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Waverly, Pennsylvania 18471

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F. J. KOSCHIER

CHO/HGPRT Mammalian Cell Forward
Gene Mutation Assay

Glyoxal 40 LF

PH 314-AC-002-82

CT-096

rec'd
3-28-85

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Submitted to
American Cyanamid Company
Wayne, New Jersey

Edmund G. Godek
Edmund G. Godek
Study Director

Robert W. Naismith
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September 28, 1982

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PHARMAKON RESEARCH INTERNATIONAL, INC.

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SUMMARY DATA

PHONE
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Study Number: PH 314-AC-002-82

Study Description: CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay with and without metabolic activation preparation according to Standard Operating Procedure PH-314 on:
Glyoxal 40 LF

Sponsor: American Cyanamid Company
Berdan Avenue
Wayne, New Jersey 07470

Purpose: To measure the ability of test article, Glyoxal 40 LF to induce mutations at the hypoxanthine-guanine phosphoribosyl transferase (hgprt) locus in Chinese Hamster Ovary (CHO) cells on the basis that presumptive mutants, by virtue of the loss of the HGPRT activity are unable to convert purine analogues such as 6-thioguanine (6-TG) to toxic metabolites and hence escape their lethal effect which is, however, encountered by the wild-type cells.

Cell Designation: CHO-K1-BH4-7182

Mycoplasma Verification: Cell line CHO-K1-BH4-7182 verified as mycoplasma free by Pharmakon Research International, Inc., August 5, 1982.

Date Cytotoxicity Assay Initiated: June 3, 1982

Date Mutagenicity Assay Initiated: August 26, 1982

Date Completed: September 20, 1982

Pharmakon Reference: Notebook #354; 66-78 and Notebook #343; 82-85

Test Article Description: Clear liquid

Test Article Preparation: Test article Glyoxal 40 LF was miscible in dH_2O and administered in a volume of 50 ul.

Cytotoxicity: Test article Glyoxal 40 LF was assayed for cytotoxicity at doses of 1000, 333.33, 100, 33.33, 10, 3.3, 1.0, 0.33, 0.1 and 0.033 ug/ml of media both with and without metabolic activation preparation for a 5 hour treatment time. Following a 19 hour recovery period, 200 cells/60 mm dish (6 dishes) were plated

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and incubated for 7 days, fixed and stained. The cytotoxicity assay resulted in no cell survival at 1000 ug/ml of media with and without metabolic activation. The 333.33 ug/ml of media level resulted in 25.92 percent relative survival with metabolic activation and 68.95 percent relative survival without metabolic activation preparation. All remaining doses showed greater than 80 percent relative survival. Data from the Cytotoxicity Assay may be found in Table I. The S-9 fraction used in the Cytotoxicity Assay contained 42 mg protein per ml, as determined by the Lowery Protein Determination Assay.

Dose Selection:

The highest concentration of test article generally used in the CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay should result in 10 percent cell survival (cloning efficiency) after treatment. For test article Glyoxal 40 LF this dose was estimated to be 600 ug/ml of media. Based on the results of the Preliminary Cytotoxicity Assay, additional doses in the Mutation Assay were 300 150, 75 and 37.5 ug/ml of media.

Mutagenicity Assay:

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 of medium F12 containing 5% fetal bovine serum. After a 24 hour growth period, the cultures were washed twice with 5 ml of Saline-G-Complete, and 4-5 ml of serum-free medium F12 were added. In cultures requiring S-9 activation, the S-9 mix was added to bring the final volume to 5 ml. The test article Glyoxal 40 LF was added in 50 ul quantities to give doses of 600, 300, 150, 75 and 37.5 ug/ml of medium. The flasks were gassed with 5 percent CO₂ in air, the caps tightened and the cells incubated at 37°C in 5% CO₂ at 90% + humidity for 5 hours. After the 5 hour treatment time, the cultures were washed three times with 5 ml of Saline-G, 5 ml of medium F12FCM5 were added to each flask, and the cultures incubated for 19 hours. On Day 1, the cells were trypsinized and plated for cloning efficiency determinations (200 cells/plate in 5 ml F12FCM5), and 1×10^6 cells/100 mm dish were plated for phenotypic expression in medium F12FCM5. Approximately 1×10^6 cells were subcultured on Days 3 and 6 to allow for phenotypic expression. On Day 8, the cells were trypsinized and the cell number determined. The cells were plated at a density of 2×10^5 cells/100 mm dish in 10 ml of hypoxanthine free medium F12FCM5 containing 10 uM 6-TG (5 dishes= 1×10^6 cells) and 200 cells/60 mm dish in hypoxanthine free medium F12FCM5 (6 dishes). The cultures were incubated for 7 days, the colonies fixed with 3.7% formalin and stained with dilute giemsa stain.

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.

Results and
Conclusions:

The results for test article Glyoxal 40 LF are as summarized in Table II. Glyoxal 40 LF was shown to produce an increased mutation frequency in the CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay at 600 ug/ml of media in the presence of a metabolic activation system under the conditions of this assay and should be considered a suspect mutagen.

TABLE I

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Mammalian Cell Forward Gene Mutation Assay
Cytotoxicity Data (5 hour Treatment)

Test Article	Dose ug/ml	S-9 (±)	Number of Surviving Colonies	Avg. No. of Surviving Colonies/6 plates	Percent Relative* Survival
untreated	-	-	183, 174, 166, 224, 178, 186	185.17	100.00
untreated	-	+	183, 192, 193, 224, 213, 185	198.33	100.00
dH ₂ O	-	-	206, 172, 173, 173, 196, 193	185.50	100.00
dH ₂ O	-	+	194, 172, 179, 183, 153, 162	173.83	93.88
Glyoxal 40 LF	1000	-	Cytotoxic	-	-
Glyoxal 40 LF	1000	+	Cytotoxic	-	-
Glyoxal 40 LF	333.33	-	117, 118, 148, 146, 109, 128	127.67	68.95
Glyoxal 40 LF	333.33	+	49, 53, 29, 39, 50, 68	48.00	25.92
Glyoxal 40 LF	100	-	121, 159, 149, 162, 182, 164	157.83	85.24
Glyoxal 40 LF	100	+	168, 158, 174, 187, 191, 14?	170.17	91.90
Glyoxal 40 LF	33.33	-	158, 151, 149, 124, 154, 160	149.33	80.65
Glyoxal 40 LF	33.33	+	148, 168, 154, 136, 131, 148	147.50	79.65

* Relative to untreated control

TABLE I (Continued)

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Mammalian Cell Forward Gene Mutator Assay
(Cytotoxicity Data (5 hour Treatment))

Test Article	Dose ug/ml	S-9 (±)	Number of Surviving Colonies	Avg. No. of Surviving Colonies/6 plates	Percent Relative* Survival
Glyoxal 40 LF	10	-	228, 221, 191, 176, 194, 197	201.17	100.00
Glyoxal 40 LF	10	+	192, 191, 172, 181, 191, 179	184.33	99.55
Glyoxal 40 LF	3.3	-	213, 226, 199, 193, 171, 189	198.50	100.00
Glyoxal 40 LF	3.3	+	202, 190, 210, 177, 176, 188	190.50	100.00
Glyoxal 40 LF	1.0	-	181, 193, 177, 188, 168, 164	178.50	96.39
Glyoxal 40 LF	1.0	+	195, 172, 186, 192, 162, 181	181.33	97.93
Glyoxal 40 LF	0.33	-	197, 196, 168, 168, 191, 192	185.33	100.00
Glyoxal 40 LF	0.33	+	160, 192, 184, 185, 192, 174	181.17	97.82
Glyoxal 40 LF	0.1	-	171, 183, 188, 152, 189, 176	176.50	95.31
Glyoxal 40 LF	0.1	+	174, 156, 173, 181, 208, 200	182.00	98.28
Glyoxal 40 LF	0.033	-	191, 147, 180, 181, 179, 163	173.50	93.69
Glyoxal 40 LF	0.033	+	182, 180, 191, 178, 180, 186	182.63	98.74

