UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

005449

SEP - 9 1986

MEMORANDUM

SUBJECT: Review of Four Mutagenicity Studies on Triforine

Caswell No. 890AA
Project No. 1433

Accession No. 260302
EPA.I.D. No. 21137-6

FROM: Whang Phang, Ph.D. 9/4/86
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THRU: Marcia van Gemert, Ph.D. 9.4.86.
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Four mutagenicity studies on triforine submitted by EM Industries, Inc. have been reviewed. Three of these studies are considered unacceptable due to various deficiencies. An Ames assay is acceptable. The data evaluation reports of these studies are attached.

005449

Data Evaluation Report (Mutagenicity Study)

Chemical: Triforine

Caswell No.: 890AA

Project No.: 1433

Formulation: Technical grade (purity unspecified)

Citation: Miltenburger, HG and Pistel, F. Chromosome Aberration in Cells of Chinese Hamster Cell Line V79. Laboratory for Mutagenicity Testing; Technische Hochschule, Darmstadt, F.P.G. Study Report No. LMP 136. Aug. 15, 1985.

Accession No.: 260302

Petitioner: EM Industries, Inc.

Reviewer: Whang Phang, Ph.D. 9/4/86
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Toxicology Branch/HED (TS-769c)

Secondary Reviewers: Marcia van Gemert, Ph.D. 9.4.86
Section Head
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Materials and Methods

1). Test system: The Chinese hamster V79 cell line was chosen because these cells are reported to have a high proliferation rate (doubling time 12-16 hrs in stock culture) and high plating efficiency (70-90%).

2). Test substances: Technical grade triforine (1,4-bis(2,2,2-trichloro-1-formamidoethyl-piperzine)) was dissolved in methanol. Maximum solubility was 50 ug/ml of methanol.

3). Positive control substances: Ethyl methanesulfonate (EMS) dissolved in nutrient medium. Cyclophosphamide was used with metabolic activation and dissolved in nutrient medium.

4). Media: Minimum essential medium (MEM) supplement with 10% fetal calf serum (FCS).

5). Cytotoxicity study: The report indicates that a cytotoxicity assay was carried out, but the details of the experiment were not reported.

6). Metabolic activation mixture (S9/S9 Mix): The S9 liver microsomal fraction was derived from the livers of Aroclor 1254 (500 mg/kg) treated male Wistar rats. After cervical dislocation the livers of the treated rats were removed, washed,

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homogenized, and centrifuged at 9000 g. The supernatant which contained the microsomal fraction was frozen and stored in ampoules of 2-5 ml.

For S9 mixture, appropriate frozen fractions were thawed and mixed with S9 cofactor solution to yield a final protein concentration of 0.3 mg/ml. The composition of the cofactor solution consisted of 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 5 mM NADP, 100 mM sodium-ortho-phosphate buffer, and pH 7.4.

7). Experimental Procedures (Submission):

A single cell suspension was prepared from trypsinizing two-day old logarithmically growing stock culture. The cells were seeded into Quadriperm dishes which contain microscopic slides (4 chambers per dish). In each chamber 3×10^4-2×10^5 cells were seeded. The medium used was MEM + 10% FCS.

After 17-24 hrs. of incubation, the medium was replaced with medium containing the control or test substance at appropriate concentrations. The preparation times were 7, 18, or 28 hr; at 5, 15.5, and 25.5 hr colcemid (0.2 ug/ml) was added to the culture medium. Subsequently, the cells were treated with a hypotonic solution (0.4 % KCl) for 20 min at 37°C on the slides. The cells were then fixed with glacial acetic acid/methanol (1:3). The following day, the cells were stained with aceto-orcein.

Results:

The concentrations of triforine used in this study were selected based upon the solubility and the cytotoxicity results. The highest soluble concentration of triforine was reported to be 50 ug/ml in methanol. The results of cytotoxicity test are presented in Table I.

TABLE I
Mitotic Index of Triforine Treated Cells†
(Data taken from Submission)

Triforine Conc.(ug/ml)	Preparation Time (hr)	Mitotic Index(absol. percentage) Without activation	With activation
0 (solvent)	7	7.80	6.60
40	7		9.63 (146)*
50	7	4.85 (62)	
0	18	9.60	9.03
0 (solvent)	18	6.15	7.65
4	18		6.20 (81)
5	18	9.25 (150)	
25	18		7.15 (93)
40	18	5.38 (87)	3.95 (52)
50	18	5.08 (83)	
Positive Control EMS (2.5 mg/ml)	18	1.90 (31)	
CPA (2.79 ug/ml)	18		3.70 (48)
0 (solvent)	28	7.03	6.10
40	28		6.98 (114)
50	28	5.80 (83)	

† Mean value of 4 slides.
* Values in the parenthesis are relative percentages.

