GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATION

EPA Reviewer: Irving Mauer, Ph.D. Signature: [signature] 10/28/90
Geneticist, Toxicology Branch (I)/HED Date:
EPA Branch Chief: Karl Baetcke, Ph.D. Signature: [signature] 11/5/91
Toxicology Branch (I)/HED Date:

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Gene mutation in cultured mammalian cells (mouse lymphoma cells)

EPA IDENTIFICATION Numbers:

Tox Chem. Number:

MRID Number: 415614-05

TEST MATERIAL: R-25788

SYNONYMS: Dichlormid; EHC-0829-33

SPONSOR: ICI Americas Inc., Wilmington, DE

STUDY NUMBER: T-13179

TESTING FACILITY: ICI Americas Inc., Farmington, CT

TITLE OF REPORT: Mutagenicity Evaluation in L5178Y Mouse Lymphoma Multiple Endpoint Test Forward Mutation Assay R-25788 T-13179

AUTHORS: Tarca, J. P., and Majeska, J. B.

REPORT ISSUED: December 31, 1987

CONCLUSIONS-EXECUTIVE SUMMARY: R-25788 was evaluated in two nonactivated and two S9-activated mouse lymphoma forward mutation assays; S9 activation was provided by either Aroclor 1254-induced rat or mouse liver microsomes. Under nonactivated conditions, levels ≥600 µg/mL were severely cytotoxic. The evidence of a mutagenic effect at 600 µg/mL in the first trial was confirmed in the repeat assay using a narrow range of doses (200, 300, 400, 550, and 600 µg/mL). Increased mutant colonies and mutation frequencies (MF) were seen at all assayed levels. However, the three highest concentrations induced clear dose-related increases in the MF; MFs were 2.1-, 2.6-, and 2.9-fold higher than the solvent control (dimethyl sulfoxide) at 400, 550, and 600 µg/mL, respectively. Relative total growth (RTG) at these levels ranged from 25% at 400 to 9% at 600 µg/mL.

In the presence of both S9 activation systems, R-25788 was more cytotoxic as indicated by the appreciably lower doses that were selected for these trials

compared to the nonactivated phase of testing: 1.0, 2.5, 5.0, and 7.5 µg/mL + rat S9 and 2.5, 5.0, 7.5, 10.0, 25.0, and 40.0 µg/mL + mouse S9. The results indicated that increased mutant colony counts and MFs accompanied exposure to 5.0 and 7.5 µg/mL + rat liver S9 and 25 and 40 µg/mL + mouse liver S9. At 5.0 and 7.5 µg/mL/+ rat S9, MFs were 1.8- and 2.8-fold higher than the solvent control; RTGs at these concentrations were ≥ 12%. In the presence of mouse liver S9 activation, the 25- and 40-µg/mL treatment levels induced 3.2- and 4.7-fold increases in the MF, respectively; RTG was 9% at 25.0 µg/mL and 5% at 40.0 µg/mL.

Although the study authors stated that the findings were inconclusive because of cytotoxicity, we assess that there was sufficient justification to include the cytotoxic levels in the interpretation of the results (see Section D--Reviewers Discussion and Interpretation of Results). We assess, therefore, that R-25788 is mutagenic both with and without S9 activation at doses that extend into the cytotoxic range. The study satisfies Guideline requirements for genetic effects Category I, Gene Mutations.

STUDY CLASSIFICATION: The study is acceptable; R-25788 is mutagenic in this cultured mammalian cell gene mutation assay.

A. MATERIALS:

1. Test Material: R-25788

Description: Amber liquid
Identification No.: WRC 4921-35-11 GGD-0101
Purity: 97.2%
Receipt date: 9/30/87
Stability: Unspecified; expiration date: 5/90
Contaminants: None listed
Solvent used: Dimethyl sulfoxide (DMSO).
Other provide information: The test material was stored in the dark at room temperature (~20°C), and at ambient humidity. The report stated that test material concentrations ≤0.8 mg/mL -S9 and 0.06 mg/mL +S9 did not "substantially" alter the pH or osmolality of the treatment medium. Solutions of the test material were used within ≈2 hours of preparation.

