLP conditions is acceptable. Since viability of cells after 3 min., 1hour and 4 hours exposure was above 50% the product GWN-10616 does not warrant classification as corrosive to skin.

A 2.5.1 Study 1

Reference	KCP 7.1.4/01
Report	*In Vitro* Skin Corrosion Test with GWN-10616 in the EPISKIN Model, Buda I., 2022, study No. 912-431-6556, Doc. No. 565-001, EFSA Study Identification: EFSA-2021-00006664
Guideline(s)	OECD No. 431 (2019), Council Regulation (EC) No 440/2008, Annex Part B, Official Journal of the European Union No. L142, amended by Commission Regulation (EU) 2019/1390 of 31 July 2019, INVITTOX Protocol No. 118 (2012)
Deviations	No
GLP	Yes
Acceptability	Yes
Duplication (if vertebrate study)	No, not applicable (in *vitro study*)

Executive summary

The purpose of this study was to determine the skin corrosion potential of the test substance GWN-10616 (batch no. P2102669001) on reconstituted human epidermis in the EPISKIN model *in vitro*.
Disks of epidermal units (2 units) were treated with the test substance (used as supplied) and incubated for 4 hours (h), 1 h and 3 minutes (min). Exposure of test substance was terminated by rinsing with 1 x phosphate buffered saline (PBS) solution. The viability of each disk was assessed by incubating the tissues for 3 h with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. The formazan precipitated was then extracted using acidified isopropanol and quantified spectrophotometrically. NaCl (0.9 %) and glacial acetic acid treated epidermis were used as negative and positive controls (2 units / positive and negative control), respectively. For each treated tissue viability was expressed as a percentage relative to negative control.

Due to the intrinsic color of the test substance (light brown), 2 additional tissues treated with the test substance were used for the non-specific optical densities (OD) evaluation (NSC_{living}).
Positive and negative controls showed the expected cell viability values within acceptable limits. The mean OD value of the two negative control tissues after 4 h exposure was 1.6 which is out of the acceptable range of 0.6 - 1.5 in the first experiment and therefore this exposure time was repeated in the additional experiment. The viability of the test substance treated tissue was above 35 % of the mean negative control value at all evaluated time points. The average test substance treated tissue relative viabilities were 101 % at 4 h, 107 % at 1 and 110 % at 3 min of exposure. All assay acceptance criteria were met, the experiments were considered to be valid.

In this *in vitro* skin corrosion test using the EPISKIN model, the mean tissue viability was > 35 % after 4 h exposure and thus the test substance GWN-10616 was classified as non-corrosive to skin.
According to the current OECD Guideline No. 431, GWN-10616 is considered as non-corrosive to skin.

I. MATERIALS AND METHODS

A. Materials

1. Test materials

Identification	GWN-10616
Batch	P2102669001
Preparation	The test substance was applied in its original form, no formulation was required.

2. Control materials

Negative control	NaCl (9 g/L saline)

Positive control	Glacial acetic acid

3. Additional materials

MTT solution	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved to the concentration of 3 mg/mL in saline buffer (1x PBS) and stored in refrigerator (2-8 °C). The MTT stock solution was diluted with pre-warmed (37 °C) assay medium to a final concentration of 0.3 mg/mL and used within 2 h for MTT incubation.

Acidified Isopropanol	Isopropanol was diluted with 12N HCl acid to a final concentration of 0.04N HCl.

4. Test system

The 3-dimensional human epidermis model EpiSkin^{TM}SM (supplied as 12-well assay plates by EPISKIN Laboratories Lyon, France, including biopsy punch, maintenance and assay medium) was obtained after a 13-day culture period of adult human derived epidermal keratinocytes on collagen matrix and comprised main basal, supra basal, spinous and granular layers and a functional *stratum corneum*.

B. STUDY DESIGN

1. Potential false viability test

Optical properties of the test substance or its chemical action on MTT may interfere with the assay and lead to a false estimate of viability. This may occur when the test substance is not completely removed from the tissue by rinsing or when it penetrates the epidermis. If the test substance acts directly on MTT (MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls are used to detect and correct for test substance interference with the viability measurement.

Check-method for possible direct MTT reduction with test substance
Approximately 50 µL test substance was added to 2 mL MTT 0.3 mg/mL (diluted with assay medium) solution and mixed. The mixture was incubated for 3 h at 37 ± 1 °C in an incubator with 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere, protected from light. Subsequently, any observed colour change of the solution was recorded. The test substance showed no direct interaction with MTT (the solution became yellow). The use of an additional control was not necessary.

Check-method to detect the colouring potential of test substance
The test substance has an intrinsic colour (light brown). Since the test substance is not white, off white, almost white and/or colorless the additional controls were automatically used and based on the

optical density (OD) results of additional controls the Non Specific Colour % (NSC_{living} %) was determined. This step prevents the false viability, which can possibly be caused by the stained surface of the tissues by the test substance.

Additional control(s) for dyes and chemicals able to colour the tissue
Two (2) additional test substance treated tissues were used for the non-specific OD evaluation. These tissues followed the same treatment steps as the other tissues except for the MTT step: MTT incubation was replaced by incubation with fresh assay medium.

2. Assay performance

The maintenance medium was pre-warmed to 37 °C. The appropriate number of assay plate wells were filled with the pre-warmed medium (2 mL per well). The epidermis units were placed above the media in a separately prepared well. The well was then incubated overnight at 37 ± 1 °C in an incubator with 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere. The pre-incubation period was extended in the additional experiment: after the overnight incubation the maintenance medium was replaced with 2 mL fresh medium in each well and after the incubation was continued overnight at the same conditions.
A volume of 50 µL test substance was applied evenly to the epidermal surface of the test skin units. A volume of 50 µL positive control or negative control was applied to the skin surface by spreading gently with the pipette tip in order to cover evenly all the epidermal surface. Two (2) replicates per test substance with each exposure time (4 h, 1 h, 3 min) and 2 replicates per controls (4 h, 1 h, 3 min for the negative and 4 h for the positive control) were used.
In addition to the normal procedure, 2 additional test substance treated tissues were used for the non-specific OD evaluation (NSC_{living}) at each exposure time (4 h, 1 h, 3 min).
Following application, the plates with the treated epidermis units were incubated for the respective time intervals at room temperature (3 min and 1 h exposure: 23.2 - 24.1 °C; 4 h exposure: 23.2 - 23.8 °C).
Exposure with the test substance was terminated by rinsing the epidermal units with 25 mL of 1 x PBS solution to remove all of the test substance from the epidermal surface. The rest of the PBS was removed from the epidermal surface with a suitable pipette tip linked to a vacuum source (care was taken to avoid damaging to the epidermis). Epidermis units were then incubated with fresh pre-warmed maintenance medium (2 mL/well) at 37 ± 1 °C for a post-incubation period of 42 ± 1 h in an incubator with 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere.
After the incubation, the viability of each disk was assessed by incubating the tissues for 3 h (± 15 min) with 2 mL of MTT solution (0.3 mg/mL) at 37 ± 1 °C in 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere and protected from light. For the additional colour control (NSC_{living}), wells were filled up with 2 mL assay medium.
The resulting formazan crystals were extracted from punched epidermis disks with 500 µL acidified isopropanol overnight and quantified by means of the OD recorded spectrophotometrically at the wavelength of 570 nm. Acidified isopropanol was used as the blank.

3. Calculation of viability

Blank

The mean of 6 blank OD values was calculated.

Test substance:

Individual test substance OD values were corrected with the mean blank OD:

$$OD\ Treated\ Tissue\ (OD_{TT}) = OD_{TTraw} - OD_{blank\ mean}$$

The corrected mean OD of the 2 test substance values was calculated.

The % viability for each test substance replicate was calculated relative to the mean negative control:

$$\% \text{ Treated Tissue 1} = (OD_{TT1} / \text{mean } OD_{NC}) \times 100$$
$$\% \text{ Treated Tissue 2} = (OD_{TT2} / \text{mean } OD_{NC}) \times 100$$

The mean value of the 2 individual viability % for test substance was calculated:

$$\text{Mean TT \%} = (\%TT1 + \%TT2) / 2$$

For test substances detected as able to stain the tissues the non-specific OD was evaluated due to the residual chemical colour (unrelated to mitochondrial activity) and subtracted before calculation of the "true" viability %

Non-Specific Colour % (NSC_{living} %):

$$\text{Tissue 1 } NSC_{living1} \% = (\text{mean } OD_{CT} / \text{mean } OD_{NC}) \times 100$$
$$\text{Tissue 2 } NSC_{living2} \% = (\text{mean } OD_{CT} / \text{mean } OD_{NC}) \times 100$$
$$\text{Mean } NSC_{living} \% = (NSC_{living1} \% + NSC_{living2} \%) / 2$$

OD_{CT}: test substance treated tissues (not incubated with MTT)
OD_{NC}: negative control OD (incubated with MTT)

True MTT metabolic conversion (TOD_{TT}) was undertaken if NSC_{living} % is > 5 % and ≤ 50 %.

$$TOD_{TT} = [OD_{TV} - \text{mean } OD_{CT}]$$

OD_{TV}: test substance treated tissues (incubated with MTT)
OD_{CT}: test substance treated tissues (not incubated with MTT)

The % relative viability (% RV) for each test substance replicate was calculated relative to the mean negative control:

$$\% \text{ RV 1} = [TOD_{TT1} / \text{mean } OD_{NC}] \times 100$$
$$\% \text{ RV 2} = [TOD_{TT2} / \text{mean } OD_{NC}] \times 100$$

The mean value of the 2 individual relative viability % for test substance was calculated:

$$\text{Mean Relative Viability \%} = (\% \text{ RV 1} + \% \text{ RV 2}) / 2$$

If NSC_{living} % is ≤ 5 % then the normal calculation mode was used. If NSC_{living} % is > 50 % relative to the negative control, additional steps must be undertaken if possible, or the test substance was considered as incompatible with the test.

Negative control (for each exposure time):

Individual negative control OD values were corrected with the mean blank OD:

$$OD\ Negative\ Control\ (OD_{NC1}) = OD_{NCraw1} - OD_{blank\ mean1}$$
$$OD\ Negative\ Control\ (OD_{NC2}) = OD_{NCraw2} - OD_{blank\ mean2}$$

The corrected mean OD of the 2 negative control values was calculated: this corresponds to 100 % viability.

$$Mean\ OD\ Negative\ Control\ (mean\ OD_{NC}) = [(OD_{NC1}) + (OD_{NC2})] / 2$$

Positive control (for the 4 h exposure time):

Individual positive control OD values were corrected with the mean blank OD:

$$OD\ Positive\ Control\ (OD_{PC}) = OD_{PCraw} - OD_{blank\ mean}$$

The corrected mean OD of the 2 positive control values was calculated.
The % viability for each positive control replicate was calculated relative to the mean negative control:

$$\%\ Positive\ Control\ 1 = (OD_{PC1} / mean\ OD_{NC}) \times 100$$
$$\%\ Positive\ Control\ 2 = (OD_{PC2} / mean\ OD_{NC}) \times 100$$

The mean value of the 2 individual viability % for positive control was calculated:

$$Mean\ PC\ \% = (\%PC1 + \%PC2) / 2$$

4. Acceptance criteria

The mean OD value of the 2 negative control tissues should be between 0.6 and 1.5.
The acceptable mean percentage viability for positive controls (mean of the two tissues) is 0 – 20 %.
In the range 20 – 100 % viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not exceed 30 %. For details on laboratory test method validation please refer to KCP 7.1.4/03, Doc. No. 565-003.

5. Evaluation of results

The prediction model below corresponds to the methods agreed by EU regulatory agencies in line with the OECD Test Guideline No. 431. The cut-off value of 35 % and classification method was validated in an international validation.

The criteria for *in vitro* interpretation are described below:

Criteria for *in vitro* interpretation	**Classification categories***
Mean tissue viability is < 35 % after 3 min exposure	Corrosive: Optional Sub- category 1A
Mean tissue viability is ≥ 35 % after 3 min exposure and < 35 % after 1 hour exposure OR Mean tissue viability is ≥ 35 % after 1 hour exposure and < 35 % after 4 hours exposure	Corrosive: A combination of optional Sub-categories 1B-and-1C
Mean tissue viability is ≥ 35 % after 4 hours exposure	Non corrosive

*: UN Globally Harmonized System of Classification and Labelling of Chemicals (GHS)

II. RESULTS AND DISCUSSION

1. Validity of the test

The mean OD value of the 2 negative control tissues was in the range of 0.6 - 1.5 at each exposure time (0.9, 1.4 and 1.2 at 4 h, 1 h and 3 min exposure respectively). The positive control result showed 3 % viability (% viability of each of two tissues was in the range of 0 – 20 %). In the range 20-100 % viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates did not exceed 30 % in any case (were in the range of 0 – 13 %). All validity criteria were within acceptable limits and therefore the study was considered to be valid.

2. Indicator for potential false viability

The test substance did not interact with the MTT, therefore additional controls and data calculations were not necessary. A false estimation of viability can be excluded.
Two (2) additional test substance-treated tissues were used for each exposure time (4 h, 1 h and 3 min) for the non-specific OD evaluation. The Non Specific Colour$_{living}$ % (NSC$_{living}$ %) was calculated as 0 %, 1 % and 2 % at 4 h, 1 h and 3 min exposure, respectively and presented in Table A 1. Therefore, additional data calculation was not necessary. A false estimation of viability was precluded.

Table A 1: Results of OD values and NSC$_{living}$ % of additional control

Additional colour control	Exposure time	OD		Non Specific Colour % (NSC$_{living}$ %)
GWN-10616[A]	4 h	Replicate 1	0.001	0
		Replicate 2	-0.002	
		Mean	-0.001	
	1 h	Replicate 1	0.013	1
		Replicate 2	0.012	
		Mean	0.012	
	3 min	Replicate 1	0.030	2
		Replicate 2	0.023	
		Mean	0.026	

[A]: Test substance treated tissues without MTT incubation
NSC$_{living}$: Non-Specific Colour in living tissues
OD: Optical density, mean value of the duplicate wells for each sample

3. Cell viability

The viability of the test substance treated tissue was above 35 % of the mean negative control value at all evaluated time points. The average test substance treated tissue relative viabilities were 101 % at 4 h, 107 % at 1 h and 110 % at 3 min of exposure. The results of the OD measured at 570 nm for each replicate and the calculated % viability of the cells are presented in Table A 2.

Table A 2: Results of OD values and viability percentages of the controls

Substance	Exposure time	OD		Viability (%)	Δ%
GWN-10616	4 h	Replicate 1	0.882	101	0
		Replicate 2	0.880	101	
		Mean[1]	0.9	101	
		SD	0.001	0.166	
		CV	0.165	0.165	
	1 h	Replicate 1	1.500	108	2
		Replicate 2	1.479	106	
		Mean[2]	1.5	107	
		SD	0.015	1.074	
		CV	1.006	1.006	
	3 min	Replicate 1	1.345	110	2
		Replicate 2	1.326	109	
		Mean[2]	1.3	110	
		SD	0.013	1.101	
		CV	1.003	1.003	
Negative control (NaCl (9 g/L saline))	4 h	Replicate 1	0.827	95	11
		Replicate 2	0.921	105	
		Mean	0.9	100	
		SD	0.067	7.607	
		CV	7.607	7.607	
	1 h	Replicate 1	1.307	94	13
		Replicate 2	1.484	106	
		Mean	1.4	100	
		SD	0.125	8.973	
		CV	8.973	8.973	
	3 min	Replicate 1	1.217	100	0
		Replicate 2	1.217	100	
		Mean	1.2	100	
		SD	0.000	0.009	
		CV	0.009	0.009	
	HCD				
	-	Mean	0.996	-	
		Min	0.571	-	
		Max	1.614	-	
Positive control (Glacial acetic acid)	4 h	Replicate 1	0.044	5	4
		Replicate 2	0.006	1	
		Mean	0.025	3	
		SD	0.027	3.061	
		CV	105.821	105.821	
	HCD				
	-	Mean	0.023	2	-
		Min	0.002	0	-
		Max	0.084	10	-

Mean blank OD value was 0.0402, used for 3 minutes and 1 hour exposure.
Mean blank OD value was 0.0375, used for 4 hours exposure.
Δ%: The difference of viability between the two relating tissues
CV: coefficient of variation; HCD: Historical control data retrieved between 2013 and 2022 (number of occasions: 75);
Max: maximum, Min: minimum; OD: Optical density, mean value of the duplicate wells for each sample;
SD: Standard deviation

III. CONCLUSION

In conclusion, in this *in vitro* skin corrosion test using the EPISKIN model, the mean tissue viability was > 35 % after 4 hours exposure and thus, according to the current OECD Guideline No. 431, GWN-10616 is considered as non-corrosive.

Comments of zRMS:	The study KCP 7.1.4/02 performed according to internationally recognised guidelines in laboratory with demonstration of proficiency and with GLP conditions is acceptable. Since the tissue viability after exposure and post-treatment incubation was above 50% the product GWN-10616 does not warrant classification as irritant to skin.

A 2.5.2 Study 2

Reference	KCP 7.1.4/02
Report	*In vitro* skin irritation test with GWN-10616 in the EPISKIN Model, Buda, I. (2022), study No. 912-439-6625, Doc. No. 565-002, EFSA Study Identification: EFSA-2022-00007879
Guideline(s)	OECD No. 439 (2021), Commission Regulation (EU) No 2019/1390 (2019), Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006, B.46, EURL ECVAM DB-ALM Protocol n° 131 (2012)
Deviations	No
GLP	Yes
Acceptability	Yes
Duplication (if vertebrate study)	No, not applicable (in *vitro study*)

Executive summary

The purpose of this study was to determine the skin irritation potential of the test substance GWN-10616 (batch no. P2102669001) on reconstituted human epidermis in the EPISKIN model *in vitro*.
Disks of epidermal units (3 units) were treated with the test substance (used as supplied) and incubated for 15 minutes (min) at room temperature. Exposure of the test substance was terminated by rinsing the epidermal units with 1 x phosphate buffered saline (PBS) solution. Epidermis units were then incubated for 42 hours (h), and the viability of each disk was assessed by incubating the tissues for 3 h with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. The resulting formazan crystals were extracted with acidified isopropanol and quantified by means of the optical densities (OD) recorded spectrophotometrically. Sodium dodecyl sulphate (SDS) 5 % aq. and 1 x PBS treated (3 units / positive and negative control) epidermis units were used as positive and negative controls, respectively. For each treated tissue, viability was expressed as a percentage relative to negative control.

Due to the intrinsic color of the test substance (light brown), 2 additional tissues treated with the test substance were used for the non-specific OD evaluation (NSC_{living}).
Positive and negative controls showed the expected OD and cell viability values within acceptable limits. Standard deviation (SD) of all calculated viability values (test substance and controls) was be-

low 18. The mean OD value of the blank samples was below 0.1. The experiment was considered to be valid.

In this *in vitro* skin irritation test using the EPISKIN model, the test substance GWN-10616 did not show significantly reduced cell viability in comparison to the negative control (mean viability: 95 %). All obtained test substance viability results were above 50 % when compared to the viability values obtained from the negative control. Therefore, the test substance was considered to be non-irritant to skin.

According to the current OECD Guideline No. 439, GWN-10616 is considered as non-irritant to skin and is therefore not classified (UN GHS No Category).

I. MATERIALS AND METHODS

A. Materials

1. Test materials

Identification	GWN-10616
Batch	P2102669001
Preparation	The test substance was applied in its original form, no formulation was required.

2. Control materials

Negative control	Phosphate buffered saline (1 x PBS)

Positive control	Sodium dodecyl sulphate (SDS) 5 % aq. solution

3. Additional materials

MTT solution	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved to the concentration of 3 mg/mL in saline buffer (1x PBS) and stored in refrigerator (2-8 °C). The MTT stock solution was diluted with pre-warmed (37 °C) assay medium to a final concentration of 0.3 mg/mL and used within 1 h for MTT incubation.

Acidified Isopropanol	Isopropanol was diluted with 12N HCl acid to a final concentration of 0.04N HCl.

4. Test system

The 3-dimensional human epidermis model EpiSkin^{TM}SM (supplied as 12-well assay plates by EPISKIN Laboratories Lyon, France, including biopsy punch, maintenance and assay medium) was obtained after a 13-day culture period of adult human derived epidermal keratinocytes on collagen matrix and comprised main basal, supra basal, spinous and granular layers and a functional *stratum corneum*.

B. STUDY DESIGN

1. Potential false viability test

Optical properties of the test substance or its chemical action on MTT may interfere with the assay and lead to a false estimate of viability. This may occur when the test substance is not completely removed from the tissue by rinsing or when it penetrates the epidermis. If the test substance acts directly on MTT (MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls are used to detect and correct for test substance interference with the viability measurement.

Check-method for possible direct MTT reduction with test substance
Approximately 10 µL test substance was added to 2 mL MTT 0.3 mg/mL (diluted with assay medium) solution and mixed. The mixture was incubated for 3 h at 37 ± 1 °C in an incubator with 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere, protected from light. Subsequently, any observed colour change of the solution was recorded. The test substance showed no direct interaction with MTT (the solution became yellow). The use of an additional control was not necessary.

Check-method to detect the colouring potential of test substance
The test substance has an intrinsic colour (light brown). Since the test substance is not pale yellow, white, off white, almost white and/or colorless the additional controls were automatically used and based on the optical density (OD) results of additional controls the Non Specific Colour % (NSCliving %) was determined. This step prevents the false viability, which can possibly be caused by the stained surface of the tissues by the test substance.

Additional control(s) for dyes and chemicals able to colour the tissue
Two (2) additional test substance treated tissues were used for the non-specific OD evaluation. These tissues followed the same treatment steps as the other tissues except for the MTT step: MTT incubation was replaced by incubation with fresh assay medium.

2. Assay performance

The maintenance medium was pre-warmed to 37 °C. The appropriate number of assay plate wells were filled with the pre-warmed medium (2 mL per well). The epidermis units were placed above the media in a separately prepared well. Contact of the epidermis units with the media was assured. The well was then incubated overnight (18 - 24 h) at 37 ± 1 °C in an incubator with 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere.
A volume of 10 µL test substance was applied evenly to the epidermal surface of the test skin units. A volume of 10 µL positive control or negative control was applied to the skin surface by spreading gently with the pipette tip in order to cover evenly all the epidermal surface. Three (3) replicates per test item and 3 replicates per controls (negative, positive controls) were used.
In addition to the normal procedure, 2 additional test substance treated tissues (10 µL/skin unit) were used for the non-specific OD evaluation (NSC_{living}).
Following application, the plates with the treated epidermis units were incubated for 15 ± 0.5 minutes at room temperature (22.7 - 23.6 °C).
Exposure with the test substance was terminated by rinsing the epidermal units with 25 mL of1 x PBS solution to remove all of the test substance from the epidermal surface. The rest of the PBS was removed from the epidermal surface with a suitable pipette tip linked to a vacuum source (care was taken to avoid damaging to the epidermis). Epidermis units were then incubated with fresh pre-warmed maintenance medium (2 mL/well) at 37 ± 1 °C for a post-incubation period of 42 ± 1 h in an incubator with 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere.
After the incubation, the viability of each disk was assessed by incubating the tissues for 3 h ± 5 minutes with 2 mL of MTT solution (0.3 mg/mL) at 37 ± 1 °C in 5 ± 1 % CO_2, ≥ 95 % humidified

atmosphere and protected from light. For the additional colour control (NSC_{living}), wells were filled up with 2 mL assay medium.
The resulting formazan crystals were extracted from punched epidermis disks with 500 µL acidified isopropanol for approximately 4 h and quantified by means of the OD recorded spectrophotometrically at the wavelength of 570 nm. Acidified isopropanol was used as the blank.

3. Calculation of viability

Blank

The mean of 6 blank OD values was calculated.

Test substance:

Individual test substance OD values were corrected with the mean blank OD:

$$OD\ Treated\ Tissue\ (OD_{TT}) = OD_{TTraw} - OD_{blank\ mean}$$

The corrected mean OD of the 3 test substance values was calculated.

The % viability for each test substance replicate was calculated relative to the mean negative control:

$$\%\ Treated\ Tissue\ 1 = (OD_{TT1} / mean\ OD_{NC}) \times 100$$
$$\%\ Treated\ Tissue\ 2 = (OD_{TT2} / mean\ OD_{NC}) \times 100$$
$$\%\ Treated\ Tissue\ 3 = (OD_{TT3} / mean\ OD_{NC}) \times 100$$

The mean value of the 3 individual viability % for test substance was calculated:

$$Mean\ TT\ \% = (\%TT1 + \%TT2 + \%TT3) / 3$$

Non-Specific Colour % (NSC_{living} %):

$$NSC_{living}\ \% = (mean\ OD_{CT} / mean\ OD_{NC}) \times 100$$

OD_{CT}: test substance treated tissues (not incubated with MTT)
OD_{NC}: negative control OD (incubated with MTT)

With a NSC_{living} % ≤ 5 % the normal calculation mode was used.

Negative control

Individual negative control OD values were corrected with the mean blank OD:

$$OD\ Negative\ Control\ (OD_{NC}) = OD_{NCraw} - OD_{blank\ mean}$$

The corrected mean OD of the 3 negative control values was calculated: this corresponds to 100 % viability.

Positive control:

Individual positive control OD values were corrected with the mean blank OD:

$$OD\ Positive\ Control\ (OD_{PC}) = OD_{PCraw} - OD_{blank\ mean}$$

The corrected mean OD of the 3 positive control values was calculated.
The % viability for each positive control replicate was calculated relative to the mean negative control:

$$\% \text{ Positive Control 1} = (OD_{PC1} / \text{mean } OD_{NC}) \times 100$$
$$\% \text{ Positive Control 2} = (OD_{PC2} / \text{mean } OD_{NC}) \times 100$$
$$\% \text{ Positive Control 3} = (OD_{PC3} / \text{mean } OD_{NC}) \times 100$$

The mean value of the 3 individual viability % for positive control was calculated:

$$\text{Mean PC \%} = (\%PC1 + \%PC2 + \%PC3) / 3$$

4. Acceptance criteria

The mean OD value of the 3 negative control tissues should be equal or between 0.6 and 1.5 and the SD of the % viability should be ≤ 18.
The acceptable mean percentage viability for positive controls is < 40 % and the SD of the % viability should be ≤ 18.
For test substances, the SD of the % viability should be ≤ 18.
The mean OD value of the blank samples should be < 0.1.

5. Evaluation of results

The test substance is identified as requiring classification and labelling according to UN GHS (Category 2 or Category 1) if the mean relative viability after 15 minutes exposure and 42 hours post incubation is less or equal (≤) to 50 % of the negative control.

The criteria for *in vitro* interpretation are described below:

Criteria for *in vitro* interpretation	**Classification**
Mean tissue viability % is ≤ 50 %	Category 2 or Category 1
Mean tissue viability % is > 50 %	No Category

II. RESULTS AND DISCUSSION

1. Validity of the test

The mean OD value of the 3 negative control tissues was 1.054. The mean OD value obtained for the positive control was 0.069 and this result corresponds to 7 % viability when compared to the results obtained from the negative controls. Each calculated SD for the % viability was below 18. The mean OD value of the blank samples was 0.0347. All validity criteria were within acceptable limits and therefore the study was considered to be valid.

2. Indicator for potential false viability

No colour change was observed after 3 hours of incubation. The test substance did not interact with the MTT, therefore additional controls and data calculations were not necessary. A false estimation of viability can be excluded.
Two (2) additional test substance-treated tissues were used for the non-specific OD evaluation. Mean OD (measured at 570 nm) of these tissues was determined as 0.014. The Non Specific Colour % (NSC_{living} %) was calculated as 1 % (below 5 %). The results are presented in Table A 3. Therefore, additional data calculation was not necessary and a false estimation of viability was precluded.

Table A 3: Results of OD values and NSC_{living} % of additional control

Additional colour control	Optical Density (OD)		Non Specific Colour % (NSC_{living} %)
GWN-10616[A]	1	0.015	**1**
	2	0.013	
	Mean	**0.014**	
	Standard deviation (SD)		0.16

[A]: Test substance treated tissues without MTT incubation
Remark: Mean blank OD value was 0.0347
NSC_{living}: Non-Specific Colour in living tissues
OD: Optical density, mean value of the duplicate wells for each sample
SD: Standard deviation

3. Cell viability

The results of the OD measured at 570 nm of each replicate and the calculated % viability of the cells are presented in Table A 4.

Table A 4: Results of OD values and viability percentages of the controls

Substance	OD		Viability (%)
GWN-10616	Replicate 1	1.004	95
	Replicate 2	1.007	96
	Replicate 3	0.997	95
	Mean	**1.003**	**95**
	SD		0.51
Negative control (1x PBS)	Replicate 1	1.045	99
	Replicate 2	1.068	101
	Replicate 3	1.048	99
	Mean	**1.054**	**100**
	SD		1.15
	HCD		
	Mean	0.9	
	Min	0.6	
	Max	1.4	
Positive control (5 % aq. SDS)	Replicate 1	0.056	5
	Replicate 2	0.079	8
	Replicate 3	0.072	7
	Mean	**0.069**	**7**
	Standard deviation (SD)		1.15
	HCD		
	Mean	0.1	13
	Min	0.0	4
	Max	0.3	37

Remark: Mean blank OD value was 0.0347
HCD: Historical control data (number of occasions: 97); OD: Optical density, mean value of the duplicate wells for each sample
Max: maximum, Min: minimum; PBS: Phosphate buffered saline; SD: Standard deviation; SDS: Sodium dodecyl sulphate

III. CONCLUSION

The results obtained from this *in vitro* skin irritation test, using the EPISKIN model, indicated that the test substance reveals no skin irritation potential under the utilised testing conditions. According to the current OECD Guideline No. 439, GWN-10616 is considered as non-irritant to skin and is therefore not classified (UN GHS No Category).

Overall conclusion on skin irritation

As no human data and no *in vivo* animal data according to OECD TG 404 existed for this product, an *in vitro* skin corrosion test using the EPISKIN model (OECD TG 431) was initially conducted, according to the OECD Guidance 203 on an integrated approach on testing and assessment (IATA) for skin corrosion and irritation (OECD, 2014).
The outcome of this *in vitro* skin corrosion test was that GWN-10616 is considered as non-corrosive to skin. Therefore, a second adopted *in vitro* test method regarding skin irritation potential was conducted. This test method (OECD TG 439), using the EPISKIN model, indicated that the test substance reveals no skin irritation potential. Therefore, GWN-10616 is considered as non-irritant to skin and is not classified (UN GHS No Category) according to the current OECD TG 439.
According to OECD (2014), the test substance was identified of no need for classification. Furthermore, according to these *in vitro* study results and considering animal welfare reasons, an experimental *in vivo* study according to OECD 404 with GWN-10616 is scientifically not justified.

A 2.6 Eye irritation (KCP 7.1.5)

Comments of zRMS:	The study KCP 7.1.5/01 performed according to internationally recognised guidelines, in laboratory with demonstration of proficiency and in GLP conditions is acceptable. The results indicate that the product GWN-10616 does neither meet criteria for Category 1 (causes serious eye damage) nor for no category according to UN GHS classification, thus according to the guideline OECD 438, GWN-10616 is categorized as “No prediction can be made”, and further assessment is required.

A 2.6.1 Study 1

Reference	KCP 7.1.5/01
Report	GWN-10616: *In vitro* Eye Irritation Test in Isolated Chicken Eyes, XXXX, (2022), study No. 912-438-6756, Doc. No. 566-001, EFSA Study Identification: EFSA-2022-00009174
Guideline(s)	OECD 438 (2018), Commission Regulation (EU) No 1152/2010 (2010) amending, Regulation (EC) No 440/2008 (REACH) B.48, Amendment Commission Regulation (EC) No 2017/735 (2017)
Deviations	No
GLP	Yes
Acceptability	Yes
Duplication (if vertebrate study)	No, not applicable (*in vitro* study)

Executive summary

The purpose of this Isolated Chicken Eye Test (ICET) was to determine the potential ocular corrosivity or severe irritancy of the test substance GWN-10616 (batch no. P2102669001) by its ability to induce toxicity in enucleated chicken eyes.
A single dose of the test substance, the positive control (5 % solution of benzalkonium chloride), and the negative control (0.9 % NaCl) were applied in a volume of 30 µL/eye, in such a way that the test and control substances evenly covered the whole cornea surface of each tested eye.
Three (3) test substance treated eyes, 3 positive control treated eyes and 1 negative control eye were used in this study. After an exposure period of 10 seconds from the end of the application, the cornea surface was rinsed thoroughly with approximately 20 mL saline solution at ambient temperature and this procedure was repeated for each eye.

The overall ICE classes of the test item were once II (based on the cornea swelling of 9 % within 240 min) and twice III (based on the corneal opacity score of 1.8 and fluorescein retention of 1.7). The positive control was classified as corrosive/severely irritating (UN GHS Classification: Category 1) and the negative control had no significant effects on the chicken eye in this study. The positive and negative controls showed the expected results and thus the experiment was considered to be valid.

According to the guideline OECD 438, GWN-10616 is categorized as “No prediction can be made”.

I. MATERIALS AND METHODS

A. Materials

1. Test materials

Identification	GWN-10616
Batch	P2102669001
Preparation	The test substance was applied in its original form, no formulation was required.

2. Control materials

Negative control	NaCl (9 g/L saline) solution

Positive control	Benzalkonium chloride solution (5%)

3. Additional materials

Fluorescein	0.1004 g of fluorescein sodium salt was diluted up to 5 mL with NaCl (9 g/L saline). The final concentration of solution was 2 % (w/v). The formulation was prepared and used on the day of treatment.

4. Test system

Chicken eyes (strain ROSS 308) were obtained from the slaughterhouse. Only the eyes of healthy animals considered suitable for entry into the human food chain were used. All eyes used in the assay were from the same groups of eyes collected on one specific day. The age and weight of the chickens used historically in this test method are that of spring chickens traditionally processed by a poultry slaughterhouse (*i.e.* approximately 7 weeks old, 1.5 - 2.5 kg).

B. STUDY DESIGN

1. Selection and preparation of eyes for the test

Cornea integrity was checked by applying one small drop of fluorescein 2 % (w/v) solution onto the cornea surface for a few seconds and subsequently rinsed off with 20 mL isotonic saline. Then the fluorescein-treated cornea was examined with a slit lamp microscope, with the eye in the head, to ensure that the cornea was not damaged. If the cornea was in good condition, the eyeball was carefully removed from the orbit. Once removed from the orbit, the eye was placed onto damp paper and the nictitating membrane was cut away with other connective tissue. The prepared eyes were kept on the wet papers in a closed box so that the appropriate humidity was maintained. The treatment group and concurrent positive control group consisted of 3 eyes. The negative control group consisted of 1 eye. The enucleated eye was placed in a steel clamp with the cornea positioned vertically with the eye in the correct relative position. The clamp with the eyeball was transferred to a chamber of the superfusion apparatus. The clamp holding the eye was positioned in such a way that the entire cornea was supplied with saline solution dripping from a stainless-steel tube, at a rate of approximately 3 to 4 drops/minute in a closed chamber at a temperature of 32 ± 1.5 °C. After being placed in the superfusion apparatus, the eyes were examined again with the slit lamp microscope to ensure that they were in good condition. Eyes with a high baseline fluorescein staining (*i.e.* > 0.5) or a high corneal opacity score (*i.e.* > 0.5) were rejected. If the selected eyes were appropriate for the test, acclimatization started and was conducted for approximately 45 to 60 minutes (min). At the end of the acclimatization period, a zero reference measurement was recorded for cornea thickness and opacity to serve as a baseline (t = 0) for each individual eye. The cornea thickness of the eyes should not change by more than ± 5 - 7 % within approximately 45 to 60 minutes before the start of application. Slight changes in thickness (0 % to 2 %) were observed in the eyes, finding considered as normal when maintaining enucleated eyes. Following the equilibration period, the fluorescein retention was measured. Baseline values were required to evaluate any potential test item related effects after treatment. None of the eyes were discarded as no eye was considered unsuitable after the baseline assessment.

2. Assay performance

After the zero reference measurements, 1 out of 3 test substance treated eyes was held in horizontal position and 30 µL of test substance was applied via a micropipette onto the center of the cornea, taking care not to damage or touch the cornea with the application equipment. This procedure was repeated with the remaining 2 test substance treated eyes. The 3 positive control eyes were treated with (30 µL/eye) Benzalkonium chloride solution (5 %) and 1 negative control eye was treated with 30 µL isotonic saline [NaCl (9 g/L saline)] according to the above procedure.
The time of application was monitored. After an exposure period of 10 seconds (s) the cornea surface was rinsed thoroughly with 20 mL isotonic saline at ambient temperature, taking care not to damage the cornea but attempting to remove the entire residual test substance, if possible. The cornea surface of negative and positive control treated corneas was also rinsed thoroughly after an exposure period of 10 s with 20 mL saline solution at ambient temperature, while taking care not to damage the cornea. The eye in the holder was then returned to its chamber. The time while the eye was out of the chamber was limited to a minimum.

3. Measurements

The control and test substance treated eyes were evaluated pre-treatment and at approximately 30, 75, 120, 180 and 240 min after the post-treatment rinse. Minor variations within ± 5 min were considered acceptable. The cornea thickness was measured, and cornea opacity and possible morphological effects were monitored at all time points. Fluorescein retention was measured on two occasions, at base line (t = 0) and 30 min after the post-treatment rinse. At the end of the procedures, the corneas were

carefully removed from the eyes and placed individually into labelled containers of preservative fluid (4 % (v/v) formaldehyde) for potential histopathology (not performed within this study).

4. Calculation of viability

The endpoints evaluated were corneal opacity, swelling, fluorescein retention, and morphological effects (*e.g.,* pitting or loosening of the epithelium). Results from corneal opacity, swelling, and fluorescein retention were evaluated separately to generate an Isolated Chicken Eye (ICE) class for each endpoint. The ICE classes for each endpoint were then combined to generate an Irritancy Classification for each test substance.

Corneal thickness or swelling
The control eye(s) and test eyes were evaluated pre-treatment and at approximately 30, 75, 120, 180 and 240 min after the post-treatment rinse. Corneal swelling was determined from corneal thickness measurements made with an optical pachymeter on a slit-lamp microscope (slit-width setting: 9½, equal to 0.095 mm). It was expressed as a percentage and is calculated from corneal thickness measurements.
Cornea swelling was calculated according to the following formulae:

$$CS \text{ at time } t = \frac{CT \text{ at time } t - CT \text{ at } t=0}{CT \text{ at } t=0} \times 100$$

$$Mean\ CS \text{ at time } t = \frac{FECS_{(at\ time\ t)} + SECS_{(at\ time\ t)} + TECS_{(at\ time\ t)}}{3}$$

CS: Cornea swelling; CT: Cornea thickness
FECS: First eye cornea swelling; SECS: Second eye cornea swelling; TECS: Third eye cornea swelling
Mean CS: The mean percentage of corneal swelling
at time t = Observation time at 30, 75, 120, 180 or 240 minutes after the post-treatment rinse; at t=0 = Reference value

For the calculation of maximum swelling, small negative numbers for swelling (0 to -5 %) following application are counted as zero. Large negative numbers (> -12 % below control) are probably due to erosion and indicate a severe effect (scored as class IV). Cases of values of -5 % to -12 % are evaluated on a case by case basis but in the absence of other findings do not indicate a severe effect.

Corneal opacity
The control eye(s) and test substance treated eyes were evaluated pre-treatment and at approximately 30, 75, 120, 180 and 240 min after the post-treatment rinse. Corneal opacity was evaluated by using the area of the cornea that was most densely opacified for scoring. Scores were taken at any given timepoint according to Table A 5.

Table A 5: Evaluation scores for corneal opacity

Score	Observation
0	No opacity
0.5	Very faint opacity
1	Scattered or diffuse areas; details of the iris are clearly visible
2	Easily discernible translucent area; details of the iris are slightly obscured
3	Severe corneal opacity; no specific details of the iris are visible; size of the pupil is barely discernible
4	Complete corneal opacity; iris invisible

Cornea opacity was calculated according to the following formulae:

$$\Delta CO \text{ at time } t = CO \text{ at time } t - CO \text{ at } t=0$$

$$Mean\ CO_{(at\ time\ t)} = \frac{FE\Delta CO_{(at\ time\ t)} + SE\Delta CO_{(at\ time\ t)} + TE\Delta CO_{(at\ time\ t)}}{3}$$

CO: Cornea opacity
ΔCO: Difference between cornea opacity and cornea opacity reference value
FEΔCO: Difference between first eye cornea opacity and first eye cornea opacity reference value
SEΔCO: Difference between second eye cornea opacity and second eye cornea opacity reference value
TEΔCO: Difference between third eye cornea opacity and third eye cornea opacity reference value
Mean CO: The mean corneal opacity value
at time t: Observation time at 30, 75, 120, 180 or 240 minutes after the post-treatment rinse; at t=0: Reference value

Fluorescein retention

The fluorescein retention was measured on two occasions: baseline (t = 0) and 30 min after the post-treatment rinse. Scores were taken at any given timepoint according to Table A 6.

