FINAL REGISTRATION REPORT

Part B

Section 6

Mammalian Toxicology

Detailed summary of the risk assessment

Product code: **T-75WG-OR2C**

Product name(s): **TOSCANA TOP 75 WG**

Chemical active substance:

Tribenuron methyl, 750 g/kg

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT/

(authorization)

Applicant: CIECH Sarzyna S.A.

Submission date: 12/2020

Correction date: 06/2021

MS Finalisation date: 15/10/2021

Version history

When	What
December 2020	First submission of product authorization.
02/2021	Dossier sent for evaluation to Merit Mark (PL)
June 2021	Correction of the first submission on product.
08/2021	zRMS finalised evaluation
10/2021	Evaluation after commenting period - RR

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Evaluator comments:
The text highlighted in grey was provided by the evaluator.

6 Mammalian Toxicology (KCP 7)

6.1 Summary

Table 6.1-1: Information on TOSCANA TOP 75 WG*

Product name and code	T-75WG-OR2-C/ TOSCANA TOP 75 WG
Formulation type	WG – water dispersible granule
Active substance(s) (incl. content)	Tribenuron methyl, 750 g/kg
Function	herbicide
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 product code/name 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 product code/name according to Regulation (EC) No 1272/2008

Hazard class(es), categories	STOT RE 2 Skin Sens. 1
Hazard pictograms or Code(s) for hazard pictogram(s)	GHS08 GHS07
Signal word	Warning
Hazard statement(s)	H373 May cause damage to organs through prolonged or repeated exposure. H317 May cause an allergic skin reaction.
Precautionary statement(s)	P260 Do not breathe dust/spray. P280 Wear protective gloves and protective clothing P314 Get medical advice/attention if you feel unwell. P302 + P352 IF ON SKIN: Wash with plenty of water
Additional labelling phrases	~~Contains: Tribenuron-methyl. May produce an allergic reaction [EUH208]~~ To avoid risks to man and the environment, comply with the instructions for use. [EUH401]

Table 6.1-3: Summary of risk assessment for operators, workers, residents and bystanders for product code/name

	Result	PPE / Risk mitigation measures
Operators	Acceptable	None
Workers	Acceptable	None

	Result	PPE / Risk mitigation measures
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.

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 g as/ha** **a) a.s. 1** **b) a.s. 2**	**Water L/ha** **min / max**			**Operator**	**Worker**	**Residents**	**Bystander**
1	Winter soft wheat (TRZAW) Winter rye (SECCW), Winter triticale (TTLWI) Winter barley (HORVW) Durum wheat (TRZDU), Spelt wheat (TRZSP), einkorn wheat (TRZMO) emmer wheat (TRZDI) Spring BBCH 13 – 39	F	Broadcast – foliar LCTM	a) 1 b) 1	a) 18.75 b) 18.75	200 / 400	n.a.	Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products; EFSA Journal 2014;12(10):3874 EUROPOEM II Project, FAIR3-CT96-1406. December 2002				
2	Miscanthus sp. (MISSS) BBCH 12 -14 Grasses grown for seeds Spring BBCH 13 – 39	F	Broadcast – foliar LCTM	a) 1 b) 1	a) 18.75 b) 18.75	200 / 400	n.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, TM: tractor-mounted, HH: hand-held

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

None.

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 substance(s)

	Tribenuron-methyl
Common Name	Tribenuron-methyl
CAS-No.	101200-48-0
Classification and proposed labelling	
With regard to toxicological endpoints (according to the criteria in Reg. 1272/2008, as amended)[1]	Hazard classes (s), categories: Skin Sens. 1 STOT RE 2 Code(s) for hazard pictogram(s): GHS07, GHS08 Signal word: WARNING Hazard statement(s): H317 - May cause an allergic skin reaction H373 -May cause damage to organs through prolonged or repeated exposure Precautionary statement(s): P260, P273, P280, P314, P391
Additional C&L proposal	RAC opinion adopted 14th of September 2018, ATP 15th to CLP
Agreed EU endpoints	
AOEL systemic	0.05 mg/kg bw/day
~~AOEL~~ (AAOEL)	0.13 mg/kg bw per day
Reference	EFSA Journal 2017;15(7):4912
Conditions to take into account/critical areas of concern with regard to toxicology	
According to Review Report/EFSA Conclusion for active substance/ RAC Opinion	Skin Sens.1 H317 "May cause an allergic skin reaction" STOT RE Cat 2 H373 "May cause

[1] Together with RAC opinion on tribenuron-methyl adopted 14th of September 2018

	damage to organs through prolonged or repeated exposure"

6.3 Toxicological Evaluation of Plant Protection Product

No acute toxicity studies were performed because of protection of animals used for experimental and other scientific purposes. According to Regulation (EC) No 1107/2009 "The use of non-animal test methods and other risk assessment strategies should be promoted". Animal testing for the purposes of registration procedure should be minimized and tests on vertebrates should be undertaken as a last resort. The same approach is strongly recommended by Regulation (EC) No 1272/2008, which advise reducing testing on vertebrate animals and the number of animals involved. Since no studies were submitted appropriate justification with the other method allowing predict properties of the product is presented in Appendix 2.
Only for eye irritation and skin sensitisation the existing studies are presented to justify the classification resulting from the calculation method. These studies prove that TOSCANA TOP 75WG is not irritant to eyes and is not a skin sensitiser.

Table 6.3-1: Summary of evaluation of the studies on acute toxicity including irritancy and skin sensitisation for TOSCANA TOP 75 WG

Type of test, species, model system (Guideline)	Result	Acceptability	Classification (acc. to the criteria in Reg. 1272/2008)	Reference
LD_{50} oral, rat	-	Yes	Not classified	Justification in Appendix 2
LD_{50} dermal, rat	-	Yes	Not classified	Justification in Appendix 2
LC_{50} inhalation, rat	-	Yes	Not classified	Justification in Appendix 2
Skin irritation	-	Yes	Not classified	Justification in Appendix 2
Eye irritation	-	Yes	Not classified	Xxxxxx, R., (2012/13) Report No: 407-1-01-5754 Appendix 2 [1]
Skin sensitisation	-	No	~~Not classified, EUH 208 statement should be only put on the label~~ Formulation is classified based on the content of active substance (75% w/w) and the harmonized classification of tribenuron-methyl as Skin Sens. 1, H317.	~~Xxxxxx, R., (2012/13) Report No: 406-1-01-5753 Appendix~~ 2 [1]
Supplementary studies for combinations of plant protection products	No data – not required	-		

1 The study was performed on formulation PP-108H which composition is the same as T-75WG-OR2-C/TOSCANA TOP 75WG.

Table 6.3-2: Additional toxicological information relevant for classification/labelling of TOSCANA TOP 75 WG

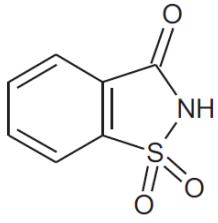
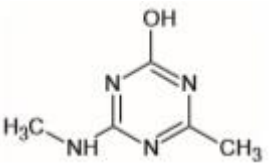
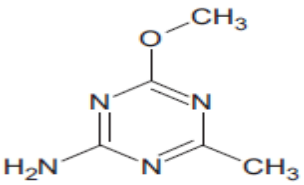
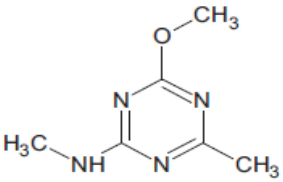
	Substance (concentration in product, % w/w)	Classification of the substance (acc. to the criteria in Reg. 1272/2008)	Reference	Classification of product (acc. to the criteria in Reg. 1272/2008)
Toxicological properties of active substance(s) (relevant for classification of product)	Tribenuron-methyl (75% (w/w))	H373 (criteria ≥ 10 %) H317	RAC opinion, 15th ATP to CLP	STOT RE 2 – H373 H317
Toxicological properties of non-active substance(s) (relevant for classification of product)	Co-formulant (>10% (w/w)	H373 (criteria ≥ 10 %)	MSDS	STOT RE 2 – H373
	Information concerning toxiclogical properties of non-active substance are presented can be found in the confidential dossier of this submission (Registration Report - Part C).			
Further toxicological information	No data – not required			

6.4 Toxicological Evaluation of Groundwater Metabolites

The following data on 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 submitted toxicological studies are summarized in this document. Full summaries of studies on the metabolite that have not previously been considered within an EU peer review process are described in detail in Appendix 2 (A 2.11 Other/Special Studies).

The metabolites listed below are predicted to occur in groundwater at concentrations above 0.1 µg/L (see dRR B section 8). Assessment of the relevance of these metabolites according to the stepwise procedure of the EC guidance document SANCO/221/2000 –rev.10 is therefore required.
General information on the metabolites is provided in **Błąd! Nie można odnaleźć źródła odwołania.**.

Table 6.4-1: General information on the metabolite(s)

Name of active substance	Metabolite name and code	Structural/molecular formula	Trigger for relevance assessment	
tribenuron-methyl	IN-00581		Max PEC_{gw} Based on:	0.304µg/L FOCUS model PELMO/ Hamburg, winter cereals 15 g a.s./ha in autumn
tribenuron-methyl	IN-R9805		Max PEC_{gw} Based on:	0.167 µg/L FOCUS model PELMO /Hamburg, winter cereals 15 g a.s./ha in autumn
tribenuron-methyl	IN-A4098		Max PEC_{gw} Based on:	0.711 µg/L FOCUS model PELMO/Hamburg, winter cereals 18.75 g a.s./ha in spring
tribenuron-methyl	IN-L5296		Max PEC_{gw} Based on:	0.264 µg/L FOCUS model PELMO/ Hamburg, winter cereals 15 g a.s./ha in autumn

6.4.1 Metabolite 1 – IN-A4098

The relevance of the groundwater metabolite IN-A4098 has not been finalized during the evaluation done for the a.i. Tribenuron during the AIR process.
According to EFSA conclusions on Tribenuron (2018), the metabolite IN-A4098 is negative for genotoxicity in gen mutation to bacteria as well as in chromosome damage.
Additionally to this information, the applicant submitted 2 negative studies on mammalian gene mutation.

Table 6.4-2: Summary of the results of toxicity studies for metabolite IN-A4098

Type of test, species (Guideline)	Result	Acceptability	Reference
In vitro Mammalian Cell Gene Mutation test (OECD 476) - genotoxicity determination of IN-A4098, IN-L9223 and IN-L9225 by Mouse Lymphoma Assay	non-genotoxic	Yes	Xxxxxx J., 2015
In vitro evaluation of IN-A4098, IN-L9223 and IN-L9225 genotoxicity	non-genotoxic	Yes	Xxxxxx, J., 2015

Type of test, species (Guideline)	Result	Acceptability	Reference
using the micronucleus assay (MNA)			

6.4.2 Metabolite 2 – IN-L5296

The relevance of the groundwater metabolite IN-L5296 has not been finalized during the evaluation done for the a.i. Tribenuron during the AIR process.
According to EFSA conclusions on Tribenuron (2017; Appendix A page 15), the metabolite IN-L5296 is negative for genotoxicity in all the studies (bone marrow exposure not demonstrated).
Additionally to this information, the applicant submitted 3 negative studies on bacterial and mammal gene mutation as well as on chromosome damage.

Table 6.4-3: Summary of the results of toxicity studies for metabolite IN-L5296

Type of test, species (Guideline)	Result	Acceptability	Reference
Bacterial reversion mutation test (OECD 471)	non-genotoxic	Yes	Xxxxxx,. 2019
In vitro chromosome aberrations test using Chinese Hamster Ovary cells (OECD 473)	non-genotoxic	Yes	Xxxxxx A., 2019
In vitro mammalian cell gene mutation test (OECD 490)	non-genotoxic	Yes	Xxxxxx C., 2019

6.5 Dermal Absorption (KCP 7.3)

A summary of the dermal absorption rates for the active substances in TOSCANA TOP 75 WG are presented in the following table.

Table 6.5-1: Dermal absorption rates for active substances in TOSCANA TOP 75 WG

	Tribenuron-methyl	
	Value	**Reference**
Concentrate	10%	Default from EFSA Guidance on dermal absorption (EFSA Journal 2017;15(6):4873)
Dilution	50%	Default from EFSA Guidance on dermal absorption (EFSA Journal 2017;15(6):4873)

6.5.1 Justification for proposed values – Tribenuron-methyl

No data on dermal absorption for Tribenuron-methyl in TOSCANA TOP 75 WG is available. Justifications for default values according to Guidance on Dermal Absorption (EFSA Journal 2012; 10(4):2665) are presented in the following table.

Table 6.5-2: Default dermal absorption rates for Tribenuron-methyl

	Value	Justification for value	Acceptability of justification
Concentrate	10%	Default dermal absorption value from EFSA Journal 2017;15(6):4873 for water-dispersible granules (WG) - concentrate.	Acceptable
Dilution	50%	Default dermal absorption value from EFSA Journal 2017;15(6):4873 for water-dispersible granules (WG)- dilution.	Acceptable

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	TOSCANA TOP 75 WG
Formulation type	WG
Category	Herbicide
Active substance(s) (incl. content)	Tribenuron-methyl 750 g/kg
AOEL systemic	0.05 mg/kg bw/d
AAOEL	0.13 mg/kg bw per day
Inhalation absorption	100%
Oral absorption	67%
Dermal absorption	Concentrate: 10% Dilution: 50 % (Defaults)

6.6.1 Selection of critical use(s) and justification

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

Justification

Critical GAP was selected due to the highest application rates recommended for use in crops, i.e. 0.025 kg of the product/ha, which is equal to 18.75 g of Tribenuron-methyl/ha. Scenario for cereals and grasslands cover the all crops to be protected as identified in the list of all intended uses from Part B, Section 0.

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 TOSCANA TOP 75 WG according to the critical use(s) is presented in Table 6.6-2. The outcome of the estimation is presented in Table 6.6-3 (acute exposure) and Table 6.6-4 (longer term exposure). Detailed calculations are in Appendix 3.

Table 6.6-2: Exposure models for intended uses

Critical use(s)	Cereals (max. 0.025 kg product/ha, equivalent to 0.01875 kg a.i./ha) Grasslands (max. 0.025 kg product/ha, equivalent to 0.01875 kg a.i./ha)
Model(s)	Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products; EFSA Journal 2014;12(10):3874 calculator version: 30/03/2015

Table 6.6-3: Estimated operator exposure (acute exposure)

		Tribenuron-methyl	
Model data	**Level of PPE**	**Total absorbed dose (mg/kg/day)**	**% of systemic AAOEL**
Tractor mounted boom spray application outdoors to low crops			
Application rate		0.01875 kg a.s./ha	
Spray application (AOEM**;** 95th percentile) Body weight: 60 kg	no PPE*	0.0623487	47.96%

* no PPE, no clothes. Potential exposure.

Table 6.6-4: Estimated operator exposure (longer term exposure)

		Tribenuron-methyl	
Model data	**Level of PPE**	**Total absorbed dose (mg/kg/day)**	**% of systemic AOEL**
Tractor mounted boom spray application outdoors to low crops			
Application rate		0.01875 kg a.s./ha	
Spray application (AOEM**;** 75th percentile) Body weight: 60 kg	no PPE*	0.0065208	13.04%

* no PPE, no clothes. Potential exposure.

6.6.2.2 Measurement of operator exposure

Since the operator exposure estimations carried out indicated that the acceptable operator exposure level

(AAOEL and AOEL) will not be exceeded under conditions of intended uses, a study to provide measurements of operator exposure was not necessary and was therefore not performed.

Comments of zRMS:	Evaluator agrees with the applicants calculations of operator exposure using the EFSA calculator as given in Table 6.6-3 and 6.6-4. The no PPE scenario modelled is the potential exposure assuming no clothing is worn. The estimated exposure to tribenuron – methyl is calculated to be equivalent to ~48% and ~13% of the AAOEL and systemic AOEL without the use of PPE.

6.6.3 Worker exposure (KCP 7.2.3)

6.6.3.1 Estimation of worker exposure

Table 6.6-5 shows the exposure model(s) used for estimation of worker exposure after entry into a previously treated area or handling a crop treated with TOSCANA TOP 75 WG according to the critical use(s). Outcome of the estimation is presented in Table 6.6-6 (longer term exposure). Detailed calculations are in Appendix 3.

Table 6.6-5: Exposure models for intended uses

Critical use(s)	Cereals (max. 0.025 kg product/ha, equivalent to 0.01875 kg a.i./ha) Grasslands (max. 0.025 kg product/ha, equivalent to 0.01875 kg a.i./ha)
Model	Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products; EFSA Journal 2014;12(10):3874 calculator version: 30/03/2015 EUROPOEM II Project, FAIR3-CT96-1406. December 2002

Table 6.6-6: Estimated worker exposure (long-term exposure)

		Tribenuron-methyl	
Model data	**Level of PPE**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
EFSA calculator Inspection and irrigation Outdoor Work rate: 2 hours/day DT_{50}: 30 days DFR: 3 µg/cm²/kg a.s./ha			
Application rate		0.01875 kg a.s./ha	
Body weight: 60 kg	Potential TC: 12500 cm²/person/h	0.0117188	23.44%
	Work wear (arms, body and legs covered) TC: 1400 cm²/person/h	0.0013125	2.63%

		Tribenuron-methyl	
Model data	**Level of PPE**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
EUROPOEM II model Inspection Outdoor Work rate: 2 hours/day, DT50: 30 days for Tribenuron-methyl DFR: 3 µg/cm2/kg a.s./ha Interval between treatments: above 365 days			
Application rate		0.01875 kg a.s./ha	
Body weight: 60 kg	Potential TC: 12500 cm^2/person/h	0.0117167	23 %
	Work wear (arms, body and legs covered) TC: 1400 cm^2/person/h	0.00131	3%

6.6.3.2 Refinement of generic DFR value (KCP 7.2)

Not relevant, default value 3 µg/cm^2/kg a.s./ha was applied for calculations.

6.6.3.3 Measurement of worker exposure

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

Comments of zRMS:	Evaluator agrees with the applicant's assessment. The estimated systemic exposure for a worker undertaking inspection to crops treated T-75WG-OR2C/TOSCANA TOP 75 WG with is within acceptable limits and no further risk assessment is required.

6.6.4 Resident and bystander exposure (KCP 7.2.2)

6.6.4.1 Estimation of resident and bystander exposure

Table 6.6-7 shows the exposure model(s) used for estimation of resident and bystander exposure to Tribenuron-methyl. The outcome of the estimation is presented in Table 6.6-9 (longer term resident exposure) and Table 6.6-8 (acute bystander exposure). Detailed calculations are in Appendix 3.

Table 6.6-7: Exposure models for intended uses

Critical use(s)	Cereals (max. 0.025 kg product/ha, equivalent to 0.01875 kg a.i./ha) Grasslands (max. 0.025 kg product/ha, equivalent to 0.01875 kg a.i./ha)
Model	Guidance on the assessment of exposure of operators, workers, residents and

	bystanders in risk assessment for plant protection products; EFSA Journal 2014;12(10):3874 calculator version: 30/03/2015

Table 6.6-8: Estimated resident exposure (longer term exposure)

		Tribenuron-methyl	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
Cereals Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no DT50: 30 days for Tribenuron-methyl DFR: 3 µg/cm^2/kg a.s./ha Interval between treatments: 365 days			
Number of applications and application rate		1 × 0.01875 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.0012590	2.52%
	Vapour (75th perc.)	0.0010700	2.14%
	Deposits (75th perc.)	0.0001467	0.29%
	Re-entry (75th perc.)	0.0015820	3.16%
	Sum (mean)	**0.0031323**	**6.26%**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0003013	0.60%
	Vapour (75th perc.)	0.0002300	0.46%
	Deposits (75th perc.)	0.0000639	0.13%
	Re-entry (75th perc.)	0.0008789	1.76%
	Sum (mean)	**0.0011207**	**2.24%**
Grasslands Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no DT50: 30 days for Tribenuron-methyl DFR: 3 µg/cm^2/kg a.s./ha Interval between treatments: 365 days			
Number of applications and application rate		1 × 0.01875 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.0012590	2.52%
	Vapour (75th perc.)	0.0010700	2.14%
	Deposits (75th perc.)	0.0001467	0.29%
	Re-entry (75th perc.)	0.0003824	0.76%
	Sum (mean)	**0.0021756**	**4.35%**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0003013	0.60%
	Vapour (75th perc.)	0.0002300	0.46%
	Deposits (75th perc.)	0.0000639	0.13%

	Re-entry (75th perc.)	0.0001426	0.29%
	Sum (mean)	**0.0005625**	**1.12%**

Table 6.6-9: Estimated bystander exposure (acute exposure)

		Tribenuron-methyl	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AAOEL**
Cereals Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no DFR: 3 µg/cm²/kg a.s./ha			
Application rate		0.01875 kg a.s./ha	
Bystander child Body weight: 10 kg	Drift (95th perc.)	0.0028549	2.20%
	Vapour (95th perc.)	0.0010700	0.82%
	Deposits (95th perc.)	0.0004411	0.34%
	Re-entry (95th perc.)	0.0015820	1.22%
Bystander adult Body weight: 60 kg	Drift (95th perc.)	0.0007759	0.60%
	Vapour (95th perc.)	0.0002300	0.18%
	Deposits (95th perc.)	0.0001926	0.15%
	Re-entry (95th perc.)	0.0008789	0.68%
Grasslands Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no DFR: 3 µg/cm²/kg a.s./ha			
Application rate		0.01875 kg a.s./ha	
Bystander child Body weight: 10 kg	Drift (95th perc.)	0.0028549	2.20%
	Vapour (95th perc.)	0.0010700	0.82%
	Deposits (95th perc.)	0.0004411	0.34%
	Re-entry (95th perc.)	0.0007036	0.54%
Bystander adult Body weight: 60 kg	Drift (95th perc.)	0.0007759	0.60%
	Vapour (95th perc.)	0.0002300	0.18%
	Deposits (95th perc.)	0.0001926	0.15%
	Re-entry (95th perc.)	0.0002832	0.22%

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 and AAOEL) for Tribenuron-methyl will not be exceeded under conditions of intended uses, a study to provide measurements of resident/bystander exposure was not necessary and was therefore not performed.

Comments of zRMS:	Evaluator agrees with the applicants calculations of resident and bystander exposure using the EFSA calculator. The estimated exposure to tribenuron-methyl are within acceptable limits and no further risk assessment is required.

6.6.5 Combined exposure

Not relevant. The product contains only one active substance.

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 7.1.5	Xxxxxx R.	2012/13	Acute eye irritation study of PP-108H (Tribenuron methyl 75% WG) in rabbits Report Nº: 407-1-01-5754 GLP, Unpublished	Y	TF PROPLAN-CHEMIROL-SARABIA
KCP 7.1.7/01	Xxxxxx J.	2015	In vitro Mammalian Cell Gene Mutation test (OECD 476) -genotoxicity determination of IN-A4098, IN-L9223 and IN-L9225 by Mouse Lymphoma Assay Selvita S.A. Report Nº: K48/JS/01 GLP, Unpublished	~~Y~~ N	TF PROPLAN-CHEMIROL-SARABIA
KCP 7.1.7/02	Xxxxxx J.	2015	In vitro evaluation of IN-A4098, IN-L9223 and IN-L9225 genotoxicity using the micronucleus assay (MNA) Selvita S.A. Report Nº: K49/JS/01 GLP, Unpublished	~~Y~~ N	TF PROPLAN-CHEMIROL-SARABIA
KCP 7.1.7/03	Xxxxxx	2019	Bacterial reversion mutation test Report Nº: B-02756 GLP, Unpublished	~~Y~~ N	TF PROPLAN-CHEMIROL-SARABIA
KCP 7.1.7/04	Xxxxxx A.	2019	In vitro chromosome aberrations test using Chinese Hamster Ovary cells (CHO) with Amendment LEMI Report Nº: ABC4-LM-18/0293	~~Y~~ N	TF PROPLAN-CHEMIROL-

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
					SARABIA
KCP 7.1.7/05	Xxxxxx C.	2019	In vitro mammalian cell gene mutation test with Amendment LEMI Report Nº: MLAl-LM-18/0293 GLP, Unpublished	~~Y~~ N	TF PROPLAN-CHEMIROL-SARABIA

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
KCP 7.1.6	Xxxxxx R	2012/13	Skin sensitization study of PP-108H (Tribenuron methyl 75% WG) in guinea pigs [Guinea pig maximization test] Report Nº: 406-1-01-5753 GLP, Unpublished	Y	TF PROPLAN-CHEMIROL-SARABIA

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
-	EFSA Panel on Plant Protection products and their Residues (EFSA PRR Panel)	2020	Scientific Opinion of the Scientific Panel on Plant Protection Products and their Residues (PPR Panel) on the genotoxic potential of triazine amine (metabolite common to several sulfonylurea active substances)	N	-

Appendix 2 Detailed evaluation of the studies relied upon

A 2.1 Statement on bridging possibilities

Bridging is not necessary since the toxicological potential of T-75WG-OR2-C/TOSCANA TOP 75WG can be predicted on the basis of toxicological data available for active substances and co-formulants included in composition of above-mentioned product.
Please notice that an eye irritation and skin sensitisation study was performed on the tested material named PP-108H. Its composition is the same as T-75WG-OR2-C/TOSCANA TOP 75WG and thus, the studies are relevant for the further assessment.

