Guideline Series 84: MUTAGENICITY

Primary Review by: Stephen C. Dapson, Ph.D. Stephen C. Dapson 8/11/88
Pharmacologist, Review Section V, Toxicology Branch/HED (TS-769C)
Secondary Review by: Kerry Dearfield, Ph.D.
SMSS, Toxicology Branch/HED (TS-769C)
Date: August 11, 1988 Kerry F Dearfield 8.16.88

DATA EVALUATION RECORD

CHEMICAL: Methoxychlor

Tox. Chem. No.: 550

Tox Branch Project No: 8-0131

STUDY TYPE: Saccharomyces cerevisiae D3 assay
mitotic recombination assay

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, NC 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 conditions of this study, Methoxychlor at doses up to 5% w/v or v/v (not stated which), did not cause an increase in mitotic recombinants in S. cerevisiae D3 assay either with or without metabolic activation. The positive control under non-metabolically activated condition did cause an increase in mitotic recombinants. There was no positive control for the metabolically activated condition. Therefore, this study is not acceptable for regulatory purposes.

Classification: Unacceptable

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 (Unpublished study including submitter summary, received Apr 3, 1980 under 279-2712; prepared by SRI International, submitted by FMC Corp., Philadelphia, Pa.; CDL:099350-A)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 (Unpublished study received Dec 5, 1983 under 239-2471; prepared by SRI International, submitted by Chevron Chemical Co., Richmond, CA; CDL:251894-F)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: E.I. du Pont de Nemours & Co.

2. Control Materials:
Negative: DMSO
Solvent/final concentration: DMSO
Positive: Non-activation:
1,2,3,4-Diepoxybutane 0.04% w/v or v/v
Activation: none used

3. Activation: S9 derived from Aroclor 1254 induced rat liver
S9 mix composition:
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 (1.65M)
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.

2

4. Test organisms: Saccharomyces cerevisiae D_3
Properly maintained? yes

5. Test compound concentrations used:
Non-activated conditions: 0.1, 0.5, 1.0, 2.0, 4.0 and 5.0 w/v or v/v
Activated conditions: 0.1, 0.5, 1.0, 2.0, 4.0 or 5.0 w/v or v/v

B. TEST PERFORMANCE

1. Type of assay: standard plate test

a. Protocol:

Tester strains are grown overnight at 30°C with aeration. The suspension is then centrifuged and resuspended in phosphate buffer, metabolic activator (as necessary) and the test substance. The suspensions are then incubated for 4 hours at 30°C on a roller drum. Then samples are diluted serially and spread on tryptone-yeast agar plates (5 plates for a 10^{-3} dilution and 3 plates for a 10^{-9} dilution). The plates are incubated for 2 days at 30°C and then 2 days at 4°C. The number of mitotic recombinants are then counted.

2. Preliminary cytotoxicity assay:

None conducted, 2 primary assays were conducted.

3. Mutagenicity assay:

Attached Table 64 presents the results of 2 experiments with Methoxychlor. Methoxychlor treated groups showed a reduction in the numbers of survivors, not dose related. There was apparently no increase in mitotic recombinants in any of the treated groups under either metabolic activation condition. The positive control under non-metabolically activated conditions produced an increase in mitotic recombinants.

4. Reviewer's discussion/conclusions:

Under conditions of this test, Methoxychlor at doses up to 5% w/v or v/v (not stated which) did not cause an increase in mitotic recombinants under either metabolic condition, whereas the positive control under non-metabolically activated conditions did cause an increase in mitotic recombinants. There was no positive controls for metabolically activated conditions. Therefore, this study is not acceptable for regulatory purposes.

5. Was test performed under GLPs (is a quality assurance statement present)? no
6. CBI appendix attached? This is a published study.

