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Toxicity Studies of Mild Gasification Products

Quarterly Report
October - December 1993

March 1994

Work Performed Under Interagency Agreement No.: DE-AI21-89MC26018

For
U.S. Department of Energy
Office of Fossil Energy
Morgantown Energy Technology Center
Morgantown, West Virginia

By
National Institute for Occupational Safety and Health (NIOSH)
Morgantown, West Virginia

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March 1994

The Genotoxicity Studies of Mild Gasification Product, Shell Oil PSIS#830331, in Mammalian Cells

INTRODUCTION

The major hypothesis of carcinogenesis is that malignancy is due to an alteration (mutation) of the genetic material in a somatic cell. Reactive electrophilic metabolites are generated from many chemicals by the action of endogenous mixed function oxidases. These reactive metabolites may bind to cellular macromolecules, such as DNA, and can, therefore, initiate a mutagenic or carcinogenic event. Prokaryotes and non-mammalian eukaryotes are used in mutation assays, while cultured mammalian cells are generally used for mutagenic as well as clastogenic tests examining alterations and damage to the DNA and/or chromosomes of somatic cells. One of the first mammalian cell lines used in genotoxicity studies is V79, which was derived from Chinese hamster lung cells.

According to the test plan on toxicity studies of mild gasification products, mammalian cell *in vitro* assays are to be performed on selected samples displaying mutagenic activity in the Ames assay. The results of the Ames testing of the mild gasification sample Shell Oil PSIS#330331 were negative. However, fractionation of the sample and Ames testing of the subfractions were performed per DOE request. None of the subfractions was mutagenic in the Ames assay, as has been previously reported. Assays for the induction of gene mutation, sister chromatid exchange and micronucleus formation in V79 cells have also been carried out for the sample. This paper reports the results of the mammalian cell assay.

METHODS AND MATERIALS

Sample description

PSIS#830331 is a low-temperature coal tar obtained from Shell Oil Company, Houston, Texas.

Sample preparation

PSIS#830331 was melted by warming to 80°C in a water bath. 0.279 g of the sample was put into a sterile vial, and 11.6 ml dimethyl sulfoxide (DMSO) was added. The combination was warmed to 50°C and sonicated in a Branson Model 3200 water bath/sonicator for 30 minutes and vortexed until a homogenous solution with a concentration of 25 mg/ml was achieved. The sample was further diluted with DMSO by one half dose in turn (12.5, 6.25, 3.12, 1.56 mg/ml etc.).

DMSO was purchased from Fisher Scientific Company, Fair Lawn, New Jersey. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), used as a positive control, was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

Cell line and culture conditions

The Chinese hamster lung fibroblast (V79) cell line was kindly supplied by Dr. C.C. Chang (Michigan State University). The cells were certified to be free of mycoplasma contamination by the standard MycoTect assay (Gibco, Gaithersburg, Maryland). Stock cultures were maintained in a monolayer in a 75 cm² flask with growth medium – minimum essential medium (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) – at 37°C and 100% humidity in a 5% CO₂ atmosphere. The cells were passaged every 3-4 days by treatment with a trypsin-EDTA solution (Gibco) in phosphate buffered saline (PBS) to prevent the cultures from becoming confluent. Working cultures of this cell line were maintained under the same conditions for the described assays.

