MEMORANDUM

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

Geoffrey No. 899
HRID No. 43082401

TO: N. Kendall/T. Myers, PM Team 51
Special Review & Re-registration Division (7505C)

FROM: Whang Phang, Ph. D.
Pharmacologist
Tox. Branch II/HRD (7505C)

THROUGH: James Rowe, Ph. D.
Section Head
and
Harcia van Gemert, Ph. D.
Branch Chief
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The registrant, Biologic, Inc., submitted a mutagenicity study on Chinese hamster ovary (CHO) cells. This study has been reviewed by Clewett International Corp. and approved by Tox. Branch II. The DER is attached. The citation and conclusion are presented below:


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 Aroclor 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 vitra mammalian cell forward gene mutation study (84-2).
DATA EVALUATION REPORT

TRIPORINE

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
Arlington, VA 22202

Prepared by

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer: Lynne T. Haber, Ph.D. Date: 5/1/94

Independent Reviewer: Nancy E. McCarron, B.S. Date: 5/9/94

QA Manager: Bill McLellan, Ph.D. Date: 5/9/94

Contract Number: 68D10075
Work Assignment Number: 3-62
Clement Number: 250
Project Officer: Caroline Gordon
Triforine

GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATION

EPA Reviewer: Whang, Pang, Ph.D.
Review Section III, Toxicology Branch II/HED 7509C
EPA Mutagenicity Reviewer: Byron Beckus, Ph.D.
Review Section II, Toxicology Branch II/HED 7509C

DATA EVALUATION REPORT

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

TOK.CHIPH. No.: 890AA
PC CODE: 107901
MAID NUMBER: 430824-01

TEST MATERIAL: Triforine

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

STUDY NUMBER(S): HRC study number SLL262/931168

SPONSOR: Shell International Chemical Co., London, England

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

TITLE OF REPORT: Chinese Hamster Ovary/HGPRT 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 forard gene mutation assays, triforine was assayed at concentrations of 25, 50, 75, 100, 150, and 200 μg/mL +/-S9. Gene mutations were assayed at all nonactivated and S9-activated levels, except 50 μg/mL +/-S9. The S9 was received 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 (250 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 (94:2).
Triforine

A. MATERIALS:

1. Test Material: Triforine
   Description: Cream-colored powder
   Identification numbers: Batch number TOX HY08/91/01
   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:

   \[ \text{Structure Image} \]

   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 methanesulphonate (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 (460 g)

   \[ \text{Activation Image} \]

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

Hyalamin Cells in Culture Gene Mutation

5. TRIFORINE

4. Test Cells: Mammalian cells in culture

- mouse lymphoma L5178Y cells
- 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 "cleaned" against high spontaneous background? Yes.

5. Loss, Examined:

- thymidine kinase (TK)
  Selection agent: bromodeoxyuridine (BrdU"
  (give concentration)" fluorodeoxyuridine (FdU"

- hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
  Selection agent: 8-azaguanine (8-AC)
  (give concentration) 10 μM
  6-thioguanine (6-TG)

- Na+/K-ATPase
  Selection agent: ouabain
  (give concentration)

6. Test Compound Concentrations Used:

   (a) Preliminary cytotoxicity assay: Nine doses (1.55, 3.125, 6.25,
   12.5, 25, 50, 100, 150, and 200 μg/mL) were evaluated using
   single cultures with and without SF 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.

   - SF-activated conditions: As above, for the nonactivated
     conditions.
B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells were exposed to the test compound, solvent, or positive controls for:
- 4 hours (nonactivated) 6 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 7 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 post-treatment 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/10^6 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. (1969)\(^1\).

C. REPORTED RESULTS:

1. Solubility Testing: The test material was soluble in DMSO up to 390 μg/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 Test: Nine doses of triforine (1.56-200 μg/ml) were evaluated with and without NH activation. There was no clear evidence of cytotoxicity at the highest nonactivated or NH-activated dose.

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 -89 and 5 µg/mL 20-KC +89) 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 µg/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 -89; 5 µg/mL 20-KC +89).

