FINAL REGISTRATION REPORT

Part B

Section 6

Mammalian Toxicology

Detailed summary of the risk assessment

Product code: MT-565SG-OR2-C

Product name(s): HAKSAR TOP 565 SG

Chemical active substance(s):

MCPA, 550 g/kg
Tribenuron methyl, 15 g/kg

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Applicant: CIECH Sarzyna S.A.

Submission date: 01/2021

MS Finalisation date: 06/12/2021

Version history

When	What
January 2021	First submission for product authorization to zRMS.
02/2021	Dossier sent for evaluation to Merit Mark (PL)
08/2021	zRMS finalised evaluation
December 2021	Final 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 MT-565SG-OR2-C/ HAKSAR TOP 565 SG *

Product name and code	MT-565SG-OR2-C/ HAKSAR TOP 565 SG *
Formulation type	SG
Active substance(s) (incl. content)	MCPA, 550 g/kg Tribenuron methyl, 15 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 MT-565SG-OR2-C/ HAKSAR TOP 565 SG *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 HAKSAR TOP 565 SG according to Regulation (EC) No 1272/2008

Hazard class(es), categories	Acute Tox. 4 Skin Irrit. 2 Skin Sens. 1 Eye Dam. 1
Hazard pictograms or Code(s) for hazard pictogram(s)	GHS05, GHS07
Signal word	Danger
Hazard statement(s)	H302 - Harmful if swallowed H315 - Causes skin irritation H317 - May cause an allergic skin reaction H318 - Causes serious eye damage
Precautionary statement(s)	P261 - Avoid breathing dust/spray. P280 - Wear protective gloves/ protective clothing/eye protection P302+P352 - IF ON SKIN: Wash with plenty of water P333+P313 - If skin irritation or rash occurs: Get medical advice/attention. P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Additional labelling phrases	To avoid risks to man and the environment, comply with the instructions for use. [EUH401]

Comments of zRMS:	Classification of HAKSAR TOP 565 SG based on the calculation method taking into consideration valid data available on each of the components in the mixture is accepted.

Table 6.1-3: Summary of risk assessment for operators, workers, residents and bystanders for HAKSAR TOP 565 SG

	Result	PPE / Risk mitigation measures
Operators	Acceptable	Working wear & gloves during mixing and loading
Workers	Acceptable	Working wear
Residents	Acceptable	Use in cereals: Application with drift-reduction nozzles or 5-meter buffer strip
Bystanders	Acceptable	Use in cereals: Application with drift-reduction nozzles or 5-meter buffer strip

No unacceptable risk for operators, workers, residents and bystanders was identified when the product is used as intended and provided that the PPE/ risk mitigation measures stated in

Comments of zRMS:	Classification of HAKSAR TOP 565 SG based on the calculation method taking into consideration valid data available on each of the components in the mixture is accepted.

Table 6.1-3 are applied.

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

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

1	2	3	4	5	6	7	8	9	10			
Use-No.*	**Crops and situation (e.g. growth stage of crop)**	**F, Fn, Fpn G, Gn, Gpn or I ****	**Application**		**Application rate**		**PHI (d)**	**Remarks:** **(e.g. safener/synergist (L/ha))** **critical gap for operator, worker, resident or bystander exposure based on [Exposure model]**	**Acceptability of exposure assessment**			
			Method / Kind **(incl. application technique ***)**	**Max. number (min. interval between applications)** **a) per use** **b) per crop/ season**	**Max. application rate kg as/ha** **a) a.s. 1** **b) a.s. 2**	**Water L/ha** **min / max**			**Operator**	**Worker**	**Residents**	**Bystander**
1.	Cereals (BBCH 13-39)	F	Spraying, LCTM	1 ; 1	a) 0.550 b) 0.015	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	Spraying, LCTM	1 ; 1	a) 0.550 b) 0.015	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

Data gaps should be listed in the summary to give an overview (especially for cMS).

Noticed data gaps are:

- data gap 1
- data gap 2
- data gap 3

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)

	MCPA	Tribenuron-methyl
Common Name	MCPA	Tribenuron-methyl
CAS-No.	94-74-6	101200-48-0
Classification and proposed labelling		
With regard to toxicological endpoints (according to the criteria in Reg. 1272/2008, as amended)	Hazard classes (s), categories: Acute Tox. 4 Skin Irrt. 2 Eye Dam.1 Codes for hazard pictograms: GHS05 GHS07 Signal word: DANGER Hazard statements: H302 - Harmful if swallowed. H315 - Causes skin irritation H318 - Causes serious eye damage Precautionary statements: P280, P301+P312, P302+P352, P305+P351+P338, P310	Hazard classes (s), categories: Skin Sens. 1 STOT RE 2* Codes for hazard pictograms: 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): P26~~0~~1, P272, P280, ~~P314~~, P333+P313, P302+P352, P362+P364
Additional C&L proposal	No additional C&L are proposed.	RAC opinion adopted 14th of September 2018, ATP 15th to CLP
Agreed EU endpoints		
AOEL systemic	0.04 mg/kg bw/d	0.05 mg/kg bw/day
~~AOEL~~AAOEL	-	0.13 mg/kg bw per day.
Reference	SANCO/4062/2001-final	EFSA Journal 2017;15(7):4912

6.3 Toxicological Evaluation of Plant Protection Product

The applicant did not perform acute toxicity studies 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. The studies were not submitted. Justification is presented in Appendix 2

Table 6.3-1: Summary of evaluation of the studies on acute toxicity including irritancy and skin sensitisation for HAKSAR TOP 565 SG

Type of test, species, model system (Guideline)	Result	Acceptability	Classification (acc. to the criteria in Reg. 1272/2008)	Reference
LD_{50} oral, rat	-	Yes	Acute Tox. Cat 4	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	Skin Irrit. Cat 2	Justification in Appendix 2
Eye irritation	-	Yes	Eye Dam Cat.1	Justification in Appendix 2
Skin sensitisation	-	Yes	Skin Sens. Cat 1	Justification in Appendix 2
Supplementary studies for combinations of plant protection products	No data – not required		-	-

Table 6.3-2: Additional toxicological information relevant for classification/labelling of HAKSAR TOP 565 SG

	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)	MCPA (55% (w/w))	-	Reg. 1272/2008	No classification
	Tribenuron-methyl (1.5% (w/w))	H373 (criteria ≥ 10 %)	RAC opinion , ATP 15th to CLP	No classification
Toxicological properties of non-active substance(s) (relevant for classification of product)	-	-	Reg. 1272/2008 / MSDS	No classification
	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			

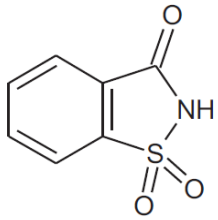
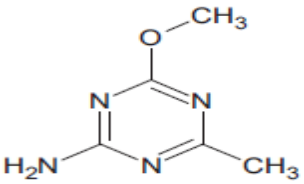
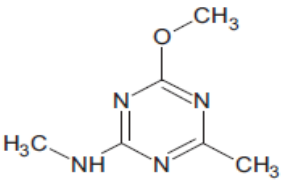
6.4 Toxicological Evaluation of Groundwater Metabolites

Comments of zRMS:	Details on the evaluation of the groundwater metabolites are included in dRR Part B, Section10.

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:	0.304 µg/L FOCUS model PELMO/ Hamburg, winter cereals 15 g a.s./ha in autumn
tribenuron-methyl	IN-A4098		Max PEC_{gw} Based on:	0.631 µg/L FOCUS model PEARL /Hamburg, spring cereals 15 g a.s./ha in spring
tribenuron-methyl	IN-L5296		Max PEC_{gw} Based on:	0.118 µg/L FOCUS model PELMO/ Hamburg, winter cereals 15 g a.s./ha in spring

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~~7~~), for the metabolite IN-A4098 ~~is negative for genotoxicity in gen mutation to bacteria as well as in chromosome damage~~ a genotoxic potential could not be excluded. In the EFSA 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) (EFSA Journal 2020;18(3):6053) was stated: "*There is no concern for the potential of triazine amine to induce gene mutations and clastogenicity; however, the potential to induce aneugenicity was not adequately investigated. For a conclusion, an in vitro micronucleus assay performed with triazine amine would be needed.*"
~~Additionally to this information, t~~The applicant submitted 2 negative studies on mammalian gene mutation. The results of *in vitro* micronucleus assay (Antonik, J., 2015) and *in vitro* mammalian cell gene mutation

test (Smagur, J., 2015) support the lack of genotoxic potential of the metabolite IN-A4098 in regards to the mammalian cells.

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 The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)	Smagur J., 2015
In vitro evaluation of IN-A4098, IN-L9223 and IN-L9225 genotoxicity using the micronucleus assay (MNA)	non-genotoxic	Yes The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)	Antonik, J., 2015

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). However, a genotoxic potential of metabolite IN-L5296 could not be excluded.
Additionally to this information, the applicant submitted 3 negative studies on bacterial and mammal gene mutation as well as on chromosome damage - bacterial reversion mutation test (De la Torre S., 2019), *in vitro* chromosome aberrations test using Chinese Hamster Ovary cells (CHO) (Peroche A., 2019) and *in vitro* mammalian cell gene mutation test (Savineau C., 2019) supporting the lack of genotoxic potential of this metabolite.

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 The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)	De la Torre, S., 2019
In vitro chromosome aberrations test using Chinese Hamster Ovary cells (OECD 473)	non-genotoxic	Yes The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-	Peroche A., 2019

Type of test, species (Guideline)	Result	Acceptability	Reference
		OR2C)	
In vitro mammalian cell gene mutation test (OECD 490)	non-genotoxic	Yes The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)	Savineau C., 2019

6.5 Dermal Absorption (KCP 7.3)

A summary of the dermal absorption rates for the active substances in HAKSAR TOP 565 SG are presented in the following table.

Table 6.5-1: Dermal absorption rates for active substances in HAKSAR TOP 565 SG

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

6.5.1 Justification for proposed values - MCPA

No data on dermal absorption for MCPA in HAKSAR TOP 565 SG is available. Justifications for default values according to Guidance on Dermal Absorption (EFSA Journal 2017;15(6):4873) are presented in the following table.

Table 6.5-2: Default dermal absorption rates for MCPA

	Value	Justification for value	Acceptability of justification
Concentrate	10%	Default dermal absorption value from EFSA Journal 2017;15(6):4873 for soluble granules (SG).	Yes
Dilution	50%	Default dermal absorption value from EFSA Journal 2017;15(6):4873 for dilution of soluble granules (SG).	Yes

6.5.2 Justification for proposed values - Tribenuron-methyl

No data on dermal absorption for Tribenuron-methyl in HAKSAR TOP 565 SG is available. Justifications for default values according to Guidance on Dermal Absorption (EFSA Journal 2017;15(6):4873) are presented in the following table.

Table 6.5-3: Default dermal absorption rates for Tribenuron-methyl

	Value	Justification for value	Acceptability of justification
Concentrate	50%	Default dermal absorption value from EFSA Journal 2017;15(6):4873 for soluble granules taking in to account also document SANTE/2018/10591 rev.1, where definition of "concentrate" and "dilution" was stated (a "dilution" when the active substance is present in the plant protection product at a concentration lower than or equal to 50 g/L (or 50g/Kg or 5%)). Concentration of Tribenuron-methyl in Tribenuron-methyl is lower than this limit therefore default value 50% as for dilution will be used also for concentrate.	Yes
Dilution	50%	Default dermal absorption value from EFSA Journal 2017;15(6):4873 for dilution of soluble granules (SG).	YES

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	Tribenuron-methyl	
Formulation type	SG	
Category	Herbicide	
Active substance(s) (incl. content)	MCPA 550 g/kg	Tribenuron-methyl 15 g/kg
AOEL systemic	0.04 mg/kg bw/d	0.05 mg/kg bw/d
AOEL	-	0.13 mg/kg bw/d
Inhalation absorption	100%	100%
Oral absorption	100%	67%
Dermal absorption	Concentrate: 10%	Concentrate: 50%

	Dilution: 50 % (Default)	Dilution: 50 % (Default)

6.6.1 Selection of critical use(s) and justification

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

Justification

Critical GAP was selected due to the highest application rates recommended for use in crops, i.e. 1 kg of the product/ha which is equal to 550 g of MCPA/ha and 15 g of Tribenuron-methyl/ha.

6.6.2 Operator exposure (KCP 7.2.1)

Comments of zRMS:	The operator exposure calculations for the proposed uses of HAKSAR TOP 565 SG conducted by the Applicant using the EFSA calculator and presented in Tables 6.6-3 and 6.6-4 are accepted. The reference value acutely toxic active substance (RVAAS) for Tribenuron-methyl is determined. Therefore the predicted acute operator exposure for Tribenuron-methyl was calculated. The estimated acute operator exposure during application for cereals and grasslands via tractor mounted boom sprayer is within acceptable limit when operator uses working wear (48.08% of the AAOEL for Tribenuron-methyl). The predicted longer term systemic operator exposure for application of the product via tractor mounted boom sprayer is within acceptable limit **when operator uses working wear and PPE (gloves) during mixing and loading** (95.73% of the AOEL for MCPA and 3.65% of the AOEL for Tribenuron-methyl). The exposure is further reduced when operator uses gloves also at the application step (16,09% of the AOEL for MCPA and 2.40% of the AOEL for Tribenuron-methyl). Taking also into consideration the classification of the product (H318 - Causes serious eye damage), additionally eye protection is recommended for operators.

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 HAKSAR TOP 565 SG according to the critical use(s) is presented in Table 6.6-2. Acute exposure calculations are only available for Tribenuron-methyl because AAOEL value was set for this active substance. 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. 1 kg product/ha) Grasslands (max. 1 kg product/ha)
Model(s)	Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products; EFSA Journal 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**
Cereals and grasslands Tractor mounted boom spray application outdoors to low crops			
Application rate		0.015 kg a.s./ha	
Spray application (AOEM**;** 95th percentile) Body weight: 60 kg	no PPE*	0.1879372	144.57
	Working wear	0.0625058	48.08

* Potential exposure

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

		MCPA		**Tribenuron-methyl**	
Model data	**Level of PPE**	**Total absorbed dose (mg/kg/day)**	**% of systemic AOEL**	**Total absorbed dose (mg/kg/day)**	**% of systemic AOEL**
Cereals and grasslands Tractor mounted boom spray application outdoors to low crops					
Application rate		0.550 kg a.s./ha		0.015 kg a.s./ha	
Spray application (AOEM**;** 75th percentile) Body weight: 60 kg	no PPE*	0.1051207	262.80	0.0192587	38.52
	Working wear	0.0660725	165.18	0.0104669	20.93
	PPE, Working wear & gloves during mixing and loading	0.0382906	95.73	0.0018269	3.65
	PPE, Working wear & gloves during mixing and application	0.0064357	16.09	0.0012019	2.40

* Potential exposure

6.6.2.2 Measurement of operator exposure

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

6.6.3 Worker exposure (KCP 7.2.3)

Comments of zRMS:	The worker exposure calculations for the proposed uses of HAKSAR TOP 565 SG conducted by the Applicant using the EUROPOEM II model and the EFSA calculator and presented in Table 6.6-7 are accepted. The potential worker exposure undertaking crop inspection activity is within acceptable limit assuming workers are wearing workwear (arms, body and legs covered). The obtained values calculated using both models are below the AOEL values. According to the calculations from EUROPOEM II model the use of gloves further reduces the worker exposure. Therefore, taking also into consideration the classification of the product (H315 - Causes skin irritation, H317 - May cause an allergic skin reaction)), the use of protective gloves is recommended for workers. As a standard rule, crops treated by HAKSAR TOP 565 SG should not be re-entered before spray deposit on leaf surfaces has completely dried. Note: In Table 6.6-7 the Applicant presented results obtained for MCPA and Tribenuron-methyl using EUROPOEM II model with the value TC of 12 500 cm^2/person/h, but the spreadsheets with calculations were not submitted. Evaluator performed and confirmed these calculations taking into consideration 60 kg bw (one value was corrected).

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 HAKSAR TOP 565 SG according to the critical use(s). Out-come 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. 1 kg product/ha) Grasslands (max. 1 kg product/ha)
Model	Post-Application Exposure of Workers to Pesticides in Agriculture – Report of the Re-entry Working Group. EUROPOEM II Project, FAIR3-CT96-1406. December 2002
	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 6.6-6: Estimated worker exposure (longer term exposure)

Cereals					
		MCPA		**Tribenuron-methyl**	
Model data	**Level of PPE**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
EUROPOEM II model Inspection Outdoor Work rate: 2 hours/day, DT50: ~~8~~ 30 days for MCPA DT50: 30 days for Tribenuron-methyl DFR: 3 µg/cm2/kg a.s./ha Interval between treatments: above 365 days					
Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	
Body weight: 60 kg	Potential TC: 12500 cm^2/person/h	~~0.0021~~ 0.3438	859	0.00938	19
	Work wear (arms, body and legs covered) TC: 1400 cm^2/person/h	0.0385	96	0.00105	2
	Work wear (arms, body and legs covered) TC: 1400 cm^2/person/h **DFR_0 = 1.76 µg/cm2/kg MCPA/ha**	0.0226	56	~~0.00105~~ n.a~~.~~	~~2~~ n.a
	Work wear (arms, body and legs covered) and gloves TC: - cm^2/person/h The use of gloves will result extra reduction	0.0077	19	0.00021~~6~~	0

	factor of 5				
EFSA calculator Inspection Outdoor Work rate: 2 hours/day, DT50: ~~8~~ 30 days for MCPA DT50: 30 days for Tribenuron-methyl DFR: 3 µg/cm2/kg a.s./ha Interval between treatments: above 365 days					
Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	
Body weight: 60 kg	Potential TC: 12500 cm^2/person/h	0.3437500	859.38	0.0093450	18.75
	Work wear (arms, body and legs covered) TC: 1400 cm^2/person/h	0.03850	96.25	0.0010500	2.10
	Work wear (arms, body and legs covered) TC: 1400 cm^2/person/h **DFR_0 = 1.76 µg/cm^2/kg MCPA/ha**	0.0225867	56.47	~~0.0010500~~ n.a.	~~2.10~~ n.a.
	Work wear (arms, body and legs covered) and gloves TC: - cm^2/person/h TC not available for this assessment	n.a.	n.a.	n.a.	n.a.

