12-15-03

DATA EVALUATION RECORD

Polymeric Xylenol Tetrasulfide (PXTS)
MRID 460626-18

Study Type: *In Vitro* Mammalian Cell Gene Mutation Test (Mouse)
OPPTS 870.5300

Prepared for

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1

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DATA EVALUATION RECORD

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

PC CODE: 006929

DP BARCODE: D299112

TEST MATERIAL (PURITY): Not reported

SYNONYMS FOR ACTIVE INGREDIENT: Polymeric Xylenol Tetrasulfide

CITATION: Cifone, M. A. (2002) L5178Y TK $^{+/-}$ Mouse Lymphoma Forward Mutation Assay with a Confirmatory Assay with Polymeric Xylenol Tetrasulfide (PXTS). Covance Laboratories Inc. (Vienna, Virginia). 23134-0-431OECD. February 1, 2002. MRID 460626-18.

SPONSOR: Akzo Nobel Functional Chemicals, Inc., Dobbs Ferry, New York

EXECUTIVE SUMMARY:

In a mammalian cell gene mutation assay TK $^{+/-}$ (MRID 460626-18), L5178Y mouse lymphoma cells cultured *in vitro* were exposed to Polymeric Xylenol Tetrasulfide (PXTS), (Lot No. 1685-11-1 Batch No. 6, Bottle No. 1) in dimethylsulfoxide (DMSO) for approximately 4 hours at concentrations of 0.498, 0.995, 1.50, 1.99, 2.99, 3.98, 4.98, 5.97, 6.97, 7.96, and 9.95 µg/mL (Trial 1, ±S9), 0.500, 1.00, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, and 6.00 (Trial 2, -S9) as well as 0.500, 1.00, 2.00, 3.00, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, and 7.00 µg/mL (Trial 2, +S9), in the presence and absence of mammalian metabolic activation derived from the livers of Aroclor 1254-induced male Sprague-Dawley rats. A dose range-finding assay, with and without metabolic activation, was performed prior to the forward mutation assay to determine test article concentrations for the mutation assay.

Initial and confirmatory mutation assays were performed with PXTS, in the presence and absence of metabolic activation. Induction of cytotoxicity ranged from none to high. The test article was found to be non-mutagenic in this *in vitro* mammalian cell gene mutation assay in the presence and absence of S9 activation; the positive controls did induce the appropriate response. **There was no evidence of a concentration- related, positive response that induced mutant colonies**

(2)

over background.

This study is classified as **Acceptable-Guideline** and satisfies the guideline requirement (OPPTS 870.5300, OECD 476) for *in vitro* mutagenicity (mammalian forward gene mutation) data.

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

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: Polymeric xylenol tetrasulfide (PXTS)

Description: Black solid

Batch/Sample #: Batch No. 6, Bottle No. 1, Lot No. 1685-11-1

Purity: Not reported; responsibility of Sponsor

CAS # of TGAI: Not reported

Structure: Not reported

Solvent Used: Dimethysulfoxide (DMSO)

2. Control Materials:

Negative control: None

Solvent control (final conc'n): DMSO, 100 µg/mL

Positive control: Nonactivation: Methyl methanesulfonate (MMS) (13 µg/mL)

Activation: Methylcholanthrene (MCA) (2 and 4 µg/mL)

3. Activation: S9 derived from

X	induced (via i.p. injection)	X	Aroclor 1254 (500 mg/kg)	X	Rat (Sprague-Dawley; males)	X	Liver
	non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Liver homogenates were checked for sterility and enzyme activity. Table 1 illustrates the S9 Metabolic Activation System for the main and preliminary toxicity trials. The S9 activation mix was prepared commercially (Molecular Toxicology, Inc., Boone, N.C., Lot No. 1111) and kept frozen (-60 to -80°C) until needed. Prior to addition to treatment medium each batch of S9 activation mix was monitored for activation with the positive control (MCA).

TABLE 1. S9 Metabolic Activation System*

Component	Final Concentration in Cultures
NADP (sodium salt)	3 mM
Isocitrate	15 mM
S9 homogenate	~10 µL/mL

* Data obtained from page 10 of study report

4. Test cells: mammalian cells in culture

X	mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells (K1)		list any others

Media: RPMI 1640 supplemented with horse serum (10% by vol.), Pluronic® F68, L-glutamine, sodium pyruvate, penicillin, and streptomycin.