Table 64

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE D3
METHOXYCHLOR

Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Survivors: Cells per ml (x 10^{-7})	Survivors: Percent	Mitotic Recombinants: Per ml (x 10^{-6})	Mitotic Recombinants: Per 10^{7} Survivors
		EXPERIMENT 1				
Negative control	-		6.8	100	7	10.3
	+		5.7	100	5	8.8
Positive control 1,2,3,4-Diepoxybutane	-	0.04	3.2	47	687	2147
Methoxychlor	-	0.1	3.7	54	7	18.9
	-	0.5	4.2	62	10	23.8
	-	1.0	4.6	68	4	8.7
	-	5.0	6.2	91	7	11.3
	+	0.1	4.7	82	4	8.5
	+	0.5	3.6	63	3	8.3
	+	1.0	5.1	89	5	9.8
	+	5.0	2.8	49	3	10.7
		EXPERIMENT 2				
Negative control	-		6.8	100	5	7.3
	+		6.1	100	3	4.9
Positive control 1,2,3,4-Diepoxybutane	-	0.04	4.8	71	1015	2114
Methoxychlor	-	1.0	5.5	81	3	5.5
	-	2.0	7.0	103	3	4.3
	-	4.0	7.1	104	3	4.2
	-	5.0	6.1	90	1	1.6
	+	1.0	5.9	97	2	3.4
	+	2.0	6.3	103	3	4.8
	+	4.0	6.1	100	5	8.2
	+	5.0	6.9	113	2	2.9

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.[2,3] 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

base-pair substitution mutation in the tryptophan operon. In addition, WP2 is deficient in the repair of some physically or chemically induced DNA damage (uvrA).[12] This uvrA mutation makes the strain more sensitive to certain mutagens.

A procedure similar to the Ames Salmonella assay is used to measure the reversion of WP2 to tryptophan independence. However, the minimal agar is supplemented with 1.25 g of Oxoid nutrient broth (CM67) per liter to provide each plate with the trace of tryptophan required for enhancement of any mutagenic effect of the test chemical.[12] No additional tryptophan is added to the top agar.

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid microorganism heterozygous for a mutation leading to a defective enzyme in the adenine-metabolizing pathway.[13] When grown on medium containing adenine, cells homozygous for this mutation produce a red pigment. These homozygous mutants can be generated from the heterozygotes by mitotic recombination. The frequency of this recombinational event may be increased by incubating the organisms with various mutagens. The degree of mutagenicity of a compound or of its metabolite is determined from the number of red-pigmented colonies appearing on the plates.[14]

The S. cerevisiae tester strain is stored at -80° C. For each experiment, the tester strain is inoculated in 1% tryptone and 0.5% yeast extract and grown overnight at 30° C with aeration.

The in vitro yeast mitotic recombination assay in suspension is conducted as follows. The overnight culture is centrifuged, and the cells are resuspended at a concentration of ~10^8 cells/ml in a 67 mM phosphate buffer (pH 7.4). To a sterile test tube are added:

- 1.30 ml of the resuspended culture
- 0.50 ml of either the metabolic activation mixture or buffer
- 0.20 ml of a solution of pesticide dissolved in DMSO or 0.20 ml of DMSO alone.

Several doses of the pesticide (up to 5%, w/v or v/v) are tested in each experiment, and appropriate controls are included.

The suspension mixture is incubated at 30° C for 4 hours on a roller drum. The sample is diluted serially in sterile physiological saline, and 0.2-ml aliquots of the 10^{-8} and 10^{-9} dilutions are spread on tryptone-yeast agar plates; five plates are used for the 10^{-9} dilution and three plates are used for the 10^{-8} dilution. The plates are incubated for 2 days at 30° C, followed by 2 days at 4° C to enhance the development of the red pigment indicative of adenine-deficient homozygosity. Plates of the 10^{-8} dilution are scanned with a dissecting microscope at 10X magnification, and the number of red colonies or red sectors (mitotic recombinants) is recorded. The surviving fraction of organisms is determined from the number of colonies appearing on the plates of the 10^{-9} dilution. The number of mitotic recombinants is calculated per 10^8 survivors.