The above results indicate that triforine at 50 ug/ml caused a decrease in mitotic index at various preparation times whereas at 40 ug/ml the results are variable. Based upon these results, the experimenters selected 40 ug/ml as the highest dose for assays with metabolic activation; 50 ug/ml, for assays without metabolic activation. However, this reviewer feels the same concentration (50 ug/ml) should be used for both types of assays.

The results of chromosomal assays are summarized in Table II.

TABLE II
Summary of Chromosomal Aberration
(Data taken from the Submission)

Triforine Conc.(ug/ml)	No. of Cells Analyzed	Preparat. Time(hr)	Percent of Aberrant Cells			
			Without Activation		With Activation	
			Excl. Gaps	Exchanges	Excl.Gap.	Exchanges
0(solvent)	400	7	0.75	0.00	0.25	2.25
40	400	7			2.25	0.25
50	400	7	2.00	0.00		
0	400	18	1.00	0.00	1.50	0.00
0(solvent)	400	18	1.25	0.25	1.25	0.25
4	400	18			1.75	0.00
5	400	18	1.75	0.00		
25	400	18			0.75	0.25
40	400	18	1.75	0.25	2.00	0.00
50	400	18	1.00	0.00		
Positive Controls						
EMS(2.50 mg/ml)	400	18	54.00	64.75*		
CPA(2.79 ug/ml)	400	18			11.5	3.00
0(solvent)	400	28	1.25	0.00	0.25	0.25
40	400	28			1.25	0.50
50	400	28	0.25	0.00		

*Value corrected by this reviewer; the reported value was 52.00%.

The results indicate that triforine at 50 ug/ml without metabolic activation at various preparation times did not cause chromosomal aberration under the experimental conditions. However, at 40 ug/ml with metabolic activation the results were variable at different preparation times; this variation could also be due to the variable results in the solvent controls (7 hr, exclusive gap = 0.25%; 18 hr, exclusive gap = 1.25%; 24 hr, exclusive gap = 0.25%).

Discussion and Conclusion:

Results from a single experiment indicate that triforine produced no chromosomal aberration on Chinese hamster V79 cell line when tested without metabolic activation whereas with metabolic activation the results were variable. A second experiment should have been carried out to verify the results obtained from the first experiment. Also the report should include individual determinations on the mitotic index and a longer sampling time (>28 hr) at which mitotic delay still evident. The report in the present form of this study is unacceptable.

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Data Evaluation Report (Mutagenicity Study) 005449

Chemical: Triforine

Caswell No.: 890AA

Project No.: 1433

Formulation: Technical grade (purity not specify)

Citation: Muller, E. et al. Mutations affecting the hypoxanthine phosphoribosyl transferase locus in V79 cells. Conducted by Laboratorium Fur Mutagenitatsprufung Technishchule Hochschule, Darmstadt and submitted by EM Industries, Inc. Study No.: LMP 076A. Dec. 12, 1984.

Accession No.: 260302

Petitioner: EM Industries, Inc.

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Secondary Reviewers: Marcia van Gemert, Ph.D. 9.4.86
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Conclusion: Under the present testing conditions, no mutagenic activity was observed in V79 cells which were treated with triforine (5 to 50 ug/ml) with or without metabolic activation.

Materials and Methods

1). Test species: The Chinese hamster V79 cell line was used. These cells were thawed from the stock cultures which were stored in liquid nitrogen in the cell bank of LMP DARMSTADT. The cells were then propagated in Eagle' minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 80 cm^2 plastic-flasks.

2). Test agent: Technical grade triforine (1,4-bis(2,2,2-trichloro-1-formamido-ethyl-piperazine) was supplied by Celamerck GmbH & Co. (batch no. 2230). Triforine was dissolved in methanol (maximum solubility, 50 ug/ml).

3). Positive control substances: Ethylmethanesulfonate (EMS) and 9,10-dimethy-1,2-benzanthracene (DMBA) were used. EMS was dissolved in nutrient medium; DMEA, in 2% DMSO.

