Guideline Series 84: MUTAGENICITY

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Date: August 11, 1988 Kerry Dearfield 8.16.88

DATA EVALUATION RECORD

CHEMICAL: Methoxychlor

Tox. Chem. No.: 550

Tox Branch Project No: 8-0131

STUDY TYPE: Mammalian cells in culture unscheduled DNA synthesis (UDS) assay in human fibroblast (WI-38) cells

MRID NUMBER(s): 28625, 132952 and 133008

SYNONYMS/CAS No.: methyl-DDT, Marlate, 72-43-5

SPONSOR: United States Environmental Protection Agency
Health Effects Research Laboratory
Research Triangle Park, N.C. 27711

TESTING FACILITY: SRI International
Menlo Park, California 94025

TITLE OF REPORT: In Vitro Microbiological Mutagenicity and Unscheduled DNA Synthesis Studies of Eighteen Pesticides

AUTHOR(S): Vincent F. Simmon, Ph.D.

STUDY NUMBER(S): EPA-600/1-79-041
Contract No. 68-01-2458

REPORT ISSUED: October 1979

CONCLUSION(S) - Executive Summary:

Under the conditions of this test, Methoxychlor at dose levels up to 1000 ug/ml, with and without metabolic activation, did not cause an increase in UDS. A precipitate was noted at the two top concentrations tested. The positive controls were adequate.

Classification: Acceptable

Bibliographic Citation

Simmon, V.F. (1979) °In vitro° Microbiological Mutagenicity and Unscheduled DNA Synthesis Studies of Eighteen Pesticides: Report No. EPA-600/1-79-041. (Unpublished study including submitter summary, received Apr 3, 1980 under 279-2712; prepared by SRI International, submitted by FMC Corp., Philadelphia, Pa.; CDL:099350-A)

Simmon, V. (1979) In vitro Microbiological Mutagenicity and Unscheduled DNA Synthesis Studies of Eighteen Pesticides: Contract No. 68-01-2458. (Unpublished study received Dec 5, 1983 under 239-2471; prepared by SRI International, submitted by Chevron Chemical Co., Richmond, CA; CDL:251894-F)

Simmon, V. (1979) In vitro microbiological mutagenicity and unscheduled DNA synthesis studies of eighteen pesticides. By SRI International. Research Triangle Park, N.C.: U.S. Environmental Protection Agency, Office of Research and Development, Health Effects Laboratory, Genetic Toxicology Div. (EPA 600/1-79-041; Contract No. 68-01-2458; also In unpublished submission received Dec 2, 1983 under 279-2038; submitted by FMC Corp., Philadelphia, PA; CDL:251984-C)

A. MATERIALS: A copy of the "materials and methods" section from the investigators report is appended.

1. Test Material: Name: Methoxychlor
Description: technical
Batch #: 6543-108 Purity: Technical
Contaminants: none reported
Solvent used: DMSO
Other comments: Manufacturer: EI du Pont de Nemours & Co.

2. Control Materials:
Negative: 0.5% DMSO
Solvent/final concentration: 0.5% DMSO
Positive:
Non-activation:
4-nitroquinoline-N-oxide (4NQO): 10^{-5} M
Activation:
dimethylnitrosamine (DMN): $5X10^{-2}$ M

3. Activation: S9 derived from Aroclor 1254 induced mouse liver
S9 mix composition:
9000xg supernatant of liver homogenate (250 mg liver/ml)
nicotinamide 3.05 mg/ml
glucose-6-phosphate 16.1 mg/ml
$MgCl_2.6H_2O$ 5.08 mg/ml
NADP 0.765 mg/ml

4. Test compound concentrations used:
Non-activated conditions: 0, 0.1, 1.0, 10, 100 and 1000 ug/ml
Activated conditions: 0, 0.1, 1.0, 10, 100 and 1000 ug/ml

5. Test Cells: mammalian cells in culture, WI-38 cells grown in T-25 culture flasks
Properly maintained? yes
Cell line or strain periodically checked for Mycoplasma contamination? yes
Cell line or strain periodically checked for karyotype stability? not stated

B. TEST PERFORMANCE

1. Cell treatment:
a. Cells exposed to test compound for: 3 hours (non-activated), 1 hour (activated).
b. Cells exposed to positive controls for: __?__ hours (non-activated), __?__ hours (activated).
c. Cells exposed to negative and/or solvent controls for: 3 hours (non-activated), 3 hours (activated).

