8-15-85
CASWELL FILE

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

004613

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

AUG 15 1985

MEMORANDUM

SUBJECT: Iprodione EPA Registration No. 359-684

FROM: Alex Arce
Toxicology Branch
Hazard Evaluation Division (TS-769)

TO: H. Jacoby, PM-21
Registration Division (TS-767)

THRU: Clint Skinner, PH.D.
Head Section lll

and

Theodore Farber, Ph.D.
Toxicology Branch (TS-769)

Compound: Iprodione

Tox Chemical No. 470 A

Accession No. 257802

Registrant: Rhone-Poulenc, Inc.

Action Requested:

To review four mutagenicity studies.

Recommendation or Conclusion:

The four submitted studies are acceptable and can be used to satisfy data gaps for iprodione. Refer to attached one-liner.

Background Information:

These studies are being submitted to fill existing data gaps.

(1)

Information Submitted:

Four Studies: Mammalian cell forward mutation; CHO metaphase analysis; DNA damage and sister chromatid exchange.

Discussion:

The product is negative in its mutagenicity potential in three of the four submitted studies. The product is positive for DNA Damage (Project # 2114).

DATA EVALUATION REPORT

1. Chemical: Iprodione

2. Test Material: Technical (white powder)

3. Study Type: Mutagenicity

4. Study ID: CHO Metaphase Analysis In Vitro Chromosome Aberration Analysis in Chinese Hamster Ovary Cells. Pharmakon Research International, Waverly, PA, PH 320-BO-001-84, January 3, 1985, Rhone-Poulenc, Inc., EPA Accession No. 257802.

5. Reviewed by: Alex Arce
Toxicologist
HED/TOX
Phone: 557-1511
Date: July 1985

6. Approved by: Clint Skinner, Ph.D.
Head, Section III
HED/TOX
Phone: 557-1511
Date: July 1985

Clint Skinner

PROTOCOL

Materials and Methods:

Iprodione

Dose Levels:

0.03, 0.1, 0.3, 1.0, 3.33, 10, 33.3, 100, and 1000 µg/ml with and without metabolic activation.

Description of the Study Design:

"Each dose level was evaluated with or without metabolic activation" as per attached protocol.

Reported Results:

"No significant increase in chromosome aberration or proportion of aberrant metaphases in cultures treated with iprodione. Thus, it is negative for this test. Did not produce chromosome aberrations in the CHO cells.

4

004613

DATA EVALUATION REPORT

1. Chemical: Iprodione

2. Test Material: Technical

3. Study Type: Mutagenicity

4. Study ID: CHO/HGPRT Mammalian Cell Forward Mutation Assay, Pharmakon Research International, Inc., Waverly, PA, PH-314-BO-001-84, January 3, 1985, Rhone-Poulenc, Inc., EPA Accession No. 257802.

5. Reviewed by: Alex Arce, Toxicology Reviewer HED/EEB — Phone: 557-1511 — Date: July 1985

6. Approved by: Clint Skinner, Ph.D., Head, Section III HED/EEB — Phone: 557-1511 — Date: July 1985

7. Conclusion:

Under the test conditions iprodione was negative in the CHO/HGPRT Mammalian Cell Forward Gene Mutation.

5

PROTOCOL

Materials and Methods:

Iprodione, a white powder

Dose Levels:

Cytotoxicity in the CHO line levels: 0.03, 0.01, 0.3, 1.0, 3.3, 10, 33.3, 3, 100, 33 and 1000 μg/ml. Mammalian forward mutation in the CHO/HGPRT dose levels: 5, 10, 50, 75, and 100 μg/ml without metabolic activation and at 100, 250, 500, 1000, and 1500 μg/ml with a 2 percent with metabolic activation.

Description of the Study Design:

The control articles were CHO-K1-BH4 cells. The positive controls were EMS; at 200 μg/ml; DMN at 100 μg/ml. The CHO cells were treated and cytotoxicity was determined as per attached protocol.

Reported Results:

The assay produced total cytotoxicity at the 333 and a 1000 μg/ml of treatment volume without metabolic activation. 51.4 percent relative cell survival at the 1000 μg/ml of treatment volume with metabolic activation. "Iprodione produced no increased mutation frequencies at treated levels when compared to negative controls."

6

004613

DATA EVALUATION REPORT

1. Chemical: Iprodione

2. Test Material: Technical

3. Study Type: Mutagenicity

4. Study ID: DNA Damage in Bacillus subtilis, Borriston, January 9, 1985, Rhone-Poulenc Inc., NJ, EPA Accession No. 257802

5. Reviewed By: Alex Arce, Toxicologist, HED/TOX, Crystal City — Phone: 557-1511 — Date: July 31, 1985

6. Approved By: Clint Skinner, Ph.D., Section III Head, HED/TOX — Phone: 557-1511 — Date: August 1985

7. Conclusion:

Iprodione, as tested, was a positive DNA damaging substance at the highest and lowest dose levels (1670 and 20.6 mg/disc) without metabolic activation.