4). Metabolic activation mixture (S9/S9 mix): The S9 liver microsomal fraction was obtained from the livers of 5 male Wistar rats which were treated with Aroclor 1254 in olive oil (500 mg/kg b.w.; i.p.). After sacrifice the livers of the rats were removed, washed, homogenized, and centrifuged at 9000 g for 10 minutes. The supernatant which contained the microsomal fraction was divided into 2 to 5 ml

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fractions and stored in ampoules in liquid nitrogen.

For S9 mixture, the frozen fraction was thawed and mixed with S9 cofactor solution to yield a final protein concentration of 0.3 mg/ml. The concentration of protein was estimated by the Lowry method. The composition of the cofactor solution consisted of 8 mM MgCl; 33 mM KCl; 5 mM glucose-6-phosphate; 5 mM NADP; 100 mM sodium-ortho-phosphate, pH 7.4.

5). Media: The treatment medium was similar to the culture maintenance medium and contained not more than 2% FCS. For assays with metabolic activation, 20 ul of S9 mix of the treatment medium was added. For the selection of mutants, the culture medium (selection medium) was supplemented with 11 ug of thioguanine/ml.

"Saline G" solution was composed of (per liter) NaCl 800 mg, KCl 400, glucose 1100 mg, $Na_2PO_4.7H_2O$ 290 mg, and KH_2PO_4 150 mg.

Ca-Mg-free salt solution consisted of (per liter) NaCl 8000 mg, KCl 400 mg, glucose 1000 mg, and $NaHCO_3$ 350 mg.

6). Cytotoxicity studies: The information derived from the cytotoxicity studies provides a basis for selecting concentrations of the test agent for the mutagenicity assays. Triforine at 50 ug/ml produced no significant toxicity on the V79 cells; however, the concentration of 50 ug/ml was the highest soluble concentration of this compound in methanol. This concentration was set as the highest testing concentration.

7). Experimental procedures: The submitted report stated that 5×10^5 cells were seeded in 15 ml of MEM-medium per flask. Two-day old logarithmically growing stock cultures were trypsinized with 2% trypsin containing Ca-Mg-free salt solulation and washed with "saline G". The cells were subcultured (1) at approximately 400 cells in 5 ml medium/25 cm^2-plastic flask for plating efficiency and (2) 1×10^6 cell in 30 ml medium/175 cm^2-plastic flask for mutagenicity test.

After 24 hrs. the medium of (1) and (2) was replaced with the treatment medium containing the appropriate concentration of the test agent with or without S9 mix. The concentrations of the test agent were 5, 10, 25, and 50 ug/ml. After 4 hrs., cells were rinsed with "saline G" solution, and the treatment medium was replaced with normal medium.

The cells in (2) were subcultured in five 80 cm^2-plastic flasks containing mutant selective medium (approximately 6×10^5 cells) and in two 25 cm^2-flasks for plating efficiency (approximately 500 cells/flask).

The cells were fixed and stained with 10% methylene blue in 0.01 N KOH solution. The stained colonies were counted with a microscope if a colony contained more than 50 cells.

Data analysis

According to the report, the toxicity of triforine was determined in a preliminary experiment by establishing the concentration related plating efficiency. If the test substance in one of the concentrations reproducibly induced mutation frequencies which were three times higher than the spontaneous mutant rates and a reproducible concentration-related increase in mutant frequency, the test chemical would be considered mutagenic.

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Results:

Based upon the concentration-related plating efficiency, triforine at 50 ug/ml which was the highest soluble concentration and the highest concentration tested produced little cytotoxicity in V79 cells except in the second experiment with metabolitic activation (Table I).

TABLE I

Cytotoxicity of triforine in V79 cells
(Before the addition of expression medium)
(Data taken from Submission, Table I and VI)

Concentration(ug/ml)	Absolute Plating Efficiency (%) Without Activation Exp. I	Without Activation Exp. II	With Activation Exp. I	With Activation Exp. II
Controls (negative)				
0	88.9	97.3	73.3	94.7
0(solvent)	90.2	97.8	74.6	87.0
0(DMSO)			74.6	89.2
Test Compound				
5.0	88.4	96.4	71.6	85.7
10.0	94.7	95.5	67.0	75.9
25.0	81.9	93.7	63.4	68.5
50.0	87.1	95.0	64.4	62.7
Positive controls				
EMS*1.0 mg/ml	72.3	62.6		
DMBA† 15.4 ug/ml			10.2	3.6

*: EMS = Ethylmethanesulfonate, which was dissolved in nutrient medium.
†: DMBA = 9, 10-dimethyl-1, 2-benzanthracene, which was dissolved in DMSO.