* relative to untreated controls

TABLE II

PH 314-AC-002-82

Mammalian Cell Forward Gene Mutation Assay

Mutagenicity Data

Test Article	ug/ml	S-9 (±)	Mutant Scoring		Post-"Expression Survival"		
			Total No. of Mutants (5 plates)	No. of cells seeded/plate $\times/6$ plates	Avg. No. of surviving cells $\times/6$ plates	Percent "expression survival"	\times Mutation Frequency (Mutants/ 10^6 "expression survivors")
untreated	-	-	6	2×10^5	205.83	102.92	
untreated	-	-	4	2×10^5	218.67	109.33	$4.75/10^6$
untreated	-	+	4	2×10^5	192.17	96.08	
untreated	-	+	6	2×10^5	191.33	95.67	$5.22/10^6$
dH ₂ O	-	-	11	2×10^5	217.67	108.83	
dH ₂ O	-	-	9	2×10^5	235.83	117.92	$8.19/10^6$
dH ₂ O	-	+	7	2×10^5	217.50	108.75	
dH ₂ O	-	+	9	2×10^5	220.83	110.42	$7.30/10^6$
Glyoxal 40 LF	600	-	22	2×10^5	166.33	83.17	
Glyoxal 40 LF	600	-	18	2×10^5	180.00	90.00	$23.23/10^6$
Glyoxal 40 LF	600	+	23	2×10^5	168.67	84.33	
Glyoxal 40 LF	600	+	52	2×10^5	148.83	74.42	$48.57/10^6$

TABLE II (Continued)

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Mammalian Cell Forward Gene Mutation Assay

Mutagenicity Data

Test Article	ug/ml	S-9 (±)	Mutant Scoring		Avg. No. of surviving cells x/6 plates	Post-"Expression Survival" Percent "expression survival"	x Mutation Frequency (Mutants/10 ⁶ "expression survivors")
			Total No. of Mutants (5 plates)	No. of cells seeded/plate			
Glyoxal 40 LF	300	-	16	2 x 10 ⁵	193.50	96.75	
Glyoxal 40 LF	300	-	17	2 x 10 ⁵	181.67	90.83	17.63/10 ⁶
Glyoxal 40 LF	300		13	2 x 10 ⁵	157.17	78.33	
Glyoxal 40 LF	300	+	12	2 x 10 ⁵	164.50	82.25	15.54/10 ⁶
Glyoxal 40 LF	150	-	28	2 x 10 ⁵	184.67	92.33	
Glyoxal 40 LF	150	-	22	2 x 10 ⁵	186.33	93.17	26.97/10 ⁶
Glyoxal 40 LF	150	+	11	2 x 10 ⁵	191.83	95.92	
Glyoxal 40 LF	150	+	27	2 x 10 ⁵	200.67	100.33	19.19/10 ⁶
Glyoxal 40 LF	75	-	26	2 x 10 ⁵	200.67	100.33	
Glyoxal 40 LF	75	-	12	2 x 10 ⁵	198.33	99.17	19.01/10 ⁶
Glyoxal 40 LF	75	+	17	2 x 10 ⁵	178.67	89.33	
Glyoxal 40 LF	75	+	11	2 x 10 ⁵	179.67	89.83	15.64/10 ⁶

TABLE I: (Continued)

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Mammalian Cell Forward Gene Mutation Assay

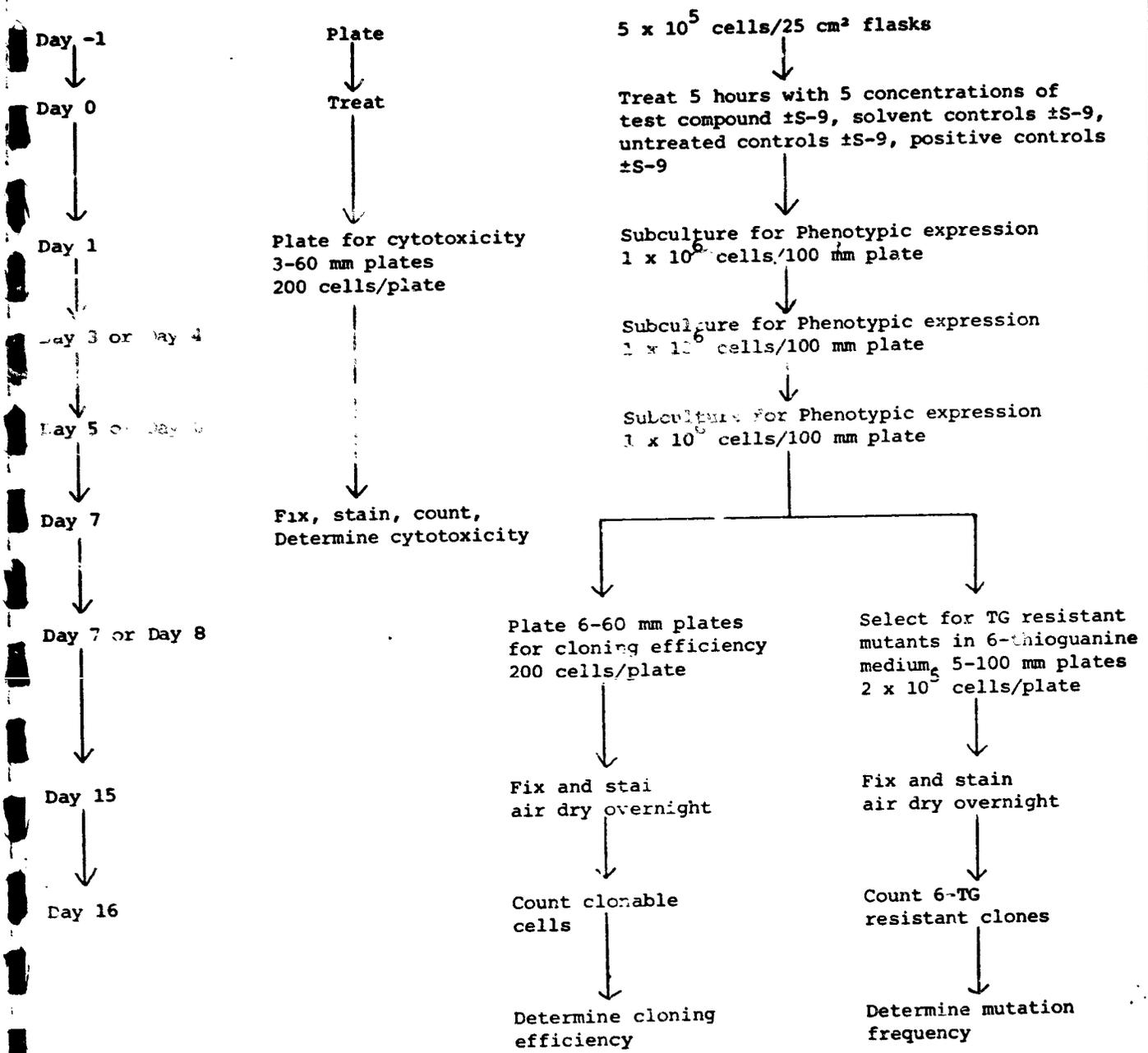
Mutagenicity Data

Test Article	ug/ml	S-9 (±)	Mutant Scoring		Post-"Expression Survival"		
			Total No. of Mutants (5 plates)	No. of cells seeded/plate $\times 10^5$	Avg. No. of surviving cells $\times 10^5$	Percent "expression survival"	Mutation Frequency (Mutants/ 10^6 "expression survivors")
Glyoxal 40 LF	37.5	-	13	2×10^5	179.83	89.92	
Glyoxal 40 LF	37.5	-	14	2×10^5	191.33	95.67	$14.55/10^6$
Glyoxal 40 LF	37.5	+	9	2×10^5	180.00	90.00	
Glyoxal 40 LF	37.5	+	8	2×10^5		91.25	$9.38/10^6$
EMS	200	-	243	2×10^5	180.67	90.33	
EMS	200	-	239	2×10^5	187.83	93.92	$261.74/10^6$ ***
DMN	100	+	155	2×10^5	127.67	63.83	
DMN	100	+	200	2×10^5	133.50	66.75	$271.23/10^6$ ***

*** denotes a positive test response

Experimental Procedure
CHO/HGPRT
Mammalian Cell Forward Gene Mutation Assay

Figure 1
Summary Flow Sheet
CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay



Experimental Procedure

CHO/HGPRT

Mammalian Cell Forward Gene Mutation Assay

Objective:

To measure the ability of a test substance to induce mutations at the hypoxanthine-guanine phosphoribosyl transferase (hgprt) locus in Chinese Hamster Ovary (CHO) cells on the basis that the presumptive mutants, by virtue of the loss of the HGPRT activity are unable to convert purine analogues such as 6-thioguanine (6-TG) to toxic metabolites and hence escape their lethal effect which is, however, encountered by the wild-type cells.

