UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

005372

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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

TO: Henry Jacoby, Product Manager (21)
Registration Division (TS-767C)

FROM: Roger Gardner, Toxicologist
Toxicology Branch
Hazard Evaluation Division (TS-769)

THRU: Jane Harris, Ph. D., Section Head
Review Section 6
Toxicology Branch
Hazard Evaluation Division (TS-769)

SUBJECT: Review of Mutagenicity Data on Technical Grade Metalaxyl. EPA Reg. No. 100-601. Tox. Chem. 375AA. (Tox. Proj. Nos. 1561 and 2125)

Actions Requested

Review of the studies listed in Section III below.

Conclusions

1. Metalaxyl did not cause gene mutations in vitro in bacteria, yeast or mouse lymphoma cells.

2. No structural or numerical chromosomal aberrations were observed in yeast or Chinese hamster ovary cells exposed to Metalaxyl in vitro, and no dominant lethal or nucleus anomaly effects were observed in treated mice or hamsters, respectively.

3. No evidence of DNA damage was observed in bacteria, human fibroblast cells, or rat primary hepatocytes exposed to Metalaxyl in vitro.

I. Background

A Toxicology Branch Peer Review of Metalaxyl was held on June 21, 1985, and the Peer Review Committee noted that there were only two mutagenicity studies available for their consideration (an Ames assay and a dominant lethal assay in mice). The committee concluded that those two assays were insufficient to fulfill the requirement for a battery of tests. A memorandum describing the Peer Review for Metalaxyl (dated December 31, 1985) noted that additional mutagenicity studies were submitted, but those reports were considered to be unacceptable.

In a letter dated November 26, 1985, the Registrant (CIBA-GEIGY) stated that eleven reports were revised to include additional information on the methods and raw individual data. These revisions were made because the level of detail in most of the reports was appropriate to the time the assays were conducted (between 1979 and 1982) rather than current standards. Three additional reports were submitted on on May 30, 1986 (one of those reports was submitted in November, 1985).

The Peer Review Committee recommended in its memorandum that issues regarding the unacceptable reports should be clarified and an evaluation of the full battery of mutagenicity studies should then be conducted. The discussion that follows (Section II) and the appended Data Evaluation Records consider each of the submitted studies and their characterization of Metalaxyl's mutagenic potential.

II. Discussion and Conclusions

A. Mutagenicity Data

1. Gene mutation

Studies were conducted in Salmonella typhimurium (1, 2, and 3) and Escherichia coli (3) at concentrations ranging from 0 to 5000 ug metalaxyl per plate with or without metabolic activation. No mutagenic activity was observed in these assays.

Two assays in Saccharomyces cerevisiae (4 and 5) were conducted with concentrations of 40 to 10,000 ug/ml, and no increase in the frequency of gene mutations for treated yeast cultures was observed. However, no response to the positive control substance, absence of toxicity in metalaxyl treated cultures, and low solubility of the test substance in growth medium suggested that the sensitivity of these assays is limited.

An assay in vitro with mouse lymphoma cells (6) also found no increases in gene mutations in treated cells at concentrations ranging from 0.125 to 1.0 mg/ml without metabolic activation or 0.0625 to 0.5 mg/ml with metabolic activation.

2. Structural and numerical chromosomal aberrations

The two yeast assays described above (4 and 5) indicated that metalaxyl did not induce mitotic gene conversion or recombination at the concentrations tested, but those assays are considered limited with respect to their sensitivity. A third assay in S. cerevisiae (7) also suggests that metalaxyl did not induce non-disjunction. The third study also has the same limitations mentioned above for the other two assays.

In an in vitro assay with Chinese hamster ovary cells exposed to concentrations ranging from 150 to 1,200 ug metalaxyl per ml (8), an increase in the number of cells with chromosomal aberrations was observed at the highest level tested without liver microsomal enzyme activation of the test substance. There was no increase in the frequency of cells with chromosomal aberrations when metalaxyl

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was added to cultures after being treated with liver microsomal enzymes. However, the results are inconclusive because there is not enough information in the report to suggest whether metalaxyl is detoxified by the microsomal enzymes or the exposure period in that portion of the assay (2 hours) is too short in comparison to that of the other phase of the experiment (17.5 hours).

Two in vivo assays were conducted with hamsters (9) and mice (10). In the first experiment, two consecutive daily oral doses of 0, 595, 1190, or 2380 mg metalaxyl per kg body weight were given to males and females. These doses did not increase the incidence of bone marrow cells with nuclear anomalies (single Jolly bodies, fragments of nuclei in erythrocytes, micronuclei in erythroblasts, micronuclei in leucopoietic cells, and polyploid cells) (9). The second assay was a dominant lethal study (10) in which no genetic effects were observed after administration of single oral doses of 0, 65, or 195 mg metalaxyl per kg body weight to male mice. Acute oral toxicity studies in hamsters and mice (11 and 12) suggest that the doses tested in the genetic toxicity assays were approximately 1/3 of the LD_{50} value for hamsters and mice.

3. DNA damage

Metalaxyl did not cause DNA damage in Bacillus subtilis (Rec assay) at levels from 20 to 5000 ug/disk (3), and the fungicide did not induce unscheduled DNA synthesis in rat primary hepatocytes at concentrations of 0 to 2000 ug/ml in two other experiments (14 and 15).

B. Conclusions

The following factors suggest that metalaxyl is not mutagenic under the conditions of the experiments described herein:

1. Metalaxyl is relatively insoluble in aqueous media. For example, solubility = 700 ug/ml in water; solubility in DMSO = 500 mg/ml; a 1:100 dilution of 500 mg/ml DMSO with culture medium caused precipitation which eventually dissolved (8).

2. The fungicide did not cause gene mutations in vitro in bacteria (1, 2, and 3), yeast (4 and 5), or mouse lymphoma cells (6).

3. No structural or numerical chromosomal aberrations were observed in treated yeast (4, 5, and 7), or Chinese hamster ovary cells (9) in vitro, and no dominant lethal or nucleus effects were observed in Metalaxyl-treated mice or hamsters, respectively (9 and 10).

4. No evidence of treatment-related DNA damage was observed in bacteria (3), human fibroblast cells (13), or rat primary hepatocytes (14 and 15) in vitro.

III. References

1. Arni, P.; Muller, D. (1978) Salmonella/Mammalian Microsome Mutagenicity Test with CGA 48 988 (Test for Mutagenic Properties in Bacteria). (Unpublished study received Nov 29, 1985 under 100-601; prepared by Pharmaceuticals Div., Toxicology/Pathology, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-A)

2. Deparade, E. (1985) Salmonella/Mammalian-Microsome Mutagenicity Test (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-B)

3. Moriya, M.; Ohta, T.; and Shirasu, Y. (1981) Report on Mutagenicity Study with CGA 48988 in a Microbial System. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Institute of Environmental Toxicology, Toxicity Dept., submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-C)

4. Arni, P.; Müller, D. (1982) Saccharomyces cerevisiae D7/Mammalian-Microsome Mutagenicity Test In Vitro with CGA 48988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-E)

5. Arni, P.; Müller, D. (1980) Mutagenicity Test on Saccharomyces cerevisiae MP-1 In Vitro with CGA 48 988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-F)

6. Strasser, F. F.; Müller, D. (1982) L5178Y/Tk$^{+/-}$ Mouse Lymphoma Mutagenicity Test: CGA 48 988. Experiment No. 811258. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-K)

7. Arni, P.; Müller, D. (1980) Test for Non-disjunction on Saccharomyces cerevisiae D 61 with CGA 48988. (Unpublished study received Nov 29, 1985 under 100601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-D)

8. Ivett, J.; Spicer, C. (1986) Clastogenic Evaluation of Metalaxyl Technical, CGA 48 988, in an in vitro cytogenetic assay Measuring Chromosomal Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells: Final Report. Project No. 20990. (Unpublished study received June 2, 1986 under 100-601; prepared by Hazleton Biotechnologies, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL: 263117-B)

9. Langauer, M.; Müller, D. (1979) Nucleus Anomaly Test in Somatic Interphase Nuclei: CGA 48 988: Chinese Hamster. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-I)

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10. Fritz, H. (1978) Mouse Dominant Lethal Study. CGA 48988 Tech. Experiment No. 32761. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-L)

11. Thomann, P.; Pericin, C. (1977) Acute Oral LD_{50} in the Chinese Hamster of CGA 48 988. Experiment No. PH 2.634. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-J)

12. Sassche, K.; Bathe, R. (1976) Acute Oral LD_{50} in the Mouse of Technical CGA 48 988. Experiment No. Siss 5 388. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-M)

13. Puri, E.; Müller, D. (1982) Autoradiographic DNA Repair Test on Human Fibroblasts with CGA 48 988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-G)

14. Puri, E.; Müller, D. (1982) Autoradiographic DNA Repair Test on Rat Hepatocytes with CGA 48 988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIG. Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-H)

15. Puri, E.; Müller, D. (1985) Autoradiographic DNA Repair Test on Rat Hepatocytes with CGA 48 988. Experiment No. 851004. (Unpublished study received June 2, 1986 under 100-601; prepared by Experimental Pathology Laboratories, CIBAGEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:263117-A)

APPENDIX

Data Evaluation Records for Studies
Cited in the Bibliography (Section III)

NOTE: Data Evaluation Records are presented in order according to the bibliography.

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DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Arni, P.; Muller, D. (1978) Salmonella/Mammalian Microsome Mutagenicity Test with CGA 48 988 (Test for Mutagenic Properties in Bacteria). (Unpublished study received Nov 29, 1985 under 100-601; prepared by Pharmaceuticals Div., Toxicology/Pathology, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-A)

REVIEW RESULTS:

VALID X INVALID _____ INCOMPLETE _____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/12/86

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1. CONCLUSIONS: Metalaxyl is not mutagenic in Salmonella typhimurium at concentrations of 25, 75, 225, 675, or 2025 ug/0.1 ml with or without metabolic activation.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch P.2; 99.8% purity)

   Reference mutagens: 9(5)-Aminoacridine hydrochloride monohydrate, N-methyl-N'-nitro-N-nitrosoguanidine, daunoblastin, cyclophosphamide, and 4-nitroquinoline-N-oxice were used as positive controls.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance and reference mutagens.

3. MATERIALS AND METHODS

Test species: The bacterial strains used were TA98, TA100, TA1535, and TA1537 of Salmonella typhimurium were used in the assays.

Bacterial culture media: Top agar for selection of histidine revertants contained 0.6% agar and 0.6% NaCl, and the medium also contained 0.05 mM L-histidine and 0.05 mM +biotin. Minimal bottom agar with salts and glucose (Vogel-Bonner Medium E) was used for plating of test strains with and without metabolic activation mixture.

Microsomal enzyme (S-9) preparation: Liver microsomal preparations were obtained from Aroclor 1254 induced rats. To each 0.3 ml sample of the S-9 was added 0.7 ml of the following: 8uM $MgCl_2$, 33 uM KCl, 4 uM NADP, 100 uM sodium phosphate buffer (pH 7.4), and 5 uM glucose-6-phosphate

Toxicity testing and dose-selection procedures: Concentrations of 25, 75, 225, 675, or 2025 ug test substance per 0.1 ml medium were tested with and without metabolic activation. The basis for dosage selection was described as the result of unspecified solubility of the test substance and toxicity to strain TA100.