Assay procedures

HGPRT gene mutation assay

Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) is an enzyme, consisting of 2-4 protein subunits that catalyzes the salvage of hypoxanthine and guanine by the conversion of these purines to the corresponding nucleoside-5-monophosphates (necessary components of DNA required for cell division and culture growth) upon reaction with phosphoribosyl pyrophosphate. The enzyme also catalyzes the conversion of the purine analogs (poisons) 6-mercaptopurine (MP), 6-thioguanine (TG), and 8-azaguanine (AG) into their lethal nucleoside-5-monophosphate forms. V79 cells with mutations that inactivate the product of the HGPRT gene locus, on the X chromosome in humans and rodents, will grow in medium containing the poisonous purine analogs that kill cells with normal HGPRT levels, because they can't metabolize exogenous purines or their deadly analogs. In order to survive, however, these mutants must rely on their ability to synthesize the required purines by a de novo pathway. Aminopterin is a substance that inhibits the de novo synthesis of nucleotides, making a cell dependent on exogenous hypoxanthine, glycine and thymidine. When cultured in medium containing hypoxanthine, aminopterin, thymidine and glycine (HAT medium), mutated V79 cells die because they cannot utilize the exogenous hypoxanthine or synthesize their own purines via the de novo pathway. V79 clones with a mutation inactivating the HGPRT gene product are selected by both 6-thioguanine resistance (TG^r) and an inability to survive in HAT medium. Induction of the TG^r mutation will be carried out according to the protocol recommended by E.R. Nestmann et al. (1991), based on a survey of current practice in genotoxicity testing laboratories.

Five chemical concentrations, a solvent control, and a positive control were tested with and without S9 metabolic activation. 5 x 10⁵ cells were seeded in 25 cm² flasks containing 5 ml growth medium and incubated at 37°C in a humid atmosphere of 5% CO₂. Twenty-four hours later, the medium was replaced with treatment medium containing 4.5 ml 5% FBS medium and 0.5 ml S9 mixture (for metabolic activation) or 5 ml 5% FBS medium without S9 (for testing direct-acting mutagens), and 50 µl of the Shell Oil sample. After a 3-hour exposure, the cells were rinsed twice with PBS, trypsinized, and replated in two 100 mm dishes containing 10 ml MEM at 2.5 x 10⁵ cells/dish for the mutation studies and in six

60 mm dishes containing 5 ml MEM at 100 cells/dish for measurement of cell survival. Following a 7-day incubation period, the cultures in the 60 mm dishes were fixed, stained and counted. Cell survival relative to the untreated controls (i.e., the relative colony forming efficiency; CFE) was determined for each dose level tested; relative CFE = (the number of colonies with treatment/the number of colonies in the control) x 100%. At least four treatment concentrations which gave relative CFEs ranging between 10 and 100% were tested.

The cultures in the 100 mm dishes, prepared for the mutation studies, were also incubated at 37°C for an expression period of 7 days; during which time the cells were passaged two or three times. After a week, the cells were washed with PBS and trypsinized in preparation for plating another CFE and expression assay. 100 cells/dish were seeded in 60 mm dishes containing 5 ml MEM. Six dishes per treatment were used. These cultures were fixed and stained with Giemsa after 7 days to determine the absolute CFE of the treated cells. Absolute CFE = (the number of colonies formed/the number of cells plated) x 100%. Approximately 2.5×10^5 cells were seeded in 100 mm dishes (six per treatment) containing 10 ml of selection medium (94% hypoxanthine-free MEM, 5% dialyzed FBS, 1% 1mM 6-thioguanine solution). The selection plates were incubated for 10 days, after which the colonies were fixed, stained, and counted by eye. The criterion for minimum acceptable colony size was a diameter of 0.5 mm or greater or a minimum of 50 cells per colony. Mutation frequency (MF) = number of mutant colonies/(total cells seeded on all replicate plates x absolute CFE). Solvent and MNNG were tested simultaneously as solvent and positive controls, respectively.

Sister Chromatid Exchange (SCE) Assay

The *in vitro* SCE assay is frequently used for evaluating the potential of chemicals to induce genetic damage. Although the molecular basis of SCE formation remains unknown, SCEs represent the breakage of 4 stands of DNA (2 double helices), a switch of those strands between chromatids of the same chromosome, and the rejoining of those strands in their new location. It is important to know whether the breakage and rejoining events occur faithfully (i.e. without producing any modification in the genetic code). Several studies have demonstrated that the frequency of SCE increases when cells are exposed to known mutagens and carcinogens.