E. QUALITY ASSURANCE MEASURE: Was the test performed under GLP? *Yes.* (A quality assurance statement signed and dated November 23, 1993 indicated that an in-lab 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

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dose (µg)</th>
<th>Relative Survival Index (control)</th>
<th>Total Mutant Colonies/Total Colonies (at selection)</th>
<th>Closing Efficiency (µg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol Control</td>
<td>10 µg</td>
<td>100.00</td>
<td>25/25</td>
<td>0.66</td>
</tr>
<tr>
<td>Ethenyl acrylate</td>
<td>30 µg</td>
<td>100.00</td>
<td>35/35</td>
<td>1.00</td>
</tr>
<tr>
<td>Positive Control</td>
<td>5 µg</td>
<td>47.00</td>
<td>26/26</td>
<td>0.54</td>
</tr>
<tr>
<td>Test Material</td>
<td>150 µg</td>
<td>66.00</td>
<td>27/27</td>
<td>0.54</td>
</tr>
<tr>
<td>Triforine</td>
<td>200 µg</td>
<td>70.00</td>
<td>25/25</td>
<td>0.49</td>
</tr>
</tbody>
</table>

*Average of 10 cultures for the test material and positive control samples, and four cultures for the solvent control.

Relative Survivial Index is calculated by dividing the average number of colonies in the solvent control by the average number of colonies in the experiment.

Closing Efficiency is calculated by dividing the average number of colonies at selection by the average number of colonies at exposure.

Mutation Frequency (MF) = Closing Efficiency x 2 x 10^5

Note: Data were extracted from the study report, pp. 20-32.
**TABLE 2. Representative Results of the C9-Activated Chinese Hamster Ovary (CHO) Cell Forward Gene Mutation Assay with Trifluorine**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative Survival (after treatment)</th>
<th>Total Mutant Colonies/Total Dishes</th>
<th>Average Mutant Colonies/Dish</th>
<th>Cloning Efficiency (at selection)</th>
<th>Mutation Frequency/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>18 µl 182.0² 186.0²</td>
<td>64/19⁰ 8/16</td>
<td>2.5</td>
<td>0.63</td>
<td>18.3</td>
</tr>
<tr>
<td>Excitation Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2H-Thymidylate</td>
<td>5 µg 76.3³</td>
<td>128/6</td>
<td>22.0</td>
<td>0.40</td>
<td>358.7**</td>
</tr>
<tr>
<td>Test Material</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifluorine</td>
<td>100 µg</td>
<td>120.3⁴</td>
<td>12/5</td>
<td>2.0</td>
<td>0.47</td>
</tr>
<tr>
<td>200 µg</td>
<td>120.3⁴</td>
<td>21/9</td>
<td>2.3</td>
<td>0.46</td>
<td>25.0</td>
</tr>
<tr>
<td>400 µg</td>
<td>122.3⁴</td>
<td>10/7</td>
<td>2.0</td>
<td>0.75</td>
<td>9.3</td>
</tr>
<tr>
<td>500 µg</td>
<td>120.0⁴</td>
<td>7/2</td>
<td>0.9</td>
<td>0.63</td>
<td>7.1</td>
</tr>
<tr>
<td>200 µg</td>
<td>147.0⁴</td>
<td>4/6</td>
<td>0.6</td>
<td>0.69</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*Average of two cultures for the test material and positive control samples, and four cultures for the solvent control.
*Relative to the average viable cells/culture of the solvent control.

Mutation Frequency (CFU) = 

\[
\frac{\text{Average Mutant Colonies/Dish}}{\text{Cloning Efficiency} \times 2 \times 10^6 \text{ cells}}
\]

Results from the initial assay:
- Three dishes/cultures were prepared; numbers <3 dishes for the solvent control or <3 dishes for all other groups indicate a loss of dishes due to contamination.
- Results from the confirmatory assay:
  - Results from the initial assay (5 µg 2H-thymidylate in both trials) did not suggest a mutagenic effect.
  - The test material prepared at concentrations 120 µg/ml was >X.

Significantly higher (p<0.05) than the solvent control.
*Significantly higher (p<0.01) than the solvent control.

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