2. Protocol:

According to the investigators:

"The contact-inhibited WI-38 cells were incubated at 37°C with dilutions of the pesticides and with 1 uCi/ml of ^{3}H-TdR (specific activity, 6.7 Ci/mmole). For testing in the absence of metabolic activation, the cells were exposed simultaneously to the pesticide and to ^{3}H-TdR for 3 hours. For testing with metabolic activation, the cells were incubated together with pesticide, ^{3}H-TdR, and the metabolic activation preparation for 1 hour. (The shorter exposure time for metabolic activation testing was used because longer exposures of WI-38 cells to the liver homogenate preparation could be cytotoxic.) In both cases, the cells were then incubated with ^{3}H-TdR and HU, but without pesticide, for an additional 3 hours.

DNA was extracted from the cells using a modification of the PCA-hydrolysis procedure; one aliquot of the DNA solution was used to measure the DNA content, after the reaction with diphenylamine, and a second aliquot was used for scintillation-counting measurements of the extent of incorporation of ^{3}H-TdR. The results were expressed as disintegrations per minute (dpm) of incorporated ^{3}H-TdR per unit of DNA and were compared with the rate of incorporation of ^{3}H-TdR per unit of DNA and were compared with the rate of incorporation of ^{3}H-TdR into cells exposed to solvent only (negative controls)."

3. Preliminary cytotoxicity assay:

No data provided, apparently the investigators did conduct a preliminary cytotoxicity assay.

4. Cytogenetics assay:

The following table presents the results of the UDS assay extracted from Tables 113 and 114 of the investigator's report.

UNSCHEDULED DNA SYNTHESIS ASSAY OF METHOXYCHLOR
(MEAN dpm/ug DNA)

Without metabolic activation

	Concentration of Compounds Tested Methoxychlor (ug/ml)						4NQO (M)
	0*	0.1	1.0	10	100±	1000±	10^{-5}
Mean++	113	79	79	99	90	106	1343
SD	18	8	13	32	15	12	78

With metabolic activation

	Concentration of Compounds Tested Methoxychlor (ug/ml)						4NQO (M)
	0*	0.1	1.0	10	100±	1000±	10^{-5}
Mean++	136	97	80	101	112	104	593
SD	27	18	19	13	20	13	78

*Negative control and compound solvent, 0.5% DMSO
+Precipitates observed at 100 and 1000 ug/ml.
++Mean of 6 samples (except 10 ug/ml with metabolic activation, 1 sample lost)

The data above indicate that at dose levels tested, Methoxychlor with and without metabolic activation did not cause increased ^{3}H-TdR incorporation.

5. Reviewer's discussion/conclusions:

From the data presented Methoxychlor at dose levels up to 1000 ug/ml either with or without metabolic activation did not cause an increase in UDS (precipitates were observed at 100 and 1000 ug/ml), whereas the positive controls did.

6. Was test performed under GLPs (is a quality assurance statement present)? no

7. CBI appendix attached? This is a published study.

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METHODS

Microbiological Assays

The *in vitro* microbiological assay systems used to examine the 18 pesticides for mutagenicity were *Salmonella typhimurium* (TA1535, TA1537, TA1538, TA98, and TA100), *Escherichia coli* WP2, repair-deficient and -proficient strains of *Bacillus subtilis* (H17 and M45) and of *E. coli* (W3110 and p3478); and the yeast *Saccharomyces cerevisiae* D3. In each procedure except the relative toxicity assays, an Aroclor 1254-stimulated, rat liver homogenate metabolic activation system was included to provide metabolic steps that the microorganisms either are incapable of conducting or do not carry out under the assay conditions.

