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OFFICE OF PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: EPA Reg. No. 100-524. Evaluation of L5178Y/TK+/- Mouse Lymphoma Mutagenicity Test with Technical Diazinon (G 24 480). EPA Guidelines No. 84-2

FROM: Krystyna K. Locke, Toxicologist Krystyna K. Locke 2/21/89
Section I, Toxicology Branch I (IRS)
Health Effects Division (H7509C)

TO: George T. LaRocca, PM Team (15)
Insecticide-Rodenticide Branch
Registration Division (H7505C)

THRU: Edwin R. Budd, Section Head
Section I, Toxicology Branch I (IRS)
Health Effects Division (H7509C)

Record No.: 234058 Tox. Chem. No.: 342
MRID/Accession No.: 406608-02 T.B. Project No.: 9-0273

Toxicology Branch I/IRS has completed an evaluation of the following study:

L5178Y/TK+/- Mouse Lymphoma Mutagenicity Test; Ciba-Geigy Limited, Basle, Switzerland; No. 840396; July 31, 1986.

Technical Diazinon (G 24 480) was not mutagenic in this test, with or without metabolic activation (S9). The concentrations of Diazinon tested were 12-120 ug/mL of assay medium without S9 and 6-60 ug/mL with S9. Concentration-dependent cytotoxicity was observed at Diazinon levels above 72 ug/mL without S9 and 12 ug/mL with S9. Diazinon concentrations of 120 ug/mL (HDT without S9) and 60 ug/mL (HDT with S9) were too cytotoxic to be evaluated for mutagenicity. Known mutagens, dimethylnitrosamine (DMN) requiring S9 and ethyl methanesulfonate (EMS) not requiring S9 were both mutagenic and cytotoxic in this test.

Classification of Study: Provisionally Acceptable, pending submission of additional (explanatory) data.

Reviewed by: Krystyna K. Locke, Toxicologist
Section I, Tox. Branch I/IRS (H7509C)
Secondary Reviewer: Edwin R. Budd, Section Head
Section I, Tox. Branch I/IRS (H7509C)
Secondary Reviewer: Irving Mauer, Geneticist
Tox. Branch I/IRS (H7509C)

Krystyna K. Locke 2/24/87

02-2-87

DATA EVALUATION REPORT

Study Type: 84-2. Mutagenic (L5178Y/TK+/- Mouse Lymphoma)

Tox. Chem. No.: 342
MRID No.: 406608-02

Test Material: Diazinon Technical (G 24 480); Batch No. P. 203008; 97.2% pure.

Study Number(s): 840396

Sponsor: Ciba-Geigy Corporation, Greensboro, NC.

Testing Facility: Ciba-Geigy Limited, Basle, Switzerland.

Title of Report: L5178Y/TK+/- Mouse Lymphoma Mutagenicity Test.

Author(s): P. Beilstein and P. Dollenmeier

Report Issued: July 31, 1986

Conclusions:

Technical Diazinon was not mutagenic in this study. It did not induce forward point mutations in the L5178Y/TK+/- mouse lymphoma cells as monitored by cell growth in the presence of 5-bromodecxyuridine (BUdR), with or without a metabolic activation system (S9).

Positive results were obtained with known mutagens, dimethylnitrosamine (DMN) requiring S9 and ethyl methanesulfonate (EMS) not requiring S9. Concentration-dependent cytotoxicity was observed at Diazinon levels above 72 µg/mL without S9 and 12 µg/mL with S9. Diazinon concentrations of 120 µg/mL (HDT without S9) and 60 µg/mL (HDT with S9) were too cytotoxic to be evaluated for mutagenicity.

Mouse lymphoma cells L5178Y/TK+/- were exposed to technical Diazinon (12-120 µg/mL and 6-60 µg/mL without and with S9, respectively) for 4 hours, centrifuged, washed (to remove test substance), maintained in exponential growth for 3 days (expression time) and then grown either in the presence of 5-bromodecxyuridine (BUdR) to determine mutations or without BUdR to determine cytotoxicity. Cultures containing BUdR were grown for 14-15 days and those without BUdR for 12 days. Solvent control (containing dimethylsulfoxide; DMSO), negative control

(without DMSO) and positive controls were treated in the same manner.

Classification of Study: Provisionally Acceptable pending submission of additional data to explain calculations for "Relative Suspension Growth" and "Mutant Frequency" (see review, COMMENTS, for details).