Comments of zRMS:	Not applicable.

A 2.2 Acute oral toxicity (KCP 7.1.1)

Acute oral toxicity value (ATE mix) can be estimated according to principles of Regulation 1272/2008, p. 3.1.3.6.1 (additivity formula) as follows:

$$\frac{100}{ATE_{mix}} = \sum_n \frac{C_i}{ATE_i}$$

Where:

C_i – concentration of ingredient i (% w/w or % v/v)
i – the individual ingredient from 1 to n
n – the number of ingredients
ATE_i – Acute Toxicity Estimate of ingredient i.

Calculations takes into account data for components which are classified to acute oral toxicity class and are present at significant concentration. None of T-75WG-OR2-C/TOSCANA TOP 75WG is classified as H300 or H301 ~~or H302.~~ One ingredient is classified with H302 statement, however its concentration (2%) is too low to affect classification of the whole product:

$$\frac{100}{ATE_{mix}} = 2/500 = 0.004 \Rightarrow \text{ATEmix} = 25\,000$$

(high above the CLP trigger value of 2000 for H302 classification)

Therefore, the product should not be classified as Acute Tox. with appropriate hazard statement. No additional studies are required.

Comments of zRMS:	The classification of T-75WG-OR2C/TOSCANA TOP 75 WG is based on the analysis of components present in the formulation. In accordance with the provisions of the Regulation EC 1272/2008, the formulation T-75WG-OR2C/TOSCANA TOP 75 WG is not classified in this class of hazard. For further details, please refer to document C.

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

Acute dermal toxicity value (ATE_{mix}) can be estimated according to principles of Regulation 1272/2008, p. 3.1.3.6.1 (additivity formula) as follows:

$$\frac{100}{ATE_{mix}} = \sum_n \frac{C_i}{ATE_i}$$

Where:

C_i – concentration of ingredient i (% w/w or % v/v)
i – the individual ingredient from 1 to n
n – the number of ingredients
ATE_i – Acute Toxicity Estimate of ingredient i.

Calculations takes into account data for components which are classified to acute dermal toxicity class and significant concentration.

Based on MSDS and harmonized CLP classification neither the active substance nor co-formulants are classified as toxic or harmful by dermal contact with hazards statement H310, H311 or H312.
Therefore, the product will not be classified in this category and no additional studies are required.

Comments of zRMS:	The classification of T-75WG-OR2C/TOSCANA TOP 75 WG is based on the analysis of components present in the formulation. In accordance with the provisions of the Regulation EC 1272/2008, the formulation T-75WG-OR2C/TOSCANA TOP 75 WG is not classified in this class of hazard.

A 2.4 Acute inhalation toxicity (KCP 7.1.3)

Acute inhalation toxicity value (ATE mix) can be estimated according to principles of Regulation 1272/2008, p. 3.1.3.6.1 (additivity formula) as follows:

$$\frac{100}{ATE_{mix}} = \sum_n \frac{C_i}{ATE_i}$$

Where:

C_i – concentration of ingredient i (% w/w or % v/v)
i – the individual ingredient from 1 to n
n – the number of ingredients
ATE_i – Acute Toxicity Estimate of ingredient i.

Calculations takes into account data for components which are classified to acute inhalation toxicity class and significant concentration.
Based on MSDS and harmonized CLP classification neither the active substance nor co-formulants are classified as toxic or harmful with hazards statement H330, H331 or H332.
Therefore, the product will not be classified in this category and no additional studies are required.

Comments of zRMS:	The classification of T-75WG-OR2C/TOSCANA TOP 75 WG is based on the analysis of components present in the formulation. In accordance with the provisions of the Regulation EC 1272/2008, the formulation T-75WG-OR2C/TOSCANA TOP 75 WG is not classified in this class of hazard.

A 2.5 Skin irritation (KCP 7.1.4)

A skin irritation potential of T-75WG-OR2-C/TOSCANA TOP 75WG can be estimated according to principles of Regulation 1272/2008 by using additivity approach.

None of components of the product T-75WG-OR2-C/TOSCANA TOP 75WG is classified as Skin Corr. 1 with hazard statement H314, therefore the product will not be classified as Skin Corr. 1 with hazard statement H314.
Two components of product are classified as Skin Irrit. 2 with hazard statement H315. Their concentrations in the product are 2% and 4%, respectively. As the sum of their concentration is below the concentration limit of 10% (stated in Table 3.2.3 of Regulation 1272/2008), therefore the product will not be classified as Skin Irrit. 2 with hazard statement H315.
No further skin corrosion/irritation study is for T-75WG-OR2-C/TOSCANA TOP 75WG necessary.

Comments of zRMS:	The classification of T-75WG-OR2C/TOSCANA TOP 75 WG is based on the analysis of components present in the formulation. In accordance with the provisions of the Regulation EC 1272/2008, the formulation T-75WG-OR2C/TOSCANA TOP 75 WG is not classified in this class of hazard.

A 2.6 Eye irritation (KCP 7.1.5)

An eye irritation potential of T-75WG-OR2-C/TOSCANA TOP 75WG can be estimated according to principles of Regulation 1272/2008 by using additivity approach.
One component of T-75WG-OR2-C/TOSCANA TOP 75WG is classified as causing serious eye damage with H318 statement. Nevertheless, as its concentration is below the trigger concentration of 3% (stated in Table 3.3.3 of Regulation 1272/2008) and no component of the product is classified to be corrosive to skin, the product T-75WG-OR2-C/TOSCANA TOP 75WG will not be classified with H318 statement.

The pH of the product of about 6 does also not influence on corrosive/irritant properties of the product.

Two components of T-75WG-OR2-C/TOSCANA TOP 75WG product are classified as Eye Irrit. 2 with hazard statement H319. Their concentrations in the product are equal to 8% and 4%, giving a sum above the trigger value of 10%, according to Table 3.3.3 of Regulation 1272/2008. In addition taking into account the additive formula from CLP Regulation of:

10×(Skin Corr. Sub-Cat. 1A/1B/1C or Skin Corr. Cat. 1 + Serious Eye Dam. (Cat. 1)) + Eye Irrit. (Cat. 2),

the sum of all relevant components in the product will be 32%, exceeding the trigger concentration of 10% according to the CLP criteria.

According to all above T-75WG-OR2-C/TOSCANA TOP 75WG would be classified as Eye Irrit. 2 with hazard statement H319. However, as there is also appropriate study available, which proves that TOSCANA TOP 75WG is not irritant to eyes, no classification is finally proposed. Summary of the study is presented below.

It is worth mentioned that "Acute eye irritation study of PP-108H (Tribenuron methyl 75% WG) in rabbits" has not been performed as the new study with a direct purpose to refine classification of TOSCANA TOP 75 WG. The study is existing one, already submitted for the older registration process of TOSCANA 75 WG (authorisation numbers: (PL) No.: R-8/2018wu; (DE): 008873-00, (HU): 0.42/3590-1/2018, (CZ): 5582-0, (RO): 180PC, (UK): 17726).

Report:	**KCP 7.1.5; Xxxxxx, R., 2012/13**
Title:	Acute eye irritation study of PP-108H (Tribenuron methyl 75% WG) in rabbits
Document No:	407-1-01-5754
Guidelines:	**OECD Nº 405**
GLP	**Yes**

SUMMARY

This study was performed to assess the acute eye irritation potential of PP-108H (tribenuron Methyl 75% WG) (supplied by PROPLAN Plant Protection Company, S.L. Spain) in New Zealand White rabbits. The method followed was as per guidelines of the OECD N° 405 (October 2012) and Commission Regulation (EC), B.5 (May 2008).

Three healthy, adult, female albino rabbits of New Zealand White strain were selected for the study. Initially one rabbit was tested. Based on the results obtained at 24 h post application observation, the irritation response was confirmed by testing two additional rabbits simultaneously. A volume of 0.1 mL PP-108H (tribenuron Methyl 75% WG) was applied into one eye of each rabbit and the contralateral eye served as the control. Observations were made following the method described in the guidelines at 1 h (on day 0), 24, 48 and 72 h and on day 7 post application.

The mean eye irritation scores (following grading at 24, 48 and 72 h post application) of the corneal opacity (0.00), iritis (0.00), conjunctival redness (1.67) and chemosis (0.33 to 1.00) were observed in all three treated rabbits.

PP-108H (Tribenuron Methyl 75% WG) is being classified as follows:

Globally Harmonized System of Classification
and Labelling of Chemicals (GHS 2011) : **Not classified as an eye irritant**

1. INTRODUCTION

1.1 Study Objective

This study was performed to assess the acute eye irritation potential of PP-108H (tribenuron Methyl 75% WG) in rabbits. The study was conducted in compliance with the OECD Principles of GLP (1998).

1.2 Study Guidelines

The present study was conducted according to:
The Organization for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals N° 405 "Acute Eye Irritation/Corrosion", adopted by the Council on October 02, 2012; and
Commission Regulation (EC) No 440/2008 of 30 May 2008: laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), B.5. Acute Toxicity: Eye Irritation/Corrosion (Official Journal No L 142, 31/05/2008 p. 0191-0201).

1.3 Justification for Selection of the Test System

The rabbit (*Oryctolagus cuniculus*) was selected as a test system because it is readily available laboratory species. It has been historically shown to be a suitable model for acute eye irritation potential assessment and is recommended by the OECD and other regulatory authorities. The results of the study are believed to be of value in predicting the eye irritation potential of the test item in humans and higher mammals.

1.4 Test Facility and Study Period

This study was performed at the xxxxxxxx.

Study Initiation	:	December 26, 2012
Experiment Start	:	March 09, 2013
Experiment Completion	:	March 25, 2013
Study Completion	:	Month/Date/Year

1.5 Archives

All original raw data including any storage medium for electronically recorded data, documentation, the signed study plan, the study plan amendment, the draft report, a copy of the final report and the representative sample of the test item will be retained in the GLP Archives at Jai Research Foundation for a period of ten years. At the end of this period, the Sponsor's instructions will be sought to either extend the archiving period or return the archived material to the Sponsor or for the material to be disposed of.

2. EXPERIMENTAL PROCEDURE

2.1 Test Item

Details of the test item provided by the Sponsor (Ref. TIDS):

Test Item Name	: PP-108H (Tribenuron-Methyl 75% WG)
IUPAC Name	: Methyl 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoylsulfamoyl]benzoate
CA Name	: Methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)methylamino]carbonyl]amino]sulfonyl]benzoate
CAS Number	: [101200-48-0]
Analysed Concentration	: 77.0 ± 0.4% w/w (Refer Certificate of Analysis in Appendix 4)
Batch N°	: 20111502
Supplied by	: PROPLAN Plant Protection Company, S.L.
Manufactured by	: PROPLAN Plant Protection Company, S.L.
Date of Manufacture	: February 2011
Date of Expiry	: February 2014
Appearance/Colour/Odour	: Solid, granular, light brown, mild, sweet
Storage Condition (at JRF)	**:** As per the instruction received from the Sponsor on storage of the test item, the test item was stored in its original container as supplied by the Sponsor at room temperature in the Test Item Control Office (TICO). The stability of the test item in storage is the responsibility of the Sponsor.

2.2 Instruments and Equipment

Tattoo Machine	:	$AIMS_{TM}$ Tattoo Machine
Balance	:	1. Electronic Weighing Scale - SMART (Capable of measuring 10.0 g to 6.0 kg) 2. Sartorius Semi Micro balance, BP 210 D (Capable of measuring 0.01 mg to 210 g)
Ophthalmoscope	:	Neitz Ophthalmoscope

Syringe : BD Disposable 1 mL syringe

2.3 Solvents and Chemicals

Ophthalmic Fluorescein Strip	:	FLUO STRIPS™, Fluorescein Sodium Ophthalmic Strips U.S.P.
Normal Saline	:	0.9% (w/v) NaCl in distilled water
Distilled Water	:	Milli Pore Elix 10 Water Purification System
Disinfectant	:	Dettol 2.5%
Sodium Chloride	:	Minimum assay 99.5%, SQ Grade
Buprenorphine Hydrochloride:		Buprenorphine Hydrochloride IP., 0.3 mg/mL Mfg by: SNG Pharmaceuticals (P) Ltd., Vapi
Meloxicam	:	Meloxicam Injection (BP), 5 mg/mL Mfg by: Intas Pharmaceuticals Ltd., Ahmedabad
Proparacaine Hydrochloride	:	Proparacaine Hydrochloride USP 0.5% Mfg by: Sunways (India) Pvt. Ltd., Mumbai

2.4 Animals

Test System	:	Rabbit (*Oryctolagus cuniculus*)
Strain	:	New Zealand White
Animal Source	:	Mahaveera Enterprises, Hyderabad
Total Number of Animals Used	:	Three
Sex	:	Female (nulliparous and non pregnant)
Body Weight Range (kg)		
on Day 0	:	Minimum : 2.572, Maximum : 2.981
Age of Rabbits Prior to Dosing	:	4 to 5 months

2.5 Acclimatisation

The rabbits were received into the experimental procedure room and allowed to acclimatise for a period of 7 days for rabbit N° 1 and 9 days for rabbit N° 2 and 3 prior to test item application.

2.6 Husbandry Practices

Caging	:	Stainless steel wire meshed cages were used.
Water Bottle	:	Each cage was supplied with a polypropylene water bottle with a stainless steel nozzle.
Housing	:	Individual rabbit.
Room Sanitation	:	Each day, the floor of experimental room was swept and all work tops and floor were mopped with a disinfectant solution.

2.7 Animal Identification

Each rabbit was serially numbered on the ear using a tattoo machine. Appropriate labels were attached to the cages indicating the study number, test item code, sex, dose, type of study, cage number and animal number.

2.8 Feed and Water

The rabbits were provided feed and water *ad libitum*. The quality of feed and water is regularly monitored at Jai Research Foundation. There were no known contaminants in the feed and water at levels that would have interfered with the experimental results obtained.

Feed : Teklad Certified Global High fibber rabbit pellet feed manufactured by Harlan, USA was provided *ad libitum.*

Water : UV sterilized drinking water filtered through Kent Reverse Osmosis water filtration system was provided *ad libitum*.

2.9 Environmental Conditions

Animal Room	:	DCR – 301, Department of Toxicology
Temperature Range	:	20 to 23 °C
Relative Humidity Range	:	65 to 67%

Photoperiod	:	The photoperiod was 12 h artificial light and 12 h darkness, light hours being 06:00 - 18:00 h except light was kept ON at the time of the subcutaneous injection during night hours.
Air Changes Rate	:	Minimum 15 air changes/h

2.10 Experimental Activities

The following activities were performed during the experimental period:

Daily

- animal observations
- providing feed and water
- floor sweeping and mopping
- rack cleaning
- recording room temperature and relative humidity
- recording photoperiod

2.11 Examination of Eyes Prior to the Treatment

Within 24 h, prior to the treatment, both the eyes of each rabbit were examined for ocular lesions to confirm that there were no pre-existing eye disease, corneal damage or any other defects. The eyes of all rabbits were found to be normal and the rabbits were accepted for the study.

2.12 Treatment

Approximately 60 minutes prior to test item application (TIA), buprenorphine 0.01 mg/kg body weight was administered by subcutaneous injection (SC) to the rabbit N° 1.

Approximately 5 minutes prior to TIA, one to two drops of 0.5% proparacaine hydrochloride was applied to each eye.

A volume of 0.1 mL of PP-108H (tribenuron Methyl 75% WG) (the test item was measured into an Eppendorf tube up to the 0.1 mL mark. The weight for the same was recorded for each rabbit separately. A volume of 0.1 mL test item was applied into the treated eye of each rabbit, which accounted for 47.15, 48.10 and 47.65 mg for rabbit N° 1, 2 and 3 respectively) (pulverized) was applied in the conjunctival sac of one eye of each rabbit after gently pulling the lower lid away from the eyeball. Then the lids were gently held together for about one second in order to prevent loss of the test item. The contra-lateral eye was served as the control.

After 8 h of TIA, buprenorphine 0.01 mg/kg SC and meloxicam 0.5 mg/kg body weight SC were administered to provide a continued therapeutic level of systemic analgesia.

After the initial 8-hour post-TIA, buprenorphine 0.01 mg/kg body weight SC was administered every 12 hours, in conjunction with meloxicam 0.5 mg/kg body weight SC every 24 hours, until the ocular lesions resolved.

A sequential testing strategy is adopted. Initially one rabbit was tested. Severe effects were not observed in the first treated rabbit, two additional rabbits were subsequently being treated in an identical manner.

At 24 h post application of the test item, both the eyes (control and treated) of the three rabbits were gently washed with 0.9% normal saline.

Note: As per OECD 405 guideline, administration of Buprenorphine to rabbits is not known to alter ocular responses. In the present study, Meloxicam injected after 8 hour of test item application hence as per OECD 405 guideline Meloxicam is not expected to interfere with the study results.

2.13 Observations

After application (day 0), the eyes of all the rabbits were observed for signs of ocular irritation at 1, 24, 48 and 72 h post application. The irritant response was scored following the method described in the guidelines.

For each observation occasion, scoring for ocular lesions was undertaken for the cornea, the iris and conjunctiva (including lids and/or nictitating membranes).

The degree of corneal opacity was graded on a scale of 0 - 4.
The reactions observed in the iris and changes in iris light response were graded 0 - 2.
The intensity of conjunctival redness was graded 0 - 3 while chemosis (swelling) was graded 0 - 4.

2.14 Corneal Observations

The eyes of each rabbit were then examined using fluorescein dye staining (Swinyard, 1990).
One to two drops of fluorescein stain was instilled into the eye. The eyelids were closed for few seconds. The eye was washed using 0.9% normal saline to remove the residual stain from the eye and the eye was examined with the aid of Neitz Ophthalmoscope through a red free filter (corneal damage showing as yellow fluorescein staining). Any loss or damage in corneal epithelium was recorded.

3. RESULTS

3.1 Mean Eye Irritation Scores

The mean eye irritation scores for corneal opacity (0.00), iritis (0.00), conjunctival redness (1.67) and chemosis (0.33 to 1.00) were observed following grading at 24, 48 and 72 h post application.

3.2 Narrative Description of Eye Irritation

Treated Eye

At 1 h post application eye examinations, the treated eyes of all three rabbits revealed conjunctival redness [some blood vessels hyperaemic (injected)] and chemosis [some swelling above normal (includes nictitating membranes)].
At 24 h post application eye examination, the treated eyes revealed conjunctival redness [some blood vessels hyperaemic (injected) in rabbit Nº 1 to diffuse, crimson colour, individual vessels not easily discernible in rabbit Nº 2 and 3] and chemosis [some swelling above normal (includes nictitating membranes) in all three rabbits] (Appendix 3). Examination at 24 h with fluorescein dye and red free filter (corneal damage showing as yellow fluorescein) revealed no corneal epithelium damage in all three rabbits.
At 48 h post application eye examination, the treated eyes revealed conjunctivae redness [diffuse, crimson colour, individual vessels not easily discernible in all three rabbits] and chemosis [some swelling above normal (includes nictitating membranes) in rabbit Nº 2 and 3].
At 72 h post application eye examination, the treated eyes revealed conjunctivae redness [some blood vessels hyperaemic (injected) in rabbit Nº 2 and 3 to diffuse, crimson colour, individual vessels not easily discernible in rabbit Nº 1] and chemosis [some swelling above normal (includes nictitating membranes) in rabbit Nº 2 and 3].
On day 7 post instillation eye examination, the treated eyes of all the three rabbits recovered completely and appeared to be normal.
Corneal opacity and iritis were not observed in any of the treated eye of all the rabbits throughout the experimental period.

Control Eye

No abnormalities were detected in the control eye of all the rabbits during the course of this study. No damage to corneal epithelium was observed during the examination with fluorescein dye and red free filter.

3.3 Clinical Observations other than Eye Irritation

No treatment related clinical signs other than eye irritation were observed in the rabbits throughout the experimental period.

3.4 Interpretation of Results

The mean eye irritation scores (following grading at 24, 48 and 72 h post instillation) of the corneal opacity (0.00), iritis (0.00), conjunctival redness (1.67) and chemosis (0.33 to 1.00) were found to be

non-significant in all three treated rabbits. Therefore, PP-108H (tribenuron Methyl 75% WG) is being classified as **"Not classified as an eye irritant"** according to Globally Harmonized System of Classification and Labelling of Chemicals (GHS 2011).

4. CONCLUSION

The mean eye irritation scores (following grading at 24, 48 and 72 h post instillation) of the corneal opacity (0.00), iritis (0.00), conjunctival redness (1.67) and chemosis (0.33 to 1.00) were observed in all three treated rabbits.
PP-108H (Tribenuron Methyl 75% WG) is being classified as follows:

Globally Harmonized System of Classification and Labelling of Chemicals (GHS 2011) : **Not classified as an eye irritant**

Comments of zRMS:	Study acceptable, without deficiencies, according to mentioned guidelines, used in evaluation. Based on study results PP-108H is not eye irritant. According to the Regulation EC No. 1272/2008 formulation T-75WG-OR2-C/ TOSCANA TOP 75 WG is not classified as an eye irritant.

A 2.7 Skin sensitisation (KCP 7.1.6)

A skin or respiratory sensitisation potential of T-75WG-OR2-C/TOSCANA TOP 75WG can be estimated according to principles of Regulation 1272/2008, which indicate that if at least one ingredient has been classified as a respiratory or skin sensitiser and is present at or above the appropriate generic concentration limit, the mixture shall be classified as a respiratory or skin sensitiser.

One component of T-75WG-OR2-C/TOSCANA TOP 75WG is classified as a respiratory sensitizers. However, its concentration in the product is 4% only, the whole product will not be classified with the H335 statement.

The only ingredient of T-75WG-OR2-C/TOSCANA TOP 75WG classified as a skin sensitiser is the active substance tribenuron-methyl (Skin Sens. 1, H317). It is present in the product in amount of approx. 75%. This concentration is above concentration limit of 1% and thus, T-75WG-OR2-C/TOSCANA TOP 75WG could be classified with the H317 statement .

However, as there is also appropriate study available, which proves that TOSCANA TOP 75WG is not a skin sensitiser, no classification is finally proposed. Instead of that only the EUH 208 statement should be stated on the label. Summary of the study is presented below.

It is worth mentioned that "Skin sensitization study of PP-108H (Tribenuron methyl 75% WG) in guinea pigs [Guinea pig maximization test]" has not been performed as the new study with a direct purpose to refine classification of TOSCANA TOP 75 WG. The study is existing one, already submitted for the older registration process of TOSCANA 75 WG (authorisation numbers: (PL) No.: R-8/2018wu; (DE): 008873-00, (HU): 0.42/3590-1/2018, (CZ): 5582-0, (RO): 180PC, (UK): 17726).

Report:	**KCP 7.1.6 Xxxxxx, R., 2012/13**
Title:	Skin sensitization study of PP-108H (Tribenuron methyl 75% WG) in guinea pigs [Guinea pig maximization test]
Document No:	406-1-01-5753
Guidelines:	**OECD Nº 406**
GLP	**Yes**

SUMMARY

This study was conducted to assess the skin sensitization potential of PP-108H (tribenuron methyl 75% WG) (supplied by PROPLAN Plant Protection Company, S.L. Spain) in guinea pigs. The method followed was as per the guidelines of OECD N° 406 (July 1992) and Commission Regulation (EC) (May 2008), B.6 using the using the Guinea-pig Maximisation Test Method.

