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**Genotoxic Potential of Reactive-Polymer in the
CHO HGPRT Forward Mutation Assay
and
CHO Sister Chromatid Exchange Assay**

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DIVISION OF TOXICOLOGY**

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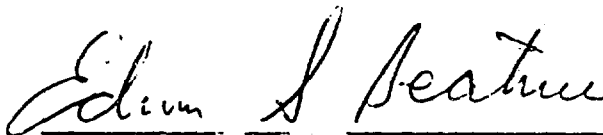
Genetic Potential of Reactive-Polymer in the CHO HGPRT Forward
Mutation Assay and CHO Sister Chromatid Exchange Assay (Harbell,
Witcher, and Korte)

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ABSTRACT

Reactive-polymer and its component parts were tested for their abilities to induce forward mutations in the CHO HGPRT Forward Mutation Assay and to damage DNA as measured by the CHO Sister Chromatid Exchange Assay. All materials were tested over dose ranges to include cytotoxic and noncytotoxic doses. Neither reactive-polymer nor its components induced a significant rise in the mutant frequency in the CHO HGPRT assay or in the frequency of sister chromatid exchanges. These data suggest a low genotoxic potential for reactive-polymer.

Key Words: Mutagenicity, Genetic Toxicology, HGPRT, Sister chromatid exchange, Chinese Hamster Ovary Cells, Reactive-polymer

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PREFACE

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STUDY NUMBER: 74028

STUDY DIRECTOR: MAJ Don W. Korte Jr., PhD, MSC

PRINCIPAL INVESTIGATOR: MAJ John W. Harbell, PhD, MSC

TEST SUBSTANCE: Reactive-polymer (TP072)

INCLUSIVE STUDY DATES: 29 Oct 86 to 8 May 87

OBJECTIVE: The objective of this study was to determine the genotoxic potential of reactive-polymer (TP072) by using the CHO HGPRT Forward Mutation Assay and the CHO Sister Chromatid Exchange Assay.

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SIGNATURES OF PRINCIPAL SCIENTISTS AND MANAGERS INVOLVED IN
THE STUDY

We, the undersigned, declare that this study number
74028 was performed under our supervision, according to the
procedures described herein, and that this report is an
accurate record of the results obtained.

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The Genotoxic Potential of Reactive-polymer in the CHO HGPRT Forward Mutation Assay and the CHO Sister Chromatid Exchange Assay -- Harbell, Witcher, and Korte

Reactive-polymer is a potential reactive barrier against chemical agents. The Division of Toxicology, LAIR, was tasked by U.S. Army Natick Research, Development, and Engineering Center to assess the genotoxic potential of this material. Two distinct genotoxicity end points were chosen for this study: forward mutation and DNA damage. The first was assessed using the Chinese Hamster Ovary (CHO) hypoxanthine-guanine phosphoribosyl transferase (HGPRT) forward mutation assay (1) while the second was tested using the CHO sister chromatid exchange assay (2). These assays are normally conducted in this laboratory by following the Good Laboratory Practices (GLP) guidelines promulgated by the EPA. Due to the security classification of the material being tested, the assays for chemical composition and the independent audit required by these guidelines could not be employed. However, with the two exceptions listed above, the studies were conducted in accordance with GLP guidelines.

Objective of the Study

To examine the cytotoxicity, mutagenicity, and DNA-damaging capacity of reactive-polymer by using the CHO HGPRT forward mutation and CHO sister chromatid exchange assays.

MATERIALS AND METHODS

Test Compound

Reactive-polymer, its active components, and matrix components were evaluated in these assays. Reactive-polymer and its matrix were received as granules while the active components were a powder. The granules were ground to a fine powder by using a Spex liquid nitrogen mill (Spex Industries, Edison, New Jersey), sterilized in 70% ethanol for 20 minutes and then washed three times with sterile glass distilled water. All other materials were dissolved in sterile glass-distilled water and filter-sterilized. All dosing materials were prepared immediately before use.

Positive Control Chemicals

Ethyl methanesulfonate (EMS) (Sigma lot# 83F-0279) was dissolved in sterile glass-distilled water, filter-sterilized

through a 0.22 μ m filter and added to the culture medium to achieve the desired final concentrations.

Cells

CHO cells, subline CHO-K₁-BH₄ used for the HGPRT assay were obtained from Dr. A. Hsie, Oak Ridge National Laboratory, Oak Ridge, Tennessee. These cells were maintained in Ham's F12 (Irvine Scientific, Irvine, California) supplemented with 5% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah). Cell stocks were stored frozen in liquid nitrogen and an aliquot was thawed as needed for each assay. CHO cells used for the sister chromatid exchange assay were obtained from Dr. S. Wolff, University of California San Francisco, San Francisco, California. These cells were grown in GEM 1717 (Irvine Scientific) supplemented with 10% FBS. No antibiotics were used in either culture system.

