Section A6.6.3 Genotoxicity in vitro

Annex Point IIA 6.6.3 **In-vitro gene mutation assay in mammalian cells**

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted]; Cypermethrin Technical – L5178Y TK +/- mouse lymphoma assay; [redacted] Dates of experimental work: 30 November 2010 – 07 February 2011.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Agriphar s.a.	
1.2.2			
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes, OECD guideline 476	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	SL25163S63	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Pale yellow viscous liquid	
3.1.2.2	Purity	93.56% w/w	
3.1.2.3	Stability	Stable	
3.2	**Study Type**	In -vitro gene mutation study in mammalian cells	
3.2.1	Organism/cell type	Mouse lymphoma cell line L5178Y TK+/- 3.7.2c	
3.2.2	Deficiencies / Proficiencies	Not applicable	
3.2.3	Metabolic activation system	S9 mix (PB/βNF S9) prepared in-house from the livers of male Sprague-Dawley rats weighing approximately 250g. These had each received, orally, three consecutive daily doses of phenobarbital/β-naphthoflavone (80/100 mg per kg per day) prior to S9 preparation on the fourth day. S9-mix was prepared by mixing S9, NADP (5 mM), G-6-P (5 mM), KCl (33 mM) and $MgCl_2$ (8 mM) in R0. 20% S9-mix (i.e. 2% final concentration of S9) was added to the cultures of the Preliminary Toxicity Test and of Experiment 1. In Experiment 2, 10% S9-mix (i.e. 1% final concentration of S9), was added.	
3.2.4	Positive control	Ethylmethanesulphonate (EMS) in the absence of metabolic activation. Cyclophosphamide (CP) in the presence of metabolic activation.	

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3.3	**Administration / Exposure; Application of test substance**	
3.3.1	Concentrations	Experiment 1 : 65 to 2080 µg/ml in both the absence and presence of metabolic activation. Experiment 2: 2.03 to 260 µg/ml in the absence of metabolic activation and 16.25 to 2080 µg/ml in the presence of metabolic activation.
3.3.2	Way of application	The study was conducted as two separate experiments <u>Experiment 1</u> Several days before starting the experiment, an exponentially growing stock culture of cells was set up so as to provide an excess of cells on the morning of the experiment. The cells were counted and processed to give 1 x 10^6 cells/ml in 10 ml aliquots in R10 medium in sterile plastic universals. The treatments were performed in duplicate (A + B), both with and without metabolic activation (S9-mix) at six dose levels of the test item (65 to 2080 µg/ml in both the absence and presence of metabolic activation), vehicle and positive controls. To each universal was added 2 ml of S9-mix if required, 0.2 ml of the treatment dilutions, (0.2 ml for the positive control) and sufficient R0 medium to bring the total volume to 20 ml. <u>Experiment 2</u> As in Experiment 1, an exponentially growing stock culture of cells was established. The cells were counted and processed to give 1 x 10^6 cells/ml in 10 ml duplicate cultures in R10 medium for the 4-hour treatment with metabolic activation cultures. In the absence of metabolic activation the exposure period was extended to 24 hours; therefore 0.3 x 10^6 cells/ml in 10 ml duplicate cultures were established in 25 cm^2 tissue culture flasks. To each culture 2 ml of S9-mix was added if required, 0.2 ml of the treatment dilutions, (0.2 ml for the positive control) and sufficient R0 medium to give a final volume of 20 ml (R10 is used for the 24-hour exposure group). The dose range of the test item was 2.03 to 260 µg/ml in the absence of metabolic activation, and 16.25 to 2080 µg/ml in the presence of metabolic activation.
3.3.3	Pre-incubation time	Experiment 1: The treatment vessels were incubated at 37°C for 4 hours. Experiment 2: The treatment vessels were incubated at 37°C with continuous shaking using an orbital shaker for 24 hours in the absence of metabolic activation and 4 hours in the presence of metabolic activation.
3.4	**Examinations**	
3.4.1	Number of cells evaluated	Cells were counted, diluted to 10^4 cells/ml and plated for mutant frequency (2000 cells/well) in selective medium containing in 96-well microtitre plates. Cells were also diluted to 10 cells/ml and plated (2 cells/well) for viability (%V) in non-selective medium. The daily cell counts were used to obtain a Relative Suspension Growth (%RSG) value.

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3.5	**Statistics**	The experimental data was analysed using a dedicated computer program, Mutant 240C by York Electronic Research, which follows the statistical guidelines recommended by the UKEMS (Robinson W D et al, 1989).
		4 RESULTS AND DISCUSSION
4.1	**Genotoxicity**	See tables A6_6_3-1 and A6_6_3-2
4.1.1	without metabolic activation	No significant increase in mutant frequency at the TK +/- locus
4.1.2	with metabolic activation	No significant increase in mutant frequency at the TK +/- locus
4.2	**Controls**	Neither of the vehicle control mutant frequency values were outside the acceptable range of 50 to 200 x 10^{-6} viable cells. Both of the positive controls produced marked increases in the mutant frequency per viable cell.