Table A 6: Evaluation scores for fluorescein retention

Score	Observation
0	No fluorescein retention
0.5	Very minor single cell staining
1	Single cell staining scattered throughout the treated area of the cornea
2	Focal or confluent dense single cell staining
3	Confluent large areas of the cornea retaining fluorescein

Fluorescein retention was calculated according to the following formulae:

$$\Delta FR \text{ at time } t = FR \text{ at time } t - FR \text{ at } t=0$$

$$Mean\ FR = \frac{FE\Delta FR_{(at\ time\ t)} + SE\Delta FR_{(at\ time\ t)} + TE\Delta FR_{(at\ time\ t)}}{3}$$

FR: Fluorescein retention
ΔFR: Difference between fluorescein retention and fluorescein retention reference value
FEΔFR: Difference between first eye fluorescein retention and first eye fluorescein retention reference value
SEΔFR: Difference between second eye fluorescein retention and second eye fluorescein retention reference value
TEΔFR: Difference between third eye fluorescein retention and third eye fluorescein retention reference value
Mean FR: The mean fluorescein retention value
at time t: Observation time at 30 minute after the post-treatment rinse; at t=0: Reference value

Morphology

Morphological effects which indicate damage, such as loss of epithelium were taken into account in making a classification. Morphological effects include “pitting” of corneal epithelial cells, “loosening” of epithelium, “roughening” of the corneal surface and “sticking” of the test item to the cornea. These findings can vary in severity and may occur simultaneously. The classification of these findings is subjective according to the interpretation of the investigator.

5. ICE and UN GHS classification

The ICE classes for each endpoint were assigned according to Table A 7, Table A 8 and Table A 9.

Table A 7: ICE classification criteria for cornea swelling

Mean corneal swelling (%)*	ICE class
0 to 5	I
>5 to 12	II
>12 to 18 (>75 min after the treatment)	II
12 to 18 (≤75 min after the treatment)	III
>18 to 26	III
>26 to 32 (>75 min after the treatment)	III
>26 to 32 (≤75 min after the treatment)	IV
>32	IV

*Highest mean score observed at any time point

Table A 8: ICE classification criteria for opacity

Maximum mean opacity score*	ICE class
0.0 – 0.5	I
0.6 – 1.5	II
1.6 – 2.5	III
2.6 – 4.0	IV

*Maximum mean score observed at any time point

Table A 9: ICE classification criteria for mean fluorescein retention

Mean fluorescein retention score at 30 minutes post - treatment	ICE class
0.0 – 0.5	I
0.6 – 1.5	II
1.6 – 2.5	III
2.6 – 3.0	IV

The ICE classes for each endpoint were then combined to generate an Irritancy Classification for each test substance, with the purpose of classify the test substance under the UN GHS classification system according to Table A 10.

Table A 10: Overall *in vitro* irritancy classifications

UN GHS classification	Combination of the 3 endpoints
No category	3×I 2×I, 1×II 1×I, 2×II
No prediction can be made	Other combinations
Category 1 (causes serious eye damage)	3×IV 2×IV, 1×III 2×IV, 1×II[1] 2×IV, 1×I[1] Corneal opacity = 3 at 30 min (in at least 2 eyes) Corneal opacity = 4 at any time point (in at least 2 eyes) Severe loosening of the epithelium (in at least 1 eye)

[1]: combination of categories less likely to occur
Note: Small negative numbers of swelling following application are counted as zero (larger negative numbers due to erosion invalidate the swelling evaluation, but indicate severe effect (scored as class IV)).

6. Acceptance criteria

A test is considered acceptable if the concurrent negative or vehicle/solvent controls and the concurrent positive controls give an Irritancy Classification that falls within non irritant and severe irritant/corrosive classes, respectively. For details on laboratory test method validation please refer to KCP 7.1.5/03, Doc. No. 566-003.

II. RESULTS AND DISCUSSION

The mean values of the treated eyes for maximum corneal thickness change, corneal opacity, fluorescein retention and other observation (morphological effect etc.) are given in Table A 11.

Table A 11: Observation and classification of the test substance and controls

Substance	Observation					
GWN-10616		Mean maximum corneal swelling at up to 75 min (%)	Mean maximum corneal swelling at up to 240 min (%)	Mean maximum corneal opacity	Mean fluorescein retention	Overall ICE Class
	Value	9	9	1.8	1.7	1xII, 2xIII
	ICE Class	II	II	III	III	
	Other observations	None				
Positive control (Benzalkonium chloride solution (5 %))	Value	29	33	3.8	2.8	3x IV
	ICE Class	IV	IV	IV	IV	
	Other observations	Loosening of the epithelium was observed in 1 eye at, 180 and 240 minutes after the post-treatment rinse. Cornea opacity score 4 was observed in all positive control treated eyes (three eyes) at 30 minutes after the post-treatment rinse.				
	HCD[1]					
	Value	39	46	4.0	3.0	-
Negative control (0.9% NaCl)	Value	2	2	0.0	0.5	3xI
	ICE Class	I	I	I	I	
	Other observations	None				
	HCD[2]					
	Value	5	5	0.5	0.1	-

HCD: Historical control data; ICE: Isolated Chicken Eye
[1]: Number of eyes = 30; period of 2021
[2]: Number of eyes = 270; period of 2011-2021

Based on the overall ICE Class the negative control NaCl (9 g/L saline) solution had no significant effects on the chicken eye in this study. Positive and negative control values were within the corresponding historical control data ranges.

III. CONCLUSION

The overall ICE classes of the test substance were once II (based on the cornea swelling of 9 % within 240 minutes) and twice III (based on the corneal opacity score of 1.8 and fluorescein retention of 1.7). The positive control was classified as corrosive/severely irritating, UN GHS Classification: Category 1 and the negative control had no significant effects on the chicken eye in this study. In summary, the positive and negative controls showed the expected results. The experiment was considered to be valid.
According to the guideline OECD 438, GWN-10616 is categorized as "No prediction can be made".

Comments of zRMS:	The study KCP 7.1.5/02 performed according to internationally recognised guidelines, in laboratory with demonstration of proficiency and in GLP conditions is acceptable. The mean tissue viability in three-dimensional reconstructed human cornea-like epithelium in the EpiOcular model *in vitro* treated with product GWN-10616 was > 60%, thus the product GWN-10616 according to the guideline OECD 492 does not require classification for eye irritation and making further in vivo test is not necessary.

A 2.6.2 Study 2

Reference	KCP 7.1.5/02
Report	*In vitro* Eye Irritation Test with GWN-10616 in the EpiOcular™ Model, Buda, I. (2022), study No. 912-492-6757, Doc. No. 566-002, EFSA Study ID: EFSA-2022-00009175
Guideline(s)	OECD No. 492 (2019)
Deviations	No
GLP	Yes
Acceptability	Yes
Duplication (if vertebrate study)	No, not applicable (in *vitro study*)

Executive summary

The purpose of this study was to determine the acute ocular irritation potential of the test substance GWN-10616 (batch no. P2102669001) on three-dimensional reconstructed human cornea-like epithelium tissue in the EpiOcular model *in vitro*.
Disks of EpiOcular (3 units) were treated with test substance (used as supplied) and incubated for 30 minutes (min) at standard culture conditions. Exposure of the test substance was terminated by rinsing the disks with $Ca^{++}Mg^{++}$ free Dulbecco's phosphate buffered saline (DPBS) solution. After rinsing, the tissues were incubated for 12 min immersion incubation and then incubated for 120 hours (h) at standard culture conditions with fresh assay medium. The viability of each disk was assessed by incubating the tissues for 3 h with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. The resulting formazan precipitated was then extracted using isopropanol and quantified by means of the optical densities (OD) recorded spectrophotometrically. Sterile deionized water and methyl acetate treated tissues were used as negative and positive controls, respectively. For each treated tissue, viability was expressed as a percentage relative to negative control.

Due to the intrinsic color of the test substance (light brown), 2 additional tissues treated with the test substance were used for the non-specific OD evaluation (NSC_{living}).

The positive and negative controls showed the expected cell viability values within acceptable limits. The test substance did not show a significantly reduced cell viability in comparison to the negative control (71.7 %). All obtained test substance viability results were above 60 % when compared to the viability values obtained from the negative control. The experiment was considered to be valid.

The results obtained from this *in vitro* eye irritation test, using the EpiOcular Model, indicated that the test substance reveals no eye irritation potential under the applied testing conditions. According to the current OECD Guideline No. 492, GWN-10616 is considered non-irritant to the eye (UN GHS No Category).

I. MATERIALS AND METHODS

A. Materials

1. Test materials

Identification	GWN-10616
Batch	P2102669001
Preparation	The test substance was applied in its original form, no formulation was required.

2. Control materials

Negative control	Sterile deionized water

Positive control	Methyl acetate (MA), 99.0%

3. Additional materials

MTT solution	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved to the concentration of 5 mg/ in Ca^{++} and Mg^{++} free Dulbecco's phosphate buffered saline (DPBS).and stored in refrigerator (2-8 °C). The MTT stock solution was diluted with pre-warmed assay medium to a final concentration of 1 mg/mL and used within few minutes for MTT check test and incubation.

4. Test system

The 3-dimensional human cornea model EpiOcular™ was supplied as 6, 12, and 24-well assay plates by MatTek In Vitro Life Science Laboratories Bratislava, Slovakia (including biopsy punch, maintenance and assay medium). The model is composed of stratified human keratinocytes in a three-dimensional structure, consisting of at least three viable layers of cells.

B. STUDY DESIGN

1. Potential false viability test

Optical properties of the test substance or its chemical action on MTT may interfere with the assay and lead to a false estimate of viability. This may occur when the test substance is not completely removed from the tissue by rinsing or when it penetrates the tissue. If the test substance acts directly on MTT (MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls are used to detect and correct for test substance interference with the viability measurement.

Check-method for possible direct MTT reduction with test substance
Approximately 50 µL test substance was added to 1 mL MTT 1 mg/mL (diluted with assay medium) solution and mixed. The mixture was incubated for 3 h at 37 ± 2 °C in an incubator with 5 ± 1 % CO_2, ≥ 95 % humidified atmosphere, protected from light. Subsequently, any observed colour change of the solution was recorded. The test substance showed no direct interaction with MTT (the solution became yellow). The use of an additional control was not necessary.

Check-method to detect the colouring potential of test substance
The test substance has an intrinsic colour (light brown). Since the test substance is not white, off white, almost white and/or colorless the additional controls were automatically used and based on the optical density (OD) results of additional controls the Non Specific Colour % (NSC_{living} %) was determined. This step prevents the false viability, which can possibly be caused by the stained surface of the tissues by the test substance.

Additional control(s) for dyes and chemicals able to colour the tissue
Two (2) additional test substance treated tissues were used for the non-specific OD evaluation. These tissues followed the same treatment steps as the other tissues except for the MTT step: MTT incubation was replaced by incubation with fresh assay medium.

2. Assay performance

Before treatment the tissues were pre-wetted with approximately 20 µL of $Ca^{++}Mg^{++}$Free-DPBS and were incubated at standard culture conditions for 30 ± 2 min.
A volume of 50 µL test substance, negative or positive control solution was applied to the tissue surface by spreading gently with the pipette tip in order to cover evenly all the tissue surface. Two (2) replicates per test substance and 2 replicates per controls (negative, positive controls) were used.
In addition to the normal procedure, 2 additional test substance treated tissues were used for the non-specific OD evaluation (NSC_{living}).
The plates with the treated tissue units were incubated for the exposure time of 30 ± 2 minutes at standard culture conditions (37 ± 2 °C in an incubator with 5 % CO_2, ≥ 95 % humidified atmosphere).
Exposure of the test substance was terminated by repeated rinsing of the disks in beakers containing $Ca^{++}Mg^{++}$ free-DPBS solution to remove all of the test substance from the tissue surface. After rinsing, the tissues were incubated for 12 ± 2 min immersion incubation and then incubated for 120 h ± 15 min at standard culture conditions with 5 mL and 1 mL of fresh assay medium, respectively.
The viability of each disk was assessed by incubating the tissues for 3 h (± 15 min) with 300 µL of MTT solution (1 mg/mL) at standard culture conditions protected from light. For the additional colour control (NSC_{living}), wells were filled up with 300 µL assay medium.
The resulting formazan crystals were extracted from punched epidermis disks with 2 mL isopropanol in each designated well. The plates were sealed with parafilm (between the plate cover and upper edge of the wells) and were stored overnight at 2-8 °C in the dark. Formazan precipitate was quantified by means of the OD recorded spectrophotometrically at the wavelength of 570 nm. Isopropanol was used as the blank.

3. Calculation of viability

Blank

The mean of 8 blank OD values was calculated.

Test substance:

Individual test substance OD values were corrected with the mean blank OD:

$$OD\ Treated\ Tissue\ (OD_{TT}) = OD_{TTraw} - OD_{blank\ mean}$$

The corrected mean OD of the 2 test substance values was calculated.

The % viability for each test substance replicate was calculated relative to the mean negative control:

$$\% \text{ Treated Tissue 1} = (OD_{TT1} / \text{mean } OD_{NC}) \times 100$$
$$\% \text{ Treated Tissue 2} = (OD_{TT2} / \text{mean } OD_{NC}) \times 100$$

The mean value of the 2 individual viability % for test substance was calculated:

$$\text{Mean TT \%} = (\%TT1 + \%TT2) / 2$$

For test substances detected as able to stain the tissues unrelated to mitochondrial activity, the non-specific OD was evaluated and subtracted before calculating the "true" viability (%)

Non-Specific Colour % (NSC_{living} %):

$$\text{Tissue 1 } NSC_{living1} \% = (\text{mean } OD_{CT} / \text{mean } OD_{NC}) \times 100$$
$$\text{Tissue 2 } NSC_{living2} \% = (\text{mean } OD_{CT} / \text{mean } OD_{NC}) \times 100$$
$$\text{Mean } NSC_{living} \% = (NSC_{living1} \% + NSC_{living2} \%) / 2$$

OD_{CT}: test substance treated tissues (not incubated with MTT)
OD_{NC}: negative control OD (incubated with MTT)

Corrected Final Viability (CFV NSC_{living}) is undertaken if NSC_{living} % is ≤ 60%:

$$\text{Tissue 1 } C_{FV1}\ NSC_{living} \% = OD_{TT1}\% - \text{Mean } NSC_{living} \%$$
$$\text{Tissue 2 } C_{FV2}\ NSC_{living} \% = OD_{TT2}\% - \text{Mean } NSC_{living} \%$$
$$\text{Mean } C_{FV}\ NSC_{living} \% = (C_{FV1}\ NSC_{living} \% + C_{FV2}\ NSC_{living} \%) / 2$$

OD_{TT}: test substance treated viable tissues
C_{FV} NSC_{living}: corrected final viability

If NSC % is > 60 % relative to the negative control, additional steps must be undertaken if possible, or the test substance must be considered as incompatible with the test.

Negative control:

Individual negative control OD values were corrected with the mean blank OD:

$$OD \text{ Negative Control } (OD_{NC1}) = OD_{NCraw1} - OD_{blank\ mean1}$$
$$OD \text{ Negative Control } (OD_{NC2}) = OD_{NCraw2} - OD_{blank\ mean2}$$

The corrected mean OD of the 2 negative control values was calculated: this corresponds to 100 % viability.

$$\text{Mean OD Negative Control (mean } OD_{NC}) = [(OD_{NC1}) + (OD_{NC2})] / 2$$

Positive control:

Individual positive control OD values were corrected with the mean blank OD:

$$OD \text{ Positive Control } (OD_{PC}) = OD_{PCraw} - OD_{blank\ mean}$$

The corrected mean OD of the 2 positive control values was calculated.

The % viability for each positive control replicate was calculated relative to the mean negative control:

$$\% \text{ Positive Control 1} = (OD_{PC1} / \text{mean } OD_{NC}) \times 100$$
$$\% \text{ Positive Control 2} = (OD_{PC2} / \text{mean } OD_{NC}) \times 100$$

The mean value of the 2 individual viability % for positive control was calculated:

Mean PC % = (%PC1 + %PC2) / 2

4. Acceptance criteria

The mean OD value of the 2 negative control tissues should be > 0.8 and < 2.8.
The acceptable percentage viability for positive control (mean of two tissues) is: below 50 % of negative control viability.
The difference of viability between the two relating tissues of a single chemical is < 20 % in the same run (for positive and negative control tissues and tissues of single chemicals). This applies also to the killed controls (single chemicals and negative killed control) and the colourant controls which are calculated as percent values related to the viability of the relating negative control.
The mean OD value of the blank samples (isopropanol) should be < 0.1.
For details on laboratory test method validation please refer to KCP 7.1.5/04, Doc. No. 566-004.

5. Evaluation of results

The test substance is identified as requiring classification and labelling according to UN GHS (Category 2 or Category 1) if the mean percent tissue viability after exposure and post-exposure incubation is less than or equal (≤) to 60 % of the negative control.

The criteria for *in vitro* interpretation are described below:

Criteria for *in vitro* interpretation	Classification
Mean tissue viability % is ≤ 60 %	to be classified but no prediction can be made between Category 2 or Category 1
Mean tissue viability % is > 60 %	No Category

II. RESULTS AND DISCUSSION

1. Validity of the test

The mean OD value of the two negative control tissues was 1.892. The positive control result showed 15.1 % viability at 30 min exposure. The difference of viability between two tissue replicates ranged from 0.3 % to 10.3 %. All validity criteria were within acceptable limits in the experiment and therefore the study was considered to be valid.

2. Indicator for potential false viability

No colour change was observed after 3 hours of incubation. The test substance did not interact with the MTT, therefore additional controls and data calculations were not necessary. A false estimation of viability can be excluded.
Two (2) additional test substance-treated tissues were used for the non-specific OD evaluation. Mean OD of these tissues was determined as 0.012. The Non Specific $Colour_{living}$ % (NSC_{living} %) was calculated as 0.6 %. The results are presented in Table A 12. Therefore, additional data calculation was not necessary and a false estimation of viability was precluded.

Table A 12: Results of OD values and NSC_{living} % of additional control

Additional colour control	OD		Non Specific Colour % (NSC_{living} %)	Δ%
GWN-10616	Replicate 1	0.009	0.6	0.3
	Replicate 2	0.014		
	Mean	0.012		-

Mean blank OD values was 0.0338
Δ%: The difference of viability between the two relating tissues
NSC_{living}: Non-Specific Colour in living tissues
OD: Optical density, mean value of the duplicate wells for each sample

3. Cell viability

The results of the OD of each replicate and the calculated % viability of the cells are presented in Table A 13.

Table A 13: Results of OD values and viability percentages of the controls

Substance	OD		Viability (%)	C_{FV} NSC_{living} (%)	Δ%
GWN-10616	Replicate 1	1.328	70.2	69.5	4.3
	Replicate 2	1.410	74.5	73.9	
	Mean	1.369	72.3	71.7	-
Negative control (Sterile deionized water)	Replicate 1	1.795	94.9	-	10.3
	Replicate 2	1.990	105.1	-	
	Mean	1.892	100.0	-	-
	HCD				
	Mean	1.989	-	-	-
	Min	1.482	-	-	-
	Max	2.700	-	-	-
Positive control (Methyl acetate)	Replicate 1	0.235	12.4	-	5.3
	Replicate 2	0.335	17.7	-	
	Mean	0.285	15.1	-	-
	HCD				
	Mean	0.292	15	-	-
	Min	0.033	2	-	-
	Max	0.884	49	-	-

Mean blank OD value was 0.0338
Δ%: The difference of viability between the two relating tissues
HCD: Historical control data retrieved between July 2016 and March 2022 (number of occasions: 18); Max: maximum, Min: minimum; OD: Optical density, mean value of the duplicate wells for each sample

III. CONCLUSION

The results obtained from this *in vitro* eye irritation test, using the EpiOcular Model, indicated that the test substance reveals no eye irritation potential under the applied testing conditions. According to the current OECD Guideline No. 492, GWN-10616 is considered as non-irritant to eye and is therefore not classified (UN GHS No Category).

Overall conclusion on eye irritation

As no human data and no *in vivo* animal data according to OECD TG 405 existed for this product and *in vitro* tests using the EPISKIN model (OECD TG 431 and 439) showed that the test substance GWN-10616 was classified as non-corrosive and non-irritant to skin, an *in vitro* OECD adopted test method (OECD TG 438) on serious eye damage and eye irritation was initially conducted, according to the OECD Guidance 263 on integrated approaches to testing and assessment (IATA) for serious eye damage and eye irritation (OECD, 2019).
The outcome of this *in vitro* isolated chicken eye (ICE) test method was "No prediction can be made". Therefore, a second adopted *in vitro* test method was conducted. This test method (OECD TG 492), using the EpiOcular Model, indicated that the test substance reveals no eye irritation potential. Therefore, GWN-10616 is considered as non-irritant to eye and is not classified (UN GHS No Category) according to the current OECD TG 492.
According to OECD (2019), the test substance was identified of no need for classification. Furthermore, according to these *in vitro* study results and considering animal welfare reasons, an experimental *in vivo* study according to OECD 405 with GWN-10616 is scientifically not justified.

A 2.7 Skin sensitisation (KCP 7.1.6)

Comments of zRMS:	The active substance Zoxamide, having a harmonised classification as Skin Sens. 1; H317, is present in the formulation above a generic concentration limit equal ≥ 1,0 % of components of a mixture classified as skin sensitisers that trigger classification of the mixture as Skin Sens. 1, therefore formulation GWN-10616 should be classified for skin sensitisation Cat 1 (H317). In addition additional ingredient is classified as skin sensitising Cat 1 and present in the formulation above the specific concentration limit (SCL) ≥ 0.05 %), therefore classification of GWN-10616 for skin sensitisation Cat 1 (H317) is warranted.

For prediction of the hazard code for skin sensitisation, each substance is compared with the generic or when available the specific concentration limit: In this case, for Skin Sensitiser Cat 1 generic concentration limit (GCL) ≥ 1.0 %.

Classification categories according to Regulation (EC) 1272/2008:

Classification ingredient	Concentration triggering classification
Skin sensitiser Category 1	≥ 1.0 %
Skin sensitiser Sub-category 1A	≥ 0.1 %
Skin sensitiser Sub-category 1B	≥ 1.0 %

Some substances that are classified as sensitisers may elicit a response, when present in a mixture in quantities below the concentrations established in the table below, in individuals who are already sensitised to the substance or mixture.

Classification ingredient	Concentration triggering classification
Skin sensitiser Category 1	≥ 0.1 %*
Skin sensitiser Sub-category 1A	≥ 0.01 %*
Skin sensitiser Sub-category 1B	≥ 0.1 %*

* This concentration limit for elicitation is used for the application of the special labelling requirements section 2.8 of Annex II to protect already sensitised individuals. A SDS is required for the mixture containing a component at or above this concentration. For sensitising substances with specific concentration limit lower than 0.1 %, the concentration limit for elicitation should be set at one tenth of the specific concentration limit.

The additivity concept does not apply for sensitising compounds; therefore, each component is evaluated separately. As Zoxamide is classified as skin sensitising Cat 1 and present above the generic concentration limit ((GCL) ≥ 1.0 %, skin sensitiser) and one additional ingredient is classified as skin sensitising Cat 1 and present above the specific concentration limit ((SCL) ≥ 0.05 %), classification of GWN-10616 for skin sensitisation Cat 1 (H317) is warranted.

A 2.8 Supplementary studies for combinations of plant protection products (KCP 7.1.7)

Not applicable.

A 2.9 Data on co-formulants (KCP 7.4)

A 2.9.1 Material safety data sheet for each co-formulant

Information regarding material safety data sheets of the co-formulants can be found in the confidential dossier of this submission (Registration Report - Part C).

A 2.9.2 Available toxicological data for each co-formulant

Available toxicological data for each co-formulant can be found in the confidential dossier of this submission (Registration Report - Part C).

A 2.10 Studies on dermal absorption (KCP 7.3)

A 2.10.1 Study 1 – *In vitro* study with GWN-10616 (analyte Zoxamide) in human skin

Dermal absorption, *in vitro* using human skin

Comments of zRMS:	The study performed on formulation GWN-10616 according to relevant OECD method and in GLP conditions is acceptable. The concentrate (60 g Zoxamide/L) and one in-use spray dilution (0.15 g Zoxamide/L) of the product were tested. The final dermal absorption rates were derived according to EFSA Guidance on Dermal Absorption (EFSA Journal 2017;15(6):4873) using for calculation of the dermal absorption of each substance a formula: mean value for a given concentrate or dilution + ks, where k is multiplication factor and s is the sample standard deviation. Since permeation (in vitro) was essentially not complete at the end of the study, all tape stripped skin material was included in the calculation of the absorbable dose fraction. Thus, the dermal penetration estimates to be used for risk assessment due to exposure to Zoxamide is 0.55% for the concentrated formulation (60 g Zoxamide/L) and 14 % for the spray dilution (0.15 g Zoxamide/L) based on the EFSA guidance criteria.

Reference	KCP 7.3/01
Report	The *In Vitro* Percutaneous Absorption of Radiolabelled GWN-8030 in a Concentrate Formulation and One In-Use Dilution Through Human Split-Thickness Skin, Finlayson, Z., 2022, study No. 787018, Doc. No. 511-001
Guideline(s)	OECD No. 428 (2004), OECD No. 28 (2004), EC - SANCO/222/2000/Rev.7 (2004), EFSA Journal, 2017; 15(6); 4873
Deviations	No
GLP	Yes
Acceptability	Yes
Duplication (if vertebrate study)	No, not applicable (*in vitro* study)

Executive summary

The aim of this study was to investigate the rate and extent of the *in vitro* dermal absorption of Zoxamide in the suspension concentrate (SC) formulation GWN-10616, following topical application of the test substance GWN-10616 (including radioactively labelled [^{14}C]-Zoxamide) to the surface of human split-thickness skin membranes mounted into a static diffusion cell. The concentrate (60 g Zoxamide/L) and one in-use spray dilution (0.15 g Zoxamide/L) of the product were tested.
For each tested concentration, 8 diffusion cells were used. A total of 16 cells were used for this study, equipped with skin from 4 human donors (2 skin membranes per donor each). Cells dosed with the formulation concentrate were left open to the atmosphere and cells dosed with the spray dilution were occluded. Test substance stability during dosing was confirmed by high performance liquid chromatography (HPLC).
The study duration was 24 h, the exposure time was 8 h and the post exposure time was 16 h. The receptor fluid was collected at 1, 2, 4, 8, 12 and 24 h post dose. The 8 h exposure period was terminated by washing the skin surface with hand wash soap followed by rinsing with a dilute soap solution and drying the surface with tissue swabs. At 24 h post dose, the skin was removed from the static cells, the *stratum corneum* tape stripped, and the skin divided into exposed and unexposed skin. The skin samples were dissolved with tissue solubilizer. The bulk receptor fluid was collected from the receptor chamber and the receptor chambers were rinsed with solvent and the samples retained for analysis. The radioactivity of all samples was measured with a liquid scintillation counter.

Results were evaluated according to the latest Guidance on Dermal Absorption (EFSA Journal 2017; 15(6): 4873) by applying the template calculator provided by EFSA as supportive information (Version 3, EFSA publication date 20 August 2018; KCA 7.3/03, Doc. No. 511-003). The mean total recovery of the radioactivity in concentrated test substance GWN-10616 was 96.88 %. Radiolabelled Zoxamide penetrates human skin in concentrated GWN-10616 with an absorbed fraction of 0.35 ± 0.23 % of the applied dose, resulting in a dermal absorption of 0.55 %.
For the spray dilution, the mean total recovery of the radioactivity was 122.04 %. The absorbed fraction of GWN-10616 in the spray dilution was 10.17 ±4.21 % of the applied dose, resulting in a dermal absorption of radiolabelled Zoxamide of 14 % within 24 hours, considering the named EFSA guidance criteria.

I. MATERIALS AND METHODS

A. Materials

1. Test materials

Non-radiolabelled test substance	
Test substance name	Zoxamide Technical (GWN-8030 technical)
Batch number	2018101001
Chemical purity	97.5 %, dose calculations corrected for purity

Non-radiolabelled active substance (analytical standard)	
Test substance name	Zoxamide
Batch number	BCBZ7753
Chemical purity	99.6 %

Radiolabelled test substance	
Test substance name	[^{14}C]-Zoxamide
Batch number	233575_Zoxamide_BX1_June21*
Specific activity	48.15 mCi/mmol
Radiochemical purity	99.7 %

* Repurified batch, the original batch number from the supplier was 11562SXG002-1. The original batch was repurified due to an unacceptable radioactive purity value of 86.8 %. The repurified batch of [^{14}C]-Zoxamide was used to conduct the study.

Blank formulation	
Test substance name	Blank formulation GWN-10616
Concentration	0 g Zoxamide / no Zoxamide
Batch number	B 2006669001

Co-active substance	
Test substance name	Potassium Phosphonate Technical
Batch number	2006648002
Chemical purity	Not reported

2. Test system

Human skin samples were obtained from abdominal surgery on 4 donors; 1 male and 3 female donors aged 35 to 55 years old.

B. STUDY DESIGN

1. Solubility assessment

[^{14}C]-Zoxamide (0.93 mg) and Zoxamide technical (59.63 mg) were added to the volumetric flask, filled up to 1 mL with acetone and mixed by vortex until homogenous. Aliquots were taken, mixed with methanol: scintillation fluid and analysed by liquid scintillation counting (LSC).
The concentration of [^{14}C]-Zoxamide in the solvent was determined to be 59.0 mg/mL. [^{14}C]-Zoxamide was homogeneously distributed in the solution with a CV of 1.84 %. The specific activity of [^{14}C]-Zoxamide was calculated to be 2.10 µCi/mg.
This [^{14}C]-Zoxamide solution (325 µL) was filled up to 25 mL with either receptor fluid or acetone (positive control) and mixed. After incubated in a water bath at 32.34°C for 1 h whilst mixing, the samples were centrifuged at *ca.* 2000 g for *ca.* 5 min. Aliquots (1 mL) of the resultant supernatant were mixed with scintillation fluid and analysed by LSC.

2. Test substance preparation

Test Preparation 1: [^{14}C]-Zoxamide in formulation concentrate (60 g/L)

Potassium phosphonate technical (750 µL) was dried under nitrogen gas, [^{14}C]-Zoxamide (950 µL, specific activity: 3.62 µCi/mg) was added to the tube and the solvent was removed under nitrogen gas. Dried potassium phosphonate technical (509.90 mg) and blank formulation (70.5 µL) was added to the tube. The contents were mixed, and ultrapure water (379.45 mg) was added and mixed.
Six (6) aliquots (6.4 µL) were collected, mixed with methanol: scintillation fluid and analysed by LSC.
Test Preparation 2: Highest concentration [^{14}C]-Zoxamide in use spray dilution (0.15 g/L)
[^{14}C]-Zoxamide in Test Preparation 1 (5.2 µL) and ultrapure water (2 mL) was mixed by vortexing. Four (4) aliquots were taken, mixed with methanol: scintillation fluid and analysed by LSC.

3. Preparation of skin membranes

Upon thawing, the skin samples were cut at a targeted thickness between 0.36 and 0.4 mm using a dermatome.

4. Static diffusion cells, receptor fluid and integrity of skin membranes

A static diffusion cell system was used. The glass static diffusion cells were placed in a manifold on a magnetic stirrer plate heated via a circulating water bath to maintain the skin surface temperature at 32 ± 1°C. The surface area of exposed skin within the cells was 0.64 cm^2 and the receptor chamber volume was nominally 5 mL. The receptor chambers were placed in a manifold and connected to a circulating water bath. Magnetic stirrer bars were placed in the receptor fluid chambers which were filled with receptor fluid. Sections of split-thickness skin (*ca.* 1.5 x 1.5 cm) were cut and mounted in the diffusion cells between the donor and receptor chamber. The donor chamber was tightened into place with a clamp.
Phosphate buffered saline (PBS) containing polyoxyethylene 20 oleyl ether (PEG, *ca.* 6 %, w/v), sodium azide (*ca.* 0.01 %, w/v), streptomycin (0.1 mg/mL) and penicillin (100 units/mL) was selected as receptor fluid. The pH of the receptor fluid was checked and adjusted to pH 7.40-7.44.

Barrier integrity was assessed by measuring the electrical resistance. Any skin sample exhibiting a resistance less than 7.7 kΩ was excluded from subsequent absorption measurements.

5. Experimental design

Application of the test substance preparations
[^{14}C]-Zoxamide in Test Preparation 1 was applied evenly over the surface of 8 split-thickness samples of human skin using a positive displacement pipette set to deliver *ca.* 6.4 µL (10 µL/cm^2). The donor chambers of the cells were not occluded. Seven (7) aliquots of Test Preparation 1 were dispensed into vials at the time of dosing, mixed with methanol: scintillation fluid and analysed by LSC.
[^{14}C]-Zoxamide in Test Preparation 2 was dosed as described above with the exception that the donor chambers of the cells were occluded with traps containing carbon filters.
The results of the aliquots are presented in Table A 14:

Table A 14: Results of test substance concentration in preparations

Test preparation	Target test substance concentration [g/L]	Test substance concentration	
		Mean [g/L]	CV [%]
[^{14}C]-Zoxamide in Test Preparation 1	60	62.6	0.51
[^{14}C]-Zoxamide in Test Preparation 2	0.15	0.129	2.03

Test Preparation 1: concentrate formulation; Test Preparation 2: spray dilution formulation

Test substance stability

Immediately after dosing, an aliquot was removed from each of the test preparations and the radiochemical purity of [^{14}C]-Zoxamide was determined using the HPLC method for all samples.

Receptor fluid sampling
Receptor fluid aliquots were collected at 1, 2, 4, 8 and 12 h post dose. All receptor fluid samples were mixed with methanol: scintillation fluid (10 mL for pre-dose – 4 h samples) and analysed by LSC.

Termination of exposure
The exposure period was terminated at 8 h post dose. For cells dosed with [^{14}C]-Zoxamide in Test Preparation 2, carbon filters were removed from the occlusive traps and retained in a vial. Commercial hand wash soap (*ca.* 50 µL) was applied to the skin and the soap gently rubbed onto the skin with a tissue swab. The skin was then rinsed with *ca.* 5 mL of a *ca.* 2 % (v/v) soap solution. The soap solution was applied in aliquots (0.5 mL) and the skin was dried with a tissue swab. The process was repeated, and the skin was dried with an additional tissue swab. Carbon filters were added to the occlusive traps and sealed.
The soap solution (skin wash) was pooled into a single vial for each cell. Each sample was split into 4 vials and scintillation fluid added to each vial. The tissue swabs were retained separately to assess the washing efficiency. The pipette tip was cut in half and retained. Methanol: scintillation fluid was added to tissue swabs and pipette tips (tissue swabs were sonicated for *ca.* 10 min). Acetone (15 mL) was added to the filter samples, sonicated for 10 min and mixed by vortex for *ca.* 30 s. The acetone was transferred to a pre-weighed pot and the process was repeated a further 3 times. Duplicate weighed aliquots (1 mL) were collected, mixed with scintillation fluid and analysed by LSC.

Termination of post exposure
After a 16 h monitoring period, *i.e.* at 24 h post dose, the donor chambers were transferred into a pot containing acetone (15 mL). For cells dosed with [^{14}C]-Zoxamide in Test Preparation 2 the donor chambers and traps were transferred into a pre-weighed pot containing acetone (40 mL). Filters were collected and analysed as described in the section "*Termination of exposure*".
Equipment was extracted in solvent for >30 min, before sonication for *ca.* 10 min. The equipment was removed from each pot, solvent samples were split and mixed with scintillation fluid. For cells dosed with [^{14}C]-Zoxamide in Test Preparation 2 the donor chambers and trap solvent samples, duplicate weighed aliquots (1 mL) were collected and mixed with scintillation fluid. The skin was removed from each cell and placed on a piece of tissue to remove any remaining receptor fluid from the underside of the skin. This tissue was placed into the receptor chamber wash pot for that particular cell.
The *stratum corneum* was removed with 20 successive tape strips using D-Squame tape discs. The skin sample was rotated 90° after each tape strip until the epidermis/dermis junction became fragile or if epidermis was removed. Each tape strip was placed into an individual vial containing methanol: scintillation fluid. The skin under the cell flange (unexposed skin) was cut away from the exposed skin. The exposed and unexposed skin samples were placed into separate vials containing Solvable™ (2 mL). The skin samples were placed into a water bath set to *ca.* 60°C to aid solubilisation. Stannous chloride (0.5 mL) and scintillation fluid was added to the skin samples.
The bulk receptor fluid was removed from each receptor chamber and retained in a vial. The samples were split and mixed with scintillation fluid.
The receptor chambers were rinsed with acetone (20 mL) and the solvent was pooled as a single sample. Receptor wash samples were then split into aliquots and mixed with scintillation fluid.
All samples were analysed by LSC.

6. Analysis of radioactivity

All samples were counted together with representative blanks using a liquid scintillation analyser with automatic quench correction by external standard. Where scintillation fluid was added to the samples, this was 10 mL. Where methanol: scintillation fluid was added, this was 12 mL.

Representative blank sample values were subtracted from sample count rates to give net disintegrations per minute (dpm) per sample. Prior to analysis, samples were allowed to stabilise with regard to light and temperature. Preliminary samples to investigate concentration and homogeneity in the dosing formulations were counted for 1 min.

Limit of reliable measurement
A limit of reliable measurement of 30 dpm above background has been established. Counts that are below 30 dpm above background represent a true value. This means that data are recorded with values that are less than the limit of reliable measurement.

7. Calculations – Distribution of radiolabelled test substance

The calculations were performed as follows:

$$\text{Sample amount [µg equiv./cm}^2\text{]} = \frac{\text{sample radioactivity [dpm]}}{\text{specific activity [dpm/µg equiv.] x exposure area [cm}^2\text{]}}$$

$$\text{Sample applied dose [\%]} = \frac{\text{sample radioactivity [dpm] x 100\%}}{\text{Applied dose [dpm]}}$$

dpm: disintegrations per minute, equiv.: equivalent

C. STATISTICS

Statistical analysis was limited to the calculation of means, standard deviations (SD) and coefficient of variation (CV).

II. RESULTS AND DISCUSSION

1. Solubility assessment

The results of the solubility assessment are presented in Table A 15.

Table A 15: Results of solubility assessment

Sample type	Concentration of Zoxamide in solution [mg/L]	% of Target Zoxamide Concentration	CV (%)
Receptor fluid	203	26.48	0.40
Acetone	792	103.13	0.08

The target concentration (768 mg/L) represented 10 times the maximum possible concentration (76.8 mg/L) of Zoxamide in the receptor fluid based on the entire applied dose being absorbed. As 26.48% of the target concentration (*i.e. ca*. 2.5 times the maximum possible concentration) was accepted into the receptor fluid, the receptor fluid was deemed not to be rate limiting to absorption.

2. Barrier integrity of skin membranes

For 2 cells the electrical resistance was < 7.7 kΩ (*i.e.* the acceptance criterion was not met), the skin was replaced with a new piece and the electrical resistance re-measured. Thereafter, the electrical resistance values of all skin membranes met the acceptance criterion (range: 10.4 – 17.03 kΩ).

3. Radiochemical stability of the formulations

The radiochemical purity of the concentrated formulation ([^{14}C]-Zoxamide in Test Preparation 1) was 99.4 % after dosing.
The radiochemical purity of the diluted formulation ([^{14}C]-Zoxamide in Test Preparation 2) was 100 % after dosing.
The results of the radiochemical purity assessment confirmed that the test substance was stable over the dosing period in all test preparations.

4. Dermal absorption of the test substance preparations

Test Preparation 1: [^{14}C]-Zoxamide in formulation concentrate (60 g/L)
The absorption profiles looked similar for all samples, with absorption from 1-12 h below the limit of reliable measurement for all samples. The mass balance for all individual samples was within 100 ± 10%. The following results are provided as mean values (n = 8).
The mean mass balance was 96.88% of the applied dose at 24 h post dose. The majority of the applied dose was washed off at 8 h post application (9.59 %, 86.36 % and 0.01 % recovered in the skin wash, tissue swab and pipette tips, respectively). At 24 h post dose, a further 0.33% was recovered in the donor chamber wash. Therefore, the total dislodgeable dose was 96.28 % of the applied dose. The mean total unabsorbed dose was 96.79 % of the applied dose. This consisted of the dislodgeable dose, unexposed skin (< 0.01 %) and the radioactivity associated with the *stratum corneum* (0.51 %). The absorbed dose (0.01 %) was the sum of the receptor fluid (< 0.01 %) and receptor chamber wash (<0.01 %). The exposed skin contained 0.09 % of the applied dose. The mean maximum flux was 2.07 ± 2.99 ng equiv./cm^2 between 0 and 2 h post dose.
The potentially absorbable dose was calculated as less than 75 % of the absorption occurred within the first half of the experiment, therefore absorption was deemed to be incomplete, as defined in the EFSA – Guidance on Dermal Absorption (2017). The potentially absorbable dose was 0.36 %.
The mass balance (total recovery), total dislodgeable dose, total unabsorbed dose, total absorbed dose, dermal delivery and potentially absorbable dose were 606, 602, 606, 0.04, 0.55 and 2.22 µg equiv./cm^2, respectively (no data table presented).

Test Preparation 2: Highest concentration [^{14}C]-Zoxamide in use spray dilution (0.15 g/L)
The absorption profiles looked similar for all samples, with absorption from 1-12 h below the limit of reliable measurement for all samples. The mass balance for all individual samples was above 110%. The receptor fluid profiles, and the risk assessment values were comparable across the cells. The majority of the mass balance was also recovered in the unabsorbed dose. The high mass balance has been accepted, with the justification that it provided the worst-case scenario risk assessment. Cell 12 and Cell 15 had high values for unexposed skin, suggesting the test preparation had leaked from the exposed area to the unexposed area. However, values for receptor fluid and all risk assessment values were consistent between cells. Therefore, the following results are provided as mean values (n = 8).
The mean mass balance was 122.03 % of the applied dose at 24 h post dose. The majority of the applied dose was washed off at 8 h post application (23.81 %, 70.00 %, 0.03 % and 1.87 % recovered in the skin wash, tissue swab, pipette tips, and filters, respectively). At 24 h post dose a further 9.30 % was recovered with the donor chamber wash + trap sample and 1.33 % was recovered in the filters. Therefore, the total dislodgeable dose was 106.33 % of the applied dose. The mean total unabsorbed dose was 115.29 % of the applied dose. This consisted of the dislodgeable dose, unexposed skin (3.63 %) and the radioactivity associated with the *stratum corneum* (5.32 %). The absorbed dose (4.50 %) was the sum of the receptor fluid (2.08 %) and receptor chamber wash (2.43 %). The exposed skin contained 2.24 % of the applied dose. Dermal delivery (6.75 %) was the sum of the absorbed dose and the exposed skin. The mean maximum flux was 3.10 ± 3.31 ng equiv./cm^2 between 0 and 2 h post dose.

