DATA EVALUATION RECORD

CHLOROTHALONIL

Study Type: §84-2; Mouse Lymphoma Cell/Mammalian Activation Gene Forward Mutation Assay (L5178Y TK$^{+/-}$)

Work Assignment No. 3-01-91 B (MRID 45710214)

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Disclaimer

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DATA EVALUATION RECORD

STUDY TYPE: *In Vitro* Mammalian Cells - Gene Mutation Assay in L5178Y Mouse Lymphoma Cells; OPPTS 870.5300 [§84-2]; OECD 476.

PC CODE: 081901

DP BARCODE: 301496

TEST MATERIAL (PURITY): Chlorothalonil (99.1% a.i., Batch # 71/96)

SYNONYMS: Tetrachloroisophthalonitrile; 2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile

CITATION: Adams, K. (1996) Chlorothalonil: mammalian cell mutation assay. Huntingdon Life Sciences Ltd., Cambridgeshire, UK. Laboratory Study ID: VCM 82/962528, October 17, 1996. MRID 45710214. Unpublished.

SPONSOR: Vischim S.r.l., Via Friuli, 55, 20031 Cesano Maderno (Milano), Italy

EXECUTIVE SUMMARY - In two independent trials of a mammalian cell gene mutation assay at the $TK^{+/-}$ locus (MRID 45710214), L5178Y mouse lymphoma cells cultured *in vitro* were exposed to Chlorothalonil (99.1% a.i., Batch # 71/96) in dimethylsulfoxide (DMSO) for 3 hours at concentrations of 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 µg/mL (Trial 1, -S9); 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, or 2.5 µg/mL (Trial 1, +S9); 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, or 0.12 µg/mL (Trial 2, -S9); and 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, or 5.0 µg/mL (Trial 2, +S9). Methyl methanesulphonate (MMS) and 20-methylcholanthrene (MCA) served as positive controls in the absence and presence of S9, respectively.

Chlorothalonil was tested up to cytotoxic concentrations (≥0.1 µg/mL both trials, -S9; and ≥2.0 µg/mL [Trial 1] and ≥4.0 µg/mL [Trial 2], +S9). In the absence of S9-activation, mean mutant frequency was increased ($p \leq 0.01$) at 0.1 µg/mL in Trial 1, and at 0.12 µg/mL in Trial 2. However, due to high levels of cytotoxicity and the lack of a dose response at these dose levels, these findings were considered not to be a direct mutagenic effect. No other significant increases in mutant frequency were observed at any dose level in the presence or absence of S9 in either trial. The positive controls induced the expected response. **There was no evidence of induced mutant colonies over background in the presence or absence of S9-activation.**

The study is classified as **acceptable/guideline** and satisfies the guideline requirements (OPPTS 870.5300, OECD 476) for *in vitro* mutagenicity (mammalian forward gene mutation) data.

COMPLIANCE - Signed and dated Data Confidentiality, GLP, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Chlorothalonil

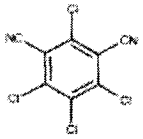
Description: White powder

Batch #: 71/96

Purity (w/w): 99.1% a.i.

CAS # of TGAI: 1897-45-6

Structure:



Solvent used: Dimethylsulfoxide (DMSO)

2. **Control materials**

Negative - The solvent alone served as the negative control.

Solvent - DMSO (1% v/v)

Positive

Non-activation - Methyl methanesulphonate (MMS, in DMSO; Final concentration: 10 µg/mL)

Activation - 20-Methylcholanthrene (MCA, in DMSO; Final concentration: 2.5 µg/mL)

3. **Activation** - The S9 fraction was derived from male Sprague Dawley rats (7-8 weeks old, weighing <300 g supplied by Harlan Olac Ltd)

X	induced	X	Aroclor 1254	X	Rat	X	Liver
	non-induced		Phenobarbital		Mouse		Lung
			β-naphthoflavone		Hamster		Other
			Other		Other		

The S9 fraction was prepared in the laboratory and stored at -80 °C until used. It was stated that all batches were checked for efficacy using 20-methylcholanthrene before use. The S9 mix contained S9 fraction (5% v/v), isocitric acid (8.7 mM), and NADP (1.6 mM) in ice cold RPMI 1640 medium. The final S9 culture concentration was approximately 2%.

