GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE CYTOGENETICS

EPA Reviewer: Irving Mauer, Ph.D.
Geneticist, Toxicology Branch (I)/HED
EPA Branch Chief: Karl Baetcke, Ph.D.
Toxicology Branch (I)/HED

Signature:
Date: 10/28/91
Signature:
Date: 11/5/91

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Mammalian cells in culture cytogenetic assay in mouse lymphoma cells

EPA IDENTIFICATION NUMBERS.:

Tox Chem. Number:

MRID Number: 415614-06

TEST MATERIAL: R-25788

SYNONYMS: Dichlormid; EHC-0829-33

SPONSOR: ICI Americas Inc., Wilmington, DE

STUDY NUMBER: T-13180

TESTING FACILITY: ICI Americas Inc., Farmington, CT

TITLE OF REPORT: Mutagenicity Evaluation in L5178Y Mouse Lymphoma Multiple Endpoint Test Cytogenetic Assay R-25788 T-13180

AUTHOR: Majeska, J. B.

REPORT ISSUED: December 31, 1987

CONCLUSIONS-EXECUTIVE SUMMARY: Five nonactivated (200, 300, 350, 400, and 500 µg/mL) and five S9-activated (8, 10, 20, 40, and 60 µg/mL) doses of R-25788 were evaluated for clastogenic effects in L5178Y mouse lymphoma cells. Under nonactivated conditions, R-25788 did not increase the frequency of structural or numerical chromosome aberrations in cells harvested 12 hours posttreatment. Cytotoxicity as indicated by a marked reduction in the mitotic index was apparent at the highest assayed level (500 µg/mL); higher concentrations were severely cytotoxic. The findings indicate that nonactivated R-25788 was tested over an adequate range of test material concentrations but failed to induce a clastogenic effect.

No conclusions can be reached; however, for the S9-activated phase of testing. No significant or dose-related increase in the percentage of cells with structural or numerical aberrations was seen. However, the presence of rare complex aberrations (i.e., triradials, quadriradials, and translocations) at

the majority of the doses, although not sufficient to conclude that R-25788 is a clastogen, should have prompted the performance of a repeat test to resolve this issue. Similarly, the lack of a marked cytotoxic effect on high-dose cultures suggest that higher concentrations could have been evaluated. The study author claimed that the number of cells completing two cell cycles (M_2) was reduced at all S9-activated assayed levels in parallel cultures incubated with BrdU for 16 hours; no data were provided to support this statement. If cell cycle delay was suspected, the rationale for proceeding with the analysis of 12-hour posttreatment cultures, presumably with a high proportion of M_1 cells, is unclear. Based on the above considerations, we assess that conditions may not have been optimal to detect the potential, if any, of S9-activated R-25788 to induce clastogenesis. Since definitive conclusions can not be reached, the study does not satisfy Guideline requirements for genetic effects Category II, Structural Chromosome Aberrations.

STUDY CLASSIFICATION: The study is unacceptable. It is recommended that the S9-activated assay be repeated using either higher test material levels and/or a prolonged cell harvest time.

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 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 pluronic, 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 1.0 µL/mL.

Activation (concentrations, solvent): Cyclophosphamide (CP) was prepared in an unspecified solvent to yield a final concentration of 20 µg/mL.

3. Activation: S9 derived from male Sprague-Dawley

x Aroclor 1254	x induced	x rat	x liver
____ phenobarbital	____ noninduced	____ mouse	____ 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.

S9 mix composition:

Component	Concentration/mL of Culture Medium
NADP	1.2 mg
Isocitrate	6.0 mg
S9	0.04 mL

4. Test Compound Concentration Used:

a. Preliminary cytotoxicity assay: Two preliminary cytotoxicity assays were performed; doses evaluated were:

(1) Initial trial: Nine doses (40, 60, 80, 100, 200, 400, 800, 1400, and 1600 µg/mL +/-S9).