2. Control Materials:

Negative: Fischer's medium supplemented with 10% horse serum, 1.9 mM glutamine, 210 µg/mL sodium pyruvate, 476 µg/mL plunonic, and antibiotics

Solvent/final concentration: DMSO/1%

Positive: Nonactivation (concentrations, solvent): Ethyl methanesulfonate (EMS) was prepared in an unspecified solvent to yield a final concentration of 0.5 µL/mL.

Activation (concentrations, solvent): N-nitrosodimethylamine (DMN) was prepared in an unspecified solvent to yield final concentrations

of 0.04 and 0.05 µl/mL. The 0.04 µl/mL solution was assayed with rat liver S9; both concentrations were assayed with mouse liver S9.

3. Activation: S9 derived from

x Aroclor 1254	x induced	x rat[a]	x liver
____ phenobarbital	____ noninduced	x mouse[b]	____ lung
____ none		____ hamster	____ other
____ other		____ other	

The rat S9 liver homogenate was prepared by the performing laboratory and was assigned Lot No. EHC-0476-25; the mouse S9 liver homogenate was purchased from Molecular Toxicology, College Park, MD and was assigned Lot No. 0223.

S9 mix composition:

Component	Concentration/mL of Culture Medium
NADP	1.2 mg
Isocitrate	6.0 mg
S9 homogenate (rat or mouse)	0.04 mL

4. Test Cells: mammalian cells in culture

x mouse lymphoma L5178Y cells
____ Chinese hamster ovary (CHO) cells
____ V79 cells (Chinese hamster lung fibroblasts)
____ other (list):

Properly maintained? Yes.
Periodically checked for mycoplasma contamination? Yes.
Periodically checked for karyotype stability? Not reported.
Periodically "cleansed" against high spontaneous background? Yes.

5. Locus Examined:

x thymidine kinase (TK)
Selection agent: (give concentration)
____ bromodeoxyuridine (BrdU)
____ fluorodeoxyuridine (FdU)
4 µg/mL trifluorothymidine (TFT)

____ hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent: (give concentration)
____ 8-azaguanine (8-AG)
____ 6-thioguanine (6-TG)

____ Na^+/K^+ATPase
Selection agent: (give concentration)
____ ouabain

____ other (locus and/or selection agent; give details):

[a]Male Sprague-Dawley rats

[b]Sex or strain not specified

6. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Nine doses (40, 60, 80, 100, 200, 400, 800, 1400, and 1600 µg/mL) were evaluated with and without rat liver S9 activation. Single cultures were prepared/dose/condition.

(b) Mutation assay:

(1) Nonactivated conditions: Two nonactivated assays were performed; doses tested were as follows:

Initial trial: 80, 100, 200, 400, and 600 µg/mL (duplicate cultures/dose).

Repeat trial: 200, 300, 400, 550, and 600 µg/mL (duplicate cultures/dose).

(2) S9-activated conditions: One S9-activated assay was performed with each metabolic activation system; doses tested were as follows:

Rat liver S9 activation: 1.0, 2.5, 5.0, and 7.5 µg/mL (duplicate cultures/dose except the high dose; only one replicate was prepared).

Mouse liver S9 activation: 2.5, 5.0, 7.5, 10.0, 25.0 and 40.0 µg/mL (duplicate cultures/dose).

B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells exposed to test compound for:
__4__ hours (nonactivated) __4__ hours (activated)

(b) Cells exposed to positive controls for:
__4__ hours (nonactivated) __4__ hours (activated)

(c) Cells exposed to negative and/or solvent controls for:
__4__ hours (nonactivated) __4__ hours (activated)

(d) After washing, cells cultured for __2__ days (expression period) before cell selection

(e) After expression, cells cultured for __10 to 12__ days in selection medium to determine numbers of mutants and for __10 to 12__ days without selection medium to determine cloning efficiency.

