1-12-87

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

005654

JAN 12 1987

MEMORANDUM

Subject: Bromochloro-5,5,dimethylhydantoin; 1-bromo-3-chloro-5,5-dimethylhydantoin
EPA ID# 8622-EL

To: Jeffrey Kempter
Barbara Pringle
PM # 32
Disinfectants Branch
Registration Division (TS-767C)

From: Joycelyn E. Stewart, Ph.D. 10/23/86
Review Section VII
Toxicology Branch/HED (TS-769C)

Thru: Albin B. Kocialski, Ph.D. 1/8/87
Supervisory Pharmacologist
Toxicology Branch/HED (TS-767C) 1/10/87

Registrant: Ameribrom, Inc
1250 Broadway
New York, N.Y. 10010

Caswell # 114A
Project # 2254

Action Requested

Review 3 mutagenicity studies for manufacturing use formulation.

Recommendations

5,5, Dimethylhydantoin, the organic moiety of bromochloro-dimethylhydantoin gave negative responses in the Ames Salmonella typhimurium assay, the chromosomal aberration test in CHO cells, and the UDS assay in human HeLa cells. These studies were conducted with and without metabolic activation. The studies are core classified as Acceptable, and satisfy the mutagenicity requirements for registration of bromochloro-5,5,-dimethylhydantoin.

Detailed reviews of the studies are attached.

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eviewed by: Joycelyn Stewart, Ph.D.
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DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity TOX. CHEM. NO.: 114A

ACCESSION NUMBER: 264468 PROJ. NO.: 2254

TEST MATERIAL: 5,5 dimethylhydantoin

SYNONYMS:

STUDY NUMBER(S): LSRI Report # DSB/094/DMH

SPONSOR: Ameribrom, Inc
1250 Broadway
New York, N.Y. 10001

TESTING FACILITY: Life Science Research Israel Ltd.
P.O. Box 139, Ness Ziona, Israel

TITLE OF REPORT: Dimethylhydantoin Assessment of Mutagenic Potential in Histidine Auxotrophs of Salmonella typhimurium (The Ames Test)

AUTHOR(S): Z. Evenchik

REPORT ISSUED: 2/24/86

CONCLUSION: 5,5,Dimethylhyantoin was negative in the Ames Salmonella typhimurium assay under the conditions of the study.

Classification: Acceptable

MATERIALS: 5,5, Dimethylhydantoin (the organic moiety of bromo-chloro dimethylhydantoin) was the test compound. Five tester strains of Salmonella typhimurium were the test system.

METHODS: Dimethylhydantoin (DMH), purity not stated was evaluated in the Ames mutagenicity assay according OECD Guideline 471, issued May 1983, in a plate incorporation assay. The compound was tested at concentrations of 5, 50, 250, 1250, and 5000 ug/plate using Salmonella typhimurium strain TA 98 without metabolic activation in the preliminary toxicity test, and at concentrations of 125, 250, 500, 1000, and 2000 ug/plate with and without metabolic activation in the main study using 5 tester strains of Salmonella typhimurium (TA 1535, TA 1537, TA 1538, TA 98, and TA 100). The cultures used in the assay were prepared from frozen stock, and were tested for the appropriate amino acid requirement and characteristic spontaneous reversion rate before use. Approx-

imately 0.1 ml of 10^6 dilution of cultures were used per plate.

The test compound was dissolved in distilled water, which served as the solvent control. Solutions were freshly prepared before each experiment. Positive control compounds used in the study were: 2 amino anthracene (2 ug/plate) for all strains when the study was done with metabolic activation; sodium azide (3 ug/plate) for strains TA 1535 and TA 100, 4-nitro-o-phenyl enediamine (NPD) 20 ug/plate for TA 1538 and TA 98, and ICR-191 2 ug/plate for TA 1537 without metabolic activation.