PROTOCOL

Materials AND Methods:

96.8% Iprodione Lot No. 83024-01

Solvent DMSO (also used as solvent control)

Concentration: 1670, 556.7, 185.6, 61.9, and 20.6 µg/ml.

Positive Control: MNNG in $^{B}(a)^{P}$

Description of the Study:

The in vitro DNA damage potential of iprodione was determined, using B. subtilis mutants.

Refer to attached protocol.

Reported Results:

Iprodione technical was a positive DNA damaging substance at the highest dose level tested (1670 and 20.6 µg/disc without metabolic activation).

It was considered to be a DNA damaging agent in the Bacillus subtilis.

8

004613

DATA EVALUATION REPORT

1. Chemical: Iprodione

2. Test Material: Technical

3. Study Type: Mutagenicity

4. Study ID: In Vitro Sister Chromatid Exchange in Chinese Hamster Ovary Cells (CHO), Pharmakon Research, Waverly, PA, PH 319-B0-001-84, August 1985, Rhone-Poulenc, Inc., EPA Accession No. 257802.

5. Reviewed by: Alex Arce
Toxicologist
HED/TOX
Crystal City
Phone: 557-1511
Date: August 1985

6. Approved by: Clint Skinner, Ph.D.
Head, Section III
HED/TOX
Phone: 557-1511
Date: August 1985

7. Conclusion:

Under the test conditions, iprodione was negative in the in vitro sister chromatid exchange in the CHO.

PROTOCOL

Materials AND Methods:

Iprodione, a white powder

Dose:

Nonactivated; 5, 10, 25, 50, 75 and 100 μg/ml.
Activated; 5, 50, 100, 200, and 400 μg/ml.

Solvent:

DMSO.

Positive Control:

Ethylmethane sulfanate
Dimethylnitrosoamine

Description of the Study Design:

Refer to attached protocol.

Tox Chem. No. 470A Iprodione Study/Lab/Study #/Date	Material	EPA Accession No.	LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grad Doc. No
Mammalian Cell Forward Gene Mutation Study Pharmakon Research Internationl, Inc. Project No. PH314-B0-001-84. January 3, 1985.	Tech	257802	Did not induce forward mutation.		Acceptabl
CHO Metaphase Analysis *In Vitro* Chromosome Abberation Analysis in Chinese Hampster Ovary Cells. Pharmakon Research International, Inc. Project No. PH320-B0-001-84. January 3, 1985.	Tech	257802	Did not increase incidence of sister chromatid exchange.		Acceptabl
DNA Damage in *Bacillus subtilis* with Iprodione Technical. Borriston Laboratories. Project No. 2214. February 9, 1985.	Tech	257802	Positive for DNA damage at the highest dose level tested, 1670 and 20.6 µg/disc.		Acceptabl
In Vitro Sister Chromatid Exchange in Chinese Hampster Ovary Cells. Pharmakon Research International, Inc. Project No. 319-B0-001-84. January 3, 1985.	Tech	257802	Did not induce DNA damage.		Acceptabl

Mamalian Cell Fordward
Gene mutation assay
PH314-BO-001-84

METHODS AND MATERIALS

Storage of Cultures

The stock cultures of CHO-K1-BH4 cell line are maintained in frozen aliquots in a Revco Ultra-low Freezer. Fresh cultures of CHO-K1-BH4 cell line were prepared from frozen stock cultures known to have a stable spontaneous mutation frequency of 0 - 10 x 10^{-6} mutants per cell, however, values up to 20 x 10^{-6} were deemed acceptable.

Control Articles

CHO-K1-BH4 cells were assayed untreated, treated with metabolic activation system only, and treated with the appropriate solvent, both with and without metabolic activation. In order to validate the integrity of the test system, known positive chemicals were evaluated concurrently.

Positive controls used in the assay were Ethylmethanesulfonate (EMS) (Sigma MO880) at 200 ug/ml of medium, a mutagen not requiring an S-9 activation system. Dimethylnitrosamine (DMN) (Aldrich - N2,500-1) at 100 ug/ml of medium, a mutagen requiring an S-9 activation system was used as the positive control.

Dose Selection

Cytotoxicity of a test substance to CHO cells was determined by a reduction in colony forming ability of the cells following a 5 hour treatment with the test substance in the presence and absence of a metabolic activation system. Test article Iprodione was evaluated at 10 doses with 1000 ug/ml per ml of media being the highest one. The remaining doses were 0.03, 0.1, 0.3, 1.0, 3.3, 10, 33.3, 100 and 333 ug/ml of media.