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4. Test cells - Mammalian cells in culture

X	mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		others (list)

Media: Culture medium - RPMI 1640 medium, supplemented with 10% heat-inactivated donor horse serum (HiDHS), 0.1% Synperonic F68, 0.011% sodium pyruvate, and 2 mM L-glutamine, 50 µg/mL gentamicin, and buffered with 2 mg/mL sodium bicarbonate; Conditioned medium - culture medium from which growing cells had been removed; and Selection medium - culture medium supplemented with 4 µg/mL trifluorothymidine (TFT).

Properly maintained?	X Yes	No
Periodically checked for mycoplasma contamination?	Yes	X Not reported
Periodically checked for karyotype stability?	Yes	X Not reported
Periodically "cleansed" against high spontaneous background?	X Yes	Not reported

5. Locus examined

	X	**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na^+/K^+ ATPase**
Selection agent:		bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		fluorodeoxyuridine (FdU)		6-thioguanine (6-TG)		
	X	trifluorothymidine (TFT, 4 µg/mL)				

6. Test compound concentrations used

a. Preliminary cytotoxicity assay - 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, or 5.0 µg/mL

b. Mutagenicity assays

Non-activated: 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 µg/mL (Trial 1)
0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, or 0.12 µg/mL (Trial 2)

Activated: 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, or 2.5 µg/mL (Trial 1)
0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, or 5.0 µg/mL (Trial 2)

B. TEST PERFORMANCE

1. Cell treatment

a. Cells were exposed to test compound, negative/solvent or positive controls for 3 hours (non-activated) and 3 hours (activated).

b. After washing, cells were cultured for 48 hours (expression period) before cell selection.

c. After expression, 2000 cells/well (2 plates/group, 192 wells/group in the treated and positive controls; 4 plates, 384 wells in the solvent controls) were cultured for 10-14 days in selection medium to determine numbers of mutants and 1.6 cells/well (1 plate/group, 96 wells/group in the treated and positive controls; 2 plates, 192 wells in the solvent controls) were cultured for 10-14 days without selective agent to determine cloning efficiency. If evidence of an increase in mutant frequency was found, colony size distribution at each dose was determined.

2. Statistical methods - It was stated that the data were analyzed using weighted analysis of variance as described by Arlett *et al* (1989); however, no further details were provided.

3. Evaluation criteria

a. Assay validity - The assay validity criteria were not reported; however, typically if the following criteria are met, the assay can be considered valid:

- The viability of the solvent controls was ≥50%.
- The positive controls (MMS and MCA) induced statistically significant increases in the mutant frequencies.

b. Positive result - The test article was considered mutagenic if all of the following criteria were met:

- A reproducible, statistically significant increase in the mutant frequency was observed.
- Evidence of a dose relationship over at least two dose levels, in any increases in mutant frequency.
- Any observed increases in mutant frequency should have corresponding mean Day_0 relative survivals of >10%.

II. REPORTED RESULTS

Dose formulations were not analyzed for actual concentrations. It was not reported if the test material had any effects on the pH or osmolality of the test cultures.

A. PRELIMINARY CYTOTOXICITY ASSAY - In the preliminary cytotoxicity test, excessive cytotoxicity (% relative survival less than 10%) was observed at doses of ≥0.5 µg/mL (-S9) and ≥2.5 µg/mL (+S9). Based on these results, doses of 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 µg/mL (-S9) and 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, or 2.5 µg/mL (+S9) were selected for the first mutagenicity assay.

