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

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JUL 27 1992

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Chloropicrin (CP): Mutagenicity studies. ID #081501
DB #D169053. HED # 1-2489
Chemical No. ~~443~~ 214

TO: Larry Schnaubelt/Karen Samek (PM 72)
Reregistration Review Branch
Registration Division (H7508W)

FROM: Stanley B. Gross, PhD, DABT, CIH
Toxicologist/Hygienist
Toxicology Branch I
Health Effects Division (H7509C)

Stanley B Gross 7/9/92

THRU: Joycelyn Stewart PhD
Acting Head, Section II, Toxicology Branch I
Health Effects Division (H7509C)

JS 7/9/92

THRU: Karl P. Baetcke, PhD
Chief, Toxicology Branch I
Health Effects Division (H7509C)

Karl P. Baetcke 7/14/92

The following mutagenicity studies have been reviewed by Clement International Corporation staff and reviewed secondarily by geneticist, Dr. Irving Mauer of Toxicology Branch 1. The Clement DER's are attached to this memo and summarized below.

A. Ames Salmonella typhimurium/mammalian microsome mutagenicity Assay.

"Salmonella typhimurium/ Mammalian-microsome plate incorporation mutagenicity assay (Ames test) with a confirmatory Assay. Microbiological Associates study #T9152.501014, June 21, 1990. MRID #41960801. Clements review 91-101.

Summary: S.typhimurium strain were tested at levels of 10 to 3333 ug/plate. Strains TA98 showed consistent and reproducible revertant with S9 activation; TA1538 strain was negative; and TA98 and TA100 strains were erratic without activation and TA1535 (with activation). The study was considered to be positive mutagenic changes in this test and the study was deemed ACCEPTABLE.

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B. Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells.

"Chromosome aberrations in Chinese hamster ovary (CHO) cells with confirmatory assay". Microbiological Associates Study number T9152-337001. May 31, 1990. MRID # 41960802. Clements review 91-102

Summary: Non-activated doses of CP ranging from 0.75 to 1 nL/mL induced a reproducible and significant clastogenic response in CHO cell harvested 12 hours posttreatment. The predominant aberration was chromatid breaks. In the presence of S9 activation, CP was clastogenic over a narrow range of cytotoxic concentrations (4 and 6 nL/mL).

Conclusions: CP was positive for clastogenic effects and the studies were ACCEPTABLE

C. Gene Mutation in cultured Mouse Lymphoma Cells.

Study: L5178Y TK+/- mouse lymphoma mutagenesis assay with confirmation. Microbiological Associates, Inc. Study No. T9152.701020, April 26, 1990. MRID #41960803. Clements review 91-103.

Summary: CP ranging from 0.038 to 0.75 nL/mL -S9 and 0.89 to 16 nL/mL +S9 did not induce a mutagenic response in two independently performed mouse lymphoma forward mutation assays. The tests were considered to adequately tests for non mutagenic changes in this test system. The study was considered ADEQUATE.

D. Unscheduled DNA Synthesis in Rat Hepatocytes.

Study: Unscheduled DNA synthesis in rat primary hapatocytes with confirmatory assay. Microbiological Associates, Inc. Study no. T9152.380009, June 28, 1990. MRID #41960804. Clements review 91-104.

Summary. CP was genotoxic in primary hepatocytes over a concentration range of 0.3 to 6 nL/mL which was moderately toxic. Higher concentrations (=>9 nL/mL) were severely toxic. The test showed no evidence of UDS as tested and the study was considered to be Acceptable.

cp12489.m 7/9/92

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FINAL

DATA EVALUATION REPORT

CHLOROPICRIN

Study Type: Mutagenicity: Gene Mutation in Cultured Mammalian Cells (Mouse Lymphoma Cells)

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 Nancy E. McCarroll, B.S. Date 6/2/92

Independent Reviewer Lynne T. Haber, Ph.D Date 6/2/92

QA/QC Manager Sharon Segal, Ph.D. Date 6/2/92

Contract Number: 68D10075
Work Assignment Number: 1-26
Clement Number: 91-103
Project Officer: James Scott

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GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATION

EPA Reviewer: Stanley Gross, Ph.D. Signature: [signature]
EPA Review Section (I), Toxicology Date: 7/5/92 [illegible]
Branch (I)/HED
EPA Acting Section Head: Joycelyn Stewart, Ph.D. Signature: _____
EPA Review Section (II), Toxicology Date: _____
Branch (I)/HED

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Gene mutation in cultured mammalian cells (mouse lymphoma cells).

EPA IDENTIFICATION NUMBER: 081501

Tox Chem. Number: 214

MRID Number: 419608-03

TEST MATERIAL: Chloropicrin technical

SYNONYMS: None listed

SPONSOR: Niklor Chemical Co., Inc., Long Beach, CA

STUDY NUMBER: T9152.701020

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD

TITLE OF REPORT: L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay with Confirmation.

AUTHORS: R.H.C. San and C.I. Sigler

REPORT ISSUED: April 26, 1990

CONCLUSIONS-EXECUTIVE SUMMARY: Doses of chloropicrin technical ranging from 0.038 to 0.75 nL/mL -S9 and 0.89 to 16 nL/mL +S9 did not induce a mutagenic response in two independently performed mouse lymphoma forward mutation assays. The relative total growth at the highest doses evaluated for mutagenesis was 16% at 0.75 nL/mL -S9 and 23% at 16 nL/mL +S9. The findings indicate, therefore, that chloropicrin technical was adequately tested to cytotoxic levels and found to be nonmutagenic in this test system. The study satisfies Guideline requirements for genetic effects Category I, Gene Mutations.

STUDY CLASSIFICATION: The study is acceptable.

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A. MATERIALS:

1. Test Material: Chloropicrin technical

Description: Colorless liquid
Identification No.: Lot no. 987
Purity: 99.5%
Receipt date: February 15, 1990
Stability: Not reported
Contaminants: None listed
Solvent used: Ethanol (ETOH)
Other provide information: The test material was stored at room temperature, protected from light.

2. Control Materials:

Negative: None

Solvent/final concentration:

Test material: ETOH/1%

Positive controls: Dimethyl sulfoxide (DMSO)/1%

Positive:
Nonactivation: (Concentrations, solvent): Ethyl methanesulfonate (EMS) was prepared in DMSO to yield final concentrations of 0.25 and 0.5 µL/mL.

Activation: (Concentration, solvent): 7,12-Dimethylbenz(a)anthracene (DMBA) was prepared in DMSO to yield final concentrations of 2.5 and 5.0 µg/mL.

3. Activation: S9 derived from adult male Sprague-Dawley

x	Aroclor 1254/1242 (1:2 mixture)	x	induced	x	rat	x	liver
_____	phenobarbital	_____	noninduced	_____	mouse	_____	lung
_____	none			_____	hamster	_____	other
_____	other			_____	other		

The S9 liver homogenate was prepared by the performing laboratory. Prior to use, the S9 fraction was characterized for its ability to convert 2-aminoanthracene and DMBA to mutagenic forms in Salmonella typhimurium TA100.

S9 mix composition:

Component	Concentration/mL
NADP	6.0 mg
Isocitric acid	11.25 mg
S9 homogenate	0.25 mL
F_0P[1]	0.75 mL

[1] F_0P = Fisher's medium supplemented with 0.1% pluronics.

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4. Test Cells: Mammalian cells in culture

__x__ 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 "cleansed" against high spontaneous background? Yes.

5. Locus Examined:

__x__ thymidine kinase (TK)
selection agent:
(give concentration)
_____ bromodoxyuridine (BrdU)
_____ fluorodeoxyuridine (FdU)
3 µg/mL trifluorothymidine (TFT)

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

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

_____ other (locus and/or selection agent; give details):

6. Test Compound Concentration Used:

(a) Cytotoxicity assay: 0.0001, 0.001, 0.01, 0.1, 1, and 10 µl/mL +/-S9.

(b) Mutation assay:

(1) Nonactivated conditions:

- Initial test: 0.038, 0.05, 0.067, 0.089, 0.12, 0.16, 0.21, 0.28, 0.38, and 0.5 nL/mL.
- Confirmatory test: 0.36, 0.46, 0.56, 0.65, and 0.75 nL/mL.

(2) S9-Activated conditions:

- Initial test: 0.89, 1.2, 1.6, 2.1, 2.8, 3.8, 5.0, 6.7, 8.9, 12, 16, and 21 nL/mL.
- Confirmatory test: 9, 10, 12, 14, and 16 nL/mL.

B. TEST PERFORMANCE:

1. Cell Treatment:

(a) Cells exposed to test compound for:
__4__ hours (nonactivated) __4__ hours (activated)

(b) Cells exposed to positive controls for:
__4__ hours (nonactivated) __4__ hours (activated)

(c) Cells exposed to negative and/or solvent controls for:
__4__ hours (nonactivated) __4__ hours (activated)

(d) After washing, cells cultured for __2__ days (expression period) before cell selection

(e) After expression, cells cultured for __10 to 12__ days in selection medium to determine numbers of mutants and for __10 to 12__ days without selection medium to determine cloning efficiency.