Study Director:

Edmund G. Godek, Pharmakon Research

Technical
Performance:

Edmund G. Godek, Ruth Sorg, and Frederika Nicholl

Cell Culture:

Chinese Hamster Ovary cells, clone K1

Cell Line
Designation:

CHO-K1-BH4

Source:

Dr. Abraham W. Hsie

Biology Division

Oak Ridge National Laboratories

P O Box Y

Oak Ridge, Tennessee 37380

Aseptic Technique:

All aseptic techniques, where possible, were carried out in a Baker NCB6 Hood.

Storage:

The stock cultures of CHO-K1-BH4 cell line are maintained in frozen aliquots in a Revco Ultra-low Freezer.

Experimental Procedure
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Mammalian Cell Forward Gene Mutation Assay

Working Cultures:

Fresh cultures of CHO-K1-BH4 cell line were prepared from frozen stock cultures known to have a stable spontaneous mutation frequency of $3 - 10 \times 10^{-6}$ mutations per cell, however, values up to 20×10^{-6} were deemed acceptable.

Negative Controls:

CHO-K1-BH4 cells were assayed untreated, treated with metabolic activation system only, and treated with the appropriate solvent, both with and without metabolic activation.

Positive Controls:

Positive controls used in the assay were: Ethylmethane sulfonate (EMS) at 200 ug/ml, a mutagen not requiring an S-9 activation system. Dimethylnitrosamine (DMN) at 100 ug/ml, a mutagen requiring an S-9 activation system.

Replication:

CHO-K1-BH4 cells were treated with five levels of the test article. All negative and positive controls were treated in duplicate. All treatment groups were tested in duplicate.

Dose Selection:

Cytotoxicity of a test substance to CHO cells was determined by a reduction in colony forming ability of the cells following a 5 hour treatment with the test substance in the presence or absence of a metabolic activation system. Generally, for test substances with unknown toxicity, they were run at 10 doses with 1000 ug/ml initially being the highest one. The remaining doses were 300, 100, 30, 10, 3, 1, 0.3, 0.1 and

Experimental Procedure
CHO/HGPRT
Mammalian Cell Forward Gene Mutation Assay

0.03 ug/ml. The highest concentration of test substance used in the CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay was that which resulted in 10% cell survival (cloning efficiency). Four additional doses which yield 10 - 100% survival were also tested.

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

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks and the cultures were washed twice with 5 ml of Saline G. After the final wash, 5 ml of serum free medium F12 was pipetted into each flask. The test substance being assayed for cytotoxicity was diluted in the proper solvent to the concentrations desired for testing and 20 - 50 µl of the test substance was added to each flask and mixed well. The flasks were gassed with 5% CO₂ in air, the caps tightened and the cells incubated for 5 hours at 37°C. After the 5 hour incubation, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours.

Treatment of CHO
Cells with Test
Substance for
Cytotoxicity
(activated):

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks, and the cultures were washed twice with 5 ml of Saline G.

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Experimental Procedure
CHO/HGPRT
Mammalian Cell Forward Gene Mutation Assay

After the final wash, 4 ml of serum free medium F12 were pipetted into each flask. The S-9 mix contained (per ml) 8 μmol MgCl_2 , 8 μmol CaCl_2 , 33 μmol KCl , 5 μmol glucose-6-phosphate, 4 μmol NADP , 50 μmol sodium phosphate buffer (pH 8.0) and 0.1 ml of the microsomal preparation containing 30 mg protein/ml. The test substance being assayed for cytotoxicity was diluted in the proper solvent to the concentrations desired for testing and 20 - 50 μl of the test substance was added to each flask and mixed well. The flasks were gassed with 5% CO_2 in air, the caps tightened and the cells incubated for 5 hours at 37°C. After the 5 hour incubation, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours. Following the 19 hour incubation after test substance treatment, the cultures were removed from the incubator and the medium aspirated from the flasks. The cultures were washed twice with 5 ml of Ca-Mg-free Saline G. The cells were then placed at ambient temperature in 0.5 ml of a 0.05% trypsin solution. The flasks were examined under an inverted microscope to ensure that the cells had rounded and 1.5 ml of

Cytotoxicity
Determination:

Experimental Procedure
CHO/SGPRT
Mammalian Cell Forward Gene Mutation Assay

medium F12FCM5 were added. The cells were washed off the surface of the flask using a plugged pasteur pipette. A cell number was determined for each culture. An aliquot of the cells was diluted to a cellular density of 1000 cells/ml in medium F12FCM5 and 0.2 ml (200 cells/plate) was added to each of 6 - 60 mm plates containing 5 ml of medium F12FCM5. The plates were incubated for 7 - 8 days at 37°C in a 5% CO₂ in air incubator. After the incubation period, the medium was removed and the colonies fixed with 3.7% formaldehyde, stained with a 1% Giemsa solution, counted and the colony numbers recorded. These survival frequencies were used to determine which levels of test substance yielded a 10 - 100% survival. Five levels of test substance yielding 10 to 100% survival were chosen to be used in the CHO Mammalian Cell Forward Gene Mutation Assay.

Plating CHO cells
for Experiment:

CHO-K1-BH4 cells for mutagenesis testing were obtained from frozen stocks of cultures known to have a stable spontaneous mutation frequency of $0 - 10 \times 10^{-6}$ mutants per cell. Exponentially growing cells were plated in 25 cm² plastic tissue culture flasks at an initial density of 5×10^5 cells in 5 ml of medium F12FCM5 and incubated for 16 - 24 hours. Normal cell

Experimental Procedure
CHO/HGPRT
Mammalian Cell Forward Gene Mutation Assay

growth was inspected using an A.O. Inverted Microscope. Cells in exponential growth state were treated.

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

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks and the cultures were washed twice with 5 ml of Saline G. After the final wash, 5 ml of serum free medium F12 were pipetted into each flask. The test or control substance being assayed was diluted in the proper solvent to the concentration desired for testing and 20 - 50 μ l of the test or control substance was added to each flask and mixed well. The flasks were gassed with 5% CO₂ in air, the caps tightened and the cells incubated for 5 hours at 37°C. After the 5 hour incubation, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours.

Treatment of CHO
with Test (& Control)
Substance (activated):

The CHO-K1-BH4 cells were removed from the incubator, the medium was aspirated from the flasks, and the cultures were washed twice with 5 ml of Saline G. After the final wash, 4 ml of serum free medium F12 were pipetted into each flask. The S-9 mix contained (per ml) 8 μ mol MgCl₂, 8 μ mol CaCl₂, 33 μ mol KCl, 5 μ mol glucose-6-phosphate, 4 μ mol NADP, 50 μ mol sodium phosphate buffer (pH 8.0) and 0.1 ml of the

Experimental Procedure
CNO/HGPRT
Mammalian Cell Forward Gene Mutation Assay

microsomal preparation containing 30 mg protein/ml. The test or control substance being assayed was diluted in the proper solvent at the concentrations desired for testing and 20 - 50 ul of the test or control substance was added to each flask and mixed well. The flasks were gassed with 5% CO₂ in air, the caps tightened and the cells incubated for 5 hours at 37°C. After the 5 hour incubation, the medium was aspirated from the flasks, the cultures washed three times with 5 ml of Saline G and 5 ml of F12FCM5 added. The cultures were incubated for an additional 19 hours.

Subculturing for
Expression of
Mutation:

Following the 19 hour incubation after test substance treatment, the cultures were removed from the incubator and the medium aspirated from the flasks. The cultures were washed twice with 5 ml of Ca-Mg-free Saline G. The cells were then placed at ambient temperature in 0.5 ml of a 0.05% trypsin solution. The flasks were examined under an inverted microscope to ensure that the cells had rounded and 1.5 ml of medium F12FCM5 was added. The cells were washed off the surface of the flask using a plugged pasteur pipette. A cell number was determined for each culture. An aliquot yielding 1×10^6 cells were subcultured into a 100 mm dish containing 10 ml of medium F12FCM5. These cultures were incubated at 37°C

Experimental Procedure
CHO/HGPRT
Mammalian Cell Forward Gene Mutation Assay

in a 5% CO₂ in air for phenotypic expression. A subculture was taken on Day 3 and Day 5. On Day 3 the subculture was performed by washing the cells twice with 5 ml of Ca-Mg-free Saline G and placing them with 0.5 ml of 0.05% trypsin solution at ambient temperature. The cells were allowed to round and 1.5 ml of F12FCM5 was added to each plate. The cells were washed off the surface of the plate using a plugged pasteur pipette. A cellular density was determined and an aliquot of the suspension containing 1×10^6 cells was added to a plate containing 10 ml of medium F12FCM5. The subculture was repeated on Day 5.