6.6.3.2 Refinement of generic DFR value (KCP 7.2)

Default value 3 µg/cm^2/kg a.s./ha was applied for calculations for both active substance. In addition DFR_0 of 1.76 µg/cm^2/kg a.s./ha for MCPA was used in the refinement step. For further details please refer to the study summary presented in Appendix 4.

6.6.3.3 Measurement of worker exposure

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

6.6.4 Resident and bystander exposure (KCP 7.2.2)

Comments of zRMS:	The bystander and resident exposure calculations for the proposed uses of HAKSAR TOP 565 SG conducted by the Applicant using the EFSA calculator presented in Tables 6.6-9 and 6.6-10 are accepted. Resident exposure: In case of grassland the predicted longer term exposure to a child and adult resident from spray drift, vapour, surface deposits, re-entry into treated crops and sum of all pathways calculated for MPCA and Tribenuron-methyl are within acceptable limits. Therefore the use of HAKSAR TOP 565 SG does not cause unacceptable health risk. In case of using the HAKSAR TOP 565 SG containing MPCA and Tribenuron-methyl for cereals the predicted longer term exposure to a child and adult resident from spray drift, vapour, surface deposits, re-entry into treated crops and sum of all pathways are within acceptable limits **after refinement** (DFR: 1.76 µg/cm^2/kg a.s./ha for MCPA) **when drift reduction technology is used or 5-meter buffer zone is applied.** Bystander exposure: For Tribenuron-methyl the reference value of acutely toxic active substance (RVAAS) is determined. Therefore the acute bystander exposure for Tribenuron-methyl was calculated. The estimated acute bystander exposure to a child and adult from spray drift, vapour, surface deposits, re-entry into treated crops for Tribenuron-methyl for both proposed uses (cereals and grasslands) is within acceptable limits. The reference value of acutely toxic active substance (RVAAS) for MPCA is not determined, therefore it is assumed that bystander exposure is covered by the resident exposure assessment for MPCA. Concluding, the bystander exposure for HAKSAR TOP 565 SG containing MPCA and Tribenuron-methyl is covered by resident exposure assessment.

6.6.4.1 Estimation of resident and bystander exposure

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

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

Acute exposure calculations are only available for Tribenuron-methyl because AAOEL value was set for this active substance.

Table 6.6-7 shows the exposure model(s) used for estimation of resident and bystander exposure to MCPA and Tribenuron-methyl. The outcome of the estimation is presented in

Grasslands		**MCPA**		**Tribenuron-methyl**	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no DT50: ~~8~~ 30 days for MCPA DT50: 30 days for Tribenuron-methyl DFR: 3 µg/cm2/kg a.s./ha Interval between treatments: 365 days					
Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.03692298	92.32	0.0010072	2.01
	Vapour (75th perc.)	0.0010700	2.68	0.0010700	2.14
	Deposits (75th perc.)	0.0044506	11.13	0.0001174	0.23
	Re-entry (75th perc.)	0.0123406	30.85	0.0003059	0.61
	Sum (mean)	0.0336077	**84.02**	0.0019544	**3.91**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0088367	22.09	0.0002410	0.48
	Vapour (75th perc.)	0.0002300	0.58	0.0002300	0.46
	Deposits (75th perc.)	0.0018737	4.68	0.0000511	0.10
	Re-entry (75th perc.)	0.0041823	10.46	0.0001141	0.23
	Sum (mean)	0.0099821	**24.96**	0.0004960	**0.99**

Table 6.6-99 (longer term resident exposure) and Table 6.6-810 (acute bystander exposure for Tribenuron-methyl). Detailed calculations are in Appendix 3.

Table 6.6-7: Exposure models for intended uses

Critical use(s)	Cereals (max. 1 kg product/ha) Grasslands (max. 1 kg product/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)

Cereals		**MCPA**		**Tribenuron-methyl**	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
Tractor mounted boom spray application outdoors to low crops					

Buffer zone: 2-3 (m)
Drift reduction technology: no
DT50: ~~8~~ 30 days for MCPA
DT50: 30 days for Tribenuron-methyl
DFR: 3 µg/cm2/kg a.s./ha
Interval between treatments: 365 days

Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.03692298	92.32	0.0010072	2.01
	Vapour (75th perc.)	0.0010700	2.68	0.0010700	2.14
	Deposits (75th perc.)	0.0044506	11.13	0.0001174	0.23
	Re-entry (75th perc.)	0.0464063	116.02	0.0012656	2.53
	Sum (mean)	0.616748	**154.18**	**0.0027198**	**5.44**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0088367	22.06	0.0002410	0.48
	Vapour (75th perc.)	0.0002300	0.58	0.0002300	0.46
	Deposits (75th perc.)	0.0018737	4.68	0.0000511	0.10
	Re-entry (75th perc.)	0.0257813	64.45	0.0007031	1.41
	Sum (mean)	**0.0263561**	**65.89**	**0.0009425**	**1.89**

Tractor mounted boom spray application outdoors to low crops
Buffer zone: 2-3 (m)
Drift reduction technology: no
DT50: ~~8~~ 30 days for MCPA
DT50: 30 days for Tribenuron-methyl
DFR: 1.76 µg/cm2/kg a.s./ha for MCPA
DFR: 3 µg/cm2/kg a.s./ha for Tribenuron-methyl
Interval between treatments: 365 days

Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.0369298	92.32	0.0010072	2.01
	Vapour (75th perc.)	0.0010700	2.68	0.0010700	2.14
	Deposits (75th perc.)	0.0044506	11.13	0.0001174	0.23
	Re-entry (75th perc.)	0.0272250	68.06	0.0012656	2.53
	Sum (mean)	**0.0463776**	**115.~~09~~94**	**0.0027198**	**5.44**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0088367	22.09	0.0002410	0.48
	Vapour (75th perc.)	0.0002300	0.58	0.0002300	0.46
	Deposits (75th perc.)	0.0018737	4.68	0.0000511	0.10
	Re-entry (75th perc.)	0.0151250	37.81	0.0007031	1.41
	Sum (mean)	**0.0178595**	**44.65**	**0.0009425**	**1.89**

Tractor mounted boom spray application outdoors to low crops
Buffer zone: 2-3 (m)
Drift reduction technology: yes
DT50: ~~8~~ 30 days for MCPA
DT50: 30 days for Tribenuron-methyl
DFR: 1.76 µg/cm2/kg a.s./ha for MCPA
DFR: 3 µg/cm2/kg a.s./ha for Tribenuron-methyl
Interval between treatments: 365 days

Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.0184649	46.16	0.0005036	1.01
	Vapour (75th perc.)	0.0010700	2.68	0.0010700	2.14
	Deposits (75th perc.)	0.0022253	5.56	0.0000587	0.12
	Re-entry (75th perc.)	0.0272250	68.06	0.0012656	2.53
	Sum (mean)	**0.0345775**	**86.44**	**0.0023995**	**4.80**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0044183	11.05	0.0001205	0.24
	Vapour (75th perc.)	0.0002300	0.58	0.0002300	0.46
	Deposits (75th perc.)	0.0009368	2.34	0.0000256	0.05
	Re-entry (75th perc.)	0.0151250	37.81	0.0007031	1.41
	Sum (mean)	**0.0150746**	**37.69**	**0.0008666**	**1.73**
Tractor mounted boom spray application outdoors to low crops Buffer zone: 5 (m) Drift reduction technology: no DT50: ~~8~~ 30 days for MCPA DT50: 30 days for Tribenuron-methyl DFR: 1.76 µg/cm2/kg a.s./ha for MCPA DFR: 3 µg/cm2/kg a.s./ha for Tribenuron-methyl Interval between treatments: 365 days					
Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	
Resident child Body weight: 10 kg	Drift (75th perc.)	0.0245699	61.42	0.0006701	1.34
	Vapour (75th perc.)	0.0010700	2.68	0.0010700	2.14
	Deposits (75th perc.)	0.0018279	4.57	0.0000482	0.10
	Re-entry (75th perc.)	0.0272250	68.06	0.0012656	2.53
	Sum (mean)	**0.0377765**	**94.44**	**0.0024869**	**4.97**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0044762	11.19	0.0001205	0.24
	Vapour (75th perc.)	0.0002300	0.58	0.0002300	0.46
	Deposits (75th perc.)	0.0007695	1.92	0.0000210	0.04
	Re-entry (75th perc.)	0.0151250	37.81	0.0007031	1.41
	Sum (mean)	**0.0152028**	**38.01**	**0.0008701**	**1.74**
Grasslands		**MCPA**		**Tribenuron-methyl**	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**	**Total absorbed dose (mg/kg bw/day)**	**% of systemic AOEL**
Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no DT50: ~~8~~ 30 days for MCPA DT50: 30 days for Tribenuron-methyl DFR: 3 µg/cm2/kg a.s./ha Interval between treatments: 365 days					
Number of applications and application rate		1×0.550 kg a.s./ha		1×0.015 kg a.s./ha	

Resident child Body weight: 10 kg	Drift (75th perc.)	0.03692298	92.32	0.0010072	2.01
	Vapour (75th perc.)	0.0010700	2.68	0.0010700	2.14
	Deposits (75th perc.)	0.0044506	11.13	0.0001174	0.23
	Re-entry (75th perc.)	0.0123406	30.85	0.0003059	0.61
	Sum (mean)	0.0336077	**84.02**	0.0019544	**3.91**
Resident adult Body weight: 60 kg	Drift (75th perc.)	0.0088367	22.09	0.0002410	0.48
	Vapour (75th perc.)	0.0002300	0.58	0.0002300	0.46
	Deposits (75th perc.)	0.0018737	4.68	0.0000511	0.10
	Re-entry (75th perc.)	0.0041823	10.46	0.0001141	0.23
	Sum (mean)	0.0099821	**24.96**	0.0004960	**0.99**

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

Cereals		**Tribenuron-methyl**	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AAOEL**
Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no			
Application rate		0.015 kg a.s./ha	
Bystander child Body weight: 10 kg	Drift (95th perc.)	0.0022839	1.76
	Vapour (95th perc.)	0.0010700	0.82
	Deposits (95th perc.)	0.0003529	0.27
	Re-entry (95th perc.)	0.0012656	0.97
Bystander adult Body weight: 60 kg	Drift (95th perc.)	0.0006208	0.48
	Vapour (95th perc.)	0.0002300	0.18
	Deposits (95th perc.)	0.0001541	0.12
	Re-entry (95th perc.)	0.0007031	0.54
Grasslands		**Tribenuron-methyl**	
Model data		**Total absorbed dose (mg/kg bw/day)**	**% of systemic AAOEL**
Tractor mounted boom spray application outdoors to low crops Buffer zone: 2-3 (m) Drift reduction technology: no			
Application rate		0.015 kg a.s./ha	
Bystander child Body weight: 10 kg	Drift (95th perc.)	0.0022839	1.76
	Vapour (95th perc.)	0.0010700	0.82
	Deposits (95th perc.)	0.0003529	0.27
	Re-entry (95th perc.)	0.0005629	0.43

Bystander adult Body weight: 60 kg	Drift (95th perc.)	0.0006208	0.48
	Vapour (95th perc.)	0.0002300	0.18
	Deposits (95th perc.)	0.0001541	0.12
	Re-entry (95th perc.)	0.0002266	0.17

The estimations performed according to EFSA calculator indicate that the systemic exposure of resident (adult and children) to Tribenuron-methyl and MCPA contained in the formulation HAKSAR TOP 565 SG does not exceed the value of AOEL for these active substances. In case of use in cereals however, the drift reduction nozzles or 5-meter buffer zone should be applied.

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 exposure level (AOEL) for MCPA and Tribenuron-methyl will not be exceeded under conditions of intended uses and considering above mentioned risk mitigation measures, a study to provide measurements of resident/bystander exposure was not necessary and was therefore not performed.

6.6.5 Combined exposure

The product is a mixture of two active substances.

6.6.5.1 Exposure assessment of MCPA and Tribenuron-methyl in HAKSAR TOP 565 SG

Comments of zRMS:	The combined exposure calculations for operator, workers and residents conducted by the Applicant presented in Table 6.6-13 are accepted. The Hazard Index is < 1, therefore combined exposure to all active substances in HAKSAR TOP 565 SG is not expected to present a risk for operators and workers and residents. The exposure assessment for residents also covers bystander exposure, therefore combined exposure is also not expected for bystanders.

At the first tier, combined exposure is calculated as the sum of the component exposures without regard to the mode of action or mechanism/target of toxicity. Initially, the individual Hazard Quotients (HQ) are calculated for all active substances in the PPP by assessing the exposure according to appropriate models and dividing the individual exposure levels by the respective systemic AOEL. This is equivalent to the predicted exposure as % of systemic AOEL from Table 6.6 3 converted to decimal. The Hazard Index (HI) is the sum of the individual HQs.

Table 6.6-10: Risk assessment from combined exposure (longer term exposure)

Application scenario	Active ingredient	Estimated exposure / AOEL (HQ)
Operators (workwear, gloves during mixing and loading) Cereals and grasslands	MCPA	0.9573
	Tribneuron-methyl	0.0365
	Cumulative risk operators (HI)	**0.9938**
Workers – inspection (workwear) Cereals and grasslands	MCPA	0.9625
	Tribneuron-methyl	0.0210
	Cumulative risk workers (HI)	**0.9835**
Resident – child 5 m buffer strip* Cereals	MCPA	
	Drift	0.6142
	Vapour	0.0268
	Deposits	0.0457
	Re-entry	0.6806
	Sum of all pathways	0.9444
	Tribneuron-methyl	
	Drift	0.0134
	Vapour	0.0214
	Deposits	0.001
	Re-entry	0.0253
	Sum of all pathways	0.0497
	Cumulative risk resident – child (HI)	
	Drift	**0.6276**
	Vapour	**0.0482**
	Deposits	**0.0467**
	Re-entry	**0.7059**
	Sum of all pathways	**0.9941**
Resident – adult 5 m buffer strip* Cereals	MCPA	
	Drift	0.1119
	Vapour	0.0058
	Deposits	0.0192
	Re-entry	0.3781
	Sum of all pathways	0.3801
	Tribenuron-methyl	
	Drift	0.0024
	Vapour	0.0046
	Deposits	0.0004

Application scenario	Active ingredient	Estimated exposure / AOEL (HQ)
	Re-entry	0.0141
	Sum of all pathways	0.0174
	Cumulative risk resident – adult (HI)	
	Drift	**0.1143**
	Vapour	**0.0104**
	Deposits	**0.0196**
	Re-entry	**0.3922**
	Sum of all pathways	**0.3975**
Resident – child Grasslands	MCPA	
	Drift	0.9232
	Vapour	0.0268
	Deposits	0.1113
	Re-entry	0.3085
	Sum of all pathways	**0.8402**
	Tribneuron-methyl	
	Drift	0.0201
	Vapour	0.0214
	Deposits	0.0023
	Re-entry	0.0061
	Sum of all pathways	**0.0391**
	Cumulative risk resident – child (HI)	
	Drift	**0.9433**
	Vapour	**0.0482**
	Deposits	**0.1136**
	Re-entry	**0.3146**
	Sum of all pathways	**0.8793**
Resident – adult Grasslands Cereals	MCPA	
	Drift	0.2209
	Vapour	~~0.2209~~0.0058
	Deposits	~~0.0058~~0.0468
	Re-entry	~~0.0468~~0.1064
	Sum of all pathways	~~0.1064~~0.2496
	Tribenuron-methyl	
	Drift	0.0048
	Vapour	0.0046

Application scenario	Active ingredient	Estimated exposure / AOEL (HQ)
	Deposits	0.001
	Re-entry	0.0023
	Sum of all pathways	0.0099
	Cumulative risk resident – adult (HI)	
	Drift	**0.2257**
	Vapour	**0.0104**
	Deposits	**0.0478**
	Re-entry	**0.1087**
	Sum of all pathways	**0.2595**

* Covers the HQ for use in cereals with drift reduction

The Hazard Index is < 1. Thus, combined exposure to all active substances in HAKSAR TOP 565 SG is not expected to present a risk for operators, workers, residents and bystanders. No further refinement of the assessment is required.

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	Verte-brate study Y/N	Owner
KCP 7.1.7/01	Smagur 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	N	TF PROPLAN CHEMIROL SARABIA
KCP 7.1.7/02	Antonik 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	N	TF PROPLAN CHEMIROL SARABIA
KCP 7.1.7/03	De la Torre S.	2019	Bacterial reversion mutation test VIVOTECNIA Research Report Nº: B-02756 GLP, Unpublished	N	TF PROPLAN CHEMIROL SARABIA
KCP 7.1.7/04	Peroche A.	2019	In vitro chromosome aberrations test using Chinese Hamster Ovary cells (CHO) with Amendment LEMI Report Nº: ABC4-LM-18/0293 GLP, Unpublished	N	TF PROPLAN CHEMIROL SARABIA
KCP 7.1.7/05	Savineau C.	2019	In vitro mammalian cell gene mutation test with Amendment LEMI Report Nº: MLAl-LM-18/0293 GLP, Unpublished	N	TF PROPLAN CHEMIROL SARABIA

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
KCP 7.2	Cirka H.	2020	Foliar dislodgeable residues dissipation in cereals after one application with the herbicide MT-565SG-OR2-C (focussed on the active substance MCPA) in Germany 2020 – Field part Report CT19-1-59 GLP, Unpublished	N	CIECH Sarzyna S.A.