(2) Repeat trial: Seven nonactivated doses (200, 300, 350, 400, 500, 550, and 600 µg/mL) and five S9-activated doses (8, 10, 20, 40, and 60 µg/mL).

Note: The repeat cytotoxicity assay was conducted concurrent with the cytogenetic assays.

b. Cytogenetic assay:

(1) Nonactivated conditions: Five doses (200, 300, 350, 400, and 500 µg/mL) with a 12-hour cell harvest.

(2) S9-activated conditions: Five doses (8, 10, 20, 40, and 60 µg/mL) with a 12-hour cell harvest.

5. Test Cells: L5178Y ($TK^{+/-}$) mouse lymphoma cells, subclone 3.7.2 were obtained from Dr. Donald Clive, Borroughs Wellcome, Research Triangle Park, NC.

Properly maintained? Yes.

Cell line or strain periodically checked for mycoplasma contamination? Yes.

Cell line or strain periodically check for karyotype stability? Not reported.

B. TEST PERFORMANCE:

1. Cell Treatment:

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)

2. Preliminary Cytotoxicity Assay: Prepared cells, seeded at a density of $6x10^5$ cells/mL, were exposed for 4 hours to the selected doses of the test material or the solvent (DMSO) with or without S9 activation. To terminate exposure, cells were washed, resuspended in growth medium, and incubated. After 24-hours, cells were counted to determine cytotoxicity (decrease in relative suspension growth). A second set of treated cultures was resuspended in growth medium containing 0.3 to $1x10^{-4}$ M BrdU and reincubated for 24 hours. Cultures were harvested and stained by the procedure of Perry and Wolff,[1] and 100 randomly selected metaphases were scored for the number of cells undergoing one (M_1), two (M_2), or three (M_3) cell cycles.

3. Cytogenetic Assay:

a. Dosing: Duplicate cultures containing $6x10^5$ cells/mL were exposed for 4 hours to the selected doses of the test material and the medium, solvent, or positive controls with or without S9 activation. At the end of exposure, cells were washed, resuspended at $3x10^5$ cells/mL, and incubated at 37°C in 5% CO_2.

b. Cytotoxicity assessment: At ≈10 hours postexposure, an aliquot of cells were counted to determine relative suspension growth. A second set of cultures, containing BrdU was harvested 24 hours after treatment to determine cell cycle kinetics.

c. Metaphase arrest/cell harvest: Approximately 10 hours postexposure, cultures with ≥$1x10^5$ cells/mL were selected for harvest, centrifuged, resuspended in growth medium containing colcemid (0.08 µg/mL), and reincubated for an additional 2 hours. Cells were swollen with distilled water, incubated at room temperature for 10 minutes, and centrifuged. Harvested cells were fixed in Carnoy's fixative and incubated overnight at 4°C.

d. Slide preparation: Fixed overnight cells were placed onto slides, air dried, and stained with 10% Giemsa. All slides were coded prior to analysis.

---

[1]Perry P., and Wolff, S. New Giemsa method for the differntial staining of sister chromatids. Nature (1974) 251:156-158.

e. Metaphase analysis: The five highest doses yielding sufficient scorable metaphases were selected for analysis. Fifty metaphases/culture were scored for structural and numerical chromosome aberrations. Chromatid and chromosome gaps were recorded in the raw data but were not reported. The mitotic index (MI) was determined for each dose by counting the number of mitoses/500 cells.

4. Statistical Analysis: Structural aberrations were analyzed on a per cell basis using Student's t-test (one-tailed) with no differentiation as to the type of aberration.