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

On Day 7 the cells were washed once with 5 ml of Ca-Mg-free Saline G and then placed in 0.5 ml of a 0.05% trypsin solution at ambient temperature. When the cells rounded up, 1.5 ml of hypoxanthine-free medium F12FCM5 was added and the cells washed from the 100 mm dish with a pasteur pipette. A cell density was determined and the cells diluted to 1×10^5 cells/ml in hypoxanthine free medium F12FCM5. This aliquot of cells was used for selection and an additional aliquot was reserved for a determination of cloning efficiency. For mutant selection, 6.25 ml of a 10^{-3} M 6-thioguanine solution was added to 494 ml of hypoxanthine free medium F12FCM5. To each of 5 100 mm plates, 8 ml of the TG medium were added and 2 ml of

Experimental Procedure
CHO/HGPRT
Mammalian Cell Forward Gene Mutation Assay

the 1×10^5 cells/ml aliquot, for a total of 2×10^5 cells/plate. The plates were incubated for 7 days at 37°C in $5\% \text{CO}_2$ in air to allow for colony formation. The colonies were then fixed in 3.7% formaldehyde, stained with a 1% Giemsa solution, counted, and the clone numbers recorded. The total number of mutant clones on the five plates was determined.

Cloning Efficiency:

From the aliquot reserved before mutant selection, a 1:100 dilution was performed in Saline G to a cellular density of 1×10^3 cells/ml. This aliquot was used to determine cloning efficiency. From this dilution, 0.2 ml was aliquoted into each of six 60 mm plates containing 5 ml of hypoxanthine free medium F12FCM5 (200 cells/plate). These plates served as viable counts (cloning efficiency) plates and were incubated for 7 days at 37°C in $5\% \text{CO}_2$ in air, fixed, stained and counted for a measure of cloning efficiency (average of the six 60 mm plates).

Calculation of Mutation Frequency:

The mutation frequency was calculated by dividing the total number of mutant clones by the number of cells plated, corrected for the cloning efficiency of the cells prior to mutant selection.

Interpretation of Results:

After treatment with the test substance, cells which had undergone mutation to hgp^{r^-} form colonies in the presence of 6-thioguanine (resistant to 6-thioguanine). The total number of mutant colonies

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(per 10^6 surviving cells) observed at each concentration of test substance was compared to the total number of mutant colonies (per 10^6 surviving cells) observed in the negative control. A test substance showing a dose-dependent increase of mutation induction with at least one dose exhibiting a mutation frequency that was greater than or equal to 50×10^{-6} per cell was considered a suspect mutagenic response, and the sponsor will be advised to repeat the assay bracketing the positive response dose. A test substance showing a true positive response in this assay should also exhibit a dose-response relationship. The spontaneous background mutation frequency (forward mutation frequency) is usually $0 - 10 \times 10^{-6}$, however, values up to 20×10^{-6} are deemed acceptable. Data is presented in this final study report in tabular form as the number of mutant colonies per 10^6 surviving cells at each concentration of test substance. In those cases where the test substance was positive, a dose-response curve was also provided showing the increase in mutant frequency vs. increasing concentration. All assays were performed in duplicate and data presented as the average number of mutants per 10^6 surviving cells.

Records
Maintained:

All correspondence pertinent to the study between the sponsor and Pharmakon Research International, Inc.,

Experimental Procedure
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protocol, amendments to the protocol, raw data, test chemical weight or volume, dispensation reports, quality assurance reports and the final report are maintained in the Pharmakon Archives.

Raw Data:

Pharmakon Research Notebook

Good Laboratory Practices Statement:

This study was conducted in compliance with the Good Laboratory Practice Regulations except if noted. There were no significant deviations from the GLP Regulations which affected the quality or integrity of the study. Q.A.U. findings derived from the inspection(s) during the conduct of this study and from the audit of the final report are documented and have been provided to the study director and the test facility management.

Test Article:

The identity, purity, quality, and strength of the test article are the responsibility of the sponsor.

Bibliography¹:

O'Neill, J. P., P. A. Brimer, R. Machanoff, G. P. Hirsch, and A. W. Hsie. 1977a. A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system. Mutation Research 45: 91-101.

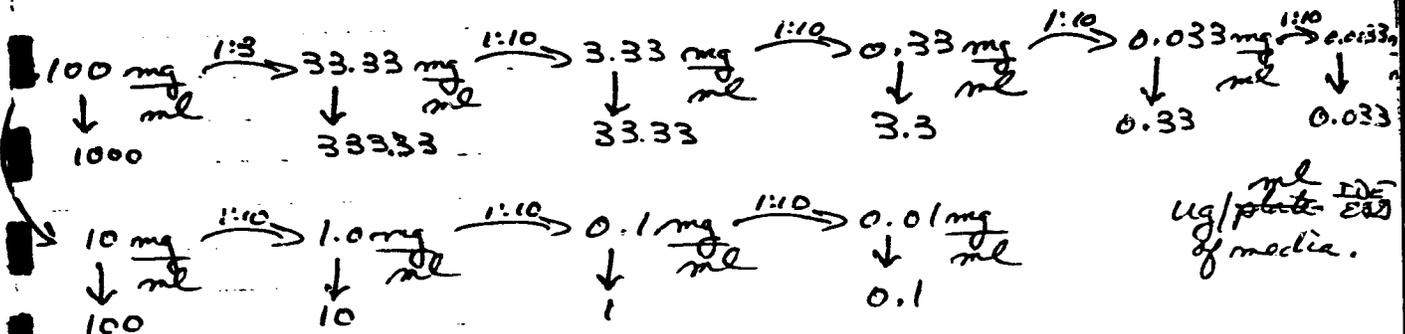
Experimental Procedure
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Mammalian Cell Forward Gene Mutation Assay

O'Neill, J. P., D. B. Couch, R. Machanoff, J. R. San Sebastian, P. A. Brimer, and A. W. Hsie. 1977b. A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Utilization with a variety of mutagenic agents. Mutation Research 45: 103-109.

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6/3/82 - Test article Preparation:

8.7890g.
 8.2260g.
 0.5630g.
 563 mg / 5.6 ml total volume. (100mg/ml).
 563mg contained in 0.45ml \Rightarrow 563mg / 0.45ml vol. + 5.15ml d. H₂O.



Mixture:

- sterile Milli Q H₂O - 10.9ml
 - 0.2M NaPO₄ (pH 8.0) - 5.0ml
 - 0.1M NADP - 0.8ml
 - 1.0M Glucose-6-Phosphate - 0.1ml
 - 1.5M KCl - 0.4ml
 - 0.5M MgCl₂ - 0.4ml
 - 0.5M CaCl₂ - 0.4ml
 - S-9 Fraction - 2.0ml
- 20ml total

S-9 prepared 4/27/82.
 at 5mg protein/ml.

Treatment time 5 hrs. 10:00 A.M. to 3:00 P.M.

All test article and control articles delivered to test system at a close volume of 50ul.

o.p.u. - All flasks washed 3X with Soline-G-Complete. 5ml Media F12FMS (5/26/82) added to each flask. All flasks incubated at 37°C, 5% CO₂ in air at 90+% humidity.

To Page No. 84

Prepared & Understood by me, <u>Ruth M. Song</u>	Date <u>6/3/82</u>	Invented by <u>Edward J. Lusk</u>	Date <u>6/3/82</u>
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Page No. 23

6/4/82 - Plate cells for initial survival. 200 cells per each of 6-60mm dishes in 5ml Media Fitzens (5/26/82).