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

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 7.1.7/01	Smagur 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	N	TF PROPLAN-CHEMIROL-SARABIA
KCP 7.1.7/02	Antonik 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	N	TF PROPLAN-CHEMIROL-SARABIA
KCP 7.1.7/03	De la Torre S.	2019	Bacterial reversion mutation test VIVOTECNIA Research Report Nº: B-02756 GLP, Unpublished	N	TF PROPLAN-CHEMIROL-SARABIA

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 7.1.7/04	Peroche A.	2019	In vitro chromosome aberrations test using Chinese Hamster Ovary cells (CHO) with Amendment LEMI Report Nº: ABC4-LM-18/0293 GLP, Unpublished	N	TF PROPLAN-CHEMIROL-SARABIA
KCP 7.1.7/05	Savineau C.	2019	In vitro mammalian cell gene mutation test with Amendment LEMI Report Nº: MLAl-LM-18/0293 GLP, Unpublished	N	TF PROPLAN-CHEMIROL-SARABIA

Appendix 2 Detailed evaluation of the studies relied upon

A 2.1 Statement on bridging possibilities

Bringing is not necessary since the toxicological potential of HAKSAR TOP 565 SG can be predicted on the basis of toxicological data available for active substances and co-formulants included in composition of above-mentioned product.

Comments of zRMS:	Explanation accepted

A 2.2 Acute oral toxicity (KCP 7.1.1)

Comments of zRMS:	Accepted. According to the Regulation (EC) No 1107/2009, Regulation (EC) 1272/2008, Regulation (EC) 1907/2006 animal testing should be minimised. Moreover, according to the Council Directive 86/609/EEC an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available. Therefore justification provided by Applicant is acceptable. Acute oral toxicity was determined taking into consideration valid data available on each of the components in the mixture. HAKSAR TOP 565 SG contains one component classified as Acute Tox. 4 with hazard statement H302 at concentration of 55%. Only for one component (5% of concentration) there is not available information on acute oral toxicity. For remain compounds LD_{50} is > 2000 mg/kg bw. Therefore the product HAKSAR TOP 565 SG should be classified as Acute Tox. 4 with hazard statement H302 according to Regulation (EC) No. 1272/2008. Note: LD_{50} in MSDS of MCPA is 500-797 mg/kg bw (no impact on conclusions)

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 account data for components which are classified to acute oral toxicity class and significant concentration. Only the active substance MCPA is classified as Acute Tox. 4, H302.
Its concentration in the product is equal to 55% and LD_{50}=962 mg/kg bw (according to SANCO/4062/2001-final). The LD 50 of the second active substance that is tribenuron methyl >5000 mg/kg bw. Based on MSDS of other co-formulants, the acute oral LD_{50} values of 38.5% components is above 2000 mg/kg bw. For one of the components the exact LD_{50} value is not stated in MSDS but these components is not classified

by producer (the concentration is equal to 5%).

Therefore, ATE_{mix} value is:

$$\text{The } ATE_{mix} = \frac{100}{\frac{55}{962}} = \sout{1754}\ 1749 \text{ mg/kg}$$

The estimated value ATE_{mix} of acute oral toxicity for HAKSAR TOP 565 SG is equal to ~~1754~~ 1749 mg/kg. Therefore, the product should be classified as Acute Tox. 4 with hazard statement H302. No additional studies are required.

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

Comments of zRMS:	Accepted. According to the Regulation (EC) No 1107/2009, Regulation (EC) 1272/2008, Regulation (EC) 1907/2006 animal testing should be minimised. Moreover, according to the Council Directive 86/609/EEC an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available. Therefore justification provided by Applicant is acceptable. Acute dermal toxicity was determined taking into consideration valid data available on each of the components in the mixture. HAKSAR TOP 565 SG does not contain any component which is classified as acute dermal toxic, therefore the product HAKSAR TOP 565 SG does not required to be classified in this category according to Regulation (EC) No. 1272/2008.

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

Based on MSDS of co-formulants, the acute dermal LD_{50} values of 80.5% components is above 2000 mg/kg bw, for other components the exact LD_{50} value is not stated in MSDS but these components are not classified by manufacturer as toxic or harmful by dermal contact with hazards statement H310, H311 or H312.

HAKSAR TOP 565 SG does not contain any component which is classified as Acute Tox. with the hazard statement H310, H311,H312 therefore, the product will not be classified in this category. No additional studies are required.

A 2.4 Acute inhalation toxicity (KCP 7.1.3)

Comments of zRMS:	Accepted. According to the Regulation (EC) No 1107/2009, Regulation (EC) 1272/2008, Regulation (EC) 1907/2006 animal testing should be minimised. Moreover, according to the Council Directive 86/609/EEC an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available. Therefore justification provided by Applicant is acceptable. Acute inhalation toxicity was determined taking into consideration valid data available on each of the components in the mixture. HAKSAR TOP 565 SG does not contain any component which is classified as acute inhalation toxic, therefore the product HAKSAR TOP 565 SG does not required to be classified in this category according to Regulation (EC) No. 1272/2008.

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 account data for components which are classified to acute inhalation toxicity class and significant concentration. Based on MSDS of co-formulants, the acute inhalation LD_{50} values of ~~79~~56.5% components is above short-cut values stated in table 3.1.2 of regulation 1272/2008, for other components the exact LD_{50} value is not stated in MSDS but these components are not classified by manufacturer as toxic or harmful by inhalation with hazards statement H330, H331 or H332

HAKSAR TOP 565 SG does not contain any component which is classified as Acute Tox. with the hazard statement H330, H331, H332 therefore, the product will not be classified in this category. No additional studies are required.

A 2.5 Skin irritation (KCP 7.1.4)

Comments of zRMS:	Accepted. According to the Regulation (EC) No 1107/2009, Regulation (EC) 1272/2008, Regulation (EC) 1907/2006 animal testing should be minimised. Moreover, according to the Council Directive 86/609/EEC an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available. Therefore justification provided by Applicant is acceptable. Skin irritation was determined taking into

	consideration valid data available on each of the components in the mixture. HAKSAR TOP 565 SG contains two components classified as Skin Irrit. 2 with hazard statement H315 at concentrations of 55% and 5%. Therefore the product HAKSAR TOP 565 SG should be classified as Skin Irrit. 2 with hazard statement H315 according to Regulation (EC) No. 1272/2008.

A skin irritation potential of HAKSAR TOP 565 SG can be estimated according to principles of Regulation 1272/2008 by using additivity approach.

None of components of the product HAKSAR TOP 565 SG 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 of the components of product are classified as Skin Irrit. 2 with hazard statement H315. Their concentrations in the product are equal to 55% and 5%. The sum of concentration is above concentration limit (10%) stated in Table 3.2.3 of Regulation 1272/2008, therefore the product will be classified as Skin Irrit. 2 with hazard statement H315.
No skin corrosion/irritation study is necessary for HAKSAR TOP 565 SG.

A 2.6 Eye irritation (KCP 7.1.5)

Comments of zRMS:	Accepted. According to the Regulation (EC) No 1107/2009, Regulation (EC) 1272/2008, Regulation (EC) 1907/2006 animal testing should be minimised. Moreover, according to the Council Directive 86/609/EEC an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available. Therefore justification provided by Applicant is acceptable. Eye irritation was determined taking into consideration valid data available on each of the components in the mixture. HAKSAR TOP 565 SG contains two components classified as Eye Irrit. 2 with hazard statement H319 at concentrations of 24% and 3% and two components classified as Eye Dam. 1 with hazard statement H318 at concentrations of 55% and 5% Therefore the product HAKSAR TOP 565 SG should be classified as Eye Dam. 1with hazard statement H318 according to Regulation (EC) No. 1272/2008

An eye irritation potential of HAKSAR TOP 565 SG can be estimated according to principles of Regulation 1272/2008 by using additivity approach.
Two of the components of product HAKSAR TOP 565 SG are classified as Eye Irrit. 2 with hazard statement H319. Their concentrations in the product are equal to 24% and 3%.
Moreover, two of the components of product HAKSAR TOP 565 SG are classified as Eye Dam. 1 with hazard statement H318. Their concentrations in the product are equal to 55% and 5% respectively. This concentration is above concentration limit (3%) stated in Table 3.3.3 of Regulation 1272/2008, therefore the product HAKSAR TOP 565 SG will be classified as Eye Dam. 1 with hazard statement H318.

A 2.7 Skin sensitisation (KCP 7.1.6)

Comments of zRMS:	Accepted. According to the Regulation (EC) No 1107/2009, Regulation (EC) 1272/2008, Regulation (EC) 1907/2006 animal testing should be minimised. Moreover, according to the Council Directive 86/609/EEC an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available. Therefore justification

	provided by Applicant is acceptable. Skin sensitisation potential of HAKSAR TOP 565 SG was determined taking into consideration valid data available on each of the components in the mixture. HAKSAR TOP 565 SG contains one component classified as skin sensitiser category 1with hazard statement H317 at concentrations of 1.5% (above concentration limit of 1%). Therefore the product HAKSAR TOP 565 SG should be classified as Skin Sens. 1 with hazard statement H317 according to Regulation (EC) No. 1272/2008.

A skin or respiratory sensitisation potential of HAKSAR TOP 565 SG 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.
The active substance tribenuron-methyl is classified as skin sensitiser category 1 (Skin Sens. 1, H317) and is present in the product in amount of approx. 1.5%. This concentration is above concentration limit of 1%. There is no need to perform additional skin sensitisation study to confirm that HAKSAR TOP 565 SG will have skin sensitisation potential.
Additionally, no of components of HAKSAR TOP 565 SG have been classified as a respiratory sensitiser and therefore the above product will not have such potential.

In conclusion HAKSAR TOP 565 SG should be classified as skin sensitiser category 1 (Skin Sens. 1, H317).

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

Not applicable.

A 2.9 Data on co-formulants (KCP 7.4)

A 2.9.1 Material safety data sheet for each co-formulant

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

A 2.9.2 Available toxicological data for each co-formulant

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

A 2.10 Studies on dermal absorption (KCP 7.3)

Comments of zRMS:	Justification accepted.

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

A 2.11 Other/Special Studies (KCP 7.1.7)

A 2.11.1 *In vitro* mammalian cell gene mutation test for metabolite IN-A4098 (KCP 7.1.7/01)

Report:	**Smagur, 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

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.

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)

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	24h

0.12; 0.04; 0.013; 0.004 [mM]		
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:

$$\mathbf{P=-\ln(EW/TW)}$$

where EW is empty wells and TW is total wells.

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

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

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:

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

from the formula for PE and with the knowledge that 2×10^3 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:

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

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. Table 6 shows the detailed information for RSG, RTG, MF and the percentage of small colonies in experimental conditions (+S9).

Table 6 Evaluation of the potential cytotoxic activity of IN-A4098, IN-L9223 and IN-L9225 in the Mouse Lymphoma Assay in the presence of S9 fraction – 4h (initial dose range-finding assay).

Cpd.	Conc.	RSG	RTG	RV	RS	Colony counts	% Small colonies	Mutation Frequency	Fold increase	GEF
PBS	**1%**	1.00	100.00	100.00	1.00	11	9.09	73.75	-	-
Cp	**3µg/mL**	0.62	37.17	59.57	0.75	134	56.72	976.98	13.25	903.24
DMSO 1	**1%**	1.00	90.38	90.38	1.14	54	33.33	132.14	-	-
DMSO 2	**1%**	1.00	109.62	109.62	0.86	28	32.14	82.97	-	-
IN-A4098	**1.07 mM**	0.66	67.41	101.53	0.81	68	41.18	231.41	2.15	123.85
	0.36 mM	0.58	50.84	87.53	0.52	43	58.14	222.00	2.06	114.45
	0.12 mM	0.95	64.57	68.18	0.91	60	50.00	223.50	2.08	115.95
	0.04 mM	0.73	59.00	81.10	0.65	50	50.00	205.58	1.91	98.02
	0.013 mM	0.53	69.28	129.82	1.26	70	42.86	158.05	1.47	50.50
	0.0044 mM	0.77	73.97	95.97	0.74	56	44.64	202.25	1.88	94.70
	0.0015 mM	0.81	78.00	95.97	0.81	64	65.63	201.37	1.87	93.81
IN-L9223	**10.00 mM**	0.37	26.38	71.16	0.65	52	50.00	200.72	1.87	93.16
	3.33 mM	0.80	71.68	90.14	0.89	71	42.25	231.27	2.15	123.72
	1.11 mM	0.44	39.78	90.14	0.72	59	55.93	231.54	2.15	123.99
	0.37 mM	0.48	55.02	113.57	0.63	49	46.94	196.62	1.83	89.07
	0.12 mM	0.55	57.05	104.14	1.04	23	30.43	55.98	0.52	-51.58
	0.04 mM	0.61	57.36	94.46	0.66	55	38.18	227.14	2.11	119.58
	0.01 mM	0.68	86.09	127.22	0.44	40	37.50	232.83	2.16	125.28
IN-L9225	**10.00 mM**	0.54	19.29	35.50	1.09	85	45.88	231.45	2.15	123.90
	3.33 mM	0.49	46.01	92.98	1.18	37	35.14	82.36	0.77	-25.20
	1.11 mM	0.66	82.75	124.72	0.91	44	43.18	130.64	1.21	23.08
	0.37 mM	0.44	33.00	75.38	0.74	55	34.55	202.25	1.88	94.70
	0.12 mM	0.51	56.41	109.62	0.46	34	44.12	194.14	1.81	86.58
	0.04 mM	0.62	62.83	101.53	0.53	37	51.35	184.79	1.72	77.24
	0.01 mM	0.75	104.16	138.38	0.40	34	58.82	220.33	2.05	112.78

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.

Table 7 Evaluation of the potential cytotoxic activity of IN-A4098, IN-L9223 and IN-L9225 in the Mouse Lymphoma Assay in the absence of S9 fraction – 4h (initial dose range-finding assay).

Cpd.	Conc.	RSG	RTG	RV	RS	Colony counts	% Small colonies	Mutation Frequency	Fold increase	GEF
PBS	**1%**	1.00	100.00	100.00	1.00	23	21.74	77.00	-	-
MMS	**14µg/mL**	0.42	54.04	129.36	0.68	97	72.16	527.59	6.85	450.59
DMSO 1	**1%**	1.17	110.73	94.99	1.04	19	57.89	64.68	-	-
DMSO 2	**1%**	0.83	87.61	105.01	0.96	28	25.00	115.16	-	-
IN-A4098	**1.07 mM**	1.13	80.29	70.94	0.89	17	41.18	75.62	0.84	-14.30
	0.36 mM	1.41	183.59	129.82	1.40	31	45.16	84.85	0.94	-5.08
	0.12 mM	1.20	162.60	135.63	0.93	28	46.43	123.22	1.37	33.30
	0.04 mM	0.67	74.39	111.30	1.00	20	40.00	80.10	0.89	-9.82
	0.013 mM	0.98	133.40	135.63	0.93	22	27.27	95.13	1.06	5.21
	0.0044 mM	0.94	111.49	118.08	0.93	25	40.00	104.38	1.16	14.46
	0.0015 mM	0.64	106.80	166.47	0.80	22	54.55	100.52	1.12	10.60
IN-L9223	**10.00 mM**	0.05	1.66	31.10	0.70	39	23.08	229.48	2.55	139.55
	3.33 mM	0.39	32.50	82.34	1.17	47	40.43	153.42	1.71	63,50
	1.11 mM	0.68	87.25	127.39	0.75	20	30.00	106.45	1.18	16.53
	0.37 mM	0.91	90.12	99.14	1.06	20	45.00	71.67	0.80	-18.25
	0.12 mM	1.04	97.75	93.65	1.09	20	40.00	65.79	0.73	-24.13
	0.04 mM	1.18	90.58	76.79	1.24	18	38.89	54.31	0.60	-35.61
	0.01 mM	1.01	100.47	99.14	1.34	23	26.09	72.51	0.81	-17.41
IN-L9225	**10.00 mM**	1.13	105.96	93.65	1.09	32	28.13	77.41	0.86	-12.51
	3.33 mM	1.31	117.31	89.75	1.07	20	35.00	70.65	0.79	-19.27
	1.11 mM	1.12	134.82	119.87	3.01	35	37.14	45.56	0.51	-44.36

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).

Table 8 Evaluation of the potential cytotoxic activity of IN-A4098, IN-L9223 and IN-L9225 in the Mouse Lymphoma Assay in the absence of S9 fraction – 24h (initial dose range-finding assay).