5. Evaluation Criteria:

a. Assay validity: The study was considered valid if (1) the results for the solvent and positive control were within the historical range of the reporting laboratory; (2) there was no significant differences between solvent control replicates; and (3) a sufficient number of cells was available from the solvent control and three doses of the test material.

b. Positive response: A test material was considered positive if it induced a dose-dependent response over three consecutive doses and the increase at the highest dose was signficantly higher ($p<0.01$) than the solvent control.

c. Protocol: See Appendix B.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: The study author stated that the first trial of the cytotoxicity assay was conducted as part of the mouse lymphoma cell gene mutation assay (see Data Evaluation Record 91-5). As shown in Table 1, ≥36% of the cells survived exposure to nonactivated levels ≥800 µg/mL. Although the cell-cycle kinetics data were difficult to interpret, we assume that no metaphases were recovered from cultures treated with 1400 and 1600 µg/mL -S9. Results for 400 and 800 µg/mL-S9 suggested a slight delay in progression through the cell cycle. In the presence of S9 activation, ≤19% of the cells survived at doses ≥60 µg/mL. The lowest dose (40 µg/mL) reduced cell survival to 27% but had no effect on cell cycling. Based on these findings, a second cytotoxicity was performed concurrent with the cytogenetic assay; these results are discussed below in conjunction with the cytogenetic assay.

2. Cytogenetic Assay:

a. Nonactivated conditions: Initially seven doses (200 to 600 µg/mL) were assayed. Although cell survival for all doses was ≥57%, the study author stated that an insufficient number of metaphases was available from cultures treated with 550 and 600 µg/mL. Accordingly, metaphases harvested from exposure levels of 200, 300, 350, 400, and 500 µg/mL were scored for chromosome aberrations.

TABLE 1. Representative Results from the Initial Cytotoxicity Assay with R-25788

Substance	Dose (µg/mL)	S9-Activation	Average Percent Relative Growth	Average Percent Cells in First Division (M_1)	Average Relative Staining Index[a]
Negative Control/Solvent Control					
Medium/Dimethyl sulfoxide	--	-	100	1	100
	--	+	100	0.5	100
Test Material					
R-25788	200[b]	-	91	--	--
	400	-	65	15	87
	800	-	36	26	76
	1400	-	34	--	--
	1600	-	34	--	--
	40	+	27	2	99
	60[c]	+	19	--	--

[a]Average Relative Staining Index = $\frac{\text{Total no. of cells in treatment groups that have completed two } (M_2) \text{ or three } (M_3) \text{ cycles in BrdU}}{\text{Total no. of } M_2 \text{ or } M_3 \text{ cells in the negative/solvent control group}} \times 100.$

[b]Cell survival at lower concentrations (40, 60, 80, and 100 µg/mL) was >100%.

[c]Cell survival at higher levels (80, 100, 200, 400, 800, 1400, and 1600 µg/mL) was ≤17%.

Cytotoxicity, as indicated by the marked reduction in the MI, was clearly apparent at 500 µg/mL. MIs were suppressed for the majority of test doses compared to the solvent control; however, there was no significant increase in the percentage of cells with either structural or numerical aberrations (Table 2). By contrast, the nonactivated positive control (1.0 µl/mL EMS) induced a significant ($p<0.01$) increase in the percentage of cells with aberrations.

b. S9-activated conditions: The five doses investigated under S9-activated conditions ranged from 8 to 60 µg/mL. Cell survival over the entire dose range was ≥71% and there were no adverse effects on the MI. The study author claimed that the number of cells that completed two rounds of DNA synthesis following treatment with all S9-activated doses was reduced after an additional 16-hour incubation in the presence of BrdU; no data were presented to support this statement. However, the relative staining indices for parallel cultures treated with the three highest doses and reincubated in culture medium containing BrdU for 22 hours (99.5% at 20 µg/mL; 98.5% at 40 µg/mL; and 96.5% at 60 µg/mL) did not indicate that M_2 or M_3 cells were reduced.

Numerical aberrations were not significantly higher than the control at any level. However, significant increases in the percentage of cells with structural aberrations were reported for single replicate cultures at 8, 10, and 20 µg/mL. We, therefore, combined the data from replicates and compared the average results to the pooled negative and solvent control values using the Student's t-test. As indicated in Table 2, when the data was combined for individual dose groups, no significant increase were seen. Similarly, neither the number of aberrations/cell nor the percentage of cells with aberrations indicated a dose-dependent effect. However, the occurrence of rare complex aberrations at the majority of test doses (1 translocation at 8 µg/mL; 1 triradial and 1 translocation at 20 µg/mL; and 1 quadriradial at 60 µg/mL) was an unusual finding that was ignored by the study author. It was, therefore, concluded that R-25788 was not clastogenic in this test system.