2. Statistical Methods: The data were not evaluated for statistical significance.

3. Evaluation Criteria:

(a) Assay validity: For the assay to be considered valid, the following criteria must be satisfied: 1) results for the negative and positive controls must be within the historical range of the reporting laboratory; 2) results for replicate solvent controls must be comparable; 3) the cloning efficiency (CE) of the solvent control should be ≥75%; and 4) the test material must be assayed to a dose causing ≈90% reduction in cell survival, to the limit of solubility, or to a maximum dose of 3 to 10 mg/mL.

b. Positive response: The test material was considered positive if it induced a dose-related increase in the mutation frequency (MF) that exceeded 2.5 times the MF of the solvent control.

4. Protocol: See Appendix B.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Nine doses of the test material (40 to 1600 µg/mL -/+ rat S9) were evaluated in the preliminary cytotoxicity assay. There was no indication in the report that the test material was insoluble at any dose. In the nonactivated assay, relative suspension growth (RSG) was reduced to ≤36% at levels ≥800 µg/mL. Below this concentration, RSG was ≥65%. In the presence of S9 activation, RSG was ≤19% for all doses except the low dose (40 µg/mL); at 40 µg/mL the RSG was 27%. By comparison to the results achieved under nonactivation, the findings suggest that the S9-activated test material was more cytotoxic.

2. Mutation Assays:

(a) Nonactivated conditions: Based on the evidence of cytotoxicity at ≥800 µg/mL -S9, the doses selected for the mutation assay without S9 activation ranged from 80 to 600 µg/mL. Average RSG ranged from >100% at levels ≤100 µg/mL to 9% at the high concentration (600 µg/mL). As shown in Table 1, there was a dose-related increase in mutant colonies and the MF at the three highest levels (200, 400, 600 µg/mL); fold increases in the MF at these concentrations ranged from 1.2 at 200 µg/mL to 4.5 at 600µg/mL. Results for the lower levels were comparable to the solvent control values.

Owing to the dose-dependent increase in MFs, the nonactivated assay was repeated with a narrower range of test material levels (200, 300, 400, 550, and 600 µg/mL). As the data presented in Table 2 show, the findings from the repeat nonactivated assay were in good agreement with the initial results and indicated that R-25788 induced a dose-related cytotoxic and mutagenic effect. Greater than 2-fold increases in the MF were obtained at

TABLE 1. Representative Results of the Initial Nonactivated Mouse Lymphoma Forward Mutation Assay with R-25788

Substance	Dose	Average Percent Relative Growth[a]	Average Mutant Colonies[a]	Average Viable Colonies[a]	Average Percent Relative Cloning Efficiency[a]	Average Percent Relative Total Growth[a]	Average Mutation Frequency[a,b] x 10^{-6}	Fold Increase[c]
Negative Control								
Medium	--	116	60	377	101	117	31.5	--
Solvent Control								
Dimethyl sulfoxide	1%	100	64	373	100	100	34.0	--
Positive Control								
Ethylmethane sulfonate	0.5 µL/mL	56	784	183	49	27	857	27.2
Test Material								
R-25788	200 µg/mL[d]	90	75	365	98	88	41.0	1.2
	400 µg/mL	38	86	329	89	34	52.0	1.5
	600 µg/mL	9	141	183	50	5	154.0	4.5

[a]Average results from duplicate cultures except the positive control; a single replicate was used. Results were calculated by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Mutant Colonies}}{\text{Viable Colonies}} \times 2 \times 10^{-4}$.

[c]Fold increase = $\frac{\text{MF of Test Dose}}{\text{MF of Solvent Control}}$. Note: It was assumed that the positive control was diluted in culture medium.

[d]Results for lower doses (80 and 100 µg/mL) did not suggest a mutagenic effect.