The potentially absorbable dose was calculated as less than 75 % of the absorption occurred within the first half of the experiment, therefore absorption was deemed to be incomplete, as defined in the EFSA Guidance on Dermal Absorption (2017). The potentially absorbable dose was 10.17 %.

The mass balance, total dislodgeable dose, total unabsorbed dose, total absorbed dose, dermal delivery and potentially absorbable dose were 1577, 1374, 1490, 58.2, 87.2 and 131 ng equiv./cm^2, respectively (no data table presented).

Post dose washing efficiency (8 h)
Overall, most of the applied dose recovered in tissue swabs 1-5 was removed with tissue swab 1. Thereafter, there was a general decrease in the percentage of the applied dose removed with tissue swabs 2-5. In the final tissue swab, a mean of 0.04 % (applied dose) for Test Preparation 1 and 0.55 % (applied dose) for Test Preparation 2 was detected, indicating that the washing process was efficient at removing the applied dose.

Table A 16: *In vitro* dermal penetration of Zoxamide formulated as GWN-10616 through human skin - Recovery data

Dose group	**High dose (Formulation concentrate) Test Preparation 1**		**Low dose (Spray dilution 1:400) Test Preparation 2**	
Target concentration [g/L]	60		0.15	
Actual concentration by radioactivity [g/L]	62.6		0.129	
Mean actual applied dose [µg/cm²]	626		1.29	
	Recovery [%]		Recovery [%]	
	Mean	S.D.	Mean	S.D.
Dislodgeable dose				
Skin washing after 8 h	9.59	3.71	23.81	8.86
Tissue swab after 8 h	86.36	4.19	70.00	11.99
Pipette tip 8 h	0.01	0.01	0.03	0.04
Dislodgeable dose 8 h	95.96	1.11	95.71	14.62
Donor chamber wash (+ Trap filters 24 h for spray dilution)	0.33	0.55	10.63	9.98
Total dislodgeable dose	96.28	1.21	106.33	20.89
Dose associated to skin				
Tape strips: 1st sample, strips 1 + 2	0.24	0.22	1.90	1.24
Tape strips: 2nd sample; strips 3 – n	0.27	0.20	3.42	1.76
Skin preparation	0.08	0.07	2.24	1.52
Absorbed dose				
Receptor fluid	0.00	0.00	2.08	0.98
Receptor chamber wash	0.00	0.00	2.43	1.33
Total recovery[1]	96.88	1.43	122.03	17.52
%Absorption at $t_{0.5}$ [2]	62.88 ± 51.24		38.35 ± 21.10	
LLC of $t_{0.5}$ absorption[2]	19.84 ± 43.04		20.62 ± 17.73	
Absorption essentially complete at end of study (>75% absorption within half the study duration)	No		No	
If no: Absorption estimates = absorbed dose + skin preparation + tape strips sample 2)[3]	0.36	0.23	10.2	4.22
If yes: Absorption estimates = absorbed dose + skin preparation	na	na	na	na
Absorption estimate normalised[4]	0.36	0.23	10.2	4.22

Relevant absorption estimate[5]	0.55	13.708
Absorption estimates used for risk assessment[6]	**0.55**	**14**

1 Values may not calculate exactly due to rounding of figures

2 Calculation of dermal absorption by the applicant according to EFSA Journal 2017; 15(6): 4873 BfR calculator, Version 3; KCA 7.3/03, Doc. No. 511-003) was performed. The dermal absorption (lower limit of the 95% confidence (LLC) interval) was calculated as follows: Absorption (mean value) + k × SD, where SD is the sample standard deviation and k is the multiplication factor for variability of results of 0.84 for 8 replicates.

2 In accordance with the EFSA Journal 2017; 15(6): 4873 the radioactivity in the second tape-strip pool (3rd to nth tape strip) is considered potentially absorbable if less than 75% of the absorption occurred in the first half of the study (see Table 7.6.2-1). The skin preparation is also considered potentially absorbable.

3 According to EFSA Journal 2017; 15(6): 4873, cells with insufficient recovery (< 95%) can be corrected by normalisation of absorption estimate to 100% recovery.

4. In accordance with EFSA Journal 2017; 15(6): 4873, one standard deviation was added to the mean% dermal absorption in cases where the standard deviation was ≥ 25% of the mean value.

5 Relevant absorption estimates were rounded to the required number of significant figures.

na: not applicable; S.D.: standard deviation

Calculation of dermal absorption by the applicant according to EFSA Journal 2017; 15(6): 4873 (BfR calculator, Version 3; KCA 7.3/03, Doc. No. 511-003) was performed taking into account a multiplication factor k for variability of results of 0.84 for 8 replicates (see Figure A.1). The dermal absorption was calculated as follows:

Absorption (mean value) + k × SD, where SD is the sample standard deviation.

Figure A.1 BfR calculation sheet (EFSA, 2017) - Results of the *in vitro* penetration of [^{14}C]Zoxamide through human skin

	Concentrate		Dilution 1 (1:400)	
Target concentration [mg/mL]	60		0.15	
Target dose [µg/cm^2]	600		1.50	
Mean actual applied dose [µg/cm^2]	626		1.29	
Recovery [%]	Mean	SD	Mean	SD
Dislodgeable dose				
Skin wash after 8 hours	95.96	1.11	99.34	10.28
Donor chamber wash	0.33	0.55	10.63	9.98
Skin associated dose				
Tape strips 1-2	0.24	0.22	1.90	1.23
Tape strips 3-x	0.27	0.20	3.43	1.75
Skin preparation	0.09	0.07	2.24	1.52
Absorbed dose				
Receptor fluid	0.01	0.00	2.08	0.98
Receptor chamber wash	N/A	N/A	2.42	1.33
Total recovery	96.88	1.43	122.04	17.52
LLC of t_0.5 absorption	19.84	43.04	20.62	17.73
Absorption complete?	No		No	
Measured absorption, if LLC of t_0.5<=75%	0.35	0.23	10.17	4.21
Measured absorption, if LLC of t_0.5>75%	N/A	N/A	N/A	N/A
Measured absorption corrected	0.35	0.23	10.17	4.21
Relevant absorption estimate	0.550		13.708	
Final estimate (rounded)	0.55		14	
Remarks				

LLC: lower limit of confidence; N/A: not applicable; SD: standard deviation

III. CONCLUSION

The mean total recovery of the radioactivity in the concentrated test substance GWN-10616 was 96.88 % for human dermatomed skin, determined *in vitro*. For the spray dilution, the mean total recovery of the radioactivity was 122.03 %.
The evaluation according to the Guidance on Dermal Absorption (EFSA Journal 2017; 15(6): 4873) indicates that radiolabelled Zoxamide penetrates human skin in concentrated GWN-10616 with an absorbed fraction of 0.35 ± 0.23 % of the applied dose. The absorbed fraction of GWN-10616 in the spray dilution was 10.17 ±4.22 % of the applied dose.

The re-calculation for human *in vitro* dermal absorption data for GWN-10616 was conducted by the applicant using the BfR sheet (Version 3, EFSA publication date 20 August 2018; KCA 7.3/03, Doc. No. 511-003) according to EFSA Journal 2017;15(6):4873).
This resulted in a dermal absorption value of 0.55 % for the concentrate and 14 % for the spray dilution of Zoxamide in GWN-10616.

A 2.10.2 Study 2 – Phosphonic acid in GWN-10616 in human skin (*in vitro*)

Comparative dermal absorption, *in vitro* using human skin

Comments of zRMS:	The study performed on formulation GWN-10616 according to relevant OECD method and in GLP conditions is acceptable. The concentrate 500 g/L (measured as Phosphonic acid) and one in-use spray dilution (1 g/L) of the product (1:500 dilution) were tested The final dermal absorption rates were derived according to EFSA Guidance on Dermal Absorption (EFSA Journal 2017;15(6):4873) using for calculation of the dermal absorption of each substance a formula: mean value for a given concentrate or dilution + ks, where k is multiplication factor and s is the sample standard deviation. Since permeation (in vitro) was essentially not complete at the end of the study, all tape stripped skin material was included in the calculation of the absorbable dose fraction. Thus, the dermal penetration estimates to be used for risk assessment due to exposure to Phosphonic acid is 0.071% for the concentrated formulation and 1.7 % for the spray dilution.

Reference	KCP 7.3/02
Report	The *In Vitro* Percutaneous Absorption of Phosphonate from a Concentrate Formulation (GWN-10616) and One In-Use Dilution through Human Split-Thickness Skin, Spa, S., 2022, study No. 20352081, Doc. No. 511-002, EFSA Study Identification: EFSA-2022-00012083
Guideline(s)	OECD No. 428 (2004), OECD No. 28 (2004), OECD ENV/JM/MONO/(2011)36 (2011), EC - SANCO/222/2000/Rev.7 (2004), EFSA Journal, 2017; 15(6); 4873
Deviations	No
GLP	Yes
Acceptability	Yes
Duplication	No, not applicable (*in vitro* study)

(if vertebrate study)

Executive summary

The objective of this study was to determine the *in vitro* dermal absorption of Phosphonic acid (phosphonate) from the agrochemical formulation (GWN-10616), following topical application of GWN-10616 to the surface of human split-thickness skin membrane mounted into flow-through diffusion cells. The concentrate 500 g/L (measured as Phosphonic acid (phosphorous acid, H_3PO_3) equivalents, equivalent to 755 g/L K-phosphonate) and one in-use spray dilution (1 g/L) of the product (1:500 dilution) were tested.
A tritiated water skin barrier integrity test was performed. The receptor fluid samples were analysed by LSC. The integrity of all human skin samples was within the acceptability criteria (absorption ≤1.6% of applied dose of tritiated water).
For each tested concentration, 8 diffusion cells were used, equipped with skin from 4 different human donors (2 skin membranes per donor each) and the cells were left open to the atmosphere.
The study duration was 24 h and the exposure time was 8 h. The receptor fluid was collected from 0-1 to 1-2 h post dose, followed by 2-hourly fractions until 24 h post-dose. The 8 h exposure period was terminated by washing the skin surface with hand wash soap followed by rinsing with a dilute soap solution and drying the surface with tissue swabs. At 24 h post dose, the skin was washed and removed from the flow-through diffusion cells, the *stratum corneum* tape stripped, and the skin divided into exposed and unexposed skin. The skin samples were cut in pieces and extracted for analysis. All samples were analysed by LC-MS analysis.

Results were evaluated according to the latest Guidance on Dermal Absorption (EFSA Journal 2017; 15(6): 4873) by applying the template calculator provided by EFSA as supportive information (Version 3, EFSA publication date 20 August 2018; KCA 7.3/04, Doc. No. 511-004).
For both test preparations, the mean absorption of Phosphonic acid into the receptor fluid within the first half of the study ($t_{0.5}$) was < 75% taking into account the corresponding confidence interval. As defined in the EFSA Guidance on Dermal Absorption (2017), the dermal absorption value for risk assessment is therefore calculated from the potentially absorbable dose (sum of: receptor fluid, the receptor wash, the exposed skin and tape-strips 3 to last). After correction for variability, the calculated dermal absorption value for risk assessment for Phosphonic acid is 0.071 % for the concentrate formulation and 1.7 % for the spray dilution.

I. MATERIALS AND METHODS

A. Materials

1. Test materials

Test substance	
Test substance name	GWN-10616
Batch number	P2102669001
Composition	Potassium phosphite content*: 507 g/L Zoxamide content: 62 g/L

* Measured as Phosphonic acid (phosphorous acid, H_3PO_3) equivalent.

Active substance (analytical standard)	
Test substance name	Phosphonic acid (phosphorous acid)
Batch number	MKCP7746
Chemical purity	98.9%

Active substance (internal standard)[#]	
Test substance name	Phosphonic acid-$^{18}O_3$
Batch number	MBBC4390
Chemical purity	99.6%

[#] The internal standard was used to prepare samples for LC-MS analysis.

2. Control material

Control material name	[^{3}H]-H_2O[##]
Batch number	3H-22-03

[##] used for the skin barrier integrity assessment

3. Test system

Human skin samples were obtained from the abdominal or breast cosmetic/reconstructive surgery of 4 female donors, aged 40 to 55 years old.

B. STUDY DESIGN

1. Solubility of the test substance in receptor fluid

Since potassium phosphonate is freely miscible with water, solubility in the receptor fluid was considered appropriate, *i.e.* diffusion into the receptor fluid was considered not a rate-limiting step. Furthermore, in the flow-through cells used, the volume of the receptor fluid in the receptor chamber beneath the skin was *ca.* 0.25 mL. At a flow rate of *ca.* 1.5 mL/h, this volume was replenished continuously (6 times per h) such that the rate of diffusion into the receptor fluid was not a rate-limiting step (i.e., sink conditions were maintained).

2. Test substance preparation

Test Preparation 1: Phosphonic acid in the formulation concentrate (500 g/L)
Test Preparation 1 was provided by the Sponsor as ready-to-use (formulation GWN-10616) and was used as such after vortex mixing.

Test Preparation 2: Phosphonic acid in in spray dilution (1 g/L)
Test Preparation 2 was prepared from Test Preparation 1 on the day of dose application by a 500-times dilution with water. Therefore, 1.4598 g of test preparation 1 was mixed with 18.9994 g of MilliQ water and stirred until visually homogenous. Subsequently 0.8079 g (800 µL) of this mixture was further diluted with 19.1990 g of MilliQ water and stirred until visually homogenous to yield Test Preparation 2.

3. Preparation of skin membranes

Upon thawing, the skin samples were cut at a thickness between 0.24 and 0.4 mm using a dermatome. The split-thickness membranes were either used immediately or wrapped in aluminium foil and stored in a freezer (-20°C) for a maximum period of 1 year.

4. Flow-through diffusion cells, receptor fluid and integrity of skin membranes

Split-thickness skin membranes (*ca.* 1.5×1.5 cm) were thawed and mounted in the diffusion cells between the donor and receptor compartments. The flow-through diffusion cells were positioned in a

manifold heated via a circulating water bath to maintain a skin surface temperature of 32 ± 1°C. The cells were connected to a peristaltic pump. Effluent from the cells dropped into vials on a fraction collector via tubing. The surface area of exposed skin within the cells was 1 cm^2, with a receptor chamber of 0.25 mL. The split-thickness skin was allowed to equilibrate for a period of *ca.* 15 minutes while receptor fluid was pumped through the receptor chamber at a flow rate of *ca.* 1.5 mL/h.

Milli-Q containing glucose (5%, w/v), was used as the receptor fluid for the test material permeability measurements. For the assessment of the skin barrier integrity, saline (0.9% (w/v) NaCl in water) was used.

The skin integrity was tested by permeation of tritiated water (^3H_2O, 250 µL/cm^2, 16.3 kBq/mL), that was applied to the skin and the donor compartment of the flow-through cells occluded. The absorption of tritiated water was assessed over 1 h by collecting a single 1 h fraction of receptor fluid. At the end of the 1 h period, residual tritiated water remaining at the donor compartment was removed with a pipette and the skin was dried with 2 cotton swabs. The skin was washed with 500 µL water and dried with 1 cotton swab, this washing procedure was repeated once. The receptor fluid samples were analysed by LSC. Tritiated water absorption (% applied dose) was calculated from the LSC data for each skin sample.

5. Experimental design

Application of the test substance preparations
A single dose (10 µL/cm^2) of each of the test preparations was applied evenly over the surface of 8 split-thickness human skin membranes using a positive displacement pipette. The donor chambers of the cells were left non-occluded. Seven (7) aliquots (10 µL) of each of the phosphonic acid containing test preparations were dispensed into 20 mL (LSC) vials, 990 µL of MilliQ water was added and the samples were kept in the freezer (-20°C) until LC-MS analysis.

The results of the aliquots are provided in the following table.

Test preparation	**Target test substance concentration [g/L]**	**Measured test substance concentration**	
		Mean [g/L]	**CV [%]**
Phosphonic acid in the formulation concentrate	500	499	4
Phosphonic acid in the spray dilution	1	1	2.14

Formulation concentrate: Test Preparation 1; spray dilution: Test Preparation 2

Receptor fluid sampling
Absorption of Phosphonate from the test preparations was assessed by collecting fractions of the receptor fluid at the following time intervals: 0-1, 1-2, followed by 2-h intervals until 24 h after dosing.

Termination of exposure
The exposure period was terminated at 8 h after dosing. Commercial hand wash soap (50 µL/cm^2) was applied to the skin and the soap gently rubbed onto the skin with a cotton swab. The skin was rinsed with approx. 5 mL of a 2% (v/v) commercial soap solution. The soap solution was applied in aliquots (approximately 0.4 mL) and each aliquot was aspirated with a pipette. The skin was dried afterwards with a cotton swab. This process was repeated once. The soap solution (skin wash) and cotton swabs samples were pooled and left to extract overnight in the refrigerator, set to maintain 4 °C.

Termination of post exposure
At 24 h post dose, *i.e.* after 16 h monitoring period, the receptor compartment was rinsed twice with *ca.* 1 mL of receptor fluid followed by air to remove all remaining receptor fluid in the compartment, and collected in a 20 mL plastic (LSC) vial. Each diffusion cell was dismantled, the skin was removed

and the donor chamber was transferred into a separate tube (donor compartment wash) and extracted using 10 mL MilliQ water.
The *stratum corneum* was removed with a maximum of 20 successive tape strips (D-Squame stripping discs). The skin sample was rotated 90° after each tape strip. Rotation was stopped if the epidermis/dermis junction became fragile or if the epidermis was removed. Where a piece of epidermis was removed this was recorded and when practically possible, the pieces of skin on the tape strip were removed using a pair of tweezers and added to the exposed skin fraction. Where all of the epidermis was removed tape stripping was stopped. Tape strips were collected (pooled) as follows: 1, 2, 3-5, 6-10, 11-15, 16-20. Pooling was done to increase the changes of detection of phosphonate above the limit of quantification (LOQ).
The skin under the cell flange (unexposed skin) was cut away from the exposed skin. The exposed and non-exposed skin samples processed and analysed individually.

Sample preparation for analysis
Mock dose samples and pipette tips were mixed/extracted with an adequate volume of MilliQ water before analysis.
Sub samples (1 mL) of the receptor fluid / receptor compartment wash were collected, centrifuged and the supernatant was analysed.
Skin wash samples were left to extract in the refrigerator overnight. Prior to analysis, samples were stirred on a multi-vortex for *ca.* 5 min. at ambient temperature and diluted, when needed.
Tape strip samples (pooled) were extracted overnight in the refrigerator using 50% aqueous methanol. Prior to analysis, samples were stirred on a multi-vortex for *ca.* 5 min. at ambient temperature and diluted, when needed.
Exposed skin samples were cut into smaller pieces, frozen using liquid nitrogen and pulverized using a tissue micro-dismembrator, extracted overnight in the refrigerator, using *ca.* 5 mL 50% methanol in water, and stored in the freezer until analysis. Prior to analysis, the skin samples were stirred on a multi-vortex for *ca.* 5 min. at ambient temperature, centrifuged, the supernatant collected and diluted, when needed.
Non-exposed skin samples were cut into small pieces and extracted overnight in the refrigerator, using *ca.* 5 mL 50% methanol in water, and stored in the freezer until analysis. Prior to analysis, the skin samples were stirred on a multi-vortex for *ca.* 5 min. at ambient temperature, centrifuged, the supernatant collected and diluted, when needed.
Donor compartments were submerged in MilliQ water (10 mL), extracted using a 15-minute sonication step at ambient temperature and diluted before analysis, when needed.

6. Analysis

Determination of radioactivity
All samples of the skin barrier integrity assessment prepared in scintillation fluid were subjected to LSC, together with representative blank samples.
Radioactivity measurements were performed by LSC using a scintillation counter. The counting time was set to 1 minute. The scintillation counter was programmed to convert counts per minute (cpm) to degradations per minute (dpm). Background signal was subtracted manually before data analysis.

LC-MS Analysis
Analyses was performed according to a validated method in compliance with SANTE/2020/12830, Rev.1 (Test Facility Study No. 20352080). Results are expressed as Phosphonic acid (phosphorous acid, H_3PO_3) equivalents.

7. Data evaluation

Dislodgeable dose (skin wash 8 h + cotton swab 8 h + dry cotton swab 8 h), unabsorbed dose (total dislodgeable dose + *stratum corneum* + unexposed skin), absorbed dose (cumulative receptor fluid +

receptor wash) and dermal delivery (total absorbed dose + exposed skin) are reported as defined in OECD guidance document No. 28. Potentially absorbable dose (complete/incomplete absorption) are reported as defined in EFSA Guidance on Dermal Absorption (2017).

Limits of quantification (LOQ) for Phosphonic acid (phosphorous acid, H_3PO_3) equivalents in the various samples was used in the calculations if the total amount measured in the sample was below LOQ. The LOQ for Phosphonic acid equivalents in the various matrices was determined during validation of the analytical method. In case measured concentrations were below LOQ, the LOQ values were used in the calculation of the total mass per sample and the outcome presented as '< calculated value'.

- If more than 50% of test material amount in the receptor fluid and/or tape strip samples of a replicate is below the LOQ, the cumulative absorption and/or total (cumulative) value for this replicate is presented as '< calculated value'.
- If more than 50% of test material amount in the samples contributing to the absorbed dose is below the LOQ, the absorbed dose is presented as '< calculated value'.
- If more than 50% of the replicates show values below the LOQ, the mean value is presented as '< calculated value'.

Samples with a mass balance outside 90% - 110% were rejected from the mean ± SD. However, if the mass balance was below 90% and the loss can be explained (*e.g.* volatilization), the samples may be accepted. These rejected data are reported. Further rejection criteria, such as mean ± 2 SD, Dixon's Q-test for outliers, stem and leaf plots or similar, may also be applied where appropriate.

C. STATISTICS

Statistical analysis was limited to the calculation of means, standard deviations (SD) and coefficient of variation (CV).

II. RESULTS AND DISCUSSION

1. Barrier integrity of skin membranes

For all test preparations, the integrity of the reported skin samples was within the acceptability criteria, *i.e.*, absorption ≤ 1.6 % of applied dose of tritiated water for the human skin.

2. Dermal absorption of the test substance preparations

Test Preparation 1: Phosphonic acid (phosphonate) in the formulation concentrate (500 g/L)
The absorption profiles looked similar for all samples, with a (minor) effect of washing visible for most replicates. The mass balance for all individual samples was within 100 ± 10% and the following results are provided as mean values (n=8).
Following the skin wash at 8 h post application, 97.1% of the applied dose was washed off, *i.e.* the 8 h dislodgeable dose. At 24 h post dose, a further 0.011% was recovered with the donor compartment wash. Therefore, the total dislodgeable dose was 97.1% of the applied dose. The mean total unabsorbed dose was 97.1%, which consisted of the total dislodgeable dose, the unexposed skin (0.000%) and the test material associated with the *stratum corneum* (0.011%), of which the first 2 tape strips contained 0.001% of the applied dose. The total absorbed dose (0.039%) was the sum of the receptor fluid (0.038%) and the receptor wash (0.000%). The exposed skin contained 0.005% of the applied dose. Dermal delivery (0.043%) was the sum of the total absorbed dose and the exposed skin. The potentially absorbable dose was 0.053%, which was the sum of the dermal delivery and the tapes trips 3-last (0.010%).

The mass balance, total dislodgeable dose, total unabsorbed dose, total absorbed dose, dermal delivery and potentially absorbable dose were 4807, 4804, 4805, 1.94, 2.17 and 2.66 µg/cm^2, respectively.
The mean absorption of Phosphonic acid (phosphonate) into the receptor fluid within the first half of the study ($t_{0.5}$) was 77.6 ± 16.1 % of the total absorption into the receptor fluid over 24 h, with a lower limit of confidence of 64.1 % (*i.e.* mean - (k × SD); 77.6 - (0.84 × 16.1). As the mean absorption at $t_{0.5}$ is near 75% and the corresponding lower limit of confidence is < 75%, as defined in the EFSA Guidance on Dermal Absorption 2017, the dermal absorption value for risk assessment is calculated from the potentially absorbable dose (sum of: receptor fluid, the receptor wash, the exposed skin and tape-strips 3-last). Based on the same EFSA Guidance, the dermal absorption value for risk assessment is corrected for variability by adding a multiple (based on the number of replicates) of the standard deviation to the mean value. After correction for variability (using a k-factor of 0.84 for 8 replicates), the calculated dermal absorption value for risk assessment of Phosphonic acid from Test Preparation 1 is 0.05% + (0.84 x 0.02%) = 0.070 %.

Test Preparation 2: Phosphonic acid in spray dilution (1 g/L)
Phosphonate concentrations, measured as Phosphonic acid (phosphorous acid, H_3PO_3) equivalents, were near the LOQ (2 ng/mL) in most receptor fluid samples, except for the samples of Cell 16. As a conservative approach, the data of Cell 16 were included in the calculations of the mean regardless of the higher absorption when compared to the other cell replicates. As its absorption profile was in line with the expectations and no apparent deviation (no obvious technical error or leakage of the cell membrane) occurred. The 22-24 h receptor fluid fraction of Cell 16 and the 10-12 h receptor fluid fraction of Cell 23 was apparently contaminated and therefore the average of the 22-24 h (for Cell 16) or 10-12 h (for Cell 23) receptor fluid fractions of the other cell replicates were inserted. The lower recovery of Cell 18 (88 %) was considered acceptable since the absorption values were comparable to the other replicates (inclusive its donor pair) and the lower recovery was clearly associated with the skin wash, suggesting the applied dose had been slightly lower than anticipated. The mass balance for all other individual samples was within 100 ± 10 % and the following results are provided as mean values (n=8).
Following the skin wash at 8 h post application, 92.8 % of the applied dose was washed off, *i.e.* the 8 h dislodgeable dose. At 24 h post dose, a further < 0.34 % was recovered with the donor compartment wash. Therefore, the total dislodgeable dose was 93.1 % of the applied dose. The mean total unabsorbed dose was 94.2 %, which consisted of the total dislodgeable dose, the unexposed skin (< 0.15 %) and the test substance associated with the *stratum corneum* (< 0.90 %), of which the first 2 tape strips contained < 0.30 % of the applied dose. The total absorbed dose (< 0.82 %) was the sum of the receptor fluid (< 0.77 %) and the receptor wash (< 0.050 %). The exposed skin contained < 0.15 % of the applied dose. Dermal delivery (< 0.96 %) was the sum of the total absorbed dose and the exposed skin. The potentially absorbable dose was 1.57 %, which was the sum of the dermal delivery and the tapes trips 3-last (< 0.60 %).
The mass balance, total dislodgeable dose, total unabsorbed dose, total absorbed dose, dermal delivery and potentially absorbable dose were 9473, 9271, 9377, < 81.2, < 96.0 and < 156 ng/cm^2, respectively.
The mean absorption of Phosphonic acid into the receptor fluid within the first half of the study ($t_{0.5}$) was 55.0 ± 6.8 % of the total absorption into the receptor fluid at 24 h. As the mean absorption at $t_{0.5}$ was < 75 %, as defined in the EFSA Guidance on Dermal Absorption 2017, the dermal absorption value for risk assessment is calculated from the potentially absorbable dose (sum of: receptor fluid, the receptor wash, the exposed skin and tape-strips 3-last). Based on the same EFSA Guidance, the dermal absorption value for risk assessment is corrected for variability by adding a multiple (based on the number of replicates) of the standard deviation to the mean value. After correction for variability (using a k factor of 0.84 for 8 replicates), the calculated dermal absorption value for risk assessment of phosphonate from Test Preparation 2 is < 1.57 % + (0.84 x 0.15 %) = < 1.7 %.

Table A 17: *In vitro* dermal penetration of Phosphonic acid formulated as GWN-10616 through human skin - Recovery data

Dose group	High dose (Formulation concentrate) Test Preparation 1		Low dose (Spray dilution 1:500) Test Preparation 2	
Target concentration [g/L]	500		1	
Target dose [µg/cm²]]	5000		10	
Mean actual applied dose [µg/cm²]	4955		9.96	
	Recovery [%]		Recovery [%]	
	Mean	S.D.	Mean	S.D.
Dislodgeable dose				
Dislodgeable dose 8 h	97.1	4.2	92.8	4.0
Donor chamber wash	0.011	0.016	< 0.34	0.38
Dose associated to skin				
Tape strips: 1st sample, strips 1 + 2	0.001	0.001	< 0.30	0.00
Tape strips: 2nd sample; strips 3 – 20	0.010	0.002	< 0.60	0.00
Skin preparation	0.005	0.002	< 0.15	0.02
Absorbed dose				
Receptor fluid	0.038	0.022	< 0.77	0.15
Receptor chamber wash	0.00	0.00	< 0.050	0.003
Total recovery[1]	97.1	4.2	95.1	4.0
%Absorption at $t_{0.5}$ [2]	77.6 ± 16.1 % (79.67 ± 13.43)		55.0 ± 6.8 % (54.97 ± 6.61)	
LLC of $t_{0.5}$ absorption[2]	64.1 % (68.38 ± 11.28)		Not stated in the report (49.41 ± 5.56)	
Absorption essentially complete at end of study (>75% absorption within half the study duration)	No		No	
If no: Absorption estimates = absorbed dose + skin preparation + tape strips sample 2)[3]	0.053	0.020	< 1.57	0.15
If yes: Absorption estimates = absorbed dose + skin preparation	na	na	na	na
Absorption estimate normalised[4]	0.053	0.020	< 1.57	0.15
Relevant absorption estimate[5]	**0.071**		1.696	
Absorption estimates used for risk assessment[6]	**0.071**		**1.7**	

1 Values may not calculate exactly due to rounding of figures

2 Calculation of dermal absorption by the applicant according to EFSA Journal 2017; 15(6): 4873 (BfR calculator, Version 3; KCA 7.3/04, Doc. No. 511-004) was performed. The dermal absorption (lower limit of the 95% confidence interval) was calculated as follows: Absorption (mean value) + k × SD, where SD is the sample standard deviation and k is the multiplication factor for variability of results of 0.84 for 8 replicates.

3 In accordance to EFSA Journal 2017; 15(6): 4873, the radioactivity in the second tape-strip pool (3rd to nth tape strip) is considered potentially absorbable if less than 75 % of the absorption occurred in the first half of the study.

4 According to EFSA Journal 2017; 15(6): 4873, cells with insufficient recovery (< 95 %) can be corrected by normalisation of absorption estimate to 100 % recovery.

5. In accordance to EFSA Journal 2017; 15(6): 4873, one standard deviation was added to the mean% dermal penetration in cases where the standard deviation was ≥ 25 % of the mean value.

6 Relevant absorption estimate was rounded to the required number of significant figures.

na: not applicable; S.D.: standard deviation

Calculation of dermal absorption by the applicant according to EFSA Journal 2017; 15(6): 4873 (BfR calculator, Version 3; KCA 7.3/04, Doc. No. 511-004) was performed taking into account a multiplication factor k for variability of results of 0.84 for 8 replicates. The summary table of the BfR calculation sheet is presented in Figure A.2.

	Concentrate		Dilution 1 (1:500)	
Target concentration [mg/mL]	500		1	
Target dose [µg/cm²]	5000		10	
Mean actual applied dose [µg/cm²]	4955		9.96	
Recovery [%]	Mean	SD	Mean	SD
Dislodgeable dose				
Skin wash after 8 hours	97.10	4.32	92.88	3.94
Donor chamber wash	0.01	0.02	0.34	0.38
Skin associated dose				
Tape strips 1-2	0.00	0.00	0.30	0.00
Tape strips 3-x	0.01	0.00	0.60	0.00
Skin preparation	0.00	0.00	0.15	0.02
Absorbed dose				
Receptor fluid	0.04	0.02	0.77	0.15
Receptor chamber wash	N/A	N/A	0.05	0.00
Total recovery	97.17	4.31	95.08	3.97
LLC of t_0.5 absorption	68.38	11.28	49.41	5.56
Absorption complete?	No		No	
Measured absorption, if LLC of t_0.5<=75%	0.05	0.02	1.57	0.15
Measured absorption, if LLC of t_0.5>75%	N/A	N/A	N/A	N/A
Measured absorption corrected	0.05	0.02	1.57	0.15
Relevant absorption estimate	0.071		1.696	
Final estimate (rounded)	0.071		1.7	

LLC: lower limit of confidence; N/A: not applicable; SD: standard deviation

Figure A.2 BfR calculation sheet (EFSA, 2017) - Results of the *in vitro* penetration of Phosphonic acid through human skin

III. CONCLUSION

The mean total recovery of the concentrated test substance GWN-10616 was 97.17 % and 95.08 % of the spray dilution for human dermatomed skin.
The evaluation according to the Guidance on Dermal Absorption (EFSA Journal 2017; 15(6): 4873) indicates that Phosphonic acid penetrates human skin in concentrated GWN-10616 with an absorbed fraction of 0.05 ± 0.02 % of the applied dose. The absorbed fraction of GWN-10616 in the spray dilution was 1.57 ± 0.15 % of the applied dose.

The results of the *in vitro* dermal absorption study with GWN-10616 through human skin were recalculated by the applicant using the BfR calculator provided by EFSA as supportive information (KCA 7.3/04, Doc. No. 511-004).

This resulted in dermal absorption values of 0.071 % for the concentrate and 1.7 % for the spray dilution for Phosphonic acid in GWN-10616.

A 2.11 Other/Special Studies

Study 1 - *In vitro* mammalian cell gene mutation RH-141452

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The study performed according to internationally recognised guideline and in GLP conditions is acceptable. The metabolite RH-141452 is not mutagenic in *in vitro* mammalian cell gene mutation test (HPRT-Locus) in Chinese Hamster V79 cells

Reference:	**KCP 7.4/01**
Report	Voges, Y., 2020: *In vitro* mammalian cell gene mutation test (HPRT-Locus) in Chinese Hamster V79 cells with RH-141452 Gowan Crop Protection Ltd., UK Eurofins BioPharma, Germany, Report No. 188620, GLP, Not published
Guideline(s):	OECD 476 (2016) EEC B.17 (2008) OPPTS 870.5300 (1998)
Deviations:	Additional test item material was required for testing (subsequent delivery). Final arrival of the Test Item: 26 November 2018 / 01 August 2019. Final batch no.: 632031-P1050-44 and 55954-24-06. These deviations did not influence the quality or integrity of the present study.
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-141452 (632031-P1050-44 and 55954-24-06)
Purity:	96.27%
Test cells	V79 cells *in vitro* Eurofins BioPharma Product Testing Munich GmbH culture, properly maintained and purified.
Medium:	Culture medium: MEM medium supplemented with 10 % fetal bovine serum (FBS) 100 U/100 µg/mL penicillin/streptomycin

Test material (Lot/Batch No.)	RH-141452 (632031-P1050-44 and 55954-24-06)
	2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B Treatment medium: MEM medium supplemented with 0 % fetal bovine serum (FBS) 100 U/100 µg/mL penicillin/streptomycin 2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B Selective medium: MEM medium supplemented with 10 % fetal bovine serum (FBS) 100 U/100 µg/mL penicillin/streptomycin 2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B 11 µg/mL 6-thioguanine (TG)
Controls	
Negative:	Medium
Solvent:	DMSO (1 %, v/v)
Positive:	*Without metabolic activation* EMS; ethylmethanesulfonate Dissolved in medium (MEM) Concentration: 300 µg/mL *With metabolic activation* DMBA; 7,12-dimethylbenz(a)anthracene Dissolved in DMSO, dimethylsulfoxide (1% in medium) Concentration: 1.0 µg/mL
Activation	Mammalian microsomal S9 mix from Eurofins Munich, Germany, derived from male Wistar rats. Protein concentration in Eurofins lot 220219 was 35.7 mg/mL. Checked for bilogical activity and sterility.
S 9 mix:	S9 cofactor solution with final protein concentration of 0.75 mg/mL, 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate and 5 mM NADP in 100 mM sodium-phosphate-buffer pH 7.4
Test concentrations	without metabolic activation: 50, 100, 150, 200, 225, 250, 300, 400, 500, 1000, 1500 and 2000 µg/mL with metabolic activation: 100, 150, 175, 200, 210, 215 and 220 µg/mL
Post exposure observation period	4 h

Test material (Lot/Batch No.)	RH-141452 (632031-P1050-44 and 55954-24-06)
Remarks	None

The test item RH-141452 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. The main experiments were carried out without and with metabolic activation. The experiments with metabolic activation were performed by including liver microsomes and NADP for efficient detection of a wide variety of carcinogens requiring metabolic activation. The test item was dissolved in DMSO. The solvent (final concentration of 1 %, v/v) was compatible with the survival of the cells and the S9 activity.

Based on the results of a pre-experiment, tests with and without metabolic activation were performed as a 4 h short-term exposure assay. The test item was investigated at the following concentrations:

without metabolic activation: 50, 100, 150, 200, 225, 250, 300, 400, 500, 1000, 1500 and 2000 µg/mL

and

with metabolic activation: 100, 150, 175, 200, 210, 215 and 220 µg/mL.

Main test:

Approx. 5 x 10^6 cells per treatment group were seeded in complete culture medium in a 175 cm^2 culture flask. Approx. 24 h after seeding, the cells were exposed to designated concentrations of the test item either in the presence or absence of metabolic activation. After 4 h the cultures were checked for precipitation and the treatment medium containing the test item (MEM without FBS) was removed. The cells were washed twice with PBS, trypsinised and counted with a cell counter.

For **expression**, one 175 cm^2 flask was seeded with at least 2 x 10^6 cells per treatment group in complete culture medium (MEM supplemented with 10% FBS). Cells were subcultured within the following (7 – 9) days after treatment in complete culture medium in a sufficient number of cells (at least 2 x 10^6 cells per treatment group).

For **survival**, two 25 cm^2 flasks were seeded with approx. 200 cells in complete culture medium for each treatment group. After incubation for an appropriate time (6 – 7 days) colonies were fixed with methanol, stained with Giemsa and counted.

For **mutant frequency**, at the end of the expression period (after 7 to 9 days) about 4 x 10^5 cells for each treatment group were seeded in 5 cell culture petri dishes (diameter 90 mm) with selective medium containing 11 µg/mL 6-thioguanine for further incubation. After incubation for 9 – 11 days colonies were fixed with methanol, stained with Giemsa and counted.

For **cloning efficiency**, at the end of the expression period (after 7 to 9 days), two 25 cm^2 flasks were seeded with approx. 200 cells in complete culture medium for each treatment group. After incubation for 6 – 8 days colonies were fixed with methanol, stained with Giemsa and counted.

The non-parametric Mann-Whitney test was applied to the mutation data to prove the concentration groups for any significant difference in mutant frequency compared to the solvent controls. Mutant frequencies of the solvent controls were used as reference.

A test chemical was considered to be clearly negative if, in all experimental conditions examined none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, there is no concentration-related increase when evaluated with an appropriate trend-test, and all results are inside the distribution of the historical negative control data. A test chemical is considered to be clearly positive if, in any of the experimental conditions examined at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, the increase is concentration-related when evaluated with an appropriate trend test, and any of the results are outside the distribution of the historical negative control data. Moreover, if there is by chance a low

spontaneous mutant frequency in the corresponding negative and solvent controls a concentration-related increase of the mutations within their range has to be discussed. According to the OECD guideline, the biological relevance is considered first for the interpretation of results.

Results and discussions

The pH-value detected with the test item was within the physiological range (pH 7.0 ± 0.4).

No precipitation of the test item was noted in the main experiments.

Toxicity: A biologically relevant growth inhibition (reduction of relative survival below 70 %) was observed after treatment with the test item in experiment with and without metabolic activation. In the experiment without metabolic activation the relative survival was 19% for the highest concentration (1500 µg/mL) evaluated. The highest biologically relevant concentration evaluated with metabolic activation was 210 µg/mL with a relative survival of 13 %.

Mutagenicity: In the main experiment metabolic activation all validity criteria were met. Most of the mutant values of the negative and solvent controls fall within the historical data range of the test facility and the cloning efficiencies of the negative and solvent controls are > 50 %. In the experiment without metabolic activation, the individual mutant value of one of the negative controls was slightly increased and outside the historic control range. Since no technical failure occurred, the data was considered acceptable for addition to the laboratory historical database. The positive controls, DMBA (1.0 µg/mL) and EMS (300 µg/mL) showed statistically significant increases in mutant frequency, thereby demonstrating both the sensitivity and validity of the test systems.