2. Statistical Methods: The data were not evaluated for statistical significance.

3. Evaluation Criteria:

(a) Assay validity: For the assay to be considered valid, the following criteria must be satisfied: (1) the cloning efficiency (CE) of the solvent control must exceed 50%; (2) the mutation frequency (MF) of the solvent control was between 0.2 and 1.0 mutant colonies/10^4 survivors; and (3) the MF of the positive controls was ≥2-fold higher than the corresponding solvent control value.

(b) Positive response: The test material was considered positive if it induced a dose-related increase in the MF that exceeded 2 times the MF of the solvent control at one or more doses with ≥10% total survival.

4. Protocol: See Appendix B.

C. REPORTED RESULTS:

1. Cytotoxicity Test: No cells survived treatment with any nonactivated dose (0.001 to 10 µl/mL). In the presence of S9 activation, the three highest levels (0.1, 1, and 10 µL/mL) were completely cytotoxic. Severe cytotoxicity (8% relative growth) was also seen at 0.01 µL/mL.

Below this level, survival was ≥85%. Based on this information, the initial nonactivated mutation assay was conducted with ten doses (0.038 to 0.5 nL/mL); 12 doses (0.89 to 21 nL/mL) were evaluated in the initial S9-activated mutation test.

2. Mutation Assay:

(a) Nonactivated conditions: In the initial nonactivated trial, relative suspension growth (RSG) and relative total growth (RTG) were 18% and 19%, respectively, for cultures tested with the highest assayed level (0.5 nL/mL). For the remaining nonactivated concentrations, cell survival was dose-related; RSG ranged from 21% at 0.38 nL/mL to 96% at 0.038 nL/mL and RTG ranged from 20% to 89% at comparable test material levels. All doses were plated for mutant colonies and survivors. Results indicated that chloropicrin was not mutagenic. To achieve a more pronounced cytotoxic effect, the maximum concentration for the confirmatory assay was increased to 0.75 nL/mL. Approximately 17% of the cells were viable immediately following treatment with 0.75 nL/mL chloropicrin. After the expression period, 93% of the treated cells were recovered; RTG was, therefore, 16%. RSG for the remaining doses was ≤25% and RTG was ≤22%. In agreement with the initial findings, chloropicrin was not mutagenic at any level. Representative results from the initial and confirmatory nonactivated assays are shown in Table 1.

(b) S9-activated conditions: The 12 doses evaluated in the initial trial ranged from 0.89 to 21 nL/mL. No cells survived treatment with the two highest doses (16 and 21 nL/mL). At 12 nL/mL, RSG was 21%; RSG for the remaining levels (0.89 to 8.9 nL/mL) was ≥55%. Representative results presented in Table 2 indicated that the highest plated concentration (12 nL/mL) did not cause an appreciable increase in either the number of mutant colonies or the MF. Similar results were observed at lower levels.

The high dose selected for the confirmatory assay was 16 nL/mL. In contrast to the earlier findings showing that 16 nL/mL was completely cytotoxic, RSG was 24% at this level in the confirmatory assay. However, in agreement with the earlier findings, none of the cloned doses (9, 10, 12, 14, or 16 nL/mL) induced a mutagenic effect.

By contrast to the test material results, the nonactivated positive control (0.25 and 0.5 µl/mL EMS) and the S9-activated positive control (2.5 and 5 µg/mL DMBA) induced dose-related increases in the MF.

From the overall results, the study authors concluded that chloropicrin technical was not mutagenic in the mouse lymphoma forward mutation assay.

TABLE 1. Representative Results of the Nonactivated Mouse Lymphoma Forward Mutation Assays with Chloropicrin Technical

Substance	Dose	Average Percent Relative Growth	Average Mutant Colonies[a]	Average Viable Colonies[a]	Average Percent Relative Cloning Efficiency	Average Percent Relative Total Growth	Average Mutation Frequency per 10^6 Survivors[b]
Solvent Control							
Ethanol	1%	100[c]	30	153	100	100	39
(Test material)	1%	100[d]	15	161	100	100	18
Dimethyl sulfoxide	1%	100[c]	29	140	100	100	43
(Positive control)	1%	100[d]	21	170	100	100	25
Positive Control[e]							
Ethyl methanesulfonate	0.25 µL/mL	81[c]	197±16	104±6	75	61	379
	0.25 µL/mL	77[d]	181±4	129±12	76	59	281
Test Material							
Chloropicrin	0.5 nL/mL[f]	18[c]	24±5	160±12	105	19	30
	0.75 nL/mL[f]	18[d]	15	150	93	16	20

[a]Means and standard deviations from the counts of triplicate plates per culture were presented. Results from duplicate solvent control cultures (both assays) and treated cultures in the confirmatory assay (3 plates/culture) were combined by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Mean Mutant Colonies}}{\text{Mean Viable Colonies}} \times 2 \times 100.$

[c]Results from the initial assay.
[d]Results from the confirmatory assay.
[e]Two levels of the positive control were assayed; results from the lower dose were selected as representative.
[f]Results for lower doses (0.038, 0.05, 0.067, 0.089, 0.12, 0.16, 0.21, 0.28, and 0.38 nL/mL in the initial test and 0.36, 0.46, 0.56, and 0.65 nL/mL in the confirmatory assay) did not suggest a mutagenic effect.

TABLE 2. Representative Results of the S9-Activated Mouse Lymphoma Forward Mutation Assays with Chloropicrin Technical

Substance	Dose	Average Percent Relative Growth	Average Mutant Colonies[a]	Average Viable Colonies[a]	Average Percent Relative Cloning Efficiency	Average Percent Relative Total Growth	Average Mutation Frequency x 10^6 Survivors[b]
Solvent Control							
Ethanol	1%	100[c]	28	160	100	100	36
(Test material)	1%	100[d]	23	172	100	100	27
Dimethyl sulfoxide	1%	100[c]	26	158	100	100	33
(Positive control)	1%	100[d]	18	160	100	100	23
Positive Control[e]							
7,12-Dimethylbenz(a)anthracene	2.5 µg/mL	81[c]	103±8	121±7	77	62	170
	2.5 µg/mL	77[d]	62±4	149±7	93	73	83
Test Material							
Chloropicrin	12 nL/mL[f,g]	21[c]	34±4	147±9	92	19	46
	16 nL/mL[f]	24[d]	26	163	95	23	31

[a]Means and standard deviations from the counts of triplicate plates per culture were presented. Results from duplicate solvent control cultures (both assays) and treated cultures in the confirmatory assay (3 plates/culture) were combined by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Mean Mutant Colonies}}{\text{Mean Viable Colonies}} \times 2 \times 100.$

[c]Results from the initial assay.
[d]Results from the confirmatory assay.
[e]Two levels of the positive control were assayed; results from the lower dose were selected as representative.
[f]Results for lower doses (0.89, 1.2, 1.6, 2.1, 2.8, 3.8, 5.0, 6.7, and 8.9 nL/mL in the initial test and 9, 10, 12, and 14 nL/mL in the confirmatory assay) did not suggest a mutagenic response.
[g]Higher doses (16 and 21 nL/mL) were severely cytotoxic.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess, in agreement with the study authors, that chloropicrin technical was not mutagenic in this test system. The lack of agreement between the preliminary assays and the mutational assays with respect to cytotoxicity may be related to test material volatility (see Data Evaluation Record 91-104) as well as the technical problems associated with the accurate preparation of low test concentrations within a narrow dose range. Nevertheless, in all trials, chloropicrin was assayed to levels that were clearly cytotoxic but failed to induce a mutagenic response. In addition, the sensitivity of the test system in detecting mutagenesis was adequately demonstrated by the response of the target cell to the nonactivated and S9-activated positive controls.

We conclude, therefore, that the study provided acceptable evidence that chloropicrin technical was not mutagenic in this cultured mammalian cell system.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement was signed and dated April 27, 1990).

F. CBI APPENDICES: Appendix A, Materials and Methods, CBI pp. 8-12; Appendix B, Protocol, CBI pp. 29-44.

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APPENDIX A

MATERIALS AND METHODS
CBI pp. 8-12

RIN 3550-99

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_____ The product confidential statement of formula.

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APPENDIX B

PROTOCOL
CBI pp. 29-44

RIN 3550-99

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_____ Description of quality control procedures.

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_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

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The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Chloropicrin

Study Type: Mutagenicity: Salmonella typhimurium/Mammalian Microsome Mutagenicity Assay

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 Date 5/28/92
Lynne T. Haber, Ph.D.

Independent Reviewer Nancy E. McCarroll Date 5-28-92
Nancy E. McCarroll, B.S.

QA/QC Manager Sharon Segal Date 5/28/92
Sharon Segal, Ph.D.