004613

Treatment of CHO Cells with Test Article for Cytotoxicity (non-activated)

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks and the cultures were washed twice with 5 ml of Saline G-Complete. After the final wash, 5 ml of serum free medium F12 was transferred into each flask. The test article being assayed for cytotoxicity was diluted in the proper solvent to the concentrations desired for testing and 50 ul of the test substance was added to each flask and mixed well. The flasks were gassed with 5% CO_2 in air, the caps tightened and the cells incubated for 5 hours at 37°C immediately after dosing. After the 5 hour incubation, the flasks were removed from the incubator, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G-Complete and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours at 37°C in 5% CO_2 in air, at 90+ % humidity.

Treatment of CHO Cells with Test Article for Cytotoxicity (activated)

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks, and the cultures were washed twice with 5 ml of Saline G-Complete. After the final wash, 4 ml of serum free medium F12 were transferred into each flask. The S-9 metabolic activation preparation was added in a total volume of 1.0 ml. The S-9 mix contained (per ml) 8 umol $MgCl_2$, 8 umol $CaCl_2$, 33 umol KCl, 5 umol glucose-6-phosphate, 4 umol NADP, 50 umol sodium phosphate buffer (pH 7.4) and 0.1 ml of the metabolic preparation containing at least 30 mg protein/ml. The test substance being assayed for cytotoxicity was diluted in the proper solvent to the concentrations desired for testing and 50 ul of the test article was added to each flask and mixed well. The flasks were gassed with 5% CO_2 in air, the caps tightened and the cells incubated for 5 hours at 37°C immediately after dosing. After the 5 hour incubation, the flasks were removed from the incubator, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G-Complete and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours at 37°C, in 5% CO_2 in air, at 90+ % humidity.

Cytotoxicity Determination

Following the 19 hour incubation after test substance treatment, the cultures were removed from the incubator and the medium aspirated from the flasks. The cultures were washed twice with 5 ml of Ca-Mg-free Saline G. The cells were then placed at 37°C in 0.5 ml of a 0.05% trypsin solution. The flasks were examined under an inverted microscope to ensure that the cells had rounded and 1.5 ml of medium F12FCM5 were added. The cells were washed off the surface of the flask using a plugged pasteur pipette. A cell number was determined for each culture. An aliquot of the cells was diluted to a cellular density of 1000 cells/ml in medium F12FCM5 and 0.2 ml (200 cells/plate) was added to each of 3 - 60 mm plates containing 5 ml of medium F12FCM5. The plates were incubated for 7 - 8 days at 37°C in a 5% CO_2 in air incubator. After the incubation period, the medium was removed and the colonies fixed with methyl alcohol, stained with a dilute crystal violet solution, counted and the colony numbers recorded. These survival frequencies were used to determine the levels of test article which yielded approximately 10 - 100% survival. Five

levels of test substance yielding 10 to 100% survival were chosen to be used in the CHO Mammalian Cell Forward Gene Mutation Assay.

Plating CHO Cells for Experiment

CHO-K1-BH4 cells for mutagenesis testing were obtained from frozen stocks of cultures known to have a stable spontaneous mutation frequency of $0 - 10 \times 10^{-6}$ mutants per cell. Exponentially growing cells were plated in 5×10^{5} 25 cm squared plastic tissue culture flasks at an initial density of cells in 5 ml of medium F12FCM5 and incubated for 16 - 24 hours. Normal cell growth was inspected using an Inverted Microscope.

Replication

CHO-K1-BH4 cells were treated with five levels of the test article both with and without a metabolic activation system. All negative and positive controls were treated in duplicate. All treatment groups were tested in duplicate.

Treatment of CHO Cells with Test or Control Substance (non-activated)

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks and the cultures were washed twice with 5 ml of Saline G-Complete. After the final wash, 5 ml of serum free medium F12 were transferred into each flask. The test substance being assayed was diluted in the proper solvent to the concentration desired for testing and 50 ul of the test or control substance was added to each flask and mixed well. The flasks were gassed with 5% CO_2 in air, the caps tightened and the cells incubated for 5 hours at 37°C in 5% CO_2 in air, at 90± % humidity. After the 5 hour incubation, the flasks were removed from the incubator, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G- Complete and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours at 37°C in 5% CO_2 in air, at 90+ % humidity.