Fifteen Hartley strain male guinea pigs were randomly divided into two groups. The control group comprised 5 guinea pigs and the treatment group comprised 10 guinea pigs. Based on the results of the pilot study, 5.0% (w/v) PP-108H (tribenuron methyl 75% WG) in distilled water was selected for intradermal injection during induction exposure on day 0. PP-108H (tribenuron methyl 75% WG) was found to be irritant when applied topically (Refer: pilot study), therefore on day 6, 0.5 mL 10% (w/v) sodium lauryl sulphate in vaseline was not applied to augment the local skin irritation. A quantity of 100 mg PP-108H (tribenuron methyl 75% WG) moistened with 0.2 mL distilled water was selected for topical application during induction on day 7 and a quantity of 75 mg PP-108H (tribenuron methyl 75% WG) moistened with 0.2 mL distilled water for challenge exposure on day 21.

The skin reactions of the guinea pigs were recorded post induction (intradermal injections/topical application) following the Xxxxxx method (Xxxxxx et al., 1944) and at 24 h and 48 h post challenge treatment following the Xxxxxx and Xxxxxx grading scale (Xxxxxx and Xxxxxx, 1969).

Well-defined erythema (in 10/10 guinea pigs) and very slight oedema (in 07/10 guinea pigs) to slight oedema (in 03/10 guinea pigs) were observed on day 1 in the guinea pigs from the treatment group following intra-dermal injection (day 0). Very slight erythema (in 08/10 guinea pigs) was observed on day 10 on the left flank of the treatment group guinea pigs following topical application on day 7. No skin reactions were observed in the guinea pigs from the control group (days 1 and 10).

Visual observation of the skin following challenge exposure did not reveal any positive skin response at 24 and 48 h post patch removal in the guinea pigs belonging to the treatment group and the control group.

No clinical signs related to treatment other than skin irritation were observed during the course of the study in guinea pigs.

The mean body weight of the treatment group guinea pigs remained comparable to that of the control group.

A sensitization rate of zero percent at 24 and 48 h post patch removal was observed using an adjuvant.

PP-108H (Tribenuron methyl 75% WG) is being classified as follows:

Globally Harmonised System of
Classification and Labelling of Chemicals (GHS 2011) : **Not considered as positive**

Positive Control Results (JRF Study Number: 408-1-01-5816, February 05, 2013) The sensitivity and reliability of the experimental technique was confirmed by conducting a positive control study using α-

Hexylcinnamaldehyde.

Visual observation of skin post challenge exposure revealed a positive response of 40% at 24 h and 35% at 48 h post patch removal in guinea pigs of the treatment group. No systemic toxicity was observed during the experimental period. The mean body weights of the treatment group (positive control) guinea pigs remained comparable to that of the control group. Hence, it has been confirmed that α-Hexylcinnamaldehyde is considered as **"positive"** to guinea pigs as per the OECD Harmonized System of Classification and labelling of Chemicals (August 2001) and **"product is a sensitizer or is positive for sensitization"** as per the EPA Toxicity Categories (December 2002), in **"Category 1"** as per the EC Classification and Labelling Requirements for Dangerous substances and Preparations (August 2008) and **"Category 1 (Subcategory 1B)"** as per Globally Harmonized System of Classification and Labelling of Chemicals (GHS 2011). The results of the positive control study confirmed the sensitivity of the Guinea Pig Maximization Test Method.

1. INTRODUCTION

1.1 Study Objective

This study was performed to determine the skin sensitization potential of PP-108H (tribenuron methyl 75% WG) in Hartley strain guinea pigs. This study was conducted in compliance with the OECD Principles of GLP (1998).

1.2 Study Guidelines

The study was conducted according to:

The Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals, N° 406 "Skin Sensitisation", adopted by the Council on July 17, 1992.

and

Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), B.6. Skin sensitisation (Official Journal No L 142, 31/05/2008 p. 0202-0209);

1.3 Justification for Selection of the Test System

The guinea pig (*Cavia porcellus*) was selected as a test system because it is a readily available species and the absence of pigmentation on the dermis facilitates evaluation of induced skin reactions. It has been historically shown to be a suitable model for skin sensitization studies and is recommended by the OECD and other regulatory authorities. The results of the study are believed to be of value in predicting the skin sensitization potential of the test item in humans and higher mammals.

1.4 Test Facility and Study Period

The study was performed at the xxxx.

Study Initiation	:	December 26, 2012
Experiment Start	:	February 12, 2013
Experiment Completion	:	March 21, 2013
Study Completion	:	Month/Date/Year

1.4.1 Study Period of Positive Control Study (JRF Study Number: 408-1-01-5816)

Study Initiation	:	December 25, 2012
Experiment Start	:	December 26, 2012
Experiment Completion	:	January 25, 2013
Study Completion	:	February 05, 2013

1.5 Archives

All original raw data including any storage medium for electronically recorded data, documentation, the signed study plan, the study plan amendment, the draft report, a copy of the final report and the representative sample of the test item will be retained in the GLP Archives at Jai Research Foundation for a period of ten years. At the end of this period, the Sponsor's instructions will be sought to either extend the archiving period or return the archived material to the Sponsor or for the material to be disposed of.

2. EXPERIMENTAL PROCEDURE

2.1 Test Item

Details of the test item provided by the Sponsor (Ref. TIDS):

Test Item Name	:	PP-108H (Tribenuron-Methyl 75% WG)
IUPAC Name	:	Methyl 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoylsulfamoyl]benzoate
CA Name	:	Methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)methylamino]carbonyl]amino]sulfonyl]benzoate
CAS Number	:	[101200-48-0]
Analysed Concentration	:	77.0 ± 0.4% w/w (Refer Certificate of Analysis in Appendix 8)
Batch N°	:	20111502
Supplied by	:	PROPLAN Plant Protection Company, S.L.
Manufactured by	:	PROPLAN Plant Protection Company, S.L.
Date of Manufacture	:	February 2011
Date of Expiry	:	February 2014
Appearance/Colour/Odour	:	Solid, granular, light brown, mild, sweet
Storage Condition (at JRF)	**:**	As per the instruction received from the Sponsor on storage of the test item, the test item was stored in its original container as supplied by the Sponsor at room temperature in the Test Item Control Office (TICO). The stability of the test item in storage is the responsibility of the Sponsor.

2.2 Details of Positive Control

Reference Substance Name	:	α-Hexylcinnamaldehyde
CAS N°	:	101-86-0
Batch N°	:	MKAA2596
Supplied by	:	Sigma Aldrich Chemie GmbH, Germany
Manufactured by	:	Sigma Aldrich Chemie GmbH, Germany
Chemical Purity	:	85% - Specified
Analysed Purity	:	95.9%
Appearance	:	Yellow liquid
Date of Receipt	:	April 28, 2012
Date of Expiry	:	December 06, 2017
Storage Condition (at JRF)	:	Room temperature

2.3 Instruments and Equipments

Balances	:	1. Electronic Weighing Scale – SMART (Capable of measuring 10.0 g to 6.0 kg) 2. Sartorius Semi Micro Balance, BP 210 D (Capable of measuring 0.01 mg to 210 g)
Clipper	:	Oster Golden A5, 5 – 65L
Syringe	:	BD 1 mL disposable syringe
Needle	:	"B-D®" Precision Glide™ needle, size: 26 G½ (0.45 mmx13 mm)

2.4 Solvent and Chemicals

Adjuvant	:	Freund's Complete Adjuvant
Distilled water	:	Milli pore Elix 10 water purification System
Vitamin-C Tablets	:	"Limcee™" Vitamin-C Chewable Tablets
Disinfectant	:	Dettol 2.5%
Distilled water	:	Minimum assay 98%, SQ grade

2.5 Details of Test System

Test System	:	Guinea pig
Species (strain)	:	*Cavia porcellus* (Hartley)
Animal Source	:	xxxxx.
Number Used	:	Fifteen (05 in control and 10 in treatment group)
Sex	:	Male
Initial Body Weight (g) on Day 0	:	Minimum: 260 Maximum: 307
Age on Day 0	:	5 to 7 weeks

2.6 Acclimatisation

The guinea pigs were received into the experimental room post veterinary examination for health and were allowed to acclimatise to the laboratory conditions for a period of 6 days prior to commencement of dosing.

2.7 Husbandry Practices

Caging	:	Solid polypropylene cages with labels were used.
Water Bottle	:	Each cage was supplied with a polypropylene water bottle with a stainless steel nozzle.
Housing	:	One guinea pig per cage.
Room Sanitation	:	Each day, the floor of the experimental room was swept and all work tops and the floor were mopped with a disinfectant solution.

2.8 Animal Identification

Guinea pigs were housed individually according to group and sex in solid polypropylene cages. Guinea pigs were identified with cage cards. The cage card showed details of study number, test item code, group number, sex and dose, type of study and cage number and animal number.

2.9 Feed and Water

The quality of feed and water is regularly monitored at Jai Research Foundation. There were no known contaminants in the feed and water at levels that would have interfered with the experimental results obtained.

Feed	:	Guinea pigs were fed with Teklad certified Global High Fiber Guinea pig diet manufactured by Harlan, U.S.A. Feed was provided *ad libitum.*
Water	:	UV sterilized water filtered through Kent Reverse Osmosis water filtration system, supplemented with vitamin-C (1 g/L), was provided *ad libitum*.

2.10 Environmental Conditions

Animal Room	:	DCR – 206 (Department of Toxicology)
Temperature Range	:	19 to 23 °C
Relative Humidity	:	64 to 65%
Photoperiod	:	The photoperiod was 12 h artificial light and 12 h darkness, light hours being 06:00 – 18:00 h.
Air Changes	:	Minimum 15 air changes/h

2.11 Randomization

After acclimatisation guinea pigs were randomised into two groups using in-house developed, validated computer software (Gad and Weil, 1994).

2.12 Experimental Activities

The following activities were performed daily during the experimental period:

- animal observations
- providing water supplemented with vitamin-C
- providing feed
- floor and work tops sweeping and mopping
- rack cleaning
- recording room temperature and relative humidity
- recording photoperiod

2.13 Pilot Study and Dose Selection

Intradermal Irritancy Test

This test was carried out to determine the suitable concentration for induction by intradermal injection which produces mild to moderate irritation reactions for sensitising the guinea pigs. Four guinea pigs (2 males and 2 females) were injected intradermally on the clipped scapular region with 0.2 mL PP-108H (tribenuron methyl 75% WG) at the concentrations of 5.0%, 2.5%, 1.0% and 0.5% (w/v) in distilled water. The reactions were examined and scored for the extent of erythema and oedema following the Xxxxxx method (Xxxxxx et al., 1944) (Refer section 2.15).

At 5.0% (w/v) concentration, well-defined erythema and very slight oedema were observed at 24 and 48 h post intradermal injection in both the sexes of guinea pigs. At 2.5% (w/v) concentration, very slight erythema and very slight oedema were observed at 24 and 48 h post intradermal injection in both the sexes of guinea pigs. At 1.0 and 0.5% (w/v) concentration, very slight erythema was observed at 24 and 48 h post intradermal injection in both the sexes of guinea pigs. As 5.0% (w/v) concentration was found to be mild to moderate irritant dose, it was selected for intradermal injection for the main study.

Topical Irritancy Test

This test was carried out to determine the highest concentration of the test item required to produce minimal irritation for induction and no irritation for the challenge phase in the main study. A patch loaded with 25, 50, 75 and 100 mg PP-108H (tribenuron methyl 75% WG) moistened with 0.2 mL distilled water was topically applied to the clipped flanks of four guinea pigs (2 males and 2 females). The patches were held in contact for a period of 24 h by an occlusive dressing (with Meditape surgical tape). The skin reactions were evaluated following the Xxxxxx method (Refer section 2.15) at 24 and 48 h post patch removal.

At 24 and 48 h post patch removal, 100 mg PP-108H (tribenuron methyl 75% WG) showed very slight erythema whereas 75, 50 and 25 mg PP-108H (tribenuron methyl 75% WG) showed no erythema and no oedema in both the sexes of guinea pigs. Based on the results of the pilot study, a quantity of 100 mg PP-108H (tribenuron methyl 75% WG) moistened with 0.2 mL distilled water was selected for topical application during induction on day 7 and a quantity of 75 mg PP-108H (tribenuron methyl 75% WG) moistened with 0.2 mL distilled water for challenge exposure on day 21.

2.14 Guinea pig Maximization Test

Fifteen guinea pigs were randomized into 2 groups by censored method of randomization and comprised 5 males in control group and 10 males in treatment group.

2.14.1 Preparation of Application Site

Approximately 24 h prior to the treatment, an area approximately 4 x 2 cm^2 across the scapular region of the guinea pigs was clipped using a clipper and was used as the injection site during induction exposure. Hair was removed from both the flanks of guinea pigs at 24 h prior to the treatment for topical induction and challenge exposure.

2.14.2 Induction Phase: Intradermal Injections

Three pairs of intradermal injections of 0.1 mL each were given in the scapular region in such a way that each pair of injections was sited contralaterally to the median line of the guinea pig.

Day 0 - Treatment Group

Injection 1 : a 1:1 mixture (v/v) of Freund's Complete Adjuvant (FCA) with distilled water
Injection 2 : 5.0% (w/v) PP-108H (tribenuron methyl 75% WG) formulated in distilled water
Injection 3 : 5.0% (w/v) PP-108H (tribenuron methyl 75% WG) formulated in distilled water and 1:1 mixture (v/v) of FCA in distilled water

Injection N° 1 and 2 were administered close to each other on the scapular region while injection N° 3 was administered towards the caudal part of the test area.

Day 0 - Control Group

Injection 1 : a 1:1 mixture (v/v) of Freund's Complete Adjuvant (FCA) with distilled water
Injection 2 : distilled water
Injection 3 : distilled water in a 1:1 mixture (v/v) of FCA in distilled water

Skin reactions were evaluated at 24 h post intradermal injections following the Xxxxxx method (1944).

2.14.3 Induction Phase: Topical Application

Day 6 - (Control and Treatment Groups)

Since PP-108H (tribenuron methyl 75% WG) was found to be irritant when applied topically during pilot study, 0.5 mL 10% (w/v) sodium lauryl sulphate in vaseline was not applied on the test area (left flank) of the guinea pigs on day 6 to augment the local skin irritation.

Day 7 - Treatment Group

A patch loaded with 100 mg PP-108H (tribenuron methyl 75% WG) moistened with 0.2 mL distilled water was applied to the left flank and was held in contact by an occlusive dressing for a period of 48 h.

Day 7 - Control Group

A patch loaded with 0.2 mL distilled water was applied to the left flank and was held in contact by an occlusive dressing for a period of 48 h.

Skin reactions were evaluated at 24 h post patch removal on day 10 following the Xxxxxx method (Refer section 2.15).

2.14.4 Challenge Phase: Topical Application

Day 21 (Treatment and Control Groups)
A patch loaded with 75 mg PP-108H (tribenuron methyl 75% WG) moistened with 0.2 mL distilled water was applied to the right flank of all the guinea pigs. The patches were held in contact by an occlusive dressing for a period of 24 h.

2.15 Observations

The guinea pigs were observed at least twice a day and clinical signs were recorded once a day during the course of study. Skin reactions were observed at 24 and 48 h post patch removal following challenge application as per the Xxxxxx and Xxxxxx grading scale. The initial (day 0) and terminal body weights (day 24) of guinea pigs were recorded.

Dermal Irritation Score
(Xxxxxx Method, Xxxxxx, et al., 1944)

Reaction	**Score**
Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to slight eschar formation (injuries in depth)	4

Oedema Formation	**Score**
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

Xxxxxx and Xxxxxx Grading Scale for the Evaluation of Challenge Patch Test Reactions (Xxxxxx and Xxxxxx, 1969)

Reaction	**Value**
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

The degree of sensitising potential was assigned according to the percentage of guinea pigs giving a

positive response in the treatment group. The skin sensitisation results was interpreted as per the Globally Harmonised System for Classification and Labelling of Chemicals (GHS 2011).

2.16 Evaluation of Results

Body weight data was statistically analysed by Student's 't' test (Gad and Weil, 1994).

3. RESULTS

3.1 Skin Reactions

Well-defined erythema (in 10/10 guinea pigs) and very slight oedema (in 07/10 guinea pigs) to slight oedema (in 03/10 guinea pigs) were observed on day 1 in the guinea pigs from the treatment group following intradermal injection (day 0). Very slight erythema (in 08/10 guinea pigs) was observed on day 10 on the left flank of the treatment group guinea pigs following topical application on day 7. No skin reactions were observed in the guinea pigs from the control group (days 1 and 10).

Skin reactions were scored at 24 and 48 h post patch removal of the challenge exposure. Visual observation of the skin following challenge exposure did not reveal any positive skin response at 24 and 48 h post patch removal in the guinea pigs belonging to the treatment group. A sensitization rate of zero percent at 24 and 48 h post patch removal was observed using an adjuvant.

3.2 Clinical Observations other than Irritation/Sensitization

No clinical signs related to treatment other than irritation were observed in any of the guinea pigs from the treated and the control groups.

3.3 Body Weight

The mean body weight of the treatment group guinea pigs remained comparable to that of the control group.

3.4 Reliability Check

The sensitivity and reliability of the experimental technique was confirmed by conducting a positive control study (JRF Study Number: 408-1-01-5816, February 05, 2013) using α-Hexylcinnamaldehyde. Visual observation of skin after challenge exposure revealed that 40% of guinea pigs exhibited a positive skin response at 24 h and 35% at 48 h post patch removal in the treatment group (Table 3). No systemic toxicity was observed during the experimental period. The mean body weight of the treatment group (positive control) guinea pigs was comparable to that of the control group.

Hence, it has been confirmed that α-Hexylcinnamaldehyde is considered as **"positive"** to guinea pigs as per the OECD Harmonized System for the Classification of Chemicals (August 2001), according to Globally Harmonized System of Classification and Labelling of Chemicals (GHS, 2011), it is classified under **"Category 1 (subcategory 1B)"** and **"product is a sensitizer or is positive for sensitization"** as per the EPA Toxicity Categories (December 2002) and **"Category 1"** as per the EC Classification and Labelling Requirements for Dangerous Substances and Preparations (August 2008).
The results of the positive control study confirmed the skin sensitization potential of α-Hexylcinnamaldehyde and the sensitivity of the test method in guinea pigs following the Guinea Pig Maximization Test.

3.5 Interpretation of the Results

A response of at least 30% is considered as positive for the adjuvant type test (FCA) as per the Globally Harmonized System of Classification and Labelling of Chemicals (GHS 2011) and a

sensitization rate of zero percent at 24 and 48 h was observed in the present study using an adjuvant (Table 2, Appendix 3). Hence, PP-108H (tribenuron methyl 75% WG) is being classified as **"Not considered as positive"** for sensitization as per Globally Harmonised System of Classification and Labelling of Chemicals (GHS 2011).

4. CONCLUSION

A sensitization rate of zero percent at 24 and 48 h post patch removal was observed using an adjuvant.

PP-108H (Tribenuron methyl 75% WG) is being classified as follows:

Globally Harmonised System of Classification and Labelling of Chemicals (GHS 2011) : **Not considered as positive**

Comments of zRMS:	According to Test Guideline No. 406: *The concentration of test chemical used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation*. In skin sensitization study of PP-108H (Tribenuron methyl 75% WG) in guinea pigs 5.0% (w/v) PP-108H (tribenuron methyl 75% WG) in distilled water was selected for intradermal injection. This concentration caused mild-to-moderate skin irritation. In the pilot study the concentrations above 5.0% in intradermal injection were not tested. Due to lack of data on higher concentrations GPMT study is not accepted. zRMS is the opinion that formulation should be classified based on the content of active substance (75% w/w) and the harmonized classification of tribenuron-methyl as Skin Sens. 1, H317. In this case additional labelling phrase - "EUH208 Contains tribenuron-methyl. May produce an allergic reaction." is not needed.

A 2.8 Additional toxicological properties - target organ toxicity after repeated exposure

According to the RAC opinion (adopted 14.09.2018) and 15th ATP to CLP tribenuron-methyl should be classified as the substance which may cause damage to organs through prolonged or repeated exposure.

In addition product T-75WG-OR2-C/TOSCANA TOP 75WG contains one co-formulant with H373 classification and is present in concentration above 10%.

Since the concentration of the active substance and co-formulant exceed the trigger value of 10% according the CLP criteria (Table 3.9.4 of Regulation 1272/2008) the product T-75WG-OR2-C/TOSCANA TOP 75WG should be classified with the H373 statement.

A 2.9 Supplementary studies for combinations of plant protection products (KCP 7.1.8)

Not applicable.

A 2.10 Data on co-formulants (KCP 7.4)

A 2.10.1 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 2.10.2 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 2.11 Studies on dermal absorption (KCP 7.3)

Not submitted. Default values according to the EFSA Guidance on dermal absorption (EFSA Journal 2017;15(6):4873) were applied.

A 2.12 Other/Special Studies (KCP 7.1.7)

A 2.12.1 *In vitro* mammalian cell gene mutation test for metabolite IN-A4098 (KCP 7.1.7/01)

Report:	**Xxxxxx, J., 2015**
Title:	*In vitro* Mammalian Cell Gene Mutation test (OECD 476) - genotoxicity determination of IN-A4098, IN-L9223 and IN-L9225 by Mouse Lymphoma Assay
Document No:	K48/JS/01
Guidelines:	OECD Nº 476
GLP	Yes

SUMMARY

Mutagenic potential of IN-A4098, IN-L9223 and IN-L9225 was evaluated trough Mouse Lymphoma Assay (MLA) in L5178Y cells. Tested compounds were analysed in MLA, in the presence and absence of exogenous metabolic activation.
Obtained results have shown that tested compounds did not exceed GEF (Global Evaluation Factor) above 126 $x10^{-6}$ in any of the tested doses both in the presence and absence of S9 exogenous activation system.
Obtained results indicate that neither tested compounds nor their metabolic derivatives were positive in Mouse Lymphoma Assay under the protocol described and according to the acceptability criteria defined in OECD guideline 476 and SPB-19.

1. INTRODUCTION

1.1 Study Objective

The scope of the project was to assess the mutagenic potential of the test compounds: IN-A4098, IN-L9223 and IN-L9225 during 4 hour incubation with and without S9 fraction and 24 hour incubation with tested compounds by using Mouse Lymphoma Assay.

Research was performed according to OECD Guideline for the Testing of Chemicals, Guideline 476 In Vitro Mammalian Cell Gene Mutation Test, updated and adopted 21 July 1997.

1.2 Study Guidelines

Research was performed according to OECD Guideline for the Testing of Chemicals, Guideline 476 In Vitro Mammalian Cell Gene Mutation Test, updated and adopted 21 July 1997.

1.3 Definitions

Mouse Lymphoma Assay (MLA) – molecular biology technique used for the quantification of forward mutations at the thymidine kinase locus of mammalian cells L5178Y from mouse lymphoma.

Expression period – time after treatment during which the genetic alteration is fixed within the genome and any preexisting gene products are depleted to the point that the phenotypic trait is altered. Each locus has a defined minimum time requirement to allow near optimal phenotypic expression of newly induced mutants. For the TK (thymidine kinase) locus determined time is 2 days.

Cloning efficiency – the percentage of cells plated at a low density that are able to grow into a colony that can be counted.

S9 fraction – a crude extract, obtained from the homogenized liver of rats previously treated with Phenobarbital/β-Naphtoflavone to enhance liver enzyme levels and activity. It contains a wide range of enzymes, to which enzyme cofactors are added.

S9 mix - mix of the S9 liver fraction and cofactors necessary for metabolic enzyme activity. S9 mix contains 40% (v/v) S9 fraction. Typically, S9 mix is added to the cells just prior to addition of the test substance solution to examine metabolites generated by liver enzymes.

h.i. HS (heat inactivated horse serum) - freshly thawed serum heat inactivated at 56°C in water bath for 30 min.

Pluronic® F-68 - non-ionic detergent, used to prevent mechanical disruption of cells during shaking, it is not necessary for stationary cultures.

GEF (Global Evaluation Factor) - parameter used to determine genotoxicity. A test compound is considered to be genotoxic if GEF exceeds predefined value 126 $x10^{-6}$. GEF is described by the formula:

$$GEF = \frac{MF\ tested\ compound}{MF\ negative\ control}$$

where :
MF – Mutant Frequency

1.4 Abstract

The evaluation of genotoxic potential of IN-A4098, IN-L9223 and IN-L9225 was carried out using in vitro mammalian Mouse Lymphoma Assay (MLA). The analysis was performed on L5178Y cell line recommended by OECD 476 and under GLP requirements.
L5178Y cells were exposed to tested compounds (IN-A4098, IN-L9223 and IN-L9225) both with an exogenous metabolic activation with S9 (short treatment) and without S9 (short and extended treatment). Obtained results have shown that tested compounds did not exceed GEF (Global Evaluation Factor) above 126 $x10^{-6}$ in any of the tested doses both in presence and absence of S9 exogenous activation system. Methyl methanesulfonate and Cyclophosphamide were used as a positive controls. Positive controls have shown increasing in Mutant Frequency (MF) compared with the negative control (PBS).