Contract Number: 68D10075
Work Assignment Number: 1-26
Clement Number: 91-101
Project Officer: James Scott

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MUTAGENICITY STUDIES

EPA Reviewer: Stanley Gross, Ph.D. Signature: Stanley Gross
Review Section II, Toxicology Branch (I)/HED Date: 7/11/91 = For Mkmm
EPA Acting Section Head: Joycelyn Stewart, Ph.D. Signature: _____
Review Section II, Toxicology Branch (I)/HED Date: _____

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Salmonella typhimurium/mammalian microsome mutagenicity assay

EPA IDENTIFICATION Numbers:

Caswell Number: 214

MRID Number: 419608-01

TEST MATERIAL: Chloropicrin technical

SYNONYMS: None listed.

SPONSOR: Niklor Chemical Co., Inc., Long Beach, CA

STUDY NUMBER: T9152.501014

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD

TITLE OF REPORT: Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test) with a Confirmatory Assay

AUTHORS: R. H. C. San and V. O. Wagner, III

REPORT ISSUED: June 21, 1990

CONCLUSIONS--EXECUTIVE SUMMARY: Chloropicrin was tested in two independently performed Salmonella typhimurium/mammalian microsome plate incorporation assays, at doses ranging from 10 to 3333 µg/plate. In S. typhimurium strain TA98, a dose-dependent positive response was observed ranging from a 1.6 to a 4.1-fold increase in mutant colonies, at S9-activated levels of 33 to 500 µg/plate, respectively. This response was unusual since it was not accompanied by a corresponding mutagenic effect in TA1538. Based on dose-dependent and reproducible increases in histidine (his^+) revertants, our reviewers also concluded, in contrast to the study authors, that S9-activated chloropicrin was mutagenic in TA100. Increases in his^+ colonies were also observed in strains TA98 and TA100 (-S9) and TA1535 (+S9); however, the results were either not dose-dependent or not reproducible. Our reviewers attribute the inconsistencies in the reported mutagenicity data to compound volatility (see

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Section D, Reviewers' Discussion/Conclusions). No information was provided in this study regarding the volatile nature of chloropicrin. However, the study author of the unscheduled DNA synthesis (UDS) assay, which was conducted by the same laboratory, noted that literature provided by the sponsor listed chloropicrin as a volatile compound (see Data Evaluation Record 91-104).

We assess, therefore, that the mutagenic potential of chloropicrin was not fully characterized by the procedures used in this study. However, there is sufficient evidence to conclude that S9-activated chloropicrin is mutagenic in S. typhimurium TA98 and TA100. The study, therefore, satisfies Guideline requirements for genetic effects Category I, Gene Mutations.

STUDY CLASSIFICATION: The study is acceptable.

A. MATERIALS:

1. Test Material: Chloropicrin technical

Description: Clear liquid
Lot number: 987
Purity: 99.5%
Receipt date: February 15, 1990
Stability: Not reported
Contaminants: None listed
Solvent used: Reported by the authors as 100% ethanol (EtOH). Presumably it was 95% ethanol, the purest form generally used in routine testing procedures.
Other provided information: The test material was stored at room temperature and diluted immediately before use.

2. Control Materials:

Solvent/final concentration: EtOH/50µl per plate

Positive:

Nonactivation:

Sodium azide	1	µg/plate TA100, TA1535
2-Nitrofluorene	1	µg/plate TA98, TA1538
ICR 191	2	µg/plate TA1537

Activation:

2-Aminoanthracene (2-AA)	0.5	µg/plate all strains

3. Activation: S9 derived from male Sprague-Dawley

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

The rat liver S9 was prepared by Microbiological Associates, Inc. Prior to use, it was characterized for its ability to convert 7,12-dimethylbenzanthracene and 2-AA to mutagenic forms.
S9 mix composition:

Component	Volume/mL
H_2O	0.56 mL
1.00 M NaH_2PO_4/K_2PO_4 (pH 7.4)	0.10 mL
0.05 M Glucose 6-phosphate	0.10 mL
0.04 M NADP	0.10 mL
0.2 M $MgCl_2$/0.825 M KCl	0.04 mL
S9	0.10 mL (10% final)

4. Test Organism Used: S. typhimurium strains
_____ TA97 __x__ TA98 __x__ TA100 _____ TA102 _____ TA104
__x__ TA1535 __x__ TA1537 __x__ TA1538
list any others:

Test organisms were properly maintained: Yes.
Checked for appropriate genetic markers (rfa mutation, R factor): Yes.

5. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Ten doses (10, 33, 67, 100, 333, 667, 1000, 3333, 6667, and 10,000 µg/plate) were evaluated with and without S9 activation in S. typhimurium strain TA100. Single plates were used per dose, per condition.

(b) Mutation assays:

(1) Initial: Five doses (10, 33, 100, 333, and 1000 µg/plate in the absence of S9 activation; and 33, 100, 333, 1000, and 3333 µg/plate in the presence of S9) were evaluated with all tester strains.

(2) Confirmatory: Five doses were tested with each strain in the absence and the presence of S9, as follows:

- Nonactivated conditions:
  Strain TA98--10, 33, 100, 333, and 500 µg/plate
  Strains TA100 and TA1538--10, 33, 100, 200, and 333 µg/plate
  Strain TA1535--10, 20, 33, 50, and 100 µg/plate
  Strain TA1537--10, 20, 33, 100, and 200 µg/plate

- S9-activated conditions:
  All strains--10, 33, 100, 333, and 500 µg/plate

(3) Selected retest: TA1535 and TA1537 were retested at seven doses in the absence of S9. For TA1535 these doses were 10, 20, 33, 50, 100, 333, and 1000 µg/plate; for TA1537 they were 10, 20, 33, 100, 200, 333, and 1000 µg/plate.

B. TEST PERFORMANCE:

1. Type of Salmonella Assay:
   - __x__ Standard plate test
   - _____ Pre-incubation (_____) minutes
   - _____ "Prival" modification
   - _____ Spot test
   - _____ Other (describe)

2. Method:

   (a) Preliminary cytotoxicity/mutation assays: Similar procedures were used for the preliminary cytotoxicity and the mutation assays.

   Approximately 1-2 x 10^8 cells (0.1 mL of a 1-2 x 10^9 cells/mL late log phase culture) of the appropriate tester strain and 50 µL of the appropriate test material dose, solvent, or positive controls were added to tubes containing 2.5-mL volumes of molten top agar. Sufficient water was added to the top agar in the nonactivated tests to ensure that equivalent concentrations of amino acid supplements were available in the nonactivated and S9-activated tests. For the S9-activated tests, 0.5 mL of the appropriate S9-cofactor mix was added to 2 mL of the top agar; tester strains and test and control solutions were added as described. The contents of the tubes were mixed, poured over Vogel-Bonner minimal medium E, and incubated at 37°C for ≈48 hours. At the end of incubation, plates were either scored immediately for revertant colonies or were refrigerated and subsequently counted. Means and standard deviations for the mutation test were determined from the counts of triplicate plates per strain, per dose, per condition.

   (b) Sterility controls: The sterility of the highest test dose and the S9 mix were determined.

3. Evaluation Criteria:

   (a) Assay validity: The assay was considered valid if it met the following criteria: (1) the presence of the appropriate genetic markers must be verified; (2) tester strain culture titers must be ≥0.6 x 10^9; (3) positive control values must show at least a tripling in the mean number of revertants for each strain; and (4) the test compound should be tested to 10 mg/plate, or at least one dose showing cytotoxicity. In addition, the spontaneous revertants for each strain should fall into the following range: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21; and TA1538, 5-35.

   (b) Positive response: The test material was considered positive if it caused a dose-related increase in the mean number of revertants per plate of at least one strain. This increase must be at least two-fold in strains TA98 and TA100, and at least three-fold in strains TA1535, TA1537, and TA1538.

4. Protocol: See Appendix B.

C. REPORTED RESULTS

1. Preliminary Cytotoxicity Assay: Ten doses of the test material ranging from 10 to 10,000 µg/plate were evaluated with and without S9 activation using strain TA100. No compound precipitation was apparent at any of the nonactivated or S9-activated doses. No revertants were observed at the three highest doses with or without S9 activation (3333, 6667, and 10,000 µg/plate). In the absence of S9 activation, the mean number of revertants was ≈40% lower than the control at 667 µg/plate and ≈90% reduced at 1000 µg/plate. An increase in the number of revertant colonies was seen at all noncytotoxic S9-activated doses of the test material (10 to 1000 µg/plate). At the high dose, mutant colonies were ≈1.5-fold higher than the solvent control. Based on these findings, the dose range selected for the nonactivated mutation assay was 10 to 1000 µg/plate, and the range selected for the S9-activated mutation assay was 33 to 3333 µg/plate.