With HGPRT assay, triforine, at all concentration tested, did not cause mutation in V79 Chinese hamster cell line (Table II).

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TABLE II

Mutagenicity of Triforine in V79 Cells
with HGLPRT Assay
(Data taken from Submission, Tables III and VI)

Concentration (ug/ml)	Mutant Colonies/10^6 Cells*			
	Without Activation		With Activation	
	Exp. I	Exp. II	Exp. I	Exp. II
Negative Controls				
0	52.1	34.7	59.1	49.1
0 (solvent)	41.0	43.7	32.6	64.1
0 (DMSO)			28.4	64.0
Test Compounds				
5.0	52.2	26.8	27.1	51.6
10.0	57.6	56.2	53.8	46.0
25.0	67.7	55.6	33.0	50.4
50.0	55.8	50.8	82.4	87.8
Positive Controls				
1.0 mg EMS	472.3	361.2		
15.4 ug DMBA			234.7	449.0

* only colonies with more than 50 cells at 7 days after seeding in selection medium were scored.

Discussion and Conclusion:

In Ames assay, DMSO was used as a solvent for triforine whereas in this assay methanol was used. Triforine may be more soluble in DMSO than in methanol, and the study did not explore this possibility. DMSO might be used as a solvent for this assay seeing the results with DMSO in Table I and in Ames assays. This study is unacceptable because inappropriate solvent is used.

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005449

Data Evaluation Report (Mutagenicity Study)

Chemical: Triforine

Caswell No.: 890AA

Project No.: 1433

Formulation: Technical grade (purity unspecified)

Citation: Kramer, P. -J. In vitro assessment for mutagenic potential in bacteria with and without addition of a metabolizing system. Institute of Toxicology; E. Merck, Darmstadt, Federal Republic of Germany. Study No. T12756. March 25, 1985.

Accession No.: 260302

Petitioner: EM Industries, Inc.

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Materials

The test agent was triforine (technical grade) which was dissolved in DMSO. The compounds for positive controls were daunomycin (DAUN), 1-ethyl-2-nitro-nitrosoguanidine (ENNG), 2-aminoanthracene (2-AA), 9-aminoacridine (9-AA), methyl methanesulfonate (MMS), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), 2-nitrofluorene (2-NF), and 4-nitro-1,2-phenylene diamine (4-NP).

The bacteria tester strains used in this study were histidine requiring mutants of Salmonella typhimurium (TA 98, TA 100, TA 1535, TA 1537, & TA 1538). The bacteria were obtained from B.N. Ames and maintained according to the methods reported by Ames et al. (1975).

Metabolic activation system consisted of S-9 mix derived from the livers of Wi-AF/Han (SPF) rats. Five days prior to sacrifice, these rats were given a single intraperitoneal injection of Aroclor 1254 (500 mg/kg body weight) to induce microsomal enzyme activity. The S-9 mix was prepared according to Ames methods, and the S-9 fraction was thawed from the batches stored at -80°C as required by the experiments.

Experimental Protocol: The assays were conducted according to the procedures described by Ames et al. (1975). For each concentration of the test agent, 8 plates were used (4 with and 4 without metabolic activation); for the solvent negative controls, 16 plates were used (8 with and 8 without metabolic activation). Fresh suspensions of the tester strains were prepared by inoculating one colony of the the corresponding master plate and incubated overnight in 37°C shaking incubator.

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Triforine was dissolved in dimethylsulfoxide (DMSO); six doses of triforine (10, 50, 250, 1250, 2500, & 5000 ug/plate) and appropriate positive controls were assayed. The revertant colonies were counted manually or using an Artek M 880 automatic colony counter.

Results:

In a preliminary experiment, toxicity of triforine was tested with a wide range of concentrations (50 - 10,000 ug/plate) for dose selection. The report stated that no toxicity was observed even in the highest dose. However, the experimental results of the preliminary study were not submitted.