Mutagenicity assay procedure: The test substance was dissolved in DMSO. Each dose and vehicle control was tested in triplicate, while positive controls were tested in duplicate. For tests without metabolic activation, 0.1 ml of each tester strain culture and 0.1 ml test or control solution were added to 2 ml selective minimal top agar. In tests with metabolic activation, 0.5 ml of the activation mixture were also added. These solutions were overlaid on minimal bottom agar, and the plates were then incubated at 37° C for 48 hours. After incubation the revertant colonies on each plate were counted, and the arithmetic mean of plate counts at each test concentration was calculated. The authors stated that the results were considered to be negative if the colony count at any test concentration was not at least double that of the vehicle control.

4. REPORTED RESULTS

No significant differences were noted at any concentration of the test substance with respect to the number of revertant colonies per plate when compared with that for the vehicle control (see Tables 1).

5. DISCUSSION

There are adequate data presented to support the conclusions of the investigators.

Table 1

Summary of revertants per plate as reported by Arne and Muller (1978) (see page 1 above)

Dose (ug per 0.1ml)	Without activation				With activation			
	TA985	TA100	TA1535	TA1537	TA985	TA100	TA1535	TA1537
0	19	167	8	6	34	146	10	11
25	25	173	12	3	32	151	11	7
75	23	162	10	7	29	167	12	6
225	21	174	7	5	31	166	13	8
675	23	166	8	4	33	187	10	8
2025	25	199	13	4	44	147	10	9
Daunoblastin*								
0	29	-	-	-	-	-	-	-
2.5	332	-	-	-	-	-	-	-
5.0	655	-	-	-	-	-	-	-
10.0	331	-	-	-	-	-	-	-
4-Nitroquinoline-N-oxice*								
0	-	191	-	-	-	-	-	-
0.0625	-	371	-	-	-	-	-	-
0.125	-	577	-	-	-	-	-	-
0.25	-	779	-	-	-	-	-	-
N-Methyl-N'-nitro-N-nitrosoguanidine*								
0	-	-	10	-	-	-	-	-
3	-	-	163	-	-	-	-	-
5	-	-	>1100	-	-	-	-	-
9(5)-Aminoacridine hydrochloridet								
0	-	-	-	4	-	-	-	-
25	-	-	-	19	-	-	-	-
50	-	-	-	122	-	-	-	-
100	-	-	-	>1200	-	-	-	-
Cyclophosphamide*								
0	-	-	-	-	-	-	11	-
100	-	-	-	-	-	-	189	-
250	-	-	-	-	-	-	517	-

*Phosphate buffer vehicle
†DMSO vehicle

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DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Deparade, E. (1985) Salmonella/Mammalian-Microsome Mutagenicity Test (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-B)

REVIEW RESULTS:

VALID X INVALID ____ INCOMPLETE ____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/12/86

1. CONCLUSIONS: Metalaxyl is not mutagenic in Salmonella typhimurium at concentrations of 20, 78, 313, 1250, or 5000 ug/0.1 ml with or without metabolic activation.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch P.503119; 95.7% purity)

   Reference mutagens: 9(5)-Aminoacridine hydrochloride, sodium azide, mitomycin-C daunoblastin, cyclophosphamide, 2-aminoanthracene, and 4-nitroquinoline-N-oxice were used as positive controls.

   Vehicle: Acetone was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: The bacterial strains used were TA98, TA100, TA102, TA1535, and TA1537 of Salmonella typhimurium were used in the assays.

Bacterial culture media: Top agar for selection of histidine revertants contained 0.6% agar and 0.6% NaCl, and the medium also contained 0.05 mM L-histidine and 0.05 mM +biotin. Minimal bottom agar with salts and glucose (Vogel-Bonner Medium E) was used for plating of test strains with and without metabolic activation mixture.

Microsomal enzyme (S-9) preparation: Liver microsomal preparations were obtained from Aroclor 1254 induced rats. To each 0.3 ml sample of the S-9 was added 0.7 ml of the following: 8uM $MgCl_2$, 33 uM KCl, 4 uM NADP, 100 uM sodium phosphate buffer (pH 7.4), and 5 uM glucose-6-phosphate

Toxicity testing and dose-selection procedures: Concentrations of 20, 78, 313, 1250, or 5000 ug test substance per 0.1 ml medium were tested with and without metabolic activation. The basis for dosage selection were results from a toxicity study of concentrations ranging from 0.08 to 5000 ug/0.1 ml.

Mutagenicity assay procedure: The test substance was dissolved in DMSO. Each dose and vehicle control was tested in triplicate, while positive controls were tested in duplicate. For tests without metabolic activation, 0.1 ml of each tester strain culture and 0.1 ml test or control solution were added to 2 ml selective minimal top agar. In tests with metabolic activation, 0.5 ml of the activation mixture were also added. These solutions were overlaid on minimal bottom agar, and the plates were then incubated at 37° C for 48 hours. After incubation the revertant colonies on each plate were counted, and the arithmetic mean of plate counts at each test concentration was calculated. The authors stated that the results were considered to be negative if the colony count at any test concentration was not at least double that of the vehicle control.

4. REPORTED RESULTS

No significant differences were noted at any concentration of the test substance with respect to the number of revertant colonies per plate when compared with that for the vehicle control (see Tables 1).

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5. DISCUSSION

There are adequate data presented to support the conclusions of the investigators.

Table 1

Summary of revertants per plate as reported by Arne and Muller (1978) (see page 1 above)

Dose (ug per 0.1ml)	TA985	TA100	TA100	TA1535	TA1537
		Without activation			
0	23	170	250	11	7
20	20	156	289	14	7
78	22	148	275	16	4
313	20	144	295	12	8
1250	28	154	292	11	6
5000	15	148	198	12	6
Daunoblastin*					
0	34	-	-	-	-
5.0	832	-	-	-	-
10.0	921	-	-	-	-
4-Nitroquinoline-N-oxice*					
0	-	156	-	-	-
0.125	-	769	-	-	-
0.25	-	1273	-	-	-
Mitomycin-C†					
0	-	-	322	-	-
3	-	-	1420	-	-
5	-	-	1579	-	-
Sodium azide†					
0	-	-	-	11	-
2.5	-	-	-	1038	-
5.0	-	-	-	1555	-
9(5)-Aminoacridine hydrochloride††					
0	-	-	-	-	5
50	-	-	-	-	104
100	-	-	-	-	890

Table 1 (continued)

Dose (ug per 0.1ml)	TA985	TA100	TA100	TA1535	TA1537
		With activation			
0	42	165	381	14	9
20	36	157	382	15	9
78	34	142	350	17	6
313	43	146	379	14	8
1250	51	143	346	13	8
5000	43	133	311	9	6
2-Aminoanthracene††					
0	40	146	-	-	7
5	1534	1246	-	-	168
2-Aminoanthracene††					
0	-	-	307	-	-
20	-	-	612	-	-
Cyclophosphamide*					
0	-	-	-	18	-
250	-	-	-	324	-

*Phosphate buffer vehicle
†Distilled water vehicle
††DMSO vehicle

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Moriya, M.; Ohta, T.; and Shirasu, Y. (1981) Report on Mutagenicity Study with CGA 48988 in a Microbial System. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Institute of Environmental Toxicology, Toxicity Dept., submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-C)

REVIEW RESULTS:

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REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/12/86

1. CONCLUSIONS: Metalaxyl did not cause DNA damage in Bacillus subtilis (Rec assay) at levels from 20 to 5000 ug/disk or induce reverse mutations in strains of Salmonella typhimurium or Escherichia coli at concentrations from 10 to 5000 ug/plate with or without metabolic activation.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; 99.5% purity)

   Reference mutagens: The report stated that kanamycin and mitomycin C were used in the Rec assay as negative and positive controls, respectively.

   For the reversion assays positive controls included 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2), N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), 9-aminoacridine (9AA), 2-nitroflourene (2-NF), and 2-aminoanthracene (2AA).

   Vehicle: Dimethyl sulfoxide was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: The bacterial strains used were H17 Rec$^+$ and M45 Rec$^-$ of Bacillus subtilis, TA98, TA100, TA1535, TA1537 and TA1538 of Salmonella typhimurium, and strain Wp2 hcr of Escherichia coli were used in the assays.

Bacterial culture media: The report stated that B-2 agar was used in the Rec assay.

Top agar for selection of histidine revertants in the S. typhimurium assay contained 0.6% agar and 0.5% NaCl, and the medium also contained 0.05 mM L-histidine and 0.05 mM +biotin. For the assay with E. coli, 0.25 mM tryptophan was added instead of the L-histidine. Minimal bottom agar with glucose was used for plating of test strains with and without metabolic activation mixture.

Microsomal enzyme (S-9) preparation: Liver microsomal preparations were obtained from Aroclor 1254 induced rats. To each 0.3 ml sample of the S-9 was added 0.7 ml of the following: 8uM $MgCl_2$, 33 uM KCl, 4 uM NADP, 100 uM sodium phosphate buffer (pH 7.4), and 5 uM glucose-6-phosphate

Rec-assay procedures: Suspensions of both strains of B.subtilis were straaked on the surface of B-2 agar plates. The origin of the streaks was covered with a 10 mm diameter paper disk containing 0, 20, 50, 100, 200, 500, 1000, 2000, or 5000 ug test substance. These plates were incubated overnight at 37° C, and after incubation the lenght of the inhibition zones was measured. Results were considered to be positive if a zone of more than 3 mm was observed for the M45 strain and that for the H17 strain was from 0 to 1 mm in length.

Reversion assay procedures: The test substance was dissolved in DMSO, and 0, 10, 50, 100, 200, 500, 1000, 2000, or 5000 ug test substance per plate was tested. Each dose and vehicle control was tested in duplicate. For tests without metabolic activation, 0.1 ml of each tester strain culture and 0.1 ml test or control solution were added to 2 ml selective minimal top agar. In tests with metabolic activation, 0.5 ml of the activation mixture were added and in those assays without metabolic activation 0.5 ml 100 mM phosphate buffer solution (pH 7.4) was added also. These solutions were overlaid on minimal bottom agar, and the plates were then incubated at 37° C for 48 hours. After

3. MATERIALS AND METHODS

incubation the revertant colonies on each plate were counted. The authors stated that the results were considered to be positive if they were reproducible and a dose-response was indicated.

4. REPORTED RESULTS

Rec assay: The report stated that no zones of inhibition for the test substance exceeded 1 mm for either test strain. The zones of inhibition for M45 and H17 strains exposed to kanamycin were 8.5 and 7.5 mm, respectively demonstrating sensitivity to toxicity unrelated to DNA damage. The respective zones for M45 and H17 exposed to mitomycin C were 10 and 2 mm showing that the test system was sensitive to differential toxicity resulting from DNA damage induced by the positive control.