In the experiment **without metabolic activation** the mean mutant values of the negative, solvent controls and all mutant values found for all applied concentrations of the test item were within the historical control data of the test facility Eurofins Munich (about 8.7 - 42.5 mutants per 10^6 cells). The positive control EMS induced a distinct increase in mutant frequency with 281.5 mutants/10^6 cells. Additionally, the individual mutant value of one of the negative controls was slightly increased (54.4 mutants/10^6 cells) and outside the historic control range. However, the mean value of this control was within the historical data range of the test facility and since no technical failure occurred, the data was considered acceptable for addition to the laboratory historical database. The mutant frequencies induced by the test item did not show a biologically relevant increase. None of the observed mutant frequencies was statistically significantly increased over those of the solvent controls and no significant concentration-related increase was determined in the χ^2 test for trend. The highest mutant frequency was observed at a concentration of 1000 µg/mL (40.9 mutants per 10^6 cells) with a relative survival of 45 %.
In the experiment **with metabolic activation** the mean mutant values of the negative, solvent controls and all mutant values found for all applied concentrations of the test item were within the historical control data of the test facility Eurofins Munich (about 9.2 - 45.3 mutants per 10^6 cells). The positive control DMBA induced a distinct increase in mutant frequency with 597.3 mutants/10^6 cells. In the experiment with metabolic activation, the individual mutant value of one of the negative controls was slightly decreased (7.0 mutants/10^6 cells) and outside the historic control range. However, the mean value of this control (9.7 mutants/10^6 cells) was within the historical data range of the test facility and therefore considered acceptable. The mutant frequencies induced by the test item did not show a biologically relevant increase. None of the observed mutant frequencies was statistically significantly increased over those of the negative controls and no significant concentration-related increase was determined in the χ^2 test for trend. The highest mutant frequency was observed at a concentration of 150 µg/mL (14.7 mutants per 10^6 cells) with a relative survival of 62 %.

The test was regarded acceptable:
- Negative and/or solvent controls fall within the performing laboratories 95th control limits of the historical control data range: 8.7 - 42.5 mutants/10^6 cells (without metabolic activation) and 9.2 - 45.3 mutants/10^6 cells (with metabolic activation) (January 2015 - December 2018).
- The absolute cloning efficiency ([number of positive cultures x 100] / total number of seeded cultures) of the negative and /or solvent controls was > 50%.

- The positive controls (EMS and DMBA) induced a statistically significant increase compared to the concurrent negative control and are compatible with the laboratory historical data base.
- Two experimental conditions (*e.g.* with and without metabolic activation) were tested unless one results in a positive response.

Conclusion

In conclusion, in the described *in vitro* cell gene mutagenicity test under the experimental conditions reported, RH-141452 is considered to be non-mutagenic in the HPRT locus using V79 cells of the Chinese Hamster.
Reference mutagens were tested in parallel to the test item and demonstrated the sensitivity of the test system. The study is valid.

Study 2 - *In vitro* mammalian cell gene mutation RH-150721

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The study performed according to internationally recognised guideline and in GLP conditions is acceptable. The metabolite RH-150721 is not mutagenic in *in vitro* mammalian cell gene mutation assay (Thymidine Kinase Locus/$TK^{+/-}$) in mouse lymphoma L5178Y cells.

Reference:	**KCP 7.4/02**
Report	Schreib, G., 2017: *In vitro* mammalian cell gene mutation assay (Thymidine Kinase Locus/$TK^{+/-}$) in mouse lymphoma L5178Y cells with RH-150721 Gowan Crop Protection Ltd., UK Eurofins BioPharma, Germany, Report No. 171360, GLP, Not published
Guideline(s):	OECD 490 (2016) EEC B.17 (2008)
Deviations:	No
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-150721 (79942-4-16)
Purity:	98.07 % (w/w)
Test cells	mouse lymphoma cell line L5178Y, culture of Eurofins, Munich, Germany
Medium:	Culture medium: RPMI 1640 complete medium with 10 % horse serum (HS) 100 U/100 µg/mL penicillin/streptomycin 1 mM sodium pyruvate 2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B Treatment medium: RPMI 1640 complete medium with 5 % horse serum (HS) for short-term exposure, 7.5 % for long-term exposure 100 U/100 µg/mL penicillin/streptomycin 2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B Selective medium: RPMI 1640 complete medium with 20 % horse serum (HS) 100 U/100 µg/mL penicillin/streptomycin 1 mM sodium pyruvate 2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B 5 µg/mL TFT
Controls	
Negative:	Medium
Solvent:	RPMI cell culture medium was used as solvent (RPMI + 5% HS)
Positive:	*Without metabolic activation* EMS; ethylmethanesulfonate Dissolved in medium Concentration: 200 and 300 µg/mL MMS; methylmethanesulfonate Dissolved in 0.9% NaCI Concentration: 8 µg/mL and 10 µg/mL

Test material (Lot/Batch No.)	RH-150721 (79942-4-16)
	With metabolic activation B(A)P; benz(a)pyrene Dissolved in DMSO, dimethylsulfoxide (1% in RPMI medium) Concentration: 1.5 and 2.5 µg/mL
Activation	Liver microsome preparations (S9 mix) prepared by Eurofins Munich, Germany, derived from male Wistar rats. Protein concentration in Eurofins lot 030217 was 32.4 mg/mL. Checked for bilogical activity and sterility.
S 9 mix:	S9 cofactor solution with final protein concentration of 0.75 mg/mL, 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate and 5 mM NADP in 100 mM sodium-phosphate-buffer pH 7.4
Test concentrations	Exposure (concentration(s), no. of applications) Experiment I without metabolic activation: 10, 20, 50, 82, 89, 112, 115 and 118µg/mL with metabolic activation: 100, 150, 200, 203, 207 and 208µg/mL Experiment II without metabolic activation: 20, 30, 35, 40, 45, 54, 60 and 64µg/mL
Post exposure observation period	4, 24, 48 h
Remarks	None

The test item RH-150721 was assessed for its potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

Concentrations used in the main experiments were selected based on data from the pre-experiments. In experiment I 118 µg/mL (without metabolic activation) and 208 µg/mL (with metabolic activation) were chosen as the highest concentrations. In experiment II 64 µg/mL (without metabolic activation) was selected as the highest concentration. Experiment I was performed without and with metabolic activation over 4 h (short-term exposure assay), experiment II without metabolic activation over 24 h (long-term exposure assay). The experiments with metabolic activation were started by including liver microsome preparations (S9 mix). The test item was dissolved in RPMI cell culture medium was used as solvent (RPMI + 5% HS).

For a short-term exposure experiment 1 x 10^7 cells were suspended in 11 ml RPMI medium with 5 % horse serum (25 cm^2 flasks) and exposed to designated concentrations of the test item either in the presence or absence of metabolic activation in the mutation experiment. After 4 h the test item was removed by centrifugation (200 x g, 10 min) and the cells were washed twice with PBS. Subsequently, the cells were suspended in 30 ml complete culture medium and incubated for an expression and growth period of 2 days in total at 37°C in 5% CO_2/95% humidified air. The cell density was determined each day and adjusted to 3 x 10^5 cells/mL in a total culture volume of 20 mL. For a long-term exposure experiment 5 x 10^6 cells were suspended in 25 mL RPMI medium with 7.5% horse serum (75 cm^2 flasks) and exposed to designated concentrations of the test item in the absence of metabolic activation. After 24 h the test item was removed by centrifugation (200 x g, 10 min) and the cells were washed twice with PBS. Subsequently, 3 x 10^5 cells/mL were suspended in 14 mL complete culture medium and incubated for an expression and growth period of 2 days at 37 °C in 5% CO_2/95% humidified air. The cell density was determined each day and adjusted to 3 x 10^5 cells/mL in a total culture volume of 14 mL. After the ex-

pression period the cloning efficiency (CE) of the cells was determined by seeding a statistical number of 1.6 cells/well in two 96-well plates. The cells were incubated for at least 6 days at 37 °C in a humidified atmosphere with 5% CO_2. Analysis of the results was based on the number of cultures with cell growth (positive wells) and those without cell growth (negative wells) compared to the total number of cultures seeded. Additionally, cultures were seeded in selective medium. Cells from each experimental group were seeded in four 96-well plates at a density of approximately 2000 cells/well in 200 µL selective medium with TFT. The plates were scored after an incubation period of about 12 days at 37 °C in 5 % CO_2/95% humidified air. The mutant frequency was calculated by dividing the number of TFT resistant colonies by the number of cells plated for selection, corrected for the plating efficiency of cells from the same culture grown in the absence of TFT. For the microwell method used here the Poisson distribution was used to calculate the plating efficiencies for cells cloned without and with TFT selection. Based on the null hypothesis of the Poisson distribution, the probable number of clones/well (P) is equal to -ln(negative wells/total wells) and the plating efficiency (PE) equals P/(number of cells plated per well). Mutant frequency then was calculated as MF = (PE(cultures in selective medium)/PE(cultures in non-selective medium)).

Suspension growth (SG) of the cell cultures reflects the number of times the cell number increases from the starting cell density. When carrying out a short-term treatment (4 h) a 2-day growth period was considered, when carrying out a long-term treatment the treatment a period of 24 h followed by a 2 days growth period. The relative total growth (RTG) was calculated as the product of the relative suspension growth (RSG; calculated by comparing the SG of the dose groups with the SG of the control) and the relative cloning efficiency (ROE) for each culture: RTG = RSG x RCE /100. The mutant frequencies obtained from the experiments were compared with the Global Evaluation Factor (GEF) from ten laboratories. It is defined as the mean of the negative/vehicle mutant frequency plus one standard deviation. Applying this definition to the collected data, the GEF was 126 for the microwell method.

The non-parametric Mann-Whitney test was applied to the mutation data to prove the dose groups for any significant difference in mutant frequency compared to the negative/solvent controls. Mutant frequencies of the solvent/negative controls were used as reference.

The test item is considered mutagenic if the induced mutant frequency meets or exceeds the Global Evaluation factor (GEF) of 126 mutants per 10^6 cells and a dose-dependent increase in mutant frequency is detected. Besides, combined with a positive effect in the mutant frequency, an increased occurrence of small colonies (>40% of total colonies) is an indication for potential clastogenic effects and/or chromosomal aberrations. According to the OECD guideline, the biological relevance is considered first for the interpretation of results. Statistical methods might be used as an aid in evaluation of the test result. A test item is considered to be negative if the induced mutant frequency is below the GEF and the trend of the test is negative.

Results and discussions

The pH-value detected with the test item and the osmolality were within the physiological range.

Precipitation of the test item was noted in the pre-experiment I without and with metabolic activation at the following concentrations: 500, 1000 and 2000 pg/mL. These concentrations were not further used in the experiments since they resulted in extremely low levels of RSG. No precipitation of the test item was noted in the main experiment I, pre-experiment II or main experiment II (without and with metabolic activation).

Toxicity: Growth inhibition was observed in experiment I and II without and with metabolic activation. In experiment I without metabolic activation the relative total growth (RTG) was 12.1% for the highest concentration (118 pg/mL) evaluated. The highest concentration evaluated with metabolic activation was 208 pg/mL with a RTG of 11.0%. In experiment II without metabolic activation the relative total growth (RTG) was 10.7% for the highest concentration (64 pg/mL) evaluated.

Mutagenicity:

The mutant frequencies obtained from all experiments were compared to the Global Evaluation Factor (GEF) of 126. Criterion for mutagenicity is the extension of the GEF by the induced mutant frequency as well as a dose-dependent increase in mutant frequency. The positive controls EMS (300 and 200 pg/mL), MMS (8 and 10 pg/mL) and B[a]P (2.5 pg/mL) showed distinct effects in mutation frequency, thus proving the ability of the test system to detect potential mutagenic effects.

In the **experiment I without metabolic activation** all validity criteria were met. The negative controls showed mutant frequencies within the acceptance range of 50-170 mutants/10^6 cells, according to the IWGT criteria. The mutant frequencies of the negative controls were 60.3 and 73.1 mutants/10^6 cells, the positive controls EMS and MMS induced a distinct increase in mutant frequency with 686.9 and 838.3 mutants/10^6 cells. The mutant frequencies induced by the test item did not show any biologically relevant increase. The GEF of 126 was not exceeded in any of the dose groups showing induced mutant frequencies between 4.0 and 28.4 mutants/10^6 cells. None of the observed mutant frequencies was statistically significantly increased over those of the negative controls.

In the **experiment I with metabolic activation** all validity criteria were met. The negative controls showed mutant frequencies within the acceptance range of 50-170 mutants/10^6 cells, according to the IWGT criteria. The mutant frequencies of the negative controls were 97.7 and 70.4 mutants/10^6 cells, the positive control B[a]P induced a distinct increase in mutant frequency with 881.0 mutants/10^6 cells. The mutant frequencies induced by the test item did not show any biologically relevant increase. The GEF of 126 was not exceeded in any of the dose groups showing induced mutant frequencies between -9.8 and 59.2 mutants/10^6 cells. Statistical analysis displayed that one of the mutant frequencies was significantly increased over those of the negative controls; however, the GEF was not exceeded. Therefore, this effect was considered as not biologically relevant.
In **experiment II without metabolic activation** all validity criteria were met. The negative controls showed mutant frequencies within the acceptance range of 50-170 mutants/106 cells, according to the IWGT criteria. The mutant frequencies of the negative controls were 57.5 and 56.7 mutants/10^6 cells, the positive controls EMS and MMS induced a distinct increase in mutant frequency with 2179.4 and 541.0 mutants/10^6 cells. The mutant frequencies induced by the test item did not show any biologically relevant increase. The GEF of 126 was not exceeded in any of the dose groups showing induced mutant frequencies between 5.5 and 63.4 mutants/10^6 cells. A statistical analysis displayed that several of the mutant frequencies were significantly increased over those of the negative controls, however the GEF was not exceeded. Therefore, this effect was considered as not biologically relevant.
Historical data for all mutant frequencies for negative and positive controls of experiment I and II were within the historical range of the test facility Eurofins Munich.

Clastogenicity:
The positive controls MMS (8 and 10 µg/mL) and B[*a*]P (2.5 µg/mL) induced a significant increase in mutant frequency and a biologically significant increase of small colonies (> 40 %), thus proving the ability of the test system to indicate potential clastogenic effects.

In **experiment I without metabolic activation** the percentage of small colonies in the negative controls was found to be 16.0 % and 23.2 %. The percentage of small colonies of the positive control MMS was found to be 41.1 %. In the highest dose groups 17.2 % (112 µg/mL), 23.6 % (115 µg/mL) and 15.4 % (118 g/mL) of small colonies were found. As none of the values exceeded 40%, all dose groups were considered as not clastogenic. **With metabolic activation** the percentage of small colonies in the negative controls was found to be 11.1 % and 15.2 %. The percentage of small colonies of the positive control B[*a*]P was found to be 41.5 %. In the highest dose groups 21.8 % (203 µg/mL), 15.3 % (207 µg/mL) and 22.2 % (208 µg/mL) of small colonies were found. As none of the values exceeded 40 %, all dose groups were considered as not clastogenic.
In **experiment II without metabolic activation** the percentage of small colonies in the negative controls was found to be 19.0 % and 13.2 %. The percentage of small colonies of the positive control MMS was found to be 47.8 %. In the highest dose groups 18.3 % (54 µg/mL), 21.1 % (60 µg/mL) and

21.7 % (64 µg/mL) of small colonies were found. As none of the values exceeded 40 %, all dose groups were considered as not clastogenic.

The test was regarded acceptable:

- At least three out of four 96-well plates from the TFT resistance-testing portion of the experiment are scorable.
- The cloning efficiency of the negative and/or solvent controls is in the range 65 % -120 %.
- The spontaneous mutant frequency in the negative and/or solvent controls is in the range 50-170 mutants per 10^6 cells
- The cell number of the negative/solvent controls should undergo 8-32-fold increase during a 2-day growth period (short-term treatment) or 32-180-fold increase during a 3-day growth period (long-term treatment)
- The clastogenic positive controls (MMS and B[a]P) have to produce an induced mutant frequency (total mutant frequency minus concurrent negative control mutant frequency) of at least 300 mutants per 10^6 cells with at least 40 % of the colonies being small colonies or with an induced small colony mutant frequency of at least 150 mutants per 10^6 cells The RTG must be greater than 10 %.

Conclusion

The test item RH-150721 is considered to be non-mutagenic in the *in vitro* mammalian cell gene mutation assay (Thymidine Kinase locus) in mouse lymphoma L5178Y cells under the experimental conditions reported. Reference mutagens were tested in parallel to the test item and demonstrated the sensitivity of the test system. The study is valid.

Study 3 - *In vitro* micronucleus assay RH-141452

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The study performed according to internationally recognised guideline and in GLP conditions is acceptable. The metabolite RH-141452 is non-mutagenic with respect to clastogenicity and/or aneugenicity in *in vitro* mammalian micronucleus assay in Chinese Hamster V79 Cells.

Reference:	**KCP 7.4/03**
Report	Donath, C., 2019: *In vitro* mammalian micronucleus assay in Chinese Hamster V79 Cells with RH-141452 Gowan Crop Protection Ltd., UK Eurofins BioPharma GmbH, Germany, Report No. 188616, GLP, Not published
Guideline(s):	OECD 487 (2016) EEC B.49 (2017)
Deviations:	No
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-141452 (632031-P1050-44)
Purity:	96.27 % (w/w)
Test cells	Chinese Hamster V79 (ATCC, CCL-93) cells *in vitro* Eurofins Munich, Germany culture Three or four day-old stock cultures (in exponential growth), more than 50% confluent, were rinsed with Ca-Mg-free PBS solution, trypsinised with a 0.05% trypsin solution in Ca-Mg-free PBS at 37°C for 5 min. By adding complete culture medium the detachment was stopped and a single cell suspension was prepared.
Medium:	Culture medium: MEM medium supplemented with 10 % fetal bovine serum (FBS) 100 U/100 µg/mL penicillin/streptomycin 2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B Treatment medium (short-term exposure): Complete culture medium with 0 % fetal bovine serum (FBS) After treatment medium (long-term exposure): Complete culture medium with 10 % fetal bovine serum (FBS) 1.5 µg/mL cytochalasin B
Controls	
Negative:	Culture medium
Solvent:	DMSO (1 %, v/v)
Positive:	**Clastogenic Controls** *Without metabolic activation* MMS (mmethylmethanesulfonate) disolved MEM Concentration: 25 µg/mL *With metabolic activation* CPA (cyclophosphamide) dissolved in MEM Concentration: 2.5 µg/mL **Aneugenic Controls** *Without metabolic activation* Colchicine dissolved in MEM Concentration: 0.08-2.0 µg/mL
Activation	S9 liver microsomal fraction from Eurofins Munich, Germany, derived from male Wistar rats. Lot 210918, protein concentration 35.7 mg/mL, checked for biological activity and sterility.

Test material (Lot/Batch No.)	RH-141452 (632031-P1050-44)
S 9 mix:	S9 cofactor solution with final protein concentration of 0.75 mg/mL, 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate and 5 mM NADP in 100 mM sodium-phosphate-buffer pH 7.4
Test concentrations	**Experiment I** without metabolic activation: 10, 20, 30, 40, 50, 60, 65, 70 µg/mL with metabolic activation: 25, 50, 100, 120, 130, 140, 150, 160, 175 µg/mL **Experiment II** without metabolic activation: 20, 30, 40, 50, 60, 65, 70, 75, 80, 85, 90 µg/mL **For the microscopic analyses of micronuclei frequencies** Experiment I with short-term exposure (4 h): without metabolic activation: 40, 50, 60, 65 µg/mL with metabolic activation: 120, 140, 150 µg/mL Experiment II with long-term exposure (24 h): without metabolic activation: 20, 50, 60 µg/mL
Post exposure observation period	4-72 h
Remarks	None

The *in vitro* micronucleus assay has been performed to assess the possible potential of RH-141452 to induce clastogenic and aneugenic activity in cells that have undergone cell division during or after exposure.

The test item was dissolved in DMSO and diluted in cell culture medium to reach a final concentration of 1 % (v/v) DMSO in the samples. The solvent was compatible with the survival of the cells and the S9 activity.

Based on the results of a pre-test, experiments with and without metabolic activation were performed in duplicate cultures:

Experiment I

without metabolic activation: 10, 20, 30, 40, 50, 60, 65, 70 µg/mL

with metabolic activation: 25, 50, 100, 120, 130, 140, 150, 160, 175 µg/mL

Experiment II without metabolic activation: 20, 30, 40, 50, 60, 65, 70, 75, 80, 85, 90 µg/mL

The following study design was performed:

	Without S9		**With S9**
	Experiment I	**Experiment II**	**Experiment I**
Exposure period	4 h	24 h	4 h
Cytochalasin B exposure	20 h	23 h	20 h
Preparation interval	24 h	24 h	24 h
Total culture period*	72 h	72 h	72 h

* Exposure started 48 h after culture initiation

Experiment I: Exponentially growing V79 cells were seeded into 25 cm^2 cell culture flasks (two flasks per test group). Approx. 50 000 cells were seeded per cell culture flask, containing 5 mL complete culture medium (minimum essential medium supplemented with 10% FBS). After an attachment period of

approx. 48 h, the complete culture medium was removed and subsequently the test item was added to the treatment medium in appropriate concentrations. The cells were incubated with the test item for 4 h in presence or absence of metabolic activation. At the end of the incubation, the treatment medium was removed and the cells were washed twice with PBS. Subsequently, the cells were incubated in complete culture medium + 1.5 µg/mL cytochalasin B for 20 h at 37 °C.

Experiment II: If negative or equivocal results are obtained, they should be confirmed using continuous treatment (long-term treatment) without metabolic activation. Approx. 50 000 exponentially growing V79 cells were seeded in 25 cm^2 cell culture flasks in absence of metabolic activation. After an attachment period of approx. 48 h the test item was added in complete culture medium. 1 h later 1.5 µg/mL cytochalasin B were added and the cells were incubated for 23 h at 37 °C. At the end of the treatment the cell culture medium was removed and the cells were prepared for microscopic analysis.

At the end of the cultivation, the complete culture medium was removed. Subsequently, cells were trypsinated and resuspended in about 9 ml complete culture medium. The cultures were transferred into tubes and incubated with hypotonic solution (0.4% KCl) for some minutes at room temperature. Prior to this an aliquot of each culture was removed to determine the cell count by a cell counter (ALSystems). After the treatment with the hypotonic solution the cells were fixed with methanol + glacial acetic acid (3+1). The cells were resuspended gently and the suspension was dropped onto clean glass slides. Consecutively, the cells were dried on a heating plate. Finally, the cells were stained with acridine orange solution.

For each experimental point, at least 2000 binucleated cells per concentration (1000 binucleated cells per slide) were analysed for micronuclei according to the criteria of Fenech (2000), i.e. clearly surrounded by a nuclear membrane, having an area of less than one-third of that of the main nucleus, being located within the cytoplasm of the cell and not linked to the main nucleus via nucleoplasmic bridges. Mononucleated and multinucleated cells and cells with more than six micronuclei were not considered.

A cytokinesis block proliferation index (CBPI) and % cytostasis were calculated.

A test item was considered clearly positive if, in any of the experimental conditions examined:

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative/solvent control,
- the increase is concentration-related in at least one experimental condition when evaluated with an appropriate trend test and
- any of the results are outside the distribution of the historical negative/solvent control data (e.g. poisson-based 95% control limits).

Results and discussions

The pH-value detected with the test item was within the physiological range (pH 7.0 ± 0.4). No precipitate of the test item was noted.

Cytotoxicity: In experiment I without metabolic activation no increase of the cytostasis above 30% was noted up to 50 µg/mL. At 60 µg/mL a cytostasis of 41% and at 65 µg/mL a cytostasis of 60% was noted. In experiment I with metabolic activation no increase of the cytostasis above 30% was noted up to 120 µg/mL. At 140 µg/mL a cytostasis of 42% and at 150 µg/mL a cytostasis of 57% was observed. In experiment II without metabolic activation no increase of the cytostasis above 30% was noted up to 20 µg/mL. At 50 µg/mL a cytostasis of 47% and at 60 µg/mL a cytostasis of 53% was observed.

Clastogenicity / aneugenicity: In experiment I without metabolic activation the micronucleated cell frequency of the negative control (0.65%) was within the historical control limits of the negative control (0.37% – 1.37%) and the micronucleated cell frequency of the solvent control (0.50%) was within the historical control limits of the solvent control (0.47% – 1.48%). The mean values of micronucleated cells found after treatment with the test item were 0.50% (40 µg/mL), 0.35% (50 µg/mL), 1.05% (60 µg/mL) and 0.55% (65 µg/mL). The numbers of micronucleated cells were within or below the historical control limits of the solvent control and did not show a biologically relevant increase compared to the concurrent solvent control. The concentration of 60 µg/mL showed a statistically significant increase of micronuclei

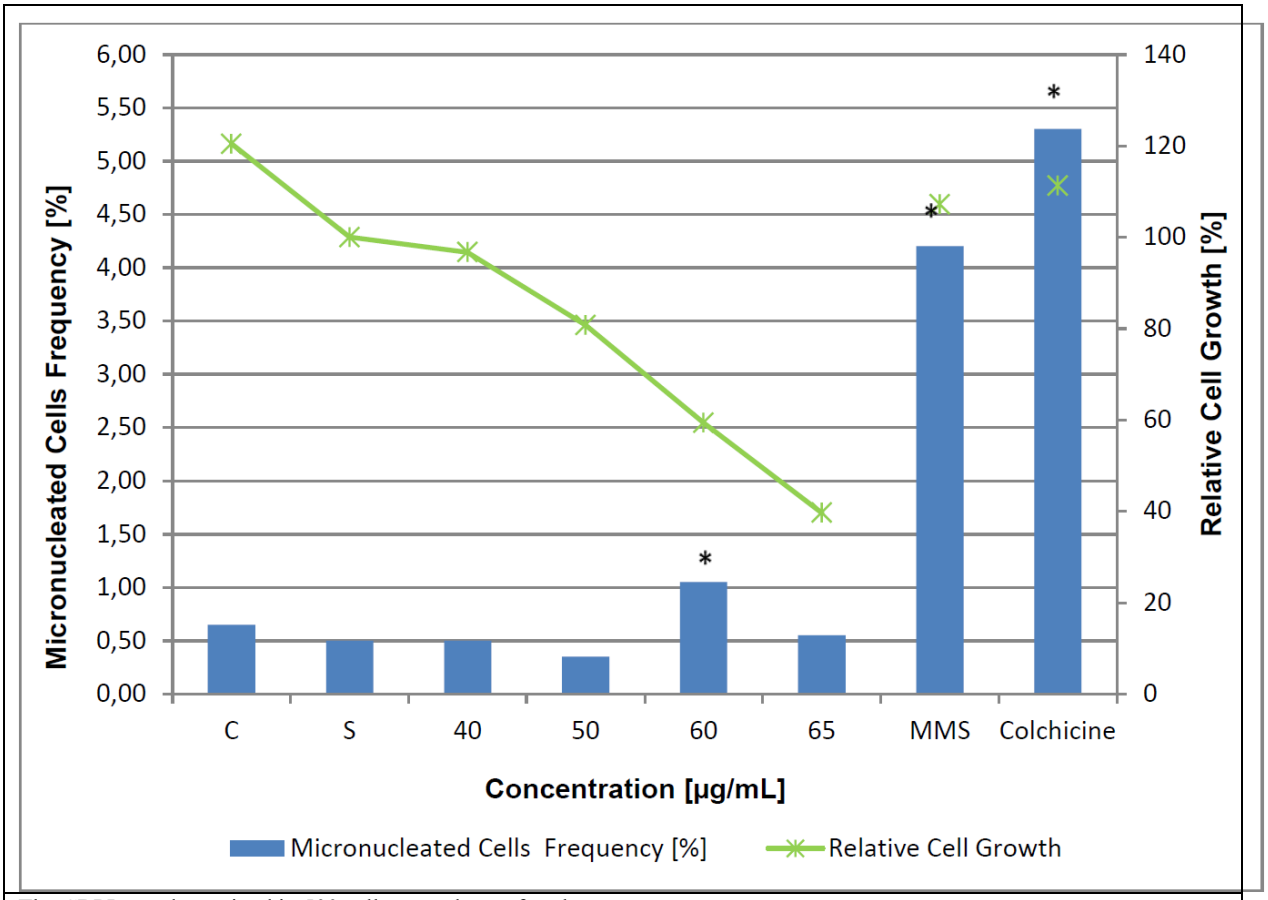
compared to the concurrent solvent control. However, the number of micronucleated cells was within the historical control limits of the solvent control. Therefore, this increase was regarded as not biologically relevant.

In experiment I with metabolic activation the micronucleated cell frequency of the negative control (0.55%) was within the historical control limits of the negative control (0.42% – 1.64%) and the micronucleated cell frequency of the solvent control (0.80%) was within the historical control limits of the solvent control (0.35% – 1.75%). The mean values of micronucleated cells found after treatment with the test item were 1.25% (120 µg/mL), 0.95% (140 µg/mL) and 0.85% (150 µg/mL). The numbers of micronucleated cells were within the historical control limits of the solvent control and did not show a biologically relevant increase compared to the concurrent solvent control. The concentration of 120 µg/mL showed a statistically significant increase of micronuclei compared to the concurrent solvent control. However, the number of micronucleated cells was within the historical control limits of the solvent control. Therefore, this increase was regarded as not biologically relevant.

In experiment II without metabolic activation the micronucleated cell frequency of the negative control (1.35%) was within the historical control limits of the negative control (0.37% – 1.37%) and the micronucleated cell frequency of the solvent control (1.00%) was within the historical control limits of the solvent control (0.47% – 1.48%). The mean values of micronucleated cells found after treatment with the test item were 1.55% (20 µg/mL), 1.30% (50 µg/mL) and 1.18% (60 µg/mL). The numbers of micronucleated cells for the concentrations of 50 and 60 µg/mL were within the historical control limits of the solvent control and did not show a biologically relevant increase compared to the concurrent solvent control. The micronuclei frequency for the concentration of 20 µg/mL was slightly above the upper historical control limit of the solvent control. However, since the exceedance was marginal and the two higher concentrations evaluated showed numbers of micronucleated cells within the historical control limits, this increase was considered as not biologically relevant.

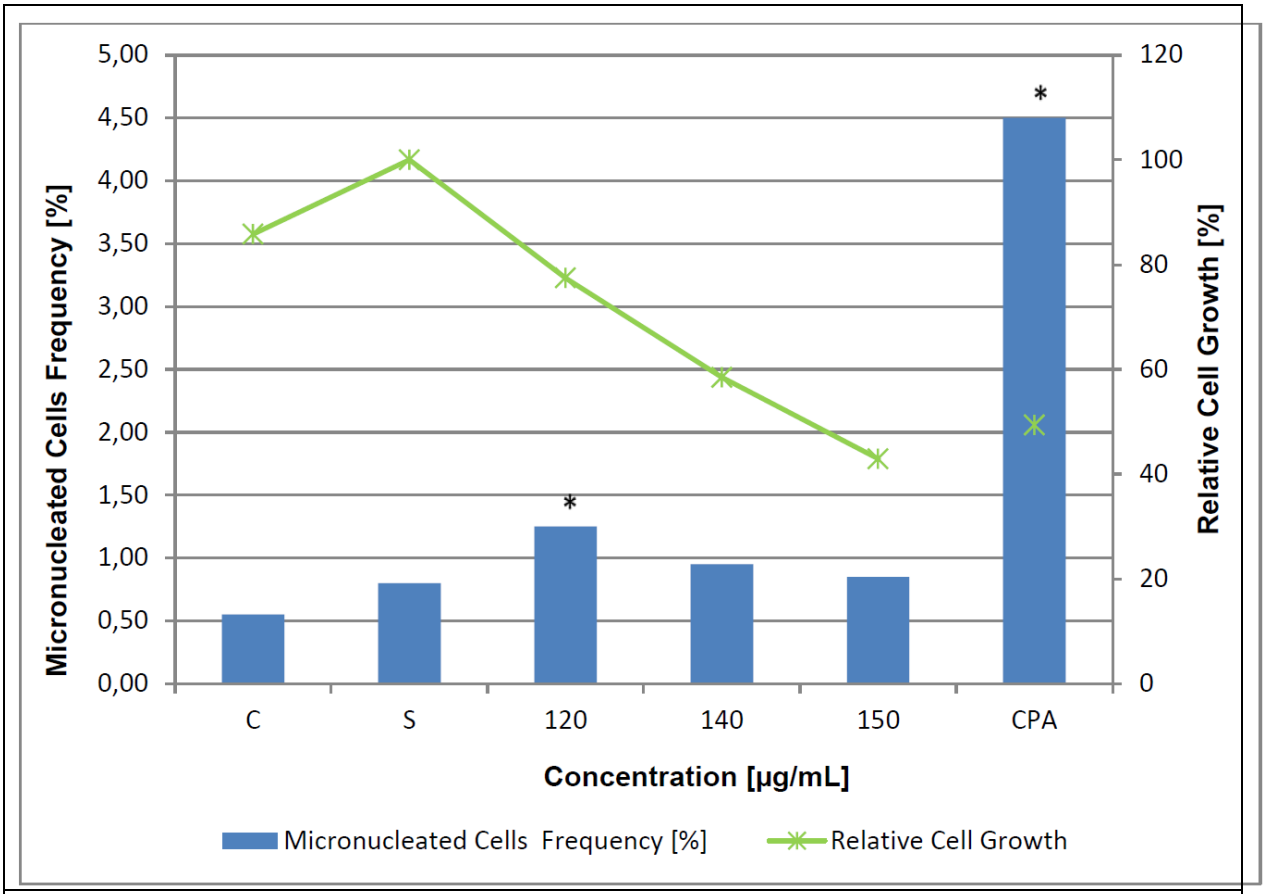
In experiment I with and without metabolic activation a statistically significant increase of cells with micronuclei was noted at a concentration of 120 µg/mL and 60 µg/mL, respectively. However, the frequency of micronucleated cells was within the historical control limits of the solvent control, did not follow a dose-response relationship and were not reproducible in experiment II. No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the concentration groups of the test item evaluated in experiment II without metabolic activation. Therefore, the increases in experiment I were regarded as not biologically relevant.

No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II. MMS (25 µg/mL) and CPA (2.5 µg/mL) were used as clastogenic controls and colchicine as aneugenic control (0.08 and 2.0 µg/mL). They induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.



The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.
Micronucleated cell frequency was determined in 2000 cells (1000 cells per slide).
C: Negative control (culture medium)
S: Solvent control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, positive control (without metabolic activation) [25 µg/mL]
Colchicine: Positive control (without metabolic activation) [2.0 µg/mL]
CBPI: Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative cell growth: 100 x ((CBPI Test conc – 1) / (CBPI control -1))
c_1: mononucleate cells
c_2: binucleate cells
c_x: multinucleate cells
n: total number of cells
*: statistically significant increase of micronucleated cells

Figure A 3: Micronucleus frequency and growth rate in the main experiment without metabolic activation



The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.
Micronucleated cell frequency was determined in 2000 cells (1000 cells per slide), except for solvent control and at a concentration of 120 µg/mL (2000 cells per slide).
C: Negative control (culture medium)
S: Solvent control (DMSO 1% v/v in culture medium)
CPA: Cyclophosphamide, positive control (with metabolic activation) [2.5 µg/mL]
CBPI: Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative cell growth: 100 x ((CBPI test conc – 1) / (CBPI control -1))
c_1: mononucleate cells
c_2: binucleate cells
c_x: multinucleate cells
n: total number of cells
*: statistically significant increase of micronucleated cells

Figure A 4: Micronucleus frequency and growth rate in the main experiment with metabolic activation

The test was regarded acceptable:

- The concurrent negative/solvent control was acceptable for addition to the laboratory historical negative/solvent control database.
- Concurrent positive controls induced responses that were compatible with those generated in the laboratory's historical positive control data base and produced a statistically significant increase compared to the concurrent negative/solvent control.
- Cell proliferation criteria in the negative/solvent control according to OECD 487 have been fulfilled (all experimental conditions were tested unless one resulted in positive results, an adequate number of cells and concentrations was analysable, criteria for the selection of top concentration were fulfilled).
- All experimental conditions were tested unless one resulted in positive results.
- Adequate number of cells and concentrations were analysable.
- Criteria for the selection of top concentration were fulfilled.

Conclusion

Under the experimental conditions of an *in vitro* mammalian micronucleus assay with Chinese Hamster V79 cells the test item did not induce structural and/or numerical chromosomal damage. Therefore, RH-141452 is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity.
Positive controls demonstrated the sensitivity of the test system.
The study is valid.

Study 4 - *In vitro* micronucleus assay RH-150721

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The study performed according to internationally recognised guideline and in GLP conditions is acceptable. The metabolite RH-150721 is non-mutagenic with respect to clastogenicity and/or aneugenicity in *in vitro* mammalian micronucleus assay in Chinese Hamster V79 Cells.

Reference:	**KCP 7.4/04**
Report	Donath, C., 2017: *In vitro* mammalian micronucleus assay in Chinese hamster V79 Cells with RH-150721 Gowan Crop Protection Ltd., UK Eurofins BioPharma, Germany, Report No. 171361, GLP, Not published
Guideline(s):	OECD 487 (2016)
Deviations:	MMS (methylmethanesulfonate) at a final concentration of 25 µg/mL instead of EMS (ethylmethanesulfonate) at a final concentration of 400-1200 µg/mL was used in the study. This typing error has been recognised in time and did not influence the quality or integrity of the present study.
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-150721 (79942-4-16)
Purity:	98.07 % (w/w)
Test cells	Chinese Hamster V79 (ATCC, CCL-93) cells *in vitro* Eurofins Munich, Germany culture Three or four day-old stock cultures (in exponential growth), more than 50% confluent, were rinsed with Ca-Mg-free PBS solution, trypsinised with a 0.2% trypsin solution in Ca-Mg-free PBS at 37°C for 5 min. By adding complete culture medium the detachment was stopped and a single cell suspension was prepared.
Medium:	Culture medium: MEM medium supplemented with 10 % fetal bovine serum (FBS) 100 U/100 µg/mL penicillin/streptomycin 2 mM L-glutamine 25 mM HEPES 2.5 µg/mL amphotericin B Treatment medium (short-term exposure): Complete culture medium with 0 % fetal bovine serum (FBS) After treatment medium (long-term exposure): Complete culture medium with 10 % fetal bovine serum (FBS) 1.5 µg/mL cytochalasin B
Controls	
Negative:	Culture medium
Solvent:	DMSO (1 %, v/v)
Positive:	**Clastogenic Controls** *Without metabolic activation* MMS (mmethylmethanesulfonate) disolved MEM Concentration: 25 µg/mL *With metabolic activation* CPA (cyclophosphamide) dissolved in MEM Concentration: 2.5 µg/mL **Aneugenic Controls** *Without metabolic activation* Colchicine dissolved in MEM Concentration: 0.16-2.0 µg/mL
Activation	S9 liver microsomal fraction from Eurofins Munich, Germany, derived from male Wistar rats. Lot 0300217, protein concentration 32.4 mg/mL, checked for biological activity and sterility.

Test material (Lot/Batch No.)	RH-150721 (79942-4-16)
S 9 mix:	S9 cofactor solution with final protein concentration of 0.75 mg/mL, 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate and 5 mM NADP in 100 mM sodium-phosphate-buffer pH 7.4
Test concentrations	**Experiment I (short-term exposure, 4 h)** without metabolic activation: 10, 25, 50, 60, 70, 80, 90, 100, 110 and 120 µg/mL with metabolic activation: 25, 50, 100, 125, 150, 175, 200, 225, 250, 275 and 300 µg/mL **Experiment II (long-term exposure, 24 h)** without metabolic activation: 0.25, 0.5, 1.0, 2.5, 5, 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL **For the microscopic analyses of micronuclei frequencies** Experiment I with short-term exposure (4 h): without metabolic activation: 50, 70, 80 µg/mL with metabolic activation: 100, 125, 150, 175 µg/mL Experiment II with long-term exposure (24 h): without metabolic activation: 30, 50, 60 µg/mL
Post exposure observation period	4- 72 h
Remarks	None

The *in vitro* micronucleus assay has been performed to assess the possible potential of RH-150721 to induce clastogenic and aneugenic activity in cells that have undergone cell division during or after exposure.

The test item was dissolved in DMSO and diluted in cell culture medium to reach a final concentration of 1 % (v/v) DMSO in the samples. The solvent was compatible with the survival of the cells and the S9 activity.

Based on the results of a pre-test, experiments with and without metabolic activation were performed in duplicate cultures:

Experiment I (short-term exposure, 4 h)

without metabolic activation: 10, 25, 50, 60, 70, 80, 90, 100, 110 and 120 µg/mL

with metabolic activation: 25, 50, 100, 125, 150, 175, 200, 225, 250, 275 and 300 µg/mL

Experiment II (long-term exposure, 24 h) without metabolic activation: 0.25, 0.5, 1.0, 2.5, 5, 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL

The following study design was performed:

	Without S9		**With S9**
	Experiment I	**Experiment II**	**Experiment I**
Exposure period	4 h	24 h	4 h
Cytochalasin B exposure	20 h	23 h	20 h
Preparation interval	24 h	24 h	24 h
Total culture period*	72 h	72 h	72 h

* Exposure started 48 h after culture initiation

Experiment I: Exponentially growing V79 cells were seeded into 25 cm^2 cell culture flasks (two flasks per test group). Approx. 50 000 cells were seeded per cell culture flask, containing 5 mL complete culture medium (minimum essential medium supplemented with 10% FBS). After an attachment period of approx. 48 h, the complete culture medium was removed and subsequently the test item was added to the treatment medium in appropriate concentrations. The cells were incubated with the test item for 4 h in presence or absence of metabolic activation. At the end of the incubation, the treatment medium was removed and the cells were washed twice with PBS. Subsequently, the cells were incubated in complete culture medium + 1.5 µg/mL cytochalasin B for 20 h at 37 °C.

