TRADE SECRET

Study Title

H-23960: *In Vitro* Mammalian Chromosome Aberration Test in Chinese Hamster Ovary (CHO) Cells

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Performing Laboratory Study Number

AA26XN.331.BTL

DuPont Project ID

DuPont-3871

Work Request Number

[redacted]

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

BioReliance Study Director: Ramadevi Gudi 04/05/00
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Approved by
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TABLE OF CONTENTS

STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: [redacted]

- H-23960

[redacted]

Haskell Number: 23960

Composition: [redacted]

Known Impurities: [redacted]

Physical Characteristics: [redacted]

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Solubility: Aquatics: soluble but cloudy in water at concentrations ≥15 mg/mL.

Sponsor: E. I. du Pont de Nemours and Company
Haskell Laboratory for Toxicology and Industrial Medicine
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Study Initiated/Completed: February 25, 2000 / (see report cover page)

In-Life Initiated/Completed: February 29, 2000 / March 27, 2000

SUMMARY

The test substance, H-23960, was tested in the *in vitro* chromosome aberration test using Chinese hamster ovary (CHO) cells in both the absence and presence of an Aroclor-induced exogenous S9 metabolic activation system. A preliminary toxicity assay was performed to establish the dose range for the chromosome aberration assay. The chromosome aberration assay was used to evaluate the clastogenic potential of the test substance.

Water was determined to be the solvent of choice based on information provided by the Sponsor, the solubility of the test substance, and compatibility with the target cells. The test substance was soluble but cloudy in water at a concentration of 50 mg/mL, the maximum concentration tested.

In the preliminary toxicity assay, the maximum dose level tested was 5000 µg/mL. The test substance was soluble in the treatment medium at all dose levels tested in the non-activated studies. Visible precipitates were observed in the treatment medium at dose levels ≥1500 µg/mL in the S9 activated study. Dose levels of ≤500 µg/mL were soluble in the treatment medium in the S9 activated study. Selection of dose levels for the chromosome aberration assay was based on total cell growth inhibition relative to the solvent control. No substantial toxicity, i.e., at least 50% cell growth inhibition, was observed at any dose level tested in either the non-activated or the S9 activated treatment groups. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 625 to 5000 µg/mL for both the non-activated and the S9 activated treatment groups.

In the chromosome aberration assay, the cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9 activated test system, and all cells were harvested at 20 hours after treatment initiation. The test substance was soluble in treatment medium at all dose levels tested in the non-activated studies. Visible precipitates were observed in the treatment medium at the dose levels ≥2500 µg/mL in the S9 activated study. The dose levels of ≤1250 µg/mL were soluble in treatment medium in the S9 activated study. No substantial toxicity (at least 50% cell growth inhibition) was observed at the highest dose level evaluated for chromosome aberrations, 5000 µg/mL, in the non-activated 4 hour and 20 hour exposure groups, respectively. No substantial toxicity (at least 50% cell growth inhibition) was observed at the highest dose level evaluated for chromosome aberrations in the S9 activated study, 2500 µg/mL. The highest dose level selected for analysis of chromosome aberrations the non-activated studies, 5000 µg/mL, was based upon the absence of at least 50% toxicity and the lack of test substance precipitation in the treatment medium. The highest dose level selected for analysis of chromosome aberrations in the S9 activated study, 2500 µg/mL, was based upon the absence of at least 50% toxicity and the presence of test substance precipitation in the treatment medium (the lowest precipitating dose was selected as the highest dose to evaluate). The non-activated and S9 activated 4 hour treatment groups were scored for structural and numerical chromosome aberrations. No statistically significant increases in structural and numerical chromosome aberrations were observed in the non-activated or S9 activated 4 hour treatment

groups relative to the solvent control group, regardless of dose level ($p>0.05$, Fisher's exact test). In the absence of a positive response in the non-activated 4 hour treatment group, the non-activated 20 hour continuous treatment group was evaluated for structural and numerical chromosome aberrations. A statistically significant increase in structural chromosome aberrations was observed in the non-activated 20 hour continuous exposure group relative to the solvent control group, at the dose level 5000 µg/mL ($p\leq0.05$, Fisher's exact test). The Cochran-Armitage test was also positive for a dose response ($p\leq0.05$). However, the percentage of structurally aberrant cells observed at the dose level 5000 µg/mL (2.5%) was within the range of structurally aberrant cells observed with the historical solvent control (0%-6%). Therefore, the statistically significant increase in the percentage of structurally aberrant cells observed at 5000 µg/mL was not considered biologically significant. No statistically significant increases in numerical chromosome aberrations were observed in the non-activated 20 hour continuous exposure group relative to the solvent control group, regardless of dose level ($p>0.05$, Fisher's exact test). The positive and solvent controls fulfilled the requirements for a valid test. Under the conditions described in this report, H-23960 was concluded to be negative for the induction of structural and numerical chromosome aberrations in the non-activated and S9 activated *in vitro* mammalian chromosome aberration test in Chinese hamster ovary (CHO) cells.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of the test substance, H-23960, based upon its ability to induce *in vitro* chromosome aberrations in the Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test substance, H-23960, was received by BioReliance on 15 February 2000 and was assigned the code number AA26XN. The test substance was characterized by the Sponsor as an [redacted] that should be stored in a well-ventilated place at temperatures below 120°F. An expiration date for the test substance was not provided.