TABLE 2. Representative Results of the Repeat Nonactivated Mouse Lymphoma Forward Mutation Assay with R-25788

Substance	Dose	Average Percent Relative Growth[a]	Average Mutant Colonies[a]	Average Viable Colonies[a]	Average Percent Relative Cloning Efficiency[a]	Average Percent Relative Total Growth[a]	Average Mutation Frequency[a,b] x 10^{-6}	Fold Increase[c]
Negative Control								
Medium	--	101	34	409	106	106	16.5	--
Solvent Control								
Dimethyl sulfoxide	1%	100	39	387	100	100	19.5	--
Positive Control								
Ethylmethane sulfonate	0.5 µL/mL	70	658	178	46	32	739	44.8
Test Material								
R-25788	200 µg/mL	62	42	360	93	58	23.5	1.2
	300 µg/mL	44	51	340	86	39	30.0	1.5
	400 µg/mL	29	68	327	85	25	41.5	2.1
	550 µg/mL	14	74	287	74	11	51.0	2.6
	600 µg/mL	12	82	293	76	9	56.0	2.9

[a]Average results from duplicate cultures except the positive control; single replicate were used. Results were calculated by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Mutant Colonies}}{\text{Viable Colonies}} \times 2 \times 10^{-4}$.

[c]Fold increase = $\frac{\text{MF of Test Dose}}{\text{MF of Solvent Control}}$. Note: It was assumed that the positive control was diluted in culture medium.

the three highest levels; the relative total growth (RTG) at these concentrations ranged from 25% at 400 µg/mL to 9% at 600 µg/mL.

(b) S9-activated conditions:

(1) Rat liver S9 activation: Results from the first trial conducted with a concentration range of 1 to 7.5 µg/mL in the presence of rat liver S9 activation are shown in Table 3. Cytotoxicity was dose related and the RSGs ranged from 81% at the low dose (1.0 µg/mL) to 10% at the highest assayed level (7.5 µg/mL); CE at all levels was >100%. The number of mutant colonies and the MFs increased with increasing doses of R-25788; MFs either approaching or >2-fold over control were obtained at the two highest levels (5.0 and 7.5 µg/mL). RTG at the mutagenic levels was ≥12%. Although mutant colony counts were increased at 1.0 and 2.0 µg/mL, the MFs were only slightly higher than the solvent control.

Mouse liver S9 activation: No explanation was provided for the use of a higher starting concentration in the mouse liver S9-activated trial; the six treatment levels that were assayed in the presence of mouse liver microsomes were 2.5, 5.0, 7.5, 10.0, 25.0, and 40.0 µg/mL. A dose-related decrease in cell survival with increasing concentrations of R-25788 was also noted in the presence of mouse S9 activation (Table 4). In contrast to the rat S9 activation results, the test material appeared less cytotoxic under mouse liver S9-activation conditions. At equivalent levels (2.5, 5.0, and 7.5 µg/mL/+ mouse S9), RSG were 98, 53, and 30%, respectively, as compared to 45, 25, and 10% RSG, respectively, in the presence of rat S9 activation. The data presented in Table 4 further show that marked increases in mutant colonies and MFs (≥3.2-fold) occurred at doses (25.0 and 40.0 µg/mL) that reduced RTG to ≤9%; however, CEs over the entire concentration range were ≥65%. No clear indication of a mutagenic effect was observed at lower levels.

Based on the overall results the study authors concluded that "R-25788 induced a mutagenic response to significant levels only at less than 20% survival. Mutagenic responses below the 20% range are difficult to interpret because chemically effected mutations can not be differentiated from possible non-specific effects also known to occur at this level.

For this reason the response observed with R-25788 appears not to be significant but can not be clearly interpreted and therefore must be considered inconclusive."

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS: We assess that the study was properly conducted; however, we disagree with the study authors

TABLE 3. Representative Results of the Rat Liver S9-Activated Mouse Lymphoma Forward Mutation Assay with R-25788

Substance	Dose	Average Percent Relative Growth[a]	Average Mutant Colonies[a]	Average Viable Colonies[a]	Average Percent Relative Cloning Efficiency[a]	Average Percent Relative Total Growth[a]	Average Mutation Frequency[a,b] x 10^{-6}	Fold Increase[c]
Negative Control								
Medium	--	98	89	342	116	114	52.0	--
Solvent Control								
Dimethyl sulfoxide	1%	100	85	296	100	100	57.5	--
Positive Control								
N-nitrosodimethylamine	0.04 µL/mL	75	276	211	71	53	262	5.0
Test Material								
R-25788	1.0 µg/mL	81	158	473	160	125	68.0	1.2
	2.5 µg/mL	45	132	336	113	51	77.5	1.3
	5.0 µg/mL	25	224	443	150	38	100.5	1.8
	7.5 µg/mL	10	283	358	121	12	158.1	2.8

