

IR-424

Container No. 10



84940000302



721-95-001244

INIT 03/28/95

PHARMAKON RESEARCH INTERNATIONAL, INC.

Waverly, Pennsylvania 18471

0 0 0 3

82-79

PHARMAKON RESEARCH INTERNATIONAL, INC.

WAVERLY, PENNSYLVANIA 18471

PHONE
(717) 586-2411

RECEIVED

SEP 08 1982

F. J. KOSCHIER

CHO/SCE

In Vitro Sister Chromatid Exchange
in Chinese Hamster Ovary Cells (CHO)

PH 319-AC-003-82

Glyoxal 40 LF

Project No. CT-096-82

~~REC-3-28-85~~

302

Submitted to

American Cyanamid Company
Wayne, New Jersey

Ruth M. Sorg

Ruth M. Sorg
Study Director

Robert W. Naismith

Robert W. Naismith, Ph.D.
Director of Toxicology

Richard J. Matthews

Richard J. Matthews, Ph.D.
Director of Research

August 31, 1982

0 0 0 9

TABLE OF CONTENTS

	PAGE
PURPOSE.....	1
BACKGROUND.....	1
METHODS.....	1
Introduction.....	1-2
Solubility and Test Article Preparation.....	2
S-9 Metabolic Activation System.....	2
CYTOTOXICITY.....	2
Dose Selection.....	2-3
ASSAY PROTOCOL.....	3
Preparation of Cells.....	3
Control Articles.....	3
Treatment.....	3-4
Slide Preparation.....	4
Differential staining of Sister Chromatids.....	4
DATA ANALYSIS.....	4
Slide Analysis.....	4
Evaluation Criteria.....	4
Criteria for a valid Assay.....	5
CONCLUSION.....	5
References.....	5
TABLES.....	6
Table I.....	6
Table II.....	7
Table III.....	8
PROTOCOL DEVIATION.....	9
QUALITY ASSURANCE STATEMENT.....	10

In Vitro Sister Chromatid Exchange in Chinese Hamster Ovary Cells

Purpose: To evaluate the ability of test article Glyoxal 40 LF to induce Sister Chromatid Exchange (SCE) in Chinese Hamster Ovary Cells In Vitro.

Date Cytotoxicity initiated: June 14, 1982

Date CHO/SCE Assay initiated: July 27, 1982, August 24, 1982 (Retest)

Date of completion: August 31, 1982

Pharmakon Reference: Notebook #332; pgs. 78-82
Notebook #357, pgs. 6-8, 10-17

Sponsor's Study Director: Francis Koschier, Ph.D.

Pharmakon Study Director: Ruth M. Sorg

Test Article Description: Glyoxal 40 LF was received as a clear liquid in a plastic jar. The bottle was stored at room temperature throughout the study.

Stability and Purity: There was no apparent change in the physical state of the test or control articles during the assay. The purity of the test article was the responsibility of the sponsor.

Cell line Designation: CHO-K1-BH4, Lot# 7182ORNL
obtained from: Dr. Abraham W. Hsie
Biology Division
Oak Ridge National Laboratories
P. O. Box Y
Oak Ridge, Tennessee 37830

Good Laboratory Practices Statement: This study was conducted in compliance with the Good Laboratory Practices Regulations as stated in the Federal Register, Vol. 43, No. 247, Friday, December 22, 1978.

Records Maintained: All correspondence pertinent to the study between the sponsor and Pharmakon Research International, Inc. protocol, amendments to the protocol, raw data, test substance weight or volume, dispensation reports, quality assurance reports, the final report as well as microscope slides scored in the study and a sample of the test chemical are maintained in the Pharmakon Archives.

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 fluorescence plus Giemsa staining technique (Perry and Wolff 1974 and Goto et al. 1978). Sister chromatid exchanges (SCE) are observed at this time as reciprocal alterations in staining pattern along the chromatids of a chromosome and can be quantitated through a compound microscope with white light illumination.

SCE are widely accepted as sensitive indicators of mutagenic and/or carcinogenic potential. A statistically significant increase in the frequency of SCE following treatment with the test article as compared to the solvent control is taken as indication of DNA damaging potential.

Solubility and Test Article Preparation: Test article Glyoxal 40 LF was evaluated in this study using distilled water as the vehicle. Glyoxal 40 LF was soluble at all levels tested. In both the cytotoxicity and the CHO/SCE assay, stock solutions were prepared based on the addition of 50 ul of Glyoxal 40 LF solution per flask giving a final solvent concentration of 1%. All dilutions were prepared from the stock prior to treatment.

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 $MgCl_2$, 8 umol $CaCl_2$, 33 umol KCl, 5 umol glucose-6-phosphate, 4 umol NADP, 50 umol sodium phosphate buffer (pH 8.0) and 0.1 ml of the microsomal preparation containing at least 30 mg protein/ml. The microsomal preparation was obtained from Aroclor 1254 induced rat liver.

Cytotoxicity: Test article Glyoxal 40 LF was assayed for cytotoxicity at doses of 1000, 750, 333, 250, 100, 33.3, 10, 3.33, 1.0 and 0.33 ug/ml of media in single 25 cm^2 cultures both with and without S-9 metabolic activation. Concurrent, distilled water and untreated controls were evaluated. Cultures were treated for 5 hours in serum free Hams F12 media followed by a 27 hour post treatment culture in the presence of 10^{-5} M BrDU (5-bromo-2'-deoxyuridine) in media Hams F12 supplemented with 5% fetal bovine serum. Colchicine (5 ug/ml) was added for the final 2 hours of culture. All cells were pooled by flask and cell counts were made. Metaphase preparations were made and stained for sister chromatid differentiation according to a modified fluorescence plus Giemsa method. Slides were evaluated for mitotic index, ploidy and for the ratios of first, second, and third division metaphases as determined by the differential staining patterns of the sister chromatids. Cytotoxicity is determined by a decrease in cell count and/or a shift in the cell cycle kinetics. At the levels tested, a dose related effect on cell cycle kinetics was noted. In the non-activated series, there were no metaphase cells at the high doses of 1000 and 750 ug/ml. Virtually all metaphases were in first division at the 333 ug/ml dose. At 250 ug/ml, 26% of metaphases were in second division and at 100 ug/ml the percentage of second division cells was 78. The remaining dose levels of 33.3, 10, 3.33, 1.0, and 0.33 ug/ml had a normal cell cycling pattern.

In the metabolically activated series the cytotoxic effect was enhanced. The top three levels of 1000, 750 and 333 ug/ml gave no metaphase cells, and 40 total metaphases (cell M-1) were seen at the 250 ug/ml levels. At 100 ug/ml, 10% second divisions were seen and at 33.3 ug/ml, 75% of the metaphases were in second division. The remaining dose levels of 10, 3.33, 1.0, and 0.33 ug/ml had a normal cell cycling pattern.