2. Mutation Assay: Due to unacceptable vehicle control values, the initial assay was repeated with strains TA98, TA100, TA1535, and TA1537 (+S9), and with TA100 (-S9). Representative results from the successfully completed nonactivated and S9-activated mutation assays with chloropicrin are presented in Tables 1 and 2. The number of revertants was markedly reduced in all strains at the highest nonactivated dose (1000 µg/plate) and at the two highest S9-activated doses (1000 and 3333 µg/plate). Positive responses were observed in the initial assay with strains TA1535 and TA1537 at 33, 100, and 333 µg/plate -S9. Maximum fold increases occurred at 33 µg/plate for strain TA1535 and at 100 µg/plate for strain TA1538. These results were not replicated in the confirmatory assay with either strain. To resolve the conflicting results, TA1535 and TA1537 were retested over an expanded range; no evidence of mutagenicity was found (Table 3). Chloropicrin was also not mutagenic in strain TA1538 in either the initial or confirmatory test.

   By contrast, results from the initial assay showed that a 1.7-fold increase in histidine revertants (his^+) of strain TA98 accompanied exposure to 100 and 333 µg/plate -S9. S9-activated doses of 33, 100, and 333 µg/plate induced 1.6-, 1.8-, and 1.9-fold increases in his^+ colonies, respectively. The mutagenic effect was reproduced in the confirmatory S9-activated test with this strain, as indicated by the dose-related positive response obtained at four concentrations (33 to 500 µg/plate); increases in his^+ colonies ranged from 1.6-fold at 33 µg/plate to 4.1-fold at 500 µg/plate (Table 2). Chloropicrin was not mutagenic in S. typhimurium TA100 under nonactivated conditions. However, in the presence of S9 activation persistent increases approaching 2-fold were observed at 33, 100, and 333 µg/plate (initial test) and at 100, 333, and 500 µg/plate (confirmatory test). Our reviewers noted that these findings were consistent with the increased number of his^+ colonies reported for the S9-activated cytotoxicity test with strain TA100. The study authors did not, however, consider the response induced in strain TA100 to be indicative of mutagenesis because the increases were <2-fold. Based on the overall results, the study

TABLE 1: Representative Results of the Initial Salmonella typhimurium/Mammalian Microsome Mutation Assay with Chloropicrin

Substance	Dose/Plate	S9 Activation	Revertants per Plate of Bacterial Tester Strain[a] TA1535	FI[b]	TA1537	FI[b]	TA1538	FI[b]	TA98	FI[b]	TA100	FI[b]
Solvent Control												
Ethanol (100%)	50 µL	-	15±2	--	6±1	--	7±1	--	19±4	--	124±23[c]	--
	50 µL	+	12±3[c]	--	5±0[c]	--	9±6	--	45±15[c]	--	166±10[c]	--
Positive Controls												
Sodium azide	1 µg	-	937±108	62.5	--	--	--	--	--	--	420±22[c]	3.4
ICR-191	2 µg	-	--	--	96±28	16.0	--	--	--	--	--	--
2-Nitrofluorene	1 µg	-	--	--	--	--	289±19	41.3	171±14	9.0	--	--
2-Aminoanthracene	0.5 µg	+	74±6[c]	6.2	67±10[c]	13.4	1015±26	112.8	593±52[c]	13.2	773±17[c]	4.6
Test Material												
Chloropicrin	10 µg	-	47±33	3.1	4±1	0.7	7±4	1.0	24±3	1.3	96±6[c]	0.8
	33 µg	-	393±36	26.2	271±284	54.2	5±2	0.7	24±3	1.3	131±4	1.0
	100 µg	-	302±26	20.1	528±184	88.0	5±1	0.7	33±11	1.7	162±26	1.3
	333 µg[d]	-	269±51	17.9	203±73	33.8	4±2	0.6	33±26	1.7	64±23	0.5
	33 µg	+	16±1[c]	1.3	5±1[c]	1.0	14±9	1.6	73±8[c]	1.6	256±29[c]	1.5
	100 µg	+	24±3	2.0	5±5	1.0	11±2	1.2	80±16	1.8	275±9	1.6
	333 µg[d]	+	22±2	1.8	7±2	1.4	11±3	1.2	87±7	1.9	267±33	1.6

[a]Means and standard deviations of the counts from triplicate plates.

[b]FI (Fold Increase) = $\frac{\text{Revertant colonies in test groups}}{\text{Revertant colonies in the solvent control group}}$

[c]Results from repeat assay; the first trial with these strains was terminated owing to unacceptable solvent control revertant frequencies.

[d]Results for higher doses (1000 µg/plate -S9 and 1000 and 3333 µg/plate +S9) were severely cytotoxic.

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authors concluded that chloropicrin was positive in strain TA98 in the presence of exogenous microsomal enzymes.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that the study authors' conclusion that chloropicrin induced a dose-related increase in his^+ revertants of strain TA98 in the presence of rat liver microsomes was correct. However, we conclude that S9-activated chloropicrin was also mutagenic in strain TA100. Although TA100 was less responsive to the mutagenic action of chloropicrin than TA98, the dose-dependency and reproducibility of the data provided convincing evidence that the increased number of his^+ colonies seen with TA100 was the result of treatment. The study findings showing that chloropicrin was negative in strain TA1538 were of interest. Since it is generally accepted that strains TA1538 and TA98 respond to the same mutagens, many investigators have dropped TA1538 from the test battery. Therefore, the positive result with strain TA98 without a corresponding genotoxic response in strain TA1538 was an unusual finding.

In addition, the overall data tend to suggest that chloropicrin may contain volatile component(s). We base our assessment on the sporatic nature of the cytotoxicity results with strain TA100. For example, 333 µg/plate -S9 was not cytotoxic in the preliminary assay, severely cytotoxic in the first mutation assay, and not cytotoxic in the confirmatory assay. In the presence of S9 activation, 1000 µg/plate caused an increase in revertant colonies in the preliminary assay, but this dose was severely cytotoxic in the first mutation assay; 1000 µg/plate was not evaluated in the final mutation assay. Similarly, the nonreproducibility of the powerful mutagenic responses induced in strains TA1535 and TA1537 is suggestive of compound volatility. Although no information was provided in the microbial assay report indicating that the test material was volatile, the study author of the unscheduled DNA synthesis (UDS) assay noted that literature provided by the sponsor reported that the compound was volatile (see Data Evaluation Record 91-104). It was further noted by our reviewers that attempts were made in the preliminary phases of the UDS assay to contain the volatile test material in a sealed chamber.

Our observations related to the negative response in strain TA1538 and compound volatility are problematic, and additional studies with protocol modifications designed to test volatile substances would be necessary to resolve these issues. Nevertheless, there is sufficient evidence to conclude that S9-activated chloropicrin is mutagenic in S. typhimurium TA98 and TA100.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLP? Yes. (A quality assurance statement was signed and dated June 22, 1990.)

F. CBI APPENDICES: Appendix A, Materials and Methods, CBI pp. 9-16; and Appendix B, Protocol CBI pp. 56-65.

L/1

TABLE 2: Representative Results of the Confirmatory Salmonella typhimurium/Mammalian Microsome Mutation Assay with Chloropicrin

Substance	Dose/Plate	S9 Activation	Revertants per Plate of Bacterial Tester Strain[a] TA1535	FI[b]	TA1537	FI[b]	TA1538	FI[b]	TA98	FI[b]	TA100	FI[b]
Solvent Control												
Ethanol (100%)	50 µL	-	12±3	--	7±1	--	8±3	--	26±3	--	133±13	--
	50 µL	+	14±2	--	8±2	--	13±3	--	26±4	--	166±17	--
Positive Controls												
Sodium azide	1 µg	-	353±38	29.4	--	--	--	--	--	--	485±21	3.6
ICR-191	2 µg	-	--	--	138±10	19.6	--	--	--	--	--	--
2-Nitrofluorene	1 µg	-	--	--	--	--	305±29	38.1	190±17	7.3	--	--
2-Aminoanthracene	0.5 µg	+	80±18	5.7	57±7	7.1	869±10	66.8	725±10	27.9	909±16	5.5
Test Material												
Chloropicrin	100 µg[c]	-	10±1	0.8	3±2	0.4	6±2	0.8	27±2	1.0	170±14	1.3
	200 µg	-	--	--	6±2	0.8	5±5	0.6	--	--	183±8	1.4
	333 µg	-	--	--	--	--	4±2	0.5	47±4	1.8	220±17	1.6
	500 µg[d]	-	--	--	--	--	--	--	28±7	1.1	--	--
	10 µg	+	13±4	0.9	6±2	0.8	11±1	0.8	37±3	1.0	167±17	1.0
	33 µg	+	14±2	1.0	7±1	0.9	12±1	0.9	41±6	1.6	187±21	1.1
	100 µg	+	17±5	1.2	5±2	0.6	14±2	1.1	70±6	2.7	271±19	1.6
	333 µg	+	21±7	1.5	9±4	1.1	7±2	0.5	94±24	3.6	298±21	1.8
	500 µg[d]	+	9±4	0.6	5±2	0.6	8±7	0.6	106±13	4.1	301±30	1.8

[a]Means and standard deviations of the counts from triplicate plates.