Treatment of CHO Cells with Test or Control Substance (activated)

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks, and the cultures were washed twice with 5 ml of Saline G-Complete. After the final wash, 4 ml of serum free medium F12 were transferred into each flask. The S-9 metabolic activation preparation was added in a total volume of 1.0 ml. The S-9 mix contained (per ml) 8 umol $MgCl_2$, 8 umol $CaCl_2$, 33 umol KCl, 5 umol glucose-6-phosphate, 4 umol NADP, 50 umol sodium phosphate buffer (pH 7.4) and 0.1 ml of the metabolic preparation containing at least 30 mg protein/ml. The test or control substance being assayed was diluted in the proper solvent at the concentrations desired for testing and 50 ul of the test or control substance was added to each flask and mixed well. The flasks were gassed with 5% CO_2 in air, the caps tightened and the cells incubated for 5 hours at 37°C in 5% CO_2 in air, at 90+ % humidity. After the 5 hour incubation, the flasks were removed from the incubator, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G-Complete and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours at 37°C in 5% CO_2 in air, at 90+ % humidity.

CHO/HGPRT
Mammalian Cell Forward Gene Mutation Assay
PH 314-BO-001-84

Subculturing for Expression of Mutation

Following the 19 hour incubation after test substance treatment, the cultures were removed from the incubator and the medium aspirated from the flasks. The cultures were washed twice with 5 ml of Ca-Mg-free Saline G. The cells were then placed at 37°C in 0.5 ml of a 0.05% trypsin solution. The flasks were examined under an inverted microscope to ensure that the cells had rounded and 1.5 ml of medium F12FCM5 was added. The cells were washed off the surface of the flask using a plugged pasteur pipette. A cell number was determined for each culture. An aliquot was diluted to 1000 cells/ml and 0.2 ml of this aliquot plated for initial survival in 3-60 mm plates containing 5 ml of media F12FCM5. The plates were incubated for seven days. The clones were then fixed, stained and counted. An additional aliquot yielding 1×10^6 cells were subcultured into a 100 mm dish containing 10 ml of medium F12FCM5. These cultures were incubated at 37°C in a 5% CO_2 in air for phenotypic expression. A subculture was taken on Day 3 and Day 5. On Day 3 the subculture was performed by washing the cells twice with 5 ml of Ca-Mg-free Saline G and placing them with 0.5 ml of 0.05% trypsin solution at 37°C. The cells were allowed to round and 1.5 ml of F12FCM5 was added to each plate. The cells were washed off the surface of the plate using a plugged pasteur pipette. A cellular density was determined and an aliquot of the suspension containing 1×10^6 cells was added to a plate containing 10 ml of medium F12FCM5. The subculture was repeated on Day 5.

Selection of Mutant CHO Cells (6-TG-Resistant)

On Day 7 the cells were washed once with 5 ml of Ca-Mg-free Saline G and then placed in 0.5 ml of a 0.05% trypsin solution at 37°C. When the cells rounded up, 1.5 ml of hypoxanthine-free medium F12FCM5 was added and the cells washed from the 100 mm dish with a pasteur pipette. A cell density was determined and the cells diluted to 1×10^5 cells/ml in hypoxanthine free medium F12FCM5. This aliquot of cells was used for selection and an additional aliquot was reserved for a determination of cloning efficiency. For mutant selection, 6.25 ml of 10^{-3} M 6-thioguanine solution was added to 494 ml of hypoxanthine free medium F12FCM5. To each of 5 100 mm plates, 8 ml of the TG medium were added and 2 ml of the 1×10^5 cells/ml aliquot, for a total of 2×10^5 cells/plate. The plates were incubated for 7 days at 37°C in 5% CO_2 in air to allow for colony formation. The colonies were then fixed in methyl alcohol, stained with a dilute crystal violet solution, counted, and the clone numbers recorded. The total number of mutant clones on the five plates was determined.

Cloning Efficiency

From the aliquot reserved before mutant selection, a 1:100 dilution was performed in Saline G to a cellular density of 1×10^3 cells/ml. This aliquot was used to determine cloning efficiency. From this dilution, 0.2 ml was aliquoted into each of three 60 mm plates containing 5 ml of hypoxanthine free medium F12FCM5 (200 cells/plate). These plates served as viable counts (cloning efficiency) plates and were incubated for 7 days at 37°C in 5% CO_2 in air, fixed, stained and counted for a measure of cloning efficiency (average of the three 60 mm plates).

A – c

CHO Metaphase Analysis
In Vitro Chromosome Aberration Analysis
in Chinese Hamster Ovary Cells (CHO)
PH 320-BO-001-84

MATERIALS AND METHODS

Introduction: CHO cells exposed in vitro to a clastogenic agent at any stage of the cell cycle may incur damage which hinders cell division resulting in loss of the cell once it has to divide. First post-treatment mitosis must be scored in order to detect primary aberrations as opposed to derived aberrations which may be transmitted to further generations. Thus, the sensitivity of this assay may be reduced if cells are permitted to cycle through a second mitosis. CHO cells have a generation time of approximately 12-14 hours. One harvest interval has been selected to maximize the probability of detecting chromosomal aberrations. This interval is 14-18 hours after 5 hours pretreatment with the test article.