The assay procedure with *S. typhimurium* has proven to be 80 to 90% accurate in detecting carcinogens as mutagens, and it has about the same accuracy in identifying chemicals that are not carcinogenic.[1,2] The assay procedure with *S. cerevisiae* is about 55% accurate in detecting carcinogens as agents that increase mitotic recombination.[4] *E. coli* WP2 and the relative toxicity assays are three additional methods of detecting mutagens; however, the reliability of these test methods has not been adequately validated yet. The combination of these five assay procedures significantly enhances the probability of detecting potentially hazardous chemicals.

Salmonella typhimurium Strains TA1535, TA1537, TA1538, TA98, and TA100

The *Salmonella typhimurium* strains used at SRI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown on a minimal media petri plate containing a trace of histidine, only those cells that revert to histidine independence (*his*$^+$) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few divisions; in many cases, this growth is essential for mutagenesis to occur. The *his*$^+$ revertants are easily scored as colonies against the slight

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test. Ames has described the liver metabolic activation system that we use.[10] In brief, adult male Sprague-Dawley rats (250 to 300 g) are given a single 500-mg/kg intraperitoneal injection of a polychlorinated biphenyl, Aroclor 1254. This treatment enhances the synthesis of enzymes involved in the metabolic conversion of chemicals. Four days after the injection, the animals' food is removed, but drinking water is provided ad libitum. On the fifth day, the rats are killed and the liver homogenate is prepared as follows.

The livers are removed aseptically and placed in a preweighed sterile glass beaker. The organ weight is determined, and all subsequent operations are conducted in an ice bath. The livers are washed in an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant, referred to as the S-9 fraction, is quickly frozen in dry ice and stored at -80° C.

The metabolic activation mixture consists of, for 10 ml:

- 1.00 ml of freshly thawed S-9 fraction
- 0.20 ml of $MgCl_2$ (0.4 M) and KCl (1.65 M)
- 0.05 ml of glucose-6-phosphate (1 M)
- 0.40 ml of NADP (0.1 M)
- 5.00 ml of sodium phosphate (0.2 M, pH 7.4)
- 3.35 ml of H_2O.

Unscheduled DNA Synthesis Assays

Many mutagenic and carcinogenic agents have been shown to induce unscheduled DNA synthesis (UDS) in an in vitro tissue culture system of mammalian cells.[10] UDS is a form of mammalian repair synthesis that involves at least two processes: first, the agent interacts with DNA, resulting in damage to the DNA; then follows incorporation of nucleotide(s) to repair the DNA. UDS, which occurs in a wide variety of mammalian cell types, is considered to be a fairly universal

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system because it has been observed in all stages of the cell cycle (G_0, G_1, G_2, and M) other than S, the normal synthetic phase.[19,20] (UDS is not observed during S-phase because the high level of incorporation of nucleotides during scheduled DNA synthesis obscures the relatively low level of incorporation of nucleotides during UDS.)

A number of chemicals have been shown to be ineffective in producing DNA damage in in vitro cultures of mammalian cells; yet in the metabolically active environment of the whole animal they are rapidly converted to mutagenic and/or carcinogenic intermediates. Therefore, the investigator must attempt to recreate this metabolic environment in vitro. This is usually done by adding a microsomal preparation from a mammalian liver homogenate to the test system. Thus, we routinely perform a parallel series of UDS assays in the presence and absence of a metabolically active environment to predict the ability of an agent to induce genetic damage.

We used the UDS assay system in the previous testing of 20 substitute pesticides.[4] Reported here are the results of UDS testing, with and without metabolic activation, of the 18 additional substitute pesticides.

Cell Culture

WI-38 cells grown in T-25 tissue culture flasks were used for the UDS assays. Replicate cultures of these cells were initiated in Eagle's Basal Medium containing 10% (v/v) fetal calf serum. The cells were grown to confluency and were maintained in medium containing 0.5% serum for 5 to 6 days preceding the UDS assays.* This produced contact-inhibited cells in synchronous cultures in the G_0 phase of the mitotic cycle. To further reduce the possibility of incorporation of ^{3}H-TdR by an occasional S-phase cell that might escape the contact-

* As a check against the presence of mycoplasma, which could incorporate tritiated thymidine (^{3}H-TdR) and thus obscure measurments of UDS, stock cultures were periodically sent to Microbiological Associates, who cultured them on Difco Beef Heart Infusion agar or broth for analysis for the presence of mycoplasma. The results of these analyses were consistently negative.