Upon receipt, the test substance was described as a [redacted] and was stored at room temperature, in a well-ventilated area, protected from exposure to light.

Based on information provided by the Sponsor, sterile distilled water (CAS 7732-18-5), obtained from the Life Technologies Company, was the solvent used to deliver H-24335 to the test system.

Mitomycin C (MMC; CAS No.: 50-07-7), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 1 and 2 µg/mL for use as the positive control in the non-activated test system. Cyclophosphamide (CP; CAS No.: 6055-19-2), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 100 and 200 µg/mL for use as the positive control in the S9 activated test system. For each positive control one dose with sufficient scorable metaphase cells was selected for analysis. The solvent for the test substance was used as the solvent control at the same concentration as that found in the test substance-treated groups.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from the American Type Culture Collection, Manassas, VA, on May 29, 1997. In order to assure the karyotypic stability of the cell line, working cell stocks were not used beyond passage 20. CHO cells at passage 6 were used for the preliminary toxicity and CHO cells at passage 12 were used for the chromosome aberration assay. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination. This cell line has an average cell cycle time of 10-14 hours with a modal chromosome number of 20. The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston et al., 1981).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the exogenous metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µL S9 per milliliter medium (McCoy's 5A serum-free medium supplemented with, 100 units penicillin and 100 µg streptomycin/mL, and 2 mM L-glutamine).

Preliminary Toxicity Assay

The preliminary toxicity assay was performed for the purpose of selecting dose levels for the chromosome aberration assay and consisted of an evaluation of test substance effect on cell growth. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 4.5 mL complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units penicillin and 100 µg streptomycin/mL, and 2 mM L-glutamine) for the non-activated study or S9 reaction mixture (3.5 mL serum-free medium plus 1 mL of 5X S9 mix) for the activated study, to which 500 µL dosing solution of test substance in solvent or solvent alone was added. The osmolality of the highest concentration of the dosing solution in the treatment medium was measured. The pH of the highest concentration of the dosing solution in the treatment medium was measured using test tape. The cells were treated for 4 hours with and without S9, and continuously for 20 hours without S9. At completion of the 4 hour exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with 5 mL complete medium and returned to the incubator for a total time period of 20 hours from the initiation of the treatment. At 20 hours after the initiation of the treatment the cells were harvested by trypsinization and counted using a Coulter counter. The presence of test substance precipitate was assessed using the unaided eye. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

Chromosome Aberration Assay

The chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of CHO cells to the test substance as well as positive and solvent

controls. For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 4.5 mL complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin and 100 µg streptomycin/mL, and 2 mM L-glutamine) for the non-activated study or 4.5 mL S9 reaction mixture for the S9 activated study, to which 500 µL of dosing solution of test or control article in solvent or solvent alone was added. The osmolality of the highest concentration of the dosing solution in the treatment medium was measured. The pH of the highest concentration of the dosing solution in the treatment medium was measured using test tape.

In the non-activated study, the cells were exposed to the test substance for 4 hours or continuously for 20 hours up to the cell harvest at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (Swierenga et al., 1991). In the 4 hour exposure group, the treatment medium was removed after the exposure period, and the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks returned to the incubator until cell collection.

In the S9 activated study, the cells were exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (Swierenga et al., 1991). After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks were returned to the incubator until cell collection.

A concurrent toxicity assay was conducted in both the non-activated and the S9 activated assay systems. After cell harvest an aliquot of the cell suspension was removed from each culture and counted using a Coulter counter. The presence of test substance precipitate was assessed using the unaided eye. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

Cell Harvest

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the non-activated and S9 activated studies by trypsinization. Cells were collected approximately 20 hours after initiation of treatment (Galloway et al., 1994). The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 mL 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 mL Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-8°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant was aspirated, and 1 mL fresh fixative was added. After additional centrifugation (at approximately 800 rpm for 5 minutes) the supernatant fluid was decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a glass slide and allowed to air dry. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. To ensure that a sufficient number of metaphase cells were present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) was determined for each treatment group. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Initially, the non-activated and S9 activated 4 hour exposure groups were evaluated for chromosome aberrations and if a positive result was obtained in the non-activated 4 hour exposure group, the non-activated 20 hour continuous exposure group was not evaluated for chromosome aberrations. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). The number of metaphase spreads that are examined and scored per duplicate flask may be reduced if the percentage of aberrant cells reaches a statistically significant level before 100 cells are scored. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥10 aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. Polyploid and endoreduplicated cells were evaluated from each treatment flask per 100 metaphase cells scored.