In the absence or presence of the metabolic activation system, triforine at various concentrations did not show mutagenic activity whereas the positive controls produced revertant colonies which were approximately 3 to 200 times greater than the solvent controls (Table I)

Table I
Summary of Revertant Colonies in Different Strains of Triforine Treated S. typhimurium
(Data taken from the submission)†

Strain		Dose (ug/plate)	Revertant Colonies: Metabolic Activation: Without	Revertant Colonies: Metabolic Activation: With
TA 98		0	22 ± 7	41 ± 10
		0	22 ± 6	38 ± 6
		10	25 ± 3	40 ± 4
		50	19 ± 5	39 ± 16
		250	24 ± 7	32 ± 4
		1250	31 ± 6	42 ± 10
		2500	29 ± 10	-------
		5000	14 ± 5	38 ± 6
	Ve-H_2O*	0	30 ± 8	
	2-AA	1	28 ± 10	387 ± 19
	DAUN	2	932 ± 142	
	2-NF	2	520 ± 258	
TA 100		0	124 ± 33	163 ± 23
		0	115 ± 24	193 ± 8
		10	117 ± 28	157 ± 44
		50	111 ± 29	185 ± 41
		250	108 ± 25	172 ± 12
		1250	122 ± 29	163 ± 13
		2500	101 ± 6	243 ± 52
		5000	121 ± 29	226 ± 11
	2-AA	1	99 ± 22	503 ± 120
	MMS	500	929 ± 401	
	ENNG	2	390 ± 47	

* Ve-H_2O = deionized H_2O

† Data of a repeat experiment have been reported, and they are similar to what are presented in Table I.

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Table I (Continued)
Summary of Revertant Colonies in Different Strains Triforine
Treated S. typhimirium
(Data taken from the submission)

Strain	Dose (ug/plate)	Revertant Colonies — Metabolic Activation: Without	Revertant Colonies — Metabolic Activation: With
TA 1535	0	12 ± 3	16 ± 5
	0	15 ± 6	17 ± 3
	10	18 ± 2	17 ± 2
	50	13 ± 6	19 ± 6
	250	13 ± 3	16 ± 11
	1250	10 ± 3	15 ± 4
	2500	10 ± 3	24 ± 8
	5000	10 ± 6	15 ± 4
2-AA	1	15 ± 7	143 ± 25
ENNG	10	1095 ± 113	
MNNG	4	>2000 ± 0	
TA 1537	0	6 ± 1	10 ± 3
	0	7 ± 2	12 ± 4
	10	6 ± 2	9 ± 6
	50	7 ± 2	17 ± 2
	250	6 ± 2	7 ± 1
	1250	7 ± 1	10 ± 4
	2500	7 ± 2	8 ± 5
	5000	5 ± 2	7 ± 1
EtOH†		5 ± 3	
2-AA	1	8 ± 1	58 ± 18
9-AA	20	38 ± 9	
9-AA	50	188 ± 21	
TA 1538	0	14 ± 3	8 ± 1
	0	12 ± 4	16 ± 3
	10	--------	13 ± 4
	50	8 ± 2	24 ± 10
	250	9 ± 3	15 ± 2
	1250	3 ± 2	16 ± 5
	2500	9 ± 2	18 ± 7
	5000	7 ± 4	14 ± 6
2-AA	1	22 ± 4	646 ± 41
4-NP	10	633 ± 44	
2-NF	5	813 ± 81	

† EtOH = ethanol

Discussion and Conclusion:

The experimental results indicate that triforine produced no mutagenic activity under these testing conditions, and the result from a repeat assay also confirmed this observation. This mutagenicity study is acceptable; however the petitioner should submit the preliminary experimental results concerning the toxicity of triforine on S. typhimurium.

005449

Data Evaluation Report (Mutagenicity Study)

Chemical: Triforine

Caswell No: 890AA

Project No: 1433

Formulation: Technical grade (purity not specified)

Citation: Miltenburger, H.G. et al. Unscheduled DNA Synthesis Assay in Hepatocytes of Male Rats (in vitro UDS Test). Laboratorium fur Mutagenitatsprufung, Technische Hochschule, Darmstadt, LMP Darmstadt. Study report no. 076B. Feb. 13, 1985.

LMP

Accession No.: 260302

Petitioner: EM Industries, Inc.

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Secondary Reviewers: Marcia van Gemert, Ph.D. 9.4.86
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Materials and Methods

1). Test species: Rat hepatocytes were isolated from the livers of 8- to 12-weeks old male Wistar rats (the number of rats used was not specified).

2). Test substance: Technical grade triforine (1,4-bis(2,2,2-trichloro-1-formamido-ethyl-piperazine) (purity unspecified) was dissolved in methanol. Maximum solubility was 50 ug/ml of methanol.

3). Positive control substances: 7,12-dimethylbenz(a)anthracene (DMBA) dissolved in DMSO.