Cell Line Designation: CHO-K1-BH4, Lot #8

obtained from: Dr. Abraham W. Hsie
Biology Division
Oak Ridge National Laboratories
P.O. Box Y
Oak Ridge, Tennessee 37830

Solubility and Test Article Preparation: Test article, Iprodione, was evaluated in this study utilizing dimethylsulfoxide (DMSO, Mallickrodt, Lot # TK1321) the vehicle. The test article was determined to be soluble at 100 mg/ml. Stock solutions were prepared based on the addition of 100 ul per 10 ml treatment volume in the aberration assay giving a final solvent concentration of 1%. All dilutions were prepared from the stock prior to treatment and excellent solutions were obtained at all levels evaluated. A white precipitate was noted at the 150 ug/ml level of both the non-activated and activated series and the 400 ug/ml level of the activated series.

16

CHO Metaphase Analysis
In Vitro Chromosome Aberration Analysis
in Chinese Hamster Ovary Cells (CHO)
PH 320-BO-001-84

004613

S-9 Metabolic Activation System: The S-9 activation mixture was prepared immediately prior to treatment. The S-9 mix contained (per ml) 8 umol $MgCl_2$ $6H_2O$, 8 umol $CaCl_2$ $2H_2O$, 33 umol KCl, 5 umol glucose-6-phosphate, 4 umol NADP (disodium salt), 50 umol sodium phosphate buffer (pH 7.4) and 0.1 ml of the microsomal preparation containing 40 mg protein/ml in the aberration assay. The microsomal preparation was obtained from Aroclor 1254 induced rat liver.

Cytotoxicity: Test article, Iprodione, was assayed for cytotoxicity utilizing the CHO/HGPRT Forward Gene Mutation Assay at doses of 0.03, 0.1, 0.3, 1.0, 3.33, 10, 33.3, 100, 333 and 1000 ug/ml of medium both with and without activation. Concurrent untreated and solvent controls were also evaluated.

Dose Selection: Generally, the highest dose selected for the CHO Chromosome Aberration Assay is that which shows approximately 40% relative cell survival in the CHO/HGPRT Mammalian Forward Gene Mutation Assay. Based on the cytotoxicity data, the doses selected for evaluation in the aberration assay for Iprodione were 15, 75 and 150 ug/ml of medium in the non-activated series and 40, 150 and 400 ug/ml of medium in the activated series.

ABERRATION ASSAY PROTOCOL

Preparation of Cells: Cells in logarithmic growth were detached with 0.05% trypsin solution and plated at a density of 8×10^5 cells/75 cm^2 flask in 15 ml medium containing 5% fetal bovine serum. Cells were grown for approximately 24 hours.

Control Articles:

(1) Solvent controls were dissolved in (DMSO) [Mallinckrodt, Lot #TK1321] which served as the solvent for the test article. 100 ul of solvent was dispensed at a final concentration of 1%.

(2) Positive controls - The following known mutagenic agents were selected:

(a) Ethylmethane Sulfonate (EMS) [Sigma Chemical Company, Lot #89C-0439] which is a direct acting mutagen was the positive control for the non-activated series. EMS was dissolved in DMSO (dimethylsulfoxide) and dispensed at 100 ul for a final treatment concentration of 1000 ug/ml of medium.

(b) Dimethylnitrosamine (DMN) [Aldrich Chemicals Lot #0220LH] which requires metabolic activation to act as a mutagen was the positive control for the activated series. DMN was dissolved in DMSO and dispensed at 100 ul for a final treatment concentration of 1000 ug/ml of media.

B-5

CHO Metaphase Analysis
In Vitro Chromosome Aberration Analysis
in Chinese Hamster Ovary Cells (CHO)
PH 320-BO-001-84

Treatment: Each level of treatment and controls were tested in duplicate both with and without S-9 metabolic activation. Following a 16-24 hour growth period, the medium was aspirated from the flasks and fresh medium was dispensed. Non-activated test and control cultures were supplied with 10.0 ml of F12FCM(5%) medium. Activated treated and control cultures were supplied with 8.0 ml F12FCM(5%) medium and 2.0 ml of S-9 mixture. Treatment was initiated by the addition of 100 ul of test article or control solutions to the appropriate cultures. Cultures were gassed with 5% CO_2 in air and tightly capped and treated for 5 hours at 37°C in 5% CO_2 at 90+ % humidity.