Properly maintained?	X Yes	No
Periodically checked for Mycoplasma contamination?	X Yes	No
Periodically checked for karyotype stability?	X Yes	No
Periodically "cleansed" against high spontaneous background?	X Yes	No

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) 20 µM		
	X	trifluorothymidine (TFT)				

6. Test compound concentrations used:

Nonactivated conditions: Precipitation test: 0.197, 0.393, 0.785, 1.57, 3.13, 6.25, 12.5, 25.0, 50.0, and 100 µg/mL

Preliminary toxicity test: 0.197, 0.393, 0.785, 1.57, 3.13, 6.25, 12.5, 25.0, 50.0, and 100 µg/mL

Mutagenicity trial I: 0.498, 0.995, 1.50, 1.99, 2.99, 3.98, 4.98*, 5.97*, 6.97*, 7.96*, 9.95* µg/mL

Mutagenicity trial II: 0.500, 1.00, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00*, 5.50*, and 6.00* µg/mL

Activated conditions: Preliminary toxicity test: 0.197, 0.393, 0.785, 1.57, 3.13, 6.25, 12.5, 25.0, 50.0, and 100 µg/mL

Mutagenicity trial I: 0.498, 0.995, 1.50, 1.99, 2.99, 3.98, 4.98, 5.97*, 6.97*, 7.96*, and 9.95* µg/mL

Mutagenicity trial II: 0.500, 1.00, 2.00, 3.00, 4.00, 4.50, 5.00**, 5.50, and 6.00, 6.50*, and 7.00* µg/mL

* Treatments terminated due to excessive cytotoxicity
** Treatment terminated due to availability of sufficient higher doses

B. TEST PERFORMANCE

1. Cell treatment:

a. Cells were exposed to the test compound in 1% DMSO for approximately 4 hours in treatment medium (final vol. 10 mL) comprised of Fischer's medium supplemented with horse serum (5% by vol.), Pluronic®F68, L-glutamine, sodium pyruvate, penicillin, and streptomycin. Cells were then centrifuged, treatment medium removed, cells washed and resuspended in culture medium. Following a 2-day expression period, cell counts were performed and cultures were selected for cloning and mutant selection. Triplicate vehicle controls, duplicate positive controls and eleven test article dose levels were initiated; 1 culture/dose level. The eleven PXTS doses were reduced in each trial due to excessive cytotoxicity (-S9: Trial 1, 6 cloned; Trial 2, 8 cloned; +S9: Trial 1, 7 cloned; Trial 2, 8 cloned). Cloning medium was RPMI 1640 supplemented with horse serum (20% by vol.), L-glutamine, sodium pyruvate, penicillin, streptomycin, and 0.24% BBL® agar (to achieve a semisolid state).

b. To calculate relative growth, cell densities greater than approximately 3 x 10^5 cells/mL were the only cultures chosen. A cell total of 3 x 10^6 were suspended in a soft agar medium selective for TFT-resistant mutants and this total was divided among three dishes (100 mm). Absolute selection cloning efficiency involved 600 cells seeded in three dishes in the cloning medium. All dishes were incubated for 13 days at 35-38°C, 95% humidified air, and 4-6% CO_2. The Loats Associates, Inc. (LAI) High Resolution Colony Counter (HRCC) System for the Mouse Lymphoma Assay (±S9 Trial 1, Version 1.05d; Trial 2, Version 1.05f) was used to count the colonies. Mutant frequency, relative cloning efficiency, and relative total growth (RTG) were measured.

c. All test cultures treated as described above including observation of appearance before and after treatment and monitoring of pH. Deviation from this involved the addition of the S9 activation mix to the treatment medium prior to test article for the metabolic activation mutation assays. Two trials (initial and confirmatory) were performed for assays with and without metabolic activation. Sizing analysis was also performed in this study with the L5178Y TK$^+$ cell line.

2. Statistical Methods: Not reported

3. Evaluation Criteria: Criteria for a valid assay were as follows: a positive response indicating a mutagen was evaluated as a dose-dependent 2-fold or greater increase in mutant frequency in relation to the background mutant frequency (vehicle control). The absence of a 2-fold increase in mutant frequency indicated a negative, nonmutagenic response for 1 of 4 situations. 1)toxicity of dose range causing 10-20% RTG, 2)test articles relatively nontoxic for doses exceeding the lower of 5 mg/mL or 10 mM, 3)dose range of 2 times the culture medium solubility limit, or 4)increases in mutant frequency that are not repeatable in confirmation trials. An equivocal response would be considered if the positive or negative evaluation was inconsistent. Additionally, any test article treatment that reduced RTG to <10% would indicate excessive

toxicity and considered biologically irrelevant and not an evaluation endpoint.