EXPERIMENTAL PROCEDURES

The mouse lymphoma cell line (L5178Y/TK+/-) used in this study was obtained from Dr. D. Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina.

The procedure used in the mutagenicity test was based on that reported by Clive and Spector (1975[1]). Exponentially growing cells (3 x 10^5 cells/mL, cleansed of spontaneous TK-/- mutants, were treated with the following concentrations of Diazinon: 12, 24, 48, 72, 96, 108 and 120 µg/mL of assay medium in the absence of S9 (metabolic activation system) and 6, 12, 24, 36, 48, 54, and 60 µg/mL with S9 (added to assay medium before Diazinon). Dimethylnitrosamine (DMN; 8 µL/mL) and ethyl methanesulfonate (EMS; 0.75 µL/mL) were used as positive controls with and without S9, respectively. Dimethylsulfoxide (DMSO) was the solvent for Diazinon, but DMN and EMS, both liquids, were apparently used in assays as purchased (undiluted). The final concentration of DMSO in the medium was 1%. The assay medium without test material and DMSO was used as a negative control. The exposure time for all cultures was 4 hours. The concentrations of Diazinon used were based on the results of the range-finding study (preliminary cytotoxicity test).

At the termination of the exposure, the cell suspensions were centrifuged and the resulting pellets washed with F10P medium (Fischer's medium with antibiotics and 10% horse serum) to remove the test material. The washed cells were resuspended in F10P medium and maintained in exponential growth for 3 days to express the induced forward TK-/- mutants.

At the end of the 3-day expression time, cells were grown either in a selective, semi-solid agar medium containing 5-bromodeoxyuridine (BUdR; 50 µg/mL) to determine mutation (mutant selection cultures) or in a nonselective medium (without BUdR) to determine cytotoxicity (viability control cultures). For mutant selection, 8 tubes were prepared at each concentration

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[1]CLIVE, D., and J.F.S. SPECTOR: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31, 17-29 (1975).

containing 4 x 10^5 cells per tube. For viability control, 4 tubes were prepared at each concentration containing 200 cells per tube.

The incubation time was 14-15 days for cultures containing BUdR and 12 days for those without BUdR. At the end of the incubation time, the cell colonies were counted with a Colony Counter (Fisher Count-All™, Model 600) and the results were expressed in terms of the number of induced TK-/- mutants/10^6 cells.

In the preliminary cytotoxicity test, exponentially growing mouse lymphoma L5178Y/TK+/- cells (3 x 10^5 cells/mL) were exposed for 4 hours to seven concentrations of Diazinon ranging from 15.6 to 1000 µg/mL of assay medium, with and without S9. After removal of Diazinon, the cells were washed with F10P medium and then incubated in the same medium for 3 days. Cell counts were performed daily and the percentages of the relative suspension growth in comparison with the solvent control (1% DMSO) were evaluated. The concentration of Diazinon calculated to produce about 85% reduction in the relative suspension growth in comparison with the solvent control was used as the highest dose in the mutagenicity test (above). Other concentrations of Diazinon used, with and without S9, corresponded to 0.9, 0.8, 0.6, 0.4, 0.2 and 0.1 of the highest concentration.

S9 (postmitochondrial) fraction, obtained from the livers of Aroclor 1254-treated male SPF RAI rats, was purchased from Analabs Inc., North Haven, Connecticut, U.S.A. S9 was used with various cofactors in the preliminary cytotoxicity test and in the mutagenicity test.

Statistical analyses were not performed for the mutagenicity test or for the preliminary cytotoxicity test.

RESULTS

Cytotoxicity

In the concentration range-finding experiment, growth of mouse lymphoma cells (relative suspension growth) was completely suppressed at Diazinon concentrations of 125-1000 µg/mL of assay medium, with and without metabolic activation (S9). At other concentrations of Diazinon tested (1.5-62.5 µg/mL), greater cytotoxicity was observed with than without S9, and cytotoxicity was concentration-dependent in the presence of S9. At a Diazinon concentration of 62.5 µg/mL, growth of mouse lymphoma cells was inhibited 92.1 and 40.3% with and without S9, respectively, relative to solvent controls. At Diazinon concentrations of 15.6 and 31.2 µg/mL, cellular growth was inhibited by 14.1 and 26.4%, respectively, with S9, but no inhibition occurred without S9.