Controls

Mitomycin C was used as the positive control in the non-activated study at final concentrations of 0.1 and 0.2 µg/mL. Cyclophosphamide was used as the positive control in the S9 activated study at final concentrations of 10 and 20 µg/mL. For both positive controls one dose level exhibiting a sufficient number of scorable metaphase cells was selected for

analysis. The solvent vehicle for the test substance was used as the solvent control at the same concentration as that found in the test substance-treated groups. DuPont studies [redacted] were conducted simultaneously and thus the same solvent and positive controls were used between these two studies.

Evaluation of Test Results

The toxic effects of treatment were based upon cell growth inhibition relative to the solvent-treated control and are presented for the toxicity and aberration studies. The number and types of aberrations found, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per cell was calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test substance dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test substance was considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). Test substances not demonstrating a statistically significant increase in aberrations will be concluded to be negative. Negative results with metabolic activation may need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not necessary, justification will be provided.

Criteria for a Valid Test

The frequency of cells with structural chromosome aberrations in the solvent control must be within the range of the historical solvent control. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control.

Deviations

No known deviations from the protocol or assay method SOPs occurred during the conduct of this study.

Archives

All raw data, the protocol, all reports, and stained and coded slides will be maintained according to Standard Operating Procedure [redacted] by the BioReliance Regulatory

Affairs/Quality Assurance Unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD, 20850.

RESULTS AND DISCUSSION

Solubility

Water was determined to be the solvent of choice based on information provided by the Sponsor, the solubility of the test substance, and compatibility with the target cells. The test substance was soluble but cloudy in water at a concentration of 50 mg/mL, the maximum concentration tested.

Preliminary Toxicity Assay

Dose levels for the chromosome aberration assay were selected following a preliminary toxicity assay and were based upon a reduction of total cell growth (cell growth inhibition) relative to the solvent control. The results of the evaluation of cell growth inhibition are presented in Tables 1-3. CHO cells were exposed to solvent alone and to nine concentrations of test substance ranging from 0.5 µg/mL to 5000 µg/mL in the absence and presence of an S9 reaction mixture. The test substance was soluble in treatment medium at all dose levels tested in the non-activated studies. Visible precipitates were observed in the treatment medium at the dose levels ≥1500 µg/mL in the S9 activated study. The dose levels ≤500 µg/mL were soluble in treatment medium in the S9 activated study. The osmolality of the solvent (water) in treatment medium was 314 mmol/kg. The osmolality in treatment medium of the highest concentration tested, 5000 µg/mL, was 315 mmol/kg, basically identical to the osmolality of the solvent. The pH of the highest concentration of test substance in treatment medium was approximately 7.0. No substantial cell growth inhibition (≥50%) relative to the solvent control was observed at any dose level tested in both the non-activated and S9 activated treatment groups. Based upon the results of the toxicity study, the dose levels selected for testing in the chromosome aberration assay were as follows:

Treatment Condition	Treatment Time (hr)	Recovery Time (hr)	Dose levels (µg/mL)
-S9	4	16	625, 1250, 2500, 5000
	20	0	625, 1250, 2500, 5000
+S9	4	16	625, 1250, 2500, 5000

Chromosome Aberration Assay

In the chromosome aberration assay, the test substance was soluble in treatment medium at all dose levels tested in the non-activated studies. Visible precipitates were observed in the

treatment medium at the dose levels ≥2500 µg/mL in the S9 activated study. The dose levels ≤1250 µg/mL were soluble in treatment medium in the S9 activated study. The osmolality in treatment medium of the highest concentration tested, 5000 µg/mL, was 226 mmol/kg, exactly the same as the osmolality of the solvent (water) in treatment medium. The pH of the highest concentration of test substance in treatment medium was approximately 7.0.

Cell growth inhibition relative to the solvent control in CHO cells was 25% after treatment with H-23960 at 5000 µg/mL for 4 hours in the absence of S9 activation (Table 4). This was the highest dose level evaluated for chromosome aberrations. The ability of H-23960 to induce chromosome aberrations is presented by treatment flask in Table 5, and summarized by treatment group in Table 10. The mitotic index was 7% reduced relative to the solvent control at the highest dose level evaluated for chromosome aberrations, 5000 µg/mL. The dose levels selected for microscopic analysis were 1250, 2500, and 5000 µg/mL. The percentage of cells with structural and numerical aberrations in the test substance-treated groups was not significantly increased above that of the solvent control ($p>0.05$, Fisher's exact test). The percentage (18.5%) of structurally damaged cells in the MMC group was found to be statistically significant.

Cell growth inhibition relative to the solvent control in CHO cells was13% after treatment with H-23960 at 2500 µg/mL for 4 hours in the presence of S9 activation (Table 6). This was the highest dose level evaluated for chromosome aberrations. The ability of H-23960 to induce chromosome aberrations is presented by treatment flask in Table 7, and summarized by treatment group in Table 10. The mitotic index at the highest dose level evaluated for chromosome aberrations, 2500 µg/mL, was 25% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 625, 1250, and 2500 µg/mL, in the absence of at least 50% cell growth inhibition. The percentage of cells with structural or numerical aberrations in the test substance-treated groups was not significantly increased above that of the solvent control ($p>0.05$, Fisher's exact test). The percentage (26.5%) of structurally damaged cells in the CP group was found to be statistically significant.