The metabolic activation system was an S-9 fraction of rat liver homogenate obtained from male CD rats using a mixed induction system of phenobarbital sodium and 3-methylcholanthrene (3MC). After i.p. administration of phenobarbital for 4 days, and 3-MC for 3 days, rats were sacrificed and the livers removed and homogenized in cold 0.15M KCl, centrifuged at 9000 x g for 10 minutes, and the supernatant collected and stored at -80°C until required. It was mixed with a cofactor solution before use. The S-9 mix contained 0.1M NADP, 2 ml; 1M glucose-6-phoshate, 0.25 ml; 0.4M $MgCl_2$/1.6M KCl, 1.0 ml; S-9 supernatant, 2 ml; 0.1M sodium orthophosphate buffer, 25 ml; and distilled water, 19.75 ml.

Cultures were grown overnight in Oxoid nutrient broth, providing approximately 2x 10^9 organisms/ml. An aliquot (0.1 ml) was treated with the appropriate concentration of test compound, positive or negative control. Mutations were quantified on duplicates for each strain treated with the test compound, and for each positive and solvent control by counting the number of revertant colonies after 48 hours incubation at 37°C on a minimum histidine deficient medium. Two separate studies were conducted. Additional plates were prepared without the test organisms to verify the sterility of the S-9 mix. Aliquots of a 10^{-6} dilution of culture were used to measure the viability and cell density of each culture by counting the total number of colonies on nutrient plates.

EVALUATION CRITERIA: The test material was considered mutagenic if numbers of revertants greater than twice the control values were obtained in consecutive dose levels or in the last non toxic dose in two independent experiments. Data were assessed using regression analysis followed by a "t" test.

RESULTS: In the preliminary study using strain TA 98, no cytotoxicity as indicated by a decline in the number of spontaneous revertants or the absence/thinning of the background lawn was observed at any dosage level.

In the main study at doses of 5 ug through 2000 ug/plate no increase over the solvent controls in the number of revert-

ant colonies was observed following exposure to the test material when the test was conducted with or without metabolic activation.

The positive control chemicals significantly increased the number of revertant colonies observed when the test was performed with and without metabolic activation.

Test solutions were reported to be free of microbial contamination, and the total colony counts demonstrated the viability and cell density of the cultures used.

DISCUSSION: Since no cytotoxicity was observed in the preliminary study at doses up to 5000 ug/plate, it is not apparent why the investigators used doses up to 2000 ug/plate in the main study, especially since only a single strain(TA 98) was used in the cytotoxicity screen. However,the study was valid in all other respects, and is therefore classified as Acceptable.

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DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity

TOX. CHEM. NO.: 114A

ACCESSION NUMBER: 264468

PROJ. NO.: 2254

TEST MATERIAL: 5,5, Dimethylhydantoin

SYNONYMS:

STUDY NUMBER(S): DSB1/86109

SPONSOR: Ameribrom, Inc.
1250 Broadway
New York, N.Y. 10001

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

TITLE OF REPORT: Analysis of Metaphase Chromosomes obtained from CHO cells cultured in vitro and treated with 5,5,dimethylhydantoin

AUTHOR(S): J.A. Allen, P.C. Brooker, and S.R. Godfrey

REPORT ISSUED: 3/4/86

CONCLUSION: Based on the results reported, 5,5, dimethylhydantoin did not cause chromosomal aberrations in Chinese hamster ovary cells under the test conditions.

Classification: Acceptable

MATERIALS: 5,5, dimethylhydantoin (DMH) batch # 85035 was the test chemical. Chinese hamster ovary (CHO) cells, strain K1-BH4 was the test system. Culture medium was Ham's F12 medium supplemented with 5% fetal calf serum (FCS). Mytomycin C (batch # 94F--02401) was the reference mutagen for the study without metabolic activation. Cyclophosphamide (batch # 33F-0157) was the reference mutagen for the study with metabolic activation. The metabolic activation system was an S-9 mixture containing: S-9 fraction, 0.1 ml; 0.4M $MgCl_2$, 0.02 ml; 0.2M Na_2HPO_4(pH 7.4) 0.5 ml; 1.0M glucose6-phosphate, 0.005 ml; 0.1M NADP, 0.04 ml; distilled water, 0.335 ml.

METHODS: Cells were routinely grown and subcultured in Ham's F 12 medium supplemented with 5% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested before reaching confluency and reseeded in fresh medium to produce 1.0 x 10^5 cells/ml.