Reverse mutation assays: No significant differences were noted at any concentration of the test substance with respect to the number of revertant colonies per plate when compared with that for the vehicle control (see Tables 1).

5. DISCUSSION

There are adequate data presented to support the conclusions of the investigators.

Table 1

Revertants per plate as reported
by Moriya et al (1981) (see page 1 above)

Dose (ug per plate)	TA98	TA100	TA1535	TA1537	TA1538	WP2 hcr
			Without activation			
0	35	95	7	6	9	11
	38	116	10	8	16	17
10	28	119	6	7	11	11
	37	105	7	13	13	13
50	12	107	6	7	7	8
	23	103	9	8	6	18
100	15	129	8	10	3	11
	25	112	7	7	8	8
500	31	99	7	4	7	16
	26	113	5	7	15	20
1000	41	100	4	6	14	16
	37	101	11	7	13	11
5000	24	95	7	2	10	14
	49	87	11	0	12	13
Pos. control ug/plate	AF-2 0.1	AF-2 0.01	ENNG 10	9-AA 90	2-NF 2	Af-2 0.04
Revertants per plate	448	608	>2000	>2000	338	341
	498	540	>2000	>2000	298	387
ug 2AA/plate	0.5	0.5	2	2	0.5	40
Revertants per plate	30	113	15	19	15	10
	35	100	8	8	15	10

Table 1 (continued)

Dose (ug per plate)	TA98	TA100	TA1535	TA1537	TA1538	WP2 hcr
			With activation			
0	38	106	2	19	32	16
	45	99	3	10	33	18
10	33	78	7	8	28	12
	34	128	6	5	40	14
50	28	90	4	11	36	13
	34	87	7	7	21	15
100	32	105	4	8	26	10
	29	109	4	10	23	11
500	34	94	6	12	28	13
	33	90	9	13	45	12
1000	37	120	4	4	39	15
	37	125	4	5	20	17
5000	39	98	4	5	24	13
	33	84	3	6	17	13
ug 2AA/plate	0.5	0.5	2	2	0.5	40
Revertants per plate	212	500	166	177	288	340
	228	506	187	158	240	408

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Arni, P.; Müller, D. (1982) Saccharomyces cerevisiae D7/Mammalian-Microsome Mutagenicity Test In Vitro with CGA 48988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-E)

REVIEW RESULTS:

VALID X INVALID ____ INCOMPLETE ____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane C Harris DATE: 8/12/86

1. CONBLUSIONS: No mutagenic activity (mitotic recombination, mitotic gene conversion, or reverse mutations) was observed at levels of 0, 400, 2000, 4000, 8000, and 10,000 ug/ml in yeast. However, marginal results with positive controls in the mitotic recombination assay and variation in positive and negative control group results limited sensitivity of the assays. The results of such a study must be confirmed by another test system sensitive to the same type of genetic toxicity.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch EN 32 212; 94.1% purity)

   Reference mutagens: 4-Nitroquinoline-N-oxice was used as positive control substance in assays without metabolic activation, and cyclophosphamide was used in assays with metabolic activation.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance, and phosphate buffer (pH 7, 0.1 M) was used for positive control substances.

3. MATERIALS AND METHODS

Test species: The yeast strain used was D 7. The genotype of the test strain was described as follows:

Alleles	For detection of:
trp5-12/trp5-27	gene conversion events
ade2-40/ade2-119	reciprocal mitotic recombinations
ivl1-92/ilv1-92	reverse mutation

The report stated that reciprocal mitotic recombinations appear as pink and red twin-sectored colonies; mitotic gene conversion is detected by the appearance of colonies on medium selective for growth of non-tryptophan-requiring cells; and mutations also appear as colonies on medium selective for non-isoleucine-requiring cells.

Culture media for yeast: Test cultures were routinely maintained at 27° C in yeast extract peptone (YEP) broth (2% peptone, 1% yeast extract, and 2% glucose in distilled water).

The solid medium used in this study consisted of yeast nitrogen base (6.7%) supplemented with the following:

L-arginine HCl	10 mg/l	L-tryptophan	10 mg/l	uracil	10 mg/l
L-isoleucine	60 mg/l	Agar	15 g/l	adenine sulfate	5 mg/l
L-leucine	60 mg/l	Glucose	20 g/l	L-histidine HCl	10 mg/l
L-lysine HCl	10 mg/l	L-valine	30 mg/l		

The tryptophan was removed from this medium to make the selective medium used in the mitotic gene conversion assay, and the isoleucine was removed from the supplemental medium to make the selective medium for detection of reverse mutations.

3. MATERIALS AND METHODS (continued)

Microsomal enzyme (S-9) preparation: Liver microsomal preparations were obtained from Aroclor 1254 induced rats. To each 0.3 ml sample of the S-9 was added 0.7 ml of a cofactor solution described only by citation of a reference (Ames et al., 1973)

Experimental procedure: Yeast inocula were grown in 150 ml broth for 3 days with agitation at 27° C for 3 days. These cultures were centrifuged to remove the cells which were then washed and resuspended in distilled water. Suspensions were adjusted to an approximate density of 6×10^8 cells/ml.

Assays without metabolic activation: One ml of the cell suspension was placed in test tubes along with 5 ml phosphate buffer (pH 7, 0.1 M) and 0.1 ml test substance dissolved in DMSO. The concentrations of the test substance evaluated were 0, 400, 2000, or 10,000 ug/ml, and those for the positive control substance were 0.25, 0.5 and 1 ug/ml. Each of these suspensions was then incubated at 27° C with agitation for 2 hours before the cells were again collected and washed with distilled water.

Assays with metabolic activation: Procedures were similar to those assays conducted without metabolic activation with the exception that only 3.5 ml phosphate buffer were added to the suspension of the test organism along with 1.5 ml of the activation mixture (S9 mixture). In addition, the report noted excessive toxicity at the highest treatment level (10,000 ug/ml), and doses were changed to 2000, 4000, and 8000 ug/ml for the metabolic activation assays. The test cultures were incubated with the test substance for 6 hours.

Observations: Plating of these suspensions was described as follows:

> ...,to determine the counts of surviving cells and the number of mutants produced: 0.1 ml of the suspension were streaked out on each of 5 plates of each of the 2 selective media. After 2 or 3 dilutions 1:10 with buffer (number of cells determined with a counting chamber), 0.1 ml were streaked out on each of 10 plates of supplemented medium to determine the number of surviving cells and recombinants. The supplement medium and the tryptophan-free agar were then incubated for 4-6 days and the isoleucine-free agar for 6-8 days at 27° C.

The colonies on each plate were counted, and from these counts the number of recombinants, convertants, and revertants per ml were determined.

Statistical analysis: The report described the procedures as follows:

> a) Mitotic crossing-over and reverse mutation
> The numbers of mutants per plate are considered to be Poisson-distributed. The object of the test is to determine whether plates containing cells treated with the test material reveal a larger number of mutants than the control group with the treated groups and a trend test. The multiple comparisons are performed with the Likelihood-Ration test (LR) (level of the individual tests is 2%, since only an increase in the number of mutants is of interest).

3. MATERIALS AND METHODS (continued)

The trend test is calculated with the C(alpha)-statistic. This test responds to monotone trends of various kinds. The test size depends on the concentrations.

Level of significance: 5%

b) Mitotic gene conversion
...The total cell count is assumed to be equal for all plates. The evaluation therefore relates to the number of mutants per plate. The square roots of the number of mutants per plate are used as single values. These values can be regarded as normally distributed with equal variances. The analysis of variance results in a decomposition of the sums of squares in orthogonal polynomes. This shows whether there is a concentration-dependent linear and/or square trend...In the case of an increasing trend, the William's test is used for the determination of the lowest effective concentration. In the other case Dunnett's test is used to determine the treated groups which differ from the control groups.

With the Shapiro-Wilk test the hypothesis of normal distribution is checked and with the Barlett test the equal variances within groups can be verified.

4. REPORTED RESULTS

The investigators noted in the text of the report that metalaxyl inhibited growth at a concentration of 10,000 ug/ml in the first trial without metabolic activation, but tabulated results (reproduced in the Appendix of this DER) indicated that the 10,000 ug/ml concentration in the first trial also inhibited growth with metabolic activation. Growth was also inhibited by the test substance at 8000 ug/ml with and without metabolic activation in the second trial. The relevant survival data for the control and high dose groups are summarized as follows:

Concentration (ug/ml)	Surviving cells (X 10^6 /ml) Experiment 1 Without S9	Experiment 1 With S9	Experiment 2 Without S9	Experiment 2 With S9
0	79.2	8.0	13.8	14.0
8000	--	--	1.6	9.6
10,000	71.8	0.2	--	--

The authors also concluded that there were no treatment-related genetic effects suggested by the incidence of convertants, recombinants, or revertants in the study (see Appendix for summary tables from the report).

With respect to the incidence of recombinants in the positive control groups, the investigators described the response as marginally increased over negative

4. REPORTED RESULTS (continued)

controls. Those results are summarized as follows:

Concentration (ug/ml)	Recombinant cells (X 10^5 /ml) Experiment 1 Without S9	Experiment 1 With S9	Experiment 2 Without S9	Experiment 2 With S9
0	0.6	1.2	0	0
4-Nitroquinoline-N-oxice				
0.25	--	--	1.6	--
0.5	3.2	--	--	--
1.0	0.7	--	--	--
cyclophosphamide				
10	--	1.2	--	--
50	--	0.4	--	0.4
250	--	--	--	0.3

5. DISCUSSION

Because the response of the test strain to the positive control substances in the mitotic recombination assay was marginal and did not appear to be dose-related, the sensitivity of that portion of the study is questionable.

The response of the test strain to 4-Nitroquinoline-N-oxice in the gene conversion assays without metabolic activation appeared to decrease as the dose increased (see Table 1 below). The negative control responses in the two trials were also widely varied (a four fold difference between the two trials). Although each trial shows a 5 to 6-fold increase in convertants for the positive control groups in comparison to their respective negative control groups, the pattern of variation from trial to trial suggests that more than two trials should have been conducted.

The responses in the positive and negative control groups with respect to the incidence of revertant cells was less variable (see Table 2 below).

The questionable sensitivity in the recombination assay and variability in control data in this study suggests that it should be confirmed by another test system appropriate to the types of genetic effects evaluated by the yeast assays described above.

6. REFERENCE

Ames, B. N., W. E. Durston, E. Yamasaki, and F. D. Lee (1973). Carcinogens Are Mutagens: A simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection. Proc. Natl. Acad. Sci. 70: 2281-2285.