2. Materials and methods

2.1 Cell lines and media used

Cell line	Origin	Cat. no	Lot No
L5178Y TK+/- clone (3.7.2C)	ATCC	CRL-9518	607 979 977

The L5178Y $TK^{+/-}$ (clone 3.7.2C) cell line, was purchased from American Type Culture Collection (ATCC) and maintained in log phase growth by serial sub-culturing. The cells were routinely cultured in RPMI 1640 supplemented with 10% (v/v) heat inactivated horse serum hereafter referred to as the medium growth (Medium10).
To reduce the frequency of spontaneous $TK^{-/-}$ mutants, cell cultures were cleansed of the pre-existing $TK^{-/-}$ mutants by exposing them to the thymidine, hypoxanthine, methotrexate and glutamine (THMG) for approximately 24 hours to select against the $TK^{-/-}$ phenotype.
During treatment with the tested compounds the concentration of heat inactivated horse serum was reduced from 10% to 5% (v/v) prior to treatment with tested compounds.
The cloning medium (Medium 20) contained RPMI 1640 supplemented with 20% (v/v) heat inactivated horse serum. For selection, the cloning media were supplemented with 3µg/mL 3-trifluorothymidine (TFT). Complete media composition is listed below.

Solution	Composition
Medium basic (medium A)	99.95% v/v RPMI 1640 0.05% v/v Pluronic® F-68 200 µg/mL Na Pyruvate 100 U/mL Penicillin 100 U/mL Streptomycin
Medium 5 (treatment medium)	94.95% v/v RPMI 1640 0.05% v/v Pluronic® F-68 5% v/v Horse Serum 200 µg/mL Na Pyruvate 100 U/mL Penicillin 100 U/mL Streptomycin
Medium 10 (growth medium)	89.95% v/v RPMI 1640 0.05% v/v Pluronic® F-68 10% v/v Horse Serum 200 µg/mL Na Pyruvate 100 U/mL Penicillin 100 U/mL Streptomycin
Medium 20 (cloning medium)	80% v/v RPMI 1640 20% v/v Horse Serum 200 µg/mL Na Pyruvate 100 U/mL Penicillin 100 U/mL Streptomycin

The cultures were tested regularly for the presence of mycoplasma contaminations.

2.2 Tested material

Sample No.	MW	CAS	Chemical name
IN-A4098	140.14	1668-54-8	2-amino-4-methoxy-6-methyl-1,3,5-triazine
IN-L9223	207.23	59337-97-2	3-(aminosulfonyl)thiophene-2-carboxylic acid
IN-L9225	373.76	79277-67-1	methyl 3-(4-methoxy-6-methyl-1,3,5-triazin-2-

			ylcarbamoylsulfamoyl)

Samples of the test compounds were provided by customer with information as provided in the table below. Dimethylsulfoxide (DMSO) was selected as a solvent. Compounds were soluble at all concentrations used.

2.3 Control material

Two positive controls were selected to be used in the assay. Methyl methanosulfonate (MMS) and Cyclophosphamide (Cp) from Sigma. MMS was used in the absence of metabolic activation (-S9) and Cp in the presence of metabolic activation (+S9). MMS is a direct acting mutagen, while Cp is promutagen that requires biotransformation with the liver enzymes to elicit a mutagenic response. Phosphate buffered saline (PBS) accounted for additional vehicle (negative) control for MMS and Cp. DMSO alone or with S9 treated cultures were used as vehicle (negative) controls for tested compounds.

Positive controls demonstrated effectiveness of the assay. Combinations of positive controls and activation conditions used in the assay are shown in the table below.

Positive controls

Positive controls (µg/mL)	
Without activation (-S9)	With activation (+S9)
Methyl methanesulfon (5 and 14µg/mL)	Cyclophosphamide (3µg/mL)

2.4 Method

The Mouse Lymphoma Assay (MLA) is a short-term assay designed to detect forward gene mutations induced by mutagens at the heterozygous thymidine kinase (TK) locus. It is capable of quantifying genetic alterations. The system, recommended by OECD 476, employed L5178Y $TK^{+/-}$ cells and the TK (thymidine kinase) locus.

5-Trifluorothymidine (TFT) is a toxic pyrimidine analogue that interferes with DNA metabolism causing cell death. However, if the functional copy of the TK gene is lost ($TK^{-/-}$) through mutation, the TFT is not metabolized and is no longer toxic. The L5178Y $TK^{+/-}$ cells are sensitive to the cytotoxic effects of the TFT. When L5178Y $TK^{+/-}$ cells are exposed on mutagenic and/or carcinogenic agents, $TK^{+/-}$ is mutated to the $TK^{-/-}$ genotype which is causing TFT resistance. The mutant cells when cloned in medium containing the selective agent TFT, proliferate and form colonies.

The mouse lymphoma TK assay uses the thymidine kinase (TK) gene (reporter of mutation) detects a broad spectrum of genetic damage, including point mutations, large scale chromosomal changes and recombination. That is why is often recommended and widely used to determine the genotoxic potential of various chemicals. This is also Gene Mutation Assay of choice at Selvita laboratory as a suitable short-term mutagenicity screening assay to predict chemical carcinogenicity.

The studies were performed according to Standard Research Procedure SPB-19D.

2.5 Preparation of cells

Before the experiment, the cells were grown for 24 hours in the medium containing THMG to select against newly arising $TK^{-/-}$ mutants, and then were placed in the medium containing THG for 2 days prior to use in mutation study.

The composition of THMG and THG stock solutions is listed below.

Stock solution for preparing 100 mL THMG 100x		
Reagent name	Final concentration	Amount needed for 100mL
Thymidine	300 µg/mL	30 mg

Hypoxanthine	500 µg/mL	50 mg
Methotrexate	10 µg/mL	1 mg
Glycine	75 µg/mL	75 mg
Stock solution for preparing 100 mL THG 100x		
Thymidine	300 µg/mL	30 mg
Hypoxanthine	500 µg/mL	50 mg
Glycine	75 µg/mL	75 mg

2.6 Treatment

2.6.1 Dose range

An initial cytotoxicity assay was performed to determine the non-cytotoxic range of the tested compounds. This experiment was assigned to be used as a mutagenicity assay in the event that sufficient number of dose levels does not exhibit cytotoxicity (please refer to the table given below). Moreover, the highest concentration of the test compounds used in the assay is limited by DMSO (solvent of test compounds) which concentration cannot exceed 1% (v/v) to have no negative effect on the assay.

Dose level used in initial dose range-finding assay.

Treatment Groups in the Cytotoxicity Assay – Initial Mutagenicity Test	**S9 concentration**
Vehicle control – DMSO [1%]	0
Vehicle control for positive control – PBS [1%]	0
Methyl Methanesulfonate [5 and 14µg/mL]	0
IN-A4098: 1.07; 0.36; 0.12; 0.04; 0.013; 0.0044; 0.0015 [mM]	0
IN-L9223: 10; 3.33; 1.11; 0.37; 0.12; 0.04; 0.014 [mM]	0
IN-L9225: 10; 3.33; 1.11; 0.37; 0.12; 0.04; 0.014 [mM]	0
Vehicle control – DMSO [1%]	2% (v/v)
Vehicle control for positive control – PBS [1%]	2% (v/v)
Cyclophosphamide [3µg/mL]	2% (v/v)
IN-A4098: 1.07; 0.36; 0.12; 0.04; 0.013; 0.0044; 0.0015 [mM]	2% (v/v)
IN-L9223: 10; 3.33; 1.11; 0.37; 0.12; 0.04; 0.014 [mM]	2% (v/v)
IN-L9225: 10; 3.33; 1.11; 0.37; 0.12; 0.04; 0.014 [mM]	2% (v/v)

Compounds were initially tested at the concentrations reaching up to 10 mM (IN-L9223 and IN-L9225) or 1.07 mM (IN-A4098) for 4 hours of incubation in presence of S9 metabolic activation and for 4 hours and 24 hours exposure in absence of S9. Based on cytotoxicity results from the initial experiments, concentrations of test compounds were selected for definitive mutagenicity assay (please refer to the table below).

Dose level used in definitive mutagenicity assay.

Treatment Groups in the Cytotoxicity Assay – Definitive Mutagenicity Test	S9 concentration	Time exposure
Vehicle control – DMSO [1%]	0	4h
Vehicle control for positive control – PBS [1%]	0	4h
Methyl Methanesulfonate [14 µg/mL]	0	4h

IN-A4098: 1.07; 0.36; 0.12; 0.04 [mM]	0	4h
IN-L9223: 10; 3.33; 1.11; 0.37; 0.12 [mM]	0	4h
IN-L9225: 10; 3.33; 1.11; 0.37 [mM]	0	4h
Vehicle control – DMSO [1%]	2% (v/v)	4h
Vehicle control for positive control – PBS [1%]	2% (v/v)	4h
Cyclophosphamide [3µg/mL]	2% (v/v)	4h
IN-A4098: 1.07; 0.36; 0.12; 0.04 [mM]	2% (v/v)	4h
IN-L9223: 10; 3.33; 1.11; 0.37 [mM]	2% (v/v)	4h
IN-L9225: 10; 3.33; 1.11; 0.37 [mM]	2% (v/v)	4h
Vehicle control – DMSO [1%]	0	24h
Vehicle control for positive control – PBS [1%]	0	24h
Cyclophosphamide [5 µg/mL]	0	24h
IN-A4098: 0.12; 0.04; 0.013; 0.004 [mM]	0	24h
IN-L9223: 3.33; 1.11; 0.37; 0.12 [mM]	0	24h
IN-L9225: 1.11; 0.37; 0.12; 0.04 [mM]	0	24h

2.6.2 Exposure Period

On day 1, L5178Y $TK^{-/-}$-clean cells growing in logarithmic phase were treated in individual 50mL falcons. Each tube contained of 9.5 mL of cell suspension ($6x10^6$ cells in total) in Medium 5. In the next step, cells were diluted to the final concentration of $6x10^5$ cells/mL by addition of 0.5 mL of S9 mixture or medium and test compound, positive control or vehicle. Following addition of the test compound, the tubes were gently mixed and placed in CO_2 incubator at 37°C for the exposure period. At the end of the exposure time, the cells were pelleted, washed with Medium A and collected by centrifugation, and then resuspended in 20 mL of Medium 10. Cultures were transferred to flasks for growth through the expression period and placed in CO_2 incubator (5% CO_2, 37°C). 1 mL of cell suspension from each culture was used for counting (post – treatment) and for plating immediately after treatment to obtain Relative Viability (RV) and Relative Total Growth (RTG) values. Portion from the cell suspension was used to prepare 3-step dilution with non-selective (with no TFT) Medium 20 to obtain concentration of 8 cells/mL. Using a multichannel pipette, 200 µL of cell suspension was dispensed to each well of two 96-well sterile flatbottom plates for each tested dose and controls.

2.6.3 Expression Period

On day 2, approximately 20-24 hours after treatment, the cultures were counted and diluted with fresh growth medium to $2x10^5$ cells/mL and placed back to CO_2 incubator. On day 3 (approx. 44-48 hours after exposure period) cells were counted and resuspended at $2x10^5$ cells/mL. Then, the Relative Suspension Growth (RSG) was established for each concentration of tested compounds. Some samples, despite exhibiting RSG less than 10% were cloned if possible as the top dose tested to obtain all spectrum of cytotoxicity for further analysis.

2.6.4 Cloning

From observations on recovery and growth of the cultures during the expression period, appropriate test dose levels demonstrating up to 90% suspension growth inhibition plus negative and positive controls were selected to be plated for viability and 5-trifluorothymidine (TFT) resistance.

The cultures were adjusted to the concentration of approximately $2x10^6$ cells/mL in Medium20, gently mixed and incubated in CO_2 incubator at 37°C for at least 30 minutes to minimize cell trauma and adopt them to new medium. In the next step, the cells were diluted to the appropriate concentration to plate for TFT resistance (2000 cells/well) and make 3-step dilution for cell viability plating (1.6 cells/well).

2.6.5 Plating for 5-triflurorthymidine (TFT) - Mutant selection

For selection of the trifluorothymidine (TFT)-resistant phenotype, the cells were agitated to form a single cell suspension. Then the cell concentration was adjusted to 1 x 10^4/mL (in 50 mL). A small amount of suspension (500 µL) was used to prepare the dilution for viability plating. The cell suspension was mixed with 500 µL of TFT stock solution (final concentration of TFT: 3 µg/mL). Using a multichannel pipette, 200 µL of cell suspension containing TFT was dispensed to each well of two 96-well sterile, flat-bottom plates for each tested dose and control compounds.

2.6.6 Plating for Viability (VP)

A portion of the cell suspension at density of $1x10^4$ cells/mL was used to prepare 3-step dilution with non-selective (with no TFT) Medium 20 to obtain concentration of 8 cells/mL. Using a multichannel pipette, 200 µL of cell suspension was dispensed to each well of two 96-well sterile, flat-bottom plates for each tested dose and controls.

2.6.7 Incubation

Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 12-14 days. Following that time, the plates were analysed - wells containing viable clones were identified in Motic reversed light microscope (large colonies confirmed by naked eye) and counted.

In plates with selective medium (with TFT) the number of wells containing large colonies and the number containing small colonies was scored for the negative and positive controls and for doses of test compounds.

2.7 Materials

Composition of solutions used in the study

Solution	Composition
S9-mix (for 0.5 mL solution)	15 µL 1 M KCl
	100 µL 0.2 M D-Glucose 6-phosphate
	100 µL 0.04 M β-NADP
	85 µL dH_2O
	200 µL S9 fraction

2.8 Data calculation and interpretation of results

2.8.1 Colony counting

The number of wells containing colonies was counted by naked eye or with the aid of a microscope. A well without colonies was classified as negative (empty wells – EW). The number of negative wells per plate was quantified for the survival (PE_{PS}), viability (PE_{OV}) and mutation (PE_2) frequency.

2.8.2 Calculations

Survival and viability

From the zero term of the Poisson distribution, the probable number of clones/well (P) on microwell plates is:

$$P=-\ln(EW/TW)$$

where EW is empty wells and TW is total wells.

The plating efficiency (PE) in any given culture is:

$$PE = P/(\text{cells planted/well})$$

when 1.6 cells/well are plated on average for all survival and viability plates:

$$\mathbf{PE = P/1.6}$$

The Relative Survival (RS) in each test culture is therefore determined by comparing plating efficiencies in test and control cultures:

$$\mathbf{RS(\%)=(PEtest/PEcontrol)\ x100}$$

Relative Total Growth (RTG)

Relative Total Growth (RTG) was calculated for estimating test chemical cytotoxicity. The Relative Suspension Growth (RSG) was first calculated by daily cell growth (DCG)

$$\mathbf{RSD=[(\ DCG_1\ x\ DCG_2\ )test]/[\ DCG_{1\,x}\ DCG_2\ )\ control]}$$

RSG [%] presented in tables with results were calculated as follows:

$$\mathbf{RSD\ [\%]=[(\ DCG_1\ x\ DCG_2\ )test]/[\ DCG_{1\,x}\ DCG_2\ )\ control]\ x\ 100}$$

DCG is the growth rate between days 1 and 2 (DCG_1) or between days 2 and (DCG_2).

The Relative Total Growth (RTG) is calculated as:

$$\mathbf{RTG\ [\%]=\ RSG\ x\ RV\ [\%]}$$

RV (relative viability) is calculated by comparing plating efficiencies in the test and control cultures at day 2 (data from plates seeded after exposure period).

Mutation Frequency

Mutation Frequency (MF) expressed as mutants/10^6 viable cells is calculated as:

$$\mathbf{MF = (PEmutant/PEviable)\ x\ 10^6}$$

from the formula for PE and with the knowledge that 2 x 10^3 cells were plated/well for mutation to TFT resistance:

$$\mathbf{PEmutant = Pmutant/2000}$$

$$\mathbf{PEviable = Pviable\ /\ 16}$$

For the TFT plates, colony size and morphology were characterized to obtain information about the mechanism of action of the tested chemical. The colonies were characterized as described below.

Parameter	**Small colony**	**Large colony**
size	Ø ≤ 1/4 of well diameter	Ø > 1/4 of well diameter
morphology	Compact	Totally or partially diffuse

Next percentage of small colonies [%SC] was counted:

$$\mathbf{\%SC = \frac{Number\ of\ wells\ with\ small\ colonies}{Number\ of\ all\ wells\ with\ colonies}}$$

2.9 Data presentation

1. Results of tested compounds, vehicle and positive controls with and without S9 (cyclophosphamide or methyl methanesulfonate, respectively) used to demonstrate mutant recovery.
2. Relative Suspension Growth (RSG) as an indicator of short term cytotoxicity.
3. Relative Total Growth (RTG) - an indicator of relative cell survival.

4. Relative Viability (RV) and Relative Survival (RS) as indicators of the cell viability just after treatment (2 days) and after cloning (2 weeks).
5. Rate (per cent) of small colonies formed on TFT resistance plates (% Small Colonies).
6. 'Fold increase' is calculated as the ratio of mutant frequency for the dose concentration to the mutant frequency of solvent control.
7. Global Evaluation Factor – determinant of positive or negative result.

2.10 Acceptance criteria

The acceptance criteria are defined in OECD 476 and SPB-19 Acceptance criteria for the MLA (criteria for accepting an experiment as valid) as well as requirements that have to be met to classify tested compound as genotoxic. Please refer to table given below:

	4-hour treatment	**24-hour treatment**
Negative controls		
Mutant Frequency (MF)	50-200 $x10^{-6}$	50-200 $x10^{-6}$
Cloning Efficiency (PEv)	65-120%	65-120%
Suspension Growth (DCG1xDCG2)	8– to 32-fold	32- to 180-fold
Positive controls		
Mutant Frequency (MF)	≥300 $x10^{-6}$	≥300 $x10^{-6}$
	≥40% of small colonies	≥40% of small colonies
MF for small colony	≥150 $x10^{-6}$	≥150 $x10^{-6}$
Relative Total Growth (RTG)	≥10%	≥10%
Positive results		
Relative Total Growth (RTG)	≥10%	≥10%
Mutant Frequency (MF)-GEF	≥126 $x10^{-6}$	≥126 $x10^{-6}$

Unless an effect is considered as clearly positive, the reproducibility of a positive effect should be confirmed. Noteworthy increases in the mutation frequency observed only at high levels of cytotoxicity (Adj. RTG lower than 10%), but with no evidence of mutagenicity at dose-level with Adj. RTG between 10% and 20%, will not be considered as positive results.

3. RESULTS

All experiments were performed in duplicates - two independent 96-well plates were seeded per single condition (compound/concentration). Then the mean values were obtained.

Both positive and negative controls met the acceptance criteria in initial cytotoxicity assay and definitive mutagenicity assay.

3.1 The cytotoxicity assay

The solubility of tested compounds: IN-A4098, IN-L9223, IN-L9225 in DMSO permitted to conduct in vitro studies at compounds concentrations up to 0.107M; 1M and 1M, respectively. The test compounds were soluble in aqueous medium up to 1.07mM; 10mM; 10mM, respectively and tested in the absence and presence of metabolic activation with 4-hour exposure and in the absence of metabolic activation with 24-hour exposure to receive a wider spectrum of cytotoxicity.

In the cytotoxicity range-finding studies cytotoxicity was assessed by comparing the RSG of the treated cultures to the vehicle control cultures. In the presence of metabolic activation with 4-hour exposure, the RSG for IN-A4098, IN-L9223 and IN-L9225 was in the range of 58%-95%, 37%-80% and 49%-75%, respectively. In the absence of S9 with 4-hour exposure, the RSG parameter for IN-A4098, IN-L9223 and IN-L9225 was in the range of 64%-141%, 5%-118% and 74%-133%, respectively. In the absence of S9 with 24-hour exposure, the RSG parameter for IN-A4098, IN-L9223 and IN-L9225 was in the range of 0.5%-68%, 1%-38% and 1%-39%, respectively. Due to cytotoxic effect on cells concentrations of compounds with RSG value ≤10% cannot be used in the mutagenicity assay.

As the sufficient number of dose levels do not exhibit cytotoxicity, the range-finding assays were assigned to be used as an initial mutagenicity assay. In the initial range–finding assay, in the presence of metabolic activation with a 4-hour exposure, the GEF did not exceed the threshold value of 126 $x10^{-6}$ in any of the dose level tested. In the initial range–finding assay, in the absence of metabolic activation with a 4-hour exposure, the GEF was higher than 126 x10-6 for IN-L9223 at 10 mM concentration. However, IN-L9223 at 10 mM exceeded the acceptable cytotoxicity limit for the cells (RTG=1.66%). The results of this experiments are shown in Table 7: RSG, RTG, MF and the percentage of small colonies in each experimental condition.

In the initial range–finding assay, in the absence of metabolic activation with a 24-hour exposure, the GEF was not higher than $126x10^{-6}$ in any of the dose level tested (GEF ranged from -30.71 to 85.40). Cells treated with 1 mM INA4098 and 10 mM IN-L9225 have not been plated for RS due to their significant cytotoxicity action at these concentrations. Table 8 shows the detailed information for RSG, RTG, MF and the percentage of small colonies in experimental conditions (-S9). MMS and Cp were used in different concentrations as positive controls without or with S9, respectively. Both positive controls elevated GEF above $126x10^{-6}$ in TFT-resistant colonies, therefore indicating the assay sensitivity and responsiveness to mutagens.

The cytotoxicity assay was conducted using the same dose range of IN-A4098, IN-L9223, IN-L9225, in the absence and presence of metabolic activation.

3.2 Mutagenicity assays

The main experiments were conducted with IN-A4098, IN-L9223, IN-L9225 using four non-cytotoxic concentrations selected based on the dose range-finding tests. Using the evaluation criteria described referred above, none of doses IN-A4098, IN-L9223, IN-L9225 induced dose-related cytotoxic and mutagenic effects in mouse lymphoma cells under experimental conditions in definitive mutagenicity assays.

In the absence and presence of metabolic activation GEF level did not exceed $126x10^{-6}$ in any of the dose level tested (GEF in the range of -28.558 to 48.98). MMS and Cp were used in different concentrations as positive controls without or with S9, respectively. Both positive controls elevated GEF above $126x10^{-6}$ in TFT-resistant colonies, therefore indicating the assay sensitivity and responsiveness to mutagens.

Obtained results indicate that the tested compounds, IN-A4098, IN-L9223, IN-L9225, are considered as non-mutagenic under the conditions employed and according to the acceptability criteria defined in OECD guideline 476 and SPB-19.

4. CONCLUSION

Obtained results indicate that neither tested compounds nor their metabolic derivatives were positive in Mouse Lymphoma Assay under the protocol described and according to the acceptability criteria defined in OECD guideline 476 and SPB-19.

Comments of zRMS:	According to data matching performed by RMS Sweden the study by Xxxxxx, J., (2015) is alternative to the Flügge, C. (2011 b) study with regard to gene mutations in mammalian cells and the endpoint was equivalent, i.e. negative.

A 2.12.2 Genotoxicity study using micronucleus assay for metabolite IN-A4098 (KCP 7.1.7/02)

Report:	**Xxxxxx, J., 2015**
Title:	*In vitro* evaluation of IN-A4098, IN-L9223 and IN-L9225 genotoxicity using the micronucleus assay (MNA)
Document No:	K49/JS/01
Guidelines:	OECD Nº 487
GLP	Yes

SUMMARY

The formation of MN is a consequence of chromosomal breakage and/or spindlefiber dysfunction induced by clastogens and/or aneuploidogens. The present study was performed in accordance with the OECD 487 and under GLP requirements.
In order to assess genotoxic potential CHO-K1 cells were exposed to tested metabolites (IN-A4098, IN-L9223 and IN-L9225) and appropriate control compounds in system with (+S9) and without (-S9 short and extended treatment) an exogenous metabolic activation.
Statistical analysis of the MN frequency and binucleate cells with MN was performed using the Chi-square test with Yates' correction. To examine the dose response relationship in frequencies of the micronuclei Chi-square test for trend was performed.
None of tested concentration of IN-A4098, IN-L9223 and IN-L9225 exhibit a statistically significant increase in MN frequency compared with the concurrent negative control ($P>0.05$). Chi-square test for trend revealed no dose-related increase in MN frequency ($P>0.05$). Results for positive control compounds (mitomycin C and cyclophosphamide) demonstrated reproducibility and sensitivity of system.
In summary, the present research has demonstrated that metabolites IN-A4098, IN-L9223 and IN-L9225 did not produce dose-dependent genetic toxicity in the CHO-K1 cells.