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A preliminary cytotoxicity study was performed in which $5x\ 10^5$ cells/flask were incubated at 37°C for 24 hours, following which the test compound was added to the flasks to achieve final concentrations of 12.5, 25, 50, 100, 200, 400, and 800 ug/ml. The test compound was dissolved in distilled water, which served as the solvent control. Untreated controls were included in the study. The preliminary study was conducted with and without metabolic activation. One flask was used for each concentration of the test compound, while duplicate flasks were used for the controls. Cultures without the S-9 mix were incubated for 20 hours. In the study with metabolic activation, 2 hours after addition of the test compound, the medium was removed and the cultures refed with fresh medium and incubated for a further 18 hours. Three hours prior to the end of the incubation, colchicine 0.25 ug/ml was added to each culture to arrest mitosis. At the end of the incubation period cells were washed with 0.25% trypsin solution and the flasks were incubated for 10 minutes at 37°C in Ham's medium with FCS. Cells were centrifuged for 10 minutes at 200 x g, suspended in 2.5 ml 0.07M KCl, then fixed in methanol: glacial acetic acid 3:1, dried and stained with 10% Giemsa, air dried and examined microscopically at x 160 magnification for the proportion of metaphase cells.

Based on the results obtained in the preliminary study, the main study was conducted similarly using duplicates of the test compound and the positive controls, and quadruplicates of the solvent controls. Cyclophosphamide 20 ug/ml was used in the study with the S-9 mix, and Mitomycin 0.4 ug/ml was used in the study without the S-9 mix.

Coded slides were examined for the following chromosomal aberrations: chromatid gaps, chromatid breaks, isochromatid gaps, isochromatid breaks, chromatid exchanges, dicentric chromosomes, acentric chromosome fragments, chromosome rings and chromosome rearrangements. One hundred metaphase cells were examined from each culture, with a maximum of 25 cells from each slide.

Results were analysed by Fisher's exact test. For each concentration of the test compound, and for the positive and negative controls, the number of aberrant cells(including and excluding gap damage), the number of aberrations/100 cells, and the frequency of structurally aberrent cells were calculated.

RESULTS: In the preliminary cytotoxicity study, the mitotic index was variable in the flasks exposed to the test compound both in the presence and in the absence of the metabolic activating system. No cytotoxicity was reported. Based on these results, 800 ug/ml was selected as the top dose in the main study.

In the main study, none of the test doses of 5,5 dimethylhydantoin produced a significant increase in the number of

cells with chromosomal aberrations when compared to the solvent controls. The positive control compounds, Mitomycin C(0.4 ug/ml) and cyclophosphamide (20 ug/ml) caused statistically significant increases ($p < 0.001$) in the number of cells with chromosomal aberrations when compared with the solvent controls.

DISCUSSION: The study as performed is adequate to detect clastogenic activity in this in vitro cytogenetic assay using CHO cells. The results reported demonstrate that 5,5, dimethylhydantoin was not mutagenic under the test conditions.

The study is Acceptable

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DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity TOX. CHEM. NO.: 114A

ACCESSION NUMBER: 264468 PROJ. NO.: 2254

TEST MATERIAL: 5,5,dimethylhydantoin

SYNONYMS:

STUDY NUMBER(S): DSB/86554

SPONSOR: Ameribrom, Inc.
1250 Broadway,
New York, N.Y. 10001

TESTING FACILITY: Huntingdon Research Centre
Cambridgeshire, England

TITLE OF REPORT: Assesment of Unscheduled DNA Repair in mammalian cells after exposure to 5,5, dimethylhydantoin (DMH).

AUTHOR(S): Allen, J.A. and Proudlock,R.J.

REPORT ISSUED: 5/19/1986

CONCLUSION: The results reported demonstrate that 5,5,dimethylhydantoin did not induce an increase in unscheduled DNA synthesis in human HeLa cells in vitro under the test conditions.

Classification: Acceptable

MATERIALS: Human HeLa epitheliod cells were the test system. Cells were maintained in Eagle's Minimum Essential Medium containing Earle's salts, gentamycin, and fetal calf serum. This medium was designated EMEM in the report. Arginine-deficient medium was EMEM without arginine, but containing dialysed fetal calf serum. This was designated ADM in the report.