A positive response in this assay is indicated by a dose-related increase in the absolute number of mitotic recombinants per milliliter as well as in the relative number of mitotic recombinants per 10^8 survivors.

Escherichia coli W3110/p3478 and Bacillus subtilis H17/M45

The E. coli strains W3110 and p3478 that are used at SRI were obtained from Dr. B. Rosenkranz, who devised the DNA polymerase repair assay.[8] Strain p3478 is a DNA polymerase-deficient (polA⁻) derivative of W3110 and is very sensitive to the effects of some physical and chemical agents that react with cellular DNA. The repair assay is based on the finding that when exposed to agents that alter the DNA, bacteria tend to protect themselves by removing the altered DNA segment and then by resynthesizing the correct DNA sequence. Thus, their survival is enhanced. The enzyme DNA polymerase is involved in this resynthesizing process.[10] The extent of chemically induced DNA damage can be measured by comparing the relative toxicity (zone of growth inhibition) of the two

strains. Therefore, if a chemical interacts with DNA, strain p3478 should be more sensitive than strain W3110 to any toxic effect due to this interaction.

The B. subtilis strains H17 and M45 were obtained from Dr. T. Kada.[17] Strain M45 (rec⁻) is derived from H17 but is deficient in the genetic recombination mechanism necessary to repair DNA damage. Cells deficient in this repair mechanism are killed more easily by chemical mutagens than are wild-type cells (rec⁺). If the chemical is toxic to rec⁻ cells but at the same concentration is not toxic to rec⁺ cells, the chemical is assumed to interact with DNA.

For each experiment, an inoculum from frozen stock cultures is grown overnight at 37° C with shaking in nutrient broth consisting of 1% tryptone and 0.5% yeast extract. A 0.1-ml aliquot of this bacterial culture (approximately 3 x 10⁸ cells) is added to 2 ml of nutrient broth containing 0.6% agar. The suspension is mixed and poured onto the surface of a plate containing the same ingredients as the broth plus 2% agar (25 ml). When the top agar has solidified, a sterile filter disc impregnated with the test substance is placed in the center of the plate. The plates are incubated at 37° C for 16 hours; then the width (diameter) of the zone of inhibition of growth is measured. Several concentrations of the substance are usually tested. We routinely use DMSO as diluent and as solvent for crystalline chemicals.

The positive control for this assay is 1-phenyl-3,3-dimethyltriazine. The negative control is chloramphenicol, which should cause equal zones of inhibition in both strains because it is toxic to bacteria but does not kill by interacting with DNA.

Aroclor 1254-Stimulated Metabolic Activation

Some carcinogenic chemical (e.g., of the aromatic amino type or polycyclic hydrocarbon type) are inactive unless they are metabolized to active forms.[7] In animals and man, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens.[8-10] Some of these intermediate metabolites are very potent mutagens in the S. typhimurium

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

9

REFERENCES

1. V. F. Simmon, A. D. Mitchell, and T. A. Jorgenson. Evaluation of select pesticides as chemical mutagens: In vitro and in vivo studies. Environmental Health Effects Research Series EPA-600/1-77-028, May 1977.

2. J. McCann, E. Choi, E. Yamasaki, and B. N. Ames. Detection of carcinogens as mutagens in the Salmonella microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. USA 72, 5135-5139 (1975).

3. I. F. H. Purchase, E. Longstaff, J. Ashby, J. A. Styles, D. Anderson, P. A. Lefevre, and F. R. Westwood. Evaluation of six short term tests for detecting organic chemical carcinogens and recommendations for their use. Nature 264, 624-627 (1976).

4. V. F. Simmon. In vitro assays for recombinogenic activity of chemical carcinogens and related compounds with Saccharomyces cerevisiae D3. J. Nat. Cancer Inst. 62 (in press) (1979).