The method followed is that described by Perry and Wolff (1974). Approximately 6×10^5 V79 cells were seeded into 100 mm dishes containing 10 ml growth medium and incubated at 37°C. Twenty-four hours later, the medium was removed and replaced with treatment medium containing 9 ml 5% FBS medium and 1 ml S9 mixture, or 10 ml 5% FBS medium without S9 treatment. The 4-6 concentrations of the Shell Oil sample were determined by the survival test included in the gene mutation assay. After a 3-hour treatment, the cells were washed with PBS, trypsinized, and reseeded into two dishes per treatment with 10 ml fresh medium. One dish was used for micronucleus induction (see below); the other for the SCE assay. Solvent and positive (MNNG) controls were run concurrently with each experiment.

For the SCE assay, 5-bromo-2'-deoxyuridine (BrdU, Sigma) was added to each dish to a final concentration of 20 μ M, followed by a 28-30-hour incubation in the dark. Two hours before harvesting, colcemid was added at a final concentration of 1 μ M. The cells were harvested by trypsinization, washed by centrifugation, resuspended in 75 μ M KCl hypotonic solution for 20 min at 37°C, and fixed 3 times in a 3:1 mixture of methanol-acetic acid. Two drops of the cell suspension was dropped onto a cold, wet slide which was then waved through a flame. After aging overnight, the slides were stained using the fluorescence-Giemsa method (Perry and Wolff, 1974). One hundred metaphase cells from each treatment were counted for replicative index determination, while 30 cells having 20-23 well-distinguished and well-spread chromosomes were scored for SCEs. The results are expressed as the mean number of SCEs per metaphase $\pm$ S.D. The grouped t test was used to compare the average frequencies of SCEs induced by the Shell Oil sample.

Micronucleus (MN) assay

Micronuclei are small chromatin-containing bodies arising from acentric chromosomal fragments or whole chromosomes that failed to be incorporated into the daughter nuclei following mitosis. The presence of MN can be taken as an indication of chromosome breakage and/or spindle dysfunction (Krishna et al., 1984). Treatment of V79 cells with the Shell Oil sample and preparation of the cell suspension after exposure are described in the procedures for the SCE assay. After treatment, cells were incubated for 24 hrs, harvested by trypsinization, washed by centrifugation, and resuspended in growth medium to an optimum density. Slides were prepared using cytocentrifugation. An aliquot of cell suspension (50-75 μ l) was loaded into a chamber, and the cells were pelleted onto slides at 600 rpm for 7 min, fixed with methanol, and stained with Diff-Quik stain (Dage Diagnostics, Aquada, Puerto Rico). The frequency of micronucleated cells in each treatment is based on 3000 cells scored. The criteria used to scored MN were the following: (1) the diameter of micronucleus must be no larger than one-fifth of the main nucleus; (2) the staining intensity must be equal to that of nucleus; (3) the micronucleus must be located in the cytoplasm; and (4) there must be no contact with the main nucleus (Roberts et al., 1986). The results are expressed as an average number of cells with MN per 1000 cells $\pm$ S.D. Statistical analysis is based on the Chi-square test.

RESULTS AND DISCUSSION

Results of the cytotoxicity testing of the Shell Oil sample indicate that the test chemical significantly reduced the survival of cultured V79 cells at the concentration range of 15.6 - 94.0 μ g/ml without S9 activation (Table 1). There was less killing effect induced by the test sample under the conditions tested in the presence of S9. This may be due to an inactivation of the cytotoxicity by the metabolic enzymes in the S9.

Results of the HGPRT gene mutation assay are shown in Table 2. V79 cells were exposed to the Shell Oil sample at doses as high as 94.0 μ g/ml, both with and without S9 activation; however, none of the concentrations tested significantly increased the frequency of TG'

mutants. No dose response or minimum of a three-fold increase over the spontaneous level was noted. Therefore, no evidence of induction of gene mutations was noted under the conditions tested. The study was repeated, and the results were confirmed.

The frequencies of MN and SCE induced by the Shell Oil sample at various concentrations are presented in Tables 3 and 4, respectively. A significant increase in the frequency of MN formation was seen when cells were treated with the Shell Oil sample at concentrations of 62.5 and 94.0 $\mu\text{g/ml}$, both with and without S9 metabolic activation. The Shell Oil sample induced a significant increase in the SCE frequency throughout the dose range tested (15.6 - 94.0 $\mu\text{g/ml}$). The observed increases in both MN and SCE are dose-dependent and statistically significant.