Cpd.	Conc.	RSG	RTG	RV	RS	Colony counts	% Small colonies	Mutation Frequency	Fold increase	GEF
PBS	**1%**	1.00	100.00	100.00	1.00	12	0.00	55.12	-	-
MMS	**5µg/mL**	0.48	27.61	57.90	1.00	151	75.50	937.27	17.01	882.16
DMSO 1	**1%**	1.00	100.00	100.00	1.00	21	47.62	85.56	-	-
DMSO 2	**1%**	1.00	100.00	100.00	1.00	14	64.29	51.57	-	-
IN-A4098	**1.07 mM**	0.005	0.03	6.56			too high cytotoxicity			
	0.36 mM	0.07	1.07	16.15	0.61	24	33.33	132.76	1.94	64.20
	0.12 mM	0.46	46.46	100.00	0.81	27	33.33	109.05	1.59	40.49
	0.04 mM	0.34	33.94	100.00	0.99	34	35.29	124.72	1.82	56.16
	0.013 mM	0.39	39.55	101.68	0.80	17	41.18	73.51	1.07	4.94
	0.0044 mM	0.68	84.42	124.22	0.94	23	34.78	85.27	1.24	16.71
	0.0015 mM	0.44	41.69	95.69	0.67	11	45.45	55.48	0.81	-13.09
IN-L9223	**10.00 mM**	0.01	0.52	81.70	1.41	58	46.55	153.97	2.25	85.40
	3.33 mM	0.31	37.38	120.24	1.24	21	33.33	59.07	0.86	-9.49
	1.11 mM	0.32	32.09	100.00	0.93	25	32.00	90.53	1.32	21.97
	0.37 mM	0.30	27.54	92.23	0.66	15	40.00	77.60	1.13	9.03
	0.12 mM	0.38	41.93	108.93	1.05	30	23.33	98.82	1.44	30.25
	0.04 mM	0.28	22.36	80.38	0.85	12	25.00	47.67	0.70	-20.89
	0.01 mM	0.31	29.23	95.69	0.93	21	42.86	78.53	1.15	9.97
IN-L9225	**10.00 mM**	0.01	0.02	1.99			too high cytotoxicity			
	3.33 mM	0.06	3.06	47.71	0.67	15	33.33	76.49	1.12	7.93
	1.11 mM	0.39	39.43	100.00	0.89	10	30.00	37.86	0.55	-30.71
	0.37 mM	0.23	13.88	59.43	0.73	18	50.00	84.98	1.24	16.41
	0.12 mM	0.26	19.06	73.61	0.64	13	61.54	68.83	1.00	0.27

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. Table 9-11 show the detailed information for RSG, RTG, RV, RS, MF and the percentage of small colonies in both with and without S9 mix.

Table 9 Evaluation of the potential mutagenic activity of IN-A4098, IN-L9223 and IN-L9225 in the Mouse Lymphoma Assay in the presence of S9- 4h (Definitive Mutagenicity Assay).

Cpd.	Conc.	RSG	RTG	RV	RS	Colony counts	% Small colonies	Mutation Frequency	Fold increase	GEF
PBS	**1%**	1.00	100.00	100.00	1.00	20	60.00	51.82	-	-
Cp	**3µg/mL**	0.70	66.47	94.48	0.58	100	64.00	526.70	10.16	**474.88**
DMSO 1	**1%**	1.00	103.36	103.36	0.93	18	61.11	53.64	-	-
DMSO 2	**1%**	1.00	96.64	96.64	1.07	20	60.00	51.70	-	-
IN-A4098	**1.07 mM**	0.85	78.60	92.07	0.68	15	60.00	60.08	1.14	**7.41**
	0.36 mM	0.88	72.90	82.60	0.85	15	40.00	45.02	0.85	**-7.65**
	0.12 mM	0.48	27.39	57.57	1.34	26	46.15	52.59	1.00	**-0.08**
	0.04 mM	0.47	51.73	110.94	0.63	14	57.14	60.89	1.16	**8.21**
IN-L9223	**10.00 mM**	0.53	34.44	64.48	0.59	21	57.14	88.68	1.68	**36.01**
	3.33 mM	0.90	54.84	60.92	0.92	18	33.33	51.12	0.97	**-1.55**
	1.11 mM	0.74	37.95	51.38	0.46	13	38.46	70.18	1.33	**17.51**
	0.37 mM	0.58	45.39	77.81	0.78	8	50.00	24.11	0.46	**-28.56**
IN-L9225	**10.00 mM**	0.39	32.71	83.86	1.04	25	56.00	67.78	1.29	**15.11**
	3.33 mM	0.94	77.70	82.60	1.09	16	31.25	40.21	0.76	**-12.46**
	1.11 mM	0.93	74.89	80.16	0.89	15	53.33	43.04	0.82	**-9.63**
	0.37 mM	0.62	88.14	142.29	0.80	23	39.13	80.47	1.53	**27.80**

Table 10 Evaluation of the potential mutagenic activity of IN-A4098, IN-L9223 and IN-L9225 in the Mouse Lymphoma Assay in the absence of S9- 4h (Definitive Mutagenicity Assay).

Cpd.	Conc.	RSG	RTG	RV	RS	Colony counts	% Small colonies	Mutation Frequency	Fold increase	GEF
PBS	**1%**	1.00	100.00	100.00	1.00	15	46.67	52.82	-	-
MMS	**14µg/mL**	0.78	48.92	62.63	0.43	101	68.32	840.61	15.92	**787.79**
DMSO 1	**1%**	0.91	85.01	93.08	1.04	19	42.11	60.13	-	-
DMSO 2	**1%**	1.09	116.19	106.92	0.96	14	21.43	51.30	-	-
IN-A4098	**1.07 mM**	1.40	144.87	101.99	1.40	22	36.36	52.45	0.94	**-3.27**
	0.36 mM	1.40	119.19	86.46	1.13	18	33.33	52.63	0.94	**-3.09**
	0.12 mM	1.80	167.14	90.36	1.45	21	52.38	45.71	0.82	**-10.01**
	0.04 mM	1.20	107.26	91.71	1.14	15	26.67	42.82	0.77	**-12.90**
IN-L9223	**3.30 mM**	0.80	86.52	101.99	1.01	26	19.23	86.52	1.55	**30.80**
	1.10 mM	1.20	158.87	133.71	0.72	21	28.57	97.19	1.74	**41.47**
	0.37 mM	1.20	143.82	122.13	0.88	10	10.00	36.79	0.66	**-18.93**
	0.12 mM	0.90	106.57	124.29	1.08	15	20.00	45.54	0.82	**-10.17**
IN-L9225	**10.00 mM**	0.80	90.14	108.64	1.01	32	40.63	104.70	1.88	**48.98**
	3.33 mM	1.00	89.28	87.74	1.67	31	22.58	61.42	1.10	**5.70**
	1.11 mM	1.10	138.97	131.23	0.85	22	45.45	77.85	1.40	**22.13**
	0.37 mM	0.80	100.86	122.13	0.78	19	5.26	80.31	1.44	**24.59**

Table 11 Evaluation of the potential mutagenic activity of IN-A4098, IN-L9223 and IN-L9225 in the Mouse Lymphoma Assay in the absence of S9 - 24h (Definitive Mutagenicity Assay).

Cpd.	Conc.	RSG	RTG	RV	RS	Colony counts	% Small colonies	Mutation Frequency	Fold increase	GEF
PBS	**1%**	1.00	100.00	100.00	1.00	18	27.78	63.73	-	-
MMS	**5µg/mL**	0.70	52.92	75.88	1.00	102	61.76	609.24	9.56	**545.51**
DMSO 1	**1%**	1.00	100.00	100.00	1.00	14	57.14	53.92	-	-
DMSO 2	**1%**	1.00	100.00	100.00	1.00	24	41.67	104.74	-	-
IN-A4098	**0.12 mM**	0.31	17.88	58.61	0.75	16	25.00	91.87	1.16	**12.54**
	0.04 mM	0.46	32.74	71.62	0.81	20	25.00	108.00	1.36	**28.67**
	0.013 mM	0.32	31.82	100.00	1.11	28	10.71	113.16	1.43	**33.83**
	0.004 mM	0.37	37.12	100.00	0.83	19	42.11	93.90	1.18	**14.57**
IN-L9223	**3.30 mM**	0.34	34.33	100.00	1.44	26	26.92	80.23	1.01	**0.91**
	1.10 mM	0.69	64.96	93.82	0.93	19	21.05	88.68	1.12	**9.35**
	0.37 mM	0.60	59.81	100.00	1.26	25	20.00	87.98	1.11	**8.65**
	0.12 mM	0.44	26.37	60.29	1.95	24	29.17	54.46	0.69	**-24.87**
IN-L9225	**1.11 mM**	0.47	30.89	65.14	0.84	14	21.43	71.19	0.90	**-8.14**
	0.33 mM	0.67	54.31	80.62	0.72	15	46.67	89.77	1.13	**10.45**
	0.11 mM	0.55	55.46	100.00	0.96	15	33.33	67.29	0.85	**-12.03**
	0.037 mM	0.50	30.04	60.29	1.00	19	26.32	82.62	1.04	**3.29**

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:	The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)

A 2.11.2 Genotoxicity study using micronucleus assay for metabolite IN-A4098 (KCP 7.1.7/02)

Report:	**Antonik, 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.

Cytotoxicity test results for IN-A4098

IN-A4098 - Cytotoxicity test

Tested compound	CBPI	RI [%]	Cytotoxicity [%]	MN [‰]	P value	Cells with MN [‰]	P value
3h (-S9)							
PBS control	1.91	100.0	0.0	7	NA	7	NA
0.1 µg/mL MMC	1.76	82.9	17.1	19	0.0323 (*)	19	0.0323 (*)
0.2 µg/mL MMC	1.66	72.7	27.3	34	<0.0001 (****)	34	<0.0001 (****)
DMSO control	1.85	100.0	0.0				
1.2 µg/mL IN-A4098	1.83	98.0	2.0				
2.3 µg/mL IN-A4098	1.81	96.1	3.9				
4.7 µg/mL IN-A4098	1.82	97.3	2.7				
9.4 µg/mL IN-A4098	1.87	102.4	-2.4				
18.7 µg/mL IN-A4098	1.87	102.7	-2.7				
37.5 µg/mL IN-A4098	1.88	103.4	-3.4				
75.0 µg/mL IN-A4098	1.88	103.7	-3.7				
150.0 µg/mL IN-A4098	1.86	101.6	-1.6				
27h (-S9)							
PBS control	1.87	100.0	0.0	5	NA	5	NA
0.05 µg/mL MMC	1.73	83.9	16.1	14	0.0355 (*)	14	0.0355 (*)
0.1 µg/mL MMC	1.64	73.4	26.6	18	0.0044 (**)	18	0.0044 (**)
DMSO control	1.84	100.0	0.0				
1.2 µg/mL IN-A4098	1.80	95.5	4.5				
2.3 µg/mL IN-A4098	1.86	102.4	-2.4				
4.7 µg/mL IN-A4098	1.86	103.0	-3.0				
9.4 µg/mL IN-A4098	1.85	101.3	-1.3				
18.7 µg/mL IN-A4098	1.84	100.2	-0.2				
37.5 µg/mL IN-A4098	1.88	105.4	-5.4				
75.0 µg/mL IN-A4098	1.91	108.9	-8.9				
150.0 µg/mL IN-A4098	1.92	109.9	-9.9				
3h (+S9)							
PBS control	1.64	100.0	0.0	7	NA	7	NA
2.5 µg/mL CP	1.65	102.8	-2.8	17	0.0064 (**)	17	0.0064 (**)
5.0 µg/mL CP	1.64	100.6	-0.6	33	<0.0001 (****)	33	<0.0001 (****)
DMSO control	1.76	100.0	0.0				
1.2 µg/mL IN-A4098	1.79	103.0	-3.0				
2.3 µg/mL IN-A4098	1.78	102.5	-2.5				
4.7 µg/mL IN-A4098	1.82	107.6	-7.6				
9.4 µg/mL IN-A4098	1.76	100.1	-0.1				
18.7 µg/mL IN-A4098	1.69	90.3	9.7				
37.5 µg/mL IN-A4098	1.71	92.8	7.2				
75.0 µg/mL IN-A4098	1.69	91.0	9.0				
150.0 µg/mL IN-A4098	1.74	97.0	3.0				

Statistically significant level: ns P>0.05; * P≤0.05; ** P≤0.01; *** P≤0.001, **** P<0.0001.

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)[1] 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).

MNA test results for IN-A4098

IN-A4098 – Genotoxicity test

Tested compound	CBPI	RI [%]	Cytotoxicity [%]	MN [‰]	P value	Cells with MN [‰]	P value	Result
3h (-S9)								
PBS control	1.69	100.0	0.0	5	NA	5	NA	NA
0.1 µg/mL MMC	1.71	102.7	-2.9	15	0.0034 (**)	14	0.0071 (**)	positive
0.2 µg/mL MMC	1.59	86.0	13.6	19	0.0001 (***)	18	0.0005 (***)	positive
DMSO control	1.72	100.0	0.0	6	NA	6	NA	NA
25 µg/mL IN-A4098	1.73	102.2	-2.5	5	0.7116	5	0.7116	negative
50 µg/mL IN-A4098	1.75	104.4	-4.6	4	0.4085	4	0.4085	negative
100 µg/mL IN-A4098	1.70	97.4	2.4	6	0.9932	6	0.9932	negative
150 µg/mL IN-A4098	1.60	83.0	16.8	5	0.8322	5	0.8322	negative
27h (-S9)								
PBS control	1.70	100.0	0.0	6	NA	6	NA	NA
0.05 µg/mL MMC	1.71	100.2	-0.2	17	0.003 (**)	15	0.0130 (*)	positive
0.1 µg/mL MMC	1.58	82.1	17.9	23	<0.0001 (****)	21	0.0001 (***)	positive
DMSO control	1.70	100.0	0.0	8	NA	8	NA	NA
25 µg/mL IN-A4098	1.68	97.4	2.6	6	0.6108	6	0.6108	negative
50 µg/mL IN-A4098	1.70	100.1	-0.1	4	0.1698	4	0.1698	negative
100 µg/mL IN-A4098	1.70	100.2	-0.2	7	0.9014	7	0.9014	negative
150 µg/mL IN-A4098	1.69	99.4	0.6	8	0.9696	8	0.9696	negative
3h (+S9)								
PBS control	1.83	100.0	0.0	5	NA	5	NA	NA
2.5 µg/mL CP	1.76	91.5	8.5	13	0.0129 (*)	12	0.0376 (*)	positive
5.0 µg/mL CP	1.75	89.7	10.3	23	<0.0001 (****)	21	<0.0001 (****)	positive
DMSO control	1.76	100.0	0.0	8	NA	8	NA	NA
25 µg/mL IN-A4098	1.71	93.6	6.4	5	0.4259	5	0.4259	negative
50 µg/mL IN-A4098	1.75	99.0	1.3	9	0.7299	9	0.7299	negative
100 µg/mL IN-A4098	1.70	92.4	8.1	9	0.7299	9	0.7299	negative
150 µg/mL IN-A4098	1.70	92.1	8.2	8	0.9989	8	0.9989	negative

Statistically significant level: ns P>0.05; * P≤0.05; ** P≤0.01; *** P≤0.001, **** P<0.0001.

[1] 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.

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:	The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)

A 2.11.3 Bacterial reversion mutation test for metabolite IN-L5296 (KCP 7.1.7/03)

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

SUMMARY

The bacterial reverse mutation test (Ames test) 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.

Results of the cytotoxicity assay

						S. typhimurium		TA100
		amount/plate		revertants/plate		mean	SD	R
Solvent:	DMSO	-	71	52	95	**82.7**	12.0	-
		1.68	70	67	76	**71.0**	4.6	**0.9**
		0.56	66	94	63	**74.3**	17.1	**0.9**
	Test item mg	0.19	94	86	99	**93.0**	6.6	**1.1**
		0.06	76	91	65	**77.3**	13.1	**0.9**
		0.02	74	104	72	**83.3**	17.9	**1.0**

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-anthracene	direct incorporation	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.

Results of the test without metabolic activation / direct incorporation procedure

B-02756 — TA98

		amount/plate	revertants/plate			mean	SD	R
Solvent:	DMSO	-	17	19	26	**20.7**	4.7	-
Reference item (µg):	2-nitrofluorene	5	675	666	631	**657.3**	23.2	**31.8**
		1.68	10	23	16	**16.3**	6.5	**0.8**
		0.56	42	40	4	**28.7**	21.4	**1.4**
Test item	mg	0.19	40	15	18	**24.3**	13.7	**1.2**
		0.06	31	31	15	**25.7**	9.2	**1.2**
		0.02	21	10	19	**16.7**	5.9	**0.8**

TA100

Solvent:	DMSO	-	76	89	87	**84.0**	7.0	-
Reference item (µg):	sodium azide	2.5	1033	1253	1286	**1190.7**	137.5	**14.2**
		1.68	90	85	67	**80.7**	12.1	**1.0**
		0.56	80	71	85	**78.7**	7.1	**0.9**
Test item	mg	0.19	81	70	84	**78.3**	7.4	**0.9**
		0.06	75	63	84	**74.0**	10.5	**0.9**
		0.02	65	72	61	**66.0**	5.6	**0.8**

TA1535

Solvent:	DMSO	-	25	17	16	**19.3**	4.9	-
Reference item (µg):	sodium azide	3.5	1020	1133	959	**1037.3**	88.3	**53.7**
		1.68	11	14	12	**12.3**	1.5	**0.6**
		0.56	12	12	15	**13.0**	1.7	**0.7**
Test item	mg	0.19	23	11	17	**17.0**	6.0	**0.9**
		0.06	12	15	14	**13.7**	1.5	**0.7**
		0.02	12	20	15	**15.7**	4.0	**0.8**

TA1537

Solvent:	DMSO	-	13	9	6	**9.3**	3.5	-
Reference item (µg):	9-aminoacridine	45	216	206	141	**187.7**	40.7	**20.1**
		1.68	5	5	8	**6.0**	1.7	**0.6**
		0.56	6	2	5	**4.3**	2.1	**0.5**
Test item	mg	0.19	8	6	2	**5.3**	3.1	**0.6**
		0.06	8	9	5	**7.3**	2.1	**0.8**
		0.02	12	4	4	**6.7**	4.6	**0.7**

WP2

Solvent:	DMSO	-	200	217	214	**210.3**	9.1	-
Reference item (µg):	4-nitroquinoline-N-oxide	0.4	2271	2050	2510	**2277.0**	230.1	**10.8**
		1.68	190	196	154	**180.0**	22.7	**0.9**
		0.56	221	195	193	**203.0**	15.6	**1.0**
Test item	mg	0.19	224	205	209	**212.7**	10.0	**1.0**
		0.06	237	252	213	**234.0**	19.7	**1.1**
		0.02	233	198	222	**217.7**	17.9	**1.0**