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5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The study was conducted according to a method that was designed to assess the potential mutagenicity of the test item on the thymidine kinase, TK +/-, locus of the L5178Y mouse lymphoma cell line.

Two independent experiments were performed. In Experiment 1, L5178Y TK +/- 3.7.2c mouse lymphoma cells (heterozygous at the thymidine kinase locus) were treated with the test item at six dose levels, in duplicate, together with vehicle (solvent) and positive controls using 4-hour exposure groups both in the absence and presence of metabolic activation (2% S9). In Experiment 2, the cells were treated with the test item at eight dose levels using a 4-hour exposure group in the presence of metabolic activation (1% S9) and a 24-hour exposure group in the absence of metabolic activation.

The dose range of test item was selected following the results of a preliminary toxicity test and for the first experiment was 65 to 2080 µg/ml in both the absence and presence of metabolic activation. For the second experiment the dose range was 2.03 to 260 µg/ml in the absence of metabolic activation, and 16.25 to 2080 µg/ml in the presence of metabolic activation.

5.2 Results and discussion

The maximum dose level used for the 4-hour exposure groups in the mutagenicity test was limited by a combination of toxicity and the presence of precipitate effectively reducing exposure of the test item to the cells, the maximum dose level in the 24-hour exposure group was limited by toxicity. The vehicle (solvent) controls had acceptable mutant frequency values that were within the normal range for the L5178Y cell line at the TK +/- locus. The positive control items induced marked increases in the mutant frequency indicating the satisfactory performance of the test and of the activity of the metabolising system.

The test item did not induce any toxicologically significant dose-related increases in the mutant frequency at any dose level, either with or without metabolic activation, in either the first or the second experiment.

5.3 Conclusion

Cypermethrin cis:trans/40:60 proved to be non-mutagenic

5.3.1 Reliability

1

5.3.2 Deficiencies

No

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Materials and Methods	*State if the applicants version is acceptable or indicate relevant discrepancies referring to the (sub) heading numbers and to applicant's summary and conclusion.*
Results and discussion	*Adopt applicant's version or include revised version. If necessary, discuss relevant deviations from applicant's view referring to the (sub)heading numbers*
Conclusion	Other conclusions: *(Adopt applicant's version or include revised version)*
Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator*
Acceptability	acceptable / not acceptable *(give reasons if necessary, e.g. if a study is considered acceptable despite a poor reliability indicator. Discuss the relevance of deficiencies and indicate if repeat is necessary.)*
Remarks	
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 6_6_3.1 Summary of results – Experiment 1

Treatment (µg/ml)		4-Hours-S-9			Treatment (µg/ml)		4-Hours+S-9		
		%RSG	RTG	MF§			%RSG	RTG	MF§
0		100	1.00	114.02	0		100	1.00	96.50
65		78	0.96	88.92	65		74	0.62	129.29
130		72	0.77	108.02	130		70	0.67	128.11
260		69	0.80	102.37	260		72	0.62	127.23
520		68	0.74	87.78	520		66	0.50	123.24
1040	X	8	0.04	124.26	1040	X	7	0.02	204.89
2080	X	6	0.03	83.83	2080	X	6	0.02	177.29
Linear trend				NS	Linear trend				NS
EMS					CP				
400		70	0.49	867.47	2		84	0.53	656.44

Table 6_6_3.2 Summary of results – Experiment 2

Treatment (µg/ml)		24-Hours-S-9			Treatment (µg/ml)		4-Hours+S-9		
		%RSG	RTG	MF§			%RSG	RTG	MF§
0		100	1.00	101.06	0		100	1.00	93.64
2.03	Ø	106			16.25		80	0.89	95.39
4.06		108	1.17	93.61	32.5		68	0.70	131.32
8.13		106	1.12	93.88	65		63	0.64	101.29
16.25		88	1.05	90.70	130		63	0.69	96.79
32.5		41	0.43	105.06	260		61	0.56	134.00
65		26	0.23	79.02	520		55	0.58	121.29
130		18	0.16	139.83	1040	X	6	0.04	153.81
260		14	0.13	104.12	2080	Ø	6		
Linear trend				NS	Linear trend				NS
EMS					CP				
150		75	0.51	958.36	2		76	0.40	863.04

%RSG = Percentage Relative Suspension Growth

RTG = Relative Total Growth

MF = Mutation Frequency