A reduction in cell count was also noted at the top levels in both series. Cytotoxicity data may be found in Table I.

Dose Selection: Since analysis of sister chromatid exchanges is dependent upon the availability of second division metaphase cells, the highest dose

generally selected in that which shows a cytotoxic effect, yet produces sufficient second division metaphases at the time of cell harvest to permit a reliable analysis. Based on the cytotoxicity data and a post treatment, culture time of 27 hours, and in discussion with the sponsor, two dosing regimes were selected for the CHO/SCE assay. Doses selected for the non-activated series were 250, 200, 100, 50, and 10 ug of Glyoxal 40 LF per ml of culture. Doses selected for the metabolically activated series were 100, 75, 50, 25, and 10 ug of Glyoxal 40 LF per ml of culture.

SISTER CHROMATID EXCHANGE ASSAY

Preparation of cells: Cells in logarithmic growth were detached with 0.05% trypsin solution and plated at a density of 5×10^5 cells/25 cm² flask in 5 ml medium containing 5% fetal bovine serum. Cells were grown for approximately 24 hours.

Control Articles:

- (1) Solvent controls were treated with distilled H₂O which served as the solvent for the test article. 50 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) which requires no metabolic activation was the positive control for the non-activated series. EMS was dissolved in DMSO (dimethylsulfoxide) and dispensed at 20 ul for a final treatment concentration of 10^{-3} M.
 - (b) Dimethylnitrosamine (DMN) 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 20 ul for a final treatment concentration of 5×10^{-4} M.

Treatment: Each level of treatment and controls were tested in duplicate both with and without S-9 metabolic activation. Dose volume of Glyoxal 40 LF and distilled water was 50 ul/5 ml total culture or 1% concentration. Positive control articles were delivered in 20 ul volumes/5 ml total culture or 0.4% solvent concentration. Following a 16-24 hour growth period, the cells were washed twice in Saline (with Ca⁺⁺ and Mg⁺⁺). Non-activated test and control cultures were supplied with 5.0 ml of serum free Hams F12 media. Activated treated and control cultures were supplied with 4.0 ml of serum free Hams F12 media and 1.0 ml of S-9 mixture.

Treatment was initiated by the addition of 50 ul of test article solutions or distilled water or 20 ul of positive control mutagen to the appropriate cultures. Cultures were gassed with 5% CO₂ in air and tightly capped and

treated for 5 hours at 37°C in 5% CO₂ at 90+ % humidity.

Following treatment, cells were washed three times in 5 ml washes of Saline-G (Ca⁺⁺ and Mg⁺⁺) and supplied with 5 ml of medium F12FCM5 (5% fetal bovine serum) containing 10⁻⁵ M BrDU (5-bromo-2'-deoxyuridine). The cultures were incubated at 37°C, 5% CO₂ and 90+ % humidity for an additional 27 hours.

During exposure to the BrDU, all cultures were protected from white light. After 25 hours of exposure to BrDU, colcemid (2 x 10⁻⁶ M final concentration) was added to the culture for an additional two hours.

Slide Preparation: At the end of incubation, cell suspensions were collected into labelled centrifuge tubes. The remaining cells were collected by adding 0.5 ml of a 0.05% trypsin solution to each flask and incubating at 37°C until the cells detached. Following the addition of 1.5 ml of medium F12FCM5, a cell count was taken on a Coulter Counter. Cells were sedimented by centrifugation and supernatant discarded. Hypotonic KCl (0.075M at 37°C) was added to swell the cells. Following centrifugation, cells were fixed three times in 3:1 methanol-glacial acetic acid and resuspended in a small amount of fixative. The cell suspension was dropped on chilled clean wet glass slides and air dried. Preparations were stained in Hoechst 33258 (5 µg/ml in Sorensens M/15 buffer) followed by incubation under UV BLAK-RAY[®] light to achieve sister chromatid differentiation and dried overnight. Slides were stained in 3% Giemsa and mounted with coverslips. Upon inspection of positive control slides, it was determined that the control article for the activated series did not produce an increase in the SCE frequency. Since DMN requires metabolic activation to produce such an effect, this lack of response invalidated this part of the study. Therefore, the activated series was repeated and this repeat study was scored.

DATA ANALYSIS

Slide Analysis: Slides were coded and 30 well spread, intact second division cells were counted for each culture. SCE were scored as reciprocal alterations in staining pattern along the chromatids of a chromosome. Cells were counted for chromosome number and data is presented as SCE/cell and SCE/chromosome. Following decoding of the slides, the mitotic index was recorded for each culture based on 500 total cells and ratios of first, second, and third metaphases are calculated based on 100 metaphases. These data are summarized in Table II.

Evaluation Criteria: Assessment of a test article as positive is based upon its ability to produce a statistically significant increase in the SCE frequency in test cultures as compared to the solvent controls. Cases with statistically significant increases which show less than a doubling are evaluated according to their ability to produce a dose response. Data is presented as SCE/cell and SCE/chromosome. The SCE/cell data was transformed by a square root transformation and a one-tailed "t" test was employed to determine significant increases of test means from solvent control means. The activated series data was analyzed separately from the non-activated series. Data is summarized in Table III.

5

Criteria for a valid assay: To be valid, the positive controls must show significant ($p < 0.05$) increase in the sister chromatid exchange frequency over the controls. This assay fits this criteria. EMS at 10^{-3} M produced a statistically significant increase in SCE frequency in the non-activated series. DMN at 5×10^{-4} M also produced a statistically significant increase in SCE frequency in the activated series, indicating that the S-9 activation system was functioning biologically.

Conclusion: A statistically significant increase in SCE/cell was found at the highest level of Glyoxal 40 LF scored in the non-activated series. (The highest level tested, 250 ug/ml, did not produce enough second division metaphases for reliable analysis). A slight increase in SCE frequency was noted with each increase in dose in the non-activated series. However, only the 200 ug/ml group showed statistical significance. The activated series also gave a dose related response to Glyoxal 40 LF. In this series, the top three dose levels (50, 75, and 100 ug/ml) produced statistically significant increases ($p < 0.05$) in the sister chromatid exchange frequency. Based upon its ability to produce a statistically significant increase in sister chromatid exchange frequency and the presence of an apparent dose related response to the test article, glyoxal 40 LF is judged positive in its ability to induce sister chromatid exchange in CHO cells in culture under the conditions tested.

References: Perry, P., and S. Wolff 1974. New Giemsa method for the differential staining of Sister Chromatids. Nature Vol. 251, p. 156-158.

Latt S. A. R. R. Schreck, K. S. Loveday, C.P. Dougherty and C. F. Shuler, 1980. Sister Chromatid Exchanges. In "Advances in Human Genetics" Volume (10); Ed. H. Harres and K. Hirschhorn Plenum Press N.Y....