Results of the test without metabolic activation / pre-incubation procedure

B-02756 TA98

		amount/plate	revertants/plate			mean	SD	R
Solvent:	DMSO	-	38	29	25	**30.7**	6.7	-
Reference item (µg):	2-nitrofluorene	5	668	518	557	**581.0**	77.8	**18.9**
		1.68	21	20	22	**21.0**	1.0	**0.7**
		0.56	17	16	27	**20.0**	6.1	**0.7**
Test item	mg	0.19	18	30	19	**22.3**	6.7	**0.7**
		0.06	14	23	21	**19.3**	4.7	**0.6**
		0.02	15	10	19	**14.7**	4.5	**0.5**

TA100

Solvent:	DMSO	-	75	70	63	**69.3**	6.0	-
Reference item (µg):	sodium azide	2.5	792	919	893	**868.0**	67.1	**12.5**
		1.68	65	64	66	**65.0**	1.0	**0.9**
		0.56	78	66	78	**74.0**	6.9	**1.1**
Test item	mg	0.19	87	85	75	**82.3**	6.4	**1.2**
		0.06	67	83	74	**74.7**	8.0	**1.1**
		0.02	77	84	82	**81.0**	3.6	**1.2**

TA1535

Solvent:	DMSO	-	12	19	24	**18.3**	6.0	-
Reference item (µg):	sodium azide	3.5	1015	1040	1064	**1039.7**	24.5	**56.7**
		1.68	10	7	10	**9.0**	1.7	**0.5**
		0.56	9	15	12	**12.0**	3.0	**0.7**
Test item	mg	0.19	18	23	14	**18.3**	4.5	**1.0**
		0.06	16	6	15	**12.3**	5.5	**0.7**
		0.02	24	13	12	**16.3**	6.7	**0.9**

TA1537

Solvent:	DMSO	-	2	9	5	**5.3**	3.5	-
Reference item (µg):	9-aminoacridine	45	121	95	137	**117.7**	21.2	**22.1**
		1.68	7	7	7	**7.0**	0.0	**1.3**
		0.56	6	3	6	**5.0**	1.7	**0.9**
Test item	mg	0.19	6	1	6	**4.3**	2.9	**0.8**
		0.06	4	5	3	**4.0**	1.0	**0.8**
		0.02	5	4	8	**5.7**	2.1	**1.1**

WP2

Solvent:	DMSO	-	356	382	309	**349.0**	37.0	-
Reference item (µg):	4-nitroquinoline-N-oxide	0.4	1938	2123	1959	**2006.7**	101.3	**5.7**
		1.68	379	351	297	**342.3**	41.7	**1.0**
		0.56	377	381	363	**373.7**	9.5	**1.1**
Test item	mg	0.19	375	363	251	**329.7**	68.4	**0.9**
		0.06	391	383	272	**348.7**	66.5	**1.0**
		0.02	365	308	365	**346.0**	32.9	**1.0**

Results of the test with metabolic activation / direct incorporation procedure

B-02756

TA98

		amount/plate	revertants/plate			mean	SD	R
Solvent:	DMSO	-	21	22	32	**25.0**	6.1	-
Reference item (µg):	2-amino-anthracene	1.5	959	839	848	**882.0**	66.8	**35.3**
		1.68	23	29	27	**26.3**	3.1	**1.1**
		0.56	29	25	24	**26.0**	2.6	**1.0**
Test item	mg	0.19	31	21	12	**21.3**	9.5	**0.9**
		0.06	27	25	25	**25.7**	1.2	**1.0**
		0.02	23	27	27	**25.7**	2.3	**1.0**

TA100

Solvent:	DMSO	-	84	69	73	**75.3**	7.8	-
Reference item (µg):	2-amino-anthracene	2.5	1008	1060	1047	**1038.3**	27.1	**13.8**
		1.68	89	96	80	**88.3**	8.0	**1.2**
		0.56	84	116	90	**96.7**	17.0	**1.3**
Test item	mg	0.19	86	92	78	**85.3**	7.0	**1.1**
		0.06	83	101	87	**90.3**	9.5	**1.2**
		0.02	84	86	82	**84.0**	2.0	**1.1**

TA1535

Solvent:	DMSO	-	24	14	25	**21.0**	6.1	-
Reference item (µg):	2-amino-anthracene	30	577	531	469	**525.7**	54.2	**25.0**
		1.68	16	10	16	**14.0**	3.5	**0.7**
		0.56	14	12	13	**13.0**	1.0	**0.6**
Test item	mg	0.19	16	17	20	**17.7**	2.1	**0.8**
		0.06	26	14	13	**17.7**	7.2	**0.8**
		0.02	17	17	13	**15.7**	2.3	**0.7**

TA1537

Solvent:	DMSO	-	9	5	10	**8.0**	2.6	-
Reference item (µg):	2-amino-anthracene	2.5	264	243	203	**236.7**	31.0	**29.6**
		1.68	5	3	5	**4.3**	1.2	**0.5**
		0.56	5	6	6	**5.7**	0.6	**0.7**
Test item	mg	0.19	3	6	11	**6.7**	4.0	**0.8**
		0.06	10	7	6	**7.7**	2.1	**1.0**
		0.02	12	9	7	**9.3**	2.5	**1.2**

WP2

Solvent:	DMSO	-	280	351	333	**321.3**	36.9	-
Reference item (µg):	2-amino-anthracene	30	1668	1546	1552	**1588.7**	68.8	**4.9**
		1.68	352	257	277	**295.3**	50.1	**0.9**
		0.56	382	343	318	**347.7**	32.3	**1.1**
Test item	mg	0.19	277	236	245	**252.7**	21.5	**0.8**
		0.06	384	374	246	**334.7**	77.0	**1.0**
		0.02	375	380	314	**356.3**	36.7	**1.1**

Results of the test with metabolic activation / pre-incubation procedure

B-02756 TA98

		amount/plate	revertants/plate			mean	SD	R
Solvent:	DMSO	-	28	26	27	**27.0**	1.0	-
Reference item (µg):	2-amino-anthracene	1.5	844	720	872	**812.0**	80.9	**30.1**
		1.68	33	32	26	**30.3**	3.8	**1.1**
		0.56	20	26	25	**23.7**	3.2	**0.9**
Test item	mg	0.19	22	25	18	**21.7**	3.5	**0.8**
		0.06	29	24	25	**26.0**	2.6	**1.0**
		0.02	24	19	24	**22.3**	2.9	**0.8**

TA100

Solvent:	DMSO	-	63	83	64	**70.0**	11.3	-
Reference item (µg):	2-amino-anthracene	2.5	1418	1619	1762	**1599.7**	172.8	**22.9**
		1.68	77	76	79	**77.3**	1.5	**1.1**
		0.56	121	84	112	**105.7**	19.3	**1.5**
Test item	mg	0.19	128	92	105	**108.3**	18.2	**1.5**
		0.06	120	129	104	**117.7**	12.7	**1.7**
		0.02	102	121	124	**115.7**	11.9	**1.7**

TA1535

Solvent:	DMSO	-	21	15	25	**20.3**	5.0	-
Reference item (µg):	2-amino-anthracene	30	573	544	479	**532.0**	48.1	**26.2**
		1.68	13	14	19	**15.3**	3.2	**0.8**
		0.56	13	17	15	**15.0**	2.0	**0.7**
Test item	mg	0.19	15	13	17	**15.0**	2.0	**0.7**
		0.06	12	16	18	**15.3**	3.1	**0.8**
		0.02	5	5	3	**4.3**	1.2	**0.2**

TA1537

Solvent:	DMSO	-	9	2	5	**5.3**	3.5	-
Reference item (µg):	2-amino-anthracene	2.5	107	119	108	**111.3**	6.7	**20.9**
		1.68	9	6	8	**7.7**	1.5	**1.4**
		0.56	2	3	4	**3.0**	1.0	**0.6**
Test item	mg	0.19	13	8	5	**8.7**	4.0	**1.6**
		0.06	10	3	4	**5.7**	3.8	**1.1**
		0.02	2	1	5	**2.7**	2.1	**0.5**

WP2

Solvent:	DMSO	-	341	302	314	**319.0**	20.0	-
Reference item (µg):	2-amino-anthracene	30	2239	2403	2084	**2242.0**	159.5	**7.0**
		1.68	334	326	293	**317.7**	21.7	**1.0**
		0.56	332	373	323	**342.7**	26.7	**1.1**
Test item	mg	0.19	342	306	331	**326.3**	18.4	**1.0**
		0.06	302	368	345	**338.3**	33.5	**1.1**
		0.02	325	327	282	**311.3**	25.4	**1.0**

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:	The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)

A 2.11.4 In vitro chromosome aberrations test for metabolite IN-L5296 (KCP 7.1.7/04)

Report:	**Peroche, 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.

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

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.

Preliminary cytotoxicity

SERIE	250 µg/mL		100 µg/mL		40 µg/mL		16 µg/mL	
	Cells/cm²	%	Cells/cm²	%	Cells/cm²	%	Cells/cm²	%
Solution of METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 Solution code: 18/0293-041218-S1	77 188 ± 5 984	88 * P < 0.05	82 656 ± 5 113	95 * NS	83 125 ± 6 271	95 * NS	85 000 ± 7 655	97 * NS
Solvent control: DMSO	87 344 ± 3 658	-						
Negative control (complete culture medium)	93 281 ± 6 929	-						
Positive control (phenol 0.64 mg/mL)	34 375 ± 1 350	37 ** P < 0.001						

* Versus solvent control
** Versus negative control
NS : non statisticaly significant ($P \geq 0.05$)

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.

Relative Increase in Cell Count – without metabolic activation

Serie		Assay	Concentration	Cells number	RICC reduction (%)***
Without metobolic activation (-S9-mix)					
Pre-incubation control****		1*	-	1 987 500	-
		2**	-	3 500 000	-
Absolute negative control		1*	-	4 387 500	-
		2**	-	7 375 000	-
Positive control	(mitomycin C)	1*	0.25 µg/mL	3 225 000	48%
		2**	0,125 µg/mL	5 425 000	50%
Solvent control	DMSO	1*	-	4 112 500	11%
		2**	-	7 437 500	-
Solution obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	Solution Code: 18/0293-201218-S1	1*	16 µg/mL	4 325 000	-
			40 µg/mL	3 962 500	7%
			100 µg/mL	4 175 000	-
			250 µg/mL	3 712 500	19%
	Solution Code: 18/0293-020119-S1	2**	16 µg/mL	7 387 500	1%
			40 µg/mL	7 387 500	1%
			100 µg/mL	7 612 500	-
			250 µg/mL	7 700 000	-

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.

Relative Increase in Cell Count – with metabolic activation

Serie		Assay	Concentration	Cells number	RICC reduction (%)***
With metabolic activation (+S9-mix)					
Pre-incubation control****		1*	-	1 987 500	-
Absolute negative control		1*	-	4 637 500	-
Positive control	(cyclophosphamide)	1*	10 µg/mL	3 387 500	47%
Solvent control	DMSO	1*	-	4 350 000	11%
Solution obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	Solution Code: 18/0293-201218-S1	1*	16 µg/mL	4 150 000	8%
			40 µg/mL	4 462 500	-
			100 µg/mL	4 337 500	1%
			250 µg/mL	4 400 000	-

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).

Chromatic aberration in CHO: without metabolic activation

Type and number of chromosome aberrations

Serie		Assay	Concentration	Cells observed	Aberrations (type and number)													
					GAP***		Chromosomal					Chromatidic					Others	
					G	g	C	M	D	CR	R	c	ic	tr	qr	d	CP	cp
Without metabolic activation (-S9-mix)																		
Absolute negative control		1*	-	300	2	5	2	0	0	0	0	3	0	0	0	1	0	0
		2**	-	301	3	5	2	0	0	0	0	1	0	0	0	0	0	0
Positive control	**(mitomycin C)**	1*	0.25 µg/mL	26	1	1	2	2	0	0	0	5	0	2	2	0	0	0
		2**	0.125 µg/mL	25	1	1	2	0	0	0	0	4	0	2	0	1	0	0
Solvent control	**DMSO**	1*	-	302	2	6	1	0	0	0	0	2	0	1	0	1	0	0
		2**	-	300	3	5	0	0	0	0	0	3	0	0	0	0	0	0
Solution obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	Solution Code: 18/0293-201218-S1	1*	40 µg/mL	306	3	5	1	0	1	0	0	2	0	0	0	0	0	0
			100 µg/mL	300	1	4	1	0	0	0	0	3	0	0	0	0	0	0
			250 µg/mL	300	3	8	0	0	0	0	0	4	0	0	0	0	0	0
	Solution Code: 18/0293-020119-S1	2**	40 µg/mL	300	4	5	1	0	0	0	0	3	0	0	0	0	0	0
			100 µg/mL	300	3	7	1	0	0	0	0	3	0	0	1	0	0	0
			250 µg/mL	300	3	9	1	0	0	0	0	2	0	0	0	0	0	0

Number and percentage of cells with aberrations

Serie		Assay	Concentration	Cells observed	Total aberrations	Number of aberrations / cell	Cells with aberration	Cells with aberration (%)	χ^2	P***
Without metabolic activation (-S9-mix)										
Absolute negative control		1*	-	300	6	0.020	6	2.0%	-	-
		2**	-	301	3	0.010	3	1.0%	-	-
Positive control	(mitomycin C)	1	0.25 µg/mL	26	13	0.500	11	42.3%	54.03	*< 0.001*
		2	0.125 µg/mL	25	9	0.360	8	32.0%	50.99	*< 0.001*
Solvent control	DMSO	1*	-	302	5	0.017	5	1.7%	-	-
		2**	-	300	3	0.010	3	1.0%	-	-
Solution obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	Solution Code: 18/0293-201218-S1	1*	40 µg/mL	306	4	0.013	4	1.3%	0.12	*NS****
			100 µg/mL	300	4	0.013	4	1.3%	0.10	*NS****
			250 µg/mL	300	4	0.013	4	1.3%	0.10	*NS****
	Solution Code: 18/0293-020119-S1	2**	40 µg/mL	300	4	0.013	4	1.3%	0.10	*NS****
			100 µg/mL	300	5	0.017	5	1.7%	0.00	*NS****
			250 µg/mL	300	3	0.010	3	1.0%	0.48	*NS****

Chromatic aberration in CHO: with metabolic activation

Type and number of chromosome aberrations

Serie		Assay	Concentration	Cells observed	Aberrations (type and number)												
					GAP***		Chromosomal					Chromatidic					Others	
					G	g	C	M	D	CR	R	c	ic	tr	qr	d	CP	cp
With metabolic activation (+S9-mix)																		
Absolute negative control		1*	-	300	1	8	1	0	0	0	0	3	0	0	0	0	0	0
Positive control	(cyclophosphamid)	1*	10 µg/mL	25	0	0	2	0	0	2	0	1	0	3	4	1	0	0
Solvent control	DMSO	1*	-	300	5	3	0	0	0	0	0	3	0	0	0	1	0	0
Solution obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	Solution Code: 18/0293-201218-S1	1*	40 µg/mL	300	2	5	1	0	0	0	0	3	0	0	0	0	0	0
			100 µg/mL	300	2	4	2	0	0	0	0	2	0	1	0	0	0	0
			250 µg/mL	300	2	6	1	0	0	0	0	5	0	0	0	1	0	0

Number and percentage of cells with aberrations

Serie		Assay	Concentration	Cells observed	Total aberrations	Aberrations /cell average	Cells with aberration	Cells with aberration (%)	χ^2	P***
With metabolic activation (+S9-mix)										
Absolute negative control		1*	-	300	4	0.013	4	1.3%	-	-
Positive control	(cyclophosphamide)	1*	10 µg/mL	25	13	0.520	10	40.0%	58.89	*< 0.001*
Solvent control	DMSO	1*	-	300	4	0.013	4	1.3%	-	-
Solution obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	Solution Code: 18/0293-201218-S1	1*	40 µg/mL	300	4	0.013	4	1.3%	0.00	*NS****
			100 µg/mL	300	5	0.017	5	1.7%	0.11	*NS****
			250 µg/mL	300	7	0.023	6	2.0%	0.39	*NS****

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:	The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)

A 2.11.5 In vitro mammalian cell gene mutation test for metabolite IN-L5296 (KCP 7.1.7/05)

Report:	**Savineau, 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[2]; Clive et al., 1987[3]). 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[4]; Aaron et al., 1994[5]) 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.
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

[2] Cole J., Arlett C.F., Green M.H.L, Lowe J., Muriel W.J., 1983: Mutation Research, 111, 371.
[3] 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.
[4] 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.
[5] 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.

adjusted by the number of colonies in non-selective medium to derive the mutant frequency (Clive et al., 1983[6]).
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[7]). Furthermore, according to Applegate et al. (1990)[8] 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.
Organoleptic specifications:	
Aspect:	Pale yellow powder
pH:	Not provided
Other physical properties:	
sterility:	Non-sterile

[6] 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.
[7] 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.
[8] 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.

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		

Stability	homogenous, pink solutions extemporaneous preparation	homogenous, pink solutions extemporaneous preparation

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[9]): 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

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).

[9] 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.

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[10] 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. 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).

[10] 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.