5. DISCUSSION (continued)

Table 1

Summary of control data for the group mean incidence of convertant cells

Concentration (ug/ml)	Convertant cells (X 10 /ml) Experiment 1 Without S9	Experiment 1 With S9	Experiment 2 Without S9	Experiment 2 With S9
0	19	25	78	82
4-Nitroquinoline-N-oxice				
0.25	--	--	480	--
0.5	101	--	--	--
1.0	6	--	--	--
cyclophosphamide				
10	--	36	--	--
50	--	48	--	133
250	--	--	--	223

Table 2

Summary of control data for the group mean incidence of revertant cells

Concentration (ug/ml)	Revertant cells (X 10 /ml) Experiment 1 Without S9	Experiment 1 With S9	Experiment 2 Without S9	Experiment 2 With S9
0	0.6	0.2	0.4	0.5
4-Nitroquinoline-N-oxice				
0.25	--	--	43.4	--
0.5	3.0	--	--	--
1.0	0.4	--	--	--
cyclophosphamide				
10	--	1.0	--	--
50	--	2.4	--	3.8
250	--	--	--	8.8

APPENDIX

Summary tables for group mean incidences of recombinants, convertants, and revertants in yeast cells treated with metalaxyl

METALAXL

Page_____ is not included in this copy.

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The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Arni, P.; Müller, D. (1980) Mutagenicity Test on Saccharomyces cerevisiae MP-1 In Vitro with CGA 48 988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-F)

REVIEW RESULTS:

VALID X INVALID ____ INCOMPLETE ____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-19-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/19/86

1. CONBLUSIONS: No mutagenic activity (mitotic recombination, mitotic gene conversion, or reverse mutations) was observed at levels of 0, 40, 200, or 1000 ug/ml in yeast. However, no response to the positive control in the mutation assay and the absence of apparent toxicity in metalaxyl-treated cultures suggested that the sensitivity of the assay is limited. The results of such a study must be considered with those from other assays sensitive to similar genetic end points.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch EN 32 212; 94.1% purity)

   Reference mutagens: 4-Nitroquinoline-N-oxice was used as the positive control substance.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: The yeast strain used was MP-1. The genotype of the test strain was described as follows:

Alleles	For detection of:
trp5-12/trp5-21	gene conversion eve.ts
wild type/ade2	reciprocal mitotic recombinations

The report stated that reciprocal mitotic recombinations appear as red or partially red colonies; mitotic gene conversion is detected by the appearance of colonies on medium selective for growth of non-tryptophan-requiring cells; and mutations also appear as colonies on medium selective for cycloheximide resistant colonies.

Culture media for yeast: Test cultures were routinely maintained at 25° C in yeast extract peptone (YEP) broth (2% peptone, 1% yeast extract, and 2% glucose in distilled water).

The supplemented medium used in this study consisted of yeast nitrogen base (6.7%) supplemented with the following:

L-arginine HCl	10 mg/l	L-methionine	10 mg/l	L-valine	30 mg/l
L-aspartic acid	10 mg/l	L-phenylalanine	50 mg/l	Agar	15 g/l
Glutamic acid	100 mg/l	L-lysine HCl	10 mg/l	Glucose	20 g/l
L-histidine	10 mg/l	L-serine	20 mg/l	uracil	10 mg/l
L-leucine	60 mg/l	L-tryptophan	10 mg/l	adenine sulfate	5 mg/l
L-lysine HCl	10 mg/l	L-tyrosine	30 mg/l		

The tryptophan was removed from this medium to make the selective medium used in the mitotic gene conversion assay, and cycloheximide (0.1 mg/l) was added to the supplemental medium to make the selective medium for detection of forward mutations.

3. MATERIALS AND METHODS (continued)

Experimental procedure: Yeast inocula were grown in 150 ml broth for 3 days with agitation at 25° C for 3 days. These cultures were centrifuged to collect the cells which were then washed and resuspended in distilled water. Suspensions were adjusted to an approximate density of 5 X 10^8 cells/ml, and 4.5 ml of the suspension was placed in test tubes. The test substance solutions in DMSO were added to the suspensions in aliquots of 0.5 ml. The concentrations of the test substance evaluated were 0, 40, 200, or 1000 ug/ml, and those for the positive control substance were 5 and 15 ug/ml. Each of the suspensions was then incubated at 25° C with agitation for 3.5 hours before the cells were again collected and washed with distilled water.

Observations: Plating of these suspensions was described as follows:

> ...,to determine the counts of surviving cells and the number of mutants produced: 0.1 ml of the suspension were streaked out on each of 40 plates of each of the 2 selective media. After 5 1:10 dilutions with twice-distilled water, 0.1 ml is streaked out on each of 10 plates of supplemented medium to determine the to determine the number of surviving cells and recombinants. The supplemented medium and the tryptophan-free agar were then incubated for '-6 days and the cycloheximide agar for about 14 days at 25° C. Inereafter the resultant colonies are counted.

The colonies on each plate were counted, and from these counts the number of recombinants, convertants, and revertants per ml were determined.

Statistical analysis: The report stated that tests for significance of differences were conducted using the Chi square test at the $p< 0.05$ level.

4. REPORTED RESULTS

The report noted that two concurrent trials were run with a single concurrent positive contgrol group. Each trial had its own vehicle control group. The mean number of surviving cells for each group in each trial is summarized in Table 1 below.

Table 2 shows the incidence of genetic events in control and treated cultures, and the authors stated that these results suggested that metalaxyl is not mutagenic in yeast under the test conditions.

5. DISCUSSION

Although the report stated that the dose range was adequate according to standard protocols at the time the study was conducted, there was no apparent compound-related effects on survival in treated yeast cultures. There was also no apparent increased incidence of mutants in the positive control cultures when compared with negative controls suggesting a further limitation of the study. Because there is no apparent toxicity in the metalaxyl-treated cultures, and because there is no apparent response in the positive control group for detection of cycloheximide-resistant mutants, the study is not considered to be complete.

Table 1

Summary of survival in the two trials

Concentration (mg/ml)	Surviving cells per ml (X 10^8)	
	Trial 1	Trial 2
CGA 48 988		
0	87.7	84.8
40	52.7	46.8
200	85.1	133.2
1000	106.5	67.1

Table 2

Summary of the group means for the incidence of recombinants, convertants, and mutants in yeast treated with metalaxyl

Concentration (mg/ml)	Recombinants per ml (X 10^4)		Convertants per ml (X 10)		Mutants per ml (X 10)	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
CGA 48 988						
0	60	90	11.3	7.8	23.3	16.5
40	60	20	10.0	11.8	16.5	16.8
200	50	130	9.5	9.5	18.0	15.8
1000	60	110	10.3	9.8	13.8	16.5
4-Nitroquino-line-N-oxice						
5	1166		240.3		18.0	
!%	402		288		16.0	

005372

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Strasser, F. F.; Müller, D. (1982) L5178Y/Tk$^{+/-}$ Mouse Lymphoma Mutagenicity Test: CGA 48 988. Experiment No. 811258. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-K)

REVIEW RESULTS:

VALID X INVALID _____ INCOMPLETE _____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane C Harris DATE: 8/12/86

31

1

1. DISCUSSION AND CONCLUSION: Concentrations of 0.125, 0.25, 0.5, and 1.0 mg/ml without metabolic activation or 0.0625, 0.125, 0.25, and 0.5 mg/ml with metabolic activation did not increase the frequency of forward mutations at the Tk locus of L5178Y mouse lymphoma cells under the test conditions.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch EN 32 212; 94.1% purity)

   Reference mutagens: Ethylmethane sulfonate (EMS) and dimethylnitrosamine (DMN) were used as positive control substances.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: L5178Y mouse lymphoma cells ($Tk^{+/-}$) were used.

Cell culture conditions: Cultures were grown in F_{10}P-medium (Fischer's mouse leukemia medium with L-glutamine supplemented with 10% horse serum, pluronic acid (1 mg/ml), sodium pyruvate (100 ug/ml), penicilin (50E/ml), and streptomycin (50 mg/ml). Cloning medium consisted of Fischer's medium supplemented with 20% horse serum, pluronic acid (200 ug/ml), sodium pyruvate (220 ug/ml), antibiotics, and agar (0.12%). The selection medium was made with cloning medium and bromodeoxyuridine (BUdR) (50 ug/ml).

Microsomal enzyme (S-9) preparation: Liver microsomal preparations were obtained from Aroclor 1254 induced rats. To each 0.5 ml sample of the S-9 was added 2.0 ml of a cofactor solution containing NADP (0.4 ml/ml), isocitric acid (0.86 mg/ml), tris-buffer (0.3 mg/ml), and $MgCl_2(*6H_2O)$ (0.034 mg/ml).

Experimental procedures---Preliminary toxicity assay: The report stated that seven concentrations from 0.0156 to 1.0 mg test substance per ml of medium were evaluated for their toxicity to the lymphoma cell cultures in the exponential phase of growth. The test substance was dissolved in DMSO and added to each culture such that the solvent concentration was 1%. These cultures were incubated for four hours. At the end of that period, the medium was removed and the cells were washed and incubated for an additional 24-hour recovery period. They were then stained with Erythrosin solution for five minutes, and fixed. Three-hundred cells were counted, and the percentage of unstained cells was noted. According to the report, the criterion for selection of doses for the main study was that the second highest dose cause no more than a 10% reduction in the viability of cells in comparison to untreated control culutres.

Experimental procedure---Main study: Stock cultures (3×10^5 cells/ml) were first incubated in F_{10}P-medium containing thymidine, hypoxanthine, methotrexate, and glycine (conecntrations not specified) for 24 hours to remove spontaneous $Tk^{-/-}$ mutants. These cells were incubated for another three days in medium with thymidine, hypoxanthine, and glycine before the mutagenicity assay was started.

3. MATERIALS AND METHODS (continued)

The four concentrations of the test substance used in the assay without metabolic activation were 0.125, 0.25, 0.5, and 1.0 mg/ml. Those selected for the two assays with metabolic activation were 0.0625, 0.125, 0.25, and 0.5 mg/ml. The control groups included an untreated control, a solvent control (1% DMSO), and EMS (0.5 ul/ml) in the assay without metabolic activation, and DMN (0.5 ul/ml) for the assay requiring metabolic activation. A 2.5 ml aliquot of the S-9 mixture was added to 7.5 ml F_0-medium for addition to 50 ml of the cell suspension used in the assay with metabolic activation. The test and control solutions were also added to 50 ml of the cell suspensions in 10 ml aliquots of the F_0-medium for the assays with and without metabolic activation.

After a four-hour exposure period, cells from these cultures were harvested and washed with F_{10P}-medium to remove the test and control substances. The cells were resuspended and incubated for three days in F_{10P}-medium so that the Tk -/- mutants could be expressed. The report stated that cell counts were obtained for these cultures on a daily basis, and they were readjusted to a density of 3×10^5 cells/ml.

After the three-day incubation period, four tubes per group containing 5 ml cloning medium were inoculated with 200 cells each for viability controls. Eight tubes per group containing 5 ml selection medium were inoculated with 4×10^5 cells for determination of mutation rates. Incubation and observation of these cultures was described in the report as follows:

> The incubation time was 14 days for selection of mutants in all cases and 11 days in the experiments without metabolic activation and 10 days in the two experiments including metabolic activation for viability controls. At the end of the incubation period, the numbers of colonies in the mutagenicity-test tubes and in viability-control cultures were determined with the aid of a Colony Counter (Fisher Count-All™ Model 600). The values obtained from the viability control served to normalize the results received from the mutagenicity test, i. e., to calculate according to a 100% viability of the cells seeded in cultures of the mutagenicity test.