1. INTRODUCTION

1.1 Study Objective

The scope of this project was to evaluate the genotoxic potential of 3 compounds (IN-A4098, IN-L9223 and IN-L9225) using the in vitro Micronucleus Assay (MNA).

1.2 Study Guidelines

The test was performed in accordance with the guideline of Organization for Economic Cooperation and Development (OECD) 487 and under GLP requirements.

1.3 Definitions

Aneugen – any substance or process that, by interacting with the components of the mitotic and meiotic cell division cycle, leads to aneuploidy in cells or organisms.

Clastogen– any substance or process which causes structural chromosomal aberrations in populations of cells or organisms.

Cytochalasin B– the agent that is most widely used to block cytokinesis because it inhibits actin assembly, and thus prevents separation of daughter cells after mitosis, leading to the formation of binucleated cells.

Cytokinesis-Block Proliferation index (CBPI) -– the proportion of second division cells in the treated population relative to the untreated control

Cytostasis - inhibition of cell growth

Growth medium (GM)- Ham's F12 medium supplemented with 10% v/v h.i. FBS, 1000 U/mL penicillin and 1000 U/mL streptomycin.

Micronuclei (MN)- small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis or meiosis by lagging chromosome fragments or whole chromosomes.

Replication Index (RI) - the proportion of cell division cycles completed in a treated culture, relative to

the untreated control, during the exposure period and recovery.

S9 liver fraction – a crude preparation of enzymes, obtained from the homogenized liver of rats previously treated with Aroclor 1254 or Phenobarbital/β-Naphtoflavone to enhance liver enzyme levels and activity.

S9 mix - mix of the S9 liver fraction and cofactors necessary for metabolic enzyme activity. S9 mix contains 10% v/v S9 fraction.

Serum-free medium (SFM) – Ham's F12 medium supplemented with 1000 U/mL penicillin and 1000 U/mL streptomycin.

1.4 Abstract

The evaluation of genotoxic potential of IN-A4098, IN-L9223 and IN-L9225 was carried out using in vitro micronucleus test (MNA). The analysis was performed on CHO-K1 cell line recommended by OECD 478, and under GLP requirements. CHO-K1 cells were exposed to tested metabolites (IN-A4098, IN-L9223 and IN-L9225) both with (+S9 short treatment) and without (-S9 short and extended treatment) an exogenous metabolic activation.

Statistical analysis of the MN frequency and binucleate cells with MN was performed using the Chi-square test with Yates' correction. To examine the dose response relationship in frequencies of MN Chi-square test for trend was performed. Statistical analysis revealed no significant differences in MN induction between tested concentration of IN-A4098, IN-L9223 and IN-L9225 and concurrent negative controls ($P>0.05$). Chi-square test for trend revealed no concentration-related increase in MN frequency in teste experimental conditions ($P>0.05$). Positive control compounds (mitomycin C and cyclophosphamide) demonstrated significant, concentration-dependent increase in MN frequency compared with the negative controls ($P<0.05$).

In summary, tested metabolites IN-A4098, IN-L9223 and IN-L9225 did not induce concentration-dependent genetic toxicity in CHO-K1 cells.

2. Materials and methods

2.1 Cell lines

Cell line	Description	Origin	Cat. no	Passage No
CHO-K1	Chinese hamster ovary cell line	CLS	603480	15-25

CHO-K1 cell line was cultivated according to the previously established SOP-01 in 25 cm2 or 75 cm2 tissue culture flasks at 37ºC in a humidified atmosphere containing 5% CO_2 using Ham's F12 medium supplemented with 10% v/v h.i. FBS and antibiotics (Penicillin and Streptomycin). The doubling time of CHO-K1 determined at Selvita is approximately 18 h. The cultures were tested regularly for the absence of mycoplasma infections.

2.2 Tested material

Sample No.
IN- A4098
IN-L9223
IN-L9225

2.3 Control material

Compound	Provider	Cat no	Batch/LOT
Mitomycin C from *Streptomyces caespitosus*	Sigma Aldich	M0503	SLBH9906V
Cyclophosphamide monohydrate	Sigma Aldich	C0768	120M1253V

2.4 Materials and Method

The in vitro Micronucleus Assay (MNA) is a mutagenic test system for the detection of chemicals that induce the formation of small membrane-bound DNA fragments (micronuclei - MN) in the cytoplasm of interphase cells. The MNA, used for regulatory purposes measures formation of chromosomal changes following DNA damage induced by the compounds under test, and is used to predict the genotoxic potential of pharmaceuticals, industrial chemicals, food additives and cosmetic ingredients. MN originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. They reflect chromosome damage and may thus provide a marker of genotoxicity and even early-stage carcinogenesis. The most commonly used method in mammalian cells is the cytokinesis-block micronucleus (CBMN) assay. In the CBMN assay, MN are scored after a single cell division using binucleated cultured cells (accumulated using cytochalasin B) to eliminate the confounding effect of altered cell division kinetics on the MN index.

Schedule of the MNA test.

Condition	**Description**
-S9 short treatment	Treatment for 3h with tested compound (at 37°C) Removal the treatment medium Addition of fresh medium and cytochalasin B (cytoB) Harvesting 1.5 – 2.0 normal cell cycles later (27h)
-S9 extended treatment	Treatment for 1.5 – 2 normal cell cycles (27h) with tested compound in the presence of cytoB (at 37°C) Harvesting at the end of the exposure period
+S9 shot treatment	Treatment for 3h with tested compound in the presence of S (at 37°C) Removal the S9 and treatment medium Addition fresh medium and cytoB Harvesting 1.5 – 2.0 normal cell cycles later (27h)

Composition of solutions used in the study

Solution	**Composition**
Growth medium (GM)	90% v/v Ham's F12 10% v/v h.i. FBS 1000 U/mL/1000 U/mL Penicillin/Streptomycin
Serum-free medium (SFM)	90% v/v Ham's F12 1000 U/mL/1000 U/mL Penicillin/Streptomycin
S9-mix (for 1mL solution)	33 µL 1 M KCl 32 µL 0.25 M MgCl2 25 µL 0.2 M D-Glucose 6-phosphate 100 µL 0.04 M β-NADP 500 µL phosphate buffer (pH 7.4) 210 µL dH_2O 10% v/v S9 fraction
Hypotonic solution	0.75 M KCl
Fixative	1:3 v/v acetic acid:methanol

Giemsa solution	15% v/v Giemsa stein (0.4% w/v stock solution) 85% v/v dH_2O

Data analysis

Analysis of the MN frequency and binucleate cells with MN was performed for each treatment using the Chi-square test with Yates' correction for α=0.05. To examine the dose-response relationship in frequencies of micronuclei Chi-square test for trend was performed (GraphPad Prism 6.00 for Windows, GraphPad Software, La Jolla California USA).

For cytotoxicity assessment, the cytotoxicity block proliferation index (CBPI) was used. The CBPI indicates the average number of cell cycles per cell during the period of exposure to cytochalasin B, and may be used to calculate cell proliferation. The RI indicates the relative number of nuclei in treated cultures compared to control cultures and can be used to calculate the % cytostasis:

$$\% \ Cytostasis = 100 - 100[(CBPI_T - 1)/(CBPI_C - 1)]$$

Where:

$$CBPI = \frac{((N^o mononucleate\ cell) + (2xN^o\ binucleate\ cells) + (3x\ N^o\ multinucleate\ cells))}{Total\ number\ of\ cells}$$

Thus, a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

$$Cytostasis = 100 - RI$$

3. RESULTS

3.1 Primary cytotoxicity test

Preliminary cytotoxicity test was performed to narrow the compound concentration range for the definitive tests. Each condition cultures were harvested and processed separately. Positive (MMC and CP) and concurrent negative controls were used to demonstrate test sensitivity and reproducibility. CBPI (Cytokinesis-Block Proliferation Index) and RI (Replication Index) was determined to assess cell proliferation (cytotoxicity) using at least 500 cells per culture. Micronucleus frequencies in MMC and CP-treated cells as well as their corresponding vehicle controls were analyzed in at least 2000 binucleated cells per concentration.

The highest tested concentrations of compounds did not reduce CBPI or RI to 45±5% of the concurrent negative control (1% v/v DMSO) in test with (+S9 short treatment) or without (-S9 short and extended treatment) metabolic activation.

Thus, based on cytotoxicity test results following concentrations of compounds was selected to be used in genotoxicity test:

· IN-A4098 – 25, 50, 100 and 150 µg/mL

· IN-L9223 – 250, 500, 1000 and 2000 µg/mL

· IN-L9225 – 250, 500, 1000 and 2000 µg/mL

Positive controls: MMC (-S9 short and extended incubation) and CP (+S9 short incubation) gave reproducible and detectable increase over background (P>0.05) and demonstrated sensitivity of the system.

3.2 Genotoxicity test (MNA)

Tested metabolites (IN-A4098, IN-L9223 and IN-L9225) were analysed in micronucleus test at 4 non-cytotoxic concentrations. DMSO (1% v/v) control were run concurrently for each experiment. Test compounds together with appropriate positive control compounds (MMC and CP) were tested in system

with and without metabolic activation

· +S9 short treatment (the final concentration of 1% v/v S9)

· -S9 short treatment

· -S9 extended exposure

The Giemsa stained slides were analysed on light microscope using criteria defined by Fenech et al. (2003)[2] for scoring micronuclei. Micronuclei were score in binucleated cells, in which both nuclei were of similar size and intensity and the micronuclei size was ≤0.33 the size of the main nuclei, and of similar intensity as the main nuclei (Fenech et al., 2003)[1]. Micronucleus frequency was analysed in at least 2000 binucleate cells per concentration and control.

MNA test results revealed no genotoxicity of tested compounds (IN-A4098 IN-L9223 and IN-L9225) in any of the experimental conditions tested Metabolites tested in system with and without metabolic activation in teste concentrations did not exhibit statistically significant increase in micronucleus frequency per culture compared with the concurrent negative control (Chi-square test with Yates' correction, P>0.05). The CHO-K1 cells treated with IN-A4098, IN-L9223 and IN-L9225, with or without S9 activation, displayed similar MN induction as concurrent negative control A significant concentration-related increase in frequency of MN was not observe in cultures treated with IN-A4098, IN-L9223 and IN-L9225 (Chi-square test for trend, P>0.05). MN formation was significantly induced in CKO-K1 cells compared to the control following exposure to positive control compounds (MMC and CP) at indicate concentrations (P<0.05). The number of CHO-K1 cells with M increased in an MMC/CP exposure concentration-dependent manner (P<0.05). Results obtained for positive control compounds (MMC and CP demonstrated reproducibility and sensitivity of system used to analyse genotoxic potential of compounds

4. CONCLUSION

The present research has demonstrated that metabolites IN-A4098, IN-L9223 and IN-L9225 did not produce dose-dependent genetic toxicity in the CHO-K1 cells.

Comments of zRMS:	According to data matching performed by RMS Sweden the study by Xxxxxx J., (2015) is alternative to the xxxx R. (2009) study with regard to *in vitro* mammalian chromosome aberration test. Study is conducted in accordance to GLP, follows OECD TG 487 and the endpoint was equivalent, i.e. negative. MNA test results revealed no genotoxicity of tested compounds (IN-A4098, IN-L9223 and IN-L9225) in any of the experimental conditions tested.

A 2.12.3 Bacterial reversion mutation test for metabolite IN-L5296 (KCP 7.1.7/03)

Report:	**Xxxxxx, S., 2019**
Title:	Bacterial reversion mutation test
Document No:	B-02756
Guidelines:	OECD Nº 471
GLP	Yes

[2] Fenech M., *et al.* (2003). HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research*, 534:65–75.

SUMMARY

assesses the mutagenic and/or pro-mutagenic potential of the test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) in several bacterial strains.
The test was performed in accordance with OECD Guideline 471 for the Testing of Chemicals (Bacterial Reverse Mutation Test. Adopted 21st July 1997).
Cytotoxicity evaluation of the test item was performed in the *S. typhimurium* TA100 strain by the direct incorporation procedure and without metabolic activation with 5 concentrations of the test item based on its solubility profile (1.68, 0.56, 0.19, 0.06 and 0.02 mg/plate).
No test item related cytotoxic activity was observed at any of the concentrations tested.
On the basis of these results, 5 test item doses ranging between 0.02 and 1.68 mg/plate were assayed in the main test. None of the concentrations assayed for the test item showed an increase in the R value either with or without S9 metabolic activation regardless of the procedure.
No dose response for the test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) was observed in any of the tested bacterial strains.
Overall interpretation of the study results suggests that the test item does not induce point mutations or frameshifts in the genome of the bacterial strains with or without metabolic activation regardless of the procedure.
Therefore, the test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) at an exposure dose range of 0.02 – 5 mg/plate is considered to be non-mutagenic / non-pro-mutagenic under the experimental conditions assayed.

INTRODUCTION

1.1 Study Objective

The objective of the bacterial reverse mutation test (Ames test) was to assess the mutagenic and/or pro-mutagenic potential of the test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) in a bacterial test system.

1.2 Study Guidelines

The test was performed in accordance with OECD Guideline 471 for the Testing of Chemicals (Bacterial Reverse Mutation Test. Adopted 21st July 1997).

1.3 Test Principle

The Ames test evaluates the potential of the test item to revert mutations present in amino acid-requiring bacterial strains. The reversion restores the functional capability of the bacteria to synthesize the essential amino acid thus enabling the bacterial culture to grow in the absence of the amino acid required by the parent bacterial strain.
Many chemicals are not mutagenic in their native forms, but are converted into mutagenic substances by metabolism in the liver. Selected bacterial strains do not produce the enzymes required to transform these chemicals. To identify the pro-mutagenic potential of a test item, the metabolic activation system (commercially available post-mitochondrial fraction (S9) from livers of rodents treated with the enzyme-inducing agent Aroclor) is also used in the test.
The mutagenic or pro-mutagenic potential of the test item is assessed by the increase in the number of revertant colonies upon exposure to the test item relative to the number of spontaneously occurring revertant colonies in the controls.

2. Materials and methods

2.1 Test Item

Test Item Name:	IN-L5296
IUPAC Name:	(2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine

Analysed Concentration: 77.0 ± 0.4% w/w (Refer Certificate of Analysis in Appendix 8)
Batch N°: 20111502
Date of Manufacture: July 2015
Date of Expiry: November 2020
Appearance/Colour: Powder, yellow
Storage Condition**:** Refrigerator (ca. 5ºC) and protected from light

2.2 Test item sterility assay

The sterility of the test item was assayed by adding 1.68 mg/plate (C5 according to the solubility profile of the test item) to a minimal agar plate and incubating at 37ºC for 48h.

2.3 Test item solubility and precipitation signs

The test item was soluble in DMSO at a concentration of 16.8 mg/mL (1.68 mg/plate), with no precipitation signs being observed in the assay final mixture with PBS.
Therefore, the C5 selected for the cytotoxicity assay was 16.8 mg/mL (1.68 mg/plate), as recommended by the OECD guideline 471.

2.4 Test item cytotoxicity assay

Cytotoxicity evaluation of the test item was performed in the *S. typhimurium* TA100 strain by the direct incorporation procedure and without metabolic activation (S9) using 5 concentrations based on the solubility profile of the test item which ranged from 0.02 up to 1.68 mg/plate. Test item solutions were prepared by 1:3 serial dilution of C5.

Formulated TI:	**C5**	**C4**	**C3**	**C2**	**C1**
Concentration (mg/plate):	1.68	0.56	0.19	0.06	0.02

On the basis of the solubility and cytotoxicity results of the test item, the C5 selected for the main test was 1.68 mg/plate. Concentrations C4 to C1 were prepared by 1:3 serial dilutions in the selected solvent from the C5 concentration.

2.5 Reference item identification

Reference item	**Solvent**	**Bacterial strain to be treated**	**Supplier**	**Reference**
2-nitrofluorene	DMSO	TA98	Sigma-Aldrich	N16754
sodium azide	Milli-Q water	TA100	Sigma-Aldrich	S2002
		TA1535	Sigma-Aldrich	
4-nitroquinoline-N-oxide	DMSO	WP2	Sigma-Aldrich	N8141
9-aminoacridine	DMSO	TA1537	MERK	8.18362.0010
2-amino-anthracene	DMSO	TA98	Sigma-Aldrich	A38800
		TA100 & TA1537	Sigma-Aldrich	
		WP2 & TA1535	Sigma-Aldrich	

2.6 Reference item formulation

		Without metabolic activation: S9 (-)			**With metabolic activation: S9 (+)**		
Bacterial	**Strain**	**Reference item**	**Solvent**	**µg/plate**	**Reference item**	**Solvent**	**µg/plate**
S. typhimurium	TA98	2-nitrofluorene	DMSO	5	2-amino-anthracene	DMSO	1.5
S. typhimurium	TA100	sodium azide	H2O	2.5			2.5

E. coli	WP2(pKM101)	4-nitroquinoline-N-oxide	DMSO	0.4			30
S. typhimurium	TA1535	sodium azide	H2O	3.5			30
S. typhimurium	TA1537	9-aminoacridine	DMSO	45			2.5

2.7 Test system characterisation

All the bacterial strains used in the Ames test carry a mutant gene that prevents them from synthesizing an essential amino acid. These strains may carry additional mutations which increase their sensitivity to different types of mutagens. All *S. typhimurium* strains used in the test carry the rfa mutation. This mutation causes an alteration in the lipopolysaccharide (LPS) layer making the bacteria more permeable to larger molecules. The uvrB and uvrA deletions eliminate the accurate excision repair mechanism resulting in an increase in the rate of mutations due to an alternative DNA repair mechanism. The plasmid pKM101 in several strains enhances the chemical mutagenesis via an increase in the error-prone recombinational DNA repair mechanism.

The strains that were used in the test are summarized in the following table.

Species	**Strain**	**Mutation**	**Others mutations Deletion**	**Plasmid**	**Reversion event**	**Target**	**Supplier**	Reference
S. typhimurium	TA98	*hisD3052*	*rfa / uvrB* deletion	pkM101	frameshift	G:C	Moltox	71-098L
S. typhimurium	TA100	*hisG46*	*rfa / uvrB* deletion	pkM101	base-pair	G:C		71-100L
E. coli	WP2(pKM101)	*trpE65*	*uvrA* deletion	pkM101	base-pair	A:T		72-003L
S. typhimurium	TA1535	*hisG46*	*rfa / uvrB* deletion	-	base-pair	G:C		71-1535L
S. typhimurium	TA1537	*hisC3076*	*rfa / uvrB* deletion	-	frameshift	G:C		71-1537L

2.8 Test system conditions

The bacterial strains used for the study were grown from controlled Working Banks obtained from Master Banks (generated in Vivotecnia) in nutrient broth supplemented with the corresponding antibiotics when required, as follows:

	TA98	**TA100**	**WP2(pKM101)**	**TA1535**	**TA1537**
Nutrient Broth #2	25 g/L	25 g/L	25 g/L	25 g/L	25 g/L
Ampicillin	0.025 mg/mL	0.025 mg/mL	0.025 mg/mL	-	-

Inoculums were liquid grown overnight up to the late exponential-early stationary phase of growth (approximately 1.2-1.4 O.D. at 660 nm). This O.D. indicated that bacteria were growing in the late exponential or early stationary phase of growth.

The following types of agar medium were used in the test:

Media	**Agar**	**Glucose**	**Vogel-Bonner 50x**	**NaCl**	**Histidine**	**Biotin**	**Tryptophan**
Minimal agar	1.5% w/v	2% w/v	2% v/v	-	-	-	-
Top agar *Salmonella*	0.54% w/v	-	-	0.45% w/v	0.05 mM	0.05 mM	-
Top agar *E. coli*	0.54% w/v	-	-	0.45% w/v	-	-	0.05 mM

2.9 Test procedure

Bacterial strains were exposed to the test item at 5 concentrations (C5 to C1) with and without metabolic activation system (S9) under the direct incorporation and the pre-incubation procedures. Plates were incubated for 48h at 37ºC and colonies were counted.
The assay was performed by triplicate along with vehicle and reference item controls.
Each bacterial strain culture was mixed with the test item either with metabolic activation system mix (S9) or without metabolic activation system mix (PBS was used instead).
In the direct incorporation procedure the mixture was immediately poured over a minimal agar medium plate and incubated at 37ºC for 48h. Whereas in the pre-incubation procedure, the mixture was incubated for 20 min at 37ºC prior to be poured over the minimal agar medium plate.

2.10 Experimental groups

Each group was assayed with 5 concentrations of the test item (C5 to C1) and with vehicle as negative control.

Test system		**Test item concentration**	**Reference items**		**Main test**	**Confirmatory test**
			S9 (-)	**S9 (+)**		
S. typhimurium	TA98	C5 to C1	2-nitrofluorene	2-amino-anthra-cene	direct in-corporation	pre-incubation
S. typhimurium	TA100		sodium azide			
E. coli	WP2(pKM101)		4-nitroquinoline-N-oxide			
S. typhimurium	TA1535		sodium azide			
S. typhimurium	TA1537		9-aminoacridine			

2.11 DEVIATIONS

No deviations were recorded throughout the study period.

3. RESULTS

The number of revertant colonies per plate was counted and recorded by an automatic colony counter. Average plate counts were presented with the mean and the standard deviation for each set of triplicates per test item concentration and was used to calculate the ratio of colonies per exposed plate compared to the corresponding negative control.

3.1 Data interpretation

The criteria used for determining a positive result take into account a dose-response effect in the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system.

A result is considered positive whenever the number of revertants of the test item-treated plates is increased when compared to the solvent-treated plates according to the following criteria:

Species	Strain	Mutagenic Ratio (R) cut-off point for considering a positive result
S. typhimurium	TA98	2 fold
S. typhimurium	TA100	2 fold
E. coli	WP2(pKM101)	2 fold
S. typhimurium	TA1535	3 fold
S. typhimurium	TA1537	3 fold

Biological relevance of the results was also considered.

Ames test acceptance criteria

The bacterial reverse mutation test for the test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) was considered valid as the following criteria were met:

− The mean solvent control counts complied with Vivotecnia historical data for each strain.

− The mean reference item control counts complied with Vivotecnia historical data for each strain.

3.2 Colony counting evaluation (R value)

Upon performance of the cytotoxicity assay on *S. typhimurium* strain TA100, following the direct incorporation procedure and in the absence of metabolic activation, no test item related cytotoxicity was observed at a concentration range from 0.02 to 1.68 mg/plate.

Nevertheless, upon performance of the main test, cytotoxic activity was observed in *S. typhimurium* strain TA1535, following the pre-incubation procedure and in the presence of metabolic activation at the concentration of 0.02 mg/plate.

3.3 Dose-response evaluation

No dose response exceeding the threshold for the test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) was observed in any of the tested bacterial strains.

4. CONCLUSION

The test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) does not induce point mutations or frameshifts in the genome of the bacterial strains with or without metabolic activation regardless of the procedure over the concentration range tested.

Therefore, the test item IN-L5296 (2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazine) at an exposure dose range of 0.02 – 1.68 mg/plate is considered to be non-mutagenic / non-pro-mutagenic under the experimental conditions assayed.

Comments of zRMS:	Study is considered acceptable. Under the experimental conditions, metabolite IN-L5296 tested up to 1680 µg/plate in the absence and in the presence of metabolic activation was negative in the bacterial reverse mutation test.

A 2.12.4 In vitro chromosome aberrations test for metabolite IN-L5296 (KCP 7.1.7/04)

Report:	**Xxxxxx, A., 2019**
Title:	*In vitro* chromosome aberrations test using Chinese Hamster Ovary cells (CHO)
Document No:	ABC4-LM-18-0293 with amendment
Guidelines:	OECD Nº 473
GLP	Yes

SUMMARY
In the study genotoxic activity by looking for chromosomal aberrations in CHO (Chinese Hamster Ovary) was determined according to OECD guideline n° 473 "In vitro mammalian chromosome aberration test" (LEMI SOP n° MB0S/120).
Solutions obtained from metabolite of tribenuron methyl IN-L5296 BATCH: MP-5248-39-5 (LEMI code: LM-18/0293) (Assay 1 and Assay 2) were tested for their ability to induce *in vitro* chromosomal aberrations in cultured CHO (Chinese Hamster Ovary). This study was carried out in the absence and presence of metabolic activation. Two independent experiments were performed.
For assay 1, CHO were exposed 4 h to solution 18/0293-201218-Sl in the absence of metabolic activation and 3 h to solution 18/0293-201218-Sl in the presence of metabolic activation (S9-mix 10 % (v/v)).