Plate	Compound	±5%	ug/ml	cell Count	# cells per ml.	# cells in suspension
ZAC-1	untreated	-	-	10800	1.08×10^6	2.16×10^6
ZAC-2	untreated	+	-	11400	1.14×10^6	2.28×10^6
ZAC-3	dH ₂ O	-	-	10900	1.09×10^6	2.18×10^6
ZAC-4	dH ₂ O	+	-	10700	1.07×10^6	2.14×10^6
ZAC-5	Hydrocortisone	-	1000	3200	3.2×10^5	6.4×10^5
ZAC-6	Hydrocortisone	+	1000	3200	3.2×10^5	6.4×10^5
ZAC-7	Hydrocortisone	-	333.33	8000	8.0×10^5	1.60×10^6
ZAC-8	Hydrocortisone	+	333.33	4000	4.0×10^5	8.0×10^5
ZAC-9	Hydrocortisone	-	100	13700	1.37×10^6	2.74×10^6
ZAC-10	Hydrocortisone	+	100	11700	1.17×10^6	2.34×10^6
ZAC-11	Hydrocortisone	-	33.33	10500	1.05×10^6	2.10×10^6
ZAC-12	Hydrocortisone	+	33.33	9800	9.8×10^5	1.96×10^6
ZAC-13	Hydrocortisone	-	10	7700	7.7×10^5	1.54×10^6
ZAC-14	Hydrocortisone	+	10	9000	9.0×10^5	1.80×10^6
ZAC-15	Hydrocortisone	-	3.33	8400	8.4×10^5	1.68×10^6
ZAC-16	Hydrocortisone	+	3.33	10600	1.06×10^6	2.12×10^6
ZAC-17	Hydrocortisone	-	1.0	10900	1.09×10^6	2.18×10^6
ZAC-18	Hydrocortisone	+	1.0	10500	1.05×10^6	2.10×10^6
ZAC-19	Hydrocortisone	-	0.33	10700	1.07×10^6	2.14×10^6
ZAC-20	Hydrocortisone	+	0.33	13800	1.38×10^6	2.76×10^6
ZAC-21	Hydrocortisone	-	0.1	13300	1.33×10^6	2.66×10^6
ZAC-22	Hydrocortisone	+	0.1	13800	1.38×10^6	2.76×10^6
ZAC-23	Hydrocortisone	-	0.033	13900	1.39×10^6	2.78×10^6
ZAC-24	Hydrocortisone	+	0.033	11700	1.17×10^6	2.34×10^6

To Page No. 3

Witnessed & Understood by me, <i>Ruth H. Song</i>	Date <i>6/4/82</i>	Invented by <i>Donald A. Audek</i>	Date <i>6/4/82</i>
	Reference		

Preliminary Cytotoxicity Screen - Glycol LF

Book No. 343

Page No. 5

6/1/82 - Plates removed from incubator, media aspirated off, plates washed with phosphate buffered saline, fixed in 3.7% formalin solution, stained with 1% Heiden stain, rinsed in running tap water and allowed to air dry. Lodick 6/1/82.

6/1/82 - Scoring for Initial Survival.

Plat#	Compound	±S ₉	cg/ml	Plate Counts	# cells seeded plate	Avg. # clonals	% survival	% relative survival
ZAC-1	untreated	-	-	183 124 166 224 178 186	200	185.17	92.59	100
ZAC-2	untreated	+	-	183 192 193 224 213 185	200	198.33	99.17	100
ZAC-3	H ₂ O	-	-	206 172 173 173 146 193	200	185.50	92.75	100
ZAC-4	H ₂ O	+	-	194 122 129 183 153 162	200	173.83	86.92	93.88
ZAC-5	Glycol ¹⁰ LF	-	1000	0 0 0 0 0 0	200	0.00	0	0
ZAC-6	Glycol ¹⁰ LF	+	1000	0 0 0 0 0 0	200	0.00	0	0
ZAC-7	Glycol ¹⁰ LF	-	333.33	117 118 148 146 109 128	200	127.67	63.84	68.95
ZAC-8	Glycol ¹⁰ LF	+	333.33	49 53 29 39 50 68	200	48.00	24.00	25.92
ZAC-9	Glycol ¹⁰ LF	-	100	131 159 149 162 182 164	200	157.83	78.92	85.24
ZAC-10	Glycol ¹⁰ LF	+	100	168 158 124 187 191 143	200	170.17	85.09	91.90
ZAC-11	Glycol ¹⁰ LF	-	33.33	158 151 149 124 154 160	200	149.33	74.67	80.65
ZAC-12	Glycol ¹⁰ LF	+	33.33	148 165 154 136 131 148	200	147.50	73.75	79.65
ZAC-13	Glycol ¹⁰ LF	-	10	228 221 191 176 194 197	200	201.17	100.59	100
ZAC-14	Glycol ¹⁰ LF	+	10	192 191 172 151 191 179	200	184.33	92.17	99.55
ZAC-15	Glycol ¹⁰ LF	-	3.33	213 226 199 193 171 189	200	198.50	99.25	100
ZAC-16	Glycol ¹⁰ LF	+	3.33	202 190 210 172 176 188	200	190.50	95.25	100
ZAC-17	Glycol ¹⁰ LF	-	1.0	181 193 172 188 165 164	200	178.50	89.25	96.39
ZAC-18	Glycol ¹⁰ LF	+	1.0	195 172 186 192 162 181	200	181.33	90.67	97.93
ZAC-19	Glycol ¹⁰ LF	-	0.33	197 196 168 168 191 192	200	185.33	92.67	100
ZAC-20	Glycol ¹⁰ LF	+	0.33	160 142 154 185 192 174	200	181.17	90.59	97.81
ZAC-21	Glycol ¹⁰ LF	-	0.1	171 183 188 152 189 176	200	176.50	88.25	95.31
ZAC-22	Glycol ¹⁰ LF	+	0.1	174 156 173 151 205 200	200	182.00	91.00	98.21
ZAC-23	Glycol ¹⁰ LF	-	0.033	191 143 180 181 179 163	200	173.50	86.75	93.69
ZAC-24	Glycol ¹⁰ LF	+	0.033	182 150 191 175 150 186	200	182.83	91.42	98.71

To Page No. 6

Witnessed & Understood by me, <i>Ruth M. Soy</i>	Date 6/14/82	Invented by <i>Donald J. Lodick</i>	Date 6/14/82	215
	Reviewed by <i>Donald J. Lodick</i>			

Page No. 50. 343

Purpose: To measure the ability of a test substance to induce mutations at the hypoxanthine guanine phosphoribosyl transferase (hgp^rt) locus in Chinese Hamster Ovary (CHO) cells on the basis that presumptive mutants, by virtue of the loss of the HGPRT activity, are unable to convert purine analogs, such as 6-thioguanine (6-TG) to toxic metabolites, and hence escape their lethal effects, which is however, encountered by the wild type cells.

Materials and Methods: Refer to Standard Operating Procedure PH314.

Source: American Cyanamid Company

Test Article: Alyosal 40 LF

Description: clear liquid

Date Preliminary Cytotoxicity Initiated: 6/3/82

Date CHO/HGPRT Forward Gene Mutation Assay Initiated: 8/26/82

CHO-K1-BH4 Lot # 7182 received from Oak Ridge National Laboratories 7/1/82. Routine subcultures were done every Friday (a.m.) and Monday (p.m.), where 1×10^5 cells were subcultured into each of 3-75 cm² flasks containing 15 ml of media F12 FCS10. CHO-K1-BH4 Lot # 7182 amniocentesis treated 7/23/82. Routine subculture regime carried out.

8/23/82 - CHO-K1-BH4 cells (Lot # 7182) subcultured into 10-T 75 cm² flasks (3×10^5 cells/flask) in 15 ml of media F12 FCS10.

W. Dodek 8/23/82.

8/25/82 - CHO-K1-BH4 cells (Lot # 7182) subcultured into 36-T 75 cm² flasks (5×10^5 cells/flask) in 5 ml of media F12 FCS5, in preparation for treatment. (7/29/82). Fetal Bovine Serum Lot # KC-321005

To Page No. 6

Witnessed & Understood by me, W. J. Beech	Date 8/25/82	Invented by	Date 8/25/82
		Recorded by Edward A. Dodek	

Page No. 66

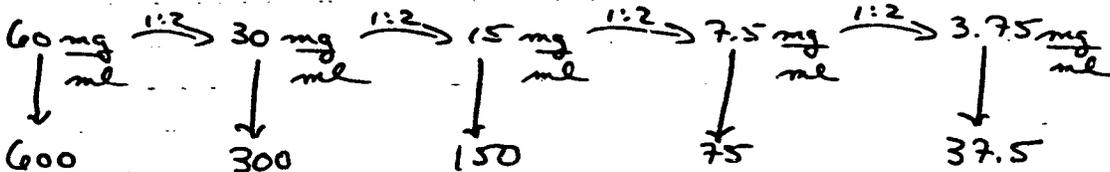
8/26/82 - Test Article Preparation:

7.7250g.