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.

pH and osmolality

Series	-S9-mix		
	Concentrations	pH	Osmolality (mosm/kg H_2O)
Negative control : culture medium	-	7.4	349
Solvent control : DMSO	-	7.5	499
Solutions obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-150119-S1	250 µg/mL	7.5	510
	100 µg/mL	7.5	500
	40 µg/mL	7.5	499
	16 µg/mL	7.5	503
	+S9-mix		
	Concentrations	pH	Osmolality (mosm/kg H_2O)
Negative control : culture medium	-	7.3	356
Solvent control : DMSO	-	7.3	504
Solutions obtained from METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-220119-S1	250 µg/mL	7.3	519
	100 µg/mL	7.3	506
	40 µg/mL	7.3	504
	16 µg/mL	7.3	502

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 %

Viability (-S9-mix)

Relative Cloning Efficiency determination

Serie		EWs	TWs	ns	c	PEs	RCE
Negative control		29 29 22 23	384	2	1.3	0.7	100%
Positive control MMS (10 µg/mL)		37 30	192	2	1.1	0.5	80%
Solvent control DMSO		26 22 12 21	384	2	1.6	0.8	100%
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-150119-S1	250 µg/mL	27 24	192	2	1.3	0.7	85%
	100 µg/mL	27 23	192	2	1.3	0.7	86%
	40 µg/mL	22 25	192	2	1.4	0.7	90%
	16 µg/mL	23 21	192	2	1.5	0.7	95%

EWs: EmptyWells for survival and viability plates at T0
TWs: Total Wells for survival and viability plates at T0
ns: number of cells / well, plating cells at 10 cells per mL, i.e. 2 cells/well
c: Probable number of clones / well = -ln (EWs / TWs)
PE: (Plating Efficiency Survival) = c / ns
RCE: % (PE positive control / PE negative control)
RCE: % (PE test substance / PE negative control)

Relative Suspension Growth

Serie		Cell number at D0	Cell number at D1	Cell number at D2	CGD1	CGD2	RSG	RSG Mean	RS	RTG	SG
Negative control		2.0 x 10^5 2.0 x 10^5	9.7 x 10^5 9.0 x 10^5	9.9 x 10^5 10.1 x 10^5	4.9 4.5	4.9 5.0	- -	-	-	-	23.2
Positive control MMS (10 µg/mL)		2.0 x 10^5 2.0 x 10^5	6.7 x 10^5 6.3 x 10^5	9.3 x 10^5 8.0 x 10^5	3.4 3.2	4.7 4.0	65.2% 55.7%	60%	80%	48%	-
Solvent control DMSO		2.0 x 10^5 2.0 x 10^5	9.8 x 10^5 9.1 x 10^5	9.8 x 10^5 9.1 x 10^5	4.9 4.6	4.9 4.5	- -	-	-	-	22.2
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-150119-S1	250 µg/mL	2.0 x 10^5 2.0 x 10^5	9.2 x 10^5 9.8 x 10^5	10.3 x 10^5 9.6 x 10^5	4.6 4.9	5.2 4.8	98.6% 114.2%	106%	85%	91%	-
	100 µg/mL	2.0 x 10^5 2.0 x 10^5	9.2 x 10^5 9.9 x 10^5	9.5 x 10^5 9.5 x 10^5	4.6 4.9	4.7 4.7	91.0% 113.0%	102%	86%	88%	-
	40 µg/mL	2.0 x 10^5 2.0 x 10^5	9.0 x 10^5 9.2 x 10^5	10.0 x 10^5 10.1 x 10^5	4.5 4.6	5.0 5.0	93.2% 112.3%	103%	90%	93%	-
	16 µg/mL	2.0 x 10^5 2.0 x 10^5	8.8 x 10^5 8.6 x 10^5	10.7 x 10^5 10.1 x 10^5	4.4 4.3	5.3 5.1	98.1% 104.9%	101%	95%	96%	-

Viability (+S9-mix)

Relative Cloning Efficiency determination

Serie		EWs	TWs	ns	c	PEs	RCE
Negative control		27 26 26 25	384	2	1.3	0.7	100%
Positive control Cyclophosphamide (2 µg/mL)		39 38	192	2	0.9	0.5	70%
Solvent control DMSO		24 32 22 24	384	2	1.3	0.7	100%
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 **LEMI code :** **18/0293-220119-S1**	250 µg/mL	33 39	192	2	1.0	0.5	74%
	100 µg/mL	35 28	192	2	1.1	0.6	84%
	40 µg/mL	35 21	192	2	1.2	0.6	93%
	16 µg/mL	24 23	192	2	1.4	0.7	106%

Relative Suspension Growth

Serie		Cell number at D0	Cell number at D1	Cell number at D2	CGD1	CGD2	RSG	Mean	RS	RTG	SG
ASSAY 1											
Negative control		2.0×10^5 2.0×10^5	9.0×10^5 9.4×10^5	10.5×10^5 11.8×10^5	4.5 4.7	5.2 5.9	- -	-	-	-	25.5
Positive control Cyclophosphamide (2 µg/mL)		2.0×10^5 2.0×10^5	9.0×10^5 8.3×10^5	10.5×10^5 11.8×10^5	4.5 4.2	5.2 5.9	99.4% 88.8%	94%	70%	66%	-
Solvent control DMSO		2.0×10^5 2.0×10^5	9.2×10^5 9.3×10^5	10.3×10^5 10.6×10^5	4.6 4.7	5.2 5.3	- -	-	-	-	24.1
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-220119-S1	250 µg/mL	2.0×10^5 2.0×10^5	8.4×10^5 8.1×10^5	10.0×10^5 11.4×10^5	4.2 4.0	5.0 5.7	88.7% 92.7%	91%	74%	67%	-
	100 µg/mL	2.0×10^5 2.0×10^5	8.7×10^5 8.4×10^5	9.7×10^5 10.9×10^5	4.4 4.2	4.8 5.5	89.1% 92.3%	91%	84%	76%	-
	40 µg/mL	2.0×10^5 2.0×10^5	9.0×10^5 8.8×10^5	9.5×10^5 11.3×10^5	4.5 4.4	4.7 5.7	89.7% 100.9%	95%	93%	89%	-
	16 µg/mL	2.0×10^5 2.0×10^5	9.0×10^5 8.7×10^5	10.8×10^5 11.2×10^5	4.5 4.4	5.4 5.6	102.1% 98.8%	100%	106%	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.

Survival (-S9-mix)

Treatment		EWs	TWs	ns	c	PE survival	RCE
Negative control		20 34 23 25	384	2	1.33	0.66	100%
Positive control : MMS 10 µg/mL		40 24	192	2	1.10	0.55	83%
Solvent control : DMSO		24 28 28 25	384	2	1.30	0.65	100%
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-150119-S1	250 µg/mL	36 27	192	2	1.11	0.56	86%
	100 µg/mL	25 36	192	2	1.15	0.57	88%
	40 µg/mL	27 22	192	2	1.37	0.68	105%
	16 µg/mL	26 30	192	2	1.23	0.62	95%

Survival (+S9-mix)

Treatment		EWs	TWs	ns	c	PE survival	RCE
Negative control		15 15 15 26	384	2	1.69	0.84	100%
Positive control : Cyclophosphamide monohydraté 2 µg/mL		32 27	192	2	1.18	0.59	70%
Solvent control : DMSO		23 21 21 19	384	2	1.52	0.76	100%
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-220119-S1	250 µg/mL	34 29	192	2	1.11	0.56	73%
	100 µg/mL	33 34	192	2	1.05	0.53	69%
	40 µg/mL	36 31	192	2	1.05	0.53	69%
	16 µg/mL	33 26	192	2	1.18	0.59	78%

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.

Mutant Frequency (-S9-mix)

Treatment		EWm	TWm	nm	PE mutant	(MF / survivors) x 10^{-6}	Induced mutants x 10^{-6}	χ^2	P
Negative control		156 153	384	2000	108.7	163.9	-	-	-
Positive control : MMS 10 µg/mL		98 65	384	2000	428.4	780.0	616.1	78.7	< 0.001
Solvent control : DMSO		156 159	384	2000	99.0	152.8	-	-	-
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	250 µg/mL	140 143	384	2000	152.6	273.9	121.1	9.2	< 0.005
	100 µg/mL	144 154	384	2000	126.8	221.1	68.4	3.5	NS*
LEMI code : 18/0293-150119-S1	40 µg/mL	156 164	384	2000	91.2	133.5	-19.2	0.4	NS*
	16 µg/mL	147 165	384	2000	103.8	168.5	15.8	0.2	NS*

Mutant Frequency (+S9-mix)

Treatment		EWm	TWm	nm	PE mutant	(MF / survivors) x 10^{-6}	Induced mutants X 10^{-6}	χ^2	P
Negative control		159 160	384	2000	92.7	109.9	-	-	-
Positive control : Cyclophosphamide monohydrate 2 µg/mL		114 117	384	2000	254.1	430.7	320.8	54.0	< 0.001
Solvent control : DMSO		146 162	384	2000	110.3	145.1	-	-	-
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5 LEMI code : 18/0293-220119-S1	250 µg/mL	156 155	384	2000	105.4	189.2	44.1	1.8	NS*
	160 µg/mL	150 162	384	2000	103.8	197.2	52.1	2.4	NS*
	40 µg/mL	154 151	384	2000	115.2	218.8	73.7	4.4	< 0.05
	16 µg/mL	169 159	384	2000	78.8	133.6	-11.5	0.2	NS*

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: (780.0×10^6 MF that of negative control 163.9×10^6 MF)
- With metabolic activation, positive control Cyclophosphamide Monohydrate induced a statistically significant increase in mutant frequency, 3.9 times that of negative control (430.7×10^6 MF that of negative control 109.9×10^6 MF).

Above the spontaneous background MF (an induced MF (IMF)) of at least 300×10^6 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:

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

Mutant frequency of the size colonies
Mutant frequency of the size colonies (-S9-mix)

Treatment		Ewm	TWm	nm	Small colonies		Large colonies	
					(MF / survivors) x 10^{-6}	Induced mutants x 10-6	(MF / survivors) x 10-6	Induced mutants x 10-6
Negative control		0.66	384	2000	63	-	92	-
Positive control : MMS 10 µg/mL		0.55	384	2000	413	349	225	133
Solvent control : DMSO		0.65	384	2000	48	-	101	-
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	250 µg/mL	0.56	384	2000	88	41	166	66
	100 µg/mL	0.57	384	2000	86	38	122	21
LEMI code : 18/0293-150119-S1	40 µg/mL	0.68	384	2000	31	-16	98	-3
	16 µg/mL	0.62	384	2000	48	0	118	17

Mutant frequency of the size colonies (+S9-mix)

Treatment		Ewm	TWm	nm	Small colonies		Large colonies	
					(MF / survivors) x 10^{-6}	Induced mutants x 10^{-6}	(MF / survivors) x 10^{-6}	Induced mutants x 10^{-6}
Negative control		0.84	384	2000	48	-	57	-
Positive control : Cyclophosphamide monohydraté 2 µg/mL		0.59	384	2000	268	219	136	80
Solvent control : DMSO		0.76	384	2000	48	-	92	-
METABOLITE OF TRIBENURON METHYL IN-L5296 BATCH: MP-5248-39-5	250 µg/mL	0.56	384	2000	55	7	125	33
	100 µg/mL	0.53	384	2000	69	21	121	29
LEMI code : 18/0293-220119-S1	40 µg/mL	0.53	384	2000	85	37	124	32
	16 µg/mL	0.59	384	2000	57	9	71	-20

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[11,12,13]). The GEF is applied as follows:

[11] 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.
[12] 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).
[13] 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.

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:	The study was evaluated and accepted during evaluation of the product TOSCANA TOP 75 WG (Product code T-75WG-OR2C)

Appendix 3 Exposure calculations

A 3.1 Operator exposure calculations (KCP 7.2.1.1)

A 3.1.1 Calculations for MCPA

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

Substance name	MCPA	
Product name	HAKSAR TOP 565 SG	
Reference value non acutely toxic active substance (RVNAS)	0,04	mg/kg bw/day
Reference value acutely toxic active substance (RVAAS)		mg/kg bw/day
Crop type	Cereals/ Grassland 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	0,55	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	100,00%	
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 23: Estimation of longer-term operator exposure towards MCPA according to EFSA guidance – potential exposure and workwear

Application rate of active substance	0,55 kg a.s./ha	*i_AppRate*
Assumed area treated	50 ha/day	*d_AreaTreated*
Amount of active substance applied	27,5 kg a.s./day	*i_AmountAS*
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	

	Exposure values	µg exposure/day mixed and loaded		Reference	Comment
		75th centile	95th centile		
Mixing and loading	Hands	16812	82558	AOEM	
	Body	12689	42091	AOEM	
	Head	179	2462	AOEM	
	Protected hands (gloves)	143	865	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	351	1711	AOEM	
	Protected head (hood and face shield)	3	139	AOEM	
	Inhalation	100	281	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	No			
	Clothing	Work wear - arms, body and legs covered		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	4079	25962	AOEM	
	Body	2281	11757	AOEM	
	Head	108	325	AOEM	
	Protected hands (gloves)	256	4905	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	63	153	AOEM	
	Inhalation	5	20	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	No			
	Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Closed cab	No		vehicle mounted upward spraying only	

1. Total

	Without RPE/PPE	With RPE/PPE	
Longer term			
Total systemic exposure from mixing, loading and application (mg a.s./day)	**6,3072406**	**3,9643508**	
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	**0,1051207**	**0,0660725**	
% of RVNAS	**262,80%**	**165,18%**	

Table A 34: Estimation of longer-term operator exposure towards MCPA according to EFSA guidance –workwear and gloves during mixing and loading

Application rate of active substance	0,55 kg a.s./ha	i_AppRate
Assumed area treated	50 ha/day	d_AreaTreated
Amount of active substance applied	27,5 kg a.s./day	i_AmountAS
Dermal absorption of the product	10,00%	i_AbsorpProduct
Dermal absorption of in-use dilution	50,00%	i_AbsorpInuse
Formulation type	Wettable granules, soluble granules	
Indoor or Outdoor application	Outdoor	
Application method	Downward spraying	
Application equipment	Vehicle-mounted	
Season	not relevant	

	Exposure values	µg exposure/day mixed and loaded 75th centile	95th centile	Reference	Comment
Mixing and loading	Hands	16812	82558	AOEM	
	Body	12689	42091	AOEM	
	Head	179	2462	AOEM	
	Protected hands (gloves)	143	865	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	351	1711	AOEM	
	Protected head (hood and face shield)	3	139	AOEM	
	Inhalation	100	281	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	Yes		Incl. in AOEM model	
	Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Water soluble bag	No		1	

	Exposure values	µg exposure/day applied 75th centile	95th centile	Reference	Comment
Application	Hands	4079	25962	AOEM	
	Body	2281	11757	AOEM	
	Head	108	325	AOEM	
	Protected hands (gloves)	256	4905	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	63	153	AOEM	
	Inhalation	5	20	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	No			
	Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Closed cab	No		vehicle mounted upward spraying only	

1. Total

	Without RPE/PPE	With RPE/PPE
Longer term		
Total systemic exposure from mixing, loading and application (mg a.s./day)	6,3072406	2,2974386
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	0,1051207	0,0382906
% of RVNAS	262,80%	95,73%

Table A 45: Estimation of longer-term operator exposure towards MCPA according to EFSA guidance –workwear and gloves during mixing and loading and application

Application rate of active substance	0,55 kg a.s./ha	*i_AppRate*
Assumed area treated	50 ha/day	*d_AreaTreated*
Amount of active substance applied	27,5 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	16812	82558	AOEM	
	Body	12689	42091	AOEM	
	Head	179	2462	AOEM	
	Protected hands (gloves)	143	865	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	351	1711	AOEM	
	Protected head (hood and face shield)	3	139	AOEM	
	Inhalation	100	281	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	Yes		Incl. in AOEM model	
	Clothing	Work wear - arms, body and legs covered		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	4079	25962	AOEM	
	Body	2281	11757	AOEM	
	Head	108	325	AOEM	
	Protected hands (gloves)	256	4905	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	63	153	AOEM	
	Inhalation	5	20	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	Yes		Incl. in AOEM model	
	Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Closed cab	No		vehicle mounted upward spraying only	

1. Total

	Without RPE/PPE	With RPE/PPE	
Longer term			
Total systemic exposure from mixing, loading and application (mg a.s./day)	6,3072406	0,3861406	
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	0,1051207	0,0064357	
% of RVNAS	262,80%	16,09%	

A 3.1.2 Calculations for Tribenuron-methyl

Table A 16: Input parameters considered for the estimation of operator exposure

Substance name	Tribenuron methyl	
Product name	HAKSAR TOP 565 SG	
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/ Grassland 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	0,015	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	50,00%	
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 57: Estimation of longer-term and acute operator exposure towards TRIBENURON-METHYL according to EFSA guidance – potential exposure and workwear

Application rate of active substance	0,015 kg a.s./ha	*i_AppRate*
Assumed area treated	50 ha/day	*d_AreaTreated*
Amount of active substance applied	0,75 kg a.s./day	*i_AmountAS*
Dermal absorption of the product	50,00%	*i_AbsorpProduct*
Dermal absorption of in-use dilution	50,00%	*i_AbsorpInuse*
Formulation type	Wettable granules, soluble granules	
Indoor or Outdoor application	Outdoor	
Application method	Downward spraying	
Application equipment	Vehicle-mounted	
Season	not relevant	

	Exposure values	µg exposure/day mixed and loaded		Reference	Comment
		75th centile	95th centile		
Mixing and loading	Hands	1051	4997	AOEM	
	Body	1009	14782	AOEM	
	Head	5	67	AOEM	
	Protected hands (gloves)	14	24	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	14	47	AOEM	
	Protected head (hood and face shield)	0	4	AOEM	
	Inhalation	34	258	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	No			
	Clothing	Work wear - arms, body and legs covered		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	111	1856	AOEM	
	Body	62	321	AOEM	
	Head	3	9	AOEM	
	Protected hands (gloves)	36	3223	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	2	4	AOEM	
	Inhalation	1	2	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	No			
	Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Closed cab	No		vehicle mounted upward spraying only	

1. Total

	Without RPE/PPE	With RPE/PPE	
Longer term			
Total systemic exposure from mixing, loading and application (mg a.s./day)	**1,1555245**	**0,6280112**	
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	**0,0192587**	**0,0104669**	
% of RVNAS	**38,52%**	**20,93%**	
Acute			
Total systemic exposure from mixing, loading and application (mg a.s./day)	**11,2762318**	**3,7503473**	
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	**0,1879372**	**0,0625058**	
% of RVAAS	**144,57%**	**48,08%**	