[a]Average results from duplicate cultures except the positive control and the high dose; no explanation was provided for the use of a single replicate for the high dose. Results were calculated by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Mutant Colonies}}{\text{Viable Colonies}} \times 2 \times 10^{-4}$.

[c]Fold increase = $\frac{\text{MF of Test Dose}}{\text{MF of Solvent Control}}$. Note: It was assumed that the positive control was diluted in culture medium.

TABLE 4. Representative Results of the Mouse Liver S9-Activated Mouse Lymphoma Forward Mutation Assay with R-25788

Substance	Dose	Average Percent Relative Growth[a]	Average Mutant Colonies[a]	Average Viable Colonies[a]	Average Percent Relative Cloning Efficiency[a]	Average Percent Relative Total Growth[a]	Average Mutation Frequency[a,b] x 10^{-6}	Fold Increase[c]
Negative Control								
Medium	--	121	44	374	86	104	23.5	--
Solvent Control								
Dimethyl sulfoxide	1%	100	67	437	100	100	30.5	--
Positive Control								
N-nitrosodimethylamine[d]	0.04 µL/mL	59	280	95	22	13	589	25.1
Test Material								
R-25788	2.5 µg/mL	98	77	408	94	91	37.5	1.2
	5.0 µg/mL	53	73	403	92	49	36.0	1.2
	7.5 µg/mL	30	64	410	94	28	31.0	1.0
	10.0 µg/mL	26	84	383	88	22	43.5	1.4
	25.0 µg/mL	13	144	297	68	9	97.5	3.2
	40.0 µg/mL	7	204	283	65	5	144.0	4.7

[a]Average results from duplicate cultures except the positive control; a single replicate was used. Results were calculated by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Mutant Colonies}}{\text{Viable Colonies}} \times 2 \times 10^{-4}$.

[c]Fold increase = $\frac{\text{MF of Test Dose}}{\text{MF of Solvent Control}}$. Note: It was assumed that the positive control was diluted in culture medium.

[d]Two levels of the positive control (0.04 and 0.05 µL/mL N-nitrosodimethylamine) were used; the lowest dose was selected as representative.

statement that the results are inconclusive because "mutagenic responses below the 20% range are difficult to interpret."

Clive et al. (1983)[1] recommend that data from doses level that reduced the RTG to 10% be included in the evaluation of a mutagenic response. The more conservative evaluation criteria of Caspary et al. (1988)[2] allow the inclusion of data from severely cytotoxic levels if the RTG is between 1 and 5%, the CE is between 10 and 20%, and the increased MF is supported by an increased mutant colony count. As the data presented in Tables 1 through 4 show, the results from the cytotoxic levels satisfy all of the above criteria. We assess, therefore, that there is sufficient justification to include the cytotoxic doses in the interpretation of the results. Based on the above considerations, it was concluded that under nonactivated and S9-activated conditions, R-25788 induced a reproducible dose-related mutagenic response at levels that extend into the cytotoxic range.

E. QUALITY ASSURANCE MEASURES: A signed quality assurance (QA) statement indicated that a QA review of the study was completed on December 29, 1987.

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 7-8; Appendix B, Protocol, CBI pp. 20-25.

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[1]Clive, D., McCuen, R., Spector, J.F.S., Piper, C., Mavournin, K.H. (1983). Specific gene mutations in L5178Y cells in culture. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 115:225-256.

[2]Caspary, W.J., Lee, Y.J., Poulton, S., Myhr, B.C., Mitchell, A.D., Rudd, C.J. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Quality-control guidelines and response categories. Environ. Mutagen. 12:19-36