7.4110g.

0.3140g.

314 mg / 5.23 ml dH₂O ⇒ 60 mg/ml.



media delivered in a volume of 50 μ l.

Positive Control Preparation:

Ems - 200 μ g/ml of media.

7.5440g.

7.4950g.

0.0490g.

49 mg / 2.45 ml total volume
eg. vol. = 0.034 ml.

density Ems = 1.418

49 mg / 0.034 ml eg. vol + 2.416 ml DMSO ⇒ 20 mg/ml
delivered in 50 μ l ⇒ 200 μ g/ml of media.

DMSO - 100 μ g/ml of media.

7.5120g.

7.4890g.

0.0230g.

23 mg / 2.3 ml DMSO ⇒ 10 μ g/ml

delivered in 50 μ l ⇒ 100 μ g/ml of media.

S-9 Mixture:

Stearic Mili QH₂O - 10.9 ml

0.2M Na₂HPO₄ · 7H₂O - (pH 7.4) - 5.0

0.1M NADP - 0.8

1.0M Glucos-6-Phosphate - 0.1

1.5M KCl - 0.4

0.5M MgCl₂ - 0.4

0.5M CaCl₂ - 0.4

S-9 Fraction - 2.0

20.0 ml total

S-9 prepared: 4/27/82
42 mg protein/ml.

Treatment time: 10:00 A.M. to 3:00 P.M.

To Page No. 6

Witnessed & Understood by me,
G. J. Mecca

Date
8/26/82

Invented by
Recorded by
L. J. Lubb

Date
8/26/82

Cholerae Mutation Assay - Alysd Wolf Book No. 229

Page No. 8/27/82 - Subculture for expression of mutation and initial survival.

Expression of Mutation - Subculture 1×10^6 cells / 100 mm plate containing 10 ml of media F12FCS.

Initial Survival - Subculture on aliquot of cells containing 1×10^3 cells/ml 0.2 ml of aliquot (200 cells) in each of 3-60 mm dishes containing 5 ml F12FCS

Plate #	Compound	±SS	ug/ml	cell Count	# cells per ml	ant. trans.	# cells in suspension
614-1-25	untreated	-	-	10500	1.05×10^6	0.95	2.10×10^6
2-25	untreated	-	-	10500	1.05×10^6	0.95	2.10×10^6
3-25	untreated	+	-	10400	1.04×10^6	0.96	2.08×10^6
4-25	untreated	+	-	10100	1.01×10^6	0.99	2.02×10^6
5-25	H ₂ O	-	-	9700	9.7×10^5	1.03	1.94×10^6
6-25	H ₂ O	-	-	10300	1.03×10^6	0.97	2.06×10^6
7-25	H ₂ O	+	-	11100	1.11×10^6	0.90	2.22×10^6
8-25	H ₂ O	+	-	9800	9.8×10^5	1.02	1.96×10^6
9-25	Alysd Wolf	-	600	4600	4.6×10^5	1.7	9.2×10^5
10-25		-	600	5100	5.1×10^5	1.7	1.02×10^6
11-25		+	600	4400	4.4×10^5	1.7	8.8×10^5
12-25		+	600	4500	4.5×10^5	1.7	9.0×10^5
13-25		-	300	7700	7.7×10^5	1.30	1.54×10^6
14-25		-	300	8500	8.5×10^5	1.18	1.70×10^6
15-25		+	300	5900	5.9×10^5	1.69	1.18×10^6
16-25		+	300	5700	5.7×10^5	1.7	1.14×10^6
17-25		-	150	11200	1.12×10^6	0.89	2.24×10^6
18-25		-	150	12600	1.26×10^6	0.79	2.52×10^6
19-25		+	150	9000	9.0×10^5	1.11	1.80×10^6
20-25		+	150	8600	8.6×10^5	1.16	1.72×10^6
21-25		-	75	11900	1.19×10^6	0.84	2.38×10^6
22-25		-	75	13900	1.39×10^6	0.72	2.78×10^6
23-25		+	75	10000	1.00×10^6	1.00	2.00×10^6
24-25		+	75	10400	1.04×10^6	0.96	2.08×10^6
25-25		-	37.5	12400	1.24×10^6	0.81	2.48×10^6
26-25		-	37.5	13400	1.34×10^6	0.75	2.68×10^6
27-25		+	37.5	12100	1.21×10^6	0.83	2.42×10^6
28-25	Alysd Wolf	+	37.5	11600	1.16×10^6	0.86	2.32×10^6

To Page No. 6

Witnessed & Understood by me,
Alysd Wolf

Date 8/27/82

Invented by

Witnessed by

Alysd Wolf

Date

8/27/82

Page No. 68

Plat#	Compound	±S9	ug/ml	cell count	# cells per ml	amt. trans.	# cells in Suspension
29-25	EMS	-	200	9200	9.2×10^5	1.09	1.84×10^6
30-25	EMS	-	200	11900	1.19×10^6	0.84	2.38×10^6
31-25	DmN	+	100	10700	1.07×10^6	0.93	2.14×10^6
32-25	DmN	+	100	10800	1.08×10^6	0.93	2.16×10^6

Model

8/28 - Subculture for expression of mutation. Subculture 1×10^6 cells into 100 mm plates containing 10 ml media F12/EMS.

#	Compound	±S9	ug/ml	cell count	# cells per ml	amt. trans.	# cells in Suspension
1	untreated	-	-	29100	2.91×10^6	0.34	5.82×10^6
2	untreated	-	-	27300	2.73×10^6	0.37	5.46×10^6
3	untreated	+	-	19700	1.97×10^6	0.51	3.94×10^6
4	untreated	+	-	23700	2.37×10^6	0.42	4.74×10^6
5	H ₂ O	-	-	26900	2.69×10^6	0.37	5.38×10^6
6	H ₂ O	-	-	24800	2.48×10^6	0.40	4.96×10^6
7	H ₂ O	+	-	25300	2.53×10^6	0.40	5.06×10^6
8	H ₂ O	+	-	24100	2.41×10^6	0.41	4.82×10^6
9	Glycol 4OLF	-	600	10600	1.06×10^6	0.94	2.12×10^6
10		-	600	10700	1.07×10^6	0.93	2.14×10^6
11		+	600	1300	1.3×10^5	1.7	2.6×10^5
12		+	600	3100	3.1×10^5	1.7	6.2×10^5
13		-	300	23000	2.30×10^6	0.43	4.60×10^6
14		-	300	25500	2.55×10^6	0.39	5.10×10^6
15		+	300	19800	1.98×10^6	0.51	3.96×10^6
16		+	300	20900	2.09×10^6	0.48	4.18×10^6
17		-	150	21600	2.16×10^6	0.46	4.32×10^6
18		-	150	23800	2.38×10^6	0.42	4.76×10^6
19		+	150	18300	1.83×10^6	0.55	3.66×10^6
20		+	150	19000	1.90×10^6	0.53	3.80×10^6
21		-	75	27800	2.78×10^6	0.36	5.56×10^6
22	Glycol 4OLF	-	75	22600	2.26×10^6	0.44	4.52×10^6

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Reviewed & Understood by me, J. Meera	Date 8/30/82	Invented by J. Meera	Date 8/30/82
		Responsible by J. Meera	

