Section A6.6.3 Genotoxicity in vitro

Annex Point IIA6.6 6.6.3 In-vitro gene mutation assay in CHO-cells (HPRT-test)

Official use only

		1 REFERENCE
1.1	**Reference**	H. Lehn, 1988, KUE 13032 C - Dichlofluanid - Mutagenicity study for the detection of induced forward mutations in the CHO-HPGRT assay in vitro, BAYER AG Institute of Toxicology, Report No. 17239, 1988-10-18 (unpublished)
1.2	**Data protection**	Yes
1.2.1	Data owner	Bayer CropScience AG
1.2.2	Companies with letter of access	Bayer Chemicals AG
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/IA.
		2 GUIDELINES AND QUALITY ASSURANCE
2.1	**Guideline study**	No The methods used are in accordance to the OECD-guideline 476.
2.2	**GLP**	Yes
2.3	**Deviations**	No
		3 MATERIALS AND METHODS
3.1	**Test material**	As given in section 2 of dossier.
3.1.1	Lot/Batch number	[redacted]
3.1.2	Specification	As given in section 2 of dossier.
3.1.2.1	Description	White powder
3.1.2.2	Purity	[redacted] (analytical result dated July 14, 1987) [redacted] (analytical result dated January 13, 1987)
3.1.2.3	Stability	The batch used was analytically examined and approved for at least the test period. A stability test in the solvent did not detect an indication of a relevant change in the active ingredient.
3.2	**Study Type**	In vitro mammalian cell gene mutation test
3.2.1	Organism/cell type	Mammalian cell lines: Chinese Hamster Ovary (CHO)
3.2.2	Deficiencies / Proficiencies	—
3.2.3	Metabolic activation system	S9 mix Livers of at least six adult Sprague Dawley rats were used to prepare the S9 mix. For enzyme induction the animals received a single intraperitoneal injection of Aroclor 1254, at dose of 500 mg/kg bw five days before preparation. For preparation, the livers were removed immediately after killing the rats. The livers were homogenised and centrifuged at 9000 × g. Then the supernatant (the S9 fraction) was diluted with a cofactor solution. The amount of S9 fraction in S9 mix is indicated in percent.

Section A6.6.3 Genotoxicity in vitro

Annex Point IIA6.6 6.6.3 In-vitro gene mutation assay in CHO-cells (HPRT-test)

3.2.4	Positive control	Without S9 mix: ethylmethane sulfonate (0.9 mg/ml) With S9 mix: 3-methylcholanthrene (5 µg/ml)
3.3	**Application of test substance**	
3.3.1	Concentrations	Without S9 mix: 0, 0.2, 0.35, 0.50, 0.65, 0.80 or 0.95 µg/ml With S9 mix: 0, 0.19, 0.38, 0.78, 1.5, 3.0, 6.0 or 12.0 µg/ml The test concentrations were based on a pilot study in which dose ranged from 0.2 µg/ml to 2.0 µg/ml without metabolic activation and from 0.098 µg/ml to 25.0 µg/ml with metabolic activation. After determination of cytotoxicity of dichlofluanid, the concentration range of dichlofluanid for the mutagenicity study was chosen ranging from approx. 0% to 90% reduction in colony forming ability.
3.3.2	Way of application	Dissolved in medium (solvent: DMSO).
3.3.3	Pre-incubation time	—
3.3.4	Other modifications	—
3.4	**Examinations**	
3.4.1	Number of cells evaluated	—

4 RESULTS AND DISCUSSION

4.1	**Genotoxicity**	
4.1.1	Without metabolic activation	No
4.1.2	With metabolic activation	No
4.2	**Cytotoxicity**	Yes Without metabolic activation: In the first assay, decreases in relative population growth up to 35 % survival were observed, but there was no dose-relationship. The second trial was not used for evaluation because no cytotoxicity was induced. In the third trial (using duplicates) dichlofluanid decreases the relative population growth up to 70 % growth inhibition. With metabolic activation: No significant cytotoxicity in all trials; in the first assay, decreases in relative population growth up to 66 % survival were observed, but there was no dose-relationship. In the second trial, decreases in relative population growth up to 63 % survival were estimated and in the third trial dichlofluanid decreases the relative population growth up to 30 % growth inhibition.

Section A6.6.3 Genotoxicity in vitro

Annex Point IIA6.6 6.6.3 In-vitro gene mutation assay in CHO-cells (HPRT-test)

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The study was done according to OECD-Guideline 476, though not stated in the study report. The methods used represent further developments of the techniques originally described in the review article of Hsie et al. (Mutation Res. 86: 193 –214, 1981). The study was carried out with a subclone of the CHO cell line designated CHO-K1-BH_4 (O' Neill et al.; Mutation Res. 45: 103 –109, 1977). This clone has been shown to be sensitive to a variety of chemical mutagens at its HGPRT gene locus (Hsie et al.; Mutation Res. 86: 193 –214, 1981). Three trials were performed each for treatment with and without metabolic activation. Under non-activation conditions, the first two trials were performed without duplicates. The third trial was performed employing duplicates. Under metabolic activation conditions, no duplicates were used in the first assay while the two other trials were performed employing duplicates.

The purpose of the test was to assess the ability of dichlofluanid to induce forward mutations at the HPRT locus in CHO cells.

5.2 Results and discussion

Under both treatment conditions, cytotoxicity was induced.

There were neither dose-related nor reproducible increases in mutant frequency which were significantly elevated over the negative controls. In contrast, the positive controls ethylmethanesulfonate (without S9 mix) and 3-methylcholanthrene (with S9 mix) revealed a clear mutagenic effect in the assay.

5.3 Conclusion

The test substance can be considered as non-mutagenic in the CHO-HPRT assay, both with and without metabolic activation.