These results indicate that the Shell Oil sample can cause DNA damage, chromosome breakage and/or spindle fiber dysfunction in V79 cells with and without metabolic activation by S9. Therefore, it appears that direct-acting genotoxic agents are present in the Shell Oil sample, although it may also contain indirect-acting mutagens.

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Table 1. Cytotoxicity of Shell Oil PSIS#830331 to V79 Cells

Chemical treatment ^a	Concentration (μg/ml)	Relative colony forming efficiency ^b			
		-S9		+S9	
		Mean ± SD	%	Mean ± SD	%
Shell Oil	0.0 ^c	96 ± 13	100	110 ± 8	100
	7.8	123 ± 7	128	116 ± 7	105
	15.6	82 ± 8	85	96 ± 7	87
	31.2	75 ± 12	78	110 ± 3	100
	62.5	63 ± 8	65	81 ± 3	73
	94.0	26 ± 5	27	61 ± 12	55
MNNG ^d	1.0	60 ± 6	63	116 ± 9	105

^a 3 h exposure

Absolute CFE (treatment)

^c DMSO solvent control^b Relative CFE = $\frac{\text{Absolute CFE (treatment)}}{\text{Absolute CFE (control)}} \times 100$ ^d N-methyl-N'-nitro-N-nitrosoguanidine**Table 2. Frequency of 6-thioguanine Resistant Mutants in V79 Cells Treated with Shell Oil PSIS#830331**

Chemical treatment ^a	Concentration (μg/ml)	Relative Survival (%)		Mutants per 10 ⁵ Survivors (Mean ± SD)	
		-S9	+S9	-S9	+S9
Shell Oil	0.0 ^b	100	100	1.9 ± 0.2	0.6 ± 0.4
	7.8	128	105	0.5 ± 0.5	2.0 ± 0.8
	15.6	85	87	0.2 ± 0.2	0.1 ± 0.1
	31.2	78	100	0.1 ± 0.2	0.2 ± 0.2
	62.5	65	73	0.9 ± 1.5	0.1 ± 0.2
	94.0	27	55	0.2 ± 0.3	0.2 ± 0.3
MNNG ^c	1.0	63	105	20.5 ± 1.7	92.1 ± 0.4

^a 3 h exposure^b DMSO solvent control^c N-methyl-N'-nitro-N-nitrosoguanidine

Table 3. Frequency of Micronuclei in V79 cells Treated with Shell Oil PSIS#830331

Chemical treatment ^a	Concentration (μg/ml)	Total Number of MN Cells ^b		Frequency of MN Cells ‰ (Mean ± SD)	
		-S9	+ S9	-S9	+ S9
Shell Oil	0.0 ^c	27	26	9.0 ± 1.7	8.7 ± 2.1
	15.6	38	36	12.7 ± 2.5	12.0 ± 2.0
	31.2	41	31	13.7 ± 0.6	10.3 ± 0.6
	62.5	50	44	16.7 ± 2.9*	14.7 ± 1.2**
	94.0	47	49	15.6 ± 2.1**	16.3 ± 3.8**
MNNG ^d	1.0	144	74	48.0 ± 2.0**	24.7 ± 3.5**

^a 3 h exposure

* p < 0.05, Chi-square test

^b 3000 cells were scored

** p < 0.01, Chi-square test

^c DMSO solvent control

^d N-methyl-N'-nitro-N-nitrosoguanidine, 2000 cells were scored.