II. REPORTED RESULTS

A. PRELIMINARY CYTOTOXICITY ASSAY

In a precipitation test, the vehicle (DMSO at 10.0 and 5.0 mg/mL) was combined with the test article (PXTS) and a dark brown suspension was formed. PXTS did not precipitate in the remaining lower concentrations of DMSO. Therefore, the maximum dose for the preliminary dose range-finding assay was 100 µg/mL.

The preliminary toxicity assay was performed using 10 doses (ranging from 0.197 to 100 µg/mL) and a 4 hour exposure time at 35-38°C, in the presence and absence of the S9 metabolic activation. Treatments with -S9 (nonactivation) exhibited no cytotoxicity (≤1.57 µg/mL), moderately high cytotoxicity (3.13 µg/mL), high cytotoxicity (6.25 µg/mL), and excessive cytotoxicity (≥12.5 µg/mL). The +S9 (activation) assay indicated results of noncytotoxic and weakly cytotoxic (≤3.13 µg/mL), highly cytotoxic (6.25 µg/mL), and excessive cytotoxic (≥12.5 µg/mL). Additional results are shown in Table 2.

B. MUTAGENICITY ASSAY

The cloning efficiency and mutant frequency data for Trial 1 are presented in Table 3.

Trial 1 - Nonactivated Conditions
A mutant frequency that was greater than 2 times the average background (Triplicate vehicle control mutant frequencies, averaged) was calculated for each assay to provide a threshold value to compare to test article and positive control mutant frequency results. The initial nonactivation average vehicle mutant frequency threshold was 130.9 x 10^{-6} was well above the mutant frequency values (52.2-100) for the test article and well below the duplicate positive controls (MMS 13 µg/mL) which were 593.4 and 574.2. Relative cloning efficiency values ranged from 83.7-106.0% of solvent controls. Mutant frequency values (per 10^6 cells) ranged from a minimum of 52.2 (at 0.498 µg/mL) to a maximum of 100.0 (at 3.98 µg/mL) with no dose-response relationship. There was a positive response with the positive control although the test article cultures indicated negative responses to the assay. The mutant frequencies were within laboratory historical control ranges.

Trial 1 - Activated Conditions
Relative cloning efficiency values ranged from 77.5-103.3% of solvent control and mutant frequency values (per 10^6 cells) ranged from a minimum of 87.0 (at 0.498 µg/mL) to a maximum of 123.4 (at 3.98 µg/mL) with no dose-response relationship in the activated cultures. The solvent control average mutant frequency threshold was 154.5 x 10^{-6}. The test article mutant frequency responses were less than the vehicle control while the positive control values were much greater at 318.4 (MCA 2 µg/mL) and 279.3 (MCA 4 µg/L). The positive control mutant frequency results indicated a positive response while all of the test article doses did not. The

(7)

mutant frequencies were within laboratory historical control ranges.

The cloning efficiency and mutant frequency data for Trial 2 are presented in Table 4.

Trial 2 - Nonactivated Conditions
Relative cloning efficiency values ranged from 97.1-129.5% of solvent control. Mutant frequency values (per 10^6 cells) ranged from a minimum of 62.6 (at 3.00 µg/mL) to a maximum of 108.6 (at 4.50 µg/mL) with no dose-response relationship in the activated cultures. The solvent control average mutant frequency threshold was 152.5 x 10^{-6} while the positive control values were 396.6 and 411.4 (MMS 13 µg/mL). The positive-control compound elicited a clear positive response, demonstrating the sensitivity of the system to detect mutagenicity. The mutant frequencies were within laboratory historical control ranges.

Trial 2 - Activated Conditions
Relative cloning efficiency values ranged from 89.0-114.6% of solvent control. Mutant frequency values (per 10^6 cells) ranged from a minimum of 74.5 (at 2.00 µg/mL) to a maximum of 135.1 (at 6.00 µg/mL) with no dose-response relationship in the activated cultures. The solvent control average mutant frequency threshold was 154.2 x 10^{-6} while the positive control values were 246.8 (MCA 2 µg/mL) and 315.1 (MCA 4 µg/mL). The mutant frequencies were within laboratory historical control ranges. There was no indication of the test article increasing mutant frequency although the positive control clearly elicited a positive response.