4). Media: Seglen's medium (details not provided) was used as the perfusion medium for rat liver.
Culture medium consisted of L-15 culture medium (GIBCO, D-7500 Karlsruhe, F.R.G.) and supplemented with HEPES (15 mM), penicillin (100 ug/ml), streptomycin (100 ug/ml), glucose (1.5 mg/ml). insulin (0.5 ug/ml), and $NaHCO_3$ (2.2 mg/ml). The pH was adjusted to 7.4.
Cell lyzing solution was composed of 10 mM Tris-HCl, 15 mM NaCl; 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, and pH 8.5
Nucleus lyzing solution was cosisted of 2.5 mM EDTA, 2% SDS, 0.1 M glycine, 1 mg/ml proteinase K, and pH 10.

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5). Cytotoxicity studies: For cytotoxicity assay, 7 concentrations of triforine were tested: 1, 5, 10, 20, 30, 40, and 50 ug/ml.

6). Experimental procedures: Male Wistar CF HB rats were anesthetized, and the liver of each rat was perfused initially with perfusion medium, and subsequently with perfusion medium containing collagenase (0.05% w/v) until the liver was swollen.

The isolated cells were washed with perfusion solution and passed through 40 mM stainless steel mesh to obtain a single cell suspension. The viability of the cells were found to be 85.3% with trypan blue. These cells were centrifuged and suspended in the culture medium. With these procedures approximately 2-5 x 10^8 cells were obtained from each liver.

Treatment processes were as follows: "aliquots containing 3.5-4 x 10^6 hepatocytes were placed into 4 ml of culture medium supplemented with hydroxyurea (15 mM) in 25 ml Erlenmeyer flasks". Flasks were agitated for 1 hr. in 37 °C water bath. Appropriate concentration of triforine was added along with 0.7 uCi/ml of tritium labeled thymidine ((^3H)-TDR) and incubated for another 3 hrs.

After 4 hrs. of incubation, the Erlenmeyer flasks were placed into an ice bath. The cells were washed with cold phosphate-buffer saline solution containing unlabeled thymidine (0.5 mg/ml). The cells were subsequently placed in the cell lysing solution for 10 min, and the nuclei were obtained by centrifugation.

The nuclei were then lysed for 30 min in 2.5 ml of nucleus lysing solution. The DNA was precipitated when 2.5 ml of trichloro-acetic acid (TCA)(10%) was added to the lysate. The DNA preparation was kept at 4 °C for overnight.

For liquid scintillation counting and DNA content analysis, 0.2 ml aliquots of the DNA preparation were used. The DNA content was determined by colorimetric method.

Results:

The cytotoxicity of the test agent was examined, the data indicated that triforine at 50 ug/ml produced toxicity resulting in 45% of the cell death relative to the controls (Table I). In addition, triforine at 50 ug/ml was also the highest soluble concentration in methanol.

TABLE I
Cytotoxicity of Troforine
(Data taken from the submission)

Triforine ug/ml	Cell Survival % (relative)	
0	100	(60% absolute)
1	104	
5	86	
10	79	
20	73	
30	77	
40	67	
50	55	

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[redacted]

Based upon the results of the cytoxicity study, 4 concentratins of the triforine were selected for the unscheduled DNA assay (Table II). Under the testing conditions of this, triforine did not produce an increase in (^{3}H)-TDR incorporation as measured by the disintegrations per minute per ug of DNA (dpm/ug) relative to the controls. In contrast, the positive control (DMBA) caused a 4.5-fold increase in the incorporation of (^{3}H)-TDR into the DNA of the DMBA treated cells.

TABLE II

^{3}H-Thymidine Incorporation in Triforine Treated Cells
(Data taken from the submission)

Triforine ug/ml	dpm/ug DNA Mean ± Stand. dev. n = 6
0	61.0 ± 13.1
0.5	58.7 ± 11.3
1.0	76.4 ± 3.5
10.0	71.3 ± 9.0
25.0	59.6 ± 6.3
50.0	64.4 ± 7.6
Positive Control DMBA 25.64 ug/ml	275.6 ± 36.9

Discussion and Conclusion:

Although the experimental results indicate that triforine, under the testing conditions presented in the report, had no effect on the unscheduled DNA synthesis, the report has not presented the analytical data on the determination of DNA. These data are important for verifying the identity of the DNA fraction and the validity of the DNA isolation procedures. In addition, the report dose not mentioned whether DMSO has ever been tried as a solvent since the test agent has limited solubility in methanol.

Core grade: The present form of the report is unacceptable because the data on the determination of DNA are missing. In addition, inappropriate solvent was used (i.e., use of methanol instead of DMSO; also see comments on Study No. LMP 076A).

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