In the mutagenicity test, without S9, growth of mouse lymphoma cells (relative suspension growth) was inhibited only at the three highest concentrations of Diazinon tested (96-120 µg/mL), relative to solvent controls. Without S9, cellular growth was inhibited at the five highest concentrations of Diazinon tested, relative to solvent controls. The inhibitions, without and with S9, were concentration-dependent. Positive controls, EMS and DMN, were also cytotoxic. These data are summarized below.

Cytotoxicity of Diazinon: Percent Inhibition of Mouse Lymphoma Cell Growth, Relative to Solvent Controls*

Diazinon (µg/mL)	Without metabolic activation (S9)
12	0
24	3.2
48	0
72	0
96	70.0
108	96.4
120	99.2
EMS (0.75 µL/mL)	88.1
	With metabolic activation (S9)
6	11.0
12	0
24	39.7
36	57.2
48	92.6
54	97.8
60	99.6
DMN (8 µL/mL)	61.0

*See Attachment I for details.

Mutation Frequency

The highest concentrations of Diazinon at which mutation frequency could be assayed were 108 µg/mL without S9 and 54 µg/mL with S9. Diazinon concentrations of 120 µg/mL (HDT) without S9 and 60 µg/mL (HDT) with S9 could not be evaluated because of high cytotoxicity.

At Diazinon concentrations of 12-96 µg/mL without S9 and 6-54 µg/mL with S9, mutation frequencies were similar for treated and untreated cells. At a Diazinon concentration of 108 µg/mL, in the absence of S9, the mutation frequency was almost twice as high as that observed with the solvent control, but was probably due to significant decrease (96.4%) in cell viability. Positive

results were obtained with known mutagens, EMS and DMN. Results obtained in the mutagenicity test are summarized below.

Diazinon (µg/mL) Without S9	Mutant Frequency x 10E -6[a]	Mutation Factor[b]
0 (SC)	54.7	--
0 (NC)	33.5	--
12	56.4	1.03
24	59.0	1.08
48	51.2	0.94
72	65.0	1.19
96	54.9	1.00
108	97.4	1.78
EMS (0.75 µL/mL)	1745.8	50.11
With S9		
0(SC)	42.9	--
0(NC)	30.6	--
6	45.9	1.07
12	42.4	0.99
24	33.0	0.77
36	43.8	1.02
48	45.9	1.07
54	51.4	1.20
DMN (8 µL/mL)	630.3	20.60

[a](Mutant clones x 800/viable clones)/3.2. See Attachment I for details.

[b]Ratio of mutant frequency for Diazinon-containing culture/mutant frequency of solvent control (SC). In the case of positive controls, EMS and DMN, negative control (NC) was used to calculate mutation factors.

According to the testing laboratory, the test substance is generally considered mutagenic in this test system if the mutant colony count exceeds that of the solvent control by a factor of more than 2.5 at any concentration. Based on this criterion and/or the similarities in mutant frequencies between the Diazinon-treated and control cultures, Diazinon was not mutagenic in the L5178Y/TK+/- mouse lymphoma cell system.

COMMENTS

The experimental procedures were reported adequately, with the following exceptions:

1. It was not stated whether positive controls (DMN and EMS), both liquids, were used in the assay medium as

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purchased or were first diluted with DMSO. Since in the RESULTS section Diazinon-treated cell cultures were compared with solvent controls and those containing DMN and EMS with negative controls, this reviewer assumed that positive controls were used as purchased.

2. Purities of the positive control substances were not reported.

The following results either cannot be verified or are ambiguous and additional data are, therefore, required:

1. Relative Susp. Growth (% of Control): Tables 1, 4 and 7. Using "Daily Counts," values reported for "Relative Susp. Growth" could not be obtained. A sample calculation is, therefore, required.

2. Mutant frequencies (Tables 4 and 7) were calculated using the following formula: (Mutant clones x 800/ viable clones)/3.2. No explanation is provided as to what "800" and "3.2" are, and this information is required.

Quality Assurance Statement, signed and dated July 30, 1986, was included in the report.

Classification of Study: Provisionally Acceptable, pending submission of the requested data.