[b]FI (Fold Increase) = $\frac{\text{Revertant colonies in test groups}}{\text{Revertant colonies in the solvent control group}}$

[c]Results for lower doses (10 and 33 µg/plate for TA98, TA100, and TA1538; 10, 20, and 33 µg/plate for TA1537; and 10, 20, 33, and 50 µg/plate for TA1535) without S9 activation did not suggest a mutagenic effect.

[d]Highest assayed level.

TABLE 3: Representative Results of Selective Retesting of Nonactivated Chloropicrin in the Salmonella typhimurium/Mammalian Microsome Mutation Assay

Substance	Dose/Plate	S9 Activation	Revertants per Plate of Bacterial Tester Strain[a] TA1535	FI[b]	TA1537	FI[b]
Solvent Control						
Ethanol (100%)	50 µL	-	9±2	--	7±0	--
Positive Controls						
Sodium azide	1 µg	-	409±10	45.4	--	--
ICR-191	2 µg	-	--	--	193±8	27.6
Test Material						
Chloropicrin	50 µg[c]	-	14±2	1.6	--	--
	100 µg	-	14±5	1.6	7±3	1.0
	200 µg	-	--	--	7±5	1.0
	333 µg[d]	-	18±5	2.0	6±3	0.8

[a]Means and standard deviations of the counts from triplicate plates.

[b]FI (Fold Increase) = $\frac{\text{Revertant colonies in test groups}}{\text{Revertant colonies in the solvent control group}}$.

[c]Results for lower doses (10, 20, and 33 µg/plate) did not suggest a mutagenic effect.
[d]The highest dose (1000 µg/plate) was cytotoxic.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 9-16

RIN 3550-99

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APPENDIX B

PROTOCOL
CBI pp. 56-65

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_____ The product confidential statement of formula.

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FINAL

DATA EVALUATION REPORT

CHLOROPICRIN

Study Type: Mutagenicity: Mammalian Cells in Culture Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells

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 Nancy E. McCarroll Date 6/2/92
Nancy E. McCarroll, B.S.

Independent Reviewer Lynne Haber Date 6/2/92
Lynne Haber, Ph.D.

QA/QC Manager Sharon A. Segal Date 6/2/92
Sharon Segal, Ph.D.

Contract Number: 68D10075
Work Assignment Number: 1-26
Clement Number: 91-102
Project Officer: James Scott

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GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE CYTOGENETICS

EPA Reviewer: Stanley Gross, Ph.D. Signature: Stanley Gross
EPA Review Section (II), Toxicology Date: 7/6/92 C.Jw Manager.
Branch { I }/HED
EPA Acting Section Head: Joycelyn Steward, Ph.D. Signature: _____
EPA Review Section (II), Toxicology Date: _____
Branch { I }/HED

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Mammalian cells in culture cytogenetic assay in Chinese hamster ovary (CHO) cells.

EPA IDENTIFICATION NUMBER: 081501

Caswell Number: 214

MRID Number: 419608-02

TEST MATERIAL: Chloropicrin technical

SYNONYMS: None listed

SPONSOR: Niklor Chemical Co., Long Beach, CA

STUDY NUMBER: T9152-337001

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD.

TITLE OF REPORT: Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells with Confirmatory Assay.

AUTHORS: Putman, D.L. and Morris, M.J.

REPORT ISSUED: May 31, 1990

CONCLUSIONS-EXECUTIVE SUMMARY: Under the conditions of this mammalian cell cytogenetic assay, nonactivated doses of chloropicrin ranging from 0.75 to 1 nL/mL induced a reproducible and significant ($p<0.01$) clastogenic response in Chinese hamster ovary (CHO) cells harvested 12 hours posttreatment; the predominant type of scored aberrations was chromatid breaks. At these levels, the mitotic indices were reduced and cytotoxic effects on monolayer cells were apparent. Higher nonactivated levels (≥1.5 nL/mL) were severely cytotoxic and not scored. In the presence of S9 activation, chloropicrin was less cytotoxic as indicated by the higher range of concentrations that could be evaluated (2 to 6 nL/mL). Although slight but significant ($p<0.05$) increases in the frequency of aberrations were observed at two doses (4 and 6 nL/mL) in the initial trial, these findings were not reproduced in the subsequently performed assay. We concluded, therefore, that nonactivated chloropicrin was

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clastogenic over a narrow range of cytotoxic concentrations. The study satisfies Guideline requirements for genetic effects, Category II, Structural Chromosome Aberrations.

STUDY CLASSIFICATION: The study is acceptable; Chloropicrin in the absence of S9 activation is a clastogen in this mammalian test system.

A. MATERIALS:

1. Test Material: Chloropicrin technical

Description: Clear colorless liquid
Identification No.: Lot no. 987
Purity: 99.5%
Receipt date: February 15, 1990
Stability: Not reported
Contaminants: None listed
Solvent used: Ethanol (ETOH)
Other provide information: The test material was stored at room temperature, protected from light. The frequency of test solution preparation was not reported. The osmolality of the highest test concentration (5 µL/mL) was determined and was found to be 385 mOsm/Kg.

2. Control Materials:

Negative: Untreated cells grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and antibiotics.

Solvent/final concentration: ETOH/1%

Positive:
Nonactivation: Triethylenemelamine (TEM) was prepared in distilled water to yield a final concentration of 0.5 µg/mL.

Activation: Cyclophosphamide (CP) was prepared in distilled water to yield a final concentration of 50 µg/mL.

3. Activation: S9 derived from male Sprague-Dawley

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. Prior to use, the ability of the fraction to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to mutagenic forms was assessed in Salmonella typhimurium strain TA100.

S9 mix composition: The S9 mix contained 15 µL S9, 1.4 mg NADP, and 2.7 mg isocitric acid per mL of McCoy's medium 5A containing 2.5% FBS.

4. Test Compound Concentration Used:

(a) Preliminary cytotoxicity assay: The nine concentrations of the test material used were 0.000005, 0.000015, 0.00005, 0.00015, 0.0005, 0.005, 0.05, 0.5, and 5 µL/mL (0.005 to 5000 nL/mL) with and without S9 activation.

(b) Cytogenetic assay:

Nonactivated conditions: Three nonactivated trials were performed; doses tested were:

- Trial 1 - 0.0002, 0.0004, 0.0008, 0.0015, and 0.003 µL/mL (0.2 to 3 nL/mL).
- Trial 2 - 0.0005, 0.00075, 0.001, 0.0015, and 0.002 µL/mL (0.5 to 2 nL/mL).
- Trial 3 - 0.0004, 0.0006, 0.0008, and 0.001 µL/mL (0.4 to 1 nL/mL).

Activated conditions: Two S9-activated trials were performed with the following doses:

- Initial trial - 0.002, 0.003, 0.004, 0.005, and 0.006 µL/mL (2 to 6 nL/mL).
- Confirmatory trial - As above

NOTE: The first attempted S9-activated assay (dose range: 0.2 to 3 nL/mL) was terminated because the test material was not cytotoxic at any level.

5. Test Cells: The Chinese hamster ovary cells (CHO-K_1) used in this assay were obtained from the American Type Culture Collection, Rockville, MD. Prior to use, the CHO cells were grown for 16 to 24 hours in McCoy's 5A complete medium.

Properly maintained? Yes.

Cell line or strain periodically checked for mycoplasma contamination? Not reported.

Cell line or strain periodically check for karyotype stability? Not reported.

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B. TEST PERFORMANCE:

1. Cell Treatment:

(a) Cells exposed to test compound for:
__10__ hours (nonactivated) __2__ hours (activated)

(b) Cells exposed to positive controls for:
__10__ hours (nonactivated) __2__ hours (activated)

(c) Cells exposed to negative and/or solvent controls for:
__10__ hours (nonactivated) __2__ hours (activated)

2. Protocol:

(a) Preliminary cytotoxicity assay: Prepared cultures, seeded at $5x10^5$ cells/flask, were exposed with or without S9 activation to nine concentrations of the test material (0.005 to 5000 nL/mL) or the solvent control (ETOH).

In the nonactivated system, cells were exposed for 6 hours to the test material; 50 µL of 1 mM BrdU were added to the cultures 2 hours after initiation. After exposure, cells were washed, refed with complete medium containing BrdU (0.01 mM), and reincubated for a total of 24 hours.

In the S9-activated system, cultures were treated for 2 hours. Cells were washed, refed and reincubated in the presence of 0.01 mM BrdU as described for the nonactivated phase of testing.

Two hours prior to the end of incubation, colcemid, at a final concentration of 0.1 µg/mL, was added to each culture. Metaphases were harvested, fixed, and stained with the modified fluorescent-plus-Giemsa technique of Perry and Wolff.[1] One hundred cells from each dose group were examined for the percentage of first (M_1), second (M_2), and third (M_3) division metaphases. Mitotic indices were determined by counting the number of mitotic cells in a population of 500 scored cells. Based on these results, the doses and harvest time for the cytogenetic assay were established.