The criterion for a positive result was described as an increase in the number of mutants per 10^6 surviving cells at any concentration that is equal to or greater than 2.5 times that observed in the solvent control.

4. REPORTED RESULTS

No results from the preliminary toxicity test were presented in the report.

Tables 1, 2, and 3 below summarizes the daily counts, relative growth parameters and mutation rates as reported for the test groups.

The authors noted that the highest mutation rate in the assay without metabolic activation was 1.03 times that for the solvent control group, and it was observed at the highest concentration tested. The mutation rate for the positive control group in the assay was 2.06 times that for the solvent control.

4. REPORTED RESULTS (continued)

The reported ratios of mutation rates for treated and solvent control groups in the first assay with metabolic activation were 1.19, 2.05, 1.52, and 2.55 for the 0.0625, 0.125, 0.25, and 0.5 mg/ml concentrations, respectively. The investigators noted that the ratio for the highest concentration tested exceeded the criterion for a positive result (2.5), and they repeated the assay. In the second assay the ratios for the 0.0625, 0.125, 0.25, and 0.5 mg/ml concentrations were 0.98, 0.98, 0.41, and 0.95, respectively. The positive control results indicated that DMN induced 13 and 11 times the mutations found in concurrent control groups in the first and second assays, respectively. Based on these results, the investigators concluded that metalaxyl did not cause forward mutations in mouse lymphoma cells in vitro.

5. DISCUSSION

There were adequate data presented in the report to support the conclusions of the authors that metalaxyl did not consistently increase the frequency of mutations at the TK locus in L5178Y cells under the test conditions. The reported increase observed in the first assay using metabolic activation was not confirmed by the second such assay nor was the increase dose related.

Table 1

Cell growth during the expression period
(from Tables 1, 2, and 3 of the original report)

Treatment	Daily count (10^5 cells/ml) 1	2	3	Relative suspension growth (% of control)
Without metabolic activation				
1% DMSO	7.4	11.0	13.1	100
Untreated	9.4	10.8	10.2	95.83
EMS (0.5 ul/ml)	6.3	8.9	8.6	47.21
CGA 48 988				
0.125 mg/ml	8.2	10.2	10.4	81.33
0.25 mg/ml	7.8	10.8	10.4	82.04
0.5 mg/ml	4.3	9.4	10.6	39.93
1.0 mg/ml	1.1	2.8	7.5	6.37
With metabolic activation (first assay)				
1% DMSO	5.1	12.7	7.7	100
Untreated	6.5	9.7	7.3	92.15
DMN (0.5 ul/ml)	4.6	11.3	7.5	84.73
CGA 48 988				
0.0625 mg/ml	5.0	13.4	7.1	95.67
0.125 mg/ml	6.0	12.8	8.3	128.03
0.25 mg/ml	5.3	12.8	7.9	107.58
0.5 mg/ml	4.8	12.5	8.0	96.37
With metabolic activation (second assay)				
1% DMSO	8.1	7.6	5.4	100
Untreated	9.0	8.3	5.7	128.48
DMN (0.5 ul/ml)	7.8	5.4	5.4	53.32
CGA 48 988				
0.0625 mg/ml	8.2	6.9	6.1	103.74
0.125 mg/ml	8.4	7.8	6.2	107.16
0.25 mg/ml	7.8	7.5	5.9	104.23
0.5 mg/ml	7.0	6.9	6.3	91.62

Table 2

Cloning efficiency and growth results
(from Tables 1, 2, and 3 of the original report)

Treatment	Total mutant clones*	Total viable clones**	Relative cloning efficiency (% of control)	Relative growth***
Without metabolic activation				
1% DMSO	125	164	100	100
Untreated	170	290	176.83	169.46
EMS (0.5 ul/ml)	312	198	68.28	32.24
CGA 48 988				
0.125 mg/ml	136	310	189.02	153.73
0.25 mg/ml	197	278	169.51	139.07
0.5 mg/ml	185	295	179.88	71.83
1.0 mg/ml	220	281	171.34	10.91
With metabolic activation (first assay)				
1% DMSO	25	502	100	100
Untreated	41	461	91.83	84.62
DMN (0.5 ul/ml)	127	196	42.52	36.03
CGA 48 988				
0.0625 mg/ml	37	620	123.51	118.16
0.125 mg/ml	51	499	99.4	127.26
0.25 mg/ml	42	531	105.78	113.8
0.5 mg/ml	55	431	85.86	82.74
With metabolic activation (second assay)				
1% DMSO	151	526	100	100
Untreated	153	363	69.01	88.66
DMN (0.5 ul/ml)	413	129	35.54	18.95
CGA 48 988				
0.0625 mg/ml	142	506	96.2	99.8
0.125 mg/ml	143	507	96.39	103.29
0.25 mg/ml	78	666	126.62	131.98
0.5 mg/ml	121	443	84.22	77.16

*From an inoculum of 4×10^5 cells.
**From an inoculum of 800 cells.
***(relative suspension growth X relative cloning efficiency)/100

Table 3

Mutation rates (per 10^6 cells)
(from Tables 1, 2, and 3 of the original report)

Treatment	Mutation rate (per 10^6 cells) Without activation	With activation Assay #1	With activation Assay #2
1% DMSO	191	12.5	71.8
Untreated	147	22.2	105
EMS (0.5 ul/ml)	394	---	---
DMN (0.5 ul/ml)	---	162	800
CGA 48 988			
0.0625 mg/ml	-	14.9	70.2
0.125 mg/ml	110	25.6	70.5
0.25 mg/ml	177	19.8	29.3
0.5 mg/ml	157	31.9	68.3
1.0 mg/ml	196	--	--

*(Mutant clones X 800/Viable clones/3.2 X 10^{-6})

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Arni, P.; Müller, D. (1980) Test for Non-disjunction on Saccharomyces cerevisiae D 61 with CGA 48988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-D)

REVIEW RESULTS: VALID X INVALID ____ INCOMPLETE ____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ____

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/13/86

1. CONCLUSIONS: Contradictory results in the positive control groups of two trials, and the wide range of survival in negative control groups of those trials (12.4 to 25.7 X 10^8 cells/ml) limit the sensitivity of the assay to detect non-disjunction in yeast. Also the complete protocol was not followed because of limited solubility of metalaxyl. However, this study is acceptable when considered with the two other studies in S. cereviciae. Together, they indicate that metalaxyl has no mutagenic activity in yeast under the test conditions of the three assays.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch EN-32-212; 94.1% purity)

   Reference mutagens: 4-Nitroquino-line-N-oxice was used as positive control substance.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: The yeast strain used was D 61 which the report stated was similar to strain D 6 of Sacch. cerevisiae. The genotype of the test strain was described as follows:

Chromosome III		Chromosome XV	Chromosome VII				
$\frac{his4}{+}$	centromere a	$\frac{ade2\text{-}40}{ade2\text{-}40}$	$\frac{ade3}{+}$	centrom.	$\frac{leu1}{+}$	$\frac{trp5}{+}$ $\frac{cyh2}{+}$	$\frac{met13}{+}$

The report stated that this test strain detects non-disjunction in yeast cells. The monosomic colonies that result from chromosomal loss appear white and are resistant to cycloheximide.

Culture media for yeast: Test cultures were maintained at 28° C in yeast extract peptone (YEP) broth (2% peptone, 1% yeast extract, and 2% glucose in distilled water). The solid medium used in this study consisted of the YEP broth with 1.5% agar. Selective medium for the D 61 strain was the solid agar with cycloheximide (see below for concentrations).

Experimental procedure: Yeast inocula were grown in 150 ml broth for 3 days with agitation at 28° C for 3 days. These cultures were centrifuged to remove the cells which were then washed and resuspended in distilled water. Suspensions were adjusted to an approximate density of 10^7 cells/ml, and 4.5 ml of the suspension was placed in test tubes. The test substance solutions in DMSO were added to the suspensions in aliquots of 0.5 ml. The concentrations of the test substance evaluated were 0, 40, 200, or 1000 ug/ml, and those for the positive control substance were 2.5 and 5.0 ug/ml. Each of the suspensions was then incubated at 28° C with agitation for 16 hours before the cells were again collected and washed with distilled water. Cells were resuspended in YEP broth and incubated under the same conditions for another 4 hours. Plating of these suspensions was described as follows:

...,to determine the counts of surviving cells and the number of

3. MATERIALS AND METHODS (continued)

monosomic cells: 6 dilutions 1:10 in phosphate buffer are prepared with each test tube. 0.1 ml of the dilutions 10^{-1} and 10^{-2} are streaked out on each of 5 plates of cycloheximide medium to determine the number of monosomic cells (white colonies). 0.1 ml of dilutions 10^{-5} and 10^{-6} are streaked out on each of 5 plates of complete medium to determine the number of surviving cells.

The inoculated plates were then incubated at 28° C for 3 days after which colonies on each plate were counted. From these counts the number of monosomic colonies per 10^7 survivors was calculated.

This experimental procedure was followed for two trials according to the individual plate data appended to the final report.

The t-test was used to identify statistically significant differences ($p<0.05$) according to the report.

The report also noted that standard protocols for this type of assay call for a 72 hour test in addition to the 16 hour test described above. The investigators stated that the longer exposure period was not attempted because of the limited solubili.y of the test substance in water.

4. REPORTED RESULTS

Reported group means from the revised summary table are as follows:

Concentration (mg/ml)	Surviving cells per ml (X 10^8)	Monosomic colonies/ml	Monosomic colonies per 10^8 surviving
CGA 48 988			
0	19.1	40	1.6
40	12.4	0	0
200	10.3	80	7.2
1000	12.6	80	6.4
4-Nitroquino-line-N-oxice			
2.5	2.4	1160	863.7
5.0	3.1	1180	939.9

The addendum to the report stated that there were no statistically significant difference between either the 200 or 1000 ug/ml group means with respect to the numbers of monosomic colonies/ml.

5. DISCUSSION

As noted above, the investigators stated that a 72 hour exposure of the test organism is usually included in these assays. Because the test substance is relatively insoluble in water, the authors stated that the longer exposure was not tested, but no explanation of why the insolubility is not a factor in the 16 hour exposure was provided.

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5. DISCUSSION (continued)

Survival was reduced by approximately 30 to 45% in the treated groups suggesting that metalaxyl may be sufficiently soluble to cause toxicity in the yeast, but there was no apparent dose-related decrease reported in the summary tables.

Based on reported individual plate counts, the survival, number of monosomic colonies per plate, and number of monosomic cells per 10^8 survivors are summarized as follows:

Concentration (mg/ml)	Surviving cells per ml (X 10^8)	Monosomic colonies/ml	Monosomic colonies per 10^8 surviving
	Trial 1		
CGA 48 988			
0	25.7	40	3.1
40	11.1	0	0
200	9.0	80	14.4
1000	16.3	10	24.5
4-Nitroquino-line-N-oxice			
2.5	0.8	47	303
5.0	5.6	98	1633
	Trial 2		
CGA 48 988			
0	12.4	0	0
40	15.7	0	0
200	9.4	0	0
1000	8.9	30	6.7
4-Nitroquino-line-N-oxice			
2.5	3.9	57	1425
5.0	0.6	55	246

These survival results further indicate that there was treatment-related toxicity observed. In addition, results suggest that there may be a dose-related trend with respect to the number of monosomic cells per 10^8 surviving cells. However, those data for the positive control substance from both trials and the range of survival suggested by the negative control results from both trials suggest that the test results are not reliable.

