UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: EPA ID# 0019565-00055 - VANCIDE TH Mutagenicity Study: CHO/HGPRT Mutation Assay with Confirmation

Record No.:	S438508
Tox. Chem. No.:	481B
Bar Code No.:	D190040
MRID No.:	415448-01

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CONCLUSIONS

R. T. Vanderbilt Company, Inc. submitted a CHO/HGPRT mutation study in response to the hexahydro-1,3,5-triethyl-s-triazine (Vancide TH, EPA Reg. No. 1965-55) data call-in notice for subchronic and chronic toxicological data for antimicrobial pesticide active ingredients of March 4, 1987.

Under the conditions of this study, Vancide TH is positive for forward mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells. When administered to cultured thioguanine-sensitive CHO cells at a concentration of 0.02 µl/ml, the test compound significantly increased the numbers of thioguanine-resistant mutants in the absence of an exogenous activator (S-9), and may increase the numbers of mutants at 0.02, 0.025 and 0.035 µl/ml with S-9 activation, and at 0.03 µl/ml without activation.

This study is acceptable for regulatory purposes.

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DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity Study - In Vitro Mammalian Cell Mutation

GUIDELINE #: 84-2

TOX. CHEM. #: 481B

MRID #: 415448-01

TEST MATERIAL: VANCIDE TH

SYNONYMS: Hexahydro-1,3,5-triethyl-s-triazine

STUDY NUMBERS: T8796.332010

SPONSOR: R. T. Vanderbilt Company, Inc.
30 Winfield Street
P.O. Box 5150
Norwalk, CT 06856

TESTING FACILITY: Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, MD 20850

TITLE OF REPORT: CHO/HGPRT Mutation Assay with Confirmation

AUTHOR: Cynthia I. Sigler, B.A.
John W. Harbell, Ph.D.

REPORT ISSUED: April 12, 1990

CONCLUSIONS: Under the conditions of this study, Vancide TH, when administered to cultured thioguanine-sensitive CHO cells at a concentration of 0.02 µl/ml, is positive for forward mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus, as it significantly increases the numbers of thioguanine-resistant mutants in the absence of an exogenous activator (S-9), and may increase the numbers of mutants at 0.02, 0.025 and 0.035 µl/ml in the presence of S-9 activation, and at 0.03 µl/ml without activation.

CLASSIFICATION: Acceptable

QUALITY ASSURANCE: A statement of quality assurance, dated April

13, 1990, and a statement of compliance with good laboratory practices, dated April 12, 1990, were included in the submission.

MATERIALS:

Mammalian Cells: Chinese Hamster Ovary (CHO)-K_1-BH_4) cells, obtained from Oak Ridge National Laboratories, Oak Ridge, TN.

Test Compound: Hexahydro-1,3,5-triethyl-s-triazine (VANCIDE TH), lot # M-TH-8K-327, a clear, colorless liquid, 98.5% pure. Stability not provided; however, it was stated in the submission that the sponsor assumed responsibility for determination of stability.

Solvent: 5 mM HEPES buffered sterile water. The test substance was dissolved in this immediately prior to dosing. The solvent concentration in the culture medium was 1% v/v concentration in the culture medium.

Positive controls: Ethyl methanesulfonate (EMS, Lot A11K, Eastman Kodak Chemical Company, Rochester, NY) was used at a concentration of 0.2 µl/ml in the nonactivated study, and benzo(a)pyrene (B(a)P, lot 57F-3434, Sigma Chemical Company, St. Louis, MO) was used at a concentration of 4 µg/ml in the activated study.

Cell Culturing Medium: Ham's F12 medium, without hypoxanthine, supplemented with 5% dialyzed fetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine (F12FBS5-Hx). This growth medium was used in the mutation assays as the untreated (negative) control.

METHODS:

S-9 Preparation: The metabolic activator, S-9, was prepared from adult male Sprague-Dawley rat livers. The rats had been given single i.p. injections of Aroclor-1254 at 500 mg/kg body weight, 5 days prior to sacrifice. The livers were removed from sacrificed rats, homogenized, centrifuged at 9000 x g for 10 min. at 4 ± 2°C, and the supernatants (S-9 fraction) collected. Just before use, the S-9 was mixed with a cofactor pool (100 µl S-9/ml 4mM NADP, 5 Mm glucose-6-phosphate, 30 Mm KCl, 10 Mm $CaCl_2$ and 50 Mm sodium phosphate buffer, Ph 8.0).