Perry, P. and H. J. Evans, 1975. Cytological detection of mutagen-carcinogen exposure by Sister Chromatid Exchange, Nature Vol. 258, p. 121-125.

Latt, S.A. (1974), Sister Chromatid Exchanges, Indices of Human Chromosome Damage and Repair; Detection by Fluorescence and Induction by Mitomycin C. Proc. National Academy Science USA Vol 71 (8) pp. 3162-3166.

Goto K, Maeder, S., Kano, Y. and Sugiyama T. 1978 Factors involved in differential Giemsa Staining of Sister Chromatids. Chromosoma 66, 351-359, 1978

PHARMAKON RESEARCH INTERNATIONAL, INC.

TABLE I

PH 319-AC-003-82

Glyoxal 40 LF, Cytotoxicity Induced (27 hours)
Cell Count, Mitotic Index and Cell Cycle Kinetics
for dose selection

Dose ug/ml	S-9 ±	Total Cell Count	Mitotic Index (%)	Cell Cycling		
				M-1	M-2	M-3
1000	-	0.53 x 10 ⁶	-	-	-	-
750	-	0.71 x 10 ⁶	-	-	-	-
333	-	1.12 x 10 ⁶	0.8	98	2	0
250	-	1.50 x 10 ⁶	1.0	74	26	0
100	-	2.06 x 10 ⁶	2.2	22	78	0
33.3	-	2.17 x 10 ⁶	1.8	9	91	0
10	-	2.19 x 10 ⁶	1.0	6	94	0
3.33	-	1.90 x 10 ⁶	1.0	10	90	0
1.0	-	2.15 x 10 ⁶	0.6	11	89	0
0.33	-	2.22 x 10 ⁶	1.8	7	93	0
dH ₂ O	-	1.80 x 10 ⁶	2.0	5	95	0
untreated	-	1.88 x 10 ⁶	2.0	8	92	0
1000	+	0.49 x 10 ⁶	-	-	-	-
750	+	0.72 x 10 ⁶	-	-	-	-
333	+	0.83 x 10 ⁶	-	-	-	-
250	+	0.80 x 10 ⁶	-	40	0	0
100	+	1.82 x 10 ⁶	1.0	90	10	0
33.3	+	2.00 x 10 ⁶	2.2	25	75	0
10	+	2.06 x 10 ⁶	2.4	8	92	0
3.33	+	1.61 x 10 ⁶	2.2	14	86	0
1.0	+	1.90 x 10 ⁶	2.0	8	92	0
0.33	+	2.16 x 10 ⁶	2.2	9	91	0
dH ₂ O	+	2.12 x 10 ⁶	2.0	12	88	0
untreated	+	2.15 x 10 ⁶	2.4	12	88	0

- No metaphase cells

PHARMAKON RESEARCH INTERNATIONAL, INC.

TABLE II

PH 319-AC-003-82

Glyoxal 40 LF, CHO/SCE Assay (27 hours)
Cell Count, Mitotic Index and Cell Cycle Kinetics

Dose ug/ml	S-9 ±	Total Cell Count	Mitotic Index (%)	Cell Cycling		
				M-1	M-2	M-3
250	-	1.98 x 10 ⁶	3.2	97	3	0
200	-	1.96 x 10 ⁶	3.6	50	50	0
100	-	2.18 x 10 ⁶	4.4	29	71	0
50	-	2.36 x 10 ⁶	6.8	15	85	0
10	-	3.02 x 10 ⁶	6.6	4	96	0
dH ₂ O	-	2.92 x 10 ⁶	8.8	5	95	0
EMS 10 ⁻³ M	-	2.40 x 10 ⁶	4.0	36	64	0
100	+	2.08 x 10 ⁶	3.0	77	23	0
75	+	2.28 x 10 ⁶	1.8	46	4	0
50	+	1.94 x 10 ⁶	4.0	37	63	0
25	+	2.51 x 10 ⁶	5.0	29	71	0
10	+	2.38 x 10 ⁶	4.6	13	87	0
dH ₂ O	+	2.49 x 10 ⁶	8.8	9	91	0
DMN 5 x 10 ⁻⁴ M	+	2.19 x 10 ⁶	3.6	91	9	0

PHARMAKON RESEARCH INTERNATIONAL, INC.

PH 319-AC-003-82

TABLE III

Results of In Vitro Sister Chromatid Exchange Assay
in Chinese Hamster Ovary Cells for test article Glyoxal 40 LP

Treatment (ug/ml)	Number of Chromosomes	Number of SCE's	SCE/Cell ± S.D.	SCE/Chromosome	Statistical Significance at p < 0.05
WITHOUT ACTIVATION					
250	-	-	-	-	-
200	592	611	20.366 ± 5.720	1.032	Positive
100	600	488	16.266 ± 5.105	0.813	N.S.
50	604	437	14.565 ± 4.591	0.724	N.S.
10	597	407	13.566 ± 4.014	0.682	N.S.
dH ₂ O	598	433	14.433 ± 3.784	0.724	-
EMS 10 ⁻³ M	600	1,957	45.283 ± 6.280	2.26	Positive
WITH METABOLIC ACTIVATION					
100	597	735	24.500 ± 6.134	1.231	Positive
75	596	720	24.000 ± 6.480	1.208	Positive
50	596	619	20.633 ± 4.172	1.039	Positive
25	600	607	20.233 ± 4.931	1.012	N.S.
10	600	545	18.166 ± 4.017	0.908	N.S.
dH ₂ O	596	556	18.533 ± 4.847	0.933	-
DMN 5x10 ⁻⁴ M*	414	1,298	61.809 ± 12.524	3.135	Positive

*Based on 21 cells scored

0013

Protocol Deviation

CHO/SCE

In Vitro Sister Chromatid Exchange
in Chinese Hamster Ovary Cells (CHO)

PN 319-AC-003-82

Glyoxal 40 LF

page 8

line 10

.....Slides are incubated on a slide warmer (55°-60°C) for 15-20 minutes in 2 x SSC with a coverslip placed on each slide to prevent evaporation.

Reason for Deviation: Obtain better differentiation at 15-20 minutes.

page 2

.....and dimethylnitrosamine (DMN 5×10^{-4} M)

line 26

which requires.....

Reason for Deviation: DMN at 5×10^{-4} M is less toxic to the cells and better yield of second mitotic division obtained. At this dose, DMN will also produce a significant increase in SCE frequency.

page 6

line 27

....hours of incubation, colcemid (2×10^{-7} M final concentration) is added to....

Reason for Deviation: Colcemid at 2×10^{-7} M also effectively blocks cells in metaphase.

Ruth M. Sorg
Ruth M. Sorg
Study Director
Pharmakon Research International, Inc.

Date: August 31, 1982

PHARMAKON RESEARCH INTERNATIONAL, INC.