Following treatment, cells were washed three times in 5 ml washes of Saline-G and supplied with 10 ml of medium F12FCM(5%). The cultures were incubated at 37°C, 5% CO_2 and 90+ % humidity for an additional 14-18 hours.

Slide Preparation: For the last 2-3 hours of incubation, colcemid (2×10^{-7} M final concentration) was added to each culture to arrest dividing cells in metaphase. At the end of incubation, cell suspensions were collected by the metaphase shake-off method. Cells were sedimented and hypotonic KCl (0.075M) added to swell the cells. Cells were fixed with three washes of methanol:glacial acetic acid (3 parts:1 part) and slides prepared by standard methods. Slides were air dried and stained with 3% Giemsa in

DNA DAMAGE IN BACILLUS SUBTILIS #2214

TESTER STRAINS

Nineteen different strains of B. subtilis, deficient in different recombination (rec⁻), excision (exc⁻), or polymerase (pol⁻) repair steps and/or combinations of these mutations were used to test for lethal DNA damage. These include Rec⁻ strains recA1, rec8, recB2, recD3, recC5, recE4, and recG13, mc-1, and m45; Pol⁻ strain T-1 provided by Dr. F. Tomita and TKJ8201 provided by Dr. H. Tanooka; exc⁻ strains, TKJ5211, TKJ8206 and hcr-9; exc⁻ and rec⁻ strain fh2006-7; exc⁻, rec⁻ and pol⁻ strain HJ15; exc⁻, pol⁻ and spore repair (spp⁻) strain TKJ6321; and repair-efficient strains HA101 and 168 WT. (For this study, the tester strains were numbered as shown in Appendix 1.) The mutants in this series are sensitive to the metabolic toxicity and/or genotoxic activity of a broad range of chemical substances at nanomolar or nanogram concentrations. Furthermore, which of the mutants will show an increased sensitivity to DNA-damaging agents compared to the wild type strains, HA101 or 168, depends upon the class of chemical and which steps in repair are required for removal and recovery from the damaging agent.

EXPERIMENTAL DESIGN

A spot test was performed by streaking overnight cultures, their cell densities adjusted so that they were in the log growth phase (OD_{540} = 0.3), onto nutrient agar or brain/heart infusion agar plates (B.B.L). Cultures were streaked radially to a centered sensitivity disc containing 10 µl of the assay solution in the presence or absence of metabolic activation. All assays were performed in duplicate. After overnight incubation at 37° C the distance (mm) of growth inhibition from the disc periphery was measured and recorded for each strain.

A quantitative assessment of DNA damage was also performed for the strains showing the greatest DNA-damaging sensitivity toward the test material in the spot test assay (if the test materials had shown no DNA-damaging or cytotoxic activity, the mutants showing the greatest DNA-damaging sensitivity to the positive control chemical would have been used). The D_{37} (the dose at which there was an average of one lethal DNA-damaging event per cell in the

population as a consequence of exposure to the test chemical) was determined in this assay. The assay was conducted in duplicate at two cell dilutions (10^5 and 10^6). The results from the 10^6 dilution were not reported because of contamination; however we were able to obtain satisfactory results from the 10^5 dilution.

For metabolic activation, liver microsomes were prepared from Sprague-Dawley rats induced with Aroclor 1254 (supplied by Litton Bionetics, Kensington, MD). The S-9 (metabolic activator) was prepared as a KCl homogenate with a protein activity of 25.0 mg/ml. The S-9 homogenate (2.0 ml) was combined with 1.0 ml salt solution, 0.25 ml glucose-6-phosphate, 2.0 ml NADP, 25.0 ml sodium phosphate buffer and 19.75 ml sterile distilled water on the day of the test. For the non-activated assay, KCl (0.15 M) was used alone.

EVALUATION CRITERIA

Data were evaluated using the two-fold rule or the modified two-fold rule, as they were applicable to the type of assay that was performed. By the two-fold rule, a single dose response must exceed the control by two-fold. By the modified two-fold rule, two consecutive dose levels must exceed the control by two-fold or the last concentration at which metabolic toxicity does not cause the test to be uninterpretable must exceed the control by two-fold.

Specific application of the two-fold criterion to the spot test (semi-quantitative assay) differs from its application to the D_{37} (quantitative assay). Methods of evaluating of the two assays are therefore presented separately.