Table 4. Frequency of Sister Chromatid Exchange in V79 Cells Treated with Shell Oil PSIS#830331

Chemical treatment ^a	Concentration (μg/ml)	SCE / Cell ^b (Mean ± SD)		Replication Index	
		-S9	+ S9	-S9	+ S9
Shell Oil	0.0 ^c	7.3 ± 2.1	7.2 ± 2.2	1.97	1.97
	15.6	10.3 ± 3.9**	8.8 ± 2.8*	2.00	1.97
	31.2	11.9 ± 3.5**	9.8 ± 2.8**	1.93	1.97
	62.5	13.3 ± 4.5**	12.6 ± 4.3**	1.69	1.93
	94.0	too toxic	15.3 ± 4.7**	toxic	1.62
MNNG ^d	1.0	48.8 ± 10.2**	36.4 ± 10.4**	1.90	1.81

^a 3 h exposure

* p < 0.05, grouped t-test

^b 30 metaphase cells were scored

** p < 0.01, grouped t-test

^c DMSO solvent control

^d N-methyl-N'-nitro-N-nitrosoguanidine

Mutagenicity of MRE 1 Subfractions in the Ames Assay

INTRODUCTION

Mild gasification of coal is a technology being developed by the United States Department of Energy (DOE) and private industry with the hope that a cleaner method of coal use can help meet future energy needs. As the technology develops and its commercial use becomes a more viable possibility, efforts are being made to study the safety and possible toxicity of the mild gasification products. DOE and the National Institute for Occupational Safety and Health (NIOSH) are cooperating through an interagency agreement to examine some of these products for their genotoxic potential.

The Ames Salmonella/microsomal assay (Maron and Ames, 1983) is widely used as a short-term test for the detection of possible genotoxic agents and potential carcinogens. Bacterial tester strains used in the Ames assay contain specific mutations (frameshift or base pair substitution) that cause the bacteria to be dependent on growth medium containing the amino acid histidine. The mutagenic activity of a test substance is measured by the number of reverse mutations eliminating the histidine requirement. DNA mutation is generally accepted to be involved in the initiation stage of carcinogenesis; therefore, the Ames assay has often been used as the first tier of testing in the evaluation of long-term health risks associated with exposure to certain chemicals. NIOSH has used this assay to study the mutagenicity of ten mild gasification products. Several of those samples, including MRE 1, have been found to be mutagenic. It is difficult to know which group of chemicals is responsible for the mutagenic activity in complex mixtures. Therefore, such mixtures are often separated by organic solvents into subfractions for testing of mutagenic potentials. This report details the results of testing the subfractions of MRE 1.

MATERIALS AND METHODS

Sample description

MRE 1 has a liquid/tar consistency and a boiling point of 216°F. The sample was made from a 50:50 mix of low- and high-sodium Montana coal and was obtained from Maumee Research and Engineering (MRE).

Chemicals

Positive controls:

2-aminoanthracene (2AA), a chemical mutagen which requires metabolic activation (+S9), and trinitrofluorenone (TNF), a direct-acting mutagen (-S9), were used in the assay system at a final concentration of 2.5 and 0.5 $\mu\text{g}/\text{plate}$, respectively. Both chemicals were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin.

Solvent control:

Dimethylsulfoxide (DMSO; sterile, spectrophotometric grade) was purchased from EM Science, Cherry Hill, New Jersey.

Sample preparation

Fractionation

The fractionation of MRE 1 was performed by Dr. Joseph Ma of the West Virginia University School of Pharmacology. Briefly, the sample was dissolved in ethyl acetate and extracted with sodium hydroxide (NaOH). The NaOH layer was neutralized with HCl and the precipitate (the acidic fraction) was collected. The ethyl acetate layer was extracted again; this time with HCl. The acidic layer was separated and neutralized with sodium bicarbonate. The precipitate, the basic fraction, was collected. The remaining ethyl acetate layer now contained only the neutral fractions of the sample. Crude silica gel was added to the solution. After stirring, the solvent, ethyl acetate, was removed under reduced pressure. An affinity column was prepared and eluted with hexane and cyclohexane to collect the nonpolar neutral fraction. The column was then eluted with cyclohexane/ethyl acetate to collect the polar neutral fraction. The solvents were removed from each fraction before testing was done. The fractions were dissolved in DMSO for the Ames assay. Care was taken to insure that homogeneous mixtures were used while preparing dilutions and during testing.