In the absence of a positive response in the non-activated 4 hour exposure group, slides from the non-activated 20 hour exposure group were evaluated for chromosome aberrations. Cell growth inhibition relative to the solvent control was 19% at 5000 µg/mL (Table 8), the highest dose level evaluated for chromosome aberrations in the non-activated 20 hour continuous exposure group. The ability of H-23960 to induce chromosome aberrations is presented by treatment flask in Table 9, and summarized by treatment group in Table 10. The mitotic index at the highest dose level evaluated for chromosome aberrations, 5000 µg/mL, was 14% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 1250, 2500, and 5000 µg/mL. The percentage of cells with structural aberrations in the test substance-treated groups was significantly increased above that of the solvent control only at dose level 5000 µg/mL ($p\leq0.05$, Fisher's exact test). The Cochran-Armitage test was also positive for a dose response ($p\leq0.05$). However, the percentage of structurally aberrant cells observed at dose level 5000 µg/mL (2.5%) was within the range of structurally aberrant cells

observed with the historical solvent control (0%-6%). Therefore, the statistically significant increase in the percentage of structurally aberrant cells observed at 5000 µg/mL was not considered biologically significant. The percentage of cells with numerical aberrations in the test substance-treated groups was not significantly increased above that of the solvent control ($p>0.05$, Fisher's exact test). The percentage (28.5%) of structurally damaged cells in the MMC group was found to be statistically significant.

The study was concluded to be negative. An independent repeat assay was not required because no unique metabolic requirements were known about the test substance and because no equivocal responses were observed.

CONCLUSION

The positive and solvent controls fulfilled the requirements for a valid test.

Under the conditions described in this report, H-23960 was concluded to be negative for the induction of structural and numerical chromosome aberrations in the non-activated and S9 activated *in vitro* mammalian chromosome aberration test in Chinese hamster ovary (CHO) cells.

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TABLE 1

PRELIMINARY TOXICITY ASSAY WITH H-23960 IN CHO CELLS IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1] (µg/mL)	Cell Count ($x10^5$)	Cell Viability[2] (%)	Mean Cells/ Flask[3] ($x10^5$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Water	2.50	99%	2.47	100%	---
H-23960 0.5	2.32	99%	2.30	93%	7%
1.5	2.94	98%	2.88	117%	-17%
5	2.89	98%	2.84	115%	-15%
15	2.87	99%	2.85	115%	-15%
50	2.85	99%	2.83	114%	-14%
150	2.83	97%	2.74	111%	-11%
500	2.88	98%	2.82	114%	-14%
1500	2.71	98%	2.65	107%	-7%
5000	2.53	97%	2.45	99%	1%

[1] CHO cells were treated in the absence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells
[4] Growth index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 2

PRELIMINARY TOXICITY ASSAY WITH H-23960 IN CHO CELLS IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1] (µg/mL)	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Cells/ Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Water	2.51	95%	2.38	100%	---
H-23960					
0.5	2.57	99%	2.54	107%	-7%
1.5	2.63	99%	2.60	109%	-9%
5	2.62	98%	2.57	108%	-8%
15	2.68	100%	2.68	113%	-13%
50	2.40	96%	2.30	97%	3%
150	2.32	99%	2.30	96%	4%
500	2.33	98%	2.28	96%	4%
1500[6]	2.28	95%	2.17	91%	9%
5000[6]	2.65	97%	2.57	108%	-8%

[1] CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells
[4] Growth index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index; not calculated for negative controls.
[6] Visible precipitates observed.

TABLE 3

PRELIMINARY TOXICITY ASSAY WITH H-23960 IN CHO CELLS IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment[1] (µg/mL)	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Cells/ Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Water	2.99	98%	2.93	100%	—
H-23960 0.5	2.94	96%	2.82	96%	4%
1.5	3.32	98%	3.19	109%	-9%
5	2.78	95%	2.64	90%	10%
15	2.79	93%	2.59	88%	12%
50	3.09	97%	2.99	102%	-2%
150	2.90	97%	2.81	96%	4%
500	3.07	95%	2.92	100%	0%
1500	3.03	98%	2.97	101%	-1%
5000	2.69	91%	2.45	84%	16%

[1] CHO cells were treated continuously in the absence of an exogenous source of metabolic activation for 20 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells
[4] Growth index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 4

CONCURRENT TOXICITY ASSAY WITH H-23960 IN CHO CELLS IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1] (µg/mL)	Flask	Cell Count Averages ($x10^5$)	Cell Viability[2]	Mean Cells per Flask[3] ($x10^5$)	Cell Growth Index[4] (%)	Cell Growth Inhibition5 (%)
Water	A	2.52	96%			
	B	3.10	98%	2.73	100%	—
H-23960						
625	A	2.85	96%			
	B	2.79	99%	2.74	101%	-1%
1250	A	2.49	97%			
	B	2.85	95%	2.56	94%	6%
2500	A	2.43	98%			
	B	2.48	95%	2.37	87%	13%
5000	A	1.99	98%			
	B	2.21	97%	2.04	75%	25%
MMC, 0.1	A	1.95	98%			
	B	2.05	97%	1.95	71%	29%
MMC, 0.2	A	2.07	97%			
	B	2.03	95%	1.97	72%	28%