(b) Cytogenetic assay:

(1) Treatment: Prepared cultures (in duplicate), seeded at $5x10^5$ cells, were exposed to the selected test material doses, the negative control (culture medium), the solvent control (ETOH), or the positive controls (0.5 µg/mL TEM -S9 and 50 µg/mL CP +S9).

[1]Perry, P., and Wolff, S. (1974). New Giemsa method for the differential staining of sister chromatids. Nature 251:156-158.

In the nonactivated assay, cells were dosed for 10 hours. Cultures were washed, refed medium containing 0.1 µg/mL colcemid, and reincubated for 2 hours. Under S9-activated conditions, cells were exposed for 2 hours, washed, refed culture medium, and incubated for an additional 8 hours. Colcemid was added 2 hours before the cultures were harvested.

Metaphase cells were collected and fixed. Slides were stained with 5% Giemsa and coded.

(2) Metaphase analysis: One hundred metaphase cells per group (50/culture) were scored for chromosome aberrations. Chromatid and chromosome gaps were counted but not included in the final analysis. Mitotic indices were calculated.

(3) Statistical methods: The data were evaluated for statistical significance at p values of 0.05 and 0.01 by the Fisher's exact test.

3. Evaluation Criteria:

(a) Assay validity: The assay was considered valid if the percent of cells with aberrations in the untreated control did not exceed 6% and the number of cells with aberrations in the positive control group was significantly higher ($p \leq 0.05$) than the solvent control.

(b) Positive response: The test material was considered positive if it caused a significant and dose-related increase in the percentage of cells with aberrations relative to the solvent control.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Doses ≥5 nL/mL +/-S9 were severely cytotoxic; MIs for these treatment groups showed a ≥95% reduction compared to the appropriate control groups. For the remaining levels, there was no appreciable decrease in the MIs or clear evidence of adverse effects on cell cycle kinetics (Table 1). Based on these results, the cytogenetic assay was initiated with a nonactivated and S9-activated concentration range of 0.2 to 3 nL/mL.

2. Cytogenetic Assay:

(a) Nonactivated conditions: The study authors stated that cytotoxic effects on the monolayers were apparent in the initial assay at 1.5 to 3 nL/mL. No metaphase plates were available from the high-dose cultures. As shown in Table 2, the reduced MI at 1.5 nL/mL supported the microscopic evidence of cytotoxicity. None of the evaluated doses caused a significant increase in the percentage of cells with aberrations or the frequency of

TABLE 1. Representative Results from the Preliminary Cytotoxicity Assay with Chloropicrin

Substance	Dose/mL	S9-activation	% Cells[a] M_1	% Cells[a] M_2	% Cells[a] M_3	Mitotic Index[b] (%)
Solvent Control						
Ethanol	1%	-	3	97	0	4.0
		+	3	95	2	7.0
Test Material						
Chloropicrin	0.15 nL/mL[c]	-	7	93	0	4.2
	0.5 nL/mL[d]	-	18	82	0	4.4
	0.15 nL/mL[c]	+	5	95	0	5.8
	0.5 nL/mL[d]	+	2	98	0	6.0

[a]Percent cells in first (M_1), second (M_2), or third (M_3) division.
[b]Percent of metaphase cells per 500 scored cells.
[c]Lower doses (0.005, 0.015, and 0.05 nL/mL +/-S9) had no appreciable cytotoxic effects.
[d]Higher doses (5, 50, 500, and 5000 nL/mL +/-S9) were severely cytotoxic.

TABLE 2. Representative Results of the CHO Cell In Vitro Nonactivated Cytogenetic Assays with Chloropicrin Following a 12-Hour Cell Harvest

Substance	Dose/mL	No. of Cells Scored	Mitotic Index (%)	Cells with Aberrations[a] (%)	Aberrations per Cell ± Standard Deviations[b]	Biologically Significant Aberrations (No/Type)[b]
Negative Control						
Untreated cells	--	100[c]	6.4	0	0.000±0.000	--
	--	100[d]	5.7	3	0.030±0.171	2TB; 1D
	--	100[e]	5.3	1	0.010±0.100	1SB
Solvent Control						
Ethanol	1%	100[c]	4.0	2	0.020±0.141	1TB; 1SB
	1%	100[d]	5.0	0	0.000±0.000	--
	1%	100[e]	3.6	1	0.010±0.100	1 SB
Positive Control						
Triethylenemelamine	0.5 µg/mL	100[c]	2.7	22*	0.230±0.446	18TB; 3TE; 2SB
	0.5 µg/mL	100[d]	3.2	17*	0.180±0.411	13TB; 3TE; 2SB
	0.5 µg/mL	100[e]	1.6	27*	0.390±1.100	21TB; 4TE; 4SB; 1SD
Test Material						
Chloropicrin	1.5 nL/mL	100[c,f,g]	2.6	2	0.020±0.141	2TB
	0.5 nL/mL	100[d]	5.4	1	0.010±0.100	1TB
	0.75 nL/mL	100	1.8	13*	0.140±0.377	9TB; 3TE; 2SB
	1 nL/mL	100[g]	0.4	12*	0.150±0.458	12TB; 1SB; 1D; 1R
	0.4 nL/mL	100[e]	4.5	3	0.030±0.171	1TB; 1SB; 1D
	0.6 nL/mL	100	1.8	4	0.050±0.261	4TB; 1TE
	0.8 nL/mL	100	1.1	10*	0.130±0.442	8TB; 4SB; 1R
	1 nL/mL	100	0.4	10*	0.100±0.302	7TB; 1TE; 1SB;1D

[a]Gaps excluded.
[b]Abbreviations used:

TB = Chromatid break SB = Chromosome break R = Ring
TE = Chromatid exchange D = Dicentric chromosome SD = Severely damaged cell (≥10 aberrations) counted as one aberration

[c]Results from trial 1.
[d]Results from trial 2.
[e]Results from trial 3.
[f]Findings for lower doses (0.2, 0.4, and 0.8 nL/mL) were negative.
[g]Higher concentrations (3 and 1.5 nL/mL in trials 1 and 2, respectively) were severely cytotoxic.

*Significantly higher than the solvent control ($p \leq 0.01$) by Fisher's exact test.

aberrations per cell. Lower doses were tested in the second trial (0.5 to 1.5 nL/mL). Owing to severe cytotoxicity, the high dose was not scored. Adverse effects on monolayer cells and reduced MIs compared to control were observed at levels ≥0.75 nL/mL. However, as further shown, significant increases ($p<0.01$) in the percentage of cells with aberrations were scored in cultures exposed to 0.75 and 1 nL/mL. The predominant type of scored aberrations was chromatid breaks. Based on this information, a third nonactivated trial was conducted with 0.4, 0.6, 0.8, and 1 nL/mL. Results from this trial, which are also presented in Table 2, confirmed the earlier results indicating that chloropicrin was clastogenic at cytotoxic doses.

(b) S9-activated conditions: The first S9-activated assay, conducted with 0.2 to 3 nL/mL, was discontinued because the test material failed to produce a cytotoxic response at these levels. Accordingly, higher doses (2, 3, 4, 5, and 6 nL/mL) were evaluated in the repeat study. Treated monolayers exposed to all levels showed slight to moderate cytotoxic effects and the MI at the high dose was reduced compared to the control. Slight increases in the percentage of cells with aberrations and aberrations per cell were scored at 4 and 6 nL/mL; the percentage of cells with aberrations was significant ($p\leq0.05$) at both levels. The data suggesting a clastogenic response was, however, not reproduced in the confirmatory trial with a concentration range of 2 to 6 nL/mL chloropicrin. Representative results from the two S9-activated assays are presented in Table 3.

Based on the overall findings, the study authors concluded that chloropicrin technical was positive in the nonactivated test but negative in the S9-activated phase of the CHO cytogenetic assay.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We agree with the study authors' conclusion that nonactivated chloropicrin was clastogenic in this mammalian cell cytogenetic assay. We further assess that the clastogenic activity of chloropicrin was confined to a narrow range of cytotoxic concentrations (0.75 to 1 nL/mL); higher levels (≥1.5 nL/mL) were severely cytotoxic. Slight but significant increases in the percentage of cells with aberrations were seen at two doses in the initial S9-activated assay; however, the confirmatory assay was negative. We conclude, therefore, that nonactivated chloropicrin is a clastogen in this mammalian cell test system.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement was signed and dated May 31, 1990).