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	28/10/04
Materials and Methods	As described above [IUCLID 5.5 10 of 12]
Results and discussion	As described above
Conclusion	As described above
Reliability	1
Acceptability	Acceptable
Remarks	The UK CA agrees with the applicant's summary and conclusions. However, the UK CA notes that the study may not have used sufficiently high concentrations.
	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 A6_6_1-1.A Table for gene mutation assay: CHO-HPRT-test

Treatment without S9 mix				
Concentration [µg/ml]	**Mutant Frequency#** (Thioguanin-resistant mutants per 10^6 clonable cells)			
	1st trial	2nd trial	3rd trial with duplicates	
Negative control	8.0	21.1	5.0	10.4
Vehicle control	16.1	20.5	9.1	2.9
0.20	20.3	19.7	15.2*	6.4
0.35	13.6	22.0	4.2	7.8
0.50	27.7	22.9	3.0	6.0
0.65	21.6	13.1	0.7	1.1
0.80	32.6	22.2	10.8	4.3
0.95	9.4	37.9*	14.4	10.1
Positive control	128.7*	103.2**	674.3**	456.9**

Table A6_6_1-1.B Table for gene mutation assay: CHO-HPRT-test

Treatment with S9 mix					
Concentration [µg/ml]	**Mutant Frequency** (Thioguanin-resistant mutants per 10^6 clonable cells)				
	1st trial	2nd trial with duplicates		3rd trial with duplicates	
Negative control	18.8	8.1	5.4	5.3	1.9
Vehicle control	20.7	1.4	3.0	2.7	4.6
0.19	26.6	3.3	8.4	0.7	0
0.38	19.8	2.2	2.9	3.0	1.2
0.78	16.3	6.2	9.7	1.0	3.3
1.5	10.5	3.2	2.3	1.2	6.4
3.0	—	7.5	0.6	4.8	7.5
6.0	30.7	9.2*	45.3**	3.4	9.7*
12.0	24.8	16.2**	43.2**	2.7	1.2
Positive control	114.7**	60.0**	85.3**	78.9**	67.2**

* = significant increase, $p < 0.05$

** = significant increase, $p < 0.01$

Mutant frequency = Total number of mutant colonies × 100/total number of cells seeded × absolute cloning efficiency

Table A6_6_1-1.C Table for gene mutation assay: CHO-HPRT-test

Treatment without S9 mix				
Concentration [µg/ml]	**Relative Survival*** (% of vehicle control)			
	1st trial	2nd trial	3rd trial with duplicates	
Negative control	96.3	162.3	119.4	75.2
Vehicle control	100.0	100.0	100.0	100.0
0.20	88.0	92.9	84.3	68.1
0.35	74.3	136.4	117.5	70.5
0.50	59.3	139.0	93.6	66.8
0.65	55.5	159.8	95.1	55.6
0.80	101.4	129.9	148.1	39.0
0.95	128.1	130.5	84.4	32.2
Positive control	93.9	64.9	26.2	18.4

Table A6_6_1-1.D Table for gene mutation assay: CHO-HPRT-test

Treatment with S9 mix			
Concentration [µg/ml]	**Relative Survival*** (% of vehicle control)		
	1st trial	2nd trial with duplicates	3rd trial with duplicates
Negative control	95.2	63.7	94.9
Vehicle control	100.0	100.0	100.0
0.19	100.0	78.6	179.7
0.38	105.5	98.7	59.1
0.78	55.8	116.8	50.0
1.5	78.8	87.5	84.6
3.0	122.4	74.4	87.4
6.0	76.4	61.8	51.2
12.0	94.5	75.7	95.4
Positive control	164.5	40.0	95.6

* Relative survival = Mean number of colonies (treated cultures) × 100/ mean number of colonies (vehicle control cultures)

Table A6_6_1-1.E Table for gene mutation assay: CHO-HPRT-test

Treatment without S9 mix				
Concentration [µg/ml]	**Relative Population Growth**** (% of vehicle control)			
	1st trial	2nd trial	3rd trial with duplicates	
Negative control	78.8	145.3	119.4	75.2
Vehicle control	100.0	100.0	100.0	100.0
0.20	81.0	236.1	84.3	68.1
0.35	46.9	322.9	117.5	70.5
0.50	35.0	391.5	93.6	66.8
0.65	55.0	204.7	95.1	55.6
0.80	50.9	163.3	148.1	39.0
0.95	88.5	250.2	84.4	32.2
Positive control	74.2	150.3	26.2	18.4

Table A6_6_1-1.F Table for gene mutation assay: CHO-HPRT-test

Treatment with S9 mix					
Concentration [µg/ml]	**Relative Population Growth**** (% of vehicle control)				
	1st trial	2nd trial with duplicates		3rd trial with duplicates	
Negative control	147.9	112.6	88.5	116.5	87.6
Vehicle control	100.0	100.0	100.0	100.0	100.0
0.19	136.4	116.0	135.1	69.1	81.6
0.38	186.8	167.3	103.8	178.5	140.9
0.78	140.5	195.3	200.4	169.9	154.5
1.5	219.7	90.0	113.8	168.0	118.2
3.0	66.0	92.8	89.2	145.6	172.7
6.0	107.2	98.5	70.6	86.0	131.8
12.0	160.5	77.4	62.9	68.6	87.0
Positive control	188.3	122.7	73.2	127.1	115.8

**Relative population growth (%) = (Treated culture population increase over the expression period/vehicle control population increase over the expression period) × 100

This parameter shows the cumulative growth of the treated cell populations, relative to the vehicle control, over the expression period and prior to mutant selection. Values lower than 100% indicate growth inhibition as result of toxicity of the test substance.