Dose Selection: A preliminary toxicity test was performed using CHO cells given the solvent or 1 of 9 different doses of VANCIDE TH, ranging from 0.0001 to 1.0 µl/ml for 5 hours at 37±1°C in the presence or absence of S-9. The following day, the cells were trypsinized and reseeded (100 cells/60 mm dish). The cloning efficiency of the test groups were compared to the solvent controls 7-10 days later.

Mutagenicity Assay: An initial and 2 confirmatory mutagenicity assays were performed, using 5 different concentrations of the test compound, positive and negative controls. The positive controls used were EMS for the nonactivated studies, and B(a)P for the activated studies; the negative or unstimulated controls were solvent and culture medium. The mutation assay was performed on exponentially growing CHO-K_1-BH_2 cells plated in F12FBS5-Hx at a density of 5 x 10^5 cells/25 cm^2 flask, at 37±1°C, 5±1% CO_2, in a humidified atmosphere for 18-24 hours. The culture medium for the cells activated with S-9 consisted of 4 ml F12FBS5-Hx, the appropriate concentration of Vancide TH, solvent, or positive control substance, and 1 ml of the S-9 activation mixture. The nonactivated culture medium contained 5 ml F12FBS5-HX plus the test or control substances. The final solvent concentration in the culture medium was 1% by volume. Untreated cells served as the negative controls. Duplicate cell cultures were exposed to the test compound or control substances for 5 hours at 37±1°C. The medium was then aspirated, and the cells washed and cultured in F12FBS5-Hx for an additional 18-24 hours at 37±1°C in a humidified chamber.

Evaluation of Cytotoxicity: Replicates from each treatment and control group were pooled, and triplicate subcultures prepared at a concentration of 100 cells/60 mm dish. The subcultures were incubated for 7-10 days at 37±1°C in 5±1% CO_2, then fixed in 95% methanol, stained with 10% Giemsa, counted, and the cytotoxicity of the treated groups compared with the controls.

Expression of Mutant Phenotype: The pooled replicates from each treatment or control group were subcultured in duplicate, at a cell density of 10^6 cells/100 mm dish. This was repeated every 2-3 days over a 7-9 day period.

Selection of mutant phenotypes was determined by pooling the duplicates within each treatment and control group, and plating 5 replicates at a density of 2 x 10^5 cells/100 mm dish, in F12FBS5-Hx medium containing 10 μM 6-thioguanine (TG, 2-amino-6-mercapto-purine). Cloning efficiency at the time of selection was determined on triplicate subcultures of the treated and control groups, plated at a density of 100 cells/60 mm dish, in a TG-free medium. The plates were incubated 7-10 days, fixed, stained for mutant phenotype selection, and cloning efficiency determined.

Criteria for Evaluating Test Results: The cytoxicity of each treatment group was compared with the solvent control, and relative cloning efficiency determined. The mutant frequency (MF) was calculated as the total number of mutant colonies per total number of cells plated (5 plates x 2 x 10^5 cells/plate = 10^6 cells), corrected for the cloning efficiency of the cells before mutant selection. A MF calculated for any treatment group with $\leq$ 10% relative cloning efficiency was considered invalid.

The mutagenic response of the treated groups was considered positive only when the MF exceeded 20 mutants per 10^6 cells, when there was a dose-dependent increase in the MF, and when the MF in 1 or more of the 5 test concentrations was significantly greater than the negative controls.

To determine statistical significance, one-sided Student's t test ($p \leq 0.05$) was used to obtain a confidence interval to compare assays with historical background MFs. In the present study, the historical control confidence interval for untreated and solvent controls was 10.8 mutants per 10^6 clonable cells; therefore, a result was considered statistically significant only when the MF was increased above that of the solvent and untreated (culture medium) controls by at least 10.8 mutants/10^6 clonable cells, and also was at least twice that of the solvent and untreated controls. Further, irrespective of the spontaneous mutant frequencies of the solvent and untreated controls, the minimum mutant frequency level used to determine significance in this study was > 20 mutants/10^6 clonable cells.

RESULTS:

The doses used were selected based on information contained in Microbiological Associates, Inc. Study No. T8796-380010. This information was not provided in the present submission, although the protocol stated that the optimal dose levels for the mutation assay should be selected from a preliminary toxicity test, based on colony-forming ability, with the highest dose used in the mutation assay as that which yields a cell survival of 10-30%, while the lowest dose should be nontoxic.