F. CBI APPENDICES: Appendix A, Materials and Methods, CBI pp 7-10; Appendix B, Protocol, CBI pp 24-30.

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TABLE 3. Representative Results of the CHO Cell In Vitro S9-Activated Cytogenetic Assays with Chloropicrin Following a 12-Hour Cell Harvest

Substance	Dose/mL	No. of Cells Scored	Mitotic Index (%)	Cells with Aberrations[a] (%)	Aberrations per Cell ± Standard Deviations[a]	Biologically Significant Aberrations (No/Type)[b]
Negative Control						
Untreated cells	--	100[c]	8.6	0	0.000±0.000	--
	--	100[d]	8.1	2	0.020±0.141	1TB; 1D
Solvent Control						
Ethanol	1%	100[c]	7.7	0	0.000±0.000	--
	1%	100[d]	8.6	1	0.010±0.100	1TB
Positive Control						
Cyclophosphamide	50 µg/mL	100[c]	4.2	16**	0.190±0.486	12TB; 3TE; 4SB
	50 µg/mL	100[d]	3.0	12**	0.130±0.367	10TB; 2TE; 1SB
Test Material						
Chloropicrin	3 nL/mL[e]	100[c]	8.9	2	0.020±0.141	1TB; 1TE
	4 nL/mL	100	7.1	5*	0.050±0.219	4TB; 1D
	5 nL/mL	100	5.3	2	0.020±0.141	2TB
	6 nL/mL	100	1.4	5*	0.060±0.278	2TB; 2TE; 2D
	4 nL/mL[f]	100[d]	7.4	2	0.020±0.141	1TB; 1TE
	5 nL/mL[g]	100	2.0	4	0.050±0.261	5TB

[a]Gaps excluded.
[b]Abbreviations used:

TB = Chromatid break SB = Chromosome break
TE = Chromatid exchange D = Dicentric chromosome

[c]Results from the initial trial.
[d]Results from the confirmatory trial.
[e]Lowest evaluated dose (2 nL/mL) was not scored.
[f]Results for lower doses (2 and 3 nL/mL) did not suggest a clastogenic effect.
[g]The highest concentration (6 nL/mL) was severely cytotoxic.

*Significantly higher than the solvent control ($p \leq 0.05$) by Fisher's exact test.
**Significantly higher than the solvent control ($p \leq 0.01$) by Fisher's exact test.

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APPENDIX A

MATERIALS AND METHODS
CBI pp. 7-10

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APPENDIX B

PROTOCOL
CBI pp. 24-30

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DATA EVALUATION REPORT

CHLOROPICRIN

Study Type: Mutagenicity: Unscheduled DNA Synthesis Assay in Primary Rat Hepatocytes

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 5/25/92

Independent Reviewer Nancy E. McCarroll B.S. Date 5/28/92

QA/QC Manager Sharon Segal, Ph.D. Date 5/25/92

Contract Number: 68D10075
Work Assignment Number: 1-26
Clement Number: 91-104
Project Officer: James Scott

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MUTAGENICITY STUDIES

EPA Reviewer: Stanley Gross, Ph.D. Signature:
Review Section II, Toxicology Branch { I }/HED Date: 9/9/92 E Iw Manuel
EPA Acting Section Head: Joycelyn Stewart, Ph.D. Signature: _____
Review Section II, Toxicology Branch { I }/HED Date: _____

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: In vitro unscheduled DNA synthesis assay in primary rat hepatocytes.

EPA IDENTIFICATION Numbers:

Caswell Number: 214

MRID Number: 419608-04

TEST MATERIAL: Chloropicrin technical

SYNONYMS: None listed

SPONSOR: Niklor Chemical Co., Inc., Long Beach, CA

STUDY NUMBER: T9152.380009

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD

TITLE OF REPORT: Unscheduled DNA Synthesis in Rat Primary Hepatocytes with a Confirmatory Assay.

AUTHOR: Curren, R. D.

REPORT ISSUED: June 28, 1990

CONCLUSIONS-EXECUTIVE SUMMARY: Chloropicrin was evaluated for the potential to cause unscheduled DNA synthesis (UDS) in two independently performed assays. Results indicate that the test material was not genotoxic in primary rat hepatocytes over a concentration range (0.3 to 6 nL/mL) that included moderately cytotoxic levels. Higher doses (≥9 nL/mL) were severely cytotoxic. Based on these findings, it was concluded that chloropicrin was tested over an appropriate range of concentrations with appropriate controls and showed no evidence of UDS. Thus the study satisfies Guideline requirements for genetic effects Category III, Other Mutagenic Mechanisms.

STUDY CLASSIFICATION: The study is acceptable.

A. MATERIALS:

1. Test Material: Chloropicrin technical

Description: Colorless liquid
Lot No: 987
Purity: 99.5%
Receipt date: February 15, 1990
Stability: Not reported
Contaminants: None listed
Solvent used: Reported by the author as 100% ethanol (EtOH). Presumably it was 95% ethanol, the purest form generally used in routine testing procedures.
Other provided information: The test material was stored at room temperature and was diluted immediately before use.

2. Indicator Cells: Primary rat hepatocytes were obtained by the in situ perfusion of the liver of an adult male Fischer 344 rat, purchased from Harlan Sprague Dawley, Inc. (Frederick, MD).

3. Control Substances: EtOH was the solvent control for the test compound; dimethyl sulfoxide (DMSO) was the solvent control for the positive control, and untreated cells served as the negative control. 7,12-Dimethylbenz(a)anthracene (DMBA) was used as the positive control at 3 and 10 µg/mL in the initial assay, and at 3 and 5 µg/mL in the confirmatory assay.

4. Medium: WME: Williams' Medium E containing 2 mM L-glutamine and gentamicin; WME+: Williams' Medium E with 10% calf serum.

5. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Three preliminary cytotoxicity assays were performed as follows:

Trial 1: Ten doses ranging from 0.3 to 10,000 nL/mL
Trial 2: Six doses ranging from 0.01 to 3 nL/mL
Trial 3: Five doses ranging from 3 to 300 nL/mL.

Note: Only results from the third trial were presented.

(b) UDS assay:

Concentrations assayed: The eight doses evaluated in the initial and confirmatory assays were 0.1, 0.3, 1, 3, 4, 5, 6, and 9 nL/mL.

Concentrations scored: 0.3, 1, 3, 4, 5, and 6 nL/mL (both trials).

B. STUDY DESIGN:

1. Cell Preparation:

(a) Perfusion technique: The animal was anesthetized with metofane (methoxyflurane) and the liver was perfused with Hanks' balanced salt solution containing 0.5 mM EGTA and Hepes buffer, pH 7.3, and with WME containing 80-100 units/ml collagenase and Hepes buffer pH 7.3. The liver was excised, and placed in WME-collagenase medium; cells were detached by combing or passage through a sieve.

(b) Hepatocyte harvest/culture preparation: Recovered cells were collected, counted, and seeded at a density of ≈5 x 10^5 cells, either into preconditioned 35-mm tissue culture dishes for the cytotoxicity assay, or onto coverslips in 35-mm tissue culture plates for the UDS assay. Cultures were placed in an incubator for 90 to 180 minutes, washed in WME+ and fed WME.

2. Preliminary Cytotoxicity Assay: Prepared primary hepatocyte cultures (in duplicate) were exposed to the selected doses of the test compound, negative control (WME), or solvent control (EtOH) for 18-20 hours. Owing to the reported volatility and severe cytotoxicity of the test material, the cultures were incubated in a sealed acrylic chamber containing 5% CO_2. Following exposure, an aliquot of culture fluid was removed, centrifuged, and the level of lactic acid dehydrogenase (LDH) activity was measured. Relative cytotoxicity was assessed by subtracting the LDH activity of the solvent control from the LDH activity in the treated cultures and comparing the values to the amount of LDH released by exposure of the solvent control cultures to 1% Triton.

3. UDS Assay:

(a) Treatment: The test compound concentrations were chosen so that five treatments ranging from ≈50% to 100% survival would be available for evaluation of net nuclear grains. Three hepatocyte cultures on coverslips were fed WME containing 10 µCi/mL [^{3}H] thymidine and exposed for 18 to 20 hours to the selected test material doses, the negative control (WME), the solvent controls (EtOH for the test material and DMSO for the positive control), and the positive control (DMBA). Due to the low doses used for the UDS assays, a sealed chamber was not used. Treated hepatocytes attached to coverslips were washed, swollen in 1% sodium citrate, fixed in ethanol-acetic acid, dried, and mounted. The parallel cytotoxicity assessment was conducted as previously described; duplicate cultures per dose were used.

(b) Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB-2 emulsion, dried, stored for 7 days at 0-4°C in light-tight desiccated boxes, developed in Kodak D-19 developer (Eastman Kodak), fixed, stained with hematoxylin-sodium acetate-eosin, coded, and counted.

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(c) Grain counting: The nuclear grains of 50 cells randomly chosen from each of three coverslips per treatment were counted. Cytoplasmic background counts were determined by counting three nuclear-sized areas adjacent to the nucleus. Net nuclear grain counts were determined by subtracting the mean cytoplasmic background count from the nuclear grain count. Nuclei exhibiting toxic effects of treatment, such as uneven staining, disrupted membranes, or irregular shape, were not counted.

4. Evaluation Criteria:

(a) Assay validity: The assay was considered valid if the positive control induced a significant increase in the net nuclear grain count, less than 15% of the cells in the negative control were in repair, and the net nuclear grain count of the vehicle control was less than one.