Experiment II: If negative or equivocal results are obtained, they should be confirmed using continuous treatment (long-term treatment) without metabolic activation. Approx. 50 000 exponentially growing V79 cells were seeded in 25 cm^2 cell culture flasks in absence of metabolic activation. After an attachment period of approx. 48 h the test item was added in complete culture medium. 1 h later 1.5 µg/mL cytochalasin B were added and the cells were incubated for 23 h at 37 °C. At the end of the treatment the cell culture medium was removed and the cells were prepared for microscopic analysis.

At the end of the cultivation, the complete culture medium was removed. Subsequently, cells were trypsinated and resuspended in about 9 ml complete culture medium. The cultures were transferred into tubes and incubated with hypotonic solution (0.4% KCl) for some minutes at room temperature. Prior to this an aliquot of each culture was removed to determine the cell count by a cell counter (ALSystems). After the treatment with the hypotonic solution the cells were fixed with methanol + glacial acetic acid (3+1). The cells were resuspended gently and the suspension was dropped onto clean glass slides. Consecutively, the cells were dried on a heating plate. Finally, the cells were stained with acridine orange solution.

For each experimental point, at least 2000 binucleated cells per concentration (1000 binucleated cells per slide) were analysed for micronuclei according to the criteria of Fenech (2000), i.e. clearly surrounded by a nuclear membrane, having an area of less than one-third of that of the main nucleus, being located within the cytoplasm of the cell and not linked to the main nucleus via nucleoplasmic bridges. Mononucleated and multinucleated cells and cells with more than six micronuclei were not considered.

A cytokinesis block proliferation index (CBPI) and % cytostasis were calculated.

A test item was considered clearly positive if, in any of the experimental conditions examined:

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative/solvent control,
- the increase is concentration-related in at least one experimental condition when evaluated with an appropriate trend test and
- any of the results are outside the distribution of the historical negative/solvent control data (e.g. poisson-based 95% control limits).

Results and discussions

The pH-value detected with the test item was within the physiological range (pH 7.0 ± 0.4). No precipitate of the test item was noted.

Cytotoxicity: In experiment I with metabolic activation no increase of the cytostasis above 30% was noted. In experiment I without metabolic activation no increase of the cytostasis above 30% was noted up to 50 pg/mL. At 70 pg/mL a cytostasis of 35% and at 80 pg/mL a cytostasis of 55% was noted. In experiment II without metabolic activation no increase of the cytostasis above 30% was noted up to 30 pg/mL At 50 pg/mL a cytostasis of 31% and at 60 pg/mL a cytostasis of 53% was noted.

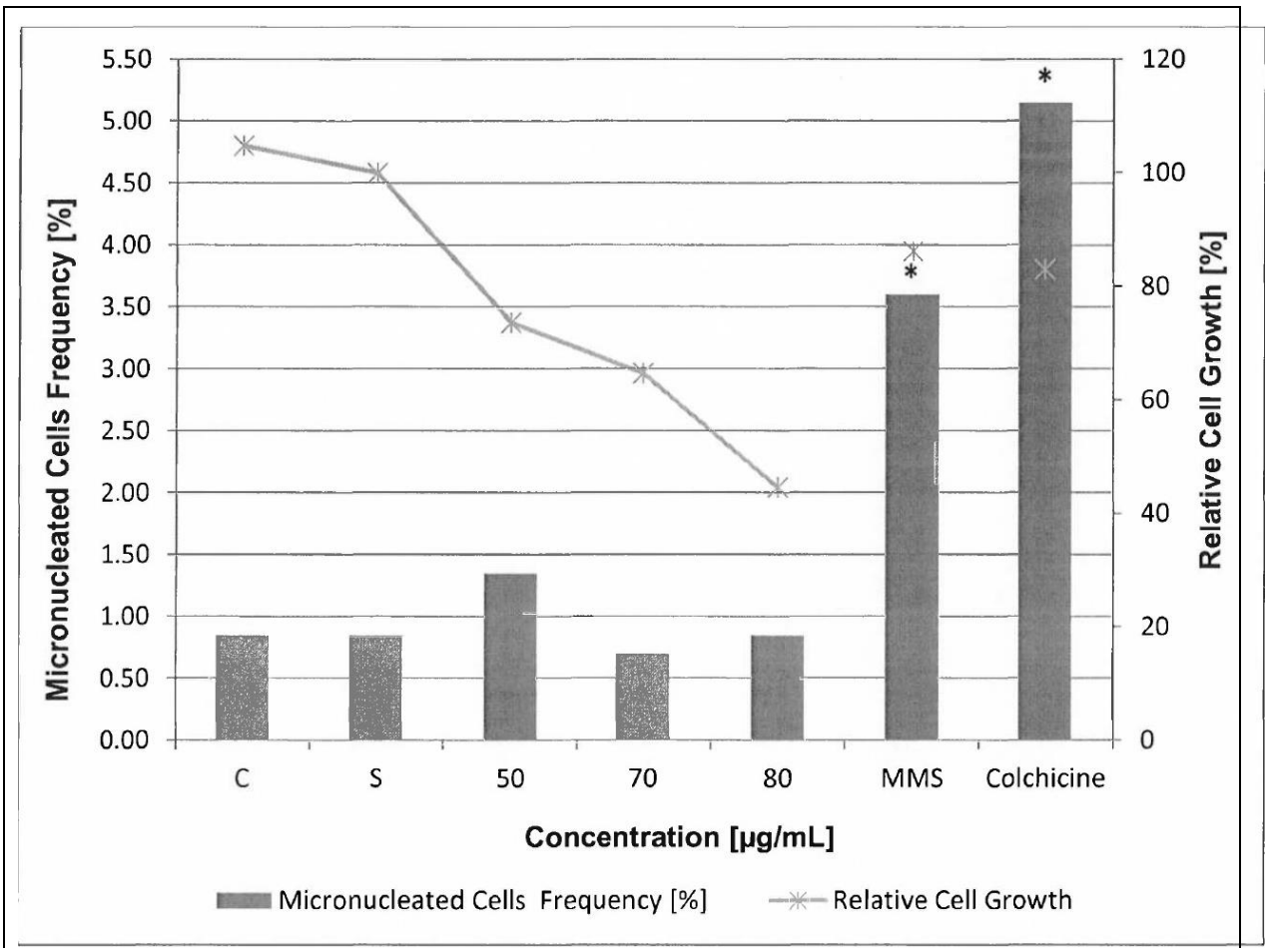
Clastogenicity / aneugenicity: In experiment I without metabolic activation the micronucleated cell frequency of the negative control (0.85%) and the solvent control (0.85%) were within the historical control limits of the negative control (0.39% - 1.40%) and the solvent control (0.45% - 1.55%), respectively. The mean values of micronucleated cells found after treatment with the test item were 1.35% (50 pg/mL), 0.70% (70 pg/mL) and 0.85% (80 pg/mL). The numbers of micronucleated cells were within the

historical control limits of the solvent control and did not show any biologically relevant increase compared to the concurrent solvent control.

In experiment I with metabolic activation the micronucleated cell frequency of the negative control (0.90%) and the solvent control (1.23%) were within the historical control limits of the negative control (0.37% - 1.68%) and the solvent control (0.23% - 1.88%), respectively. The mean values of micronucleated cells found after treatment with the test item were 1.35% (100 pg/mL), 0.90% (125 pg/mL), 1.30% (150 pg/mL) and 1.30% (175 pg/mL). The numbers of micronucleated cells were within the historical control limits of the solvent control and did not show any biologically relevant increase compared to the concurrent solvent control.

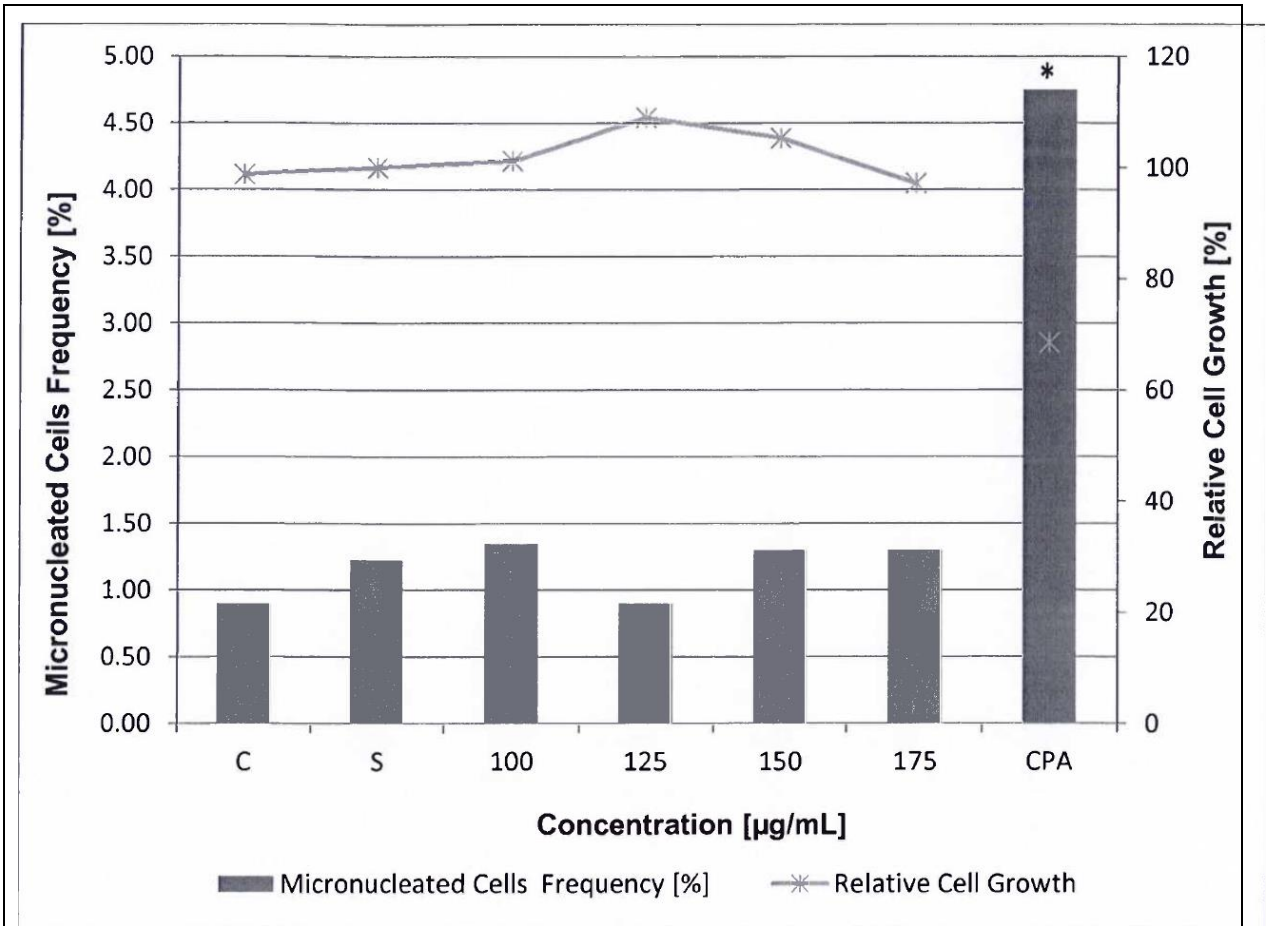
In experiment II without metabolic activation the micronucleated cell frequency of the negative control (0.95%) and the solvent control (1.05%) were within the historical control limits of the negative control (0.39% - 1.40%) and the solvent control (0.45% - 1.55%), respectively. The mean values of micronucleated cells found after treatment with the test item were 1.50% (30 pg/mL), 0.45% (50 pg/mL) and 0.73% (60 pg/ml). The numbers of micronucleated cells were within historical control limits of the solvent control and did not show any biologically relevant increase compared to the concurrent solvent control.

No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I and II. A Chi^2 test demonstrated no statistically significant increase in the frequency of micronucleated cells in experiment I and II. MMS (25 pg/mL) and CPA (2.5 pg/mL) were used as clastogenic controls and colchicine as aneugenic control (0.16 and 2.0 pg/mL). They induced distinct and statistically significant increases of the micronucleus frequency, demonstrating the validity of the assay.



The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.
Micronucleated Cell frequency was determined in 2000 cells (1000 cells per slide).
C: Negative control (culture medium)
S: Solvent control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, positive control (without metabolic activation) [25 µg/mL]
Colchicine: Positive Control (without metabolic activation) [2.0 µg/mL]
CBPI: Cytokinesis Block Proliferation Index, CBPI = ((ci x 1) + (c2 x 2) + (cx x 3))/n
Relative cell growth: 100 x ((CBPI rest conc - 1) / (CBPI control -1))
ci: mononucleate cells
C2~. binucleate cells
cx: multinucleate cells
n: total number of cells
*: statistically significant increase of micronucleated cells

Figure A 5: Micronucleus frequency and growth rate in the main experiment without metabolic activation



The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.
Micronucleated Cell Frequency was determined in 2000 cells (1000 cells per slide), except for solvent control (2000 cells per culture).
C: Negative control (culture medium)
S: Solvent control (DMSO 1% v/v in culture medium)
CPA: Cyclophosphamide, positive control (with metabolic activation) [2.5 µg/mL]
CBPI: Cytokinesis Block Proliferation Index, CBPI = ((c1 x 1) + (c2 x 2) + (cx x 3))/n
Relative Cell Growth: 100 x ((CBPI Test conc – 1) / (CBPI control -1))
c1: mononucleate cells
c2: binucleate cells
cx: multinucleate cells
n: total number of cells
*: statistically significant increase of micronucleated cells

Figure A 6: Micronucleus frequency and growth rate in the main experiment with metabolic activation

The test was regarded acceptable:

- The concurrent negative/solvent control was acceptable for addition to the laboratory historical negative/solvent control database.
- Concurrent positive controls induced responses that were compatible with those generated in the laboratory's historical positive control data base and produced a statistically significant increase compared to the concurrent negative/solvent control.
- Cell proliferation criteria in the negative/solvent control according to OECD 487 have been fulfilled (all experimental conditions were tested unless one resulted in positive results, an adequate number of cells and concentrations was analysable, criteria for the selection of top concentration were fulfilled).
- All experimental conditions were tested unless one resulted in positive results.
- Adequate number of cells and concentrations were analysable.
- Criteria for the selection of top concentration were fulfilled.

Conclusion

Under the experimental conditions of an *in vitro* mammalian micronucleus assay with Chinese Hamster V79 cells the test item did not induce structural and/or numerical chromosomal damage. Therefore, RH-150721 is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity.
Positive controls demonstrated the sensitivity of the test system.
The study is valid.

Study 5 - RH-141455: 2 days oral dietary pharmacokinetic

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The 2d oral study is acceptable. The metabolite RH-141455 was detectable in rat plasma after dietary exposure at concentrations of 0.3 to 1.5 µg/ml (AUC_{last}: 34.5 µg h/mL). The T_{max} of RH-141455 was observed on day 3 with a peak plasma concentration (C_{max}) of 1.4 µg/mL.

Reference:	**KCP 7.4/05**
Report	XXXX, 2019: RH-141455: 2 days oral dietary pharmacokinetic study in Sprague Dawley Rats XXXX, Report No. U-19044, No GLP, Not published
Guideline(s):	None (investigative study)
Deviations:	No
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-141455 (HHGCP017-00-1)
Purity:	99.6 % (w/w)

Vehicle:	rodent powder diet
Test organisms	Albino Rat, Sprague Dawley purchased from Envigo, The Netherlands
Age:	7-10 weeks
Body weight:	Body weights did not differ by more than 20% in the group at study initiation.
Acclimation period:	1 day
Water:	Tap water, *ad libitum*
Diet:	rodent powder diet *ad libitum*
Housing:	1 animal per cage in polycarbonate rat cages with bedding materials
Test design	
Total number of animals:	4 (males)
Environmental conditions	
Temperature:	21.6 to 22.6°C
Humidity:	Relative humidity ranged from 51 to 66%
Air changes:	17 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	15000 ppm in diet
Exposure time	2 days
Remarks	Designed as a pre-test to assess plasma exposures and pharmacokinetic profiles of RH- 141455 following oral dietary administration.

The study was designed to assess plasma exposures and pharmacokinetic profiles of RH- 141455 following oral dietary administration at 15000 ppm for two days in male Sprague Dawley rats. A total of 4 male rats were used. RH-141455 was formulated in powdered diet at concentration of 15000 ppm and fed from day 1 through day 3. The observations included daily morbidity/mortality check, clinical signs, body weights, feed consumption, bioanalytical & TK parameters (days 2 & 3), and gross pathology. A total of 10 rat plasma samples were analysed. Blood was collected via retro-orbital plexus puncture under mild isoflurane anaesthesia at time points from 4 pm on day 2 till 4 pm on day 3. Plasma was separated by centrifuging the whole blood sample and stored at <- 60°C until bioanalysis. The bioanalysis was performed by LC-MS/MS method. After extraction and centrifugation, the supernatant was transferred into auto sampler vials and volumes of 10 µL were injected on a HPLC column with LC-MS/MS detection. The samples were analysed using a calibration curve range from 0.064 µg/mL to 50.370 µg/mL. Pharmacokinetics parameters were evaluated using validated Phoenix® WinNonlin® (version 6.3) software.

Results and discussions

All rats in the study survived until scheduled sacrifice and no clinical signs were observed. Body weights were slightly increased on day 3 as compared to day 1. There were no test item related effects on body weight. The average feed consumption amounted to approximately 26 g/rat/day. There were no test item-related gross changes in any of the organs examined. Blood collection times were considered from the time of feed input. RH-141455 was detectable in RH-141455plasma after dietary exposure at concentrations of 0.3 to 1.5 µg/ml (AUC_{last}: 34.5 µg h/mL). The T_{max} of RH-141455 was observed on day 3 with a peak plasma concentration (C_{max}) of 1.4 µg/mL.

Table A 18: Pharmacokinetic parameters of RH-141455

Group No.	Dose	Sex	T_{max} (h)	C_{max} (µg/mL)	AUC_{last} (µg h/mL)	T_{last} (h)	C_{last} (ug/mL)
Group 1	15000 ppm	Male	53.5	1.4	34.5	53.5	1.4

Conclusion

The plasma pharmacokinetics profile of RH-141455 was determined when formulated powdered diet was fed to male Sprague Dawley rats at 15000 ppm for two days. No test item related effects were seen. Evidence of systemic plasma exposure to RH-141455 was observed ($AUCl_{ast}$: 34.5 µg h/mL), the maximum concentration was observed at 53.5 hours (on day 3).

Study 6 - RH-141455: 14 days oral dietary toxicity

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The 14d oral study performed according internationally recognised guidelines and in GLP conditions is acceptable. There was no morbidity or mortality or adverse clinical sign up to 15000 ppm in both sexes. The No-Observed-Adverse-Effect Level (NOAEL) of RH-141455 was found to be 5000 ppm, which is equivalent to 368 mg/kg body weight/day for the males. It is also noted that in REVIEW OF THE EXISTING MRLs FOR ZOXAMIDE AND SETTING OF AN IMPORT TOLERANCE FOR BULB VEGETABLES (EFSA Journal. 2023;21:e8427) for **RH-141455**, an ADI of 0.3 mg/kg bw per day was derived, based on the NOAEL of 368 mg/kg bw per day for reduced body weight gain in males in a 14-day toxicity study in rats and applying an UF of 1000. This ADI of 0.3 mg/kg bw per day is supported by the lowest observed adverse effect level (LOAEL) of 924 mg/kg bw per day in the 90-day toxicity study in rats and applying an additional UF of 3 to account for the LOAEL. Thus ADI for RH-141455 is set as used in this registration report.

Reference:	**KCP 7.4/6**
Report	XXXX, 2020: RH-141455: 14-day oral dietary dose range finding study in Sprague Dawley rats XXXX, Report No. U-19071, No GLP, Not published
Guideline(s):	OECD No 407 (2008) EEC B.7 (2008) OPPTS 870.3050 (2000) (except for duration of dosing)
Deviations:	The blood collection for toxicokinetics on day 2 (6 P.M) & day 3 (6 A.M) and on day 14 (6 A.M) was delayed between 1- 18 minutes from the

scheduled time (± 10 minutes) across the groups. However, this deviation is regarded to have no impact on the integrity of the study.

Acceptability: Yes

Duplication (if vertebrate study) No

Materials and methods

Test material (Lot/Batch No.)	RH-141455 (HHGCP017-00-1)
Purity:	99.6 % (w/w)
Vehicle:	Teklad Global 18% Protein Rodent Diet (meal) manufactured by Envigo
Test organisms	Albino Rat, Sprague Dawley purchased from Envigo, Italy
Age:	6-7 weeks
Body weight:	Male:199.25 – 228.38 g Female: 145.82 – 176.08 g
Acclimation period:	3 days
Water:	Tap water, *ad libitum*
Diet:	Teklad Global 18% Protein Rodent Diet (meal) *ad libitum* (except overnight fasting prior to clinical pathology blood collection) manufactured by Envigo
Housing:	2 per sex per cage in polycarbonate rat cages with bedding materials
Test design	
No. of groups:	4
No. of animals/group:	6 rats/sex/group
Number of animals:	48 (24 Males + 24 Females)
Extra animals:	6 (3 Males + 3 Females)
Total number of animals:	54 (27 Males + 27 Females)
Environmental conditions	
Temperature:	21.0 to 24.1℃
Humidity:	Relative humidity ranged from 45 to 68%
Air changes:	16 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	1000, 5000 and 15000 ppm in diet
Exposure time	14 days
Remarks	Designed as range-finding study for the 90 days oral toxicity study.

In a dose range finding study, RH-141455 was administered to rats for 14 days at levels of 1000, 5000 and 15000 ppm for male and female to groups of Sprague Dawley rats (6 rats/sex/group). The dose in ppm were equivalent to 76, 368 and 1123 mg/kg body weight/day in males and 86, 457 and 1069

mg/kg body weight/day in females, respectively. The control group was fed with basal diet for same duration of study.

Table A 19: Study design

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	6	6
2	1000	76	86	6	6
3	5000	368	457	6	6
4	15000	1123	1069	6	6

There were 6 animals of each sex per group. Males and females were randomised into 4 dose groups using Pristima, based on body weight to control bias. RH-141455 was administered in the diet for 14 days to Sprague Dawley rats – at 0, 1000, 5000 and 15000 ppm. Husbandry conditions were in accordance with the recommendations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

The required amount of test material was weighed and mixed with the diet using electric mixer. Diet were prepared approximately weekly. Prepared diets were stored at room temperature before use. The concentration of the test substance in the feed was analytically verified weekly by reverse phase HPLC-DAD. The method has been validated according to SANCO/3029/99 rev. 4 (2000); see analytical method validation report no. U-19069 for the estimation of RH-141455 in rat feed.

The animals were observed twice daily for morbidity and mortality. All rats were observed for clinical signs at least once daily. Detailed clinical examinations were done once on day 1 of dosing and weekly thereafter.

Body weights were recorded on days 1, 2, 4, 8, 11 and 14.

Food consumption and compound intake was recorded on days 1, 2, 4, 8, 11. Food left over was measured on days 4, 8, 11 and 14. Test substance intake was calculated.

Ophthalmoscopic examination was not reported.

Blood samples were collected from three rats per sex per time point as per the sampling schedule. Blood was collected via retro-orbital plexus puncture under mild isoflurane anaesthesia. The sampling intervals were as follows: 6 P.M (Day 2 and 13) and 6 A.M, 9 A.M, 12 P.M and 6 P.M on Day 3 and 14. Toxicokinetics evaluation was performed by validated Phoenix® WinNonlin® software (Pharsight Corporation, USA).

Blood plasma samples prepared with K_2EDTA as anti-coagulant and analysed by LC-MS/MS for RH-141455 and an internal standard (tolbutamide). A total of 186 (96 for day 1 and 90 from day 14) samples were analysed. The method has been validated according to SANCO/3029/99 rev. 4 (2000) for a concentration range of 0.064 µg/mL to 50.007 µg/mL.

Blood was collected from all animals at the terminal sacrifice (day 15) for haematology, coagulation and clinical chemistry evaluations. The animals were fasted overnight prior to terminal blood collection. Blood was collected via retro-orbital plexus puncture of right eye under mild isoflurane anaesthesia. The following haematology, coagulation and clinical chemistry parameters were measured: haematology – red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, reticulocyte count, total white blood cell count, leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils); coagulation – active partial thromboplastin time, prothrombin time and fibrinogen; clinical chemistry – albumin/globulin ratio, alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, creatinine, creatinine

kinase, gamma-glutamyl trans-peptidase, globulin, glucose, high-density lipoprotein, inorganic phosphorus, low-density lipoprotein, potassium, sodium, total bilirubin, total cholesterol, total protein and triglycerides.

Urine was collected from all animals at the end of dosing phase (day 15). During urine collection, animals were fasted overnight in metabolic cages. The following parameters were measured: volume, colour, appearance, glucose, bilirubin, ketone, blood, pH, protein, urobilinogen, nitrite, leukocyte and specific gravity.

Gross necropsy was conducted on all animals at the end of dosing phase (day 15). The following organs were collected, weighed, assessed histopathologically after fixation in 10% neutral buffered formalin and staining with haematoxylin and eosin: adrenals, brain, epididymites, heart, kidneys, liver, ovaries, pituitary, prostate, seminal vesicles and coagulating glands, spleen, testes, thymus, thyroid with parathyroid and uterus with cervix. The other organs or tissues such as aorta, biceps femoris muscles, cecum, colon, diaphragm, duodenum, oesophagus, eyes, femur bone with joint, gastrocnemius muscle, gross changes, harderian glands, ileum with Peyer's patches, jejunum, lungs, lymph nodes (mandibular and mesenteric), mammary gland, optic nerve, oviducts, pancreas, Quadriceps muscle, rectum, salivary glands (mandibular, sublingual and parotid), sciatic nerve, skin (inguinal region), soleus muscle, spinal cord, sternum with marrow, stomach, tongue, trachea, urinary bladder and vagina, were also examined histopathologically. Histopathology was performed on all tissues of animals from control and high dose groups. Peer review was performed by another in-house pathologist.

For comparative statistics, data were evaluated using Levene's Test for homogeneity of variance, with significance at 5% level. Data determined to be homogeneous was evaluated for analysis of variance (ANOVA). When the ANOVA verified significance at 5% level, pairwise comparisons of each treated group with the control group was made using a Dunnett Test, to identify statistical differences (at 5% level). When the data were found to be non-homogeneous, ANOVA was done using suitable transformation. When the homogeneity tests were significant even after transformation, data were evaluated using a Kruskal-Wallis Test for group factor significance. When significance (at 5% level) existed between groups, Dunn's pairwise comparison was performed.

Results and discussions

The dose formulation analysis results of Day 1 were within the acceptable limits i.e., 99.60 to 100.94 % (< ±20 % of nominal concentrations). The control samples analysed did not show presence of test item.

There were no treatment-related clinical signs of toxicity observed in animals at any dose.

No mortalities occurred in the test animals at any dose level.

No test substance-related changes were noticed in body weight and body weight gain parameters in any of the dose groups when compared to control group.

There were no test substance-related changes in feed consumption in any of the dose groups when compared to control group. Statistically significant increase in feed consumption on Days 4-8 (27%) was noticed in low dose females (1000 ppm) when compared to control group. Above mentioned changes in feed consumption were considered as an incidental, as the magnitude of changes were minimal and occurred during dosing phase. The test substance intake achieved over the entire treatment period (Day 1 - 14) for 1000, 5000 and 15000 ppm concentration levels in diet was equivalent to 76, 368 and 1123 mg/kg body weight/day in males and 86, 457 and 1069 mg/kg body weight/day in females, respectively.

There were no test substance-related changes in haematology and coagulation parameters in the study. Minimal but statistically significant differences in the haematology parameters (RBC, HGB, HCT, WBC and lymphocytes) in the low dose (1000 ppm) females compared to the concurrent controls were considered incidental because of the lack of dose-response relationship.

There were no test substance-related changes in clinical chemistry parameters in the study.

There were no test substance-related changes in urinalysis parameters in the study.

There were no test substance-related changes in organ weight changes in the study. All changes in organ weight parameters were considered incidental and not test substance-related because the magnitude of changes was comparable to concurrent controls and/or lacked a dose-response relationship. Variations in the uterine with cervical weights among the treated groups compared the concurrent controls were likely related to the changes during normal reproductive cycle.

There were no test substance-related gross changes in the study. Uterine dilation in two controls and one mid dose (5000 ppm) females correlated with the microscopic finding of luminal dilation of the uterine horns consistent with changes associated with normal physiological reproductive cycle. There were no test substance-related microscopic findings in the study. All microscopic changes were considered incidental or spontaneous and not related to the test substance, because they were randomly distributed across the test substance and control groups and were generally observed in the rats of this age and strain (McInnes, 2012[1]).

Following oral feeding of RH-141455 in rats, T_{max} was observed at 42 hours in males and 42 to 54 hours in females for day 2. For day 13, T_{max} was observed at 294 to 306 hours in males and 294 to 309 hours in females. Plasma concentrations of RH-141455 were observed at all time points in treated groups. T_{last} was observed at 54 hours for day 2 and 318 hours for day 13. Increase in AUC_{24h} and C_{max} were less than dose proportional over 1000 to 15000 ppm dose range in males and females in day 2 and day 13. No sex related difference was observed in RH-141455 concentrations. Repeat dose feeding of RH-141455 in diet showed evidence of systemic plasma exposure to RH-141455 and increase in exposure was less than dose proportional at 1000, 5000 and 15000 ppm in Sprague Dawley rats. RH-141455 exposure found comparable between males and females. No accumulation of RH-141455 was observed in male and female rats on day 13 following daily feeding for 2 weeks.

Table A 20: The toxicokinetic parameters of RH-141455

Day Nominal	Sex	Dose in ppm (mg/kg/day)	T_{max} in h (time from start of exposure)	C_{max} (ug/mL)	AUC_{24h} (h*ug/mL)	T_{last} in h (time from start of exposure)	C_{last} (ug/mL)
Day 2	Male	1000 (76)	12 (42)	0.700	13.33	24 (54)	0.493
		5000 (368)	12 (42)	1.480	28.70	24 (54)	1.047
		15000 (1123)	12 (42)	3.064	68.84	24 (54)	1.948

[1] McInnes, E. F. (2012). Background lesions in laboratory animal, A color atlas. Chapter 2 Wistar and Sprague-Dawley rats (pp. 17-36). Saunders Elsevier

Day Nominal	Sex	Dose in ppm (mg/kg/day)	T_{max} in h (time from start of exposure)	C_{max} (ug/mL)	AUC_{24h} (h*ug/mL)	T_{last} in h (time from start of exposure)	C_{last} (ug/mL)
Day 2	Female	1000 (86)	15 (45)	0.596	12.79	24 (54)	0.445
		5000 (442)	24 (54)	1.287	28.30	24 (54)	1.287
		15000 (1069)	12 (42)	2.650	52.68	24 (54)	2.218
Day 13	Male	1000 (76)	0 (294)	1.152	16.23	24 (318)	0.505
		5000 (368)	0 (294)	0.986	21.80	24 (318)	0.977
		15000 (1123)	12 (306)	2.378	51.38	24 (318)	2.177
Day 13	Female	1000 (86)	15 (309)	0.598	11.34	24 (318)	0.416
		5000 (442)	0 (294)	1.096	21.81	24 (318)	0.911
		15000 (1069)	0 (294)	2.606	50.62	24 (318)	2.083

Conclusion

In a dose range finding study, RH-141455 was administered to rats for 14 days at levels of 1000, 5000 and 15000 ppm for male and female to groups of Sprague Dawley rats (6 rats/sex/group). The dose in ppm were equivalent to 76, 368 and 1123 mg/kg body weight/day in males and 86, 457 and 1069 mg/kg body weight/day in females, respectively. The control group was fed with basal diet for same duration of study.

There was no morbidity or mortality or adverse clinical sign up to 15000 ppm in both sexes. There were no test material-related changes in mean body weight and food consumption in both sexes compared with control group animals. There was no test item related change in hematology, coagulation, clinical chemistry and urinalysis parameter. No test item related gross findings, organ weight changes or histopathology findings were observed in the study. The No-Observed-Adverse-Effect Level (NOAEL) of RH-141455 was found to be 15000 ppm, which is equivalent to 1123 and 1069 mg/kg body weight/day for the males and females, respectively.

Study 7 - RH-141455: 90 days oral dietary toxicity

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The 90-day oral dietary study performed according internationally recognised guidelines and in GLP conditions is acceptable. LOAEL for metabolite RH-141455 was 16000 ppm, which is equivalent to 924 and 1119 mg/kg body weight/day for the males and females, respectively, based on: -Decrease in body weight gain in males (-10.1%) compared to control group between day 1 and day 90, which is adverse; - Increased incidence of soft stool in both sexes (and cecal enlargement in 8 of 10 males and 3 of 10 females) accompanied by signs of dehydration (significant increase in serum creatinine in both sexes (m: +12%, f: +12%) and significant increase in BUN in females (+19%), decrease in mean urine volume and increase in urine specific gravity). The lowest observed adverse effect level (LOAEL) of 924 mg/kg bw per day in the 90-day toxicity study in rats when applied an additional UF of 3 to account

	for the LOAEL supports ADI of 0.3 mg/kg bw per day.

Reference: **KCP 7.4/07**

Report: XXXX, 2020: RH-141455: 90-day oral dietary toxicity study with toxico-kinetics and 28-day recovery period in Sprague Dawley rats
XXXX, Report No. U-19102, GLP, Not published

Guideline(s): OECD 408 (2018)
OPPTS 870.3100 (1998)
ICH Harmonized Tripartite Guideline S3A (Toxicokinetics, 1994)
Notification No. 12-Nousan-8147, MAFF, Japan (2000)

Deviations: No

Acceptability: Yes

Duplication (if vertebrate study): No

Materials and methods

Test material (Lot/Batch No.)	RH-141455 (HHGCP017-00-1)
Purity:	99.6 % (w/w)
Vehicle:	Teklad Global 18% Protein Rodent Diet (meal) manufactured by Envigo
Test organisms	Albino Rat, Sprague Dawley purchased from Envigo, Netherlands
Age:	6-7 weeks
Body weight:	Males:193.75 to 222.07 g Females:148.36 to 170.32 g The weight variation of the animals at randomisation was within ± 20% of the mean weight for each sex.
Acclimation period:	5 days
Water:	Tap water, *ad libitum*
Diet:	Teklad Global 18% Protein Rodent Diet (meal) manufactured by Envigo
Housing:	one or two per sex per cage in polycarbonate rat cages with bedding materials
Test design	
No. of groups:	6 (2 main groups + 2 recovery groups + 2 TK groups)
No. of animals/group:	10 rats/sex/group in main groups 6 rats/sex/group in recovery groups 6 rats/sex/group in test item TK group and 3 rats/sex/group in control TK group
Number of animals:	82 (41 Males + 41 Females)
Extra animals:	20 (10 Males + 10 Females)
Total number of animals:	102 (51 Males + 51 Females)
Environmental conditions	

Test material (Lot/Batch No.)	RH-141455 (HHGCP017-00-1)
Temperature:	20.1 – 24.3°C
Humidity:	Relative humidity ranged from 47 – 69%
Air changes:	22 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	16000 mg/kg in diet
Exposure time	90 days
Remarks	None.

This study was designed to assess the potential sub-chronic toxicity and toxicokinetics (TK) profile of RH-141455 when fed in formulated diet to Sprague Dawley rats for 90 days. A total of 41 males and 41 females Sprague Dawley rats were randomly assigned to 2 main groups (10 rats/sex/group), 2 recovery groups (6 rats/sex/group) and 2 TK groups (3/6 rats/sex/group for control and test-substance related, respectively). The dose of 16000 ppm was aimed to reach the guideline recommended limit dose of 1000 mg/kg body weight/day. The control group was fed with basal diet for same duration of study.

Table A 21: Study design

Main Groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	10	10
2	16000	924	1119	10	10

Recovery Groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	6	6
2	16000	930	1090	6	6

TK Groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	3	3
2	16000	912	1102	6	6

Males and females were randomised into 2 main groups (10 rats/sex/group), 2 recovery groups (6 rats/sex/group) and 2 TK groups (3 rats/sex/group for control and 6 rats/sex/group for RH-141455) using Pristima, based on body weight to control bias. RH-141455 was incorporated in the diet and fed at concentration level of 16000 ppm for 90 days to Sprague Dawley rats. Care of animals compiled with the recommendations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The principles of OECD Series on Testing and Assessment Document No. 19: Environment Directorate, Guidance Document on The Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation were followed.

The required amount of test material was weighed and mixed with the diet using electric mixer. Diet formulations were prepared under monochromatic light and used within the stability period (stable up to 8 days) of the test substance in the diet. The concentration of the test substance in the feed were analysed on days 1, 42 and 84 of dosing period by reverse phase high performance liquid chromatography with UV detection. The method has been validated according to SANCO/3029/99 rev. 4 (2000) under Syngene study no. U-19069. The results confirmed the test item concentrations in the rat diet at 80 - 120 % with a relative standard deviation (% RSD) ≤ 20 %.

The animals were observed for morbidity and mortality twice daily during study period and once on the day of terminal and recovery sacrifice. All rats were observed for clinical signs once daily during dosing period and recovery period. Detailed clinical examinations were done prior to initiation of treatment and weekly once thereafter all main and recovery group animals until scheduled necropsy. Detailed clinical examinations were not performed for the toxicokinetics groups.

Body weight were recorded for all animals on first day of treatment (Day 1) and weekly thereafter. Terminal (fasting) body weights were measured on the days of scheduled necropsy (Day 91 for main group and Day 119 for recovery group).

Food input and food left over was measured for all animals at once weekly interval from day of start of treatment until scheduled sacrifice.

Ophthalmological examination was performed using an ophthalmoscope once for all animals during acclimatization period and at the last week of dosing phase (day 87). Mydriasis was induced before examination of eyes using a mydriatic agent.

Following examinations were conducted on day 86 of the dosing phase in the control and test substance group. Neurological evaluation for individual animals, as applicable, were recorded in the form of scores as per test facility SOP.

The following neuromuscular observations were performed: 1. Grip strength, 2. Hind limb foot spray.

Rectal temperature was recorded in Fahrenheit (°F) using digital thermometer.

The following motor activity parameters were observed: 1. Distance travelled, 2. Horizontal counts, 3. Ambulatory counts, and 4. Vertical counts.

Blood samples were collected from three rats per sex per time point from the TK groups as per the sampling schedule. Blood was collected via retro-orbital plexus puncture under mild isoflurane anaesthesia. The sampling intervals for test substance treated group were as follows: 5 P.M and 9 P.M (day 2 and 86), 7 A.M, 10 A.M, 2 P.M and 5 P.M on Day 3 and 87. The sampling intervals for control group were as follows: 5 P.M (day 2 and 86), 7 A.M and 2 P.M and 5 P.M, on day 3 and 87. The bioanalysis was performed by LC-MS/MS method using protein precipitation as sample extraction technique. The supernatant - after extraction and centrifugation - was transferred into auto sampler vials. Volumes of 10 µL of the supernatant were injected onto a HPLC column for chromatographic separation, followed by mass spectrometer detection. The samples were analysed with a validated method (Syngene Study No. U-19104) at a LOQ of 0.100 µg/mL. Toxicokinetic evaluation was performed by validated Phoenix® WinNonlin® software (Pharsight Corporation, USA).

Blood was collected from all animals of main groups at the dosing phase (day 91) and for recovery groups at the end of recovery phase (day 29) for haematology, coagulation and clinical chemistry evaluations. The animals were fasted overnight prior to terminal blood collection. Blood was collected via retro-orbital plexus puncture of right eye under mild isoflurane anaesthesia. The following haematology, coagulation and clinical chemistry parameters were measured: haematology – red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, reticulocyte count, total white blood cell count, leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils); coagulation – active partial thromboplastin time, prothrombin time and fibrinogen; clinical chemistry – albumin/globulin ratio, alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, creatinine, creatinine kinase, gamma-

glutamyl trans-peptidase, globulin, glucose, high-density lipoprotein, inorganic phosphorus, low-density lipoprotein, potassium, sodium, total bilirubin, total cholesterol, total protein, triglycerides and T3, T4 and TSH.

Urine was collected from all animals at the end of dosing period (day 91 of dosing phase) and at the end of recovery period (day 29 of recovery phase). During urine collection, animals were fasted overnight in metabolic cages. The following parameters were measured: volume, colour, appearance, glucose, bilirubin, ketone, blood, pH, protein, urobilinogen, nitrite, leukocyte and specific gravity.

Gross necropsy was conducted on all animals at the end of dosing phase for main groups (day 91; terminal sacrifice) and at the end of recovery phase of recovery group (Day 29; recovery phase). The following organs were collected, weighed, assessed histopathologically after fixation in 10% neutral buffered formalin and staining with haematoxylin and eosin: adrenals, brain, epididymites, heart, kidneys, liver, ovaries, pituitary, prostate, seminal vesicles and coagulating glands, spleen, testes, thymus, thyroid with parathyroid and uterus with cervix. The other organs or tissues such as aorta, biceps femoris muscles, cecum, colon, diaphragm, duodenum, oesophagus, eyes, femur bone with joint, gastrocnemius muscle, gross changes, harderian glands, ileum with Peyer's patches, jejunum, lacrimal gland, lungs, lymph nodes (mandibular and mesenteric), mammary gland, optic nerve, oviducts, pancreas, Quadriceps muscle, rectum, salivary glands (mandibular, sublingual and parotid), sciatic nerve, skin (inguinal region), soleus muscle, spinal cord, sternum with marrow, stomach, tongue, trachea, urinary bladder and vagina, were also examined histopathologically. Histopathology was performed on all tissues of animals from control and test substance treated groups on main group. Histopathology was not extended to recovery groups, because there were no target organs in the test substance treated main group. Peer review was performed by another in-house pathologist.

