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

MEMORANDUM

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

SUBJECT: Triforine: Review of a gene mutation assay in Chinese hamster ovary cells

Caswell No: 889 DP Barcode:. D198852
MRID No. 43082401 PC Code: 107901

TO: R. Kendall/T. Myers, PM Team 51
Special Review & Re-registration Division (7508C)

FROM: Whang Phang, Ph.D. 5/31/94
Pharmacologist
Tox. Branch II, HED (7509C)

THROUGH: James Rowe, Ph.D. James N. Rowe 5/31/94
Section Head
and
Marcia van Gemert, Ph.D. 5/31/94
Branch Chief
Tox. Branch II/HED (7509C)

The registrant, Biologic, Inc., submitted a mutagenicity study on Chinese hamster ovary (CHO) cells. This study has been reviewed by Clement International Corp. and approved by Tox. Branch II. The DER is attached. The citation and conclusion are presented below:

Adams, K., Ransome, S., Anderson, A., & Dawe, I. S. (1993). Chinese hamster ovary/HGPRT locus assay triforine. Unpublished study conducted by Huntingdon Research Centre Ltd., England. Study No. SLL282/931168. Dec 8, 1993. Submitted to EPA by Biologic, Inc.; EPA MRID No. 430824-01.

In 2 independent CHO cell HGPRT forward gene mutation assays, triforine was tested at concentrations of 25, 50, 75, 100 and 200 µg/ml with or without S9 (metabolic activation). The S9 was derived from Arochlor 1254-induced rat livers, and triforine was delivered in dimethyl sulfoxide. Triforine was negative in the presence or absence of S9 up to the limit of solubility (200 µg/ml -S9, 150 µg/ml +S9).

This study is classified as acceptable and satisfies the data requirements for an in vitro mammalian cell forward gene mutation study (84-2).

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FINAL

DATA EVALUATION REPORT

TRITORINE

Study Type: Mutagenicity: Gene Mutation in Cultured Chinese Hamster Ovary Cells (CHO/HGPRT)

Prepared for:

Health Effects Division
Office of Pesticide Programs
Environmental Protection Agency
1921 Jefferson Davis Highway
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Prepared by

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Principal Reviewer Lynne T. Haber Date 5/4/94
Lynne T. Haber, Ph.D.

Independent Reviewer Nancy E. McCarroll Date 5/4/94
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Contract Number: 68D10075
Work Assignment Number: 3-62
Clement Number: 250
Project Officer: Caroline Gordon

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Triforine

GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATION

EPA Reviewer: Whang Phang, Ph.D.
Review Section III,
Toxicology Branch II/HED 7509C
Signature: [signature]
Date: 5/11/94

EPA Mutagenicity Reviewer:
Byron Backus, Ph.D.
Review Section II,
Toxicology Branch II/HED 7509C
Signature: Byron T. Backus
Date: 5/12/94

DATA EVALUATION REPORT

STUDY TYPE: Mammalian cells in culture gene mutation assay in Chinese hamster ovary cells (CHO/HGPRT)

TOX. CHEM. NO.: 890AA

PC CODE: 107901

MRID NUMBER: 430824-01

TEST MATERIAL: Triforine

SYNONYM(S): N,N'-[piperazine-1,4-diylbis(trichloromethyl)methylene] diformamide

STUDY NUMBER(S): HRC study number SLL282/931168

SPONSOR: Shell International Chemical Co., London, England

TESTING FACILITY: Huntingdon Research Centre Ltd., Huntingdon, Cambridgeshire, England

TITLE OF REPORT: Chinese Hamster Ovary/HPRT Locus Assay Triforine

AUTHOR(S): Adams, K., Ransome, S., Anderson, A., and Dawe, I.S.

REPORT ISSUED: December 8, 1993

EXECUTIVE SUMMARY: In two independent Chinese hamster ovary (CHO) cell HGPRT forward gene mutation assays, triforine was assayed at concentrations of 25, 50, 75, 100, 150, and 200 µg/mL +/-S9. Gene mutations were assessed at all nonactivated and S9-activated levels, except 50 µg/mL +/-S9. The S9 was derived from Arochlor 1254-induced rat livers, and triforine was delivered in dimethyl sulfoxide.