For assay 2, CHO were exposed 20 h to solution 18/0293-020119-Sl in absence of metabolic activation.
For the two assays, positive and negative controls were carried out in parallel. Both assays positive controls induced a statistically significant increase in the number of chromosomal aberrations in comparison with corresponding negative controls. The values of negative and positive controls do not show a significant difference with the historical experimental values of the laboratory. Negative controls and positive controls validate the two assays.

According to the criteria of conclusion of the study protocol and OECD 473, solutions obtained from metabolite of tribenuron methyl IN-L5296 BATCH: MP-5248-39-5 (LEMI Code: LM 18/0293) provided by VIVOTECNIA, are not considered clastogenic in the test system used (CHO) in the conditions of the assay.

INTRODUCTION

1.1 Study Objective

The purpose of the study was to identify agents that cause structural chromosome aberrations in cultured mammalian cells according to the method of Evans and O'Riordan2, in compliance with OECD guideline n° 473.

The assay was performed in both the absence and the presence of an appropriate metabolic activation system (Rat liver microsome fraction) to detect pro-mutagens agents.

Chinese Hamster Ovary (CHO- Kl (ATCC CCL 61, ECACC 85051005)) were exposed to the test item for 4 hours and 20 hours in the absence of metabolic activation and for 3 hours in the presence of metabolic activation. The cultures were then treated by Colcemid®, to block cells in metaphase. Two hours and a half later cells were harvested, and stained with Giemsa. The metaphases were analyzed microscopically (xl000) for identifying and counting chromosomal aberrations, polyploidy and endoreduplications.

1.2 Study Guidelines

The test was performed in accordance with OECD guideline n° 473 "In vitro mammalian chromosome aberration test".

2. Material and methods

2.1 Item received

Name:	**IN-L5296**
Batch:	MP-5248-39-5
Container:	Plastic flask
Quantity:	13.05196 g (content+ container)
Category:	Chemical substance
Date of reception:	15.11.2018
LEMI Code:	LM-18/0293
Concentration:	NA
Composition:	2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazone
Purity:	99.1 %

Stability: Stable under normal storage conditions and handling

Expiry date: 07.2015

Production date: 11.2020

Solubility: DMSO

Organoleptic specifications:

Aspect: Pale yellow powder

pH: NA

Other physical properties:

sterility: Non-sterile

density: Unknown

CASN°: 5248-39-5

EINECSN°: Unknown

Storage conditions: Fridge (2°C to 8°C)

2.2 Negative control

	Absolute negative control	**Solvent control (DMSO)**
Name (Supplier - Ref. - Batch)	McCoy's GIBCO-26600-023-2010306	McCoy's GIBCO-26600-023-2010306
Physical state	liquid	liquid
Color	pink (pH 7.2)	pink (pH 7.2)
Stability	stable under normal storage and handling	stable under normal storage and handling
FCS (10%) (Supplier- Ref. - Batch)	GIBCO-10270-098-42G3075K	GIBCO-10270-098-42G3075K
DMSO (1%) (Supplier - Ref. - Batch)	NA	SIGMA- 41639 - BCBW9035
Antibiotics (1%) (Supplier - Ref. - Batch)	GIBCO- 15240-096-1981203	GIBCO-15240-096- 1981203
Storage conditions	between 2°C and 8°C	between 2°C and 8°C
Expiry date	30.04.2019	30.04.2019
Safety precautions	Standard laboratory conditions	Standard laboratory conditions

2.3 Positive controls

	Without metabolic activation	**With metabolic activation**
Name	MitomycinC	Cyclophosphamide monohydrate
CASN°	50-07-07	6055-19-2
Supplier - Ref. - Batch	Bioaustralis - BIA-Ml 183 - EL 4.109	Acros organics - 203960010 - A0355340
Physical state	powder	powder
Color	blue	white
Solvent or vehicle	McCoy's	McCoy's

Stability or expiry date	30.06.2020	--.01.2020
Storage conditions	- 20 °C	between 2 °C and 8 °C
Safety precautions	mutagenic agent	mutagenic agent
After solubilisation		
Visual aspect	homogenous, pink-blue solutions extemporaneous preparation	homogenous, pink solutions extemporaneous preparation
Stability		

2.4 Test system and rationale for the choice of test system

Chinese Hamster Ovary (CHO) cultures are recommended by the OECD guideline n° 473. Moreover, CHO are currently used in standard protocols for *in vitro* cytogenetic tests. CHO are tested for absence of mycoplasma and population doubling.

Cell type used	CHO-Kl
Origin*	ATCC CCL 61, ECACC 85051005
Caryotype	stable
Chromosome modal number	20
Mycoplasma research	30.05.2018
Cell Passage	17.4 H
Passage number	18** - 21***
Maintenance of cell cultures	Mc Coy' s + 10 % FCS

* Criteria meet the requirements of OECD n° 473. **Assay I *** Assay 2

2.5 Solutions preparation

A preliminary solubility test determined a maximum solubility in DMSO of25 mg I mL (test item is poorly soluble in water; the solubility limit is less than 2.5 mg/mL).

Solutions of the test item, metabolite IN-L5296 BATCH: MP-5248-39-5 (LEMI code: LM-18/0293), were prepared at 25 mg/mL in DMSO, in sterile conditions.

	ASSAYn°1	**ASSAYn°2**
Solutions identification	Solutions realized from LM-18/0293: 18/0293-201218-S1 With and without metabolic activation	Solutions realized from LM-18/0293: 18/0293- 020119-Sl without metabolic activation
Solution vehicles:	DMSO	DMSO
Visual aspect	Pink orange - transparent homogeneous for solution at 250 µg/mL in complete culture medium	Pink orange - transparent homogeneous for solution at 250 µg/mL in complete culture medium
Stability	Extemporaneous preparation	Extemporaneous preparation

Assay conditions

Cell culture

Before exposure to test item, cells were seeded in a 25 cm^2 culture flask at the starting density of 10.10^3 cells/cm^2 into 5 ml of complete culture medium (McCoy's supplemented with 10 % (v/v) Fetal Calf Serum (FCS)).

Cell cultures were incubated at 37°C in a humid atmosphere containing 5% (v/v) CO2, for 40 hours. Two cultures were carried out for each concentration and each control.

Exposure concentrations used

Controls

Absolute negative control	Culture medium	10 %FCS	n*=2
Negative control (solvent)	Solvent of test item: DMSO	1 %maximum	n*=2
Positive control (without metabolic activation)	MitomycinC (CAS n° [50-07-7])	0.25 µg/mL (Assay 1) 0.125 µg/mL (Assay 2)	n*= 2 n*=2
Positive control (with metabolic activation)	Cyclophosphamid monohydrate (CAS n° [6055-19-2])	10 µg/mL	n*=2

* number of flasks per vessel

2.6 Test item

Test was performed in duplicate at each tested concentrations. The different solutions of the test item were prepared extemporaneously.

A preliminary cytotoxicity test (using Balb/c 3T3 mouse embryo fibroblast, by the Trypan blue exclusion test - LEMI SOP MB08/33) was performed to obtain a first estimate of the maximum concentration which should be tested in the chromosomal aberration test.

It was assessed by the determination of the Relative Increase in Cell Count (RICC) with and without metabolic activation. This parameter evaluates the cytotoxicity of the four tested concentrations and allows the final selection of the concentrations to be tested in the chromosomal aberration test.

If cytotoxicity observed, analysable concentrations should cover a range from the maximum to little or no toxicity. The highest concentration used should induce cytotoxicity less than 50 %.

Note: the osmolality and pH of the highest concentration studied should be compatible with cell culture.

2.7 Exposure of test item (solutions)

Selected concentrations of test item solutions are placed in contact with the test system. Two independent tests were carried out.

Assay 1: short-term treatment

• Without metabolic activation: 4 hours exposure followed by 18 hours of expression

• With metabolic activation (S9-mix 10 % (v/v)): 3 hours exposure followed by 18 hours of expression

Assay 2: long-term treatment

• Without metabolic activation: 20 hours of exposition

2.7.1 Without metabolic activation (assay 1 and assay 2)

40 hours after the seeding, the complete cell culture is removed and replaced by:

Complete culture medium	Complete culture medium*
Vehicle	DMSO
Test item	250 µg/mL - 100 µg/mL - 40 µg/mL - 16 µg/mL

* Complete culture medium is Mc Coye's supplemented with 10 % (v/v) Feta! Calf Serum (FCS), 1 % (v/v) antibiotics (penicillin JO 000 U/mL, streptomycin 10 000 µg/mL, amphotericin B 25 µg/mL).

Short-term treatment (Assay 1):

4 hours later at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2, the culture medium was discarded and the cells were washed twice with culture medium. 5 mL of fresh complete culture medium were added and the cells were incubated, 18 hours at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2.

Long-term treatment (Assay 2):

the cells were incubated, 20 hours at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2.

2.7.2 With metabolic activation (Assay 1)

Preparation of S9 fraction

S9 (microsome fraction from the liver of Sprague Dawley rats treated with Aroclor 1254 (500 mg/kg over a 5 day period)) was prepared in compliance with Ames and al[4], and provided by MOLTOX™ (POB Box 1189 - 157 Industrial Park Dr - Boone, NC 28607 - USA). The S9 fraction (Ref: 11-101.5 - Batch: 3919 - Expiry date: 07.02.2020) was previously validated on 06.07.2018 in the laboratory according to the LEMI SOP n° MB06/009.

Preparation of S9-mix

S9-mix composition is presented in the following table:

S9 fraction	10 % (v/v)
$MgCL_2$-$6H_2O$	8 mM
KCl	33 mM
Glucose-6-Phosphate Na2	5 mM
$NADPNa_2$	4 mM
Phosphate buffer pH 7.4	0.1 M

Exposition

40 hours after the seeding, the complete cell culture was discarded, cells layer washed with culture medium and incubated with reaction mixture composed by culture medium supplemented with 10 % (v/v) S9-mix.

S9 medium	**S9 medium***
Vehicle	McCoy's
Test item	250 ug/mL - I00 ug/mL - 40 ug/mL - 16 ug/mL

* S9 medium is Mc Coy's supplemented with JO % (v/v) S9-mix (final concentration: 1.5 %) and 1 % (v/v) antibiotics (penicillin 10000 U/mL, streptomycin 10000 µg/mL, amphotericin B 25 µg/mL).

3 hours later at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2, the culture medium was discarded and the cells layer washed twice with culture medium. 5 mL of fresh complete culture medium were added and the cells layer incubated, 18 hours at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2.

2.9 Harvest and microscope slides preparation

At the end of the incubation period (18h and 20h), Colcemid® (SIGMA-Dl925-RNBF6984) (0.15 µg/mL) was added in each flask. Cells were incubated at 37° C for 2.5 hours in a humidified atmosphere containing 5 % (v/v) CO_2 in order to block the cell division in the metaphase stage, then collected:

- culture medium was removed
- cells layer was washed once with PBS

- cells were detached (about 2 minutes at 37°C) using 0.5 mL trypsin (0.05 % (w/v) in Hank's balanced solution Ca^{2+} and Mg^{2+} free supplemented with I mM EDTA)
- then 4.5 mL of McCoy's supplemented with *5* % (v/v) Fetal Calf Serum (FCS) were added
- 100 µL of cell suspension and 100 µL of trypan blue solution at 0.2 % (w/v) in 0.15 M NaCl were added (incubation for 2 minutes).
- thereafter the living cells (Trypan blue exclusion test - LEMI SOP n° MB08/023) were counted using an haemocytometer (Malassez cell)
- hypotonic shock (KCI 0.075 M) at 37° C for 10 minutes
- fixation (2 to 3 x *5* min) using the Carnoy mixture (methanol: acetic acid, 3:I) spread on coded microscope slides
- stained using Giemsa stain at 0.4 % (w/v) in phosphate buffer (0.0I M, pH 6.8).

Metaphases were analyzed under a microscope (Zeiss), magnification x1000 for the detection of chromosomal aberrations, polyploidy and endoreduplications.

2.10 Evaluation criteria

Relative Increase in Cell Count (RICC).

The RICC corresponds to the relative increase in the number of cells in exposed cultures versus increase in non- treated cultures, a ratio expressed as a percentage.

If the RICC is above 50% (or RICC reduction below 50%), lower doses are not scored.

$$\text{RICC} = \frac{\text{Increase in number of cells in treated cultures (final - starting)}}{\text{Increase in number of cells in control cultures (final - starting)}} \times 100$$

RICC reduction = 100 - RICC

"Starting" corresponding to the cell number before incubation(= pre incubation control).

For positive controls, RICC must be not less than 50%.

If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve *55* +/- *5* % cytotoxicity (i.e. 45 +/- *5* % RICC reduction).

Detection of chromosomal aberrations (Assays n°1 and n°2)

Search for chromosomal aberrations:

- 300 metaphases minimum were analysed for the highest non-cytotoxic concentration of each solution (if necessary),
- 300 metaphases minimum were analysed for the absolute negative control,
- 25 metaphases minimum were analysed for the positive controls.

The following changes were identified according to Savage*:

Aberrations of chromosome type	Aberrations of chromatidic type	Other events

1. Gap or achromatic lesion (G*).	1. Gap (g*).	1. Pulverised chromosome (PC).
2. Break or terminal deletion (C).	2. Break (c).	2. Pulverised cell (pc).
3. Exchanges:	3. Median deletion (d).	
* dicentric chromosome (D or DF),	4. Exchanges:	
* complex rearrangement (CR),	* chromosome intrachange (ci),	
* ring (R or RF).	* triradial (tr),	
4. Minutes (M).	* quadriradial (qr).	

***Remark:** Chromosome gap (G) and chromatid gap (g) are not taken into account. These events are rather associated with cytotoxicity than with genotoxicity.

The number of cells presenting one, or more, aberration was considered as a direct response and evaluated statistically using the X^2 trend test.

The results were considered significant if $P < 0.05$ comparing cultures treated with different solutions of test item with their corresponding negative control.

The result was considered significant if $P < 0.05$ comparing positive control with their corresponding negative control.

Detection of polyploidy and endoreduplications

- 300 metaphases minimum were analysed for the highest non-cytotoxic concentration of each solution (if necessary),
- 300 metaphases minimum were analysed for the absolute negative control.

The following changes were identified according to OECD 473:

Polyploidy: numerical chromosomal aberrations in cells or organisms involving entire set(s) of chromosomes, as opposed to an individual chromosome or chromosomes (aneuploidy).

Endoreduplication: a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with 4, 8, 16... , chromatids.

Remark: polyploidy alone does not indicate aneugenic potential and can simply indicate cell cycle perturbation or cytotoxicity.

2.11 Criteria conclusion

The test item is considered as clastogen in vitro with regards to CHO cells according to the following criteria:

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent absolute negative control,
- the increase is dose-related when evaluated with an appropriate trend test,
- any of the results are outside the distribution of the historical negative control data.

The test item for which the results do not meet the all above criteria is considered non-clastogenic in this system. Positive results indicate that the test item induces structural chromosome aberrations in CHO cell cultures.

The positive control shall largely fulfil to all three criteria.

The number of cells with chromosomal aberrations in the negative control shall be less than 5 %.

Equivocal or discutable results may not allow a clear positive response. Results shall be clarified by further testing using modification of experimental conditions (concentration spacing and metabolic activation conditions).

3. RESULTS

3.1 Discussion of results

3.1.1 Cytotoxicity:

Preliminary study using Balb/c 3T3

The solutions at 250, 100, 40 and 16 µg/mL did not show any inhibition of cell growth statistically significant superior to 30 %. According to the evaluation criteria of the cytotoxicity, the solution prepared from the test item is not cytotoxic at 250 µg/mL.

Positive control (phenol 0.64 mg/mL) induced a 63 % ($P < 0.001$) inhibition on cell growth which validates the study.

RICC

Assay n°1 (short-term treatment) without metabolic activation

The solution at 250 µg/mL and 40 µg/mL of test item reduced the RICC by 19% and 7%, respectively. These concentrations were compatible with the study.

The solution at 100 µg/mL and 16 µg/mL of test item did not reduce the RICC. These concentrations were compatible with the study.

The positive control reduced the RICC by 48%. This RICC reduction was compatible with the study.

Therefore, the concentrations 250, 100 and 40 µg/mL was further used to determine the genotoxic effects.

Assay n°1 (short-term treatment) with metabolic activation

The solution at 250 µg/mL and 40 µg/mL of test item did not reduce the RICC. These concentrations were compatible with the study.

The solution at 100 µg/mL and 16 µg/mL of test item reduced the RICC by 1% and 8%, respectively. These concentrations were compatible with the study.

The positive control reduces the RICC by 47 %. This RICC reduction is compatible with the study.

Therefore, the concentrations 250, 100 and 40 µg/mL was further used to determine the genotoxic effects.

Assay n°2 (long-term treatment) without metabolic activation

The solution at 250 µg/mL and 100 µg/mL of test item did not reduce the RICC. These concentrations were compatible with the study.

The solution at 40 µg/mL and 16 µg/mL of test item reduced both the RICC by 1%. These concentrations were compatible with the study.

The positive control reduced the RICC by 50 %. This RICC reduction was compatible with the study.

Therefore, the concentrations 250, 100 and 40 µg/mL was further used to determine the genotoxic effects.

3.1.2 Genotoxicity:

Absolute negative control

The percentage of cells with aberrations was equal to 2.0 % for assay 1 and 1.0 % for assay 2 in the absence of metabolic activation and equal to 1.3 % in the presence of metabolic activation.

Solvent control

The percentage of cells with aberrations was equal to 1.7 % for assay 1 and 1.0 % for assay 2 in the absence of metabolic activation and equal to 1.3 % in the presence of metabolic activation.

Positive controls

Without metabolic activation: Mitomycin C significantly increased the percentage of cells with aberrations compared to absolute negative control (P < 0.001). This percentage was 42.3 % for assay 1 and 32.0 % for assay 2.

With metabolic activation: Cyclophosphamide significantly increased the percentage of cells with aberrations compared to absolute negative control (P < 0.001). This percentage was equal to 40.0 %.

Test item

Assay n°1 (short-term treatment) without metabolic activation

The all solutions of the test item (at 250, 100 and 40 µg/mL) did not significantly increase the percentage of cells with aberrations. These percentages were equal to 1.3 %.

Assay n°1 (short-term treatment) with metabolic activation

The all solutions of the test item (at 250, 100 and 40 µg/mL) did not significantly increase the percentage of cells with aberrations. These percentages were equal to 2.0, 1.7 and 1.3%, respectively.

Assay n°2 (long-term treatment) without metabolic activation

The all solutions of the test item (at 250, 100 and 40 µg/mL) did not significantly increase the percentage of cells with aberrations. These percentages were equal to 1.0, 1.7 and 1.3%, respectively.

No concentration exhibited a statistically significant increase compared with the co current negative control. The test item was considered non-clastogenic in this test system (human lymphocytes).

3.1.3 Polyploidy and endoreduplication cases:

No increase in polyploidy an endoreduplication was observed compared to negative control.

Interpretation does not take into account the measurement uncertainties. These uncertainties are available and can be provided on request.

4. CONCLUSIONS

The values of absolute negative and positive controls did not show a significant difference with the historical experimental values of the laboratory.

Negative controls and positive controls validate the two assays.

According to the criteria of conclusion of the study protocol and OCDE 473, solutions obtained from metabolite of tribenuron methyl IN-L5296 BATCH: MP-5248-39-S (LEMI Code: LM-18/0293) provided by VIVOTECNIA, are not considered clastogenic in the test system used (CHO) in the conditions of the assay.

Comments of zRMS:	Study is considered acceptable. Metabolite IN-L5296 is not considered clastogenic in the test system used (CHO) in the conditions of the assay.

A 2.12.5 In vitro mammalian cell gene mutation test for metabolite IN-L5296 (KCP 7.1.7/05)

Report:	**Xxxxxx, C., 2019**
Title:	*In vitro* mammalian cell gene mutation test
Document No:	MLAl-LM-18/0293 with amendment MLA2-LM-18/0293
Guidelines:	OECD Nº 490
GLP	Yes

SUMMARY

Solutions obtained from metabolite of tribenuron methyl IN-L5296 BATCH: MP 5248-39-5 were tested for their capacity to induce mutagenic activity in L5178Y Mouse Lymphoma cells. No long-term treatment has been conducted, only short-term treatment without or with metabolic activation was carried out according to the acceptability criteria of OECD 490.
250 - 100 - 40 and 16 µg/mL of IN-L5296 BATCH: MP 5248-39-5 were evaluated in contact with the cells in the absence of a metabolic activation system.
250 - 100 - 40 and 16 µg/mL of IN-L5296 BATCH: MP 5248-39-5 were evaluated in contact with the cells in the presence of metabolic activation. (S9-mix 2.5 % (v/v)).
For short-term treatment without or with metabolic activation studies, negative and positive controls were carried out in parallel. Positive controls induced a significant increase in the number of colonies compared to negative controls. These results validate the assays.
In the absence of the metabolic activation system and in presence of metabolic activation system (S9-mix 2.5% (v/v)) no concentration-related increase in the mutant frequency was measured in presence of IN-L5296 BATCH: MP 5248-39-5 at these doses.

In the framework of OECD 490 under the described experimental conditions, solutions obtained from metabolite of tribenuron methyl IN-L5296 BATCH: MP 5248-39-5 (LEMI Code: LM-18/0293) provided by VIVOTECNIA do not induce a mutagenic effect in L5178Y TK+/-Mouse lymphoma cells in the absence or in the presence of metabolic activation (2.5% S9-mix) at these doses.

INTRODUCTION

1.1 Study Objective

The aim of the test was to evaluate the mutagenic potential of a test item using a mammalian cell line (LS178Y mouse lymphoma cells) by measuring mutations at the thymidine kinase (TK) locus. Test item was studied in the absence or presence of metabolic activation (using a microsomal fraction of rat liver) in order to identify direct mutagens and promutagens, respectively.
LS178Y mouse lymphoma cells have been used for many years to detect genetic damage to mammalian cells *in vitro* (Cole et al., 1983[3]; Clive et al., 1987[4]). These assays were recommended by OECD guideline n°490. Various protocols were developed, and particularly a fluctuation test protocol using plating into microtiter plates instead of soft agar. This technique is greatly validated and is the subject of many publications (Cole et al., 1990[5]; Aaron et al., 1994[6]) and used in this study. According to Cole et al. (1983), the microtiter cloning technique gives results which are comparable to the agar cloning method, not only for mutant frequency, but also for the proportion of large and small colonies.
The heterozygous LS178Y $TK^{+/-}$ cells are exposed to the test item for 4 hours (short-term treatment without metabolic activation) or for 3 hours (short-term treatment with metabolic activation). The cells are then resuspended in order to determine their survival rate and to allow the phenotypic expression of the mutation. At the end of the expression time (2 days), the cells are exposed to a selective agent for $TK^{-/-}$ mutant cells: trifluorothymidine (TFT). TK catalyses the conversion of TFT to its cytostatic and cytotoxic trifluorothymidine monophosphate derivative. Cells deficient in the heterozygous TK-locus due to the forward mutation $TK^{+/-}$ => $TK^{-/-}$ are resistant to the cytotoxic effects of pyrimidine analogues such as TFT. The deficiencies of the "salvage" enzyme TK means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained only from "*de novo*" synthesis. On the other hand, in the presence of TK, TFT is incorporated into the nucleotides, resulting in inhibition of cellular metabolism, and cytotoxicity. Thus, mutant cells are able to proliferate in the presence of TFT, whereas normal cells which contain TK, are not.

[3] Cole J., Arlett C.F., Green M.H.L, Lowe J., Muriel W.J., 1983: Mutation Research, 111, 371.
[4] Clive D. Caspary W., Kirby P.E., Krehl R., Moore M., Mayo J., Oberly T.J, 1987: Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity. Mutation Research, 189, 143-156.
[5] Cole J., McGregor D.B., Fox M., Thacker J., Garner R.C., 1990: Gene mutation assays in cultured mammalian cells. In Kirkland D.J. (ed.), UKMS Recommended Procedures, Cambridge University Press. Cambridge UK, 87-114.
[6] Aron C.S., Bolcsfoldi D., Black H. R., Moore M., Nishi Y., Stankowski L., Theiss J., Thompson E., 1994: Mammalian cell gene mutation assays working group report. Mutation Research 312 No. 3, 235-240.