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inhibition synchrony and thus obscure measurements of UDS, the cultures were preincubated for 1 hour with 10^{-3} M hydroxyurea (HU) before each assay and 10^{-3} M HU was added during each subsequent step of the assays.

Dilution of Compounds

Immediately prior to each assay, the pesticide was diluted in an appropriate solvent (ethanol or DMSO) to form a series of concentrations that, when diluted into culture medium, yielded the appropriate set of test concentrations. To facilitate solubilization or achieve an even suspension of the stock solutions of the compounds in solvent, some of the compounds were sonicated for a brief period of time prior to dilution. The final concentration of solvent was maintained at 1% or less, which we have previously found to be not cytotoxic.

Metabolic Activation

For testing with metabolic activation, a preparation consisting of the 9000 x g supernatant of a liver homogenate (250 mg of liver/ml) from adult Swiss-Webster mice was used. To this was added the following cofactors: nicotinamide, 3.05 mg/ml; glucose-6-phosphate, 16.1 mg/ml; $MgCl_2 \cdot 6H_2O$, 3.08 mg/ml; and NADP, 0.765 mg/ml.

Controls

The positive controls were 4-nitroquinoline-N-oxide (4NQO), a compound that induces UDS in the absence of a metabolic activation system, and dimethylnitrosamine (DMN), a compound that induces UDS in vitro only when an exogenous metabolic activation system is incorporated into the treatment protocol. The negative control was the solvent diluted in culture medium.

Test Procedure

The contact-inhibited WI-38 cells were incubated at 37° C with dilutions of the pesticides and with 1 μCi/ml of ^{3}H-TdR (specific activity, 6.7 Ci/mmole). For testing in the absence of metabolic activation, the cells were exposed simultaneously to the pesticide and to ^{3}H-TdR for 3 hours. For testing with metabolic activation, the cells were incubated

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together with pesticide, ^{3}H-TdR, and the metabolic activation preparation for 1 hour. (The shorter exposure time for metabolic activation testing was used because longer exposures of WI-38 cells to the liver homogenate preparation could be cytotoxic.) In both cases, the cells were then incubated with ^{3}H-TdR and HU, but without pesticide, for an additional 3 hours.

DNA was extracted from the cells using a modification of the PCA-hydrolysis procedure;[84] one aliquot of the DNA solution was used to measure the DNA content, after the reaction with diphenylamine,[88] and a second aliquot was used for scintillation-counting measurements of the extent of incorporation of ^{3}H-TdR. The results were expressed as disintegrations per minute (dpm) of incorporated ^{3}H-TdR per unit of DNA and were compared with the rate of incorporation of ^{3}H-TdR into cells exposed to solvent only (negative controls).

We have defined as an acceptable assay one in which the response of the positive control compound is predicted, within the 95% confidence limits, by regressions of average dpm/ug DNA versus average dpm/ug for background.[88] The regressions that follow are based on data that we have acquired in previous testing:

Type of Testing	Regression*	Sample Size (n)	Correlation Coefficient (r)
Without metabolic activation	$Y_1 = 629 + 16.42\ (X)$†	55	0.8066
With metabolic activation	$Y_2 = 212 + 2.11\ (X)$†	25	0.8307

If the observed average level of incorporation for the positive control compound is outside the 95% confidence limits of the regression, we assume that some variation has occurred in the experimental procedures and the test is repeated.

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* Regressions over a range of background dpm/ug DNA of 0 to 450.

† Y_1 = Average dpm/ug DNA for 10^{-6} M 4NQO (positive control).
Y_2 = Average dpm/ug DNA for 5×10^{-6} M DMN (positive control).
X = Average dpm/ug DNA for background (negative control).

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