Body weight and body weight gain, food consumption, grip strength, hind limb foot spray, body temperature, organ weights, organ weight ratios and laboratory parameters (haematology, coagulation and clinical chemistry) were analysed using Student's t-test.

Results and discussions

No mortality was observed during the study.

Soft stool was observed in males from day 56 of dosing phase of 16000 ppm main, recovery and TK groups. Soft stool was observed in females from day 70 of dosing phase of 16000 ppm main group, and also observed from day 62 of dosing phase of 16000 ppm recovery and TK groups. However, these clinical signs were recovered in recovery groups from day 2 of recovery phase in males and females. Soft stool was considered as test substance related. This clinical sign was not associated adverse changes in body weights and other parameters, hence, not considered as adverse change.
No test substance-related changes were noticed in overall body weight and body weight gain parameters in test substance treated group when compared to control group.

Table A 22: Cumulative body weight gain

main group males

Dose (ppm)	Day 1-8 n=10	Day 29-36 n=10	Day 57-64 n=10	Day 1-90 n=10
0	40.08[1] (10.75)[2]	22.96 (4.30)	13.45 (4.50)	237.75 (35.40)
16000	45.19 (3.92)	20.97 (3.52)	13.36 (6.51)	213.80 (20.74)

[1]: Weight in gains; [2]: Standard deviation

main group females

Dose	Day 1-8	Day 29-36	Day 57-64	Day 1-90

(ppm)	n=10	n=10	n=10	n=10
0	22.27[1] (4.61)[2]	7.65 (2.86)	4.91 (2.70)	97.87 (5.29)
16000	27.41+ (2.68)	12.31 (10.78)	3.35 (3.83)	101.28 (4.27)

[1]: Weight in gains; [2]: Standard deviation, +: Significantly higher than the control group at 5% level.

There were no test substance-related changes in feed consumption in females, when compared to control. Minimal reduction (5%) on overall food consumption was observed in 16000 ppm main group males as compared to control group. Statistically significant decrease in feed consumption on days 8-15 (14%), days 36-43 (10%), days 43-50 (7%) was noticed in 16000 ppm main group males when compared to control group. Statistically significant increase in feed consumption on day 15-22 (16%) and on recovery day 1-8 (5%) were observed in 16000 ppm recovery female group. Minimal reduction on overall food consumption in males was considered to as test substance related. Above mentioned changes in feed consumption in females were considered as an incidental, as the magnitude of changes were minimal and occurred randomly during dosing phase. The test substance intake achieved over the entire treatment period (day 1-90) for 16000 ppm concentration levels in diet was equivalent to 924 mg/kg body weight/day in males and 1119 mg/kg body weight/day in females, respectively. In 16000 ppm recovery group, the test substance intake achieved over the entire treatment period (day 1-90) was equivalent to 930 mg/kg body weight/day in males and 1090 mg/kg body weight/day in females, respectively. In 16000 ppm TK group, the test substance intake achieved over the entire treatment period (day 1-85) was equivalent to 912 mg/kg body weight/day in males and 1102 mg/kg body weight/day in females, respectively.

No eye abnormalities were observed in ophthalmological examination performed toward the end of treatment period in males and females.

No test substance-related changes were observed in functional observation battery (FOB) parameters for main group animals as compared to control group.

No test substance-related changes were observed in grip strength and landing foot splay parameters as compared to control group.

No test substance-related changes were observed in rectal temperature as compared to control group.

No test substance-related changes were observed in motor activity parameters as compared to control group.

Following oral feeding of RH-141455 in rats, T_{max} was observed on both day 2 and 86 at 7 A.M in males and females, i.e. 45/2061 hours from first exposure of animals. Plasma concentrations of RH-141455 were noted up to last time points in treated groups. T_{last} was observed at 55 hours for day 2 and 2071 hours for day 86. No concentrations in control group were observed. No sex related difference was observed in RH-141455 concentrations on day 2. Male to female AUC_{24h} and C_{max} ratio on day 86 was 0.81 and 0.84, respectively. Day 86/day 2 AUC_{24h} ratios were 0.48 in males and 0.65 in females at 16000 ppm. Repeat dose feeding of RH-141455 at 16000 ppm in diet for 90 days showed evidence of systemic plasma exposure to RH-141455 in Sprague Dawley rats.

A statistically significant but minimal decrease in mean blood neutrophil counts (38%) was seen in females administered RH-141455 at terminal sacrifice, which was recovered at the end of recovery period. This change in neutrophils was considered toxicologically not significant because of low magnitude, lack of microscopic correlate and occurrence in only one sex.

A statistically significant minimal increase in serum creatine in males (12%) and females (12%) and statistically significant minimal increase in BUN in females (19%) were observed at the terminal sacrifice in 16000 ppm. The mean creatinine values were within the historical control data range. At the end of recovery period, changes in creatinine and BUN were not evident. These small differences in BUN and creatinine values were considered likely relevant to in-life observation of soft stools during the dosing phase (possibly due to a minimal water loss via soft stools). These changes were considered toxicologically not relevant because of very lower magnitude of change, absence of renal pathology,

and no evidence of such changes at the endo of recovery period. A statistically significant minimal increase in total bilirubin (14%) was noted in males at the terminal sacrifice in 16000 ppm. The bilirubin values were out of historical control data. In recovery males, although mean bilirubin values were slightly higher (31%) at the end of recovery period compared to concurrent controls, the bilirubin values were within the range of historical control data. This change was considered toxicologically not relevant because of its lower magnitude, lack of correlative microscopic findings, absence of concurrent increases in ALT and GGT (markers for hepatobiliary injury) and occurrence in only one sex. Marginal decrease (11%) in mean values of T4 was noted in males and females of 16000 ppm, when compared to the concurrent control group at the terminal sacrifice. This change in T4 was considered toxicologically not significant because of concurrent increase in TSH and its complete recovery at the end of recovery period. These changes were recovered at the end of recovery period.

At terminal sacrifice, slight decrease in mean urine volume was noted in 16000 ppm group. In addition, a marginal increase in mean urine specific gravity was noted in males of 16000 ppm group (control group mean: 1.0188; 16000 ppm group mean: 1.0328). These changes were recovered at the end of recovery period. These small changes in urine volume and specific gravity were considered likely related to in-life observation of soft stools but were considered toxicologically not significant because of their lower magnitude, lack of correlative microscopic findings, and complete recovery at the end of recovery period.

All the differences in organ weight parameters at the end and recovery sacrifices were considered incidental, as the magnitude of differences were marginal, lacked microscopic correlate and/or occurred only in one of three parameters (absolute weight, relative to body weight or relative to brain weight).

RH-141455-related cecal enlargement (with contents) was noted in males (8/10) and females (3/10) of 16000 ppm group at the terminal sacrifice. This finding had no microscopic correlate and recovered the end of recovery period, therefore, considered as non-adverse finding. Other gross findings at the end of terminal sacrifice such as a yellow focus in the liver of one male in 16000 ppm, or uterine dilation in female control group was considered an incidental finding or normal physiological uterine change. There were no test substance-related microscopic findings in the study. All microscopic changes were considered incidental or spontaneous and not related to the test substance, because they were randomly distributed across the test substance and control groups and/or were generally observed in the rats of this age and strain.

Conclusion

This study was designed to assess the potential sub-chronic toxicity and toxicokinetics (TK) profile of RH-141455 when fed in formulated diet to Sprague Dawley rats for 90 days. A total of 41 males and 41 females Sprague Dawley rats were randomly assigned to 2 main groups (10 rats/sex/group), 2 recovery groups (6 rats/sex/group) and 2 TK groups (3/6 rats/sex/group for control and test-substance related, respectively). The dose of 16000 ppm was aimed to reach the guideline recommended limit dose of 1000 mg/kg body weight/day. The control group was fed with basal diet for same duration of study.

There was no morbidity or mortality or ophthalmic changes at 16000 ppm in both sexes. Soft stool was observed in males from day 56 of dosing phase and day 70 of dosing phase in females of 16000 ppm. However, these clinical signs recovered in recovery groups. RH-141455 administration did not result in any effects on the functional observation battery, grip strength, landing foot splay, rectal temperature and motor activity.

There were no test material-related changes in overall mean body weight and body weight gain at 16000 ppm in both sexes when compared to control group animals. Minimal reduction (5%) on overall food consumption and food conversion efficiency was observed test substance treated main group males as compared to control group.

There was no relevant test material-related change in hematology and coagulation parameters. Minimal increase in serum creatine in both sexes and blood urea nitrogen (BUN) in females were not con-

sidered adverse, as creatinine values were within the historical control data range. At the end of recovery period, changes in creatinine and BUN were not evident and these small differences in BUN and creatinine value were considered likely related to in-life observation of soft stools during the dosing phase (possibly due to a minimal water loss via soft stools). These changes were considered toxicologically not relevant because of very lower magnitude of changes, absence of renal pathology, and no evidence of such changes at the end of recovery period. A statistically significant marginal increase in total bilirubin was considered toxicologically not relevant because its lower magnitude, lack of correlative microscopic findings, absence of concurrent increase in ALT and GGT (markers for hepatobiliary injury) and occurrence in only one sex. At terminal sacrifice, cecal enlargement (with contents) was noticed in males (8/10) and females (3/10) administered RH-141455 group. This finding had no microscopic correlate and recovered at the recovery period, therefore, considered as non-adverse findings. No test substance-related microscopic findings noted in the study.
NOAEL of RH-141455 was 16000 ppm, which is equivalent to 924 and 1119 mg/kg body weight/day for the males and females, respectively. This NOAEL was based on absence of adverse effects at 16000 ppm in male and female rats.

Study 8 - RH-150721: 2 days oral dietary pharmacokinetic

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The study is acceptable.

Reference:	**KCP 7.4/08**
Report	XXXX, 2020: RH-150721: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats XXXX, Report No. U-19134, No GLP, Not published
Guideline(s):	OECD 408
Deviations:	The draft report was later than expected by the sponsor. This deviation is not regarded to have an influence on the integrity of the study.
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-150721 (HHGCP004-00-3)
Purity:	99.9%
Reference material:	RH-141455 (HHGCP014-00-1)
Purity:	99.6%
Vehicle:	Teklad certified irradiated global 18% protein rodent diet (meal)
Test organisms	Albino rat, Sprague Dawley purchased from Envigo, The Netherlands
Age:	10-11 weeks

Test material (Lot/Batch No.)	RH-150721 (HHGCP004-00-3)
Body weight:	Body weights did not differ by more than 20% in the group at study initiation.
Acclimation period:	1 day
Water:	Tap water, *ad libitum*
Diet:	Teklad certified irradiated global 18% protein rodent diet (meal) diet *ad libitum*
Housing:	1 animal per cage in polycarbonate rat cages with bedding materials
Test design	
Total number of animals:	4 (males)
Environmental conditions	
Temperature:	20.3 to 24.1°C
Humidity:	Relative humidity ranged from 54-64 %
Air changes:	22 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	15000 ppm in diet
Exposure time	2 days
Remarks	None

The study was designed to assess plasma exposures and pharmacokinetic profiles of RH-150721 and its downstream metabolite RH-141455 following oral dietary administration at 15000 ppm for two days in male Sprague Dawley rats. A total of 4 male Sprague Dawley rats were used for pharmacokinetic evaluation. RH-150721 was formulated in powdered diet at concentration of 15000 ppm and fed from day 1 through day 3. The observations included daily morbidity/ mortality check, clinical signs, body weights, feed consumption, bioanalytical & PK parameters (days 2 & 3), and gross pathology. Blood samples were collected at 4 PM, 10 PM (day 2), 7 AM, 12 PM and 4 PM (day 3) via retro-orbital plexus puncture under mild isoflurane anesthesia, plasma was separated and a method for the determination of RH-150721 and RH-141455 via LC-MS/MS, using an internal standard (tolbutamide) was developed. Pharmacokinetic parameters were evaluated.

Results and discussions

No mortality/morbidity was observed during in-life phase of the study. No clinical signs were observed during in-life phase of the study. A test item related decrease in day 3 body weights (8%) compared to day 1 was noticed. At terminal sacrifice on day 3, there were no test item-related gross changes in any of the organs examined.

A total of 10 rat plasma samples were analysed with a validated LCMS/MS method using an internal standard (tolbutamide) at a calibration range of 0.057 µg/mL to 44.583 µg/mL. The resulting concentration data were below the quantification limits for both RH-150721 and RH-141455. Hence, pharmacokinetic (PK) evaluation using Phoenix® WinNonlin® software was not performed.

Conclusion

The study was designed to assess plasma exposures and pharmacokinetic profiles of RH-150721 (and downstream metabolite RH-141455) following oral dietary administration of RH-150721 at 15000 ppm for two days in male Sprague Dawley rats. The resulting concentrations were below the quantification limits for both RH-150721 and RH-141455. Pharmacokinetic parameters could not be calculat-

ed.

Study 9 - RH-150721: 14 days oral dietary toxicity

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The 14-day oral dietary dose range finding study in Sprague Dawley rats was performed in line with internationally recognised guidelines and is acceptable. 14d No-Observed-Adverse-Effect-Level (NOAEL) of RH-150721 in the Sprague Dawley rats is 5000 ppm, which is equivalent to appr. 334 mg/kg bw/day in males and 382 mg/kg bw/day in females.

Reference:	**KCP 7.4/09**
Report	XXXX, 2020: RH-150721: 14-day oral dietary dose range finding study in Sprague Dawley rats XXXX, Report No. U-19189, GLP, Not published
Guideline(s):	OECD 407
Deviations:	The draft report was provided to the sponsor with a delay of 1 month. On day 14 (7 A.M), blood sampling has been delayed between 4-27 minutes from the scheduled time (± 25 minutes) across all the groups. These findings will not change the findings and the integrity of the study.
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-150721 (HHGCP004-00-3)
Purity:	99.9 % (w/w)
Vehicle:	Teklad Global 18% Protein Rodent Diet (meal) manufactured by Envigo
Stability:	Test material was stable for at least 8 days at room temperature.
Test organisms	Albino rat, Sprague Dawley purchased from Envigo, Netherlands
Age:	7-8 weeks
Body weight:	Males: 245.26- 274.99 g Females: 187.88 – 205.94 g The weight variation of the animals at randomisation was within ± 20% of the mean weight for each sex.
Acclimation period:	4 days
Water:	Tap water, *ad libitum* (except overnight fasting prior to clinical pathology blood

Test material (Lot/Batch No.)	RH-150721 (HHGCP004-00-3)
	collection)
Diet:	Teklad Global 18% Protein Rodent Diet (meal) manufactured by Envigo
Housing:	2 per sex per cage in polycarbonate rat cages with bedding materials
Test design	
No. of groups:	4 groups
No. of animals/group:	6 rats/sex/group
Number of animals:	48 (24 Males + 24 Females)
Extra animals:	12 (6 Males + 6 Females)
Total number of animals:	60 (30 Males + 30 Females)
Environmental conditions	
Temperature:	20.1 – 23.8°C
Humidity:	Relative humidity ranged from 51 - 67%
Air changes:	20 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	1000, 5000 and 16000 ppm in diet
Exposure time	14 days
Remarks	None

In a dose range finding study, RH-150721 was administered to rats for 14 days at levels of 1000, 5000 and 16000 ppm for male and female to groups of Sprague Dawley rats (6 rats/sex/group). The high dose group was administered with 16000 ppm for 7 days. The dose was reduced to 8000 ppm from day 8 due to unpalatability. The concentrations of 1000 and 5000 ppm in the diet were equivalent to 66 and 334 mg/kg body weight/day in males and 77 and 382 mg/kg body weight/day in females, respectively. Test substance intake achieved over the treatment period (day 1-7) for 1600 ppm was equivalent to 911 mg/kg body weight/day in males and 1051 mg/kg body weight/day in females, respectively. Test substance intake achieved over the treatment period (day 8-14) for 8000 ppm was equivalent to 618 mg/kg body weight/day in males and 664 mg/kg body weight/day in females, respectively. The control group was fed with basal diet for same duration of study.

There were 6 animals of each sex per group. Males and females were randomised into 4 dose groups using Pristima, based on body weight to control bias. RH-150721 was administered in the diet for 14 days to Sprague Dawley rats – 0, 1000, 5000 and 16000/8000 ppm. Husbandry conditions were in accordance with the recommendations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The principles of OECD Series on Testing and Assessment Document No. 19: Environment Directorate, Guidance Document on The Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation were followed.

Table A 23: Study design

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female

1	0	0	0	6	6
2	1000	66	77	6	6
3	5000	334	382	6	6
4	15000 (Day 1-7)	911	1051	6	6
	8000 (Day 8-14)*	618	664		

* Due to significant reduction in body weight and food consumption, the high dose was reduced from 16000 ppm to 8000 ppm in diet from Day 8 of the dosing phase.

The required amount of test material was weighed and mixed with the diet using electric mixer. Diet was prepared approximately weekly. Prepared diets were stored at room temperature before use. The concentration of the test substance in the feed was analytically verified with a reverse phase analytical method using UV-DAD detection. The method has been validated according to SANCO 3099 rev. 4 under study no. U-19162. The active ingredient content in the prepared dose formulations were analysed 99.96-102.7 % of nominal, and there within the acceptance limits of 80 to 120 % with % RSDs ≤ 20 %.

The animals were observed twice daily for morbidity and mortality. All rats were observed for clinical signs at least once daily. Detailed clinical examinations were done once on day 1 of dosing and weekly thereafter.

Body weight were recorded on days 1, 2, 3, 7, 11 and 14.

Food input was recorded on Days 1, 3, 4, 7, 8, 9, 11, 13 and food leftover was measured on days 3, 5, 7, 8, 9, 11,13,14.

Ophthalmoscopic examinations were not performed.

Blood samples were collected from three rats per sex per time point as per the sampling schedule. Blood was collected via retro-orbital plexus puncture under mild isoflurane anesthesia. The sampling intervals were as follows: 5 P.M (day 2 and 13) and 7 A.M, 10 A.M, 2 P.M and 5 P.M on days 3 and 14. Toxicokinetic evaluation was performed by validated Phoenix® WinNonlin® software (Pharsight Corporation, USA).

Blood was collected from all animals at the terminal sacrifice (day 15) for haematology, coagulation and clinical chemistry evaluations. The animals were fasted overnight prior to terminal blood collection. Blood was collected via retro-orbital plexus puncture of right eye under mild isoflurane anaesthesia. The following haematology, coagulation and clinical chemistry parameters were measured: haematology – red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, reticulocyte count, total white blood cell count, leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils); coagulation – active partial thromboplastin time, prothrombin time and fibrinogen; clinical chemistry – albumin/globulin ratio, alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, creatinine, creatinine kinase, gamma-glutamyl trans-peptidase, globulin, glucose, high-density lipoprotein, inorganic phosphorus, low-density lipoprotein, potassium, sodium, total bilirubin, total cholesterol, total protein and triglycerides.

Urine was collected from all animals at the end of dosing phase (day 15). During urine collection, animals were fasted overnight in metabolic cages. The following parameters were measured: volume, colour, appearance, glucose, bilirubin, ketone, blood, pH, protein, urobilinogen, nitrite, leukocyte and specific gravity.

Gross necropsy was conducted on all animals at the end of dosing phase (day 15). The following organs were collected, weighed, assessed histopathologically after fixation in 10% neutral buffered formalin and staining with haematoxylin and eosin: adrenals, brain, epididymites, heart, kidneys, liver, ovaries, pituitary, prostate, seminal vesicles and coagulating glands, spleen, testes, thymus, thyroid with parathyroid and uterus with cervix. The other organs or tissues such as aorta, biceps femoris mus-

cles, cecum, colon, diaphragm, duodenum, oesophagus, eyes, femur bone with joint, gastrocnemius muscle, gross changes, harderian glands, ileum with Peyer's patches, jejunum, lungs, lymph nodes (mandibular and mesenteric), mammary gland, optic nerve, oviducts, pancreas, Quadriceps muscle, rectum, salivary glands (mandibular, sublingual and parotid), sciatic nerve, skin (inguinal region), soleus muscle, spinal cord, sternum with marrow, stomach, tongue, trachea, urinary bladder and vagina, were also examined histopathologically. Histopathology was performed on all tissues of animals from control and high dose groups. Peer review was performed by another in-house pathologist.

For comparative statistics, data were evaluated using Levene's Test for homogeneity of variance, with significance at 5% level. Data determined to be homogeneous was evaluated for analysis of variance (ANOVA). When the ANOVA verified significance at 5% level, pairwise comparisons of each treated group with the control group was made using a Dunnett Test, to identify statistical differences (at 5% level). When the data were found to be non-homogeneous, ANOVA was done using suitable transformation. When the homogeneity tests were significant even after transformation, data were evaluated using a Kruskal-Wallis Test for group factor significance. When significance (at 5% level) existed between groups, Dunn's pairwise comparison was performed.

Results and discussions

In control, 1000 ppm, 5000 ppm groups, there were no clinical signs of toxicity. In 16000/8000 ppm group, clinical signs of thin appearance were observed from day 7 to 10 in males and females. From days 11-14, there were no clinical signs of toxicity in both sexes in this group.

No mortalities or morbidity was observed in any dose group during the study.

In 1000 ppm group, no test substance-related changes were noticed in body weight and body weight gain parameters in any of the dose groups when compared to control group. In 5000 ppm group, statistically significant but mild reduction was noticed in overall body weight (7%) and body weight gain (38%) in males. Similarly, non-significant mild reduction in overall body weight (4%) and significant reduction in body weight gain (52%) was observed in females in females. The changes noted in 5000 ppm males and females were not considered adverse because the body weight decreases were <10%. Administration of RH-150721 at 16000 ppm for 7 days resulted in significant reduction in body weight gain (males -312%; females -894%) on day 1-7. However, upon reducing the dose to 8000 ppm from day 8 to day 14 of the study, a non-significant increase in body weight gain on days 7-11 (males -13%; females -74%) and significant increase in body weight gain on days 11-14 (males -69%; females -178%) was observed when compared to control group. The changes in the body weight parameters at 16000/8000 ppm group were considered test substance related and adverse in both sexes.

Table A 24: Body weight and body weight change

Group #		Dosing Phase					
		Day: 1	**Day: 2**	**Day: 3**	**Day: 7**	**Day: 11**	**Day: 14**
		Session 1	**Session 1**	**Session 1**	**Session 1**	**Session 1**	**Session 1**
Males							
Control	n	6	6	6	6	6	6
	Means	261.55	268.39	267.06	284.00	304.64	314.15
	SD	6.45	6.09	7.33	7.14	5.61	9.82
2	n	6	6	6	6	6	6
	Means	261.09	265.72	265.51	280.49	296.26	304.72
	SD	7.00	6.60	10.01	12.91	14.40	17.81
3	n	6	6	6	6	6	6
	Means	260.43	**252.48-**	**251.45-**	**271.50-**	290.49	**292.87-**
	SD	7.59	7.41	8.05	5.22	11.10	10.36
4	n	6	6	6	6	6	6
	Means	260.80	**245.45-**	**232.55-**	**213.28-**	**236.54-**	**252.60-**
	SD	10.08	11.46	9.95	6.64	11.84	14.52
Females							
Control	n	6	6	6	6	6	6
	Means	195.95	196.64	197.16	201.35	213.10	216.58
	SD	6.28	4.95	6.50	5.91	6.13	6.66
2	n	6	6	6	6	6	6
	Means	195.98	195.13	195.49	202.15	207.35	212.74
	SD	5.34	3.49	6.03	7.62	7.77	9.66
3	n	6	6	6	6	6	6
	Means	198.41	190.41	189.63	203.39	209.20	208.30
	SD	5.96	5.48	6.51	8.64	4.35	5.53
4	n	6	6	6	6	6	6
	Means	196.95	**184.02-**	**173.98-**	**154.07-**	**174.51-**	**184.17-**
	SD	5.01	5.76	5.19	5.46	5.63	5.47

Control: G1 (0 ppm in diet); 2: G2 (1000 ppm in diet); 3: G3 (5000 ppm in diet); 4: G4 (16000/8000 ppm in diet) -: Significantly lower than the control group at 5 % level.

Table A 25: Body weight change

Group #		D:1-D:2	D:2-D:3	D:3-D:7	D:1-D:7	D:7-D:11	D:11-D:14	D:1-D:14
Males								
Control	n	6	6	6	6	6	6	6
	Means	6.84	-1.34	16.94	22.45	20.64	9.50	52.60
	SD	1.38	3.49	4.02	2.78	2.44	4.36	6.50
2	n	6	6	6	6	6	6	6
	Means	4.62	-0.21	14.99	19.40	15.76	8.46	43.62
	SD	2.20	4.20	3.37	7.54	1.97	4.15	12.09
3	n	6	6	6	6	6	6	6
	Means	**-7.96^{-}**	-1.02	20.05	11.07	18.99	**2.39^{-}**	**32.44^{-}**
	SD	2.46	3.59	5.67	4.81	7.40	2.87	11.25
4	n	6	6	6	6	6	6	6
	Means	**-15.35-**	**-12.90-**	**-19.27-**	**-47.52-**	23.26	**16.06^{+}**	**-8.20-**
	SD	2.15	2.67	6.25	5.41	5.77	5.24	7.65
Females								
Control	n	6	6	6	6	6	6	6
	Means	0.69	0.51	4.19	5.40	11.75	3.48	20.63
	SD	1.86	2.09	3.96	4.29	4.63	3.08	3.67
2	n	6	6	6	6	6	6	6
	Means	-0.85	0.36	6.66	6.17	5.20	5.39	16.76
	SD	2.42	4.71	4.36	7.91	2.67	4.57	10.64
3	n	6	6	6	6	6	6	6
	Means	**-8.00^{-}**	-0.77	**13.76^{+}**	4.98	5.81	-0.90	**9.90^{-}**
	SD	2.61	2.63	5.39	6.55	5.59	2.50	3.39
4	n	6	6	6	6	6	6	6
	Means	**-12.93-**	**-10.04-**	**-19.91^{-}**	**-42.88^{-}**	20.43	**9.67^{+}**	**-12.77^{-}**
	SD	2.00	2.49	6.16	5.53	10.51	3.79	5.92

Group #: Control: G1 (0 ppm in diet); 2: G2 (1000 ppm in diet); 3: G3 (5000 ppm in diet); 4: G4 (16000/8000 ppm in diet)
+/-: Significantly higher / lower than the control group at 5 % level.

In 1000 ppm group, no test substance-related changes were noticed in mean food when compared to control group. In 5000 ppm group, non-significant reduction was noticed in feed consumption (males -7%) when compared to control group. In 16000/8000 ppm group, significant reduction was noticed in overall mean food consumption (males -22%; females -21%) when compared to control group. The reduced food consumption correlated with changes in body weight parameters and might be attributed to palatability of test substance. The dose in ppm in diet for 1000 and 5000 ppm were equivalent to 66 and 334 mg/kg body weight/day in males and 77 and 382 mg/kg body weight/day in females, respectively. Test substance intake achieved over the treatment period (day 1-7) for 1600 ppm was equivalent to 911 mg/kg body weight/day in males and 1051 mg/kg body weight/day in females, respectively. Test substance intake achieved over the treatment period (day 8-14) for 8000 ppm was equivalent to 618 mg/kg body weight/day in males and 664 mg/kg body weight/day in females, respectively.

There were no test substance-related changes in haematology and coagulation parameters. In 16000/8000 ppm group, minimal, statistically non-significant decrease in mean total WBC (males: 10%, females: 18%), lymphocytes count (males: 19%, females: 21%) were noted. These changes correlated with the light microscopic findings of decreased lymphocytes in the thymus and spleen, and decreased cellularity in the bone marrow of femur and sternum bones. These hematologic changes

were considered likely related to decreased food consumption and body weight gain observed during first week of the study (Levin et al., 1993[2], Hubert et al., 2000[3]). Similar changes were not evident at 1000 and 5000 ppm groups.

There were no test substance-related changes in clinical chemistry parameters. In 16000/8000 ppm group, statistically significant minimal decrease in mean serum BUN, total protein, albumin, globulin, increase in serum triglycerides, ALT, ALP was noticed. These changes were likely related to decreased food consumption and body weight gain observed the first week of the study (Levin et al., 1993[4], Hubert et al., 2000[5]).

There were no test substance-related changes in urinalysis parameters.

In 16000/8000 ppm group, decreased weights of thymus and spleen were observed. In 5000 and 16000/8000 ppm group, decreased weights of prostate, seminal vesicles, coagulation glands, ovaries were observed. These changes correlated with the light microscopic findings in these organs and were considered related to decreased food consumption and body weight gain during in-life phase (Rehm et al., 2008[6], Levin et al., 1993).

There were no test substance-related gross changes in 1000 and 5000 ppm groups. In 16000/8000 ppm group, gross finding of small size thymus, seminal vesicles, prostate, ovaries, uterus were observed. These changes correlated with decreased organ weights and/or light microscopic changes in these organs. These gross changes were considered likely related to the decreased food consumption and body weight gain during in-life phase. Uterine dilation observed randomly across the groups was a common change due to normal reproductive cycling in rats to this age. In 5000 and 16000/8000 ppm groups, acinar hypertrophy was observed in mandibular and parotid salivary glands. The acinar cell size was increased with increased secretary material. However, there was no increased salivation observed in these animals during in-life phase of the study. The biological significance of these findings is unknown (NTP Technical Report on Toxicity Studies of Glyphosate, 1992[7]); however, these findings were considered as adaptive change (Inoue et al., 2014[8]). The incidence and/or severity of this change was lower in 5000 ppm group than in 16000/8000 ppm group. This change was not observed in 1000 ppm group. In 16000/8000 ppm group, decreased lymphocytes in thymus and spleen, decreased secretion in prostate, seminal vesicles, coagulation glands, decreased size of *corpora lutea* in ovaries, decreased cellularity of bone marrow in sternum and femur bones, atrophy of mammary gland, decreased secretion in ducts of mandibular salivary gland were observed. All these changes were considered likely related to decreased food consumption [Levin et al. (1993), Rehm et al., (2008), Everds et al., (2013)[9]]. These light microscopic changes were not observed in 1000 and 5000 ppm groups.

RH-150721 plasma concentration at all-time points were below limit of quantification, hence, toxicokinetic evaluation was not performed.

Conclusion

Under the conditions of the study, the No-Observed-Adverse-Effect-Level (NOAEL) of RH-150721 in

[2] Levin S, Semler D, Ruben Z. Effects of two weeks of feed restriction on some common toxicologic parameters in Sprague-Dawley rats. Toxicol Pathol.1993; 21 (1):1-14.
[3] Hubert MF, Laroque P, Gillet JP, Keenan KP. The effects of diet, ad Libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters, and cause of death in control Sprague-Dawley rats. Toxicol Sci. 2000 Nov;58(1):195-207.
[4] Levin S, Semler D, Ruben Z. Effects of two weeks of feed restriction on some common toxicologic parameters in Sprague-Dawley rats. Toxicol Pathol.1993; 21 (1):1-14.
[5] Hubert MF, Laroque P, Gillet JP, Keenan KP. The effects of diet, ad Libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters, and cause of death in control Sprague-Dawley rats. Toxicol Sci. 2000 Nov;58(1):195-207.
[6] Rehm S, White TE, Zahalka EA, Stanislaus DJ, Boyce RW, Wier PJ. Effects of food restriction on testis and accessory sex glands in maturing rats. Toxicol Pathol. 2008 Jul; 36(5):687-94.
[7] NTP Technical Report on Toxicity Studies of Glyphosate (CAS No. 1071836) Administered in Dosed Feed to F344/N Rats and B6C3F1 Mice, NIH Publication 92-3135, July 1992.
[8] Inoue K, Morikawa T, Matsuo S, Tamura K, Takahashi M, Yoshida M. Adaptive parotid gland hypertrophy induced by dietary treatment of GSE in rats. Toxicol Pathol. 2014 Aug;42 (6):1016-23.
[9] Everds NE, Snyder PW, Bailey KL, Bolon B, Creasy DM, Foley GL, Rosol TJ,Sellers T. Interpreting stress responses during routine toxicity studies: a review of the biology, impact, and assessment. Toxicol Pathol.2013; 41 (4):560-614.

the Sprague Dawley rats is 5000 ppm, which is equivalent to appr. 334 mg/kg bw/day in males and 382 mg/kg bw/day in females. This NOAEL is based on reduction in body weight, food consumption, food efficiency at 16000/ 8000 ppm in males and females. Acinar hypertrophy of mandibular and parotid salivary glands at mid and high doses were considered likely related to test item. However, these findings were considered as adaptive change. The other changes noted in clinical pathology and anatomic pathology parameters at high and mid doses were considered related to decreased food consumption and/or body weights.

Study 10 - RH-150721: 90 days oral dietary toxicity

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The 90-day oral dietary study performed according internationally recognised guidelines and in GLP conditions is acceptable. 90d No-Observed-Adverse-Effect-Level (NOAEL) of metabolite RH-150721 in is 2000 ppm, which is equivalent to 111 mg/kg body weight/day in males and 134 mg/kg body weight/day in females.

Reference:	**KCP 7.4/10**
Report	XXXX, 2020: RH-150721: 90-day oral dietary toxicity study and 28-day recovery period in Sprague Dawley rats XXXX, Report No. U-19235, GLP, Not published
Guideline(s):	OECD 408 (2018) OPPTS 870.3100 (1998) ICH Harmonized Tripartite Guideline S3A (Toxicokinetics, 1994) Notification No. 12-Nousan-8147, MAFF, Japan (2000)
Deviations:	No
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-150721 (HHGCP004-00-3)
Purity:	99.9 % (w/w)
Vehicle:	Teklad Global 18% Protein Rodent Diet (meal) manufactured by Envigo
Stability:	Test material was stable for at least 2 days at room temperature.

Test material (Lot/Batch No.)	RH-150721 (HHGCP004-00-3)
Test organisms	Albino rat, Sprague Dawley, males and females purchased from Envigo, Netherlands
Age:	7-9 weeks
Body weight:	Males: 253.47 – 317.61 g Females: 177.08 – 205.74 g The weight variation of the animals at randomisation was within ± 20% of the mean weight for each sex.
Acclimation period:	5 days
Water:	Tap water, *ad libitum* (except overnight fasting prior to clinical pathology blood collection)
Diet:	Teklad Global 18% Protein Rodent Diet (meal) manufactured by Envigo
Housing:	2 per sex per cage in polycarbonate rat cages with bedding materials
Test design	
No. of groups:	6 (4 main groups + 2 recovery groups)
No. of animals/group:	10 rats/sex/group in main groups 6 rats/sex/group in recovery groups
Number of animals:	104 (52 Males + 52 Females)
Extra animals:	20 (10 Males + 10 Females)
Total number of animals:	124 (62 Males + 62 Females)
Environmental conditions	
Temperature:	19.1 – 23.7°C
Humidity:	Relative humidity ranged from 41 – 66 %
Air changes:	19 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	670, 2000 and 6000 ppm in diet
Exposure time	90 days
Remarks	None

This study was designed to assess the potential sub-chronic toxicity of RH-150721 when fed in formulated diet to Sprague Dawley rats for 90 days. Rats were randomly assigned to 4 main groups (10 rats/sex/group) and 2 recovery groups (6 rats/sex/group), and – based on the results of a range-finding study - exposed to diet formulated with RH-150721 at 670, 2000 and 6000 ppm for 90 consecutive days, followed by a 28-day treatment-free period for the high dose group.

Males and females were randomised into 4 main groups (10 rats/sex/group), 2 recovery groups (6 rats/sex/group) using Pristima, based on body weight to control bias. RH-150721 was mixed with the diet and fed at concentration level of 670, 2000 and 6000 ppm for 90 days to Sprague Dawley rats. Care of animals compiled with the recommendations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The principles of OECD Series on Testing and Assessment Document No. 19: Environment Directorate, Guidance Document on The

Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation were followed.

Table A 26: Study design

Main Groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	10	10
2	670	38	44	10	10
3	2000	111	134	10	10
4	6000	335	404	10	10

Recovery Groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	6	6
2	6000	334	408	6	6

The required amount of test material was weighed and mixed with the diet using electric mixer. The test item was proofed to be stable in the diet for at least 2 days, and therefore diet was prepared every 2 days. Prepared diets were stored at room temperature before use. The concentration of the test substance in the feed were analysed on days 1, 31 and 63 of dosing period, and was verified.

The animals were observed for morbidity and mortality twice daily during study period and once on the day of terminal and recovery sacrifice. All rats were observed for clinical signs once daily during dosing period and recovery period. Detailed clinical examinations were done prior to initiation of treatment and weekly once thereafter all main and recovery group animals until scheduled necropsy. Detailed clinical examinations were not performed for the toxicokinetics groups.

Body weight were recorded for all animals on first day of treatment (day 1) and weekly thereafter. Terminal (fasting) body weights were measured on the days of scheduled necropsy (day 91 for main group and day 119 for recovery group).

Food input and food leftover was measured for all animals once in two days of start of treatment until scheduled sacrifice during dosing phase. Additional food leftover was measured on day 90 of dosing phase. For recovery group, food input and food leftover were measured once weekly during recovery phase.

Ophthalmological examination was performed using an ophthalmoscope once for all animals during acclimatization period and at the last week of dosing phase (day 88) for control and high dose groups. Mydriasis was induced before examination of eyes using a mydriatic agent.

Following examinations were conducted on Day 86 of the dosing phase in the control and test substance group. Neurological evaluation for individual animals, as applicable, were recorded in the form of scores as per test facility SOP.

The following neuromuscular observations were performed: 1. Grip strength, 2. Hind limb foot spray.

Rectal temperature was recorded in Fahrenheit (°F) using digital thermometer.

The following motor activity parameters were observed: 1. Distance travelled, 2. Horizontal counts, 3. Ambulatory counts, 4. Vertical counts.

Blood was collected from all animals of main groups at the end of dosing phase (day 91) and for recovery groups at the end of recovery phase (day 29) for haematology, coagulation and clinical chemis-

try evaluations. The animals were fasted overnight prior to terminal blood collection. Blood was collected via retro-orbital plexus puncture of right eye under mild isoflurane anaesthesia. The following haematology, coagulation and clinical chemistry parameters were measured: haematology – red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, reticulocyte count, total white blood cell count, leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils); coagulation – active partial thromboplastin time, prothrombin time and fibrinogen; clinical chemistry – albumin/globulin ratio, alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, creatinine, creatinine kinase, gamma-glutamyl trans-peptidase, globulin, glucose, high-density lipoprotein, inorganic phosphorus, low-density lipoprotein, potassium, sodium, total bilirubin, total cholesterol, total protein, triglycerides and T3, T4 and TSH.

Urine was collected from all animals at the end of dosing period (day 91 of dosing phase) and at the end of recovery period (day 29 of recovery phase). During urine collection, animals were fasted overnight in metabolic cages. The following parameters were measured: volume, colour, appearance, glucose, bilirubin, ketone, blood, pH, protein, urobilinogen, nitrite, leukocyte and specific gravity.

Gross necropsy was conducted on all animals at the end of dosing phase for main groups (day 91; terminal sacrifice) and at the end of recovery phase of recovery group (day 29; recovery phase). The following organs were collected, weighed, assessed histopathologically after fixation in 10% neutral buffered formalin and staining with haematoxylin and eosin: adrenals, brain, epididymites, heart, kidneys, liver, ovaries, pituitary, prostate, seminal vesicles and coagulating glands, spleen, testes, thymus, thyroid with parathyroid and uterus with cervix. The other organs or tissues such as aorta, biceps femoris muscles, cecum, colon, diaphragm, duodenum, oesophagus, eyes, femur bone with joint, gastrocnemius muscle, gross changes, harderian glands, ileum with Peyer's patches, jejunum, lacrimal gland, lungs, lymph nodes (mandibular and mesenteric), mammary gland, optic nerve, oviducts, pancreas, Quadriceps muscle, rectum, salivary glands (mandibular, sublingual and parotid), sciatic nerve, skin (inguinal region), soleus muscle, spinal cord, sternum with marrow, stomach, tongue, trachea, urinary bladder and vagina, were also examined histopathologically. Histopathology was performed on all tissues of animals from control and high dose groups on main group. All gross changes in all animals were processed for histopathology and evaluated. Thyroid and parotid salivary gland were also evaluated from low dose, mid dose and recovery groups, because test substance-related microscopic changes were noted in the high dose group. Peer review was performed by another in-house pathologist.

For comparative statistics, data were evaluated using Levene's Test for homogeneity of variance, with significance at 5% level. Data determined to be homogeneous was evaluated for analysis of variance (ANOVA). When the ANOVA verified significance at 5% level, pairwise comparisons of each treated group with the control group was made using a Dunnett Test, to identify statistical differences (at 5% level). When the data were found to be non-homogeneous, ANOVA was done using suitable transformation. When the homogeneity tests were significant even after transformation, data were evaluated using a Kruskal-Wallis Test for group factor significance. When significance (at 5% level) existed between groups, Dunn's pairwise comparison was performed.

Results and discussions

There were no clinical signs were noticed in any of the treated groups during the study.
There was no mortality during the study.
In the 670 ppm group, no test substance-related changes were noticed in body weight and overall body weight (day 1-90) when compared to control group. In male 2000 ppm group, no test substance-related changes were noticed in body weight and overall body weight (day 1-90) when compared to control group. In female 2000 ppm group, statistically significant decrease in day 90 body weight (5%), was observed as compared to control group. In 6000 ppm group, statistically significant decrease in day 90 body weight (male: 11%; female: 11%) was observed. Similarly, statistically significant decrease in overall body weight gain (day 1-90) was also noticed in males (32%) and females (37%) in high dose

group. In high dose recovery group, significant improvement in body weight gain (63%) was observed in males at the end of recovery phase. On recovery phase day 28, statistical decrease in body weight in female (8%) was noticed as compared to control recovery group. The changes in the body weight parameters at 6000 ppm group were considered test substance-related in both sexes and adverse in males and females. The changes noted in females at 2000 ppm were not considered adverse because the body weight decrease were <10% compared to controls.