[1] CHO cells were treated in the absence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4] Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 5

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH H-23960 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1,8] (µg/mL)	Flask	Mitotic Index[2] (%)	Cells Scored	% Aberrant Cells[3]		Total Number of Structural Aberrations						Severely Damaged Cells[6]	Average Aberrations Per Cell[7]
				Numerical	Structural	Gaps	Chromatid[4]		Chromosome[5]				
							Br	Ex	Br	Dic	Ring		
Water	A	11.0	100	2	0	1	0	0	0	0	0	0	0.000
	B	11.4	100	3	1	0	0	0	0	1	0	0	0.010
H-23960													
1250	A	10.6	100	4	1	1	0	0	0	1	0	0	0.010
	B	9.8	100	0	1	0	1	0	0	0	0	0	0.010
2500	A	8.8	100	4	1	0	0	0	0	1	0	0	0.010
	B	7.8	100	2	1	0	1	0	0	0	0	0	0.010
5000	A	10.8	100	2	1	1	1	0	0	0	0	0	0.010
	B	10.0	100	3	1	1	1	0	0	0	0	0	0.010
MMC, 0.2	A	9.6	100	2	16	1	14	11	4	0	0	0	0.290
	B	10.6	100	5	21	0	19	7	1	3	0	0	0.300

1 CHO cells were treated for 4 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
2 Mitotic index = number mitotic figures x 100/500 cells counted.
3 Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.
4 Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
5 Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
6 Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
7 Severely damaged cells and pulverizations were counted as 10 aberrations.
8 An additional dose level of 625 µg/mL was tested as a safeguard against excessive toxicity at higher dose levels but was not required for microscopic examination.

TABLE 6

CONCURRENT TOXICITY ASSAY WITH H-23960 IN CHO CELLS IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1] (µg/mL)	Flask	Cell Count Averages ($x10^5$)	Cell Viability[2]	Mean Cells per Flask[3] ($x10^5$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Water	A	2.84	98%			
	B	2.35	98%	2.55	100%	—
H-23960						
625	A	2.40	98%			
	B	2.56	97%	2.42	95%	5%
1250	A	2.11	98%			
	B	2.08	97%	2.04	80%	20%
2500[6]	A	2.03	98%			
	B	2.12	97%	2.02	79%	21%
5000[6]	A	1.68	98%			
	B	2.04	97%	1.81	71%	29%
CP, 10	A	1.62	97%			
	B	1.71	99%	1.63	64%	36%
CP, 20	A	1.36	98%			
	B	1.50	96%	1.38	54%	46%

[1] CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4] Growth Index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index; not calculated for negative controls.
[6] Visible precipitates observed.

TABLE 7

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH H-23960 IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1,8] (µg/mL)	Flask	Mitotic Index[2] (%)	Cells Scored	% Aberrant Cells[3]		Total Number of Structural Aberrations						Severely Damaged Cells[6]	Average Aberrations Per Cell[7]
				Numerical	Structural	Gaps	Chromatid[4]		Chromosome[5]				
							Br	Ex	Br	Dic	Ring		
Water	A	12.0	100	3	2	1	2	0	0	0	0	0	0.020
	B	11.2	100	4	3	0	2	0	0	1	0	0	0.030
H-23960													
625	A	9.4	100	4	3	2	1	2	0	0	0	0	0.030
	B	10.4	100	4	4	0	4	0	0	0	0	0	0.040
1250	A	8.6	100	4	6	0	5	1	0	0	0	0	0.060
	B	9.8	100	5	5	0	6	0	0	0	0	0	0.060
2500[9]	A	8.4	100	8	6	1	6	0	0	0	0	0	0.060
	B	9.0	100	7	5	2	4	1	0	0	0	0	0.050
CP, 10	A	7.6	100	2	25	5	35	6	0	0	0	0	0.410
	B	8.2	100	1	28	2	39	3	0	0	0	0	0.420

[1] CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2] Mitotic index = number mitotic figures x 100/500 cells counted.
[3] Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.
[4] Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5] Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6] Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7] Severely damaged cells and pulverizations were counted as 10 aberrations.
[8] Dose level 5000 µg/mL was not analyzed due to test substance precipitation in the treatment medium.
[9] Lowest precipitating dose level.