WAVELEN, PENNSYLVANIA 19621

PHOTO
017 196 211

QUALITY ASSURANCE STATEMENT

This study was performed in accordance with the Good Laboratory Practices Regulation for non-clinical laboratory studies as developed by the U.S. Food and Drug Administration, as indicated in the Federal Register, Part II of December 22, 1978; Part 58, Title 21.

Study No. PH 319-AC-003-82

The following inspections were performed:

Interval	Date
<u>Treatment Phase</u>	<u>7/27/82</u>
<u>Slide Preparation & Staining Phase</u>	<u>7/28/82</u>
<u>Reporting</u>	<u>8/31/82</u>
_____	_____
_____	_____
_____	_____

Results of the above inspections were submitted to the Study Director and Management during the course of the study.

8/31/82
Date

[Signature]
Quality Assurance Unit

90 No. _____

Purpose: To determine the cytotoxicity of a test article towards the CHO cell line. Cytotoxicity of a test substance is determined by a reduction in mitotic index and/or a shift in the ratios of first, second, and third division metaphases as determined by the staining pattern of the chromosomes when stained for sister chromatid differentiation. Treatment is for 5 hours both with and without metabolic activation followed by 24-28 hours incubation. ~~Then to~~

S.O.F. # PH 319

Date of initiation: 6/14/82

Sponsor: American Cyanamid Company

Date of completion: 7/26/82

Test Article: CYANAMID glyoxal 40 LF

Cell Culture: Chinese Hamster ovary cells, clone K₁, cell line CHO-K₁-BH₄.

Vehicle: distilled H₂O

Solubility: Soluble at all levels tested

No. units of test article delivered: 50 ul

Test article description: Clear liquid

Post treatment culture time: 27 hours

6/3/82: Frozen aliquot of cell line CHO-K₁-BH₄ Lot # 11882 inoculated into 15 ml media F12 FCS 10 in T-75cm² flask
Amphotericin treated: 1/11/82 Frozen: 1/19/82
Lot 11882 certified mycoplasma free
F12 FCS 10⁺ prep 3/25/82
Fetal Bovine Serum Lot # KC 32 1002

6/14/82: CHO-K₁-BH₄ cells subcultured into 2 T-75cm² flasks at 2x10⁵ cells/flask in 15 ml F12 FCS 10⁺.

6/22/82: Cells subcultured into 2 T-75cm² flasks at 5x10⁵ cells/flask in 5 ml F12 FCS 10⁺.

6/29/82: Cells subcultured into 4 T-75cm² flasks at 5x10⁵ cells/flask in 15 ml F12 FCS 10⁺.

6/11/82: Cells subcultured into 10 T-75cm² flasks at 2x10⁵ cells/flask in 15 ml F12 FCS 10⁺.

To Page No. 79

Reviewed & Understood by me,

Date

Invented by

Date

Edward J. Jodel

7/26/82

Recorded by Ruth M. Gray

7/26/82

Page No. 236/14/82
RMSS-9 Mixture:

full Q H₂O — 8.75 ml
 0.2 M Na₂PO₄ (pH 8.6) — 3.75 ml
 0.1 M NADP — 0.6 ml
 1.0 M G-6-P — 0.075 ml
 1.5 M HCl — 0.3 ml
 0.5 M H₂Cl₂ — 0.3 ml
 0.5 M CaCl₂ · 2H₂O — 0.3 ml
 S-9 Fraction — 1.5 ml
 15.58 ml

S-9 fraction prep:
 4/27/82 by EGG

Contains 42 mg/ml
 protein

6/14/82 Treatment:

Following 2 washes with Saline-G-Complete as above cultures were treated for 5 hours in serum-free F₁₂ media (prep 11/4/81 Ⓢ) both with and without S-9 metabolic activation. _{by EGG}

Non activated series: were dispensed 5 ml serum free F₁₂ media and 50 ul of test article or control.

Activated series: were dispensed 4 ml serum free F₁₂ + 1.0 ml of the S-9 mixture, and 50 ul of test article or control.

Cultures held at 37°C, 5% CO₂ in air and 90+ % humidity following gassing of flask with 5% CO₂ in air. Caps were tightened and cultures incubated 5 hours.

Treatment time: 8:20 AM - 1:20 PM

6/14/82 1:20 PM Cultures washed 3X with Saline-G-Complete (prep 4/27/82 Ⓢ) _{by EGG}

Then 5 ml F₁₂FCMS containing 10⁻⁵ M BrDU dispensed to each flask and cells returned to incubate at 37°C, 5% CO₂ and 90+ % humidity for an additional 27 hours.

BrDU stock prep 6/14/82

Media F₁₂FCMS prep 5/28/82

10⁻⁵ M BrDU in media prep 6/14/82

To Page No. 81

Initiated & Understood by me,

Date

Initiated by

Date

Edmund A. Lodek

7/26/82

Recorded by

Ruth M. Song

7/26/82

Page No. 12

6/15/82 2:20PM 62.5ul colchicine (stock 0.4mg/ml prep 6/15/82) added to each flask → saylal. Flasks inoculated an additional 2 hours at 37°C, 5% CO₂, 90% humidity

6/15/82 Culture harvested. Cells collected into labeled centrifuge tubes and 0.5ml of 0.05% trypsin dispensed to each flask and incubated at 37°C until cells detached. 1.5ml F12 PCMS added to flask + cells pooled with original suspensions. A 200ul sample was taken and counted in 10ml EGTON on a Cottle counter. VOLUME = 7.0ml.

GROUP	TREATMENT	±S-g	Dose (ug/ml)	cell count	# cells / ml	Total cell culture
(-)	glyoxal	-	1000	759	75900	0.53 x 10 ⁶
		-	750	1012	101200	0.71 x 10 ⁶
		-	333	1605	160500	1.12 x 10 ⁶
		-	250	2149	214900	1.50 x 10 ⁶
		-	100	2939	293900	2.06 x 10 ⁶
		-	33	3103	310300	2.17 x 10 ⁶
		-	10	3135	313500	2.19 x 10 ⁶
		-	3.3	2708	270800	1.90 x 10 ⁶
		-	1.0	3074	307400	2.15 x 10 ⁶
		-	0.33	3174	317400	2.22 x 10 ⁶
	dH ₂ O	-	dH ₂ O	2568	256800	1.80 x 10 ⁶
	untreated	-	untreated	2690	269000	1.88 x 10 ⁶
(+))	glyoxal	+	1000	694	69400	0.49 x 10 ⁶
		+	750	1023	102300	0.72 x 10 ⁶
		+	333	1179	117900	0.83 x 10 ⁶
		+	250	1142	114200	0.80 x 10 ⁶
		+	100	2595	259500	1.82 x 10 ⁶
		+	33	2851	285100	2.00 x 10 ⁶
		+	10	2943	294300	2.06 x 10 ⁶
		+	3.3	2306	230600	1.61 x 10 ⁶
		+	1.0	2718	271800	1.90 x 10 ⁶
		+	0.33	3088	308800	2.16 x 10 ⁶
	dH ₂ O	+	dH ₂ O	3031	303100	2.12 x 10 ⁶
	untreated	+	untreated	3069	306900	2.15 x 10 ⁶