Cells as suspension cultures are exposed to test item for a defined period of time. Cytotoxicity is determined by measuring the growth rate of cultures. At the end of treatment period, cells are cultured 48H to allow near optimal phenotypic expression of newly induced mutants.
Mutant frequency is determined by seeding a known number of cells in medium containing the selective agent (TFT) to detect mutant cells, and in medium without the selective agent to determine cloning efficiency. After a suitable incubation time all colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in non-selective medium to derive the mutant frequency (Clive et al., 1983[7]).
In TK mutants resistant to the selective agent TFT, a bimodal distribution of size is classified as small or large (a large colony is defined as one that is over one-fourth of the diameter of the well):

- large colonies with normal growth kinetics,
- small colonies with slow growth kinetics.

Research has indicated that large colony TK mutations represent events within the gene (base-pair substitutions or deletions) that affect the expression of the TK locus, whereas small-colony mutants carry large genetic changes involving chromosome 11b, the chromosome which carries the active TK gene (Hozier et al., 1985[8]). Furthermore, according to Applegate et al. (1990)[9] the diversity of mutagen damages affecting the heterozygous TK locus can be considered as representative of some events found in human cancer and so more numerous mutation events than those observed in a homozygous locus will be detected. Thus, by scoring large and small colonies at the same time in one cell line, conclusions may be drawn about the type of damage (gene or chromosomal mutation) induced by a test compound within one study. Reference mutagens are tested in parallel to the test item in order to demonstrate the sensitivity of the test system.

1.2 Study Guidelines

This study was conducted according to the OECD Guideline n° 490 "*In vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene" adopted 29 July, 2016.

2. Materials and methods

2.1 Item received

Name:	Metabolite of tribenuron methyl IN-L5296 BATCH: MP 5248-39-5
Container:	Plastic flask
Quantity:	13.05196 g (content+ container)
Category:	Chemical substance
Date of reception*:	15.11.2018
LEMI Code:	LM-18/0293
Concentration:	Not provided
Composition:	2-methoxy-4-methyl-6-(methylamino)-1,3,5-triazone
Purity:	99.1 %
Stability:	Stable under normal storage conditions and handling
Batch production date:	-.07.2015
Expiry date:	-.11.2020
Solubility:	DMSO According to the sponsor this solvent does not have any reactivity with the test item.

[7] Clive D., McCuen R., Spector J.F.S., Piper C., Mavournin K.H., 1983: Specific gene mutation in L5718Y cells in culture: Report of the U.S. EPA Gen-tox Program. Mutation Research 115, 25-251.
[8] Hozier J., Sawyer J., Clive D., Moore M.M., 1985: Chromosome 11 aberrations in small colony L5718Y $TK^{+/-}$ mutants early in their clonal history. Mutation Research 147, 237-242.
[9] Applegate M.L., Moore M.M., Broder C.B., Burrel A., Hozier J.C., 1990: Molecular dissection of mutations at the heterozygous thymidine kinase locus in Mouse lymphoma cells. Proc. Natl. Acad. Sci. USA, 87, 51-55.

Organoleptic specifications:

Aspect:	Pale yellow powder
pH:	Not provided
Other physical properties:	
sterility:	Non-sterile
density:	Unknown
CASN°:	5248-39-5
EINECSN°:	Unknown
Storage conditions:	Fridge (2°C to 8°C)

2.2 Test Items: Solutions obtained from the item received

Test item was soluble in DMSO. A test conducted by LEMI pre-determined value of 25 mg/mL in DMSO (very light precipitates visible by eye).
For the assay, to obtain a final concentration at 250 µg/mL (slightly higher than the maximum soluble concentration visible to the naked eye, in accordance with OECD requests) a homogenous suspension at 25 mg/mL in DMSO of the test item in sterile conditions was used. The highest concentration chosen was slightly higher than the maximum soluble concentration visible to the naked eye, in accordance with OECD 490 requests (§29 "For poorly soluble test chemicals that are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analyzed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical.").

	Assay (-S9 mix)	**Assay (+S9 mix)**
LEMI code	18/0293-150119-St	18/0293-220119-St
Aspect- Colour	Light yellow - slightly cloudy homogeneous	Light yellow - slightly cloudy homogeneous
Solubility	DMSO	DMSO
Stability	extemporaneous solution	extemporaneous solution

2.3 Negative solvent control

	Absolute negative control
Name (Supplier - Ref. - Batch)	DMSO Sigma-41639-BCBW9035
Physical state	liquid
Color	colorless
Stability	stable under normal conditions
Storage conditions	Room temperature
Expiry date	-.09.2022
Safety precautions	Standard laboratory conditions

2.4 Positive controls

	Without metabolic activation	**With metabolic activation**
Name Supplier Ref. – Batch CAS No	Cyclophosphamide monohydrate Acros organics 203960010 - A0355340 6055-19-2	Methyl methanesulfonate (MMS) Sigma 129925-MKCD8572 66-27-3
Physical state	powder	liquid
Color	white	colorless
Solvent or vehicle	culture medium	culture medium

Stability and expire date	Stable under normal conditions -.01.2020	Stable under normal conditions 07.12.2019
Storage conditions	extemporaneous between 2°C and 8°C	extemporaneous room temperature (15°C and 25°C)
Safety precautions	mutagenic agent	mutagenic agent
After solubilisation		
Visual aspect	homogenous, pink solutions extemporaneous preparation	homogenous, pink solutions extemporaneous preparation
Stability		

2.5 Test system: mouse lymphoma (L5178Y) cells

2.5.1 Test system description

Mouse lymphoma L5178Y $TK^{+/-}$ cells (ATCC-CRL-9518) purchased from ATCC (American Type Culture Collection-Rockeville, MD 20852 - USA) have been used successfully in "*in vitro*" experiments for many years. These cells are characterized by their high proliferation rate (10-12 h doubling time of the stock cultures) and their cloning efficiency, usually more than 50%. They possess a nearly diploid karyotype (40 ± 2 chromosomes). They are heterozygous at the thymidine kinase (TK) locus which allows to detect mutation events at the TK^- locus.

Cells from the cell bank stored at - 80°C were systematically checked to be free from mycoplasma contamination (LEMI operating procedure MB05/02).

2.5.2 Test system purification

To prevent background arising from spontaneous mutation, cells lacking TK have to be eliminated by culturing them in a culture medium (Dulbecco's modified Eagle's medium (DMEM) GlutaMAX™ - I) supplemented with 10% (v/v) of inactivated horse serum containing HMTG (Cole et a1., 1986[10]): 15 µg/mL hypoxanthine, 0.3 µg/mL methotrexate, 9 µg/mL thymidine, 22.5 µg/mL glycine.

After 24 hours incubation at 37°C in a humidified atmosphere containing 5% (v/v) CO_2, the culture was centrifuged (200 x G, 10 min) in order to eliminate methotrexate, and the cell pellet was suspended in medium, without methotrexate, containing HTG (hypoxanthine, thymidine and glycine) and incubated at 37° C in a humidified atmosphere containing 5% (v/v) CO_2 for 1 day to 3 days.

Cleaned cells were stored at -80°C. Each cell batch was checked free from mycoplasma contamination. Thawed cultures were maintained in complete culture medium (CCM).

2.5.3. Test system conditions

Test system:	L5178Y TK+/- mouse lymphoma cell line
Viability assay:	RSG (relative suspension growth)
Concentrations of IN-L5296 tested with S-9 mix and without S-9 mix (2.5%):	16, 40, 100 and 250 µg/mL
Gene mutation assays:	according to acceptability criteria of OECD No 490 two experimental conditions were tested – short treatment with and without metabolic activation
Number of culture/ concentration:	2
Duration of treatment:	4h without S-9 mix 3h with S-9 mix
Positive controls without S-9 mix with S-9 mix	Methyl methanesulfonate (MMS) CAS: 66-27-3 10 µg/mL Cyclophosphamide monohydrate CAS: 6055-19-2 2 µg/mL

[10] Cole J., Muriel W.F., Bridges B.A., 1986: The mutagenicity od sodium fluoride to L5178Y (wild-type and $TK^{+/-}$ (3.7.2.c) mouse lymphoma cells. Mutagenesis 1, 157-167.

2.6 Culture media

Complete culture medium (CCM):

Dulbecco's modified Eagle's medium (DMEM) GlutaMAX™ - I supplemented with 10 % (v/v) (GIBCO - ref. 31966021- batch 2007769 and 2007849) inactivated horse serum (10 %), (GIBCO - ref. 16050-130 - batch 1972988) and 1 % (v/v) antibiotics (GIBCO - ref. 15240-096 - batch 1981203).

Treatment medium ™:

DMEM GlutaMAX™ - I supplemented with 5 % (v/v) inactivated horse serum and 1 % (v/v) antibiotics.

Cloning medium (CM):

DMEM GlutaMAX™ supplemented with 20 % (v/v) inactivated horse serum and 1 % (v/v) antibiotics.

Selective medium (SM):

CCM supplemented with TFT (3 µg/mL) (Sigma - RefT2255 - batch BCBW1167).

2.7 Pre-test for viability

Prior to mutagenesis assay a pre-test was carried out in order to determine the viability of IN-L5296 BATCH: MP 5248-39-5. This pre-test showed a high toxicity of test item from 128 to 5 000 µg/mL (data not showed).

1 x 10^6 L5178Y cells /mL of TM were exposed to a range of concentrations of test item for 4 or 3 hours (without or with metabolic activation). Following treatment, cells were rinsed twice with complete culture medium (CCM) (10 mL) followed by centrifugation (200 g, 10 min). Subsequently, the cells were resuspended in 20 mL CCM for a 2 days growth period. Cell density was determined at days D1, D2 and adjusted to 2 x 10^5 cells/mL.

The relative suspension growth (RSG) and the relative total growth (RTG) of the treated cell cultures was calculated at the end of the growth period according to the method of Clive and Spector[11] as follows:

$$RSG = \frac{\text{Daily growth at day 1 x Daily growth at day 2) in treated culture}}{\text{(Daily growth at day 1 x Daily growth at day 2) in control culture}}$$

% RSG = RSGx 100

2.8 Preparation of the test item

- concentrations (n=2) of the test item were:
- without S9-mix: 16 - 40 - 100 - and 250 µg/mL
- with S9-mix (2.5 %): 16 - 40 - 100 - and 250 µg/mL
- the upper limit of cytotoxicity observed in experimental cultures should not be less than 10 % RTG, which is the case for the highest concentrations tested
- the test item is soluble in DMSO, the dissolution was performed in this solvent
- the different solutions of the test item were prepared extemporaneously
- Note: the osmolality and pH of the highest concentration studied should be compatible with cell culture.

2.9 Experimental protocol

Assays were run independently using duplicate cultures. Only short-term treatments were planned.

2.9.1 Assay without metabolic activation (4 hours)

Viability: the methodology of the pre-test (described above) was applied in the main experiment.

Treatment: 1x10^6 cells/mL of TM supplemented with 5 % (v/v) were exposed to each concentration of IN-L5296 BATCH: MP 5248-39-5 at 37° in a humidified atmosphere containing 5 % (v/v) CO_2.

[11] Clive D., Spector J.F.S., 1975: Laboratory procedure for assessing specific locus mutation at the TK locus in cultured L5178Y mouse lymphoma. Mutation Research 31, 17-29.

After 4 hours, cells were rinsed twice by centrifugation (200g, 10 min). It was necessary to treat at least 6 $x10^6$ cells.

Plating for viability: Cell pellet was suspended in complete culture medium (CCM) and plated, at a statistical mean of 2 cells per well in 2 plates of 96 wells per concentration, in order to determine viability at TO (colony counting 9 to 11 days later).

Expression period: Concurrent cells were suspended in CCM and incubated in order to allow TK locus phenotypic expression over 48 hours to 72 hours. The cell density was determined every day, and adjusted to $2x10^5$ cells/mL, if necessary.

Mutagenesis test:

- **Plating for survival:**

After the expression period, the relative cloning efficiency (RCE; percentage cloning efficiency of the test group in relation to the control) of the cells is determined according to Cole et al. (1990)[3] by seeding a statistical number of 2 cells/well in two 96-well-plates. Cells were incubated for 10 -12 days at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2. Analysis of results was based on the number of cultures with cell growth (positive cultures) and/or those without cell growth (negative cultures) compared to the total number of cultures seeded. Relative total growth (RTG) of the treated cell cultures was calculated according to the method of Clive and Spector[9] as follows:

RTG = % RSGx % RCE

where RCE (Relative Cloning Efficiency) was determined by comparing plating efficiency PE in the test cultures and control cultures at day 2 and RSG (Relative Suspension Growth) is calculated from the equation presented in §6.3.

- **Plating for 5-trijluorothymidine (TFT) resistance:**

Cultures were resuspended in selective medium with TFT at 3 µg/mL. Cells from each experimental group were seeded in four 96-well plates at a density of approximately 2 000 cells/well in 200 µL selective medium. The plates were scored after an incubation period of 10 - 12 days at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2.

- **Criteria for scoring mutation plates:**

The number of wells containing colonies were counted. A well without colonies was classified as negative. Colonies were characterized as follows:

- small colonies: colonies having a diameter less than 25 % of the diameter of the well. A small colony should also have a dense morphology and a clear contour;
- large colonies: colonies having a diameter greater than 25 % of the diameter of the well. Morphology is totally or partially diffuse.

Any well which contained:

- one or more than one small colony was scored as positive for small colony;
- one or more than one large colony was scored as positive for large colony;
- a combination of large and small colonies was scored as large colony and small colony.

The mutation frequencies were then calculated from the data obtained from cultures used for the cloning efficiency (cultures with non-selective medium) and those used for selection (cultures with selective medium) as follows:

$$\text{MUTATION FREQUENCY} = \frac{-\ln[EW/TW(\text{selective medium})]/nm}{-\ln[EW/TW(\text{non selective medium})]/nm} \times 10^6$$

EW: Empty Wells
TW: Total Wells seeded

nm: number of cells /well
In: neperian logarithm

A negative control (Complete Culture Medium DMEM glutamax), a solvent control (DMSO) and a positive control (Methylmethanesulfonate at 10 µg/mL) were carried out in parallel under the same conditions.

2.9.2 Assay with metabolic activation (3 hours)

S9 fraction provider

S9 fraction, microsome fraction prepared from Sprague Dawley rat liver homogenate, was provided by MOLTOX™ (POB Box 1189 - 157 Industrial Park Dr - Boone, NC 28607 - USA (S9 Moltox-11101-5-3919 validated on 06.07.2018- expiry date: 07.02.2020).

Preparation of S9-mix

S9-mix was prepared at 4 °C on the day of use, as presented in table below. The final concentration of cofactors and salts is as follow:

S9 fraction	2.5 % (v/v)
MgCl2-6H_20	8 mM
KCl	33 mM
Glucose-6-phosphate Na_2	5 mM
NADP Na_2	4 mM
Phosphate buffer pH 7.4	0.1 M

Assay

The test was identical to the one described in the absence of metabolic activation, except cells were treated for 3 hours in the presence of 2.5 % S9-mix.

A negative control (Complete Culture Medium DMEM glutamax), a solvent control (DMSO) and a positive control, (Cyclophosphamide monohydrate at 2 µg/mL) were carried out in parallel under the same conditions.

2.10 Acceptability criteria

2.10.1 Acceptability criteria for the assay

A gene mutation assay is considered acceptable if it meets the following criteria:

- the test must be conducted under two experimental conditions (short treatment without and with metabolic activation) unless one resulted in positive results
- adequate number of cells (a minimum of $6x10^6$ cells) and concentrations should be analysable

2.10.2 Acceptability criteria for negative and positive controls

Every experiment should be evaluated as to whether the untreated control meets the IWGT MLA Workgroup acceptance criteria, below:

- Mutant Frequency 50 - 170 x 10^{-6}
- Cloning Efficiency 65 - 120%
- Suspension Growth:

- 8 - 32 fold (3-4-hour treatment)
- 32 - 180 fold (24-hour treatment, if conducted)

Every experiment should also be evaluated as to whether the positive controls meets at least one of the following two acceptance criteria:

- The positive control should demonstrate an absolute increase in total MF, that is, an increase above the spontaneous background MF [an induced MF (IMF)] of at least 300 x 10^{-6}
- At least 40 % of the IMF should be reflected in the small colony MF.
- The positive control has an increase in the small colony MF of at least 150 x 10^{-6} above that seen in the concurrent untreated control (a small colony IMF of 150 x 10^{-6})
- The upper limit of cytotoxicity observed in the positive control culture should be the same as of the experimental cultures. In other words, the RTG/RS should not be less than 10 %.

2.11 Evaluation and interpretation of the results

An approach for defining positive and negative responses is recommended to assure that the increased MF is biologically relevant.

In place of statistical analysis generally used for other tests, it relies on the use of a predefined induced mutant frequency (i.e. increase in MF above concurrent control), designated the Global Evaluation Factor (GEF), which is based on the analysis of the distribution of the negative control MF data from participating laboratories. For the microwell version of the MLA the GEF is 126 x 10^{-6}.

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined, the increase in MF above the concurrent background exceeds the GEF and the increase is concentration related. The test chemical is then considered able to induce mutation in this test system.

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly negative if, in all experimental conditions examined there is no concentration related response or, if there is an increase in MF, it does not exceed the GEF. The test chemical is then considered unable to induce mutations in this test system.

There is no requirement for verification of a clearly positive or negative response**.**

In cases when the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions [e.g. concentration spacing to increase the probability of attaining data points within the 10-20 % RTG/RS range, using other metabolic activation conditions (i.e. S9 concentration or S9 origin) and duration of treatment] could be useful.

In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore, the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

3 RESULTS

3.1 Viability study

For the assay in absence of S9-mix, the osmolality and pH of the highest concentration studied were compatible with cell culture. The pH values were 7.4 and 7.5 for negative and solvent controls and 7.5 for test item.

Osmolality value was 349 and 499 mOsm/Kg H_2O for negative and solvent, respectively and between 499 and 510 mOsm/Kg H_2O for IN-L5296.

For the assay in presence of S9-mix, the osmolality and pH of the highest concentration studied were compatible with cell culture. The pH values were 7.3 for negative and solvent controls and 7.3 for the test item.

Osmolality value was 356 and 504 mOsm/Kg H_2O for negative and solvent control respectively and between 502 and 519 mOsm/Kg H_2O for IN-L5296.

The percentage relative cloning efficiency (% RCE) in each test was calculated by comparing plating efficiency in treated and control cultures.

- RTG values without metabolic activation were in the range from 48 to 96 %
- RTG values with metabolic activation - in the range from 66 to 107 %

These values were compatible with the acceptability criteria described in OECD 490 for all concentrations evaluated and positive controls.

3.2 Discussion

3.3.1 Negative control

The plating efficiency (PE) for the negative and solvent controls should be 65 to 120 % for viability. In the two independent assays, PE values were in the acceptable range 66-65 % (negative and solvent controls) in the absence of metabolic activation and 84-76 % (negative and solvent controls) in the presence of metabolic activation for untreated controls.

Spontaneous mutant frequencies of negative control were:

- 163.9 x 10^6; 152.8 x 10^6 (negative and solvent controls),
- 109.9 x 10·6; 145.1 x 10^6 (negative and solvent controls)

which is within the range 50 - 170 x 10·6 (acceptability criteria described in OECD 490 and also in the range of the historical values of the laboratory.

The suspension growth (SG) values of untreated controls were 23.2 - 22.2 fold in the absence of metabolic activation (negative and solvent controls) and 25.5 - 24.1. fold in the presence of metabolic activation (negative and solvent controls).

In these two independent assays SG values were in the acceptable range, 8 - 32 for untreated and solvent controls.

These results for untreated and solvent controls were in accordance with the acceptability criteria described in OECD 490.

3.3.2 Positive controls

Without and with metabolic activation, positive controls produced a statistically significant increase in mutant frequency in the two independent assays. MF: 4.8 and 3.9 times that of negative control (without and with metabolic activation) ($P < 0.001$).

The positive controls used in the assay demonstrated an absolute increase in total MF:

- Without metabolic activation, positive control Methylmethanesulfonate induces a statistically significant increase in mutant frequency, 4.7 times that of negative control: (780.0x10^6 MF that of negative control 163.9 x 10^6 MF)
- With metabolic activation, positive control Cyclophosphamide Monohydrate induced a statistically significant increase in mutant frequency, 3.9 times that of negative control (430.7x10^6 MF that of negative control 109.9 x 10^6 MF).

Above the spontaneous background MF (an induced MF (IMF)) of at least 300 x 10^6 was measured to be:

- 616.1 x 10·6 in presence of Methylmethanesulfonate
- 320.8 x 10·6 in presence of Cyclophosphamide Monohydrate

These values were in the range of the historical values of the laboratory.

At least 40 % of the IMF for positive controls were reflected in the small colony:

- 349 x 10^6 in presence of Methylmethanesulfonate (72.4 % of the IMF);
- 219 x 10^6 in presence of Cyclophosphamide Monohydrate (73.2 % of the IMF).

These results for positive controls were in accordance with the acceptability criteria described in OECD 490.

3.3.3 Test item: IN-L5296

In the absence of metabolic activation -4 hours treatment:

a) A light increase in the mutant frequency is observed.

Moreover, the GEF (Global Evaluating Factor) was calculated in these experimental conditions, since the GEF was recommended by the OECD 490 to help in evaluating the test results (Moore et al. 2003, 2006, 2007[12,13,14]). The GEF is applied as follows:

if the negative control mutant frequency (MF) in a microwell experiment is 100 x 10^{-6}, then one of the treatment groups must have a MF of at least [100+126 (the microwell GEF) = 226] x 10^{6} in order to meet the GEF criterion for a positive call.

The above criteria, was not met at any concentration tested, in the absence of metabolic activation for the short exposure time. The measured MF ranged from 133.5 to 273.9 x 10^{6} and fell below GEF criterion of [126+152.8]278.8 x 10^{6}.

b) Analysis of the size of colonies showed a light increase in induced small (0 to 41), and in induced large colony for any concentration tested (0 to 66) compared to positive control.

In the presence of metabolic activation - 3 hours treatment

a) In the presence of 2.5 % S9-mix, a light increase in the mutant frequency was observed.

The criteria was not met for any concentration tested, in the presence of metabolic activation. The measured MF range from 133.6 to 218.8 x 10^{6} fell below GEF criterion of [126+145.1]271.l x 10^{6}.

b) Analysis of the size of colonies showed a light increase in induced small colonies (7 to 37) and a light increase in induced large colony (0 to 33) for any concentration tested compared to positive control.

4. CONCLUSIONS

In the framework of OECD 490 under the described experimental conditions, solutions obtained from metabolite of tribenuron methyl IN-L5296 BATCH: MP 5248-39-5 (LEMI Code: LM-18/0293) provided by VIVOTECNIA, do not induce a mutagenic effect in L5178Y TK+/-Mouse lymphoma cells in the absence or in the presence of metabolic activation (2.5% S9-mix) at these doses.

Comments of zRMS:	Study is considered acceptable. Under the experimental conditions, metabolite IN-L5296 do not induce a mutagenic effect in L5178Y TK+/-Mouse lymphoma cells in the absence or in the presence of metabolic activation.

[12] Moore et al., 2003: Mouse Lymphoma Thymidine Kinase Gene Mutation Assay: International Workshop on Genotoxicity Tests Workgroup Report. Plymouth UK, 2002. Mutation Research 540 (2003) 127-140.
[13] Moore et al., 2006: Mouse Lymphoma Thymidine Kinase Gene Mutation Assay: Follow-up Meeting of the International Workshop on Genotoxicity Testing. Aberdeen, Scotland, 2003. Assay Acceptance Criteria , Positive Control and data Evaluation Environmental and Mutagenesis 47: 1-5 (2006).
[14] Moore et al., 2007: Mouse Lymphoma Thymidine Kinase Gene Mutation Assay: Meeting of the International Workshop on Genotoxicity Testing. San Francisco, 2005. Recommendations for 24h- treatment. . Mutation Research 627 (2007) 36-40.