Spot Assay

The inhibition zone produced by any substance tested, including positive control chemicals, must be greater than 1 mm (distance from the last point of growth to the disc periphery) and the inhibition zone must be at least twice that produced by the solvent (if any zone is produced). To be positive for DNA damage, the average zone of inhibition produced in a repair-deficient mutant must exceed that produced in at least one wild type strain by more than

1 mm. If the zone of inhibition in the repair-deficient mutant exceeds that in the wild type by 2 mm or more, the result is considered to be positive at the concentration tested. If the difference in inhibition is between 1 mm and 2 mm, the result is considered questionable and the test must be repeated. However, if a questionable result is again obtained, only a quantitative assay can be used to make an appropriate interpretation.

The strains selected for the D_{37} assay were chosen on the following basis: Strain 2, which has a recA repair-deficient mutation, was positive at the highest and lowest dose levels (1670 and 20.6 µg/disc) in the DNA spot assay, without metabolic activation. Strain 2 was equivocal in the presence of S-9 activation at the 1670 µg/disc dose level.

Because there were sporadic positives in strains which had either rec⁻ or exc⁻ repair deficiencies, it was decided to perform the assay with strain 10. Strain 10, which has two repair deficiencies, Rec⁻ and Exc⁻, was equivocal for Iprodione at 1670 µg/disc and positive at the 20.6 µg/disc level without metabolic activation, it was also positive in the presence of S-9 activation at the highest dose level.

Strain 11, wild type, has all repair intact and was used as the control because strains 2 and 10 are isogenic with B. subtilis strain 11.

Quantitative DNA-damaging assay (D_{37})

The fractional survival (N/N_0) is plotted versus all compound concentrations on semilog graph paper for the wild type bacteria and for repair-deficient mutants showing the greatest DNA-damaging sensitivity toward the test material. The concentration that gives 37% survival with the wild type bacteria and the concentration that gives 37% in the mutant bacteria are determined and compared. If the dose at which 37% of the wild type bacteria survive is two-fold or greater than the dose giving 37% survival in the mutant and a dose-related response is obtained then the test material is considered to be active in the DNA-damaging assay.

In Vitro Sister Chromatid Exchange in
Chinese Hamster Ovary Cells (CHO)
PH 319-BO-001-84

MATERIALS AND METHODS

Introduction: Chinese Hamster Ovary cells (CHO) when grown in culture in the presence of the base analog 5-bromo-2'-deoxyuridine for two consecutive replication cycles exhibit differential staining of their sister chromatids when stained with a Flourescence-plus-Giemsa (FPG) staining technique (Perry and Wolff, 1974 and Goto et al, 1978). This allows identification of each chromatid at the second mitotic division. Sister chromatid exchanges (SCE) are observed at this time as reciprocal alterations in staining pattern along the two chromatids of a chromosome. Chinese Hamster Ovary Cells are known to have an average cell cycle time of 12-14 hours.

Cell Line Designation: CHO-K1-BH4, Lot #8

Obtained from: Dr. Abraham W. Hsie
Biology Division
Oak Ridge National Laboratories
P.O. Box Y
Oakridge, Tennessee 37830

Subcloned by: Pharmakon Research International, Inc.

Solubility and Test Article Preparation: Test article Iprodione was evaluated in this study utilizing DMSO as the vehicle and the test article was determined to be soluble at 100 mg/ml. Stock solutions were prepared on the addition of 100 ul per 10 ml media in the SCE assay giving a final solvent concentration of 1%. All dilutions were prepared from the stock prior to treatment. White precipitate was noted at the 200 and 400 ug/ml in the activated series of the CHO-SCE assay.

S-9 Metabolic Activation System: The S-9 activation mixture was prepared immediately prior to treatment. The S-9 mix contained (per ml) 8 umol $MgCl_2$ $6H_2O$, 8 umol $CaCl_2$ $2H_2O$, 33 umol KCl, 5 umol glucose-6-phosphate, 4 umol NADP (disodium salt), 50 umol sodium phosphate buffer (pH 7.4) and 0.1 ml of the microsomal preparation containing 40 mg protein/ml in the SCE-CHO assay. The microsomal preparation was obtained from Aroclor 1254 induced rat liver.

In Vitro Sister Chromatid Exchange in
Chinese Hamster Ovary Cells (CHO)
PH 319-BO-001-84

004613

Cytotoxicity: Cytotoxicity for the test article, Iprodione, was based upon the relative cell survival data in the CHO/HGPRT Mammalian Forward Gene Mutation Assay, Study Number, PH 314-BO-001-84 conducted May 1, 1984-July 30, 1984 at Pharmakon Research International, Inc. In this assay, Iprodione was tested for cytotoxicity at doses of 0.03, 0.1, 0.3, 1.0, 3.33, 10, 33.3, 100, 333 and 1000 ug/ml of medium both with and without activation. Concurrent and solvent controls were evaluated.