For the initial mutation studies, the following concentrations of the test compound were used: 0.03, 0.02, 0.01, 0.007, and 0.003 µl/ml in the nonactivated study, and 0.035, 0.025, 0.015, and 0.007, and 0.0035 µl/ml in the presence of S9 (activated study). In the confirmatory assays, the test compound was used at the following concentrations: 0.03, 0.02, 0.015, 0.01, and 0.005 µl/ml (both confirmatory, nonactivated studies) and 0.035, 0.03, 0.025, 0.002*, and 0.001* µl/ml in the first confirmatory activated study, and 0.035, 0.03, 0.025, 0.02, and 0.01 µl/ml in the second confirmatory activated study.

The mutagenicity data is presented in the table below. In the initial assay, the relative cloning efficiencies (comparing the test groups with the solvent controls) were 8, 20, 59, 93 and 101% without activation, and 17, 53, 90, 124 and 119% with S-9 activation (highest to lowest doses).

In the initial nonactivated mutation assay, the MF was 2.0, 5.4, 18.9, 19.4, 13.0 and < 0.9 TG-resistant mutants per 10^6 clonable cells in the solvent control, culture-medium control, 0.03, 0.02, 0.01, 0.007 and 0.003 µl/ml of Vancide TH groups,

respectively. EMS yielded a MF of 285.5 mutants per 10^6 clonable cells. Since none of the MFs of the treated groups exceeded 20 mutants per 10^6 clonable cell, the differences were not considered significant between the test groups and controls.

The activities of the test and control compounds in the initial activated study were as follows. The MF of the solvent and cell-medium controls were 6.5 and 16.1 mutants per 10^6 clonable cells, while Vancide TH yielded 11.3, 5.7, 6.1, 5.2, and 10.6 TG-resistant mutants per 10^6 clonable cells, in the 0.035, 0.025, 0.015, 0.007, and 0.0035 µl/ml groups, respectively. B(a)P yielded an MF of 261.4. None of the MF values of the treated groups were significantly different than the control groups.

In the first confirmatory assay, the relative cloning efficiencies (relative to the solvent controls) were 15, 26, 30, 60 and 111% without activation, and 14, 16, 22, 49 and 76% with activation (highest to lowest dose).

The MF values in the first nonactivated confirmatory mutation assay were < 0.8, 7.7, 7.4, 43.3, 15.8, 1.1, and 2.9 TG-resistant mutants per 10^6 clonable cells in the solvent control, culture-medium control, 0.03, 0.02, 0.015, 0.01 and 0.005 µl/ml of Vancide TH groups, respectively. EMS yielded a MF of 234 mutants per 10^6 clonable cells. The MF of the group treated with 0.02 µl/ml Vancide TH was significantly greater than the spontaneous mutant frequency of the solvent control.

In the presence of S-9 activator, the MF values in the first activated confirmatory assay were 1.9, 5.0, 5.9, 43.3, 28.0, 5.7, and 2.3 mutants per 10^6 clonable cells for the solvent control, culture medium control, and the 0.035, 0.03, 0.025, 0.002, 0.001 µl/ml test groups. B(a)P yielded 190.6 mutants per 10^6 clonable cells. The MFs of the 0.03 and 0.025 µl/ml groups of Vancide TH were significantly greater than the spontaneous MF of the solvent control.

These amounts should have been 0.02 and 0.01 µl/ml, but due to a dilution error, 0.002 and 0.001 µl/ml were tested instead.

In the second confirmatory assay, the relative cloning efficiencies (relative to the solvent controls) were 18, 31, 44, 79 and 83% without activation, and 31, 29, 41, 54 and 98% with activation (highest to lowest dose).

The MF values in the second nonactivated confirmatory mutation assay were 1.7, 6.7, 78.4, 35.9, 4.7, 3.9 and 8.9 TG-resistant mutants per 10^6 clonable cells in the solvent control, culture-medium control, 0.03, 0.02, 0.015, 0.01 and 0.005 µl/ml of Vancide TH groups, respectively. EMS yielded a MF of 180 mutants per 10^6 clonable cells. The MFs of the groups treated with 0.02 and 0.03 µl/ml Vancide TH were significantly greater than the spontaneous mutant frequency of the solvent control.