TABLE 8

CONCURRENT TOXICITY ASSAY WITH H-23960 IN CHO CELLS IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment[1] (µg/mL)	Flask	Cell Count Averages ($x10^5$)	Cell Viability[2]	Mean Cells per Flask[3] ($x10^5$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Water	A	2.34	97%			
	B	2.79	99%	2.51	100%	—
H-23960						
625	A	2.52	95%			
	B	2.82	99%	2.59	103%	-3%
1250	A	2.59	99%			
	B	2.85	97%	2.66	106%	-6%
2500	A	2.20	100%			
	B	2.38	97%	2.25	90%	10%
5000	A	2.30	96%			
	B	1.94	97%	2.04	81%	19%
MMC, 0.1	A	2.43	99%			
	B	2.06	95%	2.18	87%	13%
MMC, 0.2	A	1.79	98%			
	B	1.66	96%	1.67	67%	33%

[1] CHO cells were treated in the absence of an exogenous source of metabolic activation for 20 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4] Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 9

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH H-23960 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment[1,8] (µg/mL)	Flask	Mitotic Index[2] (%)	Cells Scored	% Aberrant Cells[3]		Total Number of Structural Aberrations						Severely Damaged Cells[6]	Average Aberrations Per Cell[7]
				Numerical	Structural	Gaps	Chromatid[4]		Chromosome[5]				
							Br	Ex	Br	Dic	Ring		
Water	A	10.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	9.6	100	0	0	0	0	0	0	0	0	0	0.000
H-23960													
1250	A	7.6	100	2	1	0	0	1	0	0	0	0	0.010
	B	7.8	100	1	0	0	0	0	0	0	0	0	0.000
2500	A	10.0	100	2	1	0	1	0	0	0	0	0	0.010
	B	9.2	100	0	0	0	0	0	0	0	0	0	0.000
5000	A	8.4	100	2	3	0	2	0	0	0	1	0	0.030
	B	9.0	100	0	2	0	1	0	0	1	0	0	0.020
MMC, 0.1	A	11.6	100	2	30	4	27	18	5	3	2	0	0.550
	B	10.2	100	1	27	0	14	16	4	2	2	0	0.380

[1] CHO cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2] Mitotic index = number mitotic figures x 100/500 cells counted.
[3] Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.
[4] Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5] Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6] Severely-damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7] Severely damaged cells and pulverizations were counted as 10 aberrations.
[8] An additional dose level of 625 µg/mL was tested as a safeguard against excessive toxicity at higher dose levels but was not required for microscopic examination.

TABLE 10

SUMMARY: CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH H-23960

Treatment (µg/mL)	S9 Activation	Treatment Time	Mean Mitotic Index	Cells Scored	Aberrations Per Cell (Mean +/- SD)		Cells With Aberrations Numerical (%)	Cells With Aberrations Structural (%)
Water	-	4	11.2	200	0.005	±0.071	2.5	0.5
H-23960								
1250	-	4	10.2	200	0.010	±0.100	2.0	1.0
2500	-	4	8.3	200	0.010	±0.100	3.0	1.0
5000	-	4	10.4	200	0.010	±0.100	2.5	1.0
MMC, 0.2	-	4	10.1	200	0.295	±0.722	3.5	18.5**
Water	+	4	11.6	200	0.025	±0.157	3.5	2.5
H-23960								
625	+	4	9.9	200	0.035	±0.184	4.0	3.5
1250	+	4	9.2	200	0.060	±0.258	4.5	5.5
2500	+	4	8.7	200	0.055	±0.229	7.5	5.5
CP, 10	+	4	7.9	200	0.415	±0.785	1.5	26.5**
Water	-	20	10.1	200	0.000	±0.000	0.0	0.0
H-23960								
1250	-	20	7.7	200	0.005	±0.071	1.5	0.5
2500	-	20	9.6	200	0.005	±0.071	1.0	0.5
5000	-	20	8.7	200	0.025	±0.157	1.0	2.5*
MMC, 0.1	-	20	10.9	200	0.465	±0.924	1.5	28.5**

[1] Cells from all treatment conditions were harvested at 20 hours after the initiation of the treatments.
[2] Severely damaged cells were counted as 10 aberrations.
[3] *, p≤0.05; **, p≤0.01; Fisher's exact test.

APPENDIX A

Historical Control Data

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST USING CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES STRUCTURAL CHROMOSOME ABERRATIONS 1996-1998

NON-ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	1.3	1.4	25.9
Standard Deviation	1.3	1.3	19.3
Range	0.0 to 6.0	0.0 to 6.0	6.5 to 100.0

S9-ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	1.5	1.6	34.0
Standard Deviation	1.3	1.4	18.0
Range	0.0 to 6.0	0.0 to 6.5	6.5 to 100.0

1 Solvents include water, saline, dimethyl sulfoxide, ethanol, acetone, non-standard solvents and Sponsor-supplied vehicles.

2 Positive control for non-activated studies, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/ml), and Mitomycin C (MMC, 0.08-0.15 µg/ml).

3 Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/ml), and benzo(α)pyrene, (B[α]P, 30 µg/ml).

IN VITRO MAMMALIAN CHROMOSOME ABERRATION USING CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES NUMERICAL CHROMOSOME ABERRATIONS 1996-1998

NON-ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	2.4	2.3	3.2
Standard Deviation	1.7	1.3	1.9
Range	0.0 to 9.5	0.0 to 8.0	0.0 to 9.5

S9-ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	2.4	3.1	3.5
Standard Deviation	1.7	2.1	2.2
Range	0.0 to 7.0	0.0 to 13.5	0.0 to 10.5

1 Solvents include water, saline, dimethyl sulfoxide, ethanol, acetone, and other non-standard solvents and Sponsor-supplied vehicles.