Page No. 11

Sub. No.	Compound	IS9	ug/ml	cell count	# cells per ml	# cells in Suspension	Cloning off. Plate
1-5	untreated	-	-	13200	1.32×10^6	5.28×10^6	AC-7g-1-CE
2-5	untreated	-	-	12000	1.20×10^6	4.80×10^6	-2-CE
3-5	untreated	+	-	13900	1.39×10^6	5.56×10^6	-3-CE
4-5	untreated	+	-	13100	1.31×10^6	5.24×10^6	-4-CE
5-5	H ₂ O	-	-	10700	1.07×10^6	4.28×10^6	-5-CE
6-5	H ₂ O	-	-	10000	1.00×10^6	4.00×10^6	-6-CE
7-5	H ₂ O	+	-	11900	1.19×10^6	4.76×10^6	-7-CE
8-5	H ₂ O	+	-	12000	1.20×10^6	4.80×10^6	-8-CE
9-5	Nigral 40LF	-	600	9200	9.2×10^5	3.68×10^6	-9-CE
10-5		-	600	10000	1.00×10^6	4.00×10^6	-10-CE
11-5		+	600	7500	7.5×10^5	3.00×10^6	-11-CE
12-5		+	600	10000	1.00×10^6	4.00×10^6	-12-CE
13-5		-	300	8400	8.4×10^5	3.36×10^6	-13-CE
14-5		-	300	10000	1.00×10^6	4.00×10^6	-14-CE
15-5		+	300	10300	1.03×10^6	4.12×10^6	-15-CE
16-5		+	300	8800	8.8×10^5	3.52×10^6	-16-CE
17-5		-	150	12300	1.23×10^6	4.92×10^6	-17-CE
18-5		-	150	13000	1.30×10^6	5.20×10^6	-18-CE
19-5		+	150	10600	1.06×10^6	4.24×10^6	-19-CE
20-5		+	150	12100	1.21×10^6	4.84×10^6	-20-CE
21-5		-	75	10000	1.00×10^6	4.00×10^6	-21-CE
22-5		-	75	11600	1.16×10^6	4.64×10^6	-22-CE
23-5		+	75	12400	1.24×10^6	4.96×10^6	-23-CE
24-5		+	75	11100	1.11×10^6	4.44×10^6	-24-CE
25-5		-	37.5	12000	1.20×10^6	4.80×10^6	-25-CE
26-5		-	37.5	11000	1.11×10^6	4.44×10^6	-26-CE
27-5		+	37.5	13900	1.39×10^6	5.56×10^6	-27-CE
28-5	Nigral 40LF	+	37.5	14000	1.40×10^6	5.60×10^6	-28-CE
29-5	EMS	-	200	12700	1.27×10^6	5.08×10^6	-29-CE
30-5	EMS	-	200	17900	1.79×10^6	5.56×10^6	-30-CE
31-5	DNA	+	100	12400	1.24×10^6	4.96×10^6	-31-CE
32-5	DNA	+	100	11100	1.11×10^6	4.44×10^6	-32-CE

To Page No. 92

Checked & Understood by me,
S. J. Mecca

Date
9/3/82

Invented by
Recorded by
Edward S. Loral

Date
9/3/82

0 0 3 6

Page No. 27

3/82 - Staining of Initial Survival Plates. Plates removed from incubator, washed, fixed, stained, rinsed in running tap water and allowed to air dry.

4/8/82 - Scoring of Initial Survival Plates:

Plate #	Compound	IS9	ug/ml	Plate Count	avg. no. \bar{x}	# cells seeded/plate	% Survival	Avg. % survival	Avg. % rel. surv.
6-1-15	untreated	-	-	140 173 165	176.00	200	88.00		
2-15	untreated	-	-	171 166 170	169.00	200	84.50	86.25	100.00
3-15	untreated	+	-	173 158 166	175.67	200	87.83		
4-15	untreated	+	-	158 170 165	164.33	200	82.17	85.00	98.55
5-15	H ₂ O	-	-	177 170 155	167.33	200	83.67		
6-15	H ₂ O	-	-	177 163 162	169.00	200	84.50	84.09	97.50
7-15	H ₂ O	+	-	163 179 151	164.33	200	82.17		
8-15	H ₂ O	+	-	152 162 152	165.33	200	82.67	82.42	95.56
9-15	<i>Myxal 4OLF</i>	-	600	58 72 53	61.00	200	30.50		
10-15		-	600	52 47 42	47.00	200	23.50	27.00	31.30
11-15		+	600	11 16 16	14.33	200	7.17		
12-15		+	600	18 22 15	19.33	200	9.67	8.42	9.76
13-15		-	300	152 172 151	170.00	200	85.00		
14-15		-	300	129 150 144	141.00	200	70.50	77.75	90.14
15-15		+	300	113 117 130	118.33	200	59.17		
16-15		+	300	127 146 125	133.67	200	66.83	63.00	73.04
17-15		-	150	135 138 137	136.67	200	68.34		
18-15		-	150	148 156 144	139.33	200	69.67	69.01	80.01
19-15		+	150	95 143 129	122.33	200	61.17		
20-15		+	150	133 143 157	144.33	200	72.17	66.67	77.30
21-15		-	75	141 130 133	134.67	200	67.34		
22-15		-	75	155 161 147	154.33	200	77.17	72.76	83.78
23-15		+	75	176 191 169	178.67	200	89.33		
24-15		+	75	154 162 145	155.33	200	77.67	83.50	96.81
25-15		-	37.5	172 170 158	168.33	200	84.17		
26-15		-	37.5	154 158 186	166.00	200	83.00	83.59	96.92
27-15		+	37.5	152 171 148	157.00	200	78.50		
28-15	<i>Myxal 4OLF</i>	+	37.5	152 168 143	154.33	200	77.17	77.84	90.25

To Page No. 28

Prepared & Understood by me,
J. Proff. Mecca

Date
9/8/82

Invented by
Recorded by
Edward J. Smith

Date
9/8/82

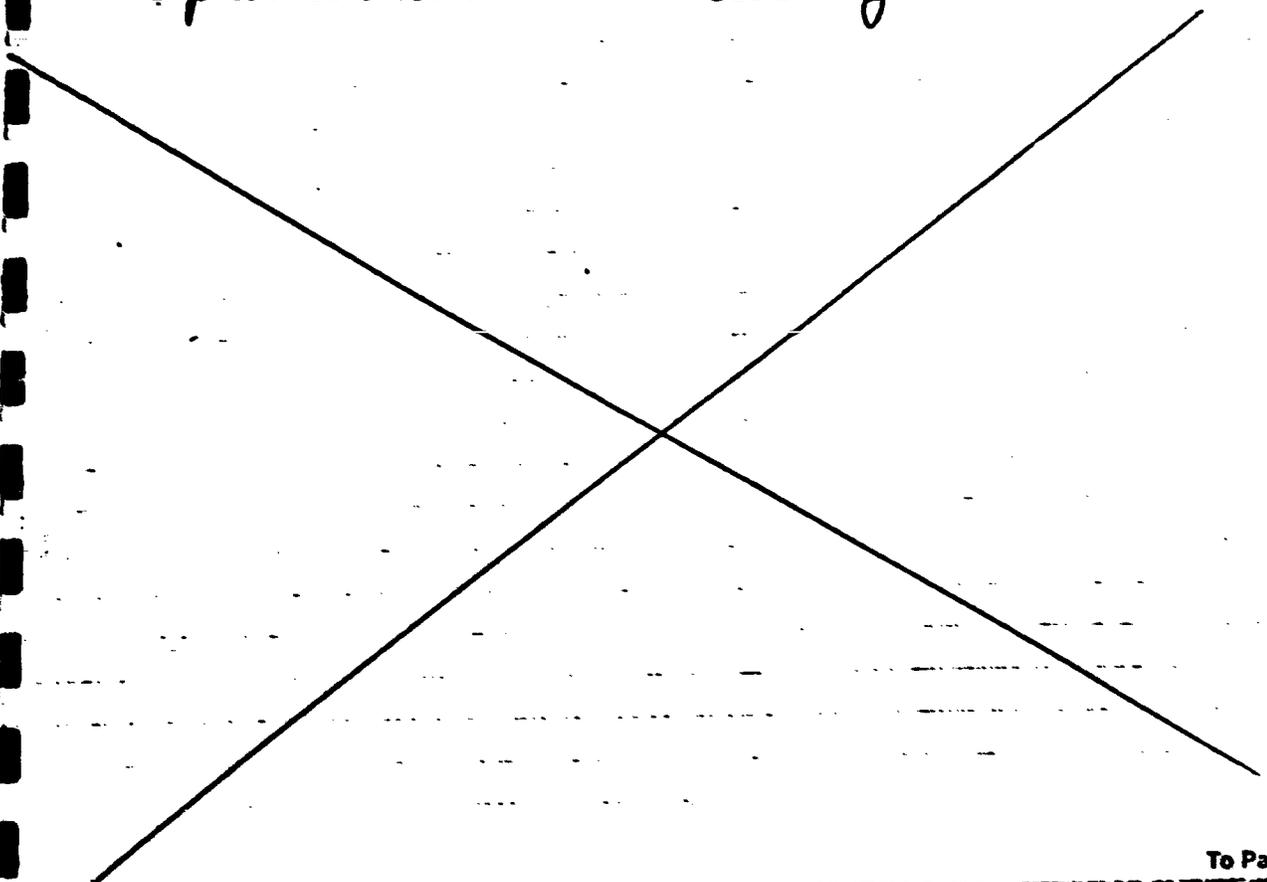
Page No. 23

Plate #	Compound	TSY	ag/ul	Plate Counts	exp. in %	#coll sides plate	% Survival	Comp. % Survival	% relative survival
29-25	EMS	-	200	54 55 54	161.00	200	80.52		
30-25	EMS	-	200	115 115 111	133.67	200	66.83	73.67	85.41
31-25	DMS	+	100	6, 62 6	61.00	200	30.50		
32-25	DMS	+	100	47 52 50	49.67	200	24.83	27.67	32.08

Table 9/8/82.