To Page No. 82

Prepared & Understood by me, <u>Edward A. Zollik</u>	Date <u>7/26/82</u>	Invented by	Date <u>7/26/82</u>
		Recorded by <u>Ruth H. Gray</u>	

Page No. 21 Cultures handled under safe lights or in subdued light.
 6/15/82 - Cells harvested previously fixed three times (after hypotonic treatment of 10 min in 3°C 0.075M KCl) in 3:1 methanol:glacial acetic acid.
 6/16/82 - Following overnight refrigeration slides prepared. Slides stained 10 min in Hoechst 33258 and incubated = 20 min. at 35-60°C under BUX-D14 UV light, and rinsed in distilled H₂O and stored overnight desiccated.
 6/17/82: Slides stained in 3% Giemsa, cleared in xylene & coverslips mounted with Permount.

GROUP	TREATMENT	±5-9	Dose (µg/ml)	MITOTIC INDEX 500 total cells			M-1; M-2; M-3 100 total METAPHASES			FLOIDY 100 TOTAL METAPHASES	
				# METAPHASE	# OTHER	MI %	M-1	M-2	M-3	# Diploid	# Polyploid (%)
(-)	GLYOXAL LF ↓ GLYOXAL LF & H ₂ O untreated	-	1000	—	no metaphase cells	—	—	—	—	—	—
		-	750	—	no metaphase cells	—	—	—	—	—	—
		-	333	4	496	0.8	98	2	0	96	4
		-	250	5	495	1.0	74	26	0	94	6
		-	100	11	489	2.2	22	78	0	96	4
		-	33	9	491	1.8	9	91	0	99	1
		-	10	5	495	1.0	6	94	0	96	4
		-	3.3	5	495	1.0	10	90	0	96	4
		-	1.0	3	497	0.6	11	89	0	99	1
		-	0.33	4	491	1.8	7	93	0	98	2
(+)	GLYOXAL LF ↓ GLYOXAL LF & H ₂ O untreated	+	1000	—	no metaphase cells	—	—	—	—	—	—
		+	750	—	no metaphase cells	—	—	—	—	—	—
		+	333	—	no metaphase cells	—	—	—	—	—	—
		+	250	0	500	—	40	0	0	32	3** (8.6%)
		+	100	5	475	1.0	90	10	0	95	5
		+	33	11	489	2.2	25	75	0	95	5
		+	10	12	488	2.4	8	92	0	100	0
		+	3.3	11	489	2.2	14	86	0	98	2
		+	1.0	10	490	2.0	8	92	0	97	3
		+	0.33	11	489	2.2	9	91	0	95	5
	GLYOXAL LF & H ₂ O untreated	+	—	10	490	2.0	12	88	0	95	5
		+	—	12	488	2.4	12	88	0	97	3

* Chromosomes darkly stained - difficult to differentiate.
 ** Only 35 cells total scored for polyploidy.

Book 357
 To Page No. 6.

Witnessed & Understood by me, <i>Edmund J. Sidel</i>	Date 7/26/82	Invented by Record 11. 829	Date 7/26/82
---	-----------------	-------------------------------	-----------------

Page No. 82

Purpose: To evaluate the ability of a test article to induce sister chromatid exchange (SCE) in CHO cells in culture.

S.O.P. # 3A

Sponsor: American Dynamid Company
Test article: CYANA CHEM. 9/10XAL 40LF

Date of initiation: 7/27/82
Date of completion: 8/6/82

Cell culture: Chinese hamster ovary cells, clone K, cell line CHO K, BH₄
- received ORNL 7/1/82.

Vehicle: Distilled H₂O

Solubility: Soluble at all levels tested

Volume of test article delivered: 50ul

Test article description: Clear liquid

Post treatment culture time: 27 hours

Length of treatment: 5 hours in serum free F12 media.

Positive control articles: DHN (N-Nitrosodimethylamine - Aldrich Chem.)
for S-9 activated control.
EMS (Ethylmethanesulphonate - Sigma)
for non activated control

7/1/82 CHO/K, BH₄ cell line received from ORNL and cultured
at 10⁵ cells / T 75 cm² flasks in F12 FCS 10 media.
Fetal Bovine Serum lot # KC 321002H

Stock maintained in culture with subculturing
each Friday AM and Monday PM at 10⁵ cells per
T 75 cm² flask in F12 FCS 10 media

7/26/82 Cells from above stock subcultured into T 25 cm² flasks
in F12 FCS 5 media (5 me per flask) at 5 x 10⁵ cells
per flask in preparation for treatment with the
test article.

To Page No. 7

Reviewed & Understood by me, <u>David H. Sadek</u>	Date <u>8/6/82</u>	Invented by	Date
		Recorded by <u>Ruth N. Day</u>	<u>8/6/82</u>

From Page No. 6

7/26/82 Cells subcultured into 33 T25cm² flasks at 5x10⁵ cells per flask in 5ml F12FCS in preparation for treatment with the test article.

F12FCS: prep.

Fetal Bovine Serum Lot # 10321005-H

7/27/82 Test article preparation:

RMS 7.6567 g

7.5658 g

0.0909 g = 90.9mg

glycol 40LF - 50ul / flask of 5ml.

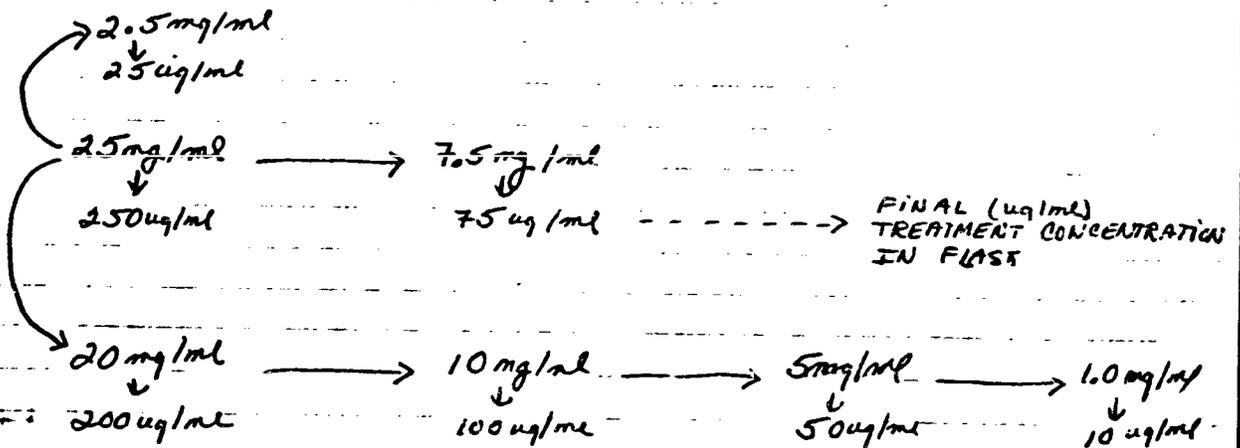
sg = 1.04 g/ml

0.09g = 0.087 ml glycol 40LF

90.9 mg / 25 mg/ml = 3.636 ml TOTAL volume.