Table A 68: Estimation of longer-term exposure towards TRIBENURON-METHYL according to EFSA guidance – workwear and gloves during mixing and loading

Application rate of active substance	0,015 kg a.s./ha	*i_AppRate*
Assumed area treated	50 ha/day	*d_AreaTreated*
Amount of active substance applied	0,75 kg a.s./day	*i_AmountAS*
Dermal absorption of the product	50,00%	*i_AbsorpProduct*
Dermal absorption of in-use dilution	50,00%	*i_AbsorpInuse*
Formulation type	Wettable granules, soluble granules	
Indoor or Outdoor application	Outdoor	
Application method	Downward spraying	
Application equipment	Vehicle-mounted	
Season	not relevant	

Mixing and loading

Exposure values	µg exposure/day mixed and loaded		Reference	Comment
	75th centile	95th centile		
Hands	1051	4997	AOEM	
Body	1009	14782	AOEM	
Head	5	67	AOEM	
Protected hands (gloves)	14	24	AOEM	
Protected body (workwear or protective garment and sturdy footwear)	14	47	AOEM	
Protected head (hood and face shield)	0	4	AOEM	
Inhalation	34	258	AOEM	
Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
Gloves	Yes		Incl. in AOEM model	
Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
Head and respiratory PPE	None		1	1
Water soluble bag	No		1	

Application

Exposure values	µg exposure/day applied		Reference	Comment
	75th centile	95th centile		
Hands	111	1856	AOEM	
Body	62	321	AOEM	
Head	3	9	AOEM	
Protected hands (gloves)	36	3223	AOEM	
Protected body (workwear or protective garment and sturdy footwear)	2	4	AOEM	
Inhalation	1	2	AOEM	
Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
Gloves	No			
Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
Head and respiratory PPE	None		1	1
Closed cab	No		vehicle mounted upward spraying only	

1. Total

	Without RPE/PPE	With RPE/PPE	
Longer term			
Total systemic exposure from mixing, loading and application (mg a.s./day)	**1,1555245**	**0,1096137**	
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	**0,0192587**	**0,0018269**	
% of RVNAS	**38,52%**	**3,65%**	

Table A 79: Estimation of longer-term exposure towards TRIBENURON-METHYL according to EFSA guidance – workwear and gloves during mixing, loading and application

Application rate of active substance	0,015	kg a.s./ha	*i_AppRate*
Assumed area treated	50	ha/day	*d_AreaTreated*
Amount of active substance applied	0,75	kg a.s./day	*i_AmoutAS*
Dermal absorption of the product	50,00%		*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 75th centile	95th centile	Reference	Comment
Mixing and loading	Hands	1051	4997	AOEM	
	Body	1009	14782	AOEM	
	Head	5	67	AOEM	
	Protected hands (gloves)	14	24	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	14	47	AOEM	
	Protected head (hood and face shield)	0	4	AOEM	
	Inhalation	34	258	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	Yes		Incl. in AOEM model	
	Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Water soluble bag	No		1	

	Exposure values	µg exposure/day applied 75th centile	95th centile	Reference	Comment
Application	Hands	111	1856	AOEM	
	Body	62	321	AOEM	
	Head	3	9	AOEM	
	Protected hands (gloves)	36	3223	AOEM	
	Protected body (workwear or protective garment and sturdy footwear)	2	4	AOEM	
	Inhalation	1	2	AOEM	
	Protective Equipment	Select for inclusion		Penetration factor	Inhalation Protection factor
	Gloves	Yes		Incl. in AOEM model	
	Clothing	Work wear - arms, body and legs covered		Incl. in AOEM model	
	Head and respiratory PPE	None		1	1
	Closed cab	No		vehicle mounted upward spraying only	

1. Total

	Without RPE/PPE	With RPE/PPE	
Longer term			
Total systemic exposure from mixing, loading and application (mg a.s./day)	1,1555245	0,0721164	
Total systemic exposure from mixing, loading and application per kg body weight (mg/kg bw/day)	0,0192587	0,0012019	
% of RVNAS	38,52%	2,40%	

A 3.2 Worker exposure calculations (KCP 7.2.3.1)

A 3.2.1 Calculations for MCPA

Table A 8: Input parameters considered for the estimation of worker exposure (DFR_0 = 3 by default)

Crop type	Cereals		
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	0,55	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	8	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)	1,65	µg a.s./cm^2	*d_DFR*
Working hours	2	hr	*d_WorkHr*
Dermal transfer coefficient - Total potential exposure	12500	cm^2/hr	*d_DermTcUCV*
Dermal transfer coefficient - arms, body and legs covered	1400	cm^2/hr	*d_DermTcCV1*
Dermal transfer coefficient - hands, arms, body and legs covered	no TC available for this assessment	cm^2/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*

Crop type	Cereals		
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	0.55	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)	1.65	µg a.s./cm^2	*d_DFR*
Working hours	2	hr	*d_WorkHr*
Dermal transfer coefficient - Total potential exposure	12500	cm^2/hr	*d_DermTcUCV*
Dermal transfer coefficient - arms, body and legs covered	1400	cm^2/hr	*d_DermTcCV1*
Dermal transfer coefficient - hands, arms, body and legs covered	no TC available for this assessment	cm^2/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 9: Estimation of longer-term exposure towards MCPA according to EFSA guidance (DFR_0 = 3 by default)

1. Total

	Potential exposure	Work wear - arms, body and legs covered	Working wear and gloves	Comments
Total systemic exposure (mg a.s./day)	20,6250000	2,3100000	no TC available for this assessment	
Total systemic exposure per kg body weight (mg/kg bw/day)	0,3437500	0,0385000		
% of RVNAS	859,38%	96,25%		

2. Details

	Systemic exposure [mg a.s. /day]	Systemic exposure [mg a.s./kg bw/day]	Formula	Comments
Dermal - Potential	20,6250000	0,3437500	*d_DermTcUCV*d_WorkHr*i_DFR*i_MAF/1000*i_AbsorpInuse*	
Dermal - Work wear - arms, body and legs covered	2,3100000	0,0385000	*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 22: Input parameters considered for the estimation of worker exposure (DFR_0 = 1.76 based on study data)

Crop type	Cereals		
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	0,55	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)	0,968	µg a.s./cm^2	*d_DFR*
Working hours	2	hr	*d_WorkHr*
Dermal transfer coefficient - Total potential exposure	12500	cm^2/hr	*d_DermTcUCV*
Dermal transfer coefficient - arms, body and legs covered	1400	cm^2/hr	*d_DermTcCV1*
Dermal transfer coefficient - hands, arms, body and legs covered	no TC available for this assessment	cm^2/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 23: Estimation of longer-term exposure towards MCPA according to EFSA guidance (DFR_0 = 1.76 based on study data)

L. Total

	Potential exposure	Work wear - arms, body and legs covered	Working wear and gloves	Comments
Total systemic exposure (mg a.s./day)	12,1000000	1,3552000	no TC available for this assessment	
Total systemic exposure per kg body weight (mg/kg bw/day)	0,2016667	0,0225867		
% of RVNAS	504,17%	56,47%		

Table A 24: Estimation of longer term worker exposure towards MCPA according to EUROPOEM II (DFR_0 = 30 mg as/m2/kg a.s./ha by default)

WORKER EXPOSURE			EUROPOEM II MODEL	
form	MT-565SG-OR2-C		Re-entry in the field	
a.s.	MCPA			
Parameter		**Value**	**Unit**	**References, comments**
Re-entry activities in the field				
AR	Application rate	0,55	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		**4,62**	mg a.s./ day	DE = DFR x AR x TC x T
Internal exposure				
DA	Dermal Absorption	50	%	
	PPE-factor dermal	5		gloves*
	AOEL	2,4	mg a.s./ day	based on 70 kg bw
		Without PPE	**With PPE**	
	Internal exposure	[mg a.s./ day]	[mg a.s./ day]	
	Inhalation	-	-	no model available
	Dermal	2,310	0,462	DE(int) = DE x (DA/100)
	Total	**2,310**	**0,462**	**sum**
	% AOEL			
	Inhalation	-	-	no model available
	Dermal	96	19	%AOEL = 100 x DE(int) / AOEL
	Total	**96**	**19**	**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.

Table A 25: Estimation of longer term worker exposure towards MCPA according to EUROPOEM II (DFR_0 = 17.6 mg as/m2/kg a.s./ha based on study data)

WORKER EXPOSURE			EUROPOEM II MODEL	
form	MCPA + tribenuron		Re-entry in the field	
a.s.	MCPA			
Parameter		**Value**	**Unit**	**References, comments**
Re-entry activities in the field				
AR	Application rate	0,55	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	17,6	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		**2,7104**	mg a.s./ day	DE = DFR x AR x TC x T
Internal exposure				
DA	Dermal Absorption	50	%	
	PPE-factor dermal	5		gloves*
	AOEL	2,4	mg a.s./ day	based on 70 kg bw
		Without PPE	**With PPE**	
	Internal exposure	[mg a.s./ day]	[mg a.s./ day]	
	Inhalation	-	-	no model available
	Dermal	1,355	0,271	DE(int) = DE x (DA/100)
	Total	**1,355**	**0,271**	**sum**
	% AOEL			
	Inhalation	-	-	no model available
	Dermal	56	11	%AOEL = 100 x DE(int) / AOEL
	Total	**56**	**11**	**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 3.2.2 Calculations for Tribenuron-methyl

Table A 26: Input parameters considered for the estimation of worker exposure

Crop type	Cereals		
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	0,015	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	50,00%		*i_AbsorpProduct*
Dermal absorption of the in-use dilution	50,00%		*i_AbsorpInuse*
Dislodgeable foliar residue (i_AppRate*i_DFR)	0,045	µg a.s./cm^2	*d_DFR*
Working hours	2	hr	*d_WorkHr*
Dermal transfer coefficient - Total potential exposure	12500	cm^2/hr	*d_DermTcUCV*
Dermal transfer coefficient - arms, body and legs covered	1400	cm^2/hr	*d_DermTcCV1*
Dermal transfer coefficient - hands, arms, body and legs covered	no TC available for this assessment	cm^2/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 27: Estimation of longer-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)	0,5625000	0,0630000	no TC available for this assessment	
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0093750	0,0010500		
% of RVNAS	18,75%	2,10%		

2. Details

	Systemic exposure [mg a.s. /day]	Systemic exposure [mg a.s./kg bw/day]	Formula	Comments
Dermal - Potential	0,5625000	0,0093750	*d_DermTcUCV*d_WorkHr*i_DFR*i_MAF/1000*i_AbsorpInuse*	
Dermal - Work wear - arms, body and legs covered	0,0630000	0,0010500	*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 28: Estimation of longer-term worker exposure towards Tribenuron-methyl according to EUROPOEM II

WORKER EXPOSURE			EUROPOEM II MODEL	
form	MT-565SG-OR2-C		Re-entry in the field	
a.s.	Tribenuron-methyl			
Parameter		**Value**	**Unit**	**References, comments**
Re-entry activities in the field				
AR	Application rate	0,015	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		**0,126**	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 70 kg bw
		Without PPE	**With PPE**	
	Internal exposure	[mg a.s./ day]	[mg a.s./ day]	
	Inhalation	-	-	no model available
	Dermal	0,063	0,013	DE(int) = DE x (DA/100)
	Total	**0,063**	**0,013**	**sum**
	% AOEL			
	Inhalation	-	-	no model available
	Dermal	2	0	%AOEL = 100 x DE(int) / AOEL
	Total	**2**	**0**	**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 MCPA - cereals

Table A 10: Input parameters considered for the estimation of longer-term resident exposure (DFR_0 = 3 by default)

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	0,55	kg a.s./ha	*i_AppRate*
Concentration of active substance (in-use dilution for liquid applications)	2,75	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	100,00%		*i_AbsorpOralInuse*
Dislodgeable foliar residue (i_AppRate*i_DFR)	1,65	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 30: Estimation of longer-term resident exposure towards MCPA according to EFSA guidance (DFR_0 = 3 by default)

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)	0,3692975	0,0107000	0,0445060	0,4640625	0,6167148
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0369298	0,0010700	0,0044506	0,0464063	0,0616715
% of RVNAS	92,32%	2,68%	11,13%	116,02%	154,18%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,5302000	0,0138000	0,1124200	1,5468750	1,5813655
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0088367	0,0002300	0,0018737	0,0257813	0,0263561
% of RVNAS	22,09%	0,58%	4,68%	64,45%	65,89%

Table A 31: Input parameters considered for the estimation of longer-term resident exposure (DFR_0 = 1.76 by study data)

Substance name	MCPA	
Product name	HAKSAR TOP 565 SG	
Reference value non acutely toxic active substance (RVNAS)	0,04	mg/kg bw/day
Reference value acutely toxic active substance (RVAAS)		mg/kg bw/day

Crop type	Cereals	
Substance properties		
Formulation type	Wettable granules, soluble granules	
Miniumum volume water for application (liquids)	200	L/ha
Maximum application rate of active substance	0,55	kg a.s. /ha
50% Dissipation Time DT50	30	days
Initial Dislodgeable Foliar Residue	1.76	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	100,00%	
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	

Application method	Downward spraying		
Application equipment	Vehicle-mounted		i_AppEquip
Formulation type	Wettable granules, soluble granules		i_FormVal
Buffer strip	5	m	i_Buffer
Application rate of the product	0,55	kg a.s./ha	i_AppRate
Concentration of active substance (in-use dilution for liquid applications)	2,75	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	100,00%		i_AbsorpOralInuse
Dislodgeable foliar residue (i_AppRate*i_DFR)	0,968	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	0,23798	ml spray dilution/person	
Resident dermal spray drift exposure 75th percentile - child	0,2175	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - adult	0,00009	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - child	0,00017	ml spray dilution/person	
Resident dermal spray drift exposure mean - adult	0,12278	ml spray dilution/person	
Resident dermal spray drift exposure mean - child	0,12	ml spray dilution/person	
Resident inhal. spray drift exposure mean - adult	0,00008	ml spray dilution/person	
Resident inhal. spray drift exposure mean - child	0,00014	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)	2,30%		
Drift percentage on surface (mean)	1,80%		
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) - adult	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops (75th percentile) - child	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 32: Estimation of longer-term resident exposure towards MCPA according to EFSA guidance (DFR_0 = 1.76 by study data)

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)	0,3692975	0,0107000	0,0445060	0,2722500	0,4637763
Total systemic exposure per kg body weight	0,0369298	0,0010700	0,0044506	0,0272250	0,0463776
% of RVNAS	92,32%	2,68%	11,13%	68,06%	115,94%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,5302000	0,0138000	0,1124200	0,9075000	1,0715705
Total systemic exposure per kg body weight	0,0088367	0,0002300	0,0018737	0,0151250	0,0178595
% of RVNAS	22,09%	0,58%	4,68%	37,81%	44,65%

Table A 11: Input parameters considered for the estimation of longer-term resident exposure (DFR_0 = 1.76 by study data and drift reduction technique)

Croptype	Cereals		
Application method	Downward spraying		
Application equipment	Vehicle-mounted-Drift Reduction		i_AppEquip
Formulation type	Wettable granules, soluble granules		i_FormVal
Buffer strip	2-3	m	i_Buffer
Application rate of the product	0,55	kg a.s./ha	i_AppRate
Concentration of active substance (in-use dilution for liquid applications)	2,75	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	100,00%		i_AbsorpOralInuse
Dislodgeable foliar residue (i_AppRate*i_DFR)	0,968	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) - adult	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops (75th percentile) - child	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 12: Estimation of longer-term resident exposure towards MCPA according to EFSA guidance (DFR_0 = 1.76 by study data and drift reduction technique)

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)	0,1846488	0,0107000	0,0222530	0,2722500	0,3457751
Total systemic exposure per kg body weight	0,0184649	0,0010700	0,0022253	0,0272250	0,0345775
% of RVNAS	46,16%	2,68%	5,56%	68,06%	86,44%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,2651000	0,0138000	0,0562100	0,9075000	0,9044752
Total systemic exposure per kg body weight	0,0044183	0,0002300	0,0009368	0,0151250	0,0150746
% of RVNAS	11,05%	0,58%	2,34%	37,81%	37,69%

Table A 35: Input parameters considered for the estimation of longer-term resident exposure (DFR_0 = 1.76 by study data and 5 m buffer strip)

Croptype	Cereals		
Application method	Downward spraying		
Application equipment	Vehicle-mounted		i_AppEquip
Formulation type	Wettable granules, soluble granules		i_FormVal
Buffer strip	5	m	i_Buffer
Application rate of the product	0,55	kg a.s./ha	i_AppRate
Concentration of active substance (in-use dilution for liquid applications)	2,75	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	100,00%		i_AbsorpOralInuse
Dislodgeable foliar residue (i_AppRate*i_DFR)	0,968	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	0,23798	ml spray dilution/person	
Resident dermal spray drift exposure 75th percentile - child	0,2175	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - adult	0,00009	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - child	0,00017	ml spray dilution/person	
Resident dermal spray drift exposure mean - adult	0,12278	ml spray dilution/person	
Resident dermal spray drift exposure mean - child	0,12	ml spray dilution/person	
Resident inhal. spray drift exposure mean - adult	0,00008	ml spray dilution/person	
Resident inhal. spray drift exposure mean - child	0,00014	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)	2,30%		
Drift percentage on surface (mean)	1,80%		
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) - adult	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops (75th percentile) - child	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 136: Estimation of longer-term resident exposure towards MCPA according to EFSA guidance (DFR_0 = 1.76 by study data and 5 m buffer strip)