In the presence of S-9 activation, the MF values in the second activated confirmatory assay were 7.6, 5.8, 51.8, 17.1, 17.8, 20.4, and 3.1 mutants per 10^6 clonable cells for the solvent control, culture medium control, and the 0.035, 0.03, 0.025, 0.02, 0.01 µl/ml test groups. B(a)P yielded 264.4 mutants per 10^6 clonable cells. The MFs of the 0.035 and 0.02 µl/ml groups of Vancide TH were significantly greater than the spontaneous MF of the solvent control.

DISCUSSION: In the nonactivated studies, the results were significant at the 0.03 µl/ml concentration of Vancide TH in 1/3 trials, while at the 0.02 µl/ml concentration, the results were significant in 2/2 trials. The relative cloning efficiencies (RCE) at the 0.03 µl/ml concentration were low; in the initial nonactivated study, this value was only 8%, which is below the Subdivision F guideline for acceptable maximum cytotoxicity levels (10-20%). The MF for that group was fairly high (18.9), though not statistically significant. Although within the guideline limits, the RCEs in the 2 nonactivated confirmatory assays were only 15% and 18%; however, the MF was very high in one of those two groups (78.4, second confirmatory study). Thus, the mutagenicity of the test compound at 0.03 µl/ml is uncertain, probably due to the cytotoxicity of the substance at that level.

In the activated studies, the results were significant in 1/3 trials at the 0.035 µl/ml and 0.025 concentrations; 1/2 trials at the 0.03 µl/ml concentration; and 1/1 trial at the 0.02 µl/ml concentration of the test substance. At the 0.035 µl/ml concentration, 2 of the 3 studies had RCEs below 20%; the MFs of those two studies were not statistically significant. The study that had a significantly greater MF (51.8) also had a fairly high (31%) RCE. At 0.03 µl/ml, RCEs were < 20% for the 2 trials, and although only one of these yielded a significantly greater MF (43.3), the second confirmatory trial also had a fairly high MF (17.1). At 0.025 µl/ml, the RCE was > 20% in all 3 trials; and although the MF was statistically significant in only one of those trials (28.0), the MF of the second confirmatory study was fairly high (17.8). At the 0.02 µl/ml concentration, the RCE and MF were both high (54% and 20.4, respectively); however, this dose was tested only once.

The results of this study indicate that Vancide TH, when tested in the CHO/HGPRT mutagenicity assay, appears to significantly increase the numbers of thioguanine-resistant mutants at 0.02 µl/ml without exogenous (S-9) activation, and may increase the numbers of TG-resistant mutants at doses of 0.02, 0.025, 0.03 and 0.035 µl/ml with activation by S-9, and at 0.03 µl/ml without activation.

VANCIDE TH - RELATIVE CLONING EFFICIENCIES (RCE) AND MUTATION FREQUENCIES (MF)
FOR THE INITIAL MUTAGENICITY ASSAY (A) AND TWO CONFIRMATORY ASSAYS (B and C)

Treatment: Vancide TH (µl/ml) or controls	A				B				C			
	RCE[1] (%)		MF[2]		RCE (%)		MF		RCE (%)		MF	
	-[3]	+	-	+	-	+	-	+	-	+	-	+
Solvent	00	100	2.0	6.5	100	100	<0.8	1.9	100	100	1.7	7.6
Untreated	128	111	5.4	16.1	96	71	7.7	5.0	105	103	6.7	5.8
0.035		17		11.3		14		5.9		31		51.8*
0.03	8		18.9		15	16	7.4	43.3*	18	29	78.4*	17.1
0.025		53		5.7		22		28.0*		41		17.8
0.02	20		**		26		43.3*		31	54	35.9*	20.4*
0.015		90		6.1	30		15.8		44		4.7	
0.01	59		19.4		60		1.1		79	98	3.9	3.1
0.007	93	124	13.1	5.2								
0.005					111		2.9		83		8.9	
0.0035		119		10.6								
0.003	101		<0.9									
0.002						49		5.7				
0.001						76		2.3				
EMS	97		285.5		90		234		90		180.0	
B(a)P		55		261.4		9		190.6		32		264.4

1 Relative cloning efficiency = (cloning efficiency of treatment group/cloning efficiency of solvent group) x 100

2 Mutant frequency = total mutant colonies/(# dishes x cloning efficiency x 2 x 10^5 cells) x 10^6

3 +/- = with or without S-9 activator

* $p < 0.05$, Student's t test

** Plates were lost due to contamination