Based on these circumstances, the conclusions of the investigators must be confirmed by additional trials under the same experimental conditions or by other experiments designed to detect non-disjunction resulting from metalaxyl treatment.

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DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Ivett, J.; Spicer, C. (1986) Clastogenic Evaluation of Metalaxyl Technical, CGA 48 988, in an in vitro cytogenetic assay Measuring Chromosomal Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells: Final Report. Project No. 20990. (Unpublished study received June 2, 1986 under 100-601; prepared by Hazleton Biotechnologies, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL: 263117-B)

REVIEW RESULTS: VALID X INVALID ____ INCOMPLETE ____

GUIDELINE: SATISFIED ___ PARTIALLY SATIS IED X NOT SATISFIED ___

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TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane C Harris DATE: 8/13/86

42

1. CONCLUSION: Concentrations of 150, 300, 900, and 1,200 ug metalaxyl per ml were tested in vitro with Chinese hamster ovary cells for clastogenic activity in the presence and absence of liver microsomal enzymes. A concentration of 1200 ug/ml increased the incidence of cells with chromatid or chromosomal breaks without metabolic activation. The assay with metabolic activation did not reveal any increases in the frequency of cells with chromosomal aberrations, but the results and test conditions were not sufficiently adequate to determine if the test substance was detoxified or if the exposure period used for the assay with microsomal enzymes was too short.

   Core classification: Unacceptable. The assay is incomplete.

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch P. 503119; 95.7% purity)

   Reference mutagens: Mitomyucin C (MMC) was used as positive control substance in the assays without metabolic activation, and cyclophosphamide (CP) was used in assays with metabolic activation.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: Chinese hamster ovary cells (CHO-WBL) were used in this assay.

Cell culture conditions: The cells were routinely grown in McCoy's 5a medium supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin. The report stated that before harvest of the cultures, they were examined for toxicity as indicated by visual assessment of the percent confluence of the monolayers and the presence of mitotic (large rounded cells) or dead cells floating in the medium of each culture flask. Metaphase cells were collected by "mitotic shake-off" and treated with 0.075 M KCl hypotonic solution. Cells to be examined were fixed with absolute methanol and glacial acetici acid (3:1), washed, and placed on slides that were air dried.

Microsomal enzyme (S-9) preparation: Liver microsomal preparations were obtained from Aroclor 1254 induced rats. Each ml sample of the S-9 mixture contained 15 ul S-9, 1.5 mg NADP, and 2.7 mg isocitric acid.

Experimental procedures---Rangefinding assays: For these assays without metabolic activation 25 cm^2 culture flasks containing 5 ml of the complete McCoy's 5a medium were seeded with 0.3 X 10^6 cells. They were incubated for 24 hours at 37° C before treatment was initiated. Concentrations of 250 ng/ml of MMC, 10 ug/ml DMSO, and test substance at concentrations of 50, 167, 500, 1670, and 5000 ug/ml were evaluated for their effect on the progress of the cell cycle. An untreated negative control culture was also evaluated concurrently. Two hours after the test and control substances were added, 5-bromo-2'-deoxyuridine (BrdUrd) was added at a concentration of 10 uM, and the cultures were incubated for approximately 23 more hours. Approximately 2.5 hours before the incubation period was ended, cells were washed with phosphate buffered saline to remove the test substance, and fresh McCoy's medium with BrdUrd and colcemid (0.1 ug/ml) was added. The culture incubation was continued until the cells were harvested and stained for evaluation of cell cycle delays.

3. MATERIALS AND METHODS (cntinued)

According to the report, a modified flourescent plus Giemsa staining technique was used for these evaluations. The technique was described as follows:

> The slides were stained for 10 minutes with Hoechst 33258 (5 ug/ml) in a pH 6.8 phosphate buffer, were mounted in the same buffer and were exposed at 55° - 60° C to "blacklight"...for the amount of time required for the differentiation of the BrdUrd incorporated sister chromatids...The slides were then stained in a 5% Giemsa solution ...

In the rangefinding assay with metabolic activation the cells were incubated at 37° C for 2 hours in the medium with S-9 and without FCS. The remainder of this phase of the rangefinding experiments with activation were similar to those described for the nonactivated assays.

Experimental Procedures---Chromosomal aberration assays: Twenty-four hour cultures were used in the assays with and without metabolic activation (S9). In the assays without activation, the cultures were incubated with the test substance at concentrations of 150, 300, 900, and 1,200 ug/ml for 17.6 hours. At the end of that incubation period, the cells were washed with phosphate buffered saline and placed in McCoy's medium 5a with 0.1 ug/ml colcemid for 2.5 hours' incubation. The cells were then harvested and placed on slides to air dry. After drying, the slides were stained with buffered 5% Giemsa solution (pH 6.8) and analyzed for chromosomal aberrations.

The assays with metabolic activation followed a similar procedure except that cultures were incubated with test substance for two hours in McCoy's medium 5a with S-9 mixture and without FCS. After the cells were washed, they were incubated for 7.5 hours in complete McCoy's medium 5a with 10% FCS followed by an additional 2.5 hours in the medium with 0.1 ug/ml colcemid. Then the cells were harvested and prepared as above for examination.

Observations---Rangefinding assays: The report stated that 100 concecutive metaphase plates were examined for the number of cell cycles through which the cultures progressed in the p;resence of BrdUrd

Observations---Chromosomal aberration assays: The report stated that 100 cells from each of two cultures for the four test substance concentrations, the untreated and vehicle controls were examined for chromosomal aberrations. Twenty-five cells from the positive control cultures were also evaluated. Slides were coded so they could be examined "blind," and the location of the microscope stage was recorded for each cell containing aberrations.

The report noted that the following factors were considered in the evaluation of the aberrations observed:

1. Overall chromosomal aberration frequencies.
2. Percentage of cells with any aberrations.
3. Percentage of cells with more than one aberration.
4. A positive dose-response relationship.
5. Estimated number of breaks associated with each type of aberration observed.

3. MATERIALS AND METHODS (cntinued)

Chromatid or isochromatid breaks were not reported in the tables, but were included in the raw data because they were not considered to be truly representative of chromosomal breaks. Tabulation of data from cells with pulverized chromasomes was also handled in a special according to the report. Those data were described as follows:

> Cells classified as PU, P+, or PC were considered to contain one aberration for statistical purposes, however, a ">" is placed before the total number of aberrations per cell in the tables to indicate that the value is a minimum number. A cell classified as "GT" is considered to contain 10 aberrations for statistical purposes but a ">" is also included in the tables for this classification to indicate that it is a minimum number.

The glossary of notation and terminology in the report is included in Appendix A below.

Statistical analyses: Comparison of the negative and solvent control results were first conducted before they were pooled for comparisons with thetreated groups. Comparisons were made using the Fisher's Exact test with an adjustment for multiple comparisons. These analyses were conducted for the five types of data considered (see previous page), and a difference was considered to be statistically significant when $p<0.05$.

4. REPORTED RESULTS

Rangefinding assays: The report stated that the test substance had a solubility of 700 ug/ml in water. The solubility in DMSO was reported to be 500 mg/ml, but the investigators observed that a 1:100 dilution of 500 mg test substance per ml DMSO with culture medium caused precipitation which dissolved within an unspecified amount of time. Based on these results, the highest concentration chosen for the rangefinding assays was 5.0 mg/ml.

There was no confluent growth reported in cultures exposed to the 5000 and 1670 ug/ml concentrations of the test substance, and at the 500 ug/ml concentration, 83% confluence was reported. The two lowest concentrations (50 and 167 ug/ml) there was 100% confluence reported. These results were seen in the assays with and without metabolic activation.

Appendix B contains the tabulated results for the percentage of cells that went through one (M1) or more (M1+ or >M2) cell cycles.

These results were used as the basis for selection of the doses used in the chromosomal aberration assays.

Chromasomal assays: The investigators noted a significant increase in the percentage of cells with chromosomal aberrations for the 1.2 mg/ml concentration without metabolic activation (see Tables 2A and 2B reproduced from the original report in Appendix C below). The authors concluded that the response was indicative of a genetic effect under the conditions of the assay.

4. REPORTED RESULTS (continued)

Results for aberrations most frequently observed in the assay without metabolic activation are summarized as follows:

Observation	Dose (mg/ml) 0	1.2	Positive control
No. cells scored	200	200	25
Cells with chromatid breaks	1	33	3
Cells with chromosome breaks	2	24	6
Aberrations/cell*	0.05	>0.37	0.64
% cells with aberrations*	0	21.0	4.0
% cells with >1 aberration*	0	7.5	0.5

*Includes other aberrations observed at much lower frequencies (1 to 4 cells/200 examined) in cultures exposed to the test substance.

In the assays with metabolic activation, the report noted no increase in the frequency of cells with chromosomal aberrations as the result of treatment with the test substance (see Tables 3A and 3B reproduced from the original report in Appendix C below).

5. DISCUSSION

There are adequate data to support the authors' conclusion that metalaxyl did not increase the frequency of Chinese hamster ovary cells with chromosomal aberrations under test conditions which included metabolic activation by liver microsomal enzymes. The results also indicated, according to the investigators, that metalaxyl at a concentration of 1.2 mg/ml significantly increased the incidence of chromosomal aberrations without liver microsomal enzyme activation.

The authors attributed the lack of a response in the assay with metabolic activation to possible detoxification of the test substance or to the shorter duration of exposure relative to the nonactivation assay (2 hours compared with 17.5 hours in the nonactivation assay). Therefore, the experiment described above should not be interpreted without other mutagenicity studies.

APPENDIX A

Glossary of notation and terminology used
(reproduced from the original report cited above)

XIV. DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA STAINED CELLS

CHROMATID TYPE

TG	Chromatid Gap:	("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
TB	Chromatid break:	An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
TD	Chromatid deletion:	A terminal piece of one chromatid is missing.
IG	Isochromatid gap:	("Chromosome gap, SG"). Same as chromatid gap but at the same locus in both sister chromatids. If the gap is large or chromosome fragment displaced, the break is included with chromosome breaks.
TI	Chromatid Intrachange:	Exchange within a chromosome involving one chromatid.
RC	Ring Chromatid:	Single chromatid ring (acentric).

CHROMATID INTERCHANGES

TR	Triradial:	An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
QR	Quadriradial:	As triradial, but resulting in a four-armed configuration.
CR	Complex Rearrangement:	An exchange among more than two chromosomes or fragments which is the result of several breaks.