B. MUTAGENICITY ASSAY - The results of the mutagenicity assays were presented in Study Report Tables 2-13 on pages 20-31, and were summarized in summary Tables 1 and 2 on pages 35. As the results of this assay were negative, these summary tables are included as an Attachment to this DER.

In the absence of S9-activation, mean mutant frequency was increased at 0.1 µg/mL (3.19×10^{-4} treated vs 1.41×10^{-4} controls, $p \leq 0.01$) in Trial 1, and at 0.12 µg/mL (7.40×10^{-4} treated vs 1.72×10^{-4} controls, $p \leq 0.001$) in Trial 2. However, cytotoxicity at these dose levels was marked (8-10% relative survival), and there was no dose response. Therefore, these findings were considered to be likely due to excessive toxicity rather than a direct mutagenic effect. No significant ($p \leq 0.05$) increases in mutant frequency were observed at any dose level in the presence of S9 in either trial. The positive controls (MMS, -S9 and MCA, +S9) induced significant ($p \leq 0.001$) increases in mutation frequency in all trials.

III. DISCUSSION and CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS - The investigator concluded that Chlorothalonil did not induce mutation at the $TK^{+/-}$ locus of L5178Y mouse lymphoma cells in the presence or absence of S9-activation.

B. REVIEWER COMMENTS - Chlorothalonil was tested up to cytotoxic concentrations (≥0.1 µg/mL both trials, -S9; and ≥2.0 µg/mL [Trial 1] and ≥4.0 µg/mL [Trial 2], +S9). In the absence of S9-activation, mean mutant frequency was increased at 0.1 µg/mL (3.19×10^{-4} treated vs 1.41×10^{-4} controls, $p \leq 0.01$) in Trial 1, and at 0.12 µg/mL (7.40×10^{-4} treated vs 1.72×10^{-4} controls, $p \leq 0.001$) in Trial 2. However, due to high levels of cytotoxicity and the lack of a dose response at these dose levels, these findings were considered not to be a direct mutagenic effect. No other significant increases in mutant frequency were observed at any dose level in the presence of S9 in either trial. The positive controls induced the expected response. **There was no evidence of induced mutant colonies over background in the presence or absence of S9-activation.**

The study is classified as **acceptable/guideline** and satisfies the guideline requirements (OPPTS 870.5300, OECD 476) for *in vitro* mutagenicity (mammalian forward gene mutation) data.

C. STUDY DEFICIENCIES - The following minor deficiency was noted, but does not affect the conclusions of this DER:

- The dose formulations were not analyzed for actual concentrations.

ATTACHMENT

The following attachment contains summary data from page 35 of MRID 45710214

Table 1 - Summary of results without S9 mix

Expression time	Test 1			Test 2		
	Dose µg/ml	Mean RTG*	Mean mutant frequency	Dose µg/ml	Day_0 relative survival	Mean mutant frequency
48 hrs	0	100	0.000141	0	100	0.000172
	0.010	86	0.000162	0.040	72	0.000187
	0.025	77	0.000184	0.060	72	0.000163
	0.050	58	0.000197	0.080	68	0.000184
	0.100	10	0.000319	0.100	39	0.000166
				0.120	8	0.000740
	MMS	60	0.000668	MMS	63	0.000684

Table 2 - Summary of results with S9 mix

Expression time	Test 1			Test 2		
	Dose µg/ml	Mean RTG*	Mean mutant frequency	Dose µg/ml	Day_0 relative survival	Mean mutant frequency
48 hrs	0	100	0.000219	0	100	0.000191
	0.100	97	0.000180	0.500	99	0.000183
	0.250	84	0.000248	1.000	86	0.000211
	0.500	88	0.000168	1.500	74	0.000240
	1.000	65	0.000279	2.000	59	0.000269
	1.500	65	0.000187	4.000	17	0.000314
	2.500	26	0.000255			
	20-MC	48	0.000931	20-MC	20	0.001391

* Due to technical problems with Day_0 plating efficiency data, relative total growth (RTG) was used as the primary measure of toxicity in Test 1