TABLE 2. Cytotoxicity Assay Summary[a]

Applied Concentration µg/mL	Nonactivated		S9 Activated	
	Cell Density/mL (x 10^5)[b]	% Relative to Vehicle Control[c]	Cell Density/mL (x 10^5)[b]	% Relative to Vehicle Control[c]
VC[d]	16.1	100.0	11.2	100.0
0.197	14.5	90.1	12.9	115.2
0.393	15.0	93.2	13.7	122.3
0.785	18.3	113.7	15.1	134.8
1.57	14.3	88.8	13.2	117.9
3.13	4.5	28.0	7.9	70.5
6.25	3.2	19.9	1.8	16.1
12.5	0.0	0.0	0.0	0.0
25.0	0.0	0.0	0.0	0.0
50.0	0.0	0.0	0.0	0.0
100	0.0	0.0	0.0	0.0

[a] Data obtained from page 20 in the study report.
[b] Cell density determined by hemacytometer.
[c] Relative to vehicle control cell density for all treatments
[d] VC=Vehicle control, 1% DMSO

TABLE 3. Initial Mutation Assay (Trial 1) With and Without Metabolic Activation[a]

Trial 1	Nonactivated		S9 Activated	
	Cloning Efficiency	Mutant Frequency (x 10^{-6})	Cloning Efficiency	Mutant Frequency (x 10^{-6})
Vehicle Control (DMSO)	83.1 73.6 78.0	71.8 68.6 55.9	87.5 55.5 79.5	74.4 80.0 77.3
Vehicle Control Average Threshold	N/A	130.9	N/A	154.5
Positive Control	44.4[c] 43.6[c]	593.4[c] 574.2[c]	65.1[d] 80.7[d]	318.4[d] 279.3[d]
Test Article (µg/mL)[b]				
0.498	106.0	52.2	88.6	87.0
0.995	83.7	72.2	90.5	95.4
1.50	96.4	78.6	86.1	91.7
1.99	96.2	78.9	85.1	95.7
2.99	99.9	87.4	90.0	103.0
3.98	100.9	100.0	77.5	123.4
4.98	N/A	N/A	103.3	106.9

[a] Data obtained from page 21 and 25 in the study report.
[b] Values excluding concentration are relative to corresponding vehicle control value.
[c] MMC 13 µg/mL(-S9, nonactivated)
[d] MCA 2 and 4 µg/mL(+S9, activated)

Table 4. Confirmatory Mutation Assay (Trial 2) With and Without Metabolic Activation[a]

Trial 2	Nonactivated		S9 Activated	
	Cloning Efficiency	Mutant Frequency (x 10-6)	Cloning Efficiency	Mutant Frequency (x 10-6)
Vehicle Control (DMSO)	71.6 71.1 61.1	76.1 74.7 78.0	80.6 67.3 75.8	69.1 86.5 75.8
Vehicle Control Average Threshold	N/A	152.5	N/A	154.2
Positive Control	43.3c 47.8c	396.6[c] 411.4[c]	73.8[d] 71.1[d]	246.8[d] 315.1[d]
Test Article (µg/mL)[b]				
0.500	129.5	69.8	N/A	N/A
1.00	107.0	75.5	102.4	77.1
2.00	110.3	73.3	114.6	74.5
2.50	123.6	70.6	N/A	N/A
3.00	122.3	62.6	99.8	79.2
3.50	120.4	75.1	N/A	N/A
4.00	97.1	104.7	98.3	95.3
4.50	109.5	108.6	89.0	91.5
5.00	N/A	N/A	105.6	117.3
5.50	N/A	N/A	90.0	116.5
6.00	N/A	N/A	89.5	135.1

[a] Data obtained from page 23 and 27 in the study report.
[b] Values excluding concentration are relative to corresponding vehicle control value.
[c] MMC 13 µg/mL(-S9, nonactivated)
[d] MCA 2 and 4 µg/mL(+S9, activated)

III. DISCUSSION and CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: The test material, PXTS, did not induce TK locus forward mutations in L5178Y mouse lymphoma cells. PXTS was evaluated as negative and not considered mutagenic in this *in vitro* mammalian cell mutation test under the test conditions of this study.

B. REVIEWER COMMENTS: PXTS was not mutagenic in this *in vitro* mammalian cell gene mutation assay in the presence or absence of S9 activation. The positive controls induced clearly positive responses, demonstrating the sensitivity of the test system to detect mutagenic activity.

C. STUDY DEFICIENCIES: The following minor deficiency was noted: (1) statistical analysis was not reported

D. STUDY CLASSIFICATION: This study is classified as **Acceptable-Guideline** and meets the guideline requirements for an *in vitro* mammalian cells in culture gene mutation assay in Chinese hamster ovary cells (OPPTS 870.5300 [§84-2]).