3.636 ml - 0.87 ml = 3.549 ml dH₂O = 3.55 ml dH₂O

7/27/82 Dilutions prepared as follows from stock solution of 25 mg/ml to be delivered in 50ul volume to 5ml volume.



To Page No. 8

Inspected & Understood by me,

Edmund J. Model

Date

8/6/82

Invented by

Recorded by Ruth M. Seng

Date

8/6/82

Page No. 3

7/27/82 Positive Control Article - Preparation - 20ul added per flask
RNS

EMS (F.W. = 124.2) $\frac{7.5607 \text{ g}}{7.5303 \text{ g}} = 1.418 \text{ g/ml}$
 $\frac{.0304 \text{ g}}{30.4 \text{ mg}} = 0.02 \text{ ml EMS}$

$10^{-3} \text{ M EMS} = 0.1242 \text{ mg/ml conc. in flask} \times 5 \text{ ml total} = 0.62 \text{ mg EMS per culture}$
Volume delivered: 20 ul / flask
 $0.62 \text{ mg in } 20 \text{ ul} = 31.05 \text{ mg/ml (STOCK)}$

$\frac{30.4 \text{ mg EMS}}{31.05 \text{ mg/ml}} = 0.98 \text{ ml TOTAL}$

$0.98 \text{ ml TOTAL} - 0.02 \text{ ml EMS} = 0.96 \text{ ml DMSO}$

7/27/82 DMN (F.W. = 74.08) at $5 \times 10^{-4} \text{ M}$ 20ul added / flask
RNS

$\frac{7.7811 \text{ g}}{7.7708 \text{ g}} = 1.006 \text{ g/ml}$
 $\frac{.0103 \text{ g}}{10.3 \text{ mg}} = 0.01 \text{ ml DMN}$

$5 \times 10^{-4} \text{ M DMN} = 37.04 \text{ ug/ml} \times 5 \text{ ml} = 185.2 \text{ ug DMN / flask}$
Volume delivered = 20 ul ; $185.2 \text{ ug in } 20 \text{ ul} = 9.26 \text{ mg/ml (STOCK)}$

$\frac{10.3 \text{ mg DMN}}{9.26 \text{ mg/ml}} = 1.11 \text{ ml TOTAL}$

$1.11 \text{ ml TOTAL} - 0.01 \text{ ml DMN} = 1.1 \text{ ml DMSO}$

EP 2MS
7/27/82

To Page No. 109

Prepared & Understood by me,

Edmund A. Sodek

Date

8/6/82

Invented by

Recorded by Robert M. Sory

Date

8/6/82

on Page No. 8

S-9 Mixture:

7/27/82

RMS	H ₂ O	—	10.22 ml
	0.2 M Na ₂ HPO ₄	—	4.69 ml
	0.1 M Na ₂ OP	—	0.75 ml
	1.0 M G-L-P	—	0.094 ml
	1.5 M KCl	—	0.375 ml
	0.5 M MgCl ₂	—	0.375 ml
	0.5 M CaCl ₂ · 2H ₂ O	—	0.375 ml
	S-9 fraction	—	1.875 ml
			<u>18.754 ml</u>

S-9 prepared: 4/27/82

Contained 42.0 mg
of protein per ml

7/27/82 Treatment:

RMS Cells washed 2x in 5 ml washes of Saline-G-Complete (prep 4/18/82 (1), (2)) and treated. Cultures run in duplicate both with and without S-9 metabolic activation in serum free F12 media (prep. 5/28/82 (1)) for 5 hours.

Non-activated series: dispensed 5 ml of serum free F12 media and 50 μ l of test article and/or negative control. EMS was dispensed at 20 μ l.

Activated series: dispensed 4 ml serum free F12 + 1.0 ml of the S-9 mixture, and 50 μ l of test article or negative control. DMN was dispensed at 20 μ l.

Cultures gassed with 5% CO₂ in air and caps tightened. Flasks incubated 5 hrs at 5% CO₂, 37°C and 90% humidity.

TREATMENT TIME: 9 AM - 2 PM

7/27/82 2 PM Cells washed 3x with 5 ml washes of Saline-G-Complete (prep 6/17/82 (1), (2)) Then 5 ml of F12 FCM5 (prep 6/12/82 (3)) containing 10⁻⁵ M BrDU (5-bromo-2'-deoxyuridine) (stock prep 7/13/82) ^{dispensed to} flasks. Flasks incubated an additional 27 hours at 37°C, 5% CO₂, 90% humidity.

10⁻⁵ M BrDU prep 7/27/82 RMS
175 ml media F12 FCM5 + 88 ml stock BrDU.

Cultures containing BrDU kept in subdued light or dark, and handled under safe light.

To Page No. 11

Informed & Understood by me,

Date

Invented by

Date

Colonel J. L. Lick

8/6/82

Recorded by

Ruth H. Sorg

8/5/82

0023

Page No. 16

7/25/82 2:30 PM. 25ul stock Colcemid (Final conc. $2 \times 10^{-9} M$) added to series A flasks. (Colcemid prep 7/25/82); 2:45 PM to series B flasks. Flasks incubated an additional 2 hours. Cultures harvested 4:30 PM (A series) and 4:45 PM (B series). Cells collected into labeled centrifuge tubes and 0.5 ml of 0.05% trypsin (prep 6/4/82) dispensed to each flask and incubated at 37°C until cells detached. Then 1.5 ml of 1% F12PCMS (012482) dispensed to each flask. Cells pooled with original suspensions. A 200 ul sample was taken and counted on a Coulter Counter (10ml isohex).
 TOTAL TUBE VOLUME = 7.0 ml

Stock colcemid prep 6/15/82
 6/22/82 500 RMS 7/12/82

GROUP	TREATMENT	± S-G	Dose (ug/ml)	Cell Count	# cells/ml	# cells/culture
A	glycol	-	250	2975	297500	2.08×10^6
		-	200	3201	320100	2.24×10^6
		-	100	3236	323600	2.27×10^6
		-	50	2505	250500	1.75×10^6
		-	10	3769	376900	2.64×10^6
		EMS dH ₂ O	-	EMS $10^{-7} M$ 500 RMS 7/25/82	3333 3832	333300 383200
A	glycol	+	100	2057	205400	1.44×10^6
		+	75	1797	179700	1.26×10^6
		+	50	2919	291900	2.04×10^6
		+	25	2986	298600	2.09×10^6
		+	10	3336	333600	2.34×10^6
		glycol DMN dH ₂ O	+	$5 \times 10^{-4} M$ -	3467 3742	346700 374200

7/28/82 Cells centrifuged at 1000 rpm for 10 min, supernatant discarded and 10 ml of 0.075% KCl (prep 7/20/82) dispensed to each culture. Incubate at 37°C for 8-10 min, centrifuge, discard supernatant and cells fixed in 5 ml washes of 3:1 methanol:glacial acetic acid. Group A fixed 3 times, Group B twice. All cells stored in dark in refrigerator overnight.