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Attachment I

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Table 1

L5178Y/TK(+/-) MOUSE LYMPHOMA TOXICITY TEST (Preliminary Study)

Test substance: G 24 480 techn. Test No.: 840396
Batch No. : P. 203008 Solvent : DMSO 1%

Concentration		Without Activation: Daily Counts (Cells/mlx10E-5)			Without Activation: Relative Susp.Growth (% of Control)	With metabolic Activation: Daily Counts (Cells/mlx10E-5)			With metabolic Activation: Relative Susp.Growth (% of Control)	% Inhibition
Solvent Control		7.1	13.1	11.8	100.0	6.0	11.9	13.2	100.0	
(5.625)	ug/ml	8.5	12.7	15.2	149.5	5.4	12.0	12.5	85.9	14.1
(11.25)	ug/ml	7.5	13.1	12.1	108.3	4.7	11.9	12.4	73.6	26.4
(62.5)	ug/ml	4.8	11.1	12.3	59.7 (40.3%)	0.6	1.8	0.3	7.9	92.1
125	ug/ml	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
250	ug/ml	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
500	ug/ml	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
1000	ug/ml	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	

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Table 4

L5178Y/TK(+/-) MOUSE LYMPHOMA MUTAGENICITY TEST

without metabolic activation

Test substance: G 24 480 techn.
Batch No. : P. 203008
Test Date : 20.1.86

Test No.: 840196
Solvent : DMSO 1%

	Daily Counts (Cells/ml x 10E5)			Rel.Susp. Growth *		Total Mutant Clones	Total Viable Clones	Relative Cloning Efficiency*	% Relative Growth**	Mutant frequency x10E-6***
					% ↓					
Solvent Control	8.5	13.4	9.7	100.0		94	430	100.0	100.0	54.7
Negative Control	9.4	13.5	12.1	139.0		84	626	145.6	202.3	33.5
EMS 0.75 ul/ml	4.8	9.3	4.1	11.9	88	412	59	9.4	1.1	1745.8
Test compound										
12 ug/ml	9.7	16.7	12.4	181.8	O	88	390	90.7	164.9	56.4
24 ug/ml	7.4	14.9	9.7	96.8	3.2	98	415	96.5	93.4	59.0
48 ug/ml	7.7	14.2	12.3	121.7	O	76	371	86.3	105.0	51.2
72 ug/ml	6.0	14.6	13.2	104.7	O	98	377	87.7	91.8	65.0
96 ug/ml	2.8	11.4	9.7	30.0	70	81	369	85.8	25.8	54.9
108 ug/ml	0.9	1.7	4.4	3.6	96.4	120	308	71.6	2.6	97.4
120 ug/ml	0.3	0.3	1.0	0.8	99.2	Insufficient cell number for mutant-selection				

* % of control
** (Relative suspension growth x relative cloning efficiency) / 100
*** (Mutant clones x 800 / viable clones) / 3.2

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Table 7

L5178Y/TK(+/-) MOUSE LYMPHOMA MUTAGENICITY TEST

with metabolic activation

Test substance: G 24 480 techn.
Batch No. : P. 203008
Test Date : 14.1.86

Test No.: 840196
Solvent : DMSO 1%

	Daily Counts (Cells/ml x 10E5)			Rel.Susp. Growth *	% ↓	Total Mutant Clones	Total Viable Clones	Relative Cloning Efficiency*	% Relative Growth**	Mutant Frequency x10E-6***
Solvent Control	9.4	12.3	11.1	100.0		84	490	100.0	100.0	42.9
Negative Control	8.9	14.9	9.5	98.2		70	572	116.7	114.6	30.6
DMN 8.00 ul/ml	7.5	9.1	7.2	39.0		295	117	20.5	8.0	630.3
Test compound										
6 ug/ml	9.8	11.1	10.5	89.0	11	91	496	101.2	90.1	45.9
12 ug/ml	8.6	10.0	16.3	109.2	↑	91	536	109.4	119.5	42.4
24 ug/ml	7.1	11.6	9.4	60.3	39.7	70	530	108.2	65.2	33.0
36 ug/ml	6.4	8.5	10.1	42.8	57.2	79	451	92.0	39.4	43.8
48 ug/ml	2.3	3.7	8.6	7.4	92.6	69	376	76.7	5.7	45.9
54 ug/ml	2.9	2.7	3.1	2.2	97.8	(60)	292	59.6	1.3	51.4
60 ug/ml	2.8	1.8	0.6	0.4	99.6	Insufficient cell number for mutant-selection				

* % of control
** (Relative suspension growth x relative cloning efficiency) / 100
*** (Mutant clones x 800 / viable clones) / 3.2