In 670 and 2000 ppm group, no test substance-related changes were noticed in overall mean food consumption (day 1-90) when compared to control group. In 6000 ppm group, significant reduction was noticed in overall mean food consumption (day 1-90) in males (10%) and females (7%) when compared to control group. In high dose recovery group, the food consumption in recovery phase was comparable with that of control recovery group. The test substance intake achieved over the entire treatment period (day 1-90) for 670, 2000 and 6000 ppm concentration levels in diet was equivalent to 38, 111 and 335 mg/kg body weight/day in males and 44, 134 and 404 mg/kg body weight/day in females, respectively. The test substance intake achieved over the entire treatment period (day 1-90) for high dose recovery group in diet was equivalent to 334 mg/kg body weight/day in males and 408 mg/kg body weight/day in females, respectively.

In 670 ppm and male 2000 ppm group, no test substance-related changes in overall food conversion efficiency (day 1-90) were noticed when compared with control group. In female 2000 ppm group, significant reduction (14%) was noticed in overall food conversion efficiency (day 1-90) as compared to control group. In 6000 ppm group, significant reduction was noticed in overall food conversion ratio (day 1-90) in males (25%) and female (32%) as compared to control group. In high dose recovery group, significant increase in food conversion efficiency (Recovery day 1-28) was noticed in males (69%) when compared with control group. The food conversion efficiency changes correlated with changes in body weight parameters and were considered test substance related. While decreases in males and females at 6000 ppm were considered adverse, decreases in 2000 ppm females were not considered adverse due to minimal nature of change.

No eye abnormalities were observed in ophthalmological examination performed towards the end of treatment period in males and females.

No test substance-related changes were observed in functional observation battery (FOB) parameters for main group animals as compared to control group.

No test substance-related changes were observed in grip strength and landing foot splay parameters as compared to control group.

No test substance-related changes were observed in rectal temperature as compared to control group.

No test substance-related changes were observed in motor activity parameters as compared to control group. Statistical significance increase (51%) in distance travelled was noticed in 670 ppm females as compared to control females. It was not considered test substance related as it occurred randomly in low dose females and lacked dose response relationship.

There were no test substance-related changes in blood coagulation parameters. In 6000 ppm group, minimal decrease in mean RBC and reticulocyte count in males, minimal decrease in haemoglobin and haematocrit in both sexes and mild decrease in eosinophil count in females were noticed. At recovery sacrifice, mean values of reticulocyte, haemoglobin and haematocrit were comparable with the concurrent control group. Minimal decrease in mean RBC in both sexes and eosinophil count in females were noted at recovery sacrifice. These findings were considered likely related to persistence of lower magnitude of decreased food consumption and body weight during recovery phase. These minor changes were considered not toxicologically relevant due to their lower magnitude of change.

In 6000 ppm group, minimal increase in mean serum ALP, minimal decrease in total protein, albumin, globulin were noticed at terminal sacrifice. In 2000 and 6000 ppm group, minimal decrease in BUN, total cholesterol, HDL in males were noticed at terminal sacrifice. At the end of recovery period, changes in ALP, total cholesterol and HDL persisted, however at a lower magnitude compared to ter-

minal sacrifice indicating trend towards recovery. Other changes in clinical chemistry parameters noted at terminal and recovery sacrifice were considered related to decreased food consumption (Levin et al., 1993[10]).

A dose-dependent minimal to mild decrease in mean serum T4 levels was noted at ≥2000 ppm at the terminal sacrifice in both sexes. Change in T4 was completely recovered to concurrent control levels at the end of recovery period. Magnitude of decrease in T4 at 2000 ppm was minimal (males 17%, females 21%) and mean values were within historical control data range. Mean value of T4 at 6000 ppm were outside historical control data range. Only effects at 6000 ppm were associated with the microscopic findings of minimal to mild colloid alteration in the thyroid follicle. Decrease in T4 occurred in isolation without concurrent changes in T3 or TSH levels and there were no morphological changes in the thyroid follicular cells. At the terminal sacrifice, there was minimal decrease in the thyroid weights in females at 6000 ppm, which was considered likely due to decreased food consumption and body weight. Thyroid weights were recovered at the end of recovery period.

There were no test substance-related changes in urinalysis parameters.

At the terminal sacrifice, decrease in organ weights (absolute, relative to brain weight and/or body weight ratios) were noted in multiple organs (males: liver and heart; Females: ovaries, pituitary, thyroid with parathyroid, heart and kidney) in 6000 ppm group. All these changes in organ weights were without any microscopic correlate and were recovered at the end of recovery period except minimal decrease in liver weights in males. These changes in organ weights were considered related to decrease in body weight and food consumption (Levin et al., 1993, Moriyama et al., 2008[11]). Decreased thyroid with parathyroid weight was limited to females and completely recovered in the recovery period. Therefore, decreased thyroid with parathyroid weight was considered more likely related to decreased food consumption and body weight than direct test substance related effect.

There were no test substance-related gross changes at the terminal and recovery sacrifice. At terminal sacrifice, microscopic findings of minimal to mild colloid alteration in the thyroid follicle were observed at 6000 ppm. Colloid alteration was present without any other related morphological changes like hypertrophy or hyperplasia of follicles. Incidence and severity of colloid alteration was likely related to decrease in T4 levels as this change can occur secondary to rapid turnover of thyroid hormones (Baiocco et al., 2018[12]). This microscopic change was not present at 670 and 2000 ppm groups and was not observed at the end of recovery period indicating complete recovery. Acinar cell hypertrophy of minimal to mild severity was noted in parotid salivary glands in 6000 ppm at the terminal sacrifice. Hypertrophy was diffuse in distribution and characterized by increased acinar cell size with increased secretary material and basophilic cytoplasm. There was no evidence of cellular injury like degeneration or necrosis in parotid salivary gland and no increased salivation observation during in-life phase of the study. These microscopic changes were completely recovered at the end of recovery period. This microscopic finding was considered as adaptive change (Greim et al., 2015[13], Inoue et al., 2014[14]).

[10] Levin S, Semler D, Ruben Z. Effects of two weeks of feed restriction on some common toxicologic parameters in Sprague-Dawley rats. Toxicol Pathol.1993; 21 (1):1-14.

[11] Moriyama T., Tsujioka S., Ohira T., Nonaka S., Ikeda H., Sugiura H., Tomohiro M., Samura K. and Nishikibe M. (2008). Effects of Reduced Food Intake on Toxicity Study Parameters in Rats. J. Toxicol. Sci., 33, 537-547.

[12] Baiocco A. B., Balme E., Bruder M., Chandra S., Hellmann J., Hoenerhoff M. J., Kambara T., Landes C., Lenz B., Mense M., Rittinghausen S., Satoh H., Schorsch F., Seeliger F., Tanaka T., Tsuchitani M., Wojcinski Z. and Rosol T. J. (2018), Nonproliferative and Proliferative Lesions of the Rat and Mouse Endocrine System. J Toxicol Pathol., 31 (3 Suppl), 1S–95S.

[13] Greim H., Saltmiras D., Mostert V. and Strupp C. (2015), Evaluation of Carcinogenic Potential of the Herbicide Glyphosate, Drawing on Tumor Incidence Data from Fourteen Chronic/Carcinogenicity Rodent Studies. Crit Rev Toxicol., 45(3), 185-208.

[14] Inoue K, Morikawa T, Matsuo S, Tamura K, Takahashi M and Yoshida M. (2014), Adaptive Parotid Gland Hypertrophy Induced by Dietary Treatment of GSE in Rats. Toxicol Pathol., 42 (6), 1016-1023.

Table A 27: Body weights (g)

Group #		Dosing Phase							
		Day: 1	**Day: 7**	**Day: 15**	**Day: 21**	**Day: 29**	**Day: 35**	**Day: 43**	**Day: 49**
		Session 1	**Session 1**	**Session 1**	**Session 1**	**Session 1**	**Session 1**	**Session 1**	**Session 1**
Males									
Control	n	10	10	10	10	10	10	10	10
	Means	284.87	314.49	340.23	356.96	375.68	387.46	402.38	410.36
	SD	16.69	15.03	18.08	17.72	20.59	18.64	19.90	21.78
2	n	10	10	10	10	10	10	10	10
	Means	290.53	313.52	338.76	353.77	370.29	379.07	390.71	395.79
	SD	16.28	15.49	18.57	19.78	370.29	21.63	21.34	23.04
3	n	10	10	10	10	10	10	10	10
	Means	289.97	311.84	339.00	355.15	370.69	380.51	393.16	402.58
	SD	17.05	15.15	15.72	17.63	20.63	21.14	23.77	25.50
4	n	10	10	10	10	10	10	10	10
	Means	288.09	**293.03-**	325.47	**332.75-**	**344.42-**	**352.47-**	**363.10-**	**366.26-**
	SD	16.37	14.01	17.85	16.30	16.30	16.42	16.51	18.52
Females									
Control	n	10	10	10	10	10	10	10	10
	Means	189.93	204.19	217.88	227.39	237.12	241.20	248.57	251.77
	SD	8.50	9.24	9.11	13.90	10.90	12.39	13.37	13.62
2	n	10	10	10	10	10	10	10	10
	Means	192.20	206.06	224.07	229.43	237.94	244.57	250.37	250.35
	SD	7.17	7.91	10.65	10.92	12.76	14.53	13.89	12.90
3	n	10	10	10	10	10	10	10	10
	Means	189.27	200.78	214.80	220.35	225.64	231.58	**234.63-**	**238.73-**
	SD	9.39	7.74	9.63	10.63	9.88	9.35	10.44	9.52
4	n	10	10	10	10	10	10	10	10
	Means	190.00	**190.16-**	210.58	**214.56-**	**217.97-**	**220.73-**	**225.16-**	**231.47-**
	SD	8.55	7.95	9.86	9.36	9.02	7.69	10.70	6.97

Group #: Control: G1 (0 ppm); 2: G2 (670 ppm); 3: G3 (2000 ppm); 4: G4 (6000 ppm)
-: Significantly lower than the control group at 5% level.

Table A 28: Body Weights (g) (continued)

Group #		Dosing Phase					
		Day: 57	**Day: 63**	**Day: 71**	**Day: 77**	**Day: 85**	**Day: 90**
		Session 1	**Session 1**	**Session 1**	**Session 1**	**Session 1**	**Session 1**
Males							
Control	n	10	10	10	10	10	10
	Means	422.61	428.00	434.57	441.53	446.11	449.02
	SD	25.20	25.38	24.32	26.30	26.14	27.95
2	n	10	10	10	10	10	10
	Means	411.18	415.51	423.72	429.11	432.45	436.61
	SD	22.38	22.93	24.98	27.16	27.87	25.09
3	n	10	10	10	10	10	10
	Means	414.41	420.70	430.18	433.55	437.13	440.70
	SD	24.68	26.37	26.30	28.21	28.98	30.15
5	n	10	10	10	10	10	10
	Means	**382.99-**	**386.27-**	**392.34-**	**396.19-**	**398.62-**	**400.04-**
	SD	19.03	19.59	18.68	18.54	17.41	18.55
Females							
Control	n	10	10	10	10	10	10
	Means	258.54	261.96	264.71	265.05	270.36	271.40
	SD	14.42	15.54	16.01	15.28	19.09	16.51
2	n	10	10	10	10	10	10
	Means	256.64	258.48	262.39	263.35	265.51	267.97
	SD	12.22	13.24	11.94	11.24	10.36	10.22
3	n	10	10	10	10	10	10
	Means	**245.40-**	249.95	253.75	255.32	256.42	**256.51-**
	SD	9.36	11.82	10.62	9.66	12.65	12.32
4	n	10	10	10	10	10	10
	Means	**239.98-**	**242.01-**	**242.47-**	**242.51-**	**240.52-**	**241.31-**
	SD	7.78	8.44	9.06	8.90	6.99	7.80

Group #: Control: G1 (0 ppm); 2: G2 (16000 ppm)
There were no statistically significant differences at 5% level

Table A 29: Body weight change (g)

Group #		D:1 – D:7	D:7 - D:15	D:15 – D:21	D:21 – D:29	D:29 – D:35	D:35 – D:43	D43 – D:49	D:59 – D:57
Males									
Control	n	10	10	10	10	10	10	10	10
	Means	29.62	25.74	16.73	18.72	11.78	14.92	7.97	12.25
	SD	9.96	5.78	5.01	5.79	4.01	2.89	4.41	5.39
2	n	10	10	10	10	10	10	10	10
	Means	22.99	25.24	15.01	16.52	8.78	11.65	5.07	15.39
	SD	8.92	7.11	3.16	3.93	3.92	2.44	3.29	4.20
3	n	10	10	10	10	10	10	10	10
	Means	21.87	27.16	16.15	15.54	9.82	12.65	9.42	11.83
	SD	5.95	4.72	3.04	5.59	3.66	4.51	3.04	3.34
4	n	10	10	10	10	10	10	10	10
	Means	**4.95-**	32.43	**7.28-**	**11.67-**	8.05	10.63	**3.16-**	16.73
	SD	6.08	11.95	4.94	5.47	2.95	4.78	4.64	4.00
Females									
Control	n	10	10	10	10	10	10	10	10
	Means	14.26	13.69	9.51	9.73	4.09	7.36	3.20	6.77
	SD	5.87	2.39	5.16	4.49	2.52	3.51	3.12	6.95
2	n	10	10	10	10	10	10	10	10
	Means	13.86	18.02	5.35	8.51	6.63	5.80	-0.02	6.29
	SD	6.28	6.52	4.37	4.13	6.13	4.41	2.39	4.99
3	n	10	10	10	10	10	10	10	10
	Means	11.51	14.02	5.55	**5.28-**	5.94	3.06	4.10	6.67
	SD	3.23	3.86	3.79	3.38	3.79	4.40	3.48	3.21
4	n	10	10	10	10	10	10	10	10
	Means	**0.15-**	**20.42+**	**3.98-**	**3.41-**	2.76	4.43	6.31	8.51
	SD	3.91	5.09	2.78	3.30	2.14	5.35	5.64	6.42

Group #: Control: G1 (0 ppm); 2: G2 (670 ppm); 3: G3 (2000 ppm); 4: G4 (6000 ppm)
-: Significantly lower than the control group at 5% level

Table A 30: Body weight change (g) (continued)

Group #		D:57-D:63	D:63-D:71	D:71-D:77	D:77-D:85	D:85-D:90	D:1-D:90
Males							
Control	n	10	10	10	10	10	10
	Means	5.39	6.58	6.95	4.58	2.91	164.15
	SD	2.01	4.19	3.49	3.53	3.27	25.87
2	n	10	10	10	10	10	10
	Means	4.34	8.21	5.39	3.34	4.16	146.07
	SD	1.95	4.10	3.87	4.91	5.22	23.37
3	n	10	10	10	10	10	10
	Means	6.29	9.48	3.37	3.58	3.57	150.73
	SD	5.86	3.10	3.46	5.18	4.03	26.25
4	n	10	10	10	10	10	10
	Means	3.28	6.07	3.85	2.43	1.42	**111.95-**
	SD	3.40	4.80	4.22	7.22	4.17	21.23
Female							
Control	n	10	10	10	10	10	10
	Means	3.42	2.75	0.34	5.31	1.04	81.47
	SD	3.83	1.96	4.41	8.17	3.72	11.66
2	n	10	10	10	10	10	10
	Means	1.84	3.92	0.96	2.16	2.46	75.77
	SD	3.40	2.50	1.26	4.39	3.04	5.67
3	n	10	10	10	10	10	10
	Means	4.55	3.81	1.56	1.11	0.09	67.24
	SD	4.13	2.46	1.89	6.80	3.80	10.49
4	n	10	10	10	10	10	10
	Means	2.02	0.47	0.04	-1.99	0.79	**51.30-**
	SD	6.42	2.29	1.70	3.49	2.59	5.78

Group #: Control: G1 (0 ppm); 2: G2 (670 ppm); 3: G3 (2000 ppm); 4: G4 (6000 ppm)
-: Significantly lower than the control group at 5% level

Table A 31: Test item intake (mg/kg/day)

Group #		Day No.						
		1-7	**7-15**	**15-21**	**21-29**	**29-35**	**35-43**	**43-49**
Males								
G2 670	Mean	45.33	42.08	41.21	39.38	39.10	37.85	37.31
	SD	2.41	2.38	2.44	2.21	2.31	1.88	2.08
	n	10	10	10	10	10	10	10
G3 2000	Mean	135.55	125.88	123.53	118.14	115.53	109.98	108.90
	SD	6.81	5.01	4.99	6.32	4.61	5.40	7.28
	n	10	10	10	10	10	10	10
G4 6000	Mean	357.32	379.26	383.45	371.63	357.35	337.21	334.98
	SD	31.64	19.22	19.82	17.07	15.45	14.67	19.60
	n	10	10	10	10	10	10	10
G4R 6000	Mean	340.71	385.56	372.74	371.56	360.20	333.48	327.98
	SD	14.02	8.27	15.91	12.07	14.22	17.56	18.20
	n	6	6	6	6	6	6	6
Females								
G2 670	Mean	50.98	47.92	47.84	45.81	45.70	43.59	44.21
	SD	2.08	1.40	1.96	1.50	2.18	2.68	2.97
	n	10	10	10	10	10	10	10
G3 2000	Mean	154.88	147.80	148.05	144.23	143.63	137.63	128.59
	SD	6.52	6.10	7.68	7.41	7.24	9.98	5.40
	n	10	10	10	10	10	10	10
G4 6000	Mean	384.94	463.17	439.07	448.93	450.42	428.16	398.53
	SD	31.98	28.65	21.91	17.64	16.46	15.87	13.12
	n	10	10	10	10	10	10	10
G4R 6000	Mean	377.73	450.12	443.87	459.52	458.13	423.31	406.92
	SD	13.67	23.85	31.05	18.83	19.84	21.19	13.60
	n	6	6	6	6	6	6	6

Table A 32: Test item intake (mg/kg/day) (continued)

Group #		Day No.					
		57-63	**63-71**	**71-77**	**77-85**	**85-90**	**1-90**
Males							
G2 670	Mean	36.04	34.84	33.88	33.76	34.04	37.75
	SD	1.83	1.88	2.20	1.92	1.98	1.93
	n	10	10	10	10	10	10
G3 2000	Mean	103.03	101.50	98.63	99.06	97.77	110.74
	SD	6.34	5.32	5.88	6.75	5.69	5.27
	n	10	6	6	6	6	6
G4 6000	Mean	314.90	307.51	303.91	310.15	297.00	334.85
	SD	19.48	14.83	14.19	11.77	17.91	13.78
	n	10	10	10	10	10	10
G4R 6000	Mean	315.15	308.39	311.05	306.62	301.98	333.90
	SD	18.20	20.10	16.81	20.07	21.03	12.51
	n	6	6	6	6	6	6
Females							
G2 670	Mean	41.98	41.52	40.77	40.88	40.65	44.23
	SD	2.68	1.94	1.81	1.17	1.42	1.69
	n	10	10	10	10	10	10
G3 2000	Mean	124.91	121.77	120.70	121.84	117.54	134.14
	SD	6.90	5.20	5.43	5.42	5.02	4.80
	n	10	10	10	10	10	10
G4 6000	Mean	375.05	368.27	373.76	373.13	366.77	403.85
	SD	14.31	15.82	13.30	11.93	12.96	13.47
	n	10	10	10	10	10	10
G4R 6000	Mean	377.72	377.37	386.14	378.22	377.48	408.01
	SD	15.95	18.66	18.31	27.15	18.06	14.95
	N	6	6	6	6	6	6

Conclusion

Based on the results, the No-Observed-Adverse-Effect-Level (NOAEL) of RH-150721 in the Sprague Dawley rats is 2000 ppm, which is equivalent to 111 mg/kg body weight/day in males and 134 mg/kg body weight/day in females. This NOAEL is based on reduction in food consumption and food efficiency, triggering changes in bodyweight, hematological and clinical chemistry parameters and multiple organ weights as a result thereof at the Lowest-Observed-Adverse-Effect-Level (LOAEL) of 6000 ppm in males and females.

Effects at 6000 ppm were partially recovered at recovery. A dose-dependent minimal to mild decrease in mean serum T4 levels was noted at ≥ 2000 ppm at the terminal sacrifice in both sexes, which were within historical control at 2000 ppm and associated with the microscopic finding of minimal to mild colloid alteration in the thyroid follicle only at 6000 ppm. Decreases in T4 and microscopic findings in the thyroid were completely recovered at the end of recovery period.

Study 11 – RH-141452: 14 days oral dietary toxicity

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The 14-day oral dietary dose range finding study in Sprague Dawley rats was performed in line with internationally recognised guidelines and is acceptable. 14 d No-Observed-Adverse-Effect-Level (NOAEL) of RH-141452 in the male and females Sprague Dawley rats is 5000 ppm, which is equivalent to 415 mg/kg bw/day in males and 432 mg/kg bw/day in females.

Reference:	**KCP 7.4/11**
Report:	XXXX, 2021: RH-141452: 14-day dietary study in Sprague Dawley rats XXXX Syngene International Ltd, India, Report No. U-20188, No GLP, Not published
Guideline(s):	OECD 407 (2008), EEC B.7 (2008), OPPTS 870.3050 (2000)
Deviations:	No
GLP:	No
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-141452, synonym 127-0007 (GCP016-00-20200930-01)
Purity:	98.6 % (w/w)
Vehicle:	Teklad certified irradiated global 18% protein rodent diet (Meal)manufactured by Envigo
Stability:	Test material was stable for at least 8 days at room temperature
Test organisms	Rat, Sprague Dawley, purchased from Envigo, The Netherlands
Age:	7 weeks
Body weight:	Male: 195.02 – 216.48 g Female: 146.66 – 168.59 g
Acclimation period:	4 days
Water:	Tap water, *ad libitum*
Diet:	Teklad certified irradiated global 18% protein rodent

Test material (Lot/Batch No.)	RH-141452, synonym 127-0007 (GCP016-00-20200930-01)
	diet (meal) *ad libitum* (except overnight fasting prior to clinical pathology blood collection) manufactured by Envigo
Housing:	2 per sex per cage in polycarbonate rat cages with bedding materials
Test design	
No. of groups:	4
No. of animals/group:	6 rats/sex/group
Number of animals:	48 (24 males + 24 females)
Extra animals:	6 (3 males + 3 females)
Total number of animals:	54 (27 males + 27 females)
Environmental conditions	
Temperature:	20.3 to 23.2°C
Humidity:	Relative humidity ranged from 41 to 65%
Air changes:	21 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	1000, 5000 and 16000 ppm in diet
Exposure time	14 days
Remarks	Designed as range-finding study for the 90 days oral toxicity study.

In a dose range finding study, RH-141452 was administered to groups of male and female Sprague Dawley rats (6 rats/sex/group) for 14 days at levels of 1000, 5000 and 16000 ppm. The doses in ppm were equivalent to 86, 416 and 1268 mg/kg body weight/day in males and 85, 432 and 1312 mg/kg body weight/day in females, respectively. The control group was fed with basal diet for same duration of study.

There were 6 animals of each sex per group. Males and females were randomised into 4 dose groups using Pristima, based on body weight to control bias. The body weight variation of the animals did not exceed ± 20% of the mean body weight per sex at the time of randomization. RH-141452 was administered in the diet for 14 days. Husbandry conditions were in accordance with the recommendations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

The test item was mixed with feed manually with spatula for approximately 5 minutes, the premix feed was homogeneously mixed with the remaining quantity of feed in a blender for approximately 20 minutes.

Diet was prepared approximately weekly. Prepared diets were stored at room temperature before use. The concentration of the test substance and the homogeneity in the feed was analytically verified one day prior to dosing by reverse phase HPLC-DAD. The method has been validated according to SANCO/3029/99 rev. 4 (2000); see analytical method validation report no. U-20192 for the estimation of RH-141452 in rat feed.

The animals were observed twice daily for morbidity and mortality. All rats were observed for clinical signs at least once daily. Detailed clinical examinations were done once on day 1 of dosing and weekly thereafter.

Body weights were recorded on days 1, 4, 8, 11 and 14. The body weight changes were calculated.

Food consumption and compound intake was recorded on days 1, 4, 8, 11. Food leftover was measured on days 4, 8, 11 and 14. Test substance intake was calculated.

Ophthalmoscopic examination was not reported.

Blood samples were collected from three rats per sex per time point as per the sampling schedule. Blood was collected via retro-orbital plexus puncture under mild isoflurane anaesthesia. The sampling intervals were as follows: 5 P.M. (day 2 and 13) and 7 A.M., 10 A.M., 2 P.M and 5 P.M (±25 min) on days 3 and 14. Toxicokinetic evaluation was performed by validated Phoenix® WinNonlin® software (Pharsight Corporation, USA).

Blood plasma samples were prepared with K_2EDTA as anti-coagulant and analysed by LC-MS/MS for RH-141455 and an internal standard (tolbutamide). A total of 186 (96 for day 1 and 90 from day 14) samples were analysed. The method has been validated according to SANCO/3029/99 rev. 4 (2000) for a concentration range of 0.095 to 49.379 µg/mL.

Blood was collected from all animals at the terminal sacrifice (day 15) for haematology, coagulation and clinical chemistry evaluations. The animals were fasted overnight prior to terminal blood collection. Blood was collected via retro-orbital plexus puncture of right eye under mild isoflurane anaesthesia. The following haematology, coagulation and clinical chemistry parameters were measured: haematology – red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, reticulocyte count, total white blood cell count, leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils); coagulation – active partial thromboplastin time, prothrombin time and fibrinogen; clinical chemistry – albumin/globulin ratio, alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, bile acids, blood urea nitrogen, calcium, chloride, creatinine, creatinine kinase, gamma-glutamyl trans-peptidase, globulin, glucose, high-density lipoprotein, inorganic phosphorus, low-density lipoprotein, potassium, sodium, total bilirubin, total cholesterol, total protein and triglycerides.

Urine was collected from all animals at the end of dosing phase (day 15). During urine collection, animals were fasted overnight in metabolic cages. The following parameters were measured: volume, colour, appearance, glucose, bilirubin, ketone, blood, pH, protein, urobilinogen, nitrite, leukocyte and specific gravity. The urinary sediments were examined by microscopy.

Gross necropsy was conducted on all animals at the end of dosing phase (day 15). The following organs were collected, weighed, assessed histopathologically after fixation in 10% neutral buffered formalin and staining with haematoxylin and eosin: adrenals, brain, epididymites, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid with parathyroid and uterus with cervix. The other organs or tissues such as aorta, biceps femoris muscles, cecum, colon, diaphragm, duodenum, oesophagus, eyes, femur bone with joint, gastrocnemius muscle, gross changes, harderian glands, ileum with Peyer's patches, jejunum, lacrimal glands, larynx & pharynx, lungs, lymph nodes (mandibular and mesenteric), mammary gland, optic nerve, oviducts, pancreas, quadriceps muscle, rectum, salivary glands (mandibular, sublingual and parotid), sciatic nerve, skin (inguinal region), soleus muscle, spinal cord, sternum with marrow, stomach, tongue, trachea, urinary bladder and vagina, were also examined histopathologically. Histopathology was performed on all tissues of animals from control and high dose groups. Peer-review was performed by another in-house pathologist.

For comparative statistics, data were evaluated using Levene's Test for homogeneity of variance, with significance at 5% level. Data determined to be homogeneous was evaluated for analysis of variance (ANOVA). When the ANOVA verified significance at 5% level, pairwise comparisons of each treated group with the control group was made using a Dunnett Test, to identify statistical differences (at 5%

level). When the data were found to be non-homogeneous, ANOVA was done using suitable transformation. When the homogeneity tests were significant even after transformation, data were evaluated using a Kruskal-Wallis Test for group factor significance. When significance (at 5% level) existed between groups, Dunn's pairwise comparison was performed.

Results and discussions

The dose formulation analysis results of day 1 were within the acceptable limits with mean recovery values of 98.46 to 100.00 % (n=5) and thus < ±20 % of nominal concentrations. The control samples analysed did not show any presence of test item.

There were no treatment-related clinical signs of toxicity observed in animals at any dose.

No mortalities occurred in the test animals at any dose level.

Test item-related effects were noted in body weight and food intake parameters at 16000 ppm dose. A test item-related statistically significant decrease in terminal body weight compared to control (male: 11%) was observed in high dose group (16000 ppm). In females, minimal decrease in body weight gain was observed at 16000 ppm. In high dose (16000 ppm), overall (days 1 - 14) food consumption was 13% lower in males and 7% lower in females as compared to control group. In the high dose (16000 ppm), overall (days 1 - 14) food conversion efficiency was 36% lower in males and 25% lower in females as compared to control group.

Table A 33 Test item intake (days 1-14):

Dietary dose (ppm)	1000	5000	16000
	Achieved Dose (mg/kg body weight/day)		
Males	86	416	1268
Females	85	432	1312

Table A 34 Summary of Body Weights (g) in male and female rats

Group #		Day no.				
		1	4	8	11	14
Males						
G1 Control	Mean	209.92	225.47	255.29	268.02	276.70
	SD	7.85	7.97	10.12	9.89	11.62
	n	6	6	6	6	6
G2 1000	Mean	209.03	222.02	251.18	263.73	273.41
	SD	7.80	8.41	8.88	9.44	11.04
	n	6	6	6	6	6
G3 5000	Mean	208.82	225.38	255.20	270.36	280.00
	SD	6.66	4.68	7.61	6.78	9.11
	n	6	6	6	6	6
G4 16000	Mean	208.44	215.01	**238.63-**	**249.07-**	**246.26-**
	SD	7.52	9.91	13.80	15.68	23.83
	n	6	6	6	6	6
Females						
G1 Control	Mean	154.02	159.97	169.97	179.62	183.48
	SD	7.68	7.64	8.14	8.41	7.27
	n	6	6	6	6	6
G2 1000	Mean	154.29	162.14	173.56	180.14	183.91
	SD	6.33	6.74	10.72	11.20	11.44
	n	6	6	6	6	6
G3 5000	Mean	154.51	160.73	172.61	180.68	185.02
	SD	7.46	6.82	7.87	8.44	8.68
	n	6	6	6	6	6
G4 16000	Mean	155.33	156.89	169.90	177.97	176.24
	SD	8.47	7.55	10.09	12.76	15.19
	n	6	6	6	6	6

Group#: Control: G1 (0 ppm); **2:** G2 (1000 ppm); **3:** G3 (5000 ppm); **4:** G4 (16000 ppm)
-: Significantly lower than the control group at 5 % level.

There were no test substance-related changes in haematology and coagulation parameters in the study. Minimal but few statistically significant differences in the haematology parameters (minimal decrease in mean reticulocyte count, WBC, lymphocyte and neutrophil) in the high dose (16000 ppm in diet) group of males compared to the controls. Similar changes were not noted in females. All these minimal changes were likely related to decreased food consumptions in animals at the high dose. These haematology changes lacked microscopic correlations.

There was a statistically significant minimal increase in mean AST, ALT and triglycerides (less than 2-fold change over concurrent control) in males at 16000 ppm. These changes were minor and not considered adverse.

There were no RH-141452- related changes in urinalysis parameters.

At 16000 ppm, gross change of small sized spleen (1/6) and thymus (2/6) were noted only in females, which correlated microscopically with decreased cellularity in thymus and spleen. These changes were considered as secondary changes related to decreased body weight/food consumption.

At 16000 ppm, minimal decreases in organ weights (absolute, relative to brain weight and/or body weight ratios) in thymus and male accessory sex glands as well as minimally decreased secretion of the accessory sex glands (prostate and seminal vesicles) were noted. These changes in organ weights were considered to be related to decrease in body weight and/or food consumption.

Test item- related minimal diffuse hypertrophy in salivary glands (parotid, sublingual and mandibular) was noted at dose levels of 5000 and 16000 ppm. There was no evidence of cellular injury like degeneration or necrosis in these glands. There was no increased salivation observed in these animals during in-life phase of the study. The minimal hypertrophy in salivary gland was considered as an adaptive change (Inoue et al., 2014[15]).

At 16000 ppm, mild vacuolation in adrenal cortex and minimal to moderate decrease in cellularity in thymus and spleen were noted in two females. All these changes in adrenals, thymus and spleen were observed in those two females in which significant decrease in body weight was observed.

Repeated dose feeding of RH-141452 at 1000, 5000 or 16000 ppm in diet for 2 weeks showed evidence of systemic plasma exposure to RH-141452 at all dose levels in Sprague Dawley rats. RH-141452 exposure (AUC_{0-24h}) increased less than or approximately proportional to dose from 1000 to 16000 ppm on day 2 and more than proportional to dose day 13 in males and females. There were no sex-specific differences.

Following repeated oral feeding of RH-141452 in rats over 14 days, T_{max} was observed at 43 hours (day 2) and 307 hours (day 13) in both males and females. Plasma concentrations of RH-141452 were observed at all time points in treated groups. Increase in AUC_{0-24h} and C_{max} were less than or approximately dose proportional from 1000 to 16000 ppm on day 2 and more than proportional to doses on day 13 in males and females. No accumulation of RH-141452 was observed in males with half-lives (as far as calculated) between 4.0 and 7.6 hours, while insufficient terminal phase data was available to reliably estimate half-lifes in female rats.

[15] Inoue K, Morikawa T, Matsuo S, Tamura K, Takahashi M, Yoshida M. Adaptive parotid gland hypertrophy induced by dietary treatment of GSE in rats. Toxicol Pathol. 2014 Aug;42 (6):1016-23.

Table A 35 The toxicokinetic parameters of RH-141452

Day nominal	Sex	Dose in ppm (mg/kg/day)	T_{max} in h (time from start of exposure)	C_{max} (µg/mL)	AUC_{0-24h} (h*µg/mL)	T_{last} in h (time from start of exposure)	C_{last} (ug/mL)
Day 2	Male	1000 (86)	14 (43)	2.265	35.777	24	0.689
		5000 (416)	14 (43)	12.222	186.102	24	3.896
		16000 (1268)	14 (43)	37.636	621.540	24	7.286
Day 2	Female	1000 (85)	14 (43)	2.898	42.593	24	0.690
		5000 (432)	14 (43)	10.936	212.851	24	4.170
		16000 (1312)	14 (43)	29.406	490.545	24	7.768
Day 13	Male	1000 (86)	14 (307)	1.827	25.443	24	0.777
		5000 (416)	14 (307)	10.086	137.587	24	2.067
		16000 (1268)	14 (307)	65.704	804.624	24	13.489
Day 13	Female	1000 (85)	14 (307)	3.081	46.969	24	1.146
		5000 (432)	14 (307)	8.903	174.184	24	1.499
		16000 (1312)	14 (307)	101.667	1312.399	24	3.219

Conclusion

In a dose range finding study, RH-141452 was administered to male and female groups of Sprague Dawley rats (6 rats/sex/group) for 14 days at levels of 1000, 5000 and 16000 ppm. The doses in ppm were equivalent to 85, 415.63 and 1267.64 mg/kg body weight/day in males and 85.36, 431.92 and 1311.64 mg/kg body weight/day in females, respectively. The control group was fed with basal diet for same duration of study.

Under the conditions of the study, the No-Observed-Adverse-Effect-Level (NOAEL) of RH-141452 in the male and females Sprague Dawley rats is 5000 ppm, which is equivalent to 415 mg/kg bw/day in males and 432 mg/kg bw/day in females. This NOAEL was based on decreased body weight, food consumption and associated microscopic changes of vacuolation in zona glomerulosa of adrenal cortex, decreased cellularity (thymus, spleen) and decreased secretion (accessory sex glands) at 16000 ppm.
Conclusion by EMS: The No-Observed-Adverse-Effect-Level (NOAEL) of RH-141452 in the male and females Sprague Dawley rats is 5000 ppm.

Study 12 – RH-141452: 90 days oral dietary toxicity

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of zRMS:	The 90-day oral dietary study in Sprague Dawley rats was performed in line with internationally recognised guidelines and in GLP conditions is acceptable. 90d No-Observed-Adverse-Effect-Level (NOAEL) of RH-141452 for male and female Sprague Dawley rats is 9000 ppm, which is equivalent to 538 mg/kg bw/day in male and 625 mg/kg bw/day in female rats.

Reference:	**KCP 7.4/12**
Report:	XXXX, 2022: RH-141452: 90-Day dietary toxicity study with 28-day recovery period in Sprague Dawley rats XXXX Syngene International Ltd, India, Report No. U-20281, GLP, Not published
Guideline(s):	OECD 408 (2018), OPPTS 870.3100 (1998), EEC B.26 (2008), ICH Harmonized Tripartite Guideline S3A (Toxicokinetics, 1994), Notification No. 12-Nousan-8147, MAFF, Japan (2000)
Deviations:	Draft report submission to sponsor was delayed. This is not considered to have any impact on the study integrity and results.
GLP:	Yes
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

Test material (Lot/Batch No.)	RH-141452, synonym 127-0007 (GCP016-00-20200930-01)
Purity:	98.9 % (w/w)
Vehicle:	Teklad certified irradiated global 18% protein rodent diet (Meal)manufactured by Envigo
Stability:	Test material was stable for at least 8 days at room temperature
Test organisms	Rat, Sprague Dawley purchased from Envigo, Netherlands
Age:	8 weeks
Body weight:	Males: 228.22 to 273.82 g (G1-G4) Females: 157.96 to 189.27 g (G1-G4) Males: 223.88 to 256.26 g (G5-G8, valid main test) Females: 156.38 to 184.12 g (G5-G8, valid main test) The weight variation of the animals at randomisation was within ± 20% of the mean weight for each sex.

Test material (Lot/Batch No.)	RH-141452, synonym 127-0007 (GCP016-00-20200930-01)
Acclimation period:	11 days / 6 days for the repitition experiment
Water:	Tap water, *ad libitum*
Diet:	Teklad certified irradiated global 18% protein rodent diet (meal) *ad libitum* (except overnight fasting prior to clinical pathology blood collection) manufactured by Envigo
Housing:	one or two per sex per cage in polycarbonate rat cages with bedding materials
Test design	
No. of groups:	10 (8 main groups + 2 recovery groups + 4 TK groups)
No. of animals/group:	10 rats/sex/group in main groups 6 rats/sex/group in recovery groups 6 rats/sex/group in test item TK groups and 3 rats/sex/group in control TK group
Number of animals:	226 (113 Males + 113 Females)
Extra animals:	40 (20 Males + 20 Females)
Total number of animals:	266 (133 Males + 133 Females)
Environmental conditions	
Temperature:	19.5 – 24.2°C
Humidity:	Relative humidity ranged from 41 – 68%
Air changes:	18-21 fresh air changes per hour
Photoperiod:	12-hour light/dark cycles
Test conentrations	1500, 4500, 9000 mg/kg in diet
Exposure time	90 days (recovery experiment + 28 days)
Remarks	None.

This study was designed to assess the potential sub-chronic toxicity and toxicokinetics (TK) profile of RH-141452 when fed in formulated diet to Sprague Dawley rats for 90 days. A total of 73 male and 73 female Sprague Dawley rats were randomly assigned to 4 main groups (10 rats/sex/group), 2 recovery groups (6 rats/sex/group) and 2 TK groups (3/6 rats/sex/group for control and test-substance related, respectively). Based on the results of a 14 days oral toxicity study (U-20188), doses of 1500, 4500 and 9000 ppm RH-141452 were chosen. The control group was fed with basal diet for same duration of study.

Due to failure of the automated room light timer, all study groups mentioned above were exposed to continuous light towards the end of dosing, leading to histopathological findings in circadian clock sensitive tissues. Hence, 4 additional rat groups (10 rats/sex/group) were added to the study by a study plan amendment number 4 to repeat the main study part. It was not considered necessary to repeat the toxicokinetic groups (no photosensitivity of the endpoint) or recovery groups (no findings in the first or repeated main groups that would trigger a repeat of the recovery groups).
The relevant study groups can be summarised as follows:

Table A 36 Study design

Main groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
5	0	0	0	10	10
6	1500	91	101	10	10
7	4500	271	308	10	10
8	9000	538	625	10	10

Recovery groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	6	6
2	9000	538	625	6	6

TK groups:

Test group	Concentration in diet (ppm)	Dose (mg/kg/day)		Animals assigned	
		Male	Female	Male	Female
1	0	0	0	3	3
2	1500	91	112	6	6
3	4500	271	332	6	6
4	9000	541	673	6	6

Males and females were randomised into 8 main groups (10 rats/sex/group), 2 recovery groups (6 rats/sex/group) and 4 TK groups (3 rats/sex/group and 6 rats/sex/group) using Pristima, based on body weight to control bias. RH-141455 was incorporated in the diet and fed at concentration level of 1500, 4500 and 9000 ppm for 90 days to Sprague Dawley rats.

The required amount of test material was weighed and mixed with feed manually with spatula for approximately 5 minutes. Premix feed was homogeneously mixed with the remaining quantity of feed in a blender for approximately 20 minutes. Diet was prepared approximately weekly. Prepared diets were stored at room temperature (max. storage period of 8 days) before use. The concentration of the test substance and the homogeneity in the feed was analytically verified one day prior to dosing by reverse phase HPLC-DAD. The method has been validated according to SANCO/3029/99 rev. 4 (2000); see analytical method validation report no. U-20236 for the estimation of RH-141452 in rat feed. The results confirmed the test item concentrations in the rat diet at 80 - 120 % of nominal (i.e. 90.8-108 % recovery) with a relative standard deviation (% RSD) ≤ 20 %.