7/29/82 Series B fixed and additional time. all cultures (A+B) resuspended in 0.25 ml fresh fix after final centrifugation, and slides prepared. slides stained in

To Page No. 12

Used & Understood by me, Edward J. Nedek	Date 8/6/82	Invented by	Date
		Recorded by Peter M. Gray	8/6/82

Page No. 11

GROUP	TREATMENT	± S-9	Dose (ug/ml)	Cell Count	# cells/ml	# cells/culture
B	glyoxal	-	250	2828	282800	1.98×10^6
		-	200	2795	279500	1.96×10^6
		-	100	3117	311900	2.18×10^6
		-	50	3371	337100	2.36×10^6
	glyoxal	-	10	4321	432100	3.02×10^6
	EMS	-	$10^{-3}M$	3423	342300	2.40×10^6
	dH ₂ O	-	-	4175	417500	2.92×10^6
B	glyoxal	+	100	2956	295600	2.07×10^6
		+	75	4048	404800	2.83×10^6
		+	50	3390	339000	2.37×10^6
		+	25	4037	403700	2.83×10^6
	glyoxal	+	10	3474	347400	2.43×10^6
	DMN	+	$5 \times 10^{-4}M$	2722	272200	1.91×10^6
	dH ₂ O	+	-	3734	373400	2.61×10^6

Hoechst 33258 in Sorenson's 7/15 buffer (20ml Hoechst stock + 180ml buffer) at 5ug/ml for 10 min, and washed in distilled water. Slides mounted in 2xSSC with coverslip and incubated on slide warmer at 55-60°C for ~20 min. under BLAK-RAY® UV light source. Slides rinsed in dH₂O and stored desiccated overnight.

7/30/82 Slides stained in 3% Giemsa for 20 min, air dried and cleared in xylene. Coverslips mounted with Permount.

** Upon inspection, the activated control DMN did not produce an increase in SCE frequency within historical data limits. Therefore the activated series is repeated. The non-activated cultures were scored and evaluated as normal.

To Page No. 13

Investigated & Understood by me,

Edward J. Lasker

Date

5/6/82

Invented by

Recorded by Ruth N. McCoy

Date

7/6/82

0025

Page No. 12

Purpose: To evaluate the ability of a test article to induce sister chromatid exchange (SCE) in CHO cells in culture. This is a repeat of original study. Positive control in activated series was not within historical limits; activated series rerun in this study.

S.O.P. # 319

Sponsor: American Cyanamid Company

Date of initiation: 8/24/82

Test article: CYANA CHEM[®] glyoxal 40%
Date of completion: 8/31/82

Cell Culture: Chinese Hamster ovary cells, clone K₁, cell line CHO-K₁-SH₂
- received ORNL 7/1/82

Vehicle: distilled H₂O

Solubility: Soluble at all levels tested

Volume of test article delivered: 50 μ l

Test article description: Clear liquid

Post treatment culture time: 27 hours

Length of treatment: 5 hours in serum free F₁₂ media.

Positive control article: DHN (N-Nitrosodimethylamine - oxidized form)
for activated control.

7/1/82 CHO-K₁-SH₂ cell line received from ORNL and cultured
at 10^5 cells per T 75 cm² flask in F₁₂ FC 510 media.
Fetal Bovine Serum 7.5% KC821002H

Stocks maintained in culture with subculturing each
Friday AM and Monday PM at 10^5 cells per T 75 cm²
flask in F₁₂ FC 510 media

8/23/82 Cells from above stock subcultured into T 25 cm²
flasks in 5 ml F₁₂ FCM 5 media at a density of
 5×10^5 cells per flask in preparation for treatment
with the test article.

To Page No. 14

Informed & Understood by me,

Date

Invented by

Date

Colonel J. J. Jank

8/31/82

Recorded by

Ruth N. Seig

8/31/82

8/24/82 Test article preparation:

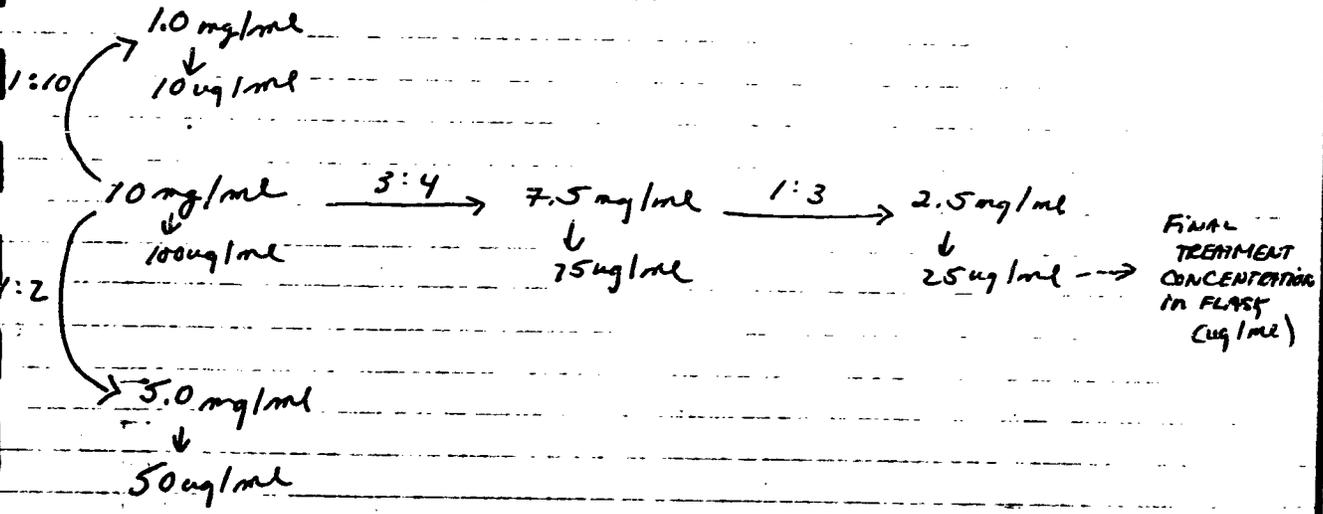
7.4873 g
7.4576 g
0.0297 g = 29.7 mg

glycol 40LF - 50ml / 5ml in flask
sg = 1.04 g/ml
g = .0286 ml glycol 40LF

29.7 mg glycol 40LF
10 mg / ml = 2.970 ml TOTAL VOLUME

2.970 ml Total - .029 ml glycol 40LF = 2.94 ml d H₂O

8/24/82 Dilutions prepared as follows from stock solution of 10 mg/ml to be delivered in 50ul volume to 5 ml volume.