Assay Formats

HGPRT forward mutation assay

This assay has 4 stages: dosing, 24-hour cytotoxicity determination, 7-day mutation expression time, and finally quantitation of the mutant frequency in the cultures.

Dosing: Twenty cultures each containing 1.5×10^6 CHO HGPRT +/- cells in Ham's F12 with 5% FBS were exposed to the controls or test compounds for five hours, washed twice, and allowed to continue growing in Ham's F12 with 5% FBS to express whatever toxicity may have been induced by the treatment. Specific doses are given in Table I of the Appendix.

Cytotoxicity: After 24 hours, the cell monolayers were removed from the flasks with Ca⁺⁺- and Mg⁺⁺- free Hanks Salt Solution, and single cell suspensions were produced by gentle pipetting. Two hundred cells from each culture were plated into each of three dishes to determine the percentage of viable cells that remained after 24 hours. These plates were incubated for 7 days, fixed, stained with crystal violet, and the colonies counted. Data are expressed in terms of mean raw count (viable count), cloning efficiency (count/200), and percent of negative controls.

Mutation expression: From the remaining cells in each group, 1.5×10^6 cells were put into a fresh flask to continue growing. Since the cells have a doubling time of about 10-12 hours, it is necessary to split the cultures again on day 4 to prevent their overgrowth. Again single

cell suspensions were produced, and 1.5×10^6 cells were seeded into fresh flasks.

Quantitation of mutant frequency On day seven, by which time the mutant cells had lost all of their HGPRT enzyme, the populations were ready to undergo selective cloning to determine the relative number of mutant cells induced by each treatment. Both viable count cloning (as on day one) and selective cloning were performed. For the selective cloning of each treatment group, 2×10^5 cells were plated into each of 7 dishes which contained selection medium (total of 1.4×10^6 cells). Selection medium consisted of Ham's F12 without hypoxanthine but supplemented with 5% dialyzed FBS and 1.34×10^{-5} M 6-thioguanine. 6-Thioguanine prevents cells that contain the HGPRT enzyme (nonmutants) from proliferating but does not harm the mutant cells. The viable count cloning was performed, as on day one, using selective medium without 6-thioguanine. After seven days, the plates were fixed, stained, and the colonies counted. Data were expressed as mutants per million viable cells. For example, if the total number of mutant colonies on the seven selective cloning plates were 178 and the viable count were 134, then the mutant frequency would be:

$$178 / (1.4 \times 10^6) \times (200/134) = 190.7 \text{ mutant clones per } 10^6 \text{ viable cells}$$

where

$1.4 \times 10^6 = 2 \times 10^5 \times 7$ was the total number of cells plated for the mutant count, and

200/134 was the inverse of the viable fraction and corrected for the percentage of dead cells in the cultures.

In order for a compound to be considered mutagenic it should induce a dose-correlated increase in mutant frequency with at least one dose yielding a threefold rise in the mutant frequency over controls. A single value in the middle of the dose range that produces a threefold increase without the correlated dose response relationship is generally not considered to signify an overall positive response.

CHO sister chromatid exchange assay

The CHO cells were grown and dosed in GEM 1717 supplemented with 10% fetal bovine serum. The sterilized test materials were dissolved directly in the medium. The dose groups and controls are given in Table II, in the Appendix. The cells were exposed for 24 hours to the compound and 5 μ M 5-bromodeoxyuridine (BrdU). During the

last 2.5 hours of the growth period, colchicine (0.4 ug/ml final) was added to the medium to arrest mitoses. Cells were then harvested, swollen in hypotonic KCl, fixed, and chromosome spreads prepared. The chromosome preparations were stained using the method of Stetka and Wolff (3) to visualize the sister chromatid exchanges. Scoring was done on coded slides. One hundred cells in each treatment group were scored to determine the percentage of first, second, and third division metaphases. Normally 40 second-division metaphases, which contained all 20 chromosomes, were scored for sister chromatid exchanges (data listed as SCEs per metaphase). Data were analyzed by t-test between the negative and positive controls and ANOVA between the negative control and the test compound groups. The level of significance required for a positive response was $p < 0.001$.

RESULTS

The specific dosing materials, dose concentrations and the resulting mutation and SCE data are given in Tables I and II of the Appendix.