5. B. N. Ames, E. G. Gurney, J. A. Miller, and H. Bartsch. Carcinogens as frameshift mutagens: Metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proc. Nat. Acad. Sci. USA 69, 3128-3132 (1972).

6. B. N. Ames, F. D. Lee, and W. E. Durston. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Nat. Acad. Sci. USA 70, 782-786 (1973).

7. J. McCann, L. E. Spingarn, J. Kobori, and B. N. Ames. The detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Nat. Acad. Sci. USA 72, 979-983 (1975).

8. B. N. Ames, W. E. Durston, E. Yamasaki, and F. D. Lee. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Nat. Acad. Sci. USA 70, 2281-2285 (1973).

9. L. D. Kier, E. Yamasaki, and B. N. Ames. Detection of mutagenic activity in cigarette smoke condensates. Proc. Nat. Acad. Sci. USA 71, 4159-4163 (1974).

10. B. N. Ames, J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res. 31, 347-364 (1975).

11. D. R. McCalla and D. Voutsinos. On mutagenicity of nitrofurans. Mutation Res. 26, 3-16 (1974).

12. B. A. Bridges. Simple bacterial systems for detecting mutagenic agents. Lab. Pract. 21, 413-419 (1972).

13. F. K. Zimmermann and R. Schwaier. A genetic effect of symmetric dimethylhydrazine: Induction of mitotic recombination. Naturwissenschaften 54, 251 (1967).

14. D. J. Brusick and V. W. Mayer. New developments in mutagenicity screening techniques with yeast. Envir. Health Perspectives 6, 83-96 (1973).

15. E. E. Slater, M. D. Anderson, and M. S. Rosenkranz. Rapid detection of mutagens and carcinogens. Cancer Res. 31, 970-973 (1971).

16. J. Cross and M. Cross. Genetic analysis of an E. coli strain with a mutation affecting DNA polymerase. Nature 224, 1166-1168 (1969).

17. T. Kada. Mutagenicity testing of chemicals in microbial systems. In New Methods in Environmental Chemistry and Toxicology. International Academic Printing Co., Totsuka, Tokyo, 1973.

18. R. M. C. Sun and N. F. Stich. DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens. Int. J. Cancer 16, 284-291 (1975).

19. B. Djordjevic and L. Tolmach. Response of synchronized populations of HeLa cells to ultraviolet irradiation at selected stages of the generation cycle. Radiat. Res. 32, 327 (1967).

20. R. E. Rasmussen and R. B. Painter. Radiation-stimulated DNA synthesis in cultured mammalian cells. J. Cell Biol. 29, 11 (1966).

21. C. Schmidt and S. J. Thannhauser. A method for the determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. 161, 83-89 (1945).

22. G. M. Richards. Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal. Biochem. 57, 369-376 (1974).

23. M. R. Spiegel. Theory and Problems of Statistics. Schaum Publishing Co., New York, 1961, pp. 241-248.

24. W. C. Guenther. Analysis of Variance. Prentice-Hall, Inc., Englewood Cliffs, N. J., 1964, pp. 20-21, 31-43.

25. S. Siegel. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill Book Company, New York, 1956, pp. 184-194.

26. S. S. Epstein, E. Arnold, J. Andrea, W. Bass, and Y. Bishop. Detection of chemical mutagens by the dominant lethal assay in the mouse. Toxicol. Appl. Pharmacol. 23, 288-325 (1972).

27. V. V. Brahmachii. Possibility of induction of mutations in Drosophila melanogaster with chlorophos. Med. Parasit. Parazit. Bolez. 42, 703-706 (1973).

28. IARC. 2,4-D and Esters. In IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man Vol. 15, IARC, Lyon, France, 1977, pp. 111-139.

29. Y. Shirasu. Significance of mutagenicity testing on pesticides. Environ. Qual. Sat. 4, 226-231 (1975).