D. REVIEWER'S DISCUSSION/CONCLUSIONS: We assess that there was sufficient evidence to conclude that nonactivated R-25788 was tested up to a cytotoxic level but failed to induce a clastogenic response in L5178Y mouse lymphoma cells. However, no conclusions can be reached regarding the S9-activated findings. The appearance of rare complex aberrations at the majority of S9-activated levels should have prompted the performance of a repeat test to determine the reproducibility of this finding. Similarly, the ≥71% cell survival at 60 µg/mL and the lack of an adverse effect on the MI suggests that higher levels could have been tested. The study author claimed that M_2 cells were reduced at all levels following a 16-hour incubation with BrdU. If cell cycle delay was suspected, it is unclear why the 12-hour harvest cytogenetic assay was continued. It would have been prudent to repeat the test using a prolonged cell harvest to ensure that conditions were optimal for the detection of a clastogen.

TABLE 2. Representative Results of the Chromosomal Aberration Assay in Mouse Lymphoma Cells Harvested 12 Hours After Treatment With R-25788

Substance	Dose	S9-Activation	Total No. of Cell Scored	Mitotic Index[a] (%)	Total No. Aberrations[a]	Number of Aberrations Cell[a]	% Cells with Aberrations[a]	% Cells with >2 Aberrations[a]	Biologically Significant Aberrations No./type
Negative Control									
Medium	--	-	100	7.0	2	0.02	2	0	2 AF
		+	100	8.0	1	0.01	1	0	1 AF
Solvent Control									
Dimethyl sulfoxide	1%	-	100	6.5	3	0.03	3	0	2 AF; 1 F
	1%	+	100	8.4	0	0.00	0	0	--
Positive Control									
Ethylmethane sulfonate	1.0 µl/mL	-	50	3.0	15	0.30	20[b]	8	1 AF; 3 TB; 6 TR; 2 QR; 3 T
Cyclophosphamide	20.0 µg/mL	+	50	5.0	6	0.12	10[c]	2	4 TB; 1 QR; 1 T
Test Material									
R-25788	500 µg/mL[d]	-	100	2.2	3	0.03	3	0	1 TB; 2 AF
	8 µg/mL	+	100	6.5	3	0.03	3	0	2 AF; 1 T; 1 AF
	10 µg/mL	+	100	6.3	2	0.02	2	0	1 AF; 1 TB
	20 µg/mL	+	100	5.9	5	0.05	5	0	2 AF; 1 TR; 1 T; 1 M
	40 µg/mL	+	100	4.4	1	0.01	1	0	1 F
	60 µg/mL	+	100	6.3	4	0.04	4	0	1 AF; 1 TB; 1 QR; 1 TB

[a]Results from replicate cultures combined and recalculated by our reviewers.
[b]Significantly higher than the pooled negative and solvent control ($p<0.05$) by Student's t-test.
[c]Significantly higher than the pooled negative and solvent control ($p<0.01$) by Student's t-test.
[d]Results for lower nonactivated levels (200, 300, 350, and 400 µg/mL) did not suggest a clastogenic effect.

Abbreviations used:
AF = Acentric fragment
F = Fragment
TB = Chromatid Break
TR = Triradial
QR = Quadriradial
T = Translocation.

We assess, therefore, that the S9-activated cytogenetic assay with R-25788 should be repeated and that consideration should be given to either increasing the starting concentration and/or using a prolonged cell harvest.

E. QUALITY ASSURANCE MEASURES: A signed quality assurance (QA) statement indicated that the QA review of the study was completed on December 30, 1987.

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 7-8; Appendix B, Protocol, CBI pp. 15-20.