Dose Selection: Generally, the highest dose selected for the CHO-SCE assay is that which gives approximately 40% relative cell survival in the CHO/HGPRT Mammalian Forward Gene Mutation assay. Iprodione was more toxic in cultures without the S-9 activation system, therefore, the levels for this series were 5, 10, 25, 50, 75 and 100 ug/ml. Dose levels for the activated series were 5, 50, 100, 200 and 400 ug/ml.

CHO-SCE ASSAY PROTOCOL

Preparation of Cells: Exponentially growing CHO-K1-BH4 cells are plated in 75 cm^2 tissue culture flasks at an initial density of 8×10^5 cells in 15 ml of medium F12FCM(5%) and incubated for 16-24 hours.

Control Articles: (1) Solvent controls were treated with DMSO, [Mallinckrodt, Inc. Lot #73560] which served as the solvent for the test article. 100 ul of solvent was dispensed at a final concentration of 1%. Untreated controls were also evaluated.
Untreated controls were also evaluated.

(2) Positive Controls - The following known mutagenic agents were selected:

(a) Ethylmethane Sulfonate (EMS) [Sigma, Lot #89C-0439], which requires no metabolic activation was the positive control for the non-activated series. EMS was dissolved in DMSO and dispensed at 100 ul for a final treatment concentration of 124 ug/ml of medium.

(b) Dimethylnitrosamine (DMN) [Aldrich CAS #62-75-9] which requires metabolic activation to act as a mutagen with positive control for the activated series. DMN was dissolved in DMSO and dispensed at 50 ul for a final treatment concentration of 25 ug/ml of medium.

Treatment: Each level of treatment and controls were tested in duplicate both with and without S-9 metabolic activation. Following a 16-24 hour growth period, the medium was aspirated from the flasks and fresh medium was dispensed. Non-activated test and control cultures were supplied with 10.0 ml of F12FCM(5%) medium. Activated treated and control cultures were supplied with 8.0 ml F12FCM(5%) medium and 2.0 ml of S-9 mixture. Treatment was initiated by the addition of 100 ul of test article or control solutions to the appropriate cultures. Cultures were gassed with 5% CO_2 in air and tightly capped and treated for 5 hours at 37°C in 5% CO_2 at 90+ % humidity.

23

Following treatment, cells were washed three times in 5 ml washes of Saline-G and supplied with 10 ml of medium F12FCM(5%) containing 5-bromo-2'-deoxyuridine (10^{-5} M final concentration). The cultures were incubated at 37°C, 5% CO_2 and 90+ % humidity for an additional 24-28 hours.

Cultures were incubated in the dark. Exposure to white light was avoided until cells were stained with Hoechst 33258 following slide preparation.

Slide Preparation: For the last 2-3 hours of incubation, colcemid (2×10^{-7} M final concentration) was added to each culture to arrest dividing cells in metaphase. At the end of incubation, cell suspensions were collected by the metaphase shake-off method. Cells were sedimented and hypotonic KCl (0.075M) added to swell the cells. Cells were fixed in three washes of methanol: glacial acetic acid (3 parts: 1 part) and slides prepared by conventional methods. Slides were stored in the refrigerator until stained. Staining of slides included: 1.0 ug/ml Hoechst 33258 stain, black light irradiation and 3% Giemsa stain. Slides were air-dried and coverslips mounted.

Coding of Slides: Slides were coded randomly by study number and number designation. Each duplicate culture was assigned a separate code and the results from duplicate cultures pooled for analysis.

Slide Analysis: A total of 50 well-spread, second division cells containing ± 2 centromeres from the modal number of 20 chromosomes were scored for each dose level. SCE are scored as reciprocal alterations in staining pattern along the chromatids of a chromosome. Cells are counted for chromosome number and data is presented as SCE/cell and SCE/chromosome. These results are summarized in Table 1. Ratios of first, second and third metaphases are recorded based on 200 metaphases. Results are summarized in Table 2.

Summary of Metaphases Scored: In this assay, a total of 50 second division cells were scored for each dose level except DMN in which 40 cells were scored.

Evaluation Criteria: Assessment of a test article as positive is based upon its ability to produce a statistically significant increase in the SCE frequency as compared to the concurrent solvent control. For biological significance, there should be a two-fold increase in SCE frequency as compared to the solvent control.

Statistical Analysis: Duplicate cultures are pooled to make a total of 50 scored cells (except DMN) per dose level. These pooled cultures were separated into activated and non-activated series. These slides were analyzed separately. The SCE/cell data was transformed by a standard square root transformation. A "t" test on the transformed data compared each dose level in a series against its concurrent solvent control.

Criteria for a Valid Assay: To be valid, the positive control must show a significant ($p \leq 0.05$) increase in SCE frequency is compared to the solvent control.