Triforine was negative in the presence and absence of S9 up to the limit of solubility (200 µg/mL -S9, 150 µg/mL +S9). Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as Acceptable and satisfies the guideline requirement for an in vitro mammalian cell forward gene mutation study (84-2).

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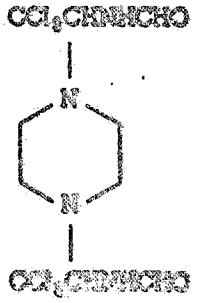
Triforine MAMMALIAN CELLS IN CULTURE GENE MUTATION

A. MATERIALS:

1. Test Material: Triforine
Description: Cream-colored powder
Identification numbers: Batch number TOX HTO8/91/01
Purity: 99.8%
Receipt date: February 5, 1992
Stability of compound: Expiration date: April 1995; the test material was stable at room temperature in the dark for at least 3 hours.
CAS number: Not provided
Structure:



Solvent used: Dimethyl sulfoxide (DMSO)
Other comments: The test material was stored at room temperature and dosing solutions were prepared the morning of the test. Analytical determinations were performed on representative stock solutions.

2. Control Materials:

Solvent/final concentration: DMSO/1% v/v

Positive: Nonactivation (concentrations, solvent): Ethyl methanesulfonate (EMS) was prepared in DMSO to yield a final concentration of 250 µg/mL.

Activation (concentrations, solvent): 20-Methylcholanthrene (20-MC) was prepared in DMSO to yield a final concentration of 5 µg/mL.

3. Activation: S9 derived from 7-8-week old male Sprague-Dawley-derived (<360 g)

X	Aroclor 1254	X	induced	X	rat	X	liver
	phenobarbital		noninduced		mouse		lung
	none				hamster		other
	other				other		

The S9 homogenate was prepared by the performing laboratory and tested for mixed function oxidase activity using 20-MC prior to use.

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S9 mix composition:

Component	Concentration in S9 Mix
NADP	8.0 mM
Isocitric acid	43.5 mM
S9 homogenate	25% (v/v)

Note: Cofactors were "neutralized" with 1N NaOH before adding to the S9 homogenate.

4. Test Cells: Mammalian cells in culture

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

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

5. Locus Examined:

____ thymidine kinase (TK)
Selection agent: _____ bromodeoxyuridine (BrdU)
(give concentration) _____ fluorodeoxyuridine (FdU)

_X_ hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent: _____ 8-azaguanine (8-AG)
(give concentration) 10 µg/mL 6-thioguanine (6-TG)

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

6. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Nine doses (1.56, 3.125, 6.25, 12.5, 25, 50, 100, 150, and 200 µg/mL) were evaluated using single cultures with and without S9 activation.

(b) Mutation assays:

- Nonactivated conditions: Six doses (25, 50, 75, 100, 150, and 200 µg/mL) were assayed in duplicate in two independent assays. Cultures exposed to 25, 75, 100, 150, or 200 µg/mL were plated in both assays.

- S9-activated conditions: As above, for the nonactivated conditions.

Triforine MAMMALIAN CELLS IN CULTURE GENE MUTATION

B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells were exposed to the test compound, solvent, or positive controls for:
__4__ hours (nonactivated) __4__ hours (activated)

(b) After washing, cells were cultured for __7__ days (expression period) before cell selection.

(c) After expression, cells seeded at $2x10^5$ cells/plate (5 dishes/culture) were cultured for __7__ days in selection medium to determine numbers of mutants, and cells seeded at 200 cells/dish (3 dishes/culture) were cultured for ____ days without selection medium to determine cloning efficiency (CE).

Note: For the assessment of post treatment survival a total of 200 cells were plated in three dishes for the preliminary cytotoxicity test. Based on the actual plate counts, our reviewers assumed that a similar procedure was used to determine posttreatment survival for the mutation assay. However, the study authors stated that "cells were seeded at 200 cells/plate."