CHROMOSOME TYPE

DM	"Double Minute" Fragment:	These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.
D	Dicentric:	An exchange between two chromosomes which results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.
DF:		Dicentric with fragment.
TC	Tricentric:	An exchange involving three chromosomes and resulting in a chromosome with three centromeres. Often associated with two to three AF. Such exchanges can involve many chromosomes and are named as follows:
QC	Quadricentric:	four centromeres, up to four AF
PC	Pentacentric:	five centromeres, up to five AF
HC	Hexacentric:	six centromeres, up to six AF
R	Ring:	A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
RF:		Ring with associated acentric fragment.
AB:		Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and often the result of a translocation, pericentric inversion, etc. Classification used if abnormality cannot be ascribed to; e.g., a reciprocal translocation.
SB	Chromosome break:	Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated but apparently related. Designated "SB" and not an "Ab+AF" which implies two aberrations.
CI	Chromosome Intra-change:	Exchange within a chromosome; e.g., a ring that does not include the entire chromosome.
T	Translocation:	Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored as "T" not "2Ab."

APPENDIX B

Summary of results from rangefinding assays
(reproduced from the original report cited above)

METALAXL

Page 10 is not included in this copy.

Pages _____ through _____ are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

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APPENDIX C

Results of the chromosomal aberration assays
(reproduced from the original report cited above)

METALAXL

Page_____ is not included in this copy.

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DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Langauer, M.; Müller, D. (1979) Nucleus Anomaly Test in Somatic Interphase Nuclei: CGA 48 988: Chinese Hamster. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-I)

REVIEW RESULTS:

VALID X INVALID ____ INCOMPLETE ____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/13/86

54

1. DISCUSSION AND CONCLUSION: The report contained adequate information to support the authors' conclusions. Two consecutive daily oral doses of 0, 595, 1190, or 2380 mg metalaxyl per kg body weight given to male and female hamsters were shown to have no effect on the incidence of cells with nuclear anomalies (single Jolly bodies, fragments of nuclei in erythrocytes, micronuclei in erythroblasts, micronuclei in leucopoietic cells, and polyploid cells.

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch EN 32 212; 94.1% purity)

   Reference mutagens: Cyclophosphamide was used as the positive control substance.

   Vehicle: According to the report, CMC was used as the vehicle for the test substance, and no further description was provided.

3. MATERIALS AND METHODS

Test species: Male and female Chinese hamsters were used. The males ranged in weight from 27 to 32 g, and females weighed from 23 to 33 g.

Preliminary considerations The investigators stated that an acute oral toxicity study was conducted in hamsters which indicated an LD_{50} of 7120 mg test substance per kg body weight with confidence limits of 5250 to 9660 mg/kg. The highest dose level to be tested was 1/3 the LD_{50}. The acute toxicity study is reviewed elsewhere.

Experimental procedure: Groups of 6 male and 6 female hamsters were given two consecutive daily doses of 0, 595, 1190, or 2380 mg test substance per kg body weight by gavage in 0.7% "CMC" solution. An additional group of 6 male and 6 female hamsters was given two consecutive daily doses of cyclophosphamide (128 mg/kg/day). Twenty-four hours after the last dose was administered, the animals were sacrificed, and the bone marrow was aspirated from both femurs of each animal. The bone marrow cells were aspirated in 0.5 ul aliquots of rat serum, and drops of this suspension were smeared onto microscope slides. These smears were air-dried overnight. The slides were then stained in undiluted MayGrunwald solution for 2 min. followed by a second staining in May-Grunwald/ water solution (1:1) for 2 min followed by 40% Giemsa stain for 20 minutes. The slides were washed with 55% methanol, rinsed with water twice, and immersed in water for 2 min. After air-drying, the slides were cleared with xylol and mounted in Eukitt.

Observations: Slides prepared from 3 animals of each sex in each group were scored for nuclear abnormalities. A thousand bone marrow cells from each animal were scored for the presence of single Jolly bodies, fragments of nuclei in erythrocytes, micronuclei in erythroblasts, micronuclei in leucopoietic cells, and polyploid cells. The report stated that the significance of differences was assessed by the Chi-square test.

4. REPORTED RESULTS

The report noted no significant differences with respect to the proportion of cells with nuclear anomalies for groups treated with the test substance and the negative control group. The positive control group exhibited an increased incidence of nuclear anomalies with respect to that reported for the negative control group. Results are shown in the Appendix.

APPENDIX

Incidence of cells with nuclear anomalies
(as reported) in hamsters treated with metalaxyl

METALAXL

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X FIFRA registration data.

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DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §81-1 Acute Oral Toxicity

MRID: Unassigned

Thomann, P.; Pericin, C. (1977) Acute Oral LD_{50} in the Chinese Hamster of CGA 48 988. Experiment No. PH 2.634. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-J)

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APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane C Harris DATE: 8/13/86

1. DISCUSSION AND CONCLUSIONS: The results of the study indicated that the acute oral LD_{50} in male and female hamsters is 7120 mg/kg suggesting that metalaxyl should be classified into Toxicity Category IV.

   Core classification: Minimum

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch no. and purity unspecified)

3. MATERIALS AND METHODS

Test species: Male and female Chinese hamsters were used. Their body weights ranged from 25 to 31 g.

Experimental procedure: Groups of 5 male and 5 female hamsters was given a single oral doses of 100, 1000, 1500, 3000, or 4500 mg test substance per kg body weight. An additional group containing 10 animals of each sex was given a 6000 mg/kg dose. The test substance was suspended in 0.5% aqueous carboxymethyl cellulose and administered by gavage. The hamsters were fasted overnight before treatment, and they were observed for mortality and appearance of toxicological and pharmacological signs at frequent but unspecified intervals during the 15 days that followed dosing. Surviving animals were sacrificed at the end of the observation period and necropsied. Postmortem examinations were limited to gross observations.

The LD_{50} was calculated by probit analysis.

4. REPORTED RESULTS

There were no deaths in the groups given doses of 100, 1000, 1500, or 3000 mg/kg. One of the five females in the 4500 mg/kg dose group died during the first day after dosing, and two of the ten females receiving the 6000 mg/kg dose died (one on the first day and the other on the second day of the observation period). Three of the ten males given the highest dose died during the first day of observation, and a fourth died on the third day of the observation period. No other mortalites were noted during the study.

There were no clinical signs reported for animals given the 100 mg/kg dose. In group given 1500 mg/kg or more, animals exhibited reduced spontaneous motility and ataxia which began 15 to 60 minutes after treatment and persisted for approximately 6 hours. As the dose increased, animals showed irregular respiration, lobored breathing, and hypoventilation. Muscular hypotonia, tremors, tetanic spasms, and prostration were also observed in animals given 3000 mg/kg or more.

Those animals that died in the 6000 mg/kg dose group had pale livers at necropsy. All other animals in the study that died during the observation period or that were sacrificed at the end of the study showed no grossly observable effects at necropsy.

The reported LD_{50} for both sexes was calculated to be 7120 mg/kg with 95% confidence limits of 5250 to 9660 mg/kg.

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Fritz, H. (1978) Mouse Dominant Lethal Study. CGA 48988 Tech. Experiment No. 32761. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-L)

REVIEW RESULTS: VALID X INVALID ____ INCOMPLETE ____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane C Harris DATE: 8/13/86

61

1. CONCLUSION: The authors do not provide a clear rationale for dose selection, and the responsiveness of the test species under the test conditions was not demonstrated by use of a positive control group. Under these limited conditions, the assay only suggests that metalaxyl is not mutagenic after a single oral dose as high as 195 mg/kg.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch no. P. 3; purity unspecified)

3. MATERIALS AND METHODS

Test species: Male and female NMRI strain mice were used.

Experimental procedures: Three groups of 20 males were given a single dose of 0, 65, or 195 mg test substance per kg body weight. The test substance was suspended in an aqueous solution of carboxymethyl cellulose and administered by gavage. Doses of the test substance were selected on the basis of results from a preliminary study which is reviewed elsewhere.

After dosing, each male was cohabitted with two untreated females for a mating period of of one week. According to the report, the females were checked daily for evidence of a successful mating as indicated by the presence of vaginal plugs. The day such evidence was discovered was designated day 0 of gestation. Each male was cohabited with two different untreated females each week for 8 consecutive weeks.

Treated animals were observed daily for condition and occurrence of clinical signs during the first week after treatment.

The report stated that untreated females were sacrificed on the 14th day of pregnancy, and the numbers of live embryos and embryonic deaths were noted. Uteri without visible implantations were placed in ammonium sulfide solution to determine the number of deciduomata.

The report stated that the Student's t test or the Mann-Whitney U test were used to evaluate group differences with respect to the number of implantations. Group totals for the numbers of mated and pregnant dams, and embryonic deaths were analyzed by the Chi-square test or the Fisher's exact test.

No other details regarding the protocol were included in the report.

4. REPORTED RESULTS

Table 1 summarizes the mating and pregnancy results, and Table 2 summarizes the litter observations. These results indicate that the treated groups remained comparable to the control group throughout the study. The authors stated that no sign of toxicity in treated males was observed after dosing. One male given the 65 mg/kg dose died during the sixth mating period, and one given the 195 mg/kg dose died during the third mating period.

4. REPORTED RESULTS (continued)

Table 1

Mating and pregnancy results
(from summary tables in original report)

Dose (mg/kg)	Mating week	Number of females	Females mated successfully		Pregnant females	
			Number	%	Number	%
0	1	40	36	90.0	31	86.1
65		40	35	87.5	32	91.4
195		40	34	85.0	31	91.2
0	2	40	37	92.5	32	86.5
65		39	34*	87.2	28	82.4
195		40	33	82.5	31	93.9
0	3	40	37	92.5	34	91.9
65		40	32	80.0	28	87.5
195		40	31	77.5	27	87.1
0	4	40	38	95.0	32	84.2
65		40	36	90.0	34	94.4
195		38	29	76.3	27	93.1
0	5	39	35	89.7	34	97.1
65		40	36	90.0	34	94.4
195		34	29	85.3	26	89.7
0	6	40	34	85.0	28	82.4
65		40	29	72.5	25	86.2
195		36	27	75.0	24	88.9
0	7	40	34*	85.0	31	91.2
65		38	33	86.8	29	87.9
195		38	31	81.6	30	96.8
0	8	40	37	92.5	34	91.9
65		38	34	89.5	27	79.4
195		37	29	78.4	26	89.7

*Includes one female with deciduomata only.

4. REPORTED RESULTS (continued)

Table 2

Litter results (from summary tables in original report)

Dose (mg/kg)	Mating week	Implantations/dam Mean	S. D.	Mean number of embryos per dam* Live	Dead
0	1	11.45	1.98	10.58	0.87
65		10.88	1.74	10.03	0.84
195		11.61	1.94	10.74	0.87
0	2	10.47	3.30	9.53	0.94
65		10.18	2.50	9.21	0.96
195		10.84	2.85	9.87	0.97
0	3	9.59	3.51	8.97	0.62
65		10.04	3.81	8.54	0.50
195		10.59	2.76	9.81	0.78
0	4	10.09	3.25	9.09	1.00
65		10.62	3.69	9.56	1.06
195		10.26	2.68	9.15	1.11
0	5	10.62	3.24	9.82	0.79
65		10.32	2.47	9.37	0.89
195		9.96	2.13	9.31	0.65
0	6	9.43	2.89	8.64	0.79
65		10.52	2.60	9.60	0.92
195		9.38	2.60	8.21	1.17
0	7	10.42	2.96	9.71	0.71
65		10.41	2.85	9.59	0.83
195		10.70	2.02	9.97	0.73
0	8	10.62	3.43	9.62	1.00
65		10.26	3.02	9.52	0.74
195		11.04	3.00	10.27	0.77

*Calculated independently by dividing the total reported number of live or dead embryos by the number of reported pregnancies in each group.