2 Positive control for non-activated studies, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/ml), and Mitomycin C (MMC, 0.08-0.15 µg/ml).

3 Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/ml), and benzo(α)pyrene (B[α]P, 30 µg/ml).

APPENDIX B

Study Protocol

H-23960 [redacted] *In Vitro* Mammalian Chromosome Aberration Test in Chinese Hamster Ovary (CHO) Cells

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test substance based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: E.I. du Pont de Nemours and Company

2.2 Address: Haskell Laboratory for Toxicology and Industrial Medicine
P.O. Box 50, Elkton Road
Newark, DE 19714-0050

2.3 Study Monitor : Maria Donner, Ph.D.

2.4 Sponsor Project #: DuPont-3871
WR#: [redacted]
Haskell #: 23960
Service Code: [redacted]

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Substance [redacted]

3.2 Test Substance Name to be used in the Report: H-23960

3.3 Controls: Solvent: Water
Positive: Mitomycin C (MMC)
Cyclophosphamide (CP)

3.4 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test substance, and the stability and strength of the test substance in the solvent (or vehicle).

3.5 Test Substance Retention Sample

The retention of a reserve sample of the test substance will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Toxicology Testing Facility BioReliance
4.2	Address:	9630 Medical Center Drive Rockville, MD 20850
4.3	Study Director:	Ramadevi Gudi, Ph.D. Phone: (301) 610-2169 Fax: (301) 738-2362 E-mail: rgudi@bioreliance.com

5.0 TEST SCHEDULE

5.1	Proposed Experimental Start Date:	2/29/2000
5.2	Proposed Experimental Termination Date:	3/31/2000
5.3	Proposed Report Date:	4/14/2000

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Manassas, VA. The stability of the modal chromosome number of the cell line is routinely checked and the cell line is routinely tested and determined to be free from mycoplasma contamination. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The chromosome aberration test will be conducted using standard procedures (Evans, 1976), by treatment cultures of CHO cells to a minimum of four concentrations of the test article as well as to positive and solvent controls. DuPont Studies 3870 and 3871 will be conducted simultaneously and thus the same solvent and positive controls will be used between these two studies. In the non-activated test system, treatment will be for 4 hours and for 20 hours; in the S9 activated test system, treatment will be for 4 hours (Swierenga et

al., 1991). To ensure evaluation of first division metaphase cells the dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 20 hours (1.5 normal cell cycles) after the initiation of treatment (Galloway et al., 1994). The clastogenic potential of the test article will be measured by its ability to induce structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group. In the event of a positive response in the 4 hour non-activated study, the prolonged treatment non-activated study may not be scored. The test article will also be assessed for its ability to induce numerical chromosome aberrations.

7.1 Solubility Determination

Water will be used as the test article solvent based on the solubility data available with the Sponsor.

7.2 Preliminary Toxicity Test for Selection of Dose Levels

Selection of the dose levels for the cytogenetics assay will be done in consultation with the Sponsor , and added to the protocol by an amendment based upon post-treatment toxicity (cell growth inhibition relative to the solvent control) and solubility of the test substance. CHO cells will be treated to solvent alone and to at least nine concentrations of test substance. The highest concentration tested will be 5 mg/ml or 10 mM whichever is lower for freely soluble test substances, or the maximum concentration resulting in a workable suspension for poorly soluble test substances not to exceed 5 mg/ml. The pH will be measured at the highest test substance treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest dose level, lowest precipitating dose level (where applicable) and the highest soluble dose level (where applicable) in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be treated for 4 hours in the absence and presence of S9 and for 20 hours in the absence of S9. Just prior to trypsinization the cell cultures will be visually inspected for the extent of monolayer confluency relative to the solvent control. Twenty hours after treatment initiation the cells will be harvested by trypsinization and counted using an automatic cell counter and the cell viability will be assessed using trypan blue dye exclusion (SOP # [redacted]). The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control (SOP # [redacted]).

Whenever possible, the high dose to evaluate chromosome aberrations will be selected to give at least 50% cytotoxicity observed as (cell growth inhibition relative to the solvent control) irrespective of solubility. The highest dose will not to exceed 5 mg/ml or 10 mM. At least two additional dose levels, demonstrating minimal or no toxicity, will be included. In the event the test substance cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, then the highest dose to be tested in the chromosome aberration assay will be the concentration resulting in minimum precipitation in test medium. Precipitation will be determined

by direct visual inspection. In the event the test substance demonstrates a dose-responsive increase in toxicity at concentrations that exceed solubility in treatment medium, then the highest dose to be tested will be the maximum concentration that results in at least 50% toxicity. In the event that neither cytotoxicity nor insolubility is observed in the preliminary test, the highest dose in the chromosome aberration assay will be 5 mg/ml or 10 mM whichever is lower. If excessive precipitation of the test substance-solvent solution occurs upon addition to treatment medium, or if the osmolality of the treatment medium is considered excessive, the Sponsor will be consulted.