2. Evaluation Criteria: The test material was considered positive if it induced a reproducible, dose-related statistically significant ($p<0.05$) increase in the mutation frequency (MF). In addition, at least one dose was required to induce a MF that was >20 mutants/10^5 survivors.

3. Statistical Analysis: The data were analyzed for statistical significance ($p<0.05$) by weighted analysis of variance according to the methods of Arlett et al. (1989)[1].

C. REPORTED RESULTS:

1. Solubility Testing: The test material was soluble in DMSO up to 390 mg/mL, but precipitation was observed when this concentration was diluted with culture medium. Therefore, the high dose selected for the preliminary cytotoxicity test was 200 µg/mL.

2. Preliminary Cytotoxicity Assay: Nine doses of triforine (1.56-200 µg/mL) were evaluated with and without S9 activation. There was no clear evidence of cytotoxicity at the highest nonactivated or S9-activated dose.

[1]Arlett, C.F., Smith, B.M., Clarke, G.M., Green, M.H.L., Cole, J., McGregor D.B. and Asquith, J.C. 1989. Mammalian cell gene mutation assays based upon colony formation. In: Kirkland, D.J. (ed.), UKEMS Sub-committee on Guidelines for Mutagenicity Testing, Report. Part III. Statistical Evaluation of Mutagenicity Test Data. Cambridge University Press, Cambridge, p. 24.

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3. Mutation Assays:

(a) Nonactivated: Similar results were observed in both the initial and confirmatory assays, which were conducted with 25-200 µg/mL. The test material precipitated at the high dose. Relative survival (RS) posttreatment was 70% at 200 µg/mL in the initial assay and 47% in the confirmatory assay (Table 1). However, there was no evidence of mutagenicity in either trial. Although a statistically significant ($p<0.05$) increase in MF was observed at 75 µg/mL in the confirmatory trial, the effect was not dose related, was not seen in the initial trial, and did not involve a MF that was >20 mutants/10^6 survivors.

(b) S9-Activated: The initial and confirmatory S9-activated assays were conducted with 25-200 µg/mL. In agreement with the preliminary cytotoxicity assay results, there was no evidence of cytotoxicity with the S9-activated test material in either trial (Table 2). Triforine precipitated at concentrations ≥150 µg/mL in both trials and there was no evidence that S9-activated triforine was mutagenic in either trial.

In both trials, the positive controls (250 µg/mL EMS -S9 and 5 µg/mL 20-MC +S9) induced significant ($p<0.001$) increases in the MFs and marked increases in the total number of mutants.

4. Analytical Determination and Stability Analysis: Stock solutions of 2.5, 10, or 20 mg/mL triforine prepared on two separate dates were determined to be within ±5% of the nominal concentration. Stock solutions containing 0.2 or 20 mg/mL in DMSO were stable at room temperature in the dark for at least 3 hours.

From the overall findings, the study authors concluded that triforine was not mutagenic in this test system.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS: We assess that the study was properly conducted and that the investigators interpreted the data correctly. Triforine was evaluated in two independently performed CHO/HGPRT assays up to the limit of solubility but failed to induce a mutagenic response in the presence or absence of S9 activation. Additionally, the sensitivity of the test system to detect a mutagenic effect was clearly demonstrated by the test results obtained with the positive controls (250 µg/mL EMS -S9; 5 µg/mL 20-MC +S9).

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLP? Yes. (A quality assurance statement signed and dated November 23, 1993 indicated that an in-life inspection was conducted on this type of assay on or about the time of the study under review. The study report was, however, audited by the quality assurance unit.)