Appendix 3 Exposure calculations

A 3.1 Operator exposure calculations (KCP 7.2.1.1)

A 3.1.1 Calculations for Tribenuron-methyl

Table A 1: Input parameters considered for the estimation of operator exposure

Substance name	Tribenuron-methyl	
Product name	TOSCANA TOP 75 WG	
Reference value non acutely toxic active substance (RVNAS)	0,05	mg/kg bw/day
Reference value acutely toxic active substance (RVAAS)	0,13	mg/kg bw/day
Crop type	Cereals/ Grasslands and lawns	
Substance properties		
Formulation type	Wettable granules, soluble granules	
Miniumum volume water for application (liquids)	200	L/ha
Maximum application rate of active substance	0,01875	kg a.s. /ha
50% Dissipation Time DT50	30	days
Initial Dislodgeable Foliar Residue	3	µg/cm2 of foliage/kg a.s. applied/ha
Dermal absorption of product	10,00%	
Dermal absorption of in-use dilution	50,00%	
Oral absorption of active substance	67,00%	
Inhalation absorption of active substance	100,00%	
Vapour pressure of active substance	low volatile substances having a vapour pressure of <5*10-3Pa	
Scenario		
Indoor or Outdoor application	Outdoor	
Application method	Downward spraying	
Application equipment	Vehicle-mounted	
Buffer strip	2-3	m
Number of applications	1	
Interval between multiple applications	365	days
Season (upward spraying orchards only)	not relevant	

Table A 2: Estimation of acute and long-term operator exposure towards Tribenuron-methyl according to EFSA guidance

Application rate of active substance	0,01875	kg a.s./ha	*i_AppRate*
Assumed area treated	50	ha/day	*d_AreaTreated*
Amount of active substance applied	0,9375	kg a.s./day	*i_AmoutAS*
Dermal absorption of the product	10,00%		*i_AbsorpProduct*
Dermal absorption of in-use dilution	50,00%		*i_AbsorInuse*
Formulation type	Wettable granules, soluble granules		
Indoor or Outdoor application	Outdoor		
Application method	Downward spraying		
Application equipment	Vehicle-mounted		
Season	not relevant		

OutdoorWettable granules, soluble granulesDownward sprayingVehicle-mounted

	Exposure values	µg exposure/day mixed and loaded		Reference	Comment
		75th centile	95th centile		
Mixing and loading	Hands	1247	5945	AOEM	
	Body	1180	15772	AOEM	
	Head	6	84	AOEM	
	Protected hands (gloves)	16	30	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	18	58	AOEM	
	Protected head (hood and face shield)	0	5	AOEM	
	Inhalation	37	259	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	No			
	Clothing	Potential exposure		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Water soluble bag	No		1	

	Exposure values	µg exposure/day applied		Reference	Comment
		75th centile	95th centile		
Application	Hands	139	2186	AOEM	
	Body	78	401	AOEM	

Head	4	11	AOEM	
Protected hands (gloves)	41	3308	AOEM	
Protected body (workwear or protective garment and sturdy footwear)	2	5	AOEM	
Inhalation	1	3	AOEM	

Protective Equipment	Select for inclusion	Penetration factor	Inhalation Protection factor
Gloves	No		
Clothing	Potential exposure	Incl. in AOEM model	
Head and respiratory PPE	None	1	1
Closed cab	No	vehicle mounted upward spraying only	

Total

	Without RPE/PPE	With RPE/PPE
Longer term		
Total systemic exposure from mixing, loading and application (mg a.s./day)	**0,3912489**	**0,3912489**
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	**0,0065208**	**0,0065208**
% of RVNAS	**13,04%**	**13,04%**
Acute		
Total systemic exposure from mixing, loading and application (mg a.s./day)	**3,7409238**	**3,7409238**
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	**0,0623487**	**0,0623487**
% of RVAAS	**47,96%**	**47,96%**

A 3.2 Worker exposure calculations (KCP 7.2.3.1)

A 3.2.1 Calculations for Tribenuron-methyl

Table A 14: Input parameters considered for the estimation of worker exposure

Crop type	Cereals/ Grasslands and lawns		
Indoor or outdoor	Outdoor		
Application method	Downward spraying		
Application equipment	Vehicle-mounted		
Worker's task	Inspection, irrigation		
Main body parts in contact with foliage	Hand and body		
Application rate of active substance	0,01875	kg a.s./ha	*i_AppRate*
Number of applications	1		*i_AppNo*
Interval between multiple applications	365	days	*i_AppInt*
Half-life of active substance	30	days	*d_HalflifeAS*
Multiple application factor	1,0		*d_MAF*
Dermal absorption of the product	10,00%		*i_AbsorpProduct*
Dermal absorption of the in-use dilution	50,00%		*i_AbsorpInuse*
Dislodgeable foliar residue (i_AppRate*i_DFR)	0,05625	µg a.s./cm²	*d_DFR*
Working hours	2	hr	*d_WorkHr*
Dermal transfer coefficient - Total potential exposure	12500	cm²/hr	*d_DermTcUCV*
Dermal transfer coefficient - arms, body and legs covered	1400	cm²/hr	*d_DermTcCV1*
Dermal transfer coefficient - hands, arms, body and legs covered	no TC available for this assessment	cm²/hr	*d_DermTcCV2*
Inhalation transfer coefficient for automated applications	NA	ha/hr*10^(-3)	*d_InhalTcAut*
Inhalation transfer coefficient for cutting ornamentals	NA	ha/hr*10^(-3)	*d_InhalTcCut*
Inhalation transfer coefficient for sorting / bundling ornamentals	NA	ha/hr*10^(-3)	*d_InhalTcSort*

Table A 35: Estimation of long-term exposure towards Tribenuron-methyl according to EFSA guidance

1. Total

	Potential exposure	Work wear - arms, body and legs covered	Working wear and gloves	Comments
Total systemic exposure (mg a.s./day)	0,7031250	0,0787500	no TC available for this assessment	
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0117188	0,0013125		
% of RVNAS	23,44%	2,63%		

2. Details

	Systemic exposure		Formula	Comments
	[mg a.s. /day]	[mg a.s./kg bw/day]		
Dermal - Potential	0,7031250	0,0117188	*d_DermTcUCV*d_WorkHr*i_DFR*i_MAF/1000*i_AbsorpInuse*	
Dermal - Work wear - arms, body and legs covered	0,0787500	0,0013125	*d_DermTcCV1*d_WorkHr*d_DFR*d_MAF/1000*i_AbsorpInuse*	
Dermal - Working wear and gloves	no TC available for this assessment		*d_DermTcCV2*d_WorkHr*d_DFR*d_MAF/1000*i_AbsorpInuse*	

Inhalation				Na for outdoor activities

Table A 46: Estimation of longer term worker exposure towards Tribenuron-methyl according to EUROPOEM II – potential exposure and workwear

WORKER EXPOSURE			**EUROPOEM II MODEL**	
form	Tribenuron methyl		**Re-entry in the field**	
a.s.	Tribenuron /methyl			
Parameter		**Value**	**Unit**	**References, comments**
Re-entry activities in the field				
AR	Application rate	0,01875	kg a.s./ha	summary of intended uses
Worker				
Duration				
T		2	hours / day	default: 6 h (Europoem II)
Inhalation Exposure				without PPE
	no model available	-		
Dermal Exposure				
DFR	Dislodgeable foliar residue	30	mg a.s./m2/kg a.s./ha	default (Europoem II)
TC	Transfer coefficient	1,25	m2/ hour	vegetable (field): 0.25; ornamentals: 0.5; small fruit: 0.3; large fruit: 0.45 (Europoem II)
Dermal Exposure		**1,40625**	mg a.s./ day	DE = DFR x AR x TC x T
Internal exposure				
DA	Dermal Absorption	50	%	
	PPE-factor dermal	5		gloves*
	AOEL	3	mg a.s./ day	based on 60 kg bw
		Without PPE	**With PPE**	
	Internal exposure	[mg a.s./ day]	[mg a.s./ day]	
	Inhalation	-	-	no model available
	Dermal	0,703	0,141	DE(int) = DE x (DA/100)
	Total	**0,703**	**0,141**	**sum**
	% AOEL			
	Inhalation	-	-	no model available
	Dermal	23	5	%AOEL = 100 x DE(int) / AOEL
	Total	**23**	**5**	**sum**

WORKER EXPOSURE		**EUROPOEM II MODEL**
form	Tribenuron methyl	**Re-entry in the field**
a.s.	Tribenuron /methyl	

Parameter		Value	Unit	References, comments
Re-entry activities in the field				
AR	Application rate	0,01875	kg a.s./ha	summary of intended uses
Worker				
Duration				
T		2	hours / day	default: 6 h (Europoem II)
Inhalation Exposure				without PPE
	no model available	-		
Dermal Exposure				
DFR	Dislodgeable foliar residue	30	mg a.s./m2/kg a.s./ha	default (Europoem II)
TC	Transfer coefficient	0,14	m2/ hour	vegetable (field): 0.25; ornamentals: 0.5; small fruit: 0.3; large fruit: 0.45 (Europoem II)
Dermal Exposure		**0,1575**	mg a.s./ day	DE = DFR x AR x TC x T
Internal exposure				
DA	Dermal Absorption	50	%	
	PPE-factor dermal	5		gloves*
	AOEL	3	mg a.s./ day	based on 60 kg bw
		Without PPE	**With PPE**	
	Internal exposure	[mg a.s./ day]	[mg a.s./ day]	
	Inhalation	-	-	no model available
	Dermal	0,079	0,016	DE(int) = DE x (DA/100)
	Total	**0,079**	**0,016**	**sum**
	% AOEL			
	Inhalation	-	-	no model available
	Dermal	3	1	%AOEL = 100 x DE(int) / AOEL
	Total	**3**	**1**	**sum**

* It is assumed in the used TC values, that body exposure is already reduced by (protective) clothing. The use of gloves will result in an extra reduction factor of 5.

A 3.3 Resident and bystander exposure calculations (KCP 7.2.2.1)

A 3.3.1 Calculations for Tribenuron-methyl

Table A 5: Input parameters considered for the estimation of longer term resident exposure - cereals

Croptype	**Cereals**	
Application method	**Downward spraying**	
Application equipment	**Vehicle-mounted**	*i_AppEquip*
Formulation type	**Wettable granules, soluble granules**	*i_FormVal*

Buffer strip	**2-3**	m	*i_Buffer*
Application rate of the product	**0,01875**	kg a.s./ha	*i_AppRate*
Concentration of active substance (in-use dilution for liquid applications)	**0,09375**	g a.s./l	*d_ConcAS*
Dermal absorption of product	**10,00%**		*i_AbsorpProduct*
Dermal absorption of in-use dilution	**50,00%**		*i_AbsorpInuse*
Oral absorption	**67,00%**		*i_AbsorpOralInuse*
Dislodgeable foliar residue (i_AppRate*i_DFR)	**0,05625**	µg a.s./cm^2	*d_DFR*
Vapour pressure of in-use dilution	**low volatile substances having a vapour pressure of <5*10-3Pa**	Pa	*i_Volat*
Concentration in air	**0,001**	mg/m^3	*d_AirCon*
Resident dermal spray drift exposure 75th percentile - adult	**0,47**	ml spray dilution/person	
Resident dermal spray drift exposure 75th percentile - child	**0,327**	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - adult	**0,00010**	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - child	**0,00022**	ml spray dilution/person	
Resident dermal spray drift exposure mean - adult	**0,22318**	ml spray dilution/person	
Resident dermal spray drift exposure mean - child	**0,18**	ml spray dilution/person	
Resident inhal. spray drift exposure mean - adult	**0,00009**	ml spray dilution/person	
Resident inhal. spray drift exposure mean - child	**0,00017**	ml spray dilution/person	
Exposure duration dermal	**2**	hours	*d_ReExpDur*
Exposure duration inhalation	**24**	hours	*d_ReExpDurInhal*
Exposure duration entry into treated crops	**0,25**	hours	*d_ExpDurTreatCrop*
Light clothing adjustment factor	**18,0%**		*d_ClothAF*
Breathing rate adult	**0,23**	m^3/day/kg	*d_BreathRAd*
Breathing rate child (1-3 year old)	**1,07**	m^3/day/kg	*d_BreathRCh*
Drift percentage on surface (75th percentile)	**5,60%**		
Drift percentage on surface (mean)	**4,10%**		
Turf transferable residues percentage	**5,00%**		*d_Turf*
Transfer coeff. of surface deposits-adult	**7300**	cm^2/hour	*d_ReTCAd*
Transfer coeff. of surface deposits-child (1-3 year old)	**2600**	cm^2/hour	*d_ReTCCh*
Saliva extraction percentage	**50,00%**		*d_SalExt*
Surface area of hands mouthed	**20**	cm^2	*d_AreaHM*
Frequency of hand to mouth activity	**9,5**	events/hour	*d_ReFreqHM*
Ingestion rate for mouthing of grass per day	**25**	cm^2	*d_MouthGrass*
Dislodgeable residues percentage transferability for object to mouth	**20,00%**		*d_DRP*
Transfer coefficient for entry into treated crops (75th percentile) - adult	**7500**	cm^2/h	*d_TcEntryAd*
Transfer coefficient for entry into treated crops (75th percentile) - child	**2250**	cm^2/h	*d_TcEntryCh*
Transfer coefficient for entry into treated crops (mean) - adult	**5980**	cm^2/h	*d_TcEntryAd*

Transfer coefficient for entry into treated crops (mean) - child	1794	cm^2/h	*d_TcEntryCh*

Table A 6: Estimation of longer term resident exposure towards Tribenuron-methyl according to EFSA guidance - cereals

1. Total

1.1 1-3 year old child

	Spray drift (75th percentile)	Vapour (75th percentile)	Surface deposits (75th percentile)	Entry into treated crops (75th percentile)	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0125897	0,0107000	0,0014670	0,0158203	0,0313228
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0012590	0,0010700	0,0001467	0,0015820	0,0031323
% of RVNAS	2,52%	2,14%	0,29%	3,16%	6,26%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0180750	0,0138000	0,0038325	0,0527344	0,0672397
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0003013	0,0002300	0,0000639	0,0008789	0,0011207
% of RVNAS	0,60%	0,46%	0,13%	1,76%	2,24%

Table A 19: Input parameters considered for the estimation of longer term resident exposure – grasslands

Croptype	Grassland and lawns		
Application method	Downward spraying		
Application equipment	Vehicle-mounted		i_AppEquip
Formulation type	Wettable granules, soluble granules		i_FormVal
Buffer strip	2-3	m	i_Buffer
Application rate of the product	0,01875	kg a.s./ha	i_AppRate
Concentration of active substance (in-use dilution for liquid applications)	0,09375	g a.s./l	d_ConcAS
Dermal absorption of product	10,00%		i_AbsorpProduct
Dermal absorption of in-use dilution	50,00%		i_AbsorpInuse
Oral absorption	67,00%		i_AbsorpOralInuse
Dislodgeable foliar residue (i_AppRate*i_DFR)	0,05625	µg a.s./cm²	d_DFR
Vapour pressure of in-use dilution	low volatile substances having a vapour pressure of <5*10-3Pa	Pa	i_Volat
Concentration in air	0,001	mg/m³	d_AirCon
Resident dermal spray drift exposure 75th percentile - adult	0,47	ml spray dilution/person	
Resident dermal spray drift exposure 75th percentile - child	0,327	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - adult	0,00010	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - child	0,00022	ml spray dilution/person	
Resident dermal spray drift exposure mean - adult	0,22318	ml spray dilution/person	
Resident dermal spray drift exposure mean - child	0,18	ml spray dilution/person	
Resident inhal. spray drift exposure mean - adult	0,00009	ml spray dilution/person	
Resident inhal. spray drift exposure mean - child	0,00017	ml spray dilution/person	
Exposure duration dermal	2	hours	d_ReExpDur
Exposure duration inhalation	24	hours	d_ReExpDurInhal
Exposure duration entry into treated crops	0,25	hours	d_ExpDurTreatCrop
Light clothing adjustment factor	18,0%		d_ClothAF
Breathing rate adult	0,23	m³/day/kg	d_BreathRAd
Breathing rate child (1-3 year old)	1,07	m³/day/kg	d_BreathRCh
Drift percentage on surface (75th percentile)	5,60%		
Drift percentage on surface (mean)	4,10%		
Turf transferable residues percentage	5,00%		d_Turf
Transfer coeff. of surface deposits-adult	7300	cm²/hour	d_ReTCAd
Transfer coeff. of surface deposits-child (1-3 year old)	2600	cm²/hour	d_ReTCCh
Saliva extraction percentage	50,00%		d_SalExt
Surface area of hands mouthed	20	cm²	d_AreaHM
Frequency of hand to mouth activity	9,5	events/hour	d_ReFreqHM
Ingestion rate for mouthing of grass per day	25	cm²	d_MouthGrass
Dislodgeable residues percentage transferability for object to mouth	20,00%		d_DRP
Transfer coefficient for entry into treated crops (75th percentile) - ad	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops (75th percentile) - ch	2250	cm²/h	d_TcEntryCh
Transfer coefficient for entry into treated crops (mean) - adult	5980	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops (mean) - child	1794	cm²/h	d_TcEntryCh

Table A 7: Estimation of longer term resident exposure towards Tribenuron-methyl according to EFSA guidance – grasslands

1. Total

1.1 1-3 year old child

	Spray drift (75th percentile)	Vapour (75th percentile)	Surface deposits (75th percentile)	Entry into treated crops (75th percentile)	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0125897	0,0107000	0,0014670	0,0038242	0,0217556
Total systemic exposure per kg body weight	0,0012590	0,0010700	0,0001467	0,0003824	0,0021756
% of RVNAS	2,52%	2,14%	0,29%	0,76%	4,35%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0180750	0,0138000	0,0038325	0,0085547	0,0337475
Total systemic exposure per kg body weight	0,0003013	0,0002300	0,0000639	0,0001426	0,0005625
% of RVNAS	0,60%	0,46%	0,13%	0,29%	1,12%

Table A 21: Input parameters considered for the estimation of acute bystander exposure - cereals

Croptype	Cereals
Application method	Downward spraying

Application equipment	**Vehicle-mounted**		*i_AppEquip*
Formulation type	**Wettable granules, soluble granules**		
Application rate of the product	**0,01875**	kg a.s./ha	*i_AppRate*
Buffer strip	**2-3**	m	*i_Buffer*
Concentration of active substance (in-use dilution for liquid applications)	**0,09375**	g a.s./l	*d_ConcAS*
Dermal absorption of product	**10,00%**		*i_AbsorpProduct*
Dermal absorption of in-use dilution	**50,00%**		*i_AbsorpInuse*
Oral absorption	**67,00%**		*i_AbsorpOralInuse*
Dislodgeable foliar residue (i_AppRate*i_DFR)	**0,05625**	µg a.s./cm^2	*d_DFR*
Vapour pressure of in-use dilution	**low volatile substances having a vapour pressure of <5*10-3Pa**	Pa	*i_Volat*
Concentration in air	**0,001**	mg/m^3	*d_AirCon*
Bystander dermal spray drift exposure - adult	**1,21**	ml spray dilution/person	
Bystander dermal spray drift exposure - child	**0,74**	ml spray dilution/person	
Bystander inhal. spray drift exposure - adult	**0,00050**	ml spray dilution/person	
Bystander inhal. spray drift exposure - child	**0,00112**	ml spray dilution/person	
Exposure duration	**2**	hours	*d_ByExpDur*
Exposure duration entry into treated crops	**0,25**	hours	*d_ExpDurTreatCrop*
Light clothing adjustment factor	**18,0%**		*d_ClothAF*
Breathing rate adult	**0,23**	m^3/kg bw/day	*d_BreathRAd*
Breathing rate child (1-3 year old)	**1,07**	m^3/kg bw/day	*d_BreathRCh*
Drift percentage on surface (90th percentile)	**8,50%**		
Turf transferable residues percentage	**5,00%**		*d_Turf*
Transfer coeff. of surface deposits-adult	**14500**	cm^2/hour	*d_ByTCAd*
Transfer coeff. of surface deposits-child (1-3 year old)	**5200**	cm^2/hour	*d_ByTCCh*
Saliva extraction percentage	**50,00%**		*d_SalExt*
Surface area of hands mouthed	**20**	cm^2	*d_AreaHM*
Frequency of hand to mouth activity	**20**	events/hour	*d_ByFreqHM*
Ingestion rate for mouthing of grass per day	**25**	cm^2	*d_MouthGrass*
Dislodgeable residues percentage transferability for object to mouth	**20,00%**		*d_DRP*
Transfer coefficient for entry into treated crops - adult	**7500**	cm^2/h	*d_TcEntryAd*
Transfer coefficient for entry into treated crops - child	**2250**	cm^2/h	*d_TcEntryCh*

Table A 22: Estimation of acute bystander exposure towards Tribenuron-methyl according to EFSA guidance - cereals

1. Total

1.1 1-3 year old child

	Spray drift	Vapour	Surface deposits	Entry into treated crops
Total systemic exposure (mg a.s./day)	**0,0285488**	**0,0107000**	**0,0044107**	**0,0158203**
Total systemic exposure per kg body weight (mg/kg bw/day)	**0,0028549**	**0,0010700**	**0,0004411**	**0,0015820**
% of RVAAS	**2,20%**	**0,82%**	**0,34%**	**1,22%**

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops
Total systemic exposure (mg a.s./day)	**0,0465563**	**0,0138000**	**0,0115547**	**0,0527344**
Total systemic exposure per kg body weight (mg/kg bw/day)	**0,0007759**	**0,0002300**	**0,0001926**	**0,0008789**
% of RVAAS	**0,60%**	**0,18%**	**0,15%**	**0,68%**

Table A 23: Input parameters considered for the estimation of acute bystander exposure - grasslands

Croptype	Grassland and lawns		
Application method	Downward spraying		
Application equipment	Vehicle-mounted		i_AppEquip
Formulation type	Wettable granules, soluble granules		
Application rate of the product	0,01875	kg a.s./ha	i_AppRate
Buffer strip	2-3	m	i_Buffer
Concentration of active substance (in-use dilution for liquid applications)	0,09375	g a.s./l	d_ConcAS
Dermal absorption of product	10,00%		i_AbsorpProduct
Dermal absorption of in-use dilution	50,00%		i_AbsorpInuse
Oral absorption	67,00%		i_AbsorpOralInuse
Dislodgeable foliar residue (i_AppRate*i_DFR)	0,05625	µg a.s./cm²	d_DFR
Vapour pressure of in-use dilution	low volatile substances having a vapour pressure of	Pa	i_Volat
Concentration in air	0,001	mg/m³	d_AirCon
Bystander dermal spray drift exposure - adult	1,21	ml spray dilution/person	
Bystander dermal spray drift exposure - child	0,74	ml spray dilution/person	
Bystander inhal. spray drift exposure - adult	0,00050	ml spray dilution/person	
Bystander inhal. spray drift exposure - child	0,00112	ml spray dilution/person	
Exposure duration	2	hours	d_ByExpDur
Exposure duration entry into treated crops	0,25	hours	d_ExpDurTreatCrop
Light clothing adjustment factor	18,0%		d_ClothAF
Breathing rate adult	0,23	m³/kg bw/day	d_BreathRAd
Breathing rate child (1-3 year old)	1,07	m³/kg bw/day	d_BreathRCh
Drift percentage on surface (90th percentile)	8,50%		
Turf transferable residues percentage	5,00%		d_Turf
Transfer coeff. of surface deposits-adult	14500	cm²/hour	d_ByTCAd
Transfer coeff. of surface deposits-child (1-3 year old)	5200	cm²/hour	d_ByTCCh
Saliva extraction percentage	50,00%		d_SalExt
Surface area of hands mouthed	20	cm²	d_AreaHM
Frequency of hand to mouth activity	20	events/hour	d_ByFreqHM
Ingestion rate for mouthing of grass per day	25	cm²	d_MouthGrass
Dislodgeable residues percentage transferability for object to mouth	20,00%		d_DRP
Transfer coefficient for entry into treated crops - a	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops - c	2250	cm²/h	d_TcEntryCh

Table A 24: Estimation of acute bystander exposure towards Tribenuron-methyl according to EFSA guidance – grasslands

1. Total

1.1 1-3 year old child

	Spray drift	Vapour	Surface deposits	Entry into treated crops
Total systemic exposure (mg a.s./day)	0,0285488	0,0107000	0,0044107	0,0070359
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0028549	0,0010700	0,0004411	0,0007036
% of RVAAS	2,20%	0,82%	0,34%	0,54%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops
Total systemic exposure (mg a.s./day)	0,0465563	0,0138000	0,0115547	0,0169922
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0007759	0,0002300	0,0001926	0,0002832

A 3.4 Combined exposure calculations for active substance 1 and active substance 2

Not relevant.

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 relevant.