(b) Positive response: The assay was considered positive if the test compound induced a dose-related response, with the mean net nuclear grain count increased by at least five counts over the control for at least one dose. In the absence of a dose-related response, the test was also considered positive if the mean net nuclear grain count increased by at least five in at least two successive doses.

5. Protocol: See Appendix B.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Owing to compound volatility, the cultures used in the cytotoxicity studies were placed in a sealed acrylic chamber after compound addition. The first assay tested ten doses of the test material, ranging from 0.03 to 10,000 nL/mL; it was found that all concentrations caused high levels of cytotoxicity, as measured by release of LDH. Doses ranging from 0.01 to 3 nL/mL were evaluated in the second assay; minimal cytotoxicity was observed. Data were not presented from the first two preliminary studies. A third cytotoxicity assay was conducted with five doses ranging from 3 to 300 nL/mL. As shown in Table 1, relative cytotoxicities of 82%, 82%, 91%, 71%, and 2% were observed at doses of 300, 100, 30, 10, and 3 nL/mL, respectively. Microscopic examination of the monolayers indicated severe cytotoxic effects at all concentrations above 3 nL/mL. Less severe cytotoxicity was observed at 3 nL/mL. Based on these results, 10 nL/mL was selected as the highest dose for the UDS assay.

2. UDS Assay: The study author stated that because lower concentrations were used in the UDS assays, sealed chambers were no longer necessary. Eight doses of chloropicrin technical, ranging from 0.03 to 10 nL/mL, were tested in the first trial, but the slides could not be evaluated due to an unspecified technical error. However, the results of the

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TABLE 1. Representative Results of the Preliminary Cytotoxicity Assay with Chloropicrin: Lactic Acid Dehydrogenase (LDH) Activity

Treatment	Dose	Average[a] LDH Activity (Units/L)	Corrected[b] LDH Activity (Units/L)	Relative Cytotoxicity[c] (%)
Negative Control				
Culture medium	--	43.0	-20.0	-4
Solvent Control				
Ethanol	10 µL/mL	63.0	0.0	0
Ethanol + 1% Triton	10 µL/mL	518.0	455.0	100
Test Compound Control				
Chloropicrin +1% Triton	300 nL/mL	473.5	410.5	90
Test Compound				
Chloropicrin	3 nL/mL	70.0	7.0	2
	10 nL/mL	387.5	324.5	71
	30 nL/mL	478.5	415.5	91
	100 nL/mL	434.0	371.0	82
	300 nL/mL	436.0	373.0	82

[a]Average of two samples.
[b]Corrected LDH = Average LDH - Solvent Control LDH.

$$[c]\text{Relative Cytotoxicity} = \frac{\text{Corrected LDH}}{\text{Corrected LDH of Solvent Control + 1\% Triton}} \times 100\%$$

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parallel cytotoxicity assays revealed severe cellular damage at 10 nL/mL and moderate cell damage at 6 nL/mL. Accordingly, the dose range was revised to 0.1 to 9 nL/mL for the repeat evaluation. As shown in Table 2, adverse compound effects were dose-related; relative cytotoxicity ranged from 86% at 9 nL/mL to 20% at 3 nL/mL. Lower levels (1, 0.3, and 0.1 nL/mL) were not cytotoxic. Microscopic examination of the cells revealed cytotoxic effects at concentrations ≥4 nL/mL, and normal cellular morphology at lower doses. Results from the cytotoxicity phase of the confirmatory trial were in general agreement with the initial assay and indicated that doses ≥5 nL/mL caused an increase in LDH release and adverse effects on the monolayer cells (Table 3).

In both trials, cultures exposed to the highest dose (9 nL/mL) were not scored because of excessive cytotoxicity. There was, however, no evidence of genotoxicity at any of the levels scored for UDS (0.3 to 6 nL/mL). By contrast, the positive control (DMBA at 3.0 and 10.0 µg/mL in the initial assay, and 3.0 and 5.0 µg/mL in the confirmatory assay) induced a marked and dose-related increase in UDS. Based on these findings, the study author concluded that chloropicrin was negative in the primary rat hepatocyte UDS assay.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that the study author's interpretation of the data was correct. Chloropicrin was assayed to cytotoxic doses but failed to induce UDS. The response of the test system to the positive control indicated that the assay was sufficiently sensitive to detect a mutagenic response. We, therefore, conclude that chloropicrin was not genotoxic in this test system.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement was signed and dated June 29, 1990.)

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 7-10; Appendix B, Protocol, CBI pp. 20-28.

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TABLE 2. Representative Results of the Initial Unscheduled DNA Synthesis Rat Hepatocyte Assay with Chloropicrin

Treatment	Dose	Cytotoxicity: Average[a] LDH Activity (units/L)	Cytotoxicity: Corrected[b] LDH Activity (units/L)	Cytotoxicity: Relative Cytotoxicity[c] (%)	UDS Activity: Number Cells Scored	UDS Activity: Mean Net Nuclear Grain Count ± S.D.[d]	UDS Activity: Percent Cells with ≥5 Grains
Negative Control							
Culture medium	--	47.0	-10.5	-2	150	-0.9±3.1	0
Solvent Controls							
Ethanol (Solvent for test compound)	10 µL/mL	57.5	0.0	0	150	-1.3±2.9	1
Ethanol +1% Triton	10 µL/mL	485.0	427.5	100	--	--	--
Dimethyl sulfoxide (Solvent for positive control)	10 µL/mL	60.0	0.0	0	150	-0.8±2.8	1
Test Compound Control							
Chloropicrin +1% Triton	9 nL/mL	477.0	419.5	98	--	--	--
Positive Control							
7,12-Dimethylbenz(a)anthracene[e]	3 µg/mL	170.0	110.0	26	150	14.8±6.8[f]	94
Test Compound							
Chloropicrin	3 nL/mL[g]	141.0	83.5	20	150	-1.9±2.8	0
	4 nL/mL	210.5	153.0	36	150	-1.5±2.9	0
	5 nL/mL	278.0	220.5	52	150	-0.5±2.7	1
	6 nL/mL	361.5	304.0	71	150	-1.3±2.5	0
	9 nL/mL	424.5	367.0	86	--	Cytotoxic	

[a]Average of two samples.
[b]Corrected LDH = Average LDH - Solvent Control LDH.
[c]Relative Cytotoxicity = $\frac{\text{Corrected LDH}}{\text{Corrected LDH of Solvent Control + 1\% Triton}} \times 100\%$
[d]Means and standard deviations from the counts of 50 cells/culture.
[e]Positive results were obtained with a 10.0 µg/mL dose.
[f]Fulfills the reporting laboratory's criterion for a positive response (i.e., ≥5 net nuclear grains).
[g]Results for lower doses (0.3 and 1 nL/mL) did not suggest a genotoxic or cytotoxic effect.

TABLE 3. Representative Results of the Confirmatory Unscheduled DNA Synthesis Rat Hepatocyte Assay with Chloropicrin

Treatment	Dose	Cytotoxicity			UDS Activity		
		Average[a] LDH Activity (units/L)	Corrected[b] LDH Activity (units/L)	Relative Cytotoxicity[c] (%)	Number Cells Scored	Mean Net Nuclear Grain Count ± S.D.[d]	Percent Cells with ≥5 Grains
Negative Control							
Culture medium	--	56.5	6.5	2%	150	-1.0±2.5	0
Solvent Controls							
Ethanol (Solvent for test compound)	10 µL/mL	50.0	0.0	0	150	-0.9±2.6	1
Ethanol +1% Triton	10 µL/mL	480.0	430.0	100	--	--	--
Dimethyl sulfoxide (Solvent for positive control)	10 µL/mL	71.0	0.0	0	150	-0.8±2.9	0
Test Compound Control							
Chloropicrin +1% Triton	9 nL/mL	404.0	354.0	82	--	--	--
Positive Control							
7,12-Dimethylbenz(a)anthracene[e]	3 µg/mL	82.5	11.5	3	150	17.5±7.7[f]	99
Test Compound							
Chloropicrin	4 nL/mL[g]	171.0	121.0	28	150	-1.8±2.7	0
	5 nL/mL	198.0	148.0	34	150	-2.3±2.8	1
	6 nL/mL	259.0	209.0	49	150	-2.1±3.3	1
	9 nL/mL	359.5	309.5	72	--	Cytotoxic	

[a]Average of two samples.
[b]Corrected LDH = Average LDH - Solvent Control LDH.
[c]Relative Cytotoxicity = $\frac{\text{Corrected LDH}}{\text{Corrected LDH of Solvent Control + 1\% Triton}} \times 100\%$
[d]Means and standard deviations from the counts of 50 cells/culture.
[e]Positive results were obtained with a 5.0-µg/mL dose.
[f]Fulfills the reporting laboratory's criterion for a positive response (i.e., ≥5 net nuclear grains).
[g]Results for lower doses (0.0003, 0.001, and 0.003 µL/mL) did not suggest a genotoxic or cytotoxic effect.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 7-10

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APPENDIX B

PROTOCOL
CBI pp. 20-28

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