The test chemical was the organic moiety of bromochloro-5,5 dimethylhydantoin, dimethylhydantoin dissolved in distilled water. Distilled water served as the solvent control.

The inducer substance was Arochlor 1254 and the reference mutagens were 4-nitroquinoline-1-oxide (4NQO) in the absence of S-9, and 2-amino anthracene(2AA)in the presence of the S-9 mix. Positive controls were dissolved in DMSO, then diluted with water to the required concentrations.

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METHODS: The S-9 mixture used in the metabolic activation system was made from livers of male SPF CD Sprague-Dawley derived rats. Animals were given a single dose of 500 mg/kg of Arochlor 1254 to induce microsomal enzyme activity and were sacrificed 5 days later. Livers were removed and homogenized in 0.15 M KCl. The homogenates were centrifuged at 9000x g for 10 minutes. The supernatant was stored at -70° C. Prior to use the fraction was mixed with a cofactor containing KCl, glucose-6-phosphate, NADP, and distilled water.

Cells were thawed and subcultured twice in fresh EMEM and incubated at 37° C in a humidified 5% CO_2 atmosphere. The cultures were harvested before the cells had grown to confluence, and the cell monolayer incubated with versene buffer at 37° C, then centrifuged. The supernatant was discarded and the cell pellet again incubated at 37° C in a humidified 5% CO_2 atmosphere. Cells were harvested and resuspended in EMEM, and the cell density adjusted to $5x\ 10^4$ cells/ml. Two ml aliquots of the cell suspension were placed in 35 mm diameter wells of multi-well tissue culture dishes and incubated for 96 hours, when the cells formed a confluent monolayer. The culture medium was removed from each well and replaced with 2 ml of ADM. The cultures were incubated for 72 hours with the ADM replaced after 24 hours.

After the 72 hour incubation, 6^3H-thymidine (0.5 mCi/ml, 20 Ci/m mole) was added to the cultures to give a final activity of 5 uCi/ml, both in the presence and absence of the S-9 mix. The compound was tested at a maximum final concentration of 20480 ug/ml, with 11 serial 2-fold dilutions starting at 10 ug/ml. The positive control doses were 2-fold dilutions from 0.02 ug/ml to 0.32 ug/ml for 4 NQO and from 2.5 ug/ml to 40 ug/ml for 2AA. Water was the solvent control. Cultures were incubated for 180 minutes in the presence of the test compound, positive control or vehicle, then washed, fixed and stained and examined by autoradiography. One hundred non-S phase nuclei were examined from each culture and the number of silver grains overlying the nuclei and a corresponding adjacent area of cytoplasm was examined. The number of non-S nuclei with more than 3 grains were also recorded. Replicate cultures were run at each dose level. Two independent tests were performed. Results were analysed using one way analysis of variance and Student's "t" test.

EVALUATION CRITERIA: The test was regarded as positive if a reproducible statistically significant increase in the number of grains per 100 nuclei of non-S-phase compound treated cells was observed when compared to the solvent controls.

RESULTS: In the DNA repair test without S-9, no significant increase in net nuclear grain count was observed after treat- with 5,5 dimethylhydantoin. The positive control 4NQO caused significant increases ($p \leq 0.001$) in nuclear grain count, in net nuclear grain count and in the number of non-S-phase nuclei. In the study with S-9, a statistically significant increase ($p < 0.05$) in net grain count was observed at 40 ug/ml, but was not observed when the study was repeated. No other increase was reported. The positive control 2AA gave significant increases in net grain count at all dosage levels($p \leq 0.05$) and in the number of non S-phase nuclei.

Based on these observations, it was concluded that 5,5 dimethylhydantoin did not increase unscheduled DNA synthesis in cultured human HeLa cells.

DISCUSSION: Although no preliminary cytotoxicity study was done, the range of dosage tested was wide enough to determine the mutagenic potential of 5,5,dimethylhydantoin in the UDS assay.

The compound was tested up to the limit of its solubility in water and the results demonstrate that 5,5, dimethylhydantoin was negative in this in vitro assay under the test conditions.

CLASSIFICATION: Acceptable