To Page No. 15

Read & Understood by me, Richard H. Arditt	Date 8/31/82	Invented by Ruth M. Gray	Date 8/31/82
		Recorded by	

Page No. 14

8/24/82 Positive Control Antibiotic DMN 20ul added per flask
at $5 \times 10^{-4} M$
(F.W. = 74.08)

sg = 1.006 g/ml

7.4620 g

7.4291 g

0.0329 g = 32.9 mg = 0.033 ml DMN

32.94 ug/ml x 5 ml = 185.2 ug DMN/flask

Volume delivered = 20ul

185 ug in 20ul = 9.26 mg/ml [STOCK]

32.9 mg DMN

9.26 mg/ml

3.553 ml TOTAL

3.553 ml TOTAL = 0.033 ml DMN = 3.52 ml DMSO

8/24/82 S-9 Mixture

ENS

H₂O — 10.22 ml

0.2 M Na₂HPO₄ — 4.69 ml

0.1 M Na DP — 0.75 ml

1.0 M G-6-P — 0.094 ml

1.5 M KCl — 0.375 ml

0.5 M MgCl₂ — 0.375 ml

0.5 M CaCl₂ · 2H₂O — 0.375 ml

S-9 fraction — 1.875 ml

18.754 ml

S-9 prepared: 4/22/82

Contained 42.0 mg of protein per ml.

8/24/82 Treatment:

Cells washed 2x in 5 ml washes of Saline-G-Complete and treated. Cultures treated in serum free F12 media (5/20/82) for 5 hours.

TREATMENT TIME: 8:45 AM - 1:45 PM

To Page No. 14

Inspected & Understood by me,

Date

Invented by

Date

Edward A. Link

8/31/82

Recorded by Ruth N. Siny

8/31/82

Page No. 15

Activated Cultures - dispensed 4 ml of serum free F₁₂ media + 1.5 ml S-9 mixture and 50 ul of test article or negative control. DMN dispensed at 20 ul.

Cultures gassed with 5% CO₂ in air and caps tightened. Flasks incubated 5 hrs at 5% CO₂, 37°C, and 90% humidity.

8/24/82 1:45 PM Cells washed with 3 x 5 ml washes of Saline G-Complete (6/4/82 ^{500 ul}) Then 5 ml of F₁₂ FCM5 (6/21/82 ²⁰) containing 10⁻⁵ M BrDU (5-Bromo-2'-deoxyuridine) dispensed to each flask. Flasks incubated an additional 27-28 hrs at 37°C, 5% CO₂, 90% humidity.
Frozen 2mM BrDU stock: 7/13/82 RMS
BrDU + Media prep: 8/24/82
(10ml stock + 100ml media.)

Cultures containing BrDU kept in dark or under safe light.

8/25/82 2:45 PM - 25 ul colcemid (Final conc. 2 x 10⁻³ M) added to each flask. Flasks incubated an additional 2 hours. (Colcemid stock prep 6/22/82 ^{RMS} and diluted 1:10 8/25/82 RMS prior to addition to flasks.) Cultures harvested 4:45 PM. Cells collected into labelled centrifuge tubes and 0.05% trypsin (0.5 ml) (prep 8/13/82) dispensed to each flask and incubated at 37°C until cells detached. Then 1.5 ml of media F₁₂ FCM5 (6/21/82 ²⁰) dispensed to each flask. Cells pooled with original suspensions. A 200 ul sample was taken and counted in 10 ml buffer on a Coulter Counter (TOTAL TUBE VOLUME = 7.0 ml).

8/25/82 Cells centrifuged at 1000 rpm for 10 min., supernatant discarded and 10 ml of 0.075 M KCl (prep. 8/25/82) dispensed to each culture - incubated 8-10 min. at 37°C. Centrifuge, discard supernatant and cell fixed in 2 x 5 ml washes of 3:1 methanol, glacial acetic acid. Cells refrigerated overnight overnight in dark.

8/26/82 Cultures fixed an additional time, then resuspended in ~ 0.25 ml fresh fix and slides prepared. Slides stained in Hoechst 33258 at 5 ug/ml in Sorenson's 1/15 buffer (20 ml Hoechst stock prep 8/26/82 + 180 ul buffer prep 8/26/82)

To Page No. 17

Used & Understood by me, Edward A. Zalk	Date 8/31/82	Invented by Rick M. Soy	Date 8/31/82
--	-----------------	----------------------------	-----------------

0029

Page No. 26

Culture Volume Total = 7 ml.

Group	TREATMENT	± S-g	Dose (ug/ml)	Well Count	# cells/ml	# cells/culture
A	glyceral	+	100	2972	297200	2.08×10^6
		+	75	3262	326200	2.28×10^6
		+	50	2768	276800	1.94×10^6
		+	25	3588	358800	2.51×10^6
	glyceral	+	10	3394	339400	2.32×10^6
	DMSO	+	$5 \times 10^{-4} M$	3131	313100	2.19×10^6
	dH ₂ O	+	—	3560	355000	2.49×10^6
B	glyceral	+	100	2196	219600	1.54×10^6
		+	75	3028	302800	2.12×10^6
		+	50	2885	288500	2.02×10^6
		+	25	2984	298400	2.09×10^6
	glyceral	+	10	3041	304100	2.13×10^6
	DMSO	+	$5 \times 10^{-4} M$	2223	222300	1.56×10^6
	dH ₂ O	+	—	2903	290300	2.03×10^6

for 10 minutes and then washed in distilled water. Slides mounted with 2x558 (pup 8/26/82) and coverslip and incubated under BLAR-RAY[®] UV light source on slide warmer at 55-60°C for \approx 18-20 min. Slides rinsed in distilled water and stored desiccated overnight.

8/27/82 Slides stained in 3% Thionin in Sorenson's 1/15 buffer, air dried and cleared in xylene. Coverslips mounted with permount. Slides coded for evaluation.

To Page No. _____

Prepared & Understood by me,

Edward J. Zitek

Date

8/31/82

Invented by

Recorded by Ruth M. Song

Date

8/5/82



CERTIFICATE OF AUTHENTICITY

THIS IS TO CERTIFY that the microimages appearing on this microfiche are accurate and complete reproductions of the records of U.S. Environmental Protection Agency documents as delivered in the regular course of business for microfilming.

Data produced 7 11 97 Barbara Smith
(Month) (Day) (Year) Camera Operator

Place Syracuse New York
(City) (State)