F. Appendix attached? No.

TABLE 1. Representative Results of the Nonactivated Chinese Hamster Ovary (CHO) Cell Forward Gene Mutation Assays with Triforine

Substance	Dose/mL	Relative % Survival (after treatment)[a,b]	Total Mutant Colonies/Total Dishes	Average Mutant Colonies/Dish	Cloning Efficiency (at selection)[a]	Mutation Frequency/ 10^6 cells[a,c]
Solvent Control						
Dimethyl sulfoxide	10 µL	100.0[d]	35/20[e]	1.75	0.65	13.5
		100.0[f]	13/19	0.58	0.64	6.5
Positive Control						
Ethyl methanesulfonate	250 µg	57.5[d]	369/10	36.9	0.42	439.3**
	250 µg	23.0[f]	134/4	33.5	0.42	339.3**
Test Material						
Triforine	150 µg[g]	66.8[d]	24/10	2.40	0.48	25.0
	200 µg[h]	70.0	14/10	1.40	0.49	14.3
	75 µg[g]	67.0[f]	14/9	1.56	0.48	16.3*
	150 µg	36.3	5/9	0.56	0.48	5.8
	200 µg[h]	47.0	6/10	0.60	0.54	5.6

[a]Average of two cultures for the test material and positive control samples, and four cultures for the solvent control

[b]Relative to the average viable cells/culture of the solvent controls

[c]Mutation Frequency (MF) = $\frac{\text{Average Mutant Colonies/Dish}}{\text{Cloning Efficiency} \times 2 \times 10^5 \text{ cells}} \times 10^6$; calculated by our reviewers.

[d]Results from the initial assay

[e]Five dishes/culture were prepared; numbers <20 dishes for the solvent control or <10 dishes for all other groups indicate a loss of dishes due to contamination.

[f]Results from the confirmatory assay

[g]Findings for lower and intermediate doses (25, 75, or 100 µg/mL in the initial trial and 25 or 100 µg/mL in the confirmatory trial) did not suggest a mutagenic effect.

[h]The test material precipitated at the high dose.

*Significantly higher (p≤.05) than the solvent control

**Significantly higher (p<0.001) than the solvent control

Note: Data were extracted from the study report, pp. 20-23.

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TABLE 2. Representative Results of the S9-Activated Chinese Hamster Ovary (CHO) Cell Forward Gene Mutation Assay with Triforine

Substance	Dose/mL	Relative % Survival (after treatment)[a,b]	Total Mutant Colonies/ Total Dishes	Average Mutant Colonies/ Dish	Cloning Efficiency (at selection)[a]	Mutation Frequency/ 10^6 cells[a,c]
Solvent Control						
Dimethyl sulfoxide	10 µL	100.0[d] 100.0[f]	44/19[e] 8/16	2.3 0.5	0.63 0.71	18.3 3.5
Positive Control						
20-Methylcholanthrene	5 µg 5 µg	76.3[d] 100.5[f]	131/4 369/9	32.8 41.0	0.49 0.68	334.7** 301.5**
Test Material						
Triforine	100 µg[g] 150 µg[h] 200 µg	120.5[d] 120.0 121.5	10/9 12/6 21/9	1.1 2.0 2.3	0.55 0.47 0.46	10.1 21.3 25.0
	100 µg[g] 150 µg[h] 200 µg	122.5[f] 150.0 147.0	10/7 7/8 4/9	1.4 0.9 0.4	0.75 0.63 0.69	9.3 7.1 2.9

[a]Average of two cultures for the test material and positive control samples, and four cultures for the solvent control
[b]Relative to the average viable cells/culture of the solvent controls

[c]Mutation Frequency (MF) = $\frac{\text{Average Mutant Colonies/Dish}}{\text{Cloning Efficiency} \times 2 \times 10^5 \text{ cells}} \times 10^6$; calculated by our reviewers.

[d]Results from the initial assay
[e]Five dishes/culture were prepared; numbers <20 dishes for the solvent control or <10 dishes for all other groups indicate a loss of dishes due to contamination.
[f]Results from the confirmatory assay
[g]Findings for lower doses (25 or 75 µg/mL in both trials) did not suggest a mutagenic effect.
[h]The test material precipitated at concentrations ≥150 µg/mL +/-S9.

*Significantly higher (p<0.05) than the solvent control
**Significantly higher (p<0.001) than the solvent control

Note: Data were extracted from the study report, pp. 24-27.

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