7.3 Frequency and Route of Administration

Target cells will be treated for 4 hours in the absence and presence of S9, and for 20 hours in the absence of S9, by incorporation of the test substance-solvent mixture into the treatment medium. This technique has been demonstrated to be an effective method of detection of chemical clastogens in this test system (Evans, 1976).

No repeats of the chromosome aberration tests will be done.

7.4 Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch preparation of S9 will be assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$,) 6 mM potassium chloride (KCl), 1mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per ml serum free medium.

7.5 Controls

7.5.1 Solvent (or Vehicle) Control

The solvent for the test substance will be used as the solvent control. For solvents other than water, physiological buffer, or medium, the final concentration in treatment medium will not exceed 1%.

7.5.2 Positive Controls

Mitomycin C will be used at a concentration within 0.05-0.3 µg/ml

as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 µg/ml as the positive control in the S9-activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 µg streptomycin/ml) for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask. The flasks will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the BioReliance study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated treatment or 5 ml S9 reaction mixture for the S9-activated treatment, to which will be added 50 µl of dosing solution of test or control substance in solvent or solvent alone. Larger volumes of dosing solution may be used if water, physiological buffer, or medium is used as the solvent.

In the non-activated study, the cells will be treated for 4 hours and for 20 hours; in the S9-activated study the cells will be treated for 4 hours. Treatment will be carried out at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the 4 hour treatment period in the non-activated and the S9-activated studies, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

7.9 Cell Harvest

Cells will be collected approximately 20 hours after initiation of treatment. This post-treatment harvest time represents approximately 1.5 normal cell cycles and was selected to ensure that the cells are analyzed in the first division metaphase after initiation of treatment. Two hours prior to cell harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 µg/ml.

Cells will be harvested by trypsinization, collected by centrifugation and an aliquot will be removed for counting using an automatic cell counter and trypan blue dye exclusion. The remainder of the cells will be swollen with 0.075M KCl, washed

H-23960 [redacted] *In Vitro* Mammalian Chromosome Aberration Test in Chinese Hamster Ovary (CHO) Cells DuPont-3871
BioReliance Study Number:AA26XN.331.BTL

with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-8°C. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control (% toxicity). To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. The suspension of fixed cells will be applied to glass microscope slides and air-dried. The slides will be identified by the experiment number, treatment condition and date. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined and recorded for each coded treatment group selected for scoring chromosome aberrations. Slides will be coded using random numbers by an individual not involved with the scoring process. In the event of a positive response in the 4 hour non-activated study, the prolonged treatmentnon-activated study may not be scored. Metaphase cells with 20 ± 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads from each dose level (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). The number of metaphase spreads that will be examined and scored per duplicate flask may be reduced if the percentage of aberrant cells reaches a statistically significant level before 100 cells are scored. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The percent polyploid and endoreduplicated cells will be evaluated per 100 cells for each dose level analyzed for structural aberrations.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Solvent Control

The frequency of cells with structural chromosome aberrations in the solvent control must be within the range of the historical solvent control and not exceed 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control.

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of the treatments are based upon cell growth inhibition relative to the solvent control and will be presented for the toxicity and aberration studies. The number and types of aberrations found, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per cell will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test substance dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test substance will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). Test substances not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by BioReliance and will accurately describe all methods used for generation and analysis of the data.

Results presented will include, but not be limited to:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test substance, if known.

- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.

- Source of cells, karyotype features (modal chromosome number) and suitability of the cell type used, absence of mycoplasma, cell cycle length, passage number.

- Test conditions: composition of medium; CO_2 concentration; incubation time; cell seeding density; solvent and solvent selection rationale; concentration of test substance and concentration selection rationale; composition and acceptability criteria for the metabolic

activation (S9) system; duration of treatment; duration of treatment with and concentration of Colcemid®; type of metabolic activation system used; positive and solvent controls; methods of slide preparation; number of cell cultures; criteria for scoring aberrations and criteria for considering studies positive, negative.

• Results: description of precipitation; pH and osmolality of the treatment medium; cell growth inhibition relative to the solvent control; mitotic index and number of metaphases analyzed; type and number of aberration (structural and numerical) given separately for each treated and control culture; concentration-response relationship; statistical analysis; historical control data.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure [redacted] by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This Non-GLP study will be performed using the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies as a general guideline.

This protocol has been written to comply with OECD Guideline 473 (*In Vitro* Mammalian Chromosome Aberration Test), February 1998 and with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

Will this study be submitted to a regulatory agency? NO

If so, to which agency or agencies? NA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

13.0 REFERENCES

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Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

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Swierenga S.H.H., J.A. Heddle, E.A. Sigal, J.P.W. Gilman, R.L. Brillinger, G.R. Douglas and E.R. Nestmann (1991) Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese lung and human lymphocyte cultures, Mutation Research 246:301-322.

14.0 APPROVAL

Maria Donner 2/25/00
STUDY MONITOR DATE

Maria Donner, Ph.D.
(Print or Type Name)

Ramadevi Gudi 2/25/00
BIORELIANCE STUDY DIRECTOR DATE

BIORELIANCE STUDY MANAGEMENT DATE