The animals were observed for morbidity and mortality twice daily during study period and once on the day of terminal and recovery sacrifice. All rats were observed for clinical signs once daily during the dosing and recovery period. Detailed clinical examination of the individual rats were done prior to initiation treatment and once weekly thereafter until scheduled necropsy.

Ophthalmological examination was performed using an ophthalmoscope once for all animals during acclimatization period and during the last week of dosing phase. Mydriasis was induced before examination of eyes using a mydriatic agent.

Body weight were recorded for all animals on first day of treatment (day 1) and weekly thereafter. Terminal (fasting) body weights were measured on the days of scheduled necropsy (day 91 for main group and day 29 of recovery phase). The body weight changes were calculated.

Food input and food left over was measured for all animals once weekly from day of start of treatment until scheduled sacrifice.

Functional observation battery and motor activity were investigated in week 12/13 during dosing phase in the control and the test substance groups (e.g. posture, respirator pattern, tremor in the cages and e.g. ease of removal or handling, salivation, piloerection, fur appearance, dehydration after removal from the cage). Individual rats were neurologically evaluated as applicable. Rats were placed in an open field standard arena and evaluated for about 2 minutes for e.g. tremors, muscle spasms, mobility, abnormal behaviour, number of defecations or urinations. Sensory observations were made, like e.g. touch response, auditory response, pupil reflex.

The following neuromuscular observations were performed: 1. Grip strength, 2. Hind limb foot splay.

Rectal temperature was recorded using digital thermometer.

The following motor activity parameters were observed via Opto-Varimex-4 Activity Meter (Columbus Instruments): Distance travelled, horizontal counts, ambulatory counts, and vertical counts.

Blood samples were collected from three rats per sex per time point from the TK animal groups. Blood was collected via retro-orbital plexus puncture under mild isoflurane anaesthesia. The sampling intervals for test substance treated group were as follows: 5 P.M. (day 2 and 87), 7 A.M., 10 A.M., 2 P.M. and 5 P.M. (±25 min) on days 3 and 88. The sampling intervals for control group were as follows: 5 P.M. (day 2 and 87), 7 A.M. and 2 P.M. on days 3 and 88. The blood samples were mixed and maintained on wet ice until centrifugation. Plasma was separated by centrifugation at 5000 rpm for 10 minutes at 2-8°C. The blood samples were stored at ≤ - 60 °C until bioanalysis.

The bioanalysis was performed by LC-MS/MS method using protein precipitation as sample extraction technique. The supernatant - after extraction and centrifugation - was transferred into auto sampler vials. Volumes of 10 µL of the supernatant were injected onto a HPLC column for chromatographic separation, followed by mass spectrometer detection. The samples were analysed with a validated method U-20272 at a lower limit of quantification (LLOQ) of 0.222 µg/mL and upper limit of 110.803 µg/mL (ULOQ). Toxicokinetic evaluation was performed by validated Phoenix® WinNonlin® software (Pharsight Corporation, USA).

Blood was collected from all animals of main groups at the dosing phase (day 91) and for recovery groups at the end of recovery phase (day 29) for haematology, coagulation and clinical chemistry evaluations. The animals were fasted overnight prior to terminal blood collection. Blood was collected via retro-orbital plexus puncture of right eye under mild isoflurane anaesthesia. The following haematology, coagulation and clinical chemistry parameters were measured: haematology – red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, reticulocyte count, total white blood cell count, leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils); coagulation – active partial thromboplastin time, prothrombin time and fibrinogen; clinical chemistry – albumin/globulin ratio, alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, creatinine, creatinine kinase, gamma-glutamyl trans-peptidase, globulin, glucose, high-density lipoprotein, inorganic phosphorus, low-density lipoprotein, potassium, sodium, total bilirubin, total cholesterol, total protein, triglycerides and T3, T4 and TSH.

Urine was collected from all animals at the end of dosing period (day 91 of dosing phase) and at the end of recovery period (day 29 of recovery phase). During urine collection, animals were fasted overnight in metabolic cages. The following parameters were measured: volume, colour, appearance, glucose, bilirubin, ketone, blood, pH, protein, urobilinogen, nitrite, leukocyte and specific gravity.

Gross necropsy was conducted on all animals at the end of dosing phase for main groups (day 91; terminal sacrifice) and at the end of recovery phase of recovery group (day 29; recovery phase). The following organs were collected, weighed, assessed histopathologically after fixation in 10% neutral buffered formalin and staining with haematoxylin and eosin: adrenals, brain, epididymites, heart, kid-

neys, liver, ovaries, pituitary, prostate, seminal vesicles and coagulating glands, spleen, testes, thymus, thyroid with parathyroid and uterus with cervix. The other organs or tissues such as aorta, biceps femoris muscles, cecum, colon, diaphragm, duodenum, oesophagus, eyes, femur bone with joint, gastrocnemius muscle, gross changes, harderian glands, ileum with Peyer's patches, jejunum, lacrimal gland, lungs, lymph nodes (mandibular and mesenteric), mammary gland, optic nerve, oviducts, pancreas, quadriceps muscle, rectum, salivary glands (mandibular, sublingual and parotid), sciatic nerve, skin (inguinal region), soleus muscle, spinal cord, sternum with marrow, stomach, tongue, trachea, urinary bladder and vagina, were also examined histopathologically. Histopathology was performed on all tissues of animals from control and test substance treated groups on main group. Histopathology was also extended to recovery groups for eye, ovaries, uterus, vagina. Peer review was performed by another in-house pathologist.

For comparative statistics, data were evaluated using Levene's Test for homogeneity of variance, with significance at 5% level. Data determined to be homogeneous was evaluated for analysis of variance (ANOVA). When the ANOVA verified significance at 5% level, pairwise comparisons of each treated group with the control group was made using a Dunnett Test, to identify statistical differences (at 5% level). When the data were found to be non-homogeneous, ANOVA was done using suitable transformation. When the homogeneity tests were significant even after transformation, data were evaluated using a Kruskal-Wallis Test for group factor significance. When significance (at 5% level) existed between groups, Dunn's pairwise comparison was performed.

Results and discussions

Results of the original study groups:

There were no test item related changes in the three main test item treated rat groups and the high dose recovery group. Changes in ovary, uterus and retina in the original control and the high dose test organism groups were considered related to excessive light exposure and not test item related. Changes in ovary and uterus were found reversible and retinal changes were not recovered at end of recovery period. All data are presented in the study report, however, only data from the repeated study part was considered reliable for NOAEL determination.

Toxicokinetic results are summarised here below:

Table A 37 The toxicokinetic parameters of RH-141452

Day nominal	Sex	Dose in ppm (mg/kg/day)	T_{max} in h (time from start of exposure)	C_{max} (µg/mL)	AUC_{0-24h} (h*µg/mL)
Day 2	Male	1500 (91)	14 (40)	4.0	60
		4500 (271)	14 (40)	9.6	166
		9000 (541)	14 (40)	21.8	306
Day 2	Female	1500 (112)	0 (26)	4.5	75
		4500 (332)	14 (40)	10.6	184
		9000 (673)	14 (40)	24.5	458
Day 87	Male	1500 (91)	0 (2066)	3.0	48
		4500 (271)	0 (2066)	9.0	178
		9000 (541)	17 (2083)	17.6	348
Day 87	Female	1500 (112)	24 (2090)	5.1	98
		4500 (332)	0 (2066)	16.8	283
		9000 (673)	0 (2066)	26.1	538

Repeat dose feeding of RH-141452 at 1500, 4500 or 9000 ppm in diet for 13 weeks showed evidence of systemic plasma exposure to RH-141452 at all dose levels in Sprague Dawley rats (see report U-20281). RH-141452 exposure (AUC_{0-24h}) and C_{max} increased dose proportionally from 1500 to 9000 ppm on day 2 and day 87 in males and females. There was no sex difference in systemic exposure. No accumulation of RH-141452 was observed on day 87 as compared to day 2 in both sexes.

Results of the main (repeated) study groups reliable for NOAEL determination

No mortality or signs of morbidity were observed during the whole study period.

No clinical signs or test item related ophthalmological lesions were observed in males or females at any test item concentration during the whole study period.

The mean test item consumption of rats after repeated intake of RH-141452 over 90 days is given in Table C.2.3.1-18 above, details in Table C.2.3.1-21 below. There were no test item-related effects in food consumption or food conversion efficiency in males and females. Statistically increased or decreased food consumption was observed during few intervals (days 43-50, 64-71, 1-90 in low or mid dose males and days 71-78 and 85-90 in low dose females). Statistically increased (males and females) or decreased (males) food conversion efficiency was observed during few intervals (days 29-36, 36-43, 43-50, 71-78 in males and days 29-36, 36-43, 85-90 in females). However, these changes were of low magnitude (<10%) and/or there was no dose response relationship. Hence, these small transitional effects were not considered test item related.

There were no test item related changes body weight and body weight gain (except sporadic statistically decreased body weights and body weight gains on days 50, 57 and 64 in low dose males – which are regarded as not test item related).

There were no test item related changes in functional observation battery, in grip strength and foot splay parameters, or motor activity at doses of 1500, 4500 and 9000 ppp in male and female rats.

No test item-related changes were noticed for rectal temperatures.

There were no RH-141452 related changes in haematology, coagulation, clinical chemistry and urinalysis parameters. In addition, no test item related gross changes and changes in organ weights and organ weight ratios (in relation to body and brain weight). At terminal sacrifice, there were no RH-141452 related microscopic changes in any of the dose groups.

Table A 38 Summary of Body Weights (g) in male and female rats in main (repeat) study groups

Group #		Day no.													
		1	8	15	22	29	36	43	50	57	64	71	78	85	90
Males															
G5 Control	Mean	243.12	284.24	316.08	338.24	357.13	373.14	380.97	397.53	410.66	420.01	424.05	430.82	436.26	439.03
	SD	9.41	10.59	15.04	16.51	17.29	16.76	17.45	21.38	21.50	20.70	21.66	21.25	21.39	21.30
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G6 1500	Mean	242.88	282.75	312.19	331.60	349.12	351.78	370.55	**370.05^{-}**	**382.21^{-}**	**389.61^{-}**	398.41	413.33	417.07	422.98
	SD	8.23	9.56	12.73	16.36	20.91	21.88	23.27	23.37	26.93	27.93	29.64	31.11	30.81	29.93
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G7 4500	Mean	242.67	283.17	314.84	335.93	354.73	365.14	380.20	392.86	406.58	413.88	418.73	428.95	431.89	438.19
	SD	10.77	11.45	11.37	11.94	12.44	14.13	16.28	16.13	16.65	18.69	21.65	23.81	23.23	23.31
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G8 9000	Mean	243.98	280.37	315.38	334.49	353.79	366.25	385.47	397.34	409.39	415.98	421.63	434.38	438.78	441.70
	SD	10.23	14.10	14.89	18.09	19.68	18.76	21.35	20.67	20.90	22.41	22.06	22.02	21.70	20.90
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Females															
G5 Control	Mean	168.95	188.76	204.10	212.81	225.81	226.77	229.52	236.72	244.40	247.51	247.80	251.31	252.25	253.98
	SD	7.20	8.18	8.75	9.86	11.77	10.60	10.96	10.15	11.83	10.29	8.80	9.77	9.54	8.90
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G6 1500	Mean	168.86	189.19	202.07	210.15	222.07	226.49	236.96	239.47	244.99	249.01	251.64	256.52	257.93	265.39
	SD	7.73	9.06	12.96	16.41	17.73	16.96	20.26	20.93	22.67	23.13	22.21	22.21	21.67	22.26
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G7 4500	Mean	168.60	186.51	197.84	208.63	219.77	223.51	231.90	236.17	240.34	242.67	245.40	247.29	249.89	257.74
	SD	7.77	7.89	9.79	10.77	11.39	10.91	12.47	12.37	12.93	11.68	11.81	11.92	12.26	12.12
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G8 9000	Mean	167.77	183.19	197.07	203.96	213.12	218.34	225.88	228.69	232.73	237.34	239.60	243.82	246.04	248.44
	SD	7.19	9.88	10.35	9.03	8.40	10.32	9.47	10.41	10.28	9.78	9.85	9.46	10.58	10.10

	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Group#: Control: G5 (0 ppm); **2:** G6 (1500 ppm); **3:** G7 (4500 ppm); **4:** G8 (9000 ppm)
-: Significantly lower than the control group at 5 % level.

Table A.39 Summary of Cage Wise Food Consumption (g/day)

Group #		Day no.													
		1-8	8-15	15-22	22-29	29-36	36-43	43-50	50-57	57-64	64-71	71-78	78-85	85-90	1-90
Males															
G5 Control	Mean	21.85	22.59	22.44	22.79	22.72	22.83	23.29	23.18	22.67	23.15	21.60	22.04	21.81	22.55
	SD	0.88	0.42	0.65	0.35	0.54	0.66	0.56	0.32	0.63	0.41	1.16	0.30	0.73	0.30
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G6 1500	Mean	21.72	21.66	22.16	22.04	22.00	22.77	**21.83^{-}**	22.79	21.97	**22.05^{-}**	21.37	21.83	22.14	**22.02^{-}**
	SD	0.41	0.53	0.24	0.70	0.57	0.40	0.79	0.44	0.40	0.54	0.54	0.35	0.82	0.25
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G7 4500	Mean	22.34	22.86	22.80	22.58	22.31	22.99	22.66	23.05	21.98	**22.13^{-}**	22.75	22.01	22.70	22.55
	SD	0.36	0.50	0.48	0.71	0.61	0.52	0.21	0.30	0.22	0.44	0.69	0.24	0.43	0.25
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G8 9000	Mean	21.79	22.75	22.46	22.89	22.72	23.03	22.48	23.20	22.27	22.49	22.14	22.18	22.20	22.51
	SD	0.42	0.90	0.49	0.47	0.39	0.52	0.32	0.33	0.42	0.46	0.65	0.35	0.60	0.34
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Females															
G5 Control	Mean	15.02	15.14	14.76	15.97	15.35	15.60	16.07	16.06	15.47	15.61	16.53	15.38	15.16	15.56
	SD	0.42	0.39	0.32	0.42	0.22	0.55	0.63	0.55	0.62	0.43	1.22	0.36	0.49	0.21
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G6 1500	Mean	14.94	15.40	15.16	15.85	15.84	16.13	15.85	16.14	15.69	15.77	**14.54^{-}**	15.38	**16.20^{+}**	15.59
	SD	0.62	0.58	0.70	0.46	0.13	0.93	0.56	0.60	0.34	0.42	1.05	0.55	0.25	0.36
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G7 4500	Mean	14.67	15.02	15.69	16.37	15.57	16.07	15.65	15.91	15.55	15.52	14.97	15.73	15.99	15.58
	SD	0.57	0.43	0.53	0.25	0.38	0.41	0.54	0.65	0.42	0.29	0.99	0.18	0.11	0.19

	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G8 9000	Mean	15.08	14.97	15.18	15.87	15.31	15.72	15.79	15.94	15.13	15.32	15.35	15.23	15.83	15.43
	SD	0.71	0.20	0.38	0.11	0.39	0.38	0.25	0.46	0.28	0.52	0.56	0.17	0.52	0.10
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group#: Control: G5 (0 ppm); **2:** G6 (1500 ppm); **3:** G7 (4500 ppm); **4:** G8 (9000 ppm)

-: Significantly lower than the control group at 5 % level.

Conclusion

This study was designed to assess the potential sub-chronic toxicity and toxicokinetics (TK) profile of RH-141452 when fed in formulated diet to Sprague Dawley rats for 90 days. A total of 73 male and 73 female Sprague Dawley rats were randomly assigned to 4 main groups (10 rats/sex/group), 2 recovery groups (6 rats/sex/group) and 2 TK groups (3/6 rats/sex/group for control and test-substance related, respectively). Based on the results of a 14 days oral toxicity study (U-20188), doses of 1500, 4500 and 9000 ppm RH-141452 were chosen. The control group was fed with basal diet for same duration of study.

Due to failure of the automated room light timer, all study groups mentioned above were exposed to continuous light towards the end of dosing, leading to histopathological findings in circadian clock sensitive tissues. Hence, 4 additional rat groups (10 rats/sex/group) were added to the study by a study plan amendment number 4 to repeat the main study part. It was not considered necessary to repeat the toxicokinetic groups (no photosensitivity of the endpoint) or recovery groups (no findings in the first or repeated main groups that would trigger a repeat of the recovery groups).

As a result, no mortality, clinical signs, or test item related ophthalmological lesions were observed in males or females at any test item concentration. There were no test item related changes in functional observation battery, motor activity, body weight, food consumption, food conversion efficiency in males and females. There were no test item related changes in hematology, coagulation, clinical chemistry, and urinalysis parameters. There were no test item related gross pathology findings and organ weight changes in males or females. No test item related histopathology findings were observed in males or females.

The No-Observed-Adverse-Effect-Level (NOAEL) of RH-141452 for male and female Sprague Dawley rats was 9000 ppm, which is equivalent to 538 mg/kg bw/day in male and 625 mg/kg bw/day in female rats.

Conclusion by EMS: The No-Observed-Adverse-Effect-Level (NOAEL) of RH-141452 for male and female Sprague Dawley rats was 9000 ppm, which is equivalent to 538 mg/kg bw/day in male and 625 mg/kg bw/day in female rats.

Appendix 3 Exposure calculations

A 3.1 Operator exposure calculations (KCP 7.2.1.1)

A 3.1.1 Calculations for Zoxamide

Table A 40: Input parameters considered for the estimation of operator exposure

Formulation type	SC		Crop type	Grapevine (high crop)
Application rate (AR)	0.18	kg a.s./ha	Application method	Upward spraying
Area treated per day (A)	10	ha	Application equipment	Vehicle-mounted
Dermal absorption (DA)	0.55	% (concentr.)	Indoor/outdoor	Outdoor
	14	% (dilution)	Closed cabin	No
Inhalation absorption (IA)	100	%	Drift reduction	No
Body weight (BW)	60	kg/person	Cultivation	Normal
AOEL	0.3	mg/kg bw/d	Water soluble bag	No
AAOEL	-	mg/kg bw/d		

Table A 41: Estimation of short term operator exposure towards Zoxamide according to EFSA guidance, vehicle-mounted

Activity	Systemic exposure per body part	With work-wear	With workwear + PPE/RPE
Mixing and loading (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**1.1**	**1.1**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.007**	**0.007**
	Head protection	*None*	*None*
	Head exposure	**0.01**	**0.01**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**0.07**	**0.07**
Application (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**9.9**	**9.9**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.5**	**0.5**
	Head protection	*None*	*None*
	Head exposure	**4.8**	**4.8**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**1.5**	**1.5**
Total	Total systemic exposure [mg/kg bw per day]	0.02	0.02
	% of AOEL	6	6

Table A 42: Input parameters considered for the estimation of operator exposure

Formulation type	SC		Crop type	Grapevine (high crop)
Application rate (AR)	0.18	kg a.s./ha	Application method	Upward spraying
Area treated per day (A)	4	ha	Application equipment	Manual-hand held
Dermal absorption (DA)	0.55	% (concentr.)	Indoor/outdoor	Outdoor
	14	% (dilution)	Closed cabin	No
Inhalation absorption (IA)	100	%	Drift reduction	No
Body weight (BW)	60	kg/person	Cultivation	Normal
AOEL	0.3	mg/kg bw/d	Water soluble bag	No
AAOEL	-	mg/kg bw/d		

Table A 43: Estimation of short term operator exposure towards Zoxamide according to EFSA guidance, manual-hand held

Activity	Systemic exposure per body part	With work-wear	With workwear + PPE/RPE
Mixing and loading (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**0.6**	**0.6**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.004**	**0.004**
	Head protection	*None*	*None*
	Head exposure	**0.004**	**0.004**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**0.05**	**0.05**
Application (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**4.8**	**4.8**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**2.4**	**2.4**
	Head protection	*None*	*None*
	Head exposure	**0.3**	**0.3**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**1**	**1**
Total	Total systemic exposure [mg/kg bw per day]	0.009	0.009
	% of AOEL	3.1	3.1

Table A 44: Input parameters considered for the estimation of operator exposure

Formulation type	SC		Crop type	Grapevine (high crop)
Application rate (AR)	0.18	kg a.s./ha	Application method	Upward spraying
Area treated per day (A)	1	ha	Application equipment	Manual-knapsack
Dermal absorption (DA)	0.55	% (concentr.)	Indoor/outdoor	Outdoor
	14	% (dilution)	Closed cabin	No
Inhalation absorption (IA)	100	%	Drift reduction	No
Body weight (BW)	60	kg/person	Cultivation	Normal
AOEL	0.3	mg/kg bw/d	Water soluble bag	No
AAOEL	-	mg/kg bw/d		

Table A 45: Estimation of short term operator exposure towards Zoxamide according to EFSA guidance, manual-knapsack

Activity	Systemic exposure per body part	With work-wear	With workwear + PPE/RPE
Mixing and loading (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**0.9**	**0.9**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.002**	**0.002**
	Head protection	*None*	*None*
	Head exposure	**0.0005**	**0.0005**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**0.6**	**0.6**
Application (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**1.5**	**1.5**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**2.4**	**2.4**
	Head protection	*None*	*None*
	Head exposure	**0.2**	**0.2**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**0.3**	**0.3**
Total	Total systemic exposure [mg/kg bw per day]	0.006	0.006
	% of AOEL	2	2

A 3.1.2 Calculations for Potassium phosphonates

~~Table A 42~~ Table A 46: Input parameters considered for the estimation of operator exposure

Formulation type	SC		Crop type	Grapevine (high crop)
Application rate (AR)	2.265	kg a.s./ha	Application method	Upward spraying
Area treated per day (A)	10	ha	Application equipment	Vehicle-mounted
Dermal absorption (DA)	0.071	% (concentr.)	Indoor/outdoor	Outdoor
	1.7	% (dilution)	Closed cabin	No
Inhalation absorption (IA)	100	%	Drift reduction	No
Body weight (BW)	60	kg/person	Cultivation	Normal
AOEL	5	mg/kg bw/d	Water soluble bag	No
AAOEL	-	mg/kg bw/d		

~~Table A 43~~ Table A 47: Estimation of short term operator exposure towards Potassium phosphonates according to EFSA guidance, vehicle-mounted

Activity	Systemic exposure per body part	With work-wear	With workwear + PPE/RPE
Mixing and loading (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**0.7**	**0.7**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.004**	**0.004**
	Head protection	*None*	*None*
	Head exposure	**0.02**	**0.02**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**0.2**	**0.2**
Application (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**11.4**	**11.4**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.7**	**0.7**
	Head protection	*None*	*None*
	Head exposure	**7.4**	**7.4**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**6.4**	**6.4**
Total	Total systemic exposure [mg/kg bw per day]	0.03	0.03
	% of AOEL	0.5	0.5

Table A 48: Input parameters considered for the estimation of operator exposure

Formulation type	SC		Crop type	Grapevine (high crop)
Application rate (AR)	2.265	kg a.s./ha	Application method	Upward spraying
Area treated per day (A)	4	ha	Application equipment	Manual-hand held
Dermal absorption (DA)	0.071	% (concentr.)	Indoor/outdoor	Outdoor
	1.7	% (dilution)	Closed cabin	No
Inhalation absorption (IA)	100	%	Drift reduction	No
Body weight (BW)	60	kg/person	Cultivation	Normal
AOEL	5	mg/kg bw/d	Water soluble bag	No
AAOEL	-	mg/kg bw/d		

Table A 49: Estimation of short term operator exposure towards Potassium phosphonates according to EFSA guidance, manual-hand held

Activity	Systemic exposure per body part	With work-wear	With workwear + PPE/RPE
Mixing and loading (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**0.4**	**0.4**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.002**	**0.002**
	Head protection	*None*	*None*
	Head exposure	**0.007**	**0.007**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**0.1**	**0.1**
Application (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**4.9**	**4.9**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.3**	**0.3**
	Head protection	*None*	*None*
	Head exposure	**0.09**	**0.09**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**8.4**	**8.4**
Total	Total systemic exposure [mg/kg bw per day]	0.01	0.01
	% of AOEL	0.3	0.3

Table A 50: Input parameters considered for the estimation of operator exposure

Formulation type	SC		Crop type	Grapevine (high crop)
Application rate (AR)	2.265	kg a.s./ha	Application method	Upward spraying
Area treated per day (A)	1	ha	Application equipment	Manual-knapsack
Dermal absorption (DA)	0.071	% (concentr.)	Indoor/outdoor	Outdoor
	1.7	% (dilution)	Closed cabin	No
Inhalation absorption (IA)	100	%	Drift reduction	No
Body weight (BW)	60	kg/person	Cultivation	Normal
AOEL	5	mg/kg bw/d	Water soluble bag	No
AAOEL	-	mg/kg bw/d		

Table A 51: Estimation of short term operator exposure towards Potassium phosphonates according to EFSA guidance, manual-knapsack

Activity	Systemic exposure per body part	With work-wear	With workwear + PPE/RPE
Mixing and loading (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**0.2**	**0.2**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.0004**	**0.0004**
	Head protection	*None*	*None*
	Head exposure	**0.0001**	**0.0001**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**0.9**	**0.9**
Application (µg/kg bw per day)	*Hand protection*	*None*	*None*
	Hands exposure	**1.5**	**1.5**
	Body protection	*Workwear*	*Workwear*
	Body exposure	**0.3**	**0.3**
	Head protection	*None*	*None*
	Head exposure	**0.06**	**0.06**
	Inhalation protection	*None*	*None*
	Inhalation exposure	**2.7**	**2.7**
Total	Total systemic exposure [mg/kg bw per day]	0.006	0.006
	% of AOEL	0.1	0.1

A 3.2 Worker exposure calculations (KCP 7.2.3.1)

A 3.2.1 Calculations for Zoxamide

~~Table A 44~~ Table A 52: Input parameters considered for the estimation of worker exposure

Intended use(s)	Grapevine (high crop), hand harvesting, outdoor		Dislodgeable foliar residue (DFR)	3	µg/cm²/kg a.s./ha
Application rate (AR)	0.18	kg a.s./ha	Dermal absorption (DA)	14	% (worst case)
Number of applications (NA)	3		Inhalation absorption (IA)	100	%
Interval between applications	8	days	Work rate per day (WR)	8	h/d
Half-life of active substance	30	days	TC dermal (potential)	30000	cm²/h
Multiple application factor (MAF)	2.52		TC dermal (work wear)	10100	cm²/h
Body weight (BW)	60	kg/person	TC dermal (work wear, gloves)	na	cm²/h
AOEL	0.3	mg/kg bw/d	Task specific factor inhalation	-	ha/h x 10^{-3}
AAOEL	-	mg/kg bw/d			

na: not applicable
- no information available

~~Table A 45~~ Table A 53: Estimation of short term worker exposure towards Zoxamide according to EFSA guidance

Exposure route	Description	Potential	Workwear	Workwear and gloves	Gloves
Dermal	Systemic dermal exposure [mg a.s. per day]	45.7	15.4	na	na
Inhalation	Systemic inhalation exposure [mg a.s. per day]	na	na	na	na
Total	Total systemic exposure [mg a.s. per day]	45.7	15.4	na	na
	Total systemic exposure [mg/kg bw per day]	0.8	0.3	na	na
	% of AOEL	254	85.5	na	na

na: not applicable

A 3.2.2 Calculations for Potassium phosphonates

~~Table A 46~~ Table A 54: Input parameters considered for the estimation of worker exposure

Intended use(s)	Grapevine (high crop), hand harvesting, outdoor		Dislodgeable foliar residue (DFR)	3	µg/cm²/kg a.s./ha
Application rate (AR)	2.265	kg a.s./ha	Dermal absorption (DA)	1.7	% (worst case)
Number of applications (NA)	3		Inhalation absorption (IA)	100	%
Interval between applications	8	days	Work rate per day (WR)	2	h/d
Half-life of active substance	30	days	TC dermal (potential)	30000	cm²/h
Multiple application factor (MAF)	2.52		TC dermal (work wear)	10100	cm²/h
Body weight (BW)	60	kg/person	TC dermal (work wear, gloves)	na	cm²/h
AOEL	5	mg/kg bw/d	Task specific factor inhalation	-	ha/h x 10^{-3}
AAOEL	-	mg/kg bw/d			

na: not applicable
- no information available

~~Table A 47~~ Table A 55: Estimation of short term worker exposure towards Potassium phosphonates according to EFSA guidance

Exposure route	Description	Potential	Workwear	Workwear and gloves	Gloves
Dermal	Systemic dermal exposure [mg a.s. per day]	69.9	23.5	na	na
Inhalation	Systemic inhalation exposure [mg a.s. per day]	na	na	na	na
Total	Total systemic exposure [mg a.s. per day]	69.9	23.5	na	na
	Total systemic exposure [mg/kg bw per day]	1.2	0.4	na	na
	% of AOEL	23.3	7.8	na	na

na: not applicable

A 3.3 Resident and bystander exposure calculations (KCP 7.2.2.1)

A 3.3.1 Calculations for Zoxamide

~~Table A 48~~ Table A 56: Input parameters considered for the estimation of short term resident exposure

Intended use(s)	Grapevine (high crop), vehicle mounted application		Drift reduction (DR)	0	%
Application rate (AR)	0.18	kg a.s./ha	Transfer coefficient surface deposits (TC)	7300	cm^2/h (adult)
				2600	cm^2/h (child)
Minimum water volume (V)	200	L/ha	Drift on surface (D) - 75th perc.	-	%
Buffer strip	5	m	Drift on surface (D) - mean	-	%
Number of applications (NA)	3		Turf Transferable Residues (TTR)	5	%
Interval between applications	8	days	Exposure duration dermal (H_D)	2	h
Half-life of active substance	30	days	Exposure duration inhal. (H_I)	24	h
Multiple application factor (MAF)	-		Exposure duration entry into treated crops (H_E)	0.25	h
Body weight (BW)	60	kg/person (adults)	Airborne Concentration of Vapour (VC)	0.001	mg/m^3
	10	kg/person (children)			
Dermal absorption (DA)	14	% ('worst case')	Dislodgeable foliar residue (DFR)	3	$\mu g/cm^2$/kg a.s./ha
Inhalation absorption (IA)	100	%	Light clothing adjustment factor (CF)	18	%
Oral absorption (OA)	60	%	Saliva Extraction Factor (SE)	50	%
AOEL	0.3	mg/kg bw/d	Surface Area of Hands (SA)	20	cm^2
Spray drift dermal (SD) - 75th perc.	-	mL spray dilution (adult)	Frequency of Hand to Mouth (Freq)	20	events/h
	-	mL spray dilution (child)			
Spray drift inhal. (SI) - 75th perc.	-	mL spray dilution (adult)	Dislodgeable residues object to mouth (DR_{OM})	20	%
	-	mL spray dilution (child)			
Spray drift dermal (SD) - mean	-	mL spray dilution (adult)	Ingestion Rate for Mouthing of Grass (IgR)	25	cm^2/d
	-	mL spray dilution (child)			
Spray drift inhal. (SD) - mean	-	mL spray dilution (adult)	TC entry into treated crops - 75th perc.	7500	cm^2/h (adult)
	-	mL spray dilution (child)		2250	cm^2/h (child)
Inhalation rate (IR)	16.2	m^3/d (adult)	TC entry into treated crops - mean:	5980	cm^2/h (adult)
	8.0	m^3/d (child)		1794	cm^2/h (child)

-: No data presented in the EFSA online calculator (version: opex 1.0.1, EFSA publication date: 01/07/2023)

~~Table A 49~~ Table A 57: Estimation of short term resident exposure towards Zoxamide according to EFSA guidance

Model data	Level of PPE	Total absorbed dose [mg/kg bw per day]	% of systemic AOEL
			Season: Not relevant Buffer zone: 5 m Drift reduction technology: 0 % Interval between treatments: 8 days Minimum volume of water: 200 l
Zoxamide			Number of applications and application rate: 3 x 0.18 kg a.s./ha Dermal absorption: 14 % DFR: 3 µg/cm² foliage per kg a.s./ha DT50: 30 days
Resident child Body weight: 10 kg	Drift (75th perc.)	0.02	5.8
	Vapour (75th perc.)	0.0008	0.3
	Deposits (75th perc.)	0.0006	0.2
	Re-entry (75th perc.)	0.01	3.6
	Sum (mean)	0.02	7.1
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.01	3.2
	Vapour (75th perc.)	0.0003	0.09
	Deposits (75th perc.)	0.0002	0.08
	Re-entry (75th perc.)	0.006	2
	Sum (mean)	0.01	3.9

A 3.3.2 Calculations for Potassium phosphonates

~~Table A 50~~ Table A 58: Input parameters considered for the estimation of short term resident exposure

Intended use(s)	Grapevine (high crop), vehicle mounted application		Drift reduction (DR)	0	%
Application rate (AR)	2.265	kg a.s./ha	Transfer coefficient surface deposits (TC)	7300	cm^2/h (adult)
				2600	cm^2/h (child)
Minimum water volume (V)	200	L/ha	Drift on surface (D) - 75th perc.	-	%
Buffer strip	5	m	Drift on surface (D) - mean	-	%
Number of applications (NA)	3		Turf Transferable Residues (TTR)	5	%
Interval between applications	8	days	Exposure duration dermal (H_D)	2	h
Half-life of active substance	30	days	Exposure duration inhal. (H_I)	24	h
Multiple application factor (MAF)	-		Exposure duration entry into treated crops (H_E)	0.25	h
Body weight (BW)	60	kg/person (adults)	Airborne Concentration of Vapour (VC)	0.001	mg/m^3
	10	kg/person (children)			
Dermal absorption (DA)	1.7	% ('worst case')	Dislodgeable foliar residue (DFR)	3	$\mu g/cm^2$/kg a.s./ha
Inhalation absorption (IA)	100	%	Light clothing adjustment factor (CF)	18	%
Oral absorption (OA)	100	%	Saliva Extraction Factor (SE)	50	%
AOEL	5	mg/kg bw/d	Surface Area of Hands (SA)	20	cm^2
Spray drift dermal (SD) - 75th perc.	-	mL spray dilution (adult)	Frequency of Hand to Mouth (Freq)	20	events/h
	-	mL spray dilution (child)			
Spray drift inhal. (SI) - 75th perc.	-	mL spray dilution (adult)	Dislodgeable residues object to mouth (DR_{OM})	20	%
	-	mL spray dilution (child)			
Spray drift dermal (SD) - mean	-	mL spray dilution (adult)	Ingestion Rate for Mouthing of Grass (IgR)	25	cm^2/d
	-	mL spray dilution (child)			
Spray drift inhal. (SD) - mean	-	mL spray dilution (adult)	TC entry into treated crops - 75th perc.	7500	cm^2/h (adult)
	-	mL spray dilution (child)		2250	cm^2/h (child)
Inhalation rate (IR)	16.2	m^3/d (adult)	TC entry into treated crops - mean:	5980	cm^2/h (adult)
	8.0	m^3/d (child)		1794	cm^2/h (child)

-: No data presented in the EFSA online calculator (version: opex 1.0.1, EFSA publication date: 01/07/2023)

~~Table A 51~~ Table A 59: Estimation of short term resident exposure towards Potassium phosphonates according to EFSA guidance

Model data	Level of PPE	Total absorbed dose [mg/kg bw per day]	% of systemic AOEL
			Season: Not relevant Buffer zone: 5 m Drift reduction technology: 0 % Interval between treatments: 8 days Minimum volume of water: 200 l
Potassium phosphonates			Number of applications and application rate: 3 x 2.265 kg a.s./ha Dermal absorption: 1.7 % DFR: 3 µg/cm² foliage per kg a.s./ha DT50: 30 days
Resident child Body weight: 10 kg	Drift (75th perc.)	0.03	0.6
	Vapour (75th perc.)	0.0008	0.02
	Deposits (75th perc.)	0.003	0.07
	Re-entry (75th perc.)	0.02	0.3
	Sum (mean)	0.03	0.7
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.02	0.3
	Vapour (75th perc.)	0.0003	0.005
	Deposits (75th perc.)	0.0004	0.007
	Re-entry (75th perc.)	0.009	0.2
	Sum (mean)	0.02	0.4

A 3.4 Combined exposure calculations for Zoxamide and Potassium phosphonates

~~Table A 52~~ Table A 60: Risk assessment for operator from combined exposure (short term exposure), vehicle mounted

Model data	Level of PPE	Total absorbed dose [mg/kg bw per day]	% of systemic AOEL
Viticulture/Outdoor/Upward spraying/Vehicle-mounted/Drift reduction: 0 %/75th percentile Crop density: Normal			
Zoxamide	Number of applications and application rate: 3 x 0.18 kg a.s./ha Dermal absorption (concentrate): 0.55 % Dermal absorption (in-use dilution): 14 %		
	M/L: Workwear App: Workwear	0.02	6
Potassium phosphonates	Number of applications and application rate: 3 x 2.265 kg a.s./ha Dermal absorption (concentrate): 0.071 % Dermal absorption (in-use dilution): 1.7 %		
	M/L: Workwear App: Workwear	0.03	0.5
Combined exposure			Hazard index
	M/L: Workwear App: Workwear		0.06

Table A 61: Risk assessment for operator from combined exposure (short term exposure), manual-hand held

Model data	Level of PPE	Total absorbed dose [mg/kg bw per day]	% of systemic AOEL
Viticulture/Outdoor/Upward spraying/Manual-hand held/Drift reduction: 0 %/75th percentile Crop density: Normal			
Zoxamide	Number of applications and application rate: 3 x 0.18 kg a.s./ha Dermal absorption (concentrate): 0.55 % Dermal absorption (in-use dilution): 14 %		
	M/L: Workwear App: Workwear	0.009	3.1
Potassium phosphonates	Number of applications and application rate: 3 x 2.265 kg a.s./ha Dermal absorption (concentrate): 0.071 % Dermal absorption (in-use dilution): 1.7 %		
	M/L: Workwear App: Workwear	0.01	0.3
Combined exposure			Hazard index
	M/L: Workwear App: Workwear		0.03

Table A 62: Risk assessment for operator from combined exposure (short term exposure), manual-knapsack

	Model data	Level of PPE	Total absorbed dose [mg/kg bw per day]	% of systemic AOEL
Viticulture/Outdoor/Upward spraying/Manual-knapsack/Drift reduction: 0 %/75th percentile Crop density: Normal				
Zoxamide	Number of applications and application rate: 3 x 0.18 kg a.s./ha Dermal absorption (concentrate): 0.55 % Dermal absorption (in-use dilution): 14 %			
		M/L: Workwear App: Workwear	0.006	2
Potassium phosphonates	Number of applications and application rate: 3 x 2.265 kg a.s./ha Dermal absorption (concentrate): 0.071 % Dermal absorption (in-use dilution): 1.7 %			
		M/L: Workwear App: Workwear	0.006	0.1
Combined exposure				Hazard index
		M/L: Workwear App: Workwear		0.02

~~Table A 56~~ Table A 63: Risk assessment for worker from combined exposure (short term exposure)

	Level of PPE	Total absorbed dose [mg/kg bw per day]	% of systemic AOEL	Re-entry re-striction [days]
				Hand harvesting / Outdoor Work rate: 8 hours/day Interval: 8 days Body weight: 60 kg TC (potential): 30000 cm²/h TC (workwear (arms, body and legs covered)): 10100 cm²/h TC (workwear (arms, body and legs covered) and gloves): na cm²/h TC (gloves): na cm²/h
	Zoxamide			Number of applications & application rate: 3 x 0.18 kg a.s./ha Dermal absorption: 14 % DFR: 3 µg/cm² foliage per kg a.s./ha DT50: 30 days
	Potential	0.8	254	41
	Workwear	0.3	85.5	0
	Workwear and gloves	na	na	na
	Potassium phosphonates			Number of applications & application rate: 3 x 2.265 kg a.s./ha Dermal absorption: 1.7 % DFR: 3 µg/cm² foliage per kg a.s./ha DT50: 30 days
	Potential	1.2	23.3	0
	Workwear	0.4	7.8	0
	Workwear and gloves	na	na	na
	Combined		Hazard index	
	potential		2.8	45
	Workwear		0.9	0
	Workwear and gloves	na	na	na

na: not applicable

~~Table A 56~~ Table A 64: Risk assessment for resident from combined exposure (short term exposure)

Model data	Level of PPE	Total absorbed dose [mg/kg bw per day]	% of systemic AOEL
			Season: Not relevant Buffer zone: 5 m Drift reduction technology: 0 % Interval between treatments: 8 days Minimum volume of water: 200 l
Zoxamide			Number of applications and application rate: 3 x 0.18 kg a.s./ha Dermal absorption: 14 % DFR: 3 µg/cm² foliage per kg a.s./ha DT50: 30 days
Potassium phosphonates			Number of applications and application rate: 3 x 2.265 kg a.s./ha Dermal absorption: 1.7 % DFR: 3 µg/cm² foliage per kg a.s./ha DT50: 30 days
Combined exposure			Hazard index
Resident child Body weight: 10 kg	Drift (75th perc.)		0.06
	Vapour (75th perc.)		0.003
	Deposits (75th perc.)		0.003
	Re-entry (75th perc.)		0.04
	Sum (mean)		0.08
Resident adult Body weight: 60 kg	Drift (75th perc.)		0.04
	Vapour (75th perc.)		0.001
	Deposits (75th perc.)		0.0009
	Re-entry (75th perc.)		0.02
	Sum (mean)		0.04

Appendix 4 Detailed evaluation of exposure and/or DFR studies relied upon (KCP 7.2, KCP 7.2.1.1, KCP 7.2.2.1, KCP 7.2.3.1)

Not applicable.