L. Total

L.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)	0,2456988	0,0107000	0,0182793	0,2722500	0,3777645
Total systemic exposure per kg body weight	0,0245699	0,0010700	0,0018279	0,0272250	0,0377765
% of RVNAS	61,42%	2,68%	4,57%	68,06%	94,44%

L.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,2685700	0,0138000	0,0461725	0,9075000	0,9121695
Total systemic exposure per kg body weight	0,0044762	0,0002300	0,0007695	0,0151250	0,0152028
% of RVNAS	11,19%	0,58%	1,92%	37,81%	38,01%

A 3.3.2 Calculations for Tribenuron-methyl– cereals

Table A 37: Input parameters considered for the estimation of longer-term resident exposure

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	0,015	kg a.s./ha	*i_AppRate*
Concentration of active substance (in-use dilution for liquid applications)	0,075	g a.s./l	*d_ConcAS*
Dermal absorption of product	50,00%		*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)	0,045	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 14: Estimation of longer-term resident exposure towards Tribenuron methyl according to EFSA guidance

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)	0,0100718	0,0107000	0,0011736	0,0126563	0,0271982
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0010072	0,0010700	0,0001174	0,0012656	0,0027198
% of RVNAS	2,01%	2,14%	0,23%	2,53%	5,44%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0144600	0,0138000	0,0030660	0,0421875	0,0565518
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0002410	0,0002300	0,0000511	0,0007031	0,0009425
% of RVNAS	0,48%	0,46%	0,10%	1,41%	1,89%

Table A 15: Input parameters considered for the estimation of longer-term resident exposure (drift reduction technique)

Croptype	Cereals		
Application method	Downward spraying		
Application equipment	Vehicle-mounted-Drift Reduction		*i_AppEquip*
Formulation type	Wettable granules, soluble granules		*i_FormVal*
Buffer strip	2-3	m	*i_Buffer*
Application rate of the product	0,015	kg a.s./ha	*i_AppRate*
Concentration of active substance (in-use dilution for liquid applications)	0,075	g a.s./l	*d_ConcAS*
Dermal absorption of product	50,00%		*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)	0,045	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) - ad	7500	cm^2/h	*d_TcEntryAd*
Transfer coefficient for entry into treated crops (75th percentile) - chi	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 16: Estimation of longer-term resident exposure towards Tribenuron methyl according to EFSA guidance (drift reduction technique)

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)	0,0050359	0,0107000	0,0005868	0,0126563	0,0239947
Total systemic exposure per kg body weight	0,0005036	0,0010700	0,0000587	0,0012656	0,0023995
% of RVNAS	1,01%	2,14%	0,12%	2,53%	4,80%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0072300	0,0138000	0,0015330	0,0421875	0,0519946
Total systemic exposure per kg body weight	0,0001205	0,0002300	0,0000256	0,0007031	0,0008666
% of RVNAS	0,24%	0,46%	0,05%	1,41%	1,73%

Table A 41: Input parameters considered for the estimation of longer-term resident exposure (5 m buffer strip)

Croptype	Cereals		
Application method	Downward spraying		
Application equipment	Vehicle-mounted		i_AppEquip
Formulation type	Wettable granules, soluble granules		i_FormVal
Buffer strip	5	m	i_Buffer
Application rate of the product	0,015	kg a.s./ha	i_AppRate
Concentration of active substance (in-use dilution for liquid applications)	0,075	g a.s./l	d_ConcAS
Dermal absorption of product	50,00%		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)	0,045	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	0,23798	ml spray dilution/person	
Resident dermal spray drift exposure 75th percentile - child	0,2175	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - adult	0,00009	ml spray dilution/person	
Resident inhal. spray drift exposure 75th percentile - child	0,00017	ml spray dilution/person	
Resident dermal spray drift exposure mean - adult	0,12278	ml spray dilution/person	
Resident dermal spray drift exposure mean - child	0,12	ml spray dilution/person	
Resident inhal. spray drift exposure mean - adult	0,00008	ml spray dilution/person	
Resident inhal. spray drift exposure mean - child	0,00014	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)	2,30%		
Drift percentage on surface (mean)	1,80%		
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) - adult	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops (75th percentile) - child	2250	cm²/h	d_TcEntryCh
Transfer coefficient for entry into treated crops (mean) - adult	5980	cm²/h	d_TcEntryAd

Table A 42: Estimation of longer-term resident exposure towards Tribenuron methyl according to EFSA guidance (5 m buffer strip)

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)	0,0067009	0,0107000	0,0004820	0,0126563	0,0248690
Total systemic exposure per kg body weight	0,0006701	0,0010700	0,0000482	0,0012656	0,0024869
% of RVNAS	1,34%	2,14%	0,10%	2,53%	4,97%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0073246	0,0138000	0,0012593	0,0421875	0,0522045
Total systemic exposure per kg body weight	0,0001221	0,0002300	0,0000210	0,0007031	0,0008701
% of RVNAS	0,24%	0,46%	0,04%	1,41%	1,74%

Table A 43: Input parameters considered for the estimation of acute bystander exposure

Croptype	Cereals		
Application method	Downward spraying		
Application equipment	Vehicle-mounted		*i_AppEquip*
Formulation type	Wettable granules, soluble granules		
Application rate of the product	0,015	kg a.s./ha	*i_AppRate*
Buffer strip	2-3	m	*i_Buffer*
Concentration of active substance (in-use dilution for liquid applications)	0,075	g a.s./l	*d_ConcAS*
Dermal absorption of product	50,00%		*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)	0,045	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 44: Estimation of acute bystander exposure towards Tribenuron methyl according to EFSA guidance

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)	0,0228390	0,0107000	0,0035286	0,0126563
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0022839	0,0010700	0,0003529	0,0012656
% of RVAAS	1,76%	0,82%	0,27%	0,97%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops
Total systemic exposure (mg a.s./day)	0,0372450	0,0138000	0,0092438	0,0421875
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0006208	0,0002300	0,0001541	0,0007031
% of RVAAS	0,48%	0,18%	0,12%	0,54%

A 3.3.3 Calculations for MCPA – grasslands

Table A 45: Input parameters considered for the estimation of longer-term resident exposure

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	0,55	kg a.s./ha	*i_AppRate*
Concentration of active substance (in-use dilution for liquid applications)	2,75	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	100,00%		*i_AbsorpOralInuse*
Dislodgeable foliar residue (i_AppRate*i_DFR)	1,65	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) - adult	7500	cm²/h	*d_TcEntryAd*
Transfer coefficient for entry into treated crops (75th percentile) - child	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 46: Estimation of longer-term resident exposure towards Tribenuron methyl according to EFSA guidance

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)	0,3692975	0,0107000	0,0445060	0,1234063	0,3360773
Total systemic exposure per kg body weight (mg/kg	0,0369298	0,0010700	0,0044506	0,0123406	0,0336077
% of RVNAS	92,32%	2,68%	11,13%	30,85%	84,02%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,5302000	0,0138000	0,1124200	0,2509375	0,5989280
Total systemic exposure per kg body weight (mg/kg	0,0088367	0,0002300	0,0018737	0,0041823	0,0099821
% of RVNAS	22,09%	0,58%	4,68%	10,46%	24,96%

A 3.3.4 Calculations for Tribenuron-methyl – grasslands

Table A 47: Input parameters considered for the estimation of longer-term resident exposure

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	0,015	kg a.s./ha	i_AppRate
Concentration of active substance (in-use dilution for liquid applications)	0,075	g a.s./l	d_ConcAS
Dermal absorption of product	50,00%		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)	0,045	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) - adult	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops (75th percentile) - child	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 48: Estimation of longer-term resident exposure towards Tribenuron methyl according to EFSA guidance

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)	0,0100718	0,0107000	0,0011736	0,0030593	0,0195445
Total systemic exposure per kg body weight (mg/kg	0,0010072	0,0010700	0,0001174	0,0003059	0,0019544
% of RVNAS	2,01%	2,14%	0,23%	0,61%	3,91%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops	All pathways (mean)
Total systemic exposure (mg a.s./day)	0,0144600	0,0138000	0,0030660	0,0068438	0,0297580
Total systemic exposure per kg body weight (mg/kg	0,0002410	0,0002300	0,0000511	0,0001141	0,0004960
% of RVNAS	0,48%	0,46%	0,10%	0,23%	0,99%

Table A 49: Input parameters considered for the estimation of acute bystander exposure

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	0,015	kg a.s./ha	i_AppRate
Buffer strip	2-3	m	i_Buffer
Concentration of active substance (in-use dilution for liquid applications)	0,075	g a.s./l	d_ConcAS
Dermal absorption of product	50,00%		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)	0,045	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
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 - adult	7500	cm²/h	d_TcEntryAd
Transfer coefficient for entry into treated crops - child	2250	cm²/h	d_TcEntryCh

Table A 50: Estimation of acute bystander exposure towards Tribenuron methyl according to EFSA guidance

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)	0,0228390	0,0107000	0,0035286	0,0056288
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0022839	0,0010700	0,0003529	0,0005629
% of RVAAS	1,76%	0,82%	0,27%	0,43%

1.2 Adult

	Spray drift	Vapour	Surface deposits	Entry into treated crops
Total systemic exposure (mg a.s./day)	0,0372450	0,0138000	0,0092438	0,0135938
Total systemic exposure per kg body weight (mg/kg bw/day)	0,0006208	0,0002300	0,0001541	0,0002266
% of RVAAS	0,48%	0,18%	0,12%	0,17%

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)

Comments of zRMS:	Study accepted.

	The study Foliar dislodgeable residues dissipation in cereals after one application with the herbicide MT-565SG-OR2-C (focussed on the active substance MCPA) in Germany 2020 – Field part was performed for formulation being under evaluation in this dRR according to the guidelines OPPTS 875.2100, OECD Guidelines for the testing of chemicals, No 509: Crop Field Trials (2009), EC document 7029/V1/95 rev. 5, 1997, Appendix B working document 1607/V1/97, rev. 2, 1999, Rückstands-versuche, Teil 1 Prüfungen an Pflanzen, A: Allgemeiner Teil, B: Spezieller Teil, IVA-Guideline, Industrieverband Agrar e. V. 1992 in compliance with present OECD, EC and German principles of Good Laboratory Practice (GLP) with not significant exceptions (weather data, soil parameter, trial site history). No deviations occurred during the conduct of the study. The trial was carried out in the crop winter wheat on open field in Lower Saxony / North Germany. The tested plot was treated with the herbicide with the rate of 1.0 kg/ha (560g MCPA/ha) (200 L/ha) at crop stage BBCH 23 by using backpack sprayer with boom, running by compressed air. Leaf samples of the untreated and treated plots were collected, dislodged by using an aqueous surfactant, deep frozed and shipped to the analytical laboratory LAUS GmbH, 67489 Kirrweiler (Pfalz) / Germany for residue analysis. The analytical results are included in the reports with study number: 19112203G926 and 19112203G405 enclosed to part B.5 (Note: An adequate description of the analytical part of the study will be provided in section 5.) After calculation the mean initial value of DFR for MCPA of 1.76 µg per cm² leaf area/1 kg MCPA applied/ha was obtained. This value was decreasing in time to <LOQ in 5 days after application.

Reference	KCP 7.2
Report	Foliar dislodgeable residues dissipation in cereals after one application with the herbicide MT-565SG-OR2-C (focussed on the active substance MCPA) in Germany 2020 – Field part Cirka H. (2020) Study No. CT19-1-59 CropTrials GmbH
Guideline(s)	Foliar Dislodgeable Residue Dissipation: OPPTS 875.2100 OECD Guidelines for the testing of chemicals, No 509: Crop Field Trials (2009) EC document 7029/V1/95 rev. 5, 1997, Appendix B working document 1607/V1/97, rev. 2, 1999: General recommendation for the design, preparation and realisation of residue trials The Application of the GLP Principles to Field Studies, OECD Consensus Document, 6, revised, ENV/JM/MONO (1999) 22, Paris 2002 Rückstandsversuche, Teil 1 Prüfungen an Pflanzen, A: Allgemeiner Teil, B: Spezieller Teil, IVA-Guideline, Industrieverband Agrar e. V. 1992
Deviations	No
GLP	Yes
Acceptability	Yes
Duplication	Non relevant (non vertebrate study)

(if vertebrate study)	

The purpose of the study was to generate dislodgeable foliar residues samples after one application with the herbicide in cereals in Germany 2019. The active substance MCPA was the subject of investigation.

Material and Methods

Trial Layout

The trial was carried out in the crop winter wheat on open field in Lower Saxony / North Germany.

Two plots were measured out: one untreated control plot (= U / plot 1) and one treated plot (= T / plot 2).
Plot 2 was treated with the herbicide with the rate of 1.0 kg/ha. The used water volume was 200 L/ha. The application was performed in spring 2020 at crop stage BBCH 23. For sampling purposes, plot 2 was divided into three subplots (2A, 2B, 2C).

Drift of spray solution during the application was avoided by choosing an adequate distance of 21 m between the untreated and treated plot. A buffer zone of 5 m was set up around the plots of the trial.

Application
One application of the test item was performed in spring 2020 at crop stage BBCH 23 by using backpack sprayer with boom, running by compressed air.

The application rate of the herbicide was 1.0 kg/ha (eq. to 560 g MCPA/ha). The water volume was 200 L/ha.

Field Sampling

Leaf samples of the untreated plot (U / plot 1) were collected at two timings:
0 DALA (at the day of the application) / prior application and 21 DALA (= days after application).

Leaf samples of the treated plot (T / plot 2) were collected at 9 timings:

0 DALA (at the day of the application): prior application
0 DALA (at the day of the application): 4 hours (+/- 30 minutes) after application
1 DALA , 2 DALA, 5 DALA, 7 DALA, 10 DALA, 14 DALA, 21 DALA (= days after application).

Each leaf sample consisted of 38 leaves, collected from 12 different areas within each plot (U) or subplot (T: 2A, 2B, 2C). The edges (0.5 m) of the plots were left out for sampling. Only fully matured leaves were sampled.

Dislodging Method

The leaf samples were dislodged by using an aqueous surfactant solution within 3 hours after each field sampling. The dislodging solution samples were stored deep frozen at the test facility in Burgwedel / Germany until shipment to the analytical laboratory.

The leaf area of the dislodged leaves was determined per leaf sample.

Field fortification samples

To assess the stability of the active substance MCPA in the dislodging solution during freezer storage, field fortification samples were prepared at the timings 0 DALA and 21 DALA.

To assess the stability of the active substance MCPA in the dislodging solution during freezer storage, field fortification samples were prepared at the timings 0 DALA and 21 DALA.

The dislodging solution samples were stored deep-frozen at the test facility in Burgwedel / Germany, together with the dislodging solution samples, until shipment to the analytical laboratory.

The dislodging solution samples and the field fortification samples were shipped deep-frozen to the analytical laboratory LAUS GmbH, 67489 Kirrweiler (Pfalz) / Germany for residue analysis.
The analysis was not part of this study, it was performed as a separate study (for further details please refer to part B.5 (LAUS GmbH, study number: 19112203G926 and 19112203G405).

Results:

At the end the following results were obtained as presented in the table below:

Sampling timing	Plot / Subplot Identification (U = Untreated, T = treated)	Leaf area per sample [cm²]	Concentration of MCPA [µg/L] in 200 mL washing solution before / after an application of 560 g MCPA/ha	Calculated concentration of MCPA [µg] per cm² leaf area before / after an application of 560 g MCPA / ha	Calculated concentration of MCPA [µg] per cm² leaf area before / after an application of 1 kg MCPA / ha (linear extrapolation)	
					Replicates	Mean
0 DALA, prior application	1 (U)	345	<LOD	<LOD	<LOD	<LOD
0 DALA, prior application	2A (T)	376	<LOD	<LOD	<LOD	<LOD
0 DALA, prior application	2B (T)	383	<LOD	<LOD	<LOD	
0 DALA, prior application	2C (T)	384	<LOD	<LOD	<LOD	
0 DALA, after application	2A (T)	408	2030	0.995	1.777	1.76
0 DALA, after application	2B (T)	399	2010	1.008	1.799	
0 DALA, after application	2C (T)	362	1720	0.950	1.697	
1 DALA	2A (T)	376	1670	0.888	1.586	1.47

1 DALA	2B (T)	364	1460	0.802	1.432	
1 DALA	2C (T)	347	1360	0.784	1.400	
2 DALA	2A (T)	435	1060	0.487	0.870	
2 DALA	2B (T)	368	1070	0.582	1.038	0.95
2 DALA	2C (T)	333	863	0.518	0.926	
5 DALA	2A (T)	289	< LOQ (0.999)	< LOQ	< LOQ	
5 DALA	2B (T)	290	< LOQ (0.890)	< LOQ	< LOQ	< LOQ
5 DALA	2C (T)	309	< LOQ (0.804)	< LOQ	< LOQ	
7 DALA	2A (T)	358	1.34	0.001	0.001	
7 DALA	2B (T)	336	< LOQ (0.546)	< LOQ	< LOQ	< LOQ
7 DALA	2C (T)	332	< LOQ (0.770)	< LOQ	< LOQ	
14 DALA	2A (T)	416	< LOQ ~~(0.314)~~	< LOQ	< LOQ	
14 DALA	2B (T)	395	< LOQ (0.509)	< LOQ	< LOQ	< LOQ
14 DALA	2C (T)	389	<LOD	<LOD	<LOD	
21 DALA	1 (U)	369	<LOD	<LOD	<LOD	<LOD
21 DALA	2A (T)	426	<LOD	<LOD	<LOD	
21 DALA	2B (T)	383	<LOD	<LOD	<LOD	<LOD
21 DALA	2C (T)	392	<LOD	<LOD	<LOD	

Conclusion/endpoint:

The mean initial DFR for MCPA was 1.76 µg per cm² leaf area/1 kg MCPA applied/ha and was decreasing in time to <LOQ in 5 days after application.