9/10/82 - Staining of resistant clones and cloning efficiency plate.

Plate removed from incubator, media aspirated off. Plates washed, fixed, and stained. Plates used in measuring top water and allowed to air dry.



To Page No. 7

Checked & Understood by me,
Prof. Beach

Date 9/10/82

Invented by

Revised by

Date

9/10/82

[Handwritten signature]

Confound	±	mg	mg/ml	# cells	mutant	total #	surviving	# early	% surviving
	SE			plated	plated	mutants	expressions	survival	survival
Ac-Hy-1-5	-	-	-	2x10 ⁵	2 2 0 1 1	6	2, 1, 1, 3, 2, 4, 3, 1, 3	200	102.92
Ac-Hy-2-3	-	-	-	2x10 ⁵	0 1 2 1 0	4	2, 2, 1, 4, 2, 1, 1, 2, 4	200	109.33
Ac-Hy-3-3	+	-	-	2x10 ⁵	1 1 0 0 2	4	1, 2, 1, 5, 2, 1, 1, 4, 3, 0	200	96.08
Ac-Hy-4-3	+	-	-	2x10 ⁵	1 1 2 1 1	6	2, 1, 1, 6, 1, 1, 5, 2, 5, 1, 3	200	95.67
Ac-Hy-5-5	-	-	-	2x10 ⁵	1 3 3 2 2	11	2, 1, 2, 3, 3, 2, 2, 4, 1, 2, 4	200	108.83
Ac-Hy-6-5	-	-	-	2x10 ⁵	1 4 0 2 2	9	2, 1, 4, 2, 3, 2, 2, 4, 2, 3	200	117.92
Ac-Hy-7-5	+	-	-	2x10 ⁵	1 1 3 2 0	7	1, 4, 2, 1, 2, 4, 3, 0, 2, 1, 2, 5	200	108.95
Ac-Hy-8-5	+	-	-	2x10 ⁵	0 1 2 2 4	9	2, 2, 4, 3, 2, 4, 2, 3, 1, 5, 4	200	110.42
Ac-Hy-9-5	-	600	-	2x10 ⁵	0 5 5 6 6	22	2, 0, 1, 2, 6, 1, 3, 1, 5, 4, 1, 4, 0	200	83.17
Ac-Hy-10-5	-	600	-	2x10 ⁵	3 3 6 3 3	18	1, 3, 1, 6, 1, 4, 2, 2, 5, 1, 2, 1, 2	200	90.00
Ac-Hy-11-5	+	600	-	2x10 ⁵	8 6 5 5 1	23	1, 2, 5, 1, 5, 1, 5, 1, 5, 3, 1, 3, 5	200	84.33
Ac-Hy-12-5	+	600	-	2x10 ⁵	9 4 12 16 11	52	1, 5, 1, 5, 1, 5, 1, 5, 1, 5, 1, 5, 1, 3, 5	200	74.42
Ac-Hy-13-5	-	300	-	2x10 ⁵	5 4 3 2 2	16	2, 0, 2, 1, 5, 1, 7, 1, 6, 1, 0, 2, 1, 6	200	96.95
Ac-Hy-14-5	-	300	-	2x10 ⁵	2 5 2 6 2	17	2, 0, 1, 7, 1, 9, 1, 5, 1, 7, 1, 2, 1, 6, 0	200	90.75

Witnessed & Understood by me,
 [Signature]

Date
 9/20/82

Invented by
 [Signature]

Date
 9/20/82

CHD/H6P27 Mutation assay - Hybrid YOLF

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Acid	Sample	Sig	To ml	Blank	Plat Counts	# mutants	Exp. min	Plat. Area	% mut
Acid 20E	EMS	-	200	2 x 10 ⁵	58 51 47 43 52	243	157 157 153 155 158 154	200	90.33
Acid 30E	EMS	-	200	2 x 10 ⁵	49 47 48 45 50	239	163 150 155 153 163 155	200	93.92
Acid 31E	DMN	+	100	2 x 10 ⁵	26 24 38 32 30	155	97 134 113 144 133 143	200	63.83
Acid 32E	DMN	+	100	2 x 10 ⁵	40 44 41 43 32	200	130 161 155 113 124 145	200	66.75

Control Colony Units calibrated
from sensitivity 6.0 - 8.3
Calibration = 419

Std. Sensitivity = 7.3
Comp. = 18%

Hand Count Control Count
211 179
187 161
183 158

To Page No. 7

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Noj. Mecca

Date 9/20/82

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Concnd	TS9	ug/ml	% relative survival (mean \bar{x})	total # mutants	Cloning efficiency (% survival)	Corrected Mutation Freq. (10^6 cells $^{-1}$)	Average Mutation Frequency
untreated	-	-	-	6	102.92	5.83 / 10^6	-
untreated	-	-	100.00	4	109.33	3.66 / 10^6	4.75 / 10^6
untreated	+	-	-	4	96.08	4.16 / 10^6	-
untreated	+	-	98.55	6	95.67	6.27 / 10^6	5.22 / 10^6
H ₂ O	-	-	-	11	105.83	10.11 / 10^6	-
H ₂ O	-	-	97.50	9	117.92	7.63 / 10^6	8.19 / 10^6
H ₂ O	+	-	-	7	108.75	6.44 / 10^6	-
H ₂ O	+	-	95.56	9	110.42	8.15 / 10^6	7.30 / 10^6
1/200	-	600	-	22	83.17	26.45 / 10^6	-
	-	600	31.30	18	90.00	20.00 / 10^6	23.23 / 10^6
	+	600	-	23	84.33	27.27 / 10^6	-
	+	600	9.76	52	74.42	69.87 / 10^6	48.57 / 10^6
	-	300	-	16	96.75	16.54 / 10^6	-
	-	300	90.14	17	90.83	18.72 / 10^6	17.63 / 10^6
	+	300	-	13	78.83	16.49 / 10^6	-
	+	300	73.04	12	82.25	14.59 / 10^6	15.54 / 10^6
	-	150	-	28	92.33	30.23 / 10^6	-
	-	150	80.01	22	93.17	23.61 / 10^6	26.97 / 10^6
	+	150	-	11	95.92	11.47 / 10^6	-
	+	150	77.30	27	100.33	26.91 / 10^6	19.19 / 10^6
	-	75	-	26	100.33	25.91 / 10^6	-
	-	75	83.78	12	99.17	12.10 / 10^6	19.01 / 10^6
	+	75	-	17	89.33	19.03 / 10^6	-
	+	75	96.81	11	89.83	12.25 / 10^6	15.64 / 10^6
	-	37.5	-	13	89.92	14.46 / 10^6	-
	-	37.5	96.92	14	95.67	14.63 / 10^6	14.55 / 10^6
	+	37.5	-	9	90.00	10.00 / 10^6	-
	+	37.5	90.25	8	91.25	8.77 / 10^6	9.38 / 10^6
EMS	-	200	-	243	90.33	269.01 / 10^6	-
EMS	-	200	85.41	239	93.92	254.47 / 10^6	261.74 / 10^6
DMU	+	100	-	155	63.83	242.83 / 10^6	-
DMU	+	100	32.08	200	66.75	299.63 / 10^6	271.23 / 10^6

Read & Understood by me,

J. Meera

Date

9/20/82

Invented by

Recorded by

J. A. Smith

Date

9/20/82

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QUALITY ASSURANCE UNIT 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 314-AC-002-82

The following inspections were performed:

Interval	Date
<u>Treatment Phase</u>	<u>8/26/82</u>
<u>Selection for Mutation Phase</u>	<u>9/3/82</u>
<u>Scoring Phase</u>	<u>9/20/82</u>
<u>Reporting Phase</u>	<u>10/1/82</u>
_____	_____
_____	_____

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

10/1/82
Date

Stephen Mass
Quality Assurance Unit