DMSO Solvation:

Each fraction was dissolved in pure DMSO to the desired concentrations. The mixture was heated to 50°C and sonicated in a Branson model 3200 water bath/sonicator for 30 minutes to facilitate homogenization. The positive controls were prepared in a manner similar to the sample to ensure consistent experimental design.

Microsuspension Assay

The mutagenicity of MRE 1 was determined using the microsuspension variant of the Ames Salmonella/microsomal assay system (Maron and Ames, 1983) that can be used when the amount of sample is very small. This test is more sensitive than the standard protocol (Kado et al., 1983), partially due to the fact that a more concentrated solution (20X) of bacteria is used. Tester strains TA98 and TA100 (kindly provided by Dr. B.N. Ames of the University of California at Berkeley) were grown and centrifuged to a density of approximately 4×10^9 cells/ml nutrient broth. A 25 μ l aliquot of the bacterial solution was added to each test tube used in the assay. To determine the metabolic activation requirements of the sample, each dose was tested both with and without S9 metabolic activation mixture (prepared from liver preparations of Arochlor-1254-treated male Wistar rats). Each of these treatments was tested in triplicate. The +S9 treatments received 65 μ l aliquots of the S9 mix, while -S9 treatments received 65 μ l physiological saline. 10 μ l of chemical solution was added to the test tubes which were then incubated at 37°C for 30 min on a rotary shaker. After the incubation 2.5 ml molten top agar was added to each tube,

the mixture was poured onto plates, and the plates were incubated at 37°C for 48 h. The large colonies that survived and grew were mutated, histidine-independent revertants. Three readings of each plate were scored on an automatic colony counter. The criteria for positive test results consist of revertant counts at least twice that of the solvent control and a clear dose-related increase in mutagenic activity.

RESULTS AND DISCUSSION

The sample MRE 1 displayed mutagenic activity only in the nonpolar neutral subfraction. This fraction was mutagenic on both tester strains, TA98 and TA100, in the presence and absence of S9 metabolic activation. Activity was weaker in TA100, strongest in TA98 with S9. These results were confirmed when the assay was repeated.

The histidine dependence in TA98 is due to a frameshift mutation, which shifts the reading frame of the genetic code, while the histidine dependence in TA100 is due to a non-specific base-pair substitution. The reversion of either mutation requires another event of the same type of mutation, so the evidence of mutagenic activity in TA98 and TA100 indicates that the nonpolar neutral sample most likely contains frameshift mutagens, but may also contain base-pair substitution mutagens. The fact that the activity is present both with and without S9 suggests that the nonpolar neutral fraction contains direct and possibly indirect mutagenic agents.

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TABLE I
Mutagenicity Testing of the Subfractions of MRE 1

Sample	Concentration $\mu\text{g/pl}$	Average Number of Revertant Colonies per Plate			
		TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
Basic	0 ^A	23	27	100	98
	62.5	26	34	77	87
	125	23	23	83	94
	250	26	24	88	95
	500	20	23	74	85
Acidic	0 ^A	23	27	100	98
	31.2	30	41	100	110
	62.5	30	43	119	98
	125	38	47	115	98
	250	49*	48	132	114
Nonpolar Neutral	0 ^A	23	27	100	98
	15.6	50*	- ^C	237*	-
	31.2	59*	101*	292*	241*
	62.5	71*	94*	387*	211*
	125	34	135*	360*	243*
	250	-	66 ^B	-	194
Polar Neutral	0 ^A	23	27	100	98
	3.9	29	-	-	-
	7.8	29	-	-	114
	15.6	30	40	121	113
	31.2	25	34	101	133
	62.5	-	38	- ^B	114
	125	-	- ^B	-	78 ^B

2AA	0 ^A	23	27	100	98
	2.5	-	1595*	-	1974*
TNF	0 ^A	23	27	100	98
	0.4	1284*	-	197	-

^A Solvent control ^B Cytotoxicity ^C Not tested * Positive response

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