In the CHO HGPRT assay, compounds A and B showed marked toxicity at the highest doses tested (1.4-0.7 mg/ml and 4.0-2.0 mg/ml, respectively). Compound C was only moderately toxic even at the highest dose tested (4.0 mg/ml). As expected, the EMS induced a dose-dependent, statistically significant rise in the mutant frequency. Control frequency was 4.6×10^{-6} while EMS at 0.075 and 0.15 mg/ml induced mutation frequencies of 190.7×10^{-6} and 375.8×10^{-6} respectively. Compound C induced greater than a threefold increase in the mutant frequency (18.3×10^{-6}) at 2.0 mg/ml, but this increase did not correlate with dose. No other significant increases in mutant frequency were observed.

In the CHO sister chromatid exchange assay, compound B was very toxic at doses above 2.0 mg/ml while the other compounds were only slightly toxic. Even at 2.0 mg/ml, cells treated with B showed 29% first division metaphases indicating a slowing of cell cycle times. EMS at 2.4 mg/flask (0.16 mg/ml) induced a statistically significant increase in the number of SCEs (control of 7.3 vs EMS of 13.3). None of the test compounds induced any significant increase in SCEs.

DISCUSSION

In the two assays employed, reactive-polymer and its components showed some cytotoxicity but did not induce significant increases in mutant frequency or SCEs. Both assays were considered valid in that the negative control values were within normal limits and the positive controls

induced statistically significant responses. These data suggest a low genotoxic potential for reactive-polymer. Its cytotoxic action, however, might have an effect on very sensitive tissues (e.g. ocular), and therefore some care in handling this material would be appropriate.

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Appendix

Table I

CHO HGPRT FORWARD MUTATION ASSAY

22 OCT 1986

Compound	Dose (mg/ml)	Viable ^a Count (day one)	CE ^b	%C ^c	Viable Count (day seven)	CE	%C	Mutant Frequency (x 10 ⁻⁶)
Med	0.0	171	86	100	174	87	100	4.6
EMS ^d	0.075	134	67	78	133	67	76	190.7 ^g
EMS	0.150	97	49	57	92	46	53	375.8 ^g
A	1.40	0	0	0	Too toxic			
A	0.70	7	4	4	108	54	62	0.0 ^f
A	0.52	111	56	65	152	76	87	7.5 ^f
A	0.35	164	82	96	147	74	84	7.8 ^f
A	0.26	179	90	105	144	72	83	11.9 ^f
A	0.175	175	88	102	143	72	83	2.0 ^f
E	4.0	0	0	0	Too toxic			
E	2.0	0	0	0	Too toxic			
B	1.5	8	4	5	121	61	70	0.0 ^f
B	1.0	86	43	50	157	79	90	5.4 ^f
B	0.75	165	83	96	158	79	91	0. ^f
B	0.5	176	88	103	168	84	97	3.4 ^f
C	4.0	114	57	67	160	80	92	5.4 ^f
C	2.0	135	67	79	164	82	94	18.3 ^g
C	1.5	150	75	88	153	77	88	4.4 ^f
C	1.0	136	68	80	158	79	91	5.4 ^f

^a Viable Count= number of colonies formed from 200 cells plated

^b CE = Cloning Efficiency = viable count/200

^c %C = percent of controls

^d EMS= ethyl methanesulfonate

^e Significant by the threefold-over-controls rule

^f Not Significant

^g Meets the threefold-over-controls rule; however, no strong correlated dose response was achieved and therefore M was not considered to be significantly mutagenic.

A= Active B= Reactive-polymer C= Matrix

Table II
SISTER CHROMATID EXCHANGE ASSAY

1 Oct 86

Compound	Dose	%FD	SCE	SD	Comment
Neg Cont.		2	7.1	3.1	
EMS	2.4 mg/flask	2	13.3	4.3	p<0.00001
A	0.75 mg/flask	15	8.0	3.7	NS
A	0.60 mg/flask	2	8.2	2.9	NS
A	0.45 mg/flask	15	7.6	2.8	NS
B	5.0 mg/flask	too toxic to score			
B	4.0 mg/flask	too toxic to score			
B	3.0 mg/flask	too toxic to score			
B	2.0 mg/flask	29	7.8	2.8	NS
B	1.0 mg/flask	12	8.4	2.8	NS
C	3.0 mg/flask	7	6.0	2.8	NS
C	2.0 mg/flask	6	7.5	2.6	NS
C	1.0 mg/flask	7	7.2	3.2	NS
Neg Cont.		6	7.4	2.8	

%FD= percent in first division increase above controls indicates cytotoxicity and slowing of cell cycle.

SCE= sister chromatid exchanges

SD= standard deviation

NS= not significant

Control and treatment groups were compared using ANOVA at $p < 0.001$. Negative control and positive controls were compared using the t-test.

A= Active B= Reactive-polymer C= Matrix

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