5. DISCUSSION

There were no signs of toxicity observed at 65 or 195 mg/kg. The highest dose administered in this study is approximately 25% of an acute oral LD_{50} reported elsewhere for both sexes of mice (788 mg/kg; Sassche and Barthe, 1976). In addition, investigators conducting the acute study indicated that there were

5. DISCUSSION (continued)

signs of toxicity at the lowest level (317 mg/kg). Those signs included sedation, dyspnea, exophthalmus, curved or ventral position, and ruffled fur. These signs were exhibited by treated mice 2 hours after dosing, and they persisted in surviving animals for 7 to 12 days. No incidence data were presented in the report on the acute study to determine the extent of the toxicity observed, and that study can not be used to support the dose selection for the dominant lethal study discussed herein.

In addition to these limitations, no positive control substance was evaluated along with the test substance.

In this assay, the responsiveness of the mice under test conditions was not demonstrated, and a clear rationale for dose selection is not provided. Given these limitations, the assay only suggests that metalaxyl may not be mutagenic after a single oral dose as high as 195 mg/kg.

6. REFERENCE

Sassche, K.; Bathe, R. (1976) Acute Oral LD_{50} in the Mouse of Technical CGA 48 988. Experiment No. Siss 5 388. 'Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-M)

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §81-1 Acute Oral Toxicity

MRID: Unassigned

Sassche, K.; Bathe, R. (1976) Acute Oral LD_{50} in the Mouse of Technical CGA 48 988. Experiment No. Siss 5 388. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-M)

REVIEW RESULTS:

VALID X INVALID _____ INCOMPLETE _____

GUIDELINE: SATISFIED X PARTIALLY SATISFIED __ NOT SATISFIED __

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/13/86

1. DISCUSSION AND CONCLUSIONS: The results of the study indicated that the acute oral LD_{50} in male and female mice is 788 mg/kg suggesting that metalaxyl should be classified into Toxicity Category III.

   Core classification: Minimum

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch no. P. 3; purity unspecified)

3. MATERIALS AND METHODS

Test species: Male and female Tif MAG (SPF) strain mice were used. Their body weights ranged from 20 to 30 g.

Experimental procedure: Groups of 5 male and 5 female mice was given a single oral doses of 317, 464, 600, 1000, or 2150 mg test substance per kg body weight. The test substance was suspended in 2% aqueous carboxymethyl cellulose and administered by gavage. The mice were fasted overnight before treatment, and they were observed for mortality and appearance of toxicological and pharmacological signs at frequent but unspecified intervals during the 14 days that followed dosing. Surviving animals were sacrificed at the end of the observation period and necropsied. Postmortem examinations were limited to gross observations.

The LD_{50} and 95% confidence limits were calculated by probit analysis.

4. REPORTED RESULTS

None of the five females given the 317 or 600 mg/kg doses died during the experiment, and all of those given the 2150 mg/kg dose died within the first hour after treatment. One female given the 464 mg/kg dose and 4 females in the 1000 mg/kg dose group died within 24 hours after treatment. No male mice in the 317 and 464 mg/kg dosed groups died, but 4 of 5 in the 600 mg/kg group and 2 of 5 in the 1000 mg/kg dose group died within 24 hours of treatment. All five males in the 2150 mg/kg dose group died during the first hour following dosing.

Toxic signs noted by the investigators included sedation, dyspnea, exophthalmus, curved or ventral position, and ruffled fur. These signs were exhibited by all treated mice 2 hours after dosing. The authors also stated that tonic-clonic muscle spasms were observed in animals at the three highest doses, and sedation the sedation was described as more accentuated with increasing dose. Surviving animals were reported to recover within 7 to 12 days after treatment. Necropsy of animals dying during the observation period or sacrificed at the end of the experiment revealed no compound related gross effects on organs according to the report.

The reported LD_{50} for both sexes was calculated to be 788 mg/kg with 95% confidence limits of 626 to 991 mg/kg.

005372

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Puri, E.; Müller, D. (1982) Autoradiographic DNA Repair Test on Human Fibroblasts with CGA 48 988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-G)

REVIEW RESULTS:

VALID X INVALID _____ INCOMPLETE _____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/13/86

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1. DISCUSSION AND CONCLUSION: There was adequate information presented in the report to support the conclusion of the investigators. Under the conditions of the experiment, metalaxyl did not induce unscheduled DNA synthesis in human fibroblast cells at concentrations of 0 to 500 ug/ml.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch EN 32 212; 94.1% purity)

   Reference mutagens: 4-Nitroquinoline-N-oxide (4NQO) was used as the positive control substance.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: Human fibroblasts (CRL 1121) were used.

Cell culture conditions: Cultures were grown in Dulbecco's Minimum Essential Medium supplemented with 10% fetal bovine serum and antibiotics (unspecified). They were incubated in an atmosphere described as humidified and containing 10% CO_2. Cultures were grown in Petri dishes with a series of compartments containing cover slips. These compartments were seeded with 1 ml of medium having a cell density of 3 X 10^4/ml, and the seeded cultures were incubated overnight before the assays were begun.

Experimental procedures---Preliminary toxicity assay: Concentrations of 31.25, 62.5, 125, 250, 500, 1000, and 2000 ug/ml were evaluated for their toxicity to the fibroblasts. Ten ul of the test substance in DMSO was added to each compartment to attain these concentrations, and the treated cultures were incubated for five hours. At the end of that period, the medium was removed from the cultures, and the cells were washed with BSS. They were then stained with Trypan-blue solution for five minutes, and fixed. One-hundred cells were counted, and the percentage of unstained cells was noted. The criterion for selection of doses for the main study was that 25% of the cells examined should be viable (unstained).

Experimental procedures---UDS assay: In this portion of the assay, procedures were similar to those for the toxicity assay. The four concentrations of the test substance used were 4, 20, 100, and 500 ug/ml, and 0.5 uM 4NQO, 10 ug/ml DMSO, and untreated cells were tested as the positive, vehicle, and negative control groups, respectively. After adding the test solutions, 4 ul (2 mCi) of ^{3}H-thymidine was added, and the cultures were incubated for 5 hours. At the end of the incubation period, the cells were washed two times with BSS and fixed with ethanol/acetic acid (3:1). The report stated that the cover-slips were mounted on microscope slides and prepared for autoradiography. After a 6 day exposure, the slides were stained with hematoxylin-eosin.

Observations: The report described the observations made as follows:

> The background in the autoradiographs was determined in cell-free areas and found to be neglibly low. From each of the treatment groups and from the positive and the negative controls, 200 nuclei

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3. MATERIALS AND METHODS (contintued)

in altogether four slides (50 cells/slide) were scored, the number of silver grains were counted, and the mean values (5/treatment) calculated.

Cells which were in the DNA-synthesis phase showed more than 120 silver grains/nucleus. These were excluded from the determiation of silver grain/nucleus count.

The authors stated that counts from treated groups at least three times greater than those in the negative control group were considered indicative of a positive response.

4. REPORTED RESULTS

According to the report, there was 100% viability in cells exposed to 31.25 to 500 ug/ml. Viability at the 1000 ug/ml concentration was 20%, and that at the 2000 ug/ml concentration was 0. Based on these results, the authors stated that the highest concentration chosen for the main study was 500 ug/ml.

The mean number of silver grains (with standard deviation) per nuclei and the background counts included in the report are summarized as follows:

Test group/ concentration (ug/ml)	Silver grains per nucleus	Silver grains per nucleus equivalent area
Negative control	1.00 (1.01)	0.10
Vehicle control	0.90 (1.06)	0.10
CGA 48 988		
4	0.32 (0.55)	0.00
20	0.62 (0.91)	0.05
100	0.92 (1.07)	0.15
500	0.80 (1.00)	0.05
Positive control	21.6 (7.01)	0.10

005372

DATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Puri, E.; Müller, D. (1982) Autoradiographic DNA Repair Test on Rat Hepatocytes with CGA 48 988. (Unpublished study received Nov 29, 1985 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:260496-H)

REVIEW RESULTS:

VALID X INVALID _____ INCOMPLETE _____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane C Harris DATE: 8/13/86

71

1

1. DISCUSSION AND CONCLUSION: There was adequate information presented in the report to support the conclusion of the investigators. Under the conditions of the experiment, metalaxyl did not induce unscheduled DNA synthesis in rat primary hepatocytes at concentrations of 0 to 2000 ug/ml.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch EN 32 212; 94.1% purity)

   Reference mutagens: 4-Nitroquinoline-N-oxide (4NQO) and dimethylnitrosamine (DMN) were used as positive control substances.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: Liver hepatocytes were freshly isolated from an adult male Tif: RAI f (SPF) strain rat. The liver was perfused in situ with enzymbe solution E (0.05% collagenase, 0.1% hyaluronidase, and 0.1% trypsin in calcium-free Hanks' solution). Then the liver ewa removed and rinsed with ice-cold calcium-free Hanks' solution. It was again perfused with trypsin solution (0.1%) and sliced thinly. Slices were further treated with enzyme solution E for 35 minutes at 37° C under oxygen. After 35 minutes of incubation, a calcium chloride solution was added, and the tissue was incubated for another 20 minutes. The solution was then filtered through a nylon stocking, and the filtrate was centrifuged. The sediment was resuspended in Williams' medium E. The cells were then analyzed for viability and used in the experiment.

Cell culture conditions: Cells were grown in Williams' Medium E supplemented with 10% fetal bovine serum and antibiotics (unspecified). They were incubated in an atmosphere described as humidified and containing 5% CO_2. Cultures were grown in Petri dishes with a series of compartments containing cover-slips. These compartments were seeded with 2 ml of medium having a cell density of 10^5/ml, and the seeded cultures were incubated for 1.5 to 2 hours to allow the cells to attach to the cover-slips. After the attachment period, the cells were washed and a fresh 1 ml aliquot of Williams' Medium E was added. These cultures were then incubated overnight before they were used in the experiment.

Experimental procedures---Preliminary toxicity assay: Concentrations of 31.25, 62.5, 125, 250, 500, 1000, and 2000 ug/ml were evaluated for their toxicity to the hepatocytes. Ten ul of the test substance in DMSO was added to each compartment to attain these concentrations, and the treated cultures were incubated for five hours. At the end of that period, the medium was rmoved from the cultures, and the cells were washed with BSS. They were then stained with Trypan-blue solution for five minutes, and fixed. One-hundred cells were counted, and the percentage of unstained cells was noted. The criterion for selection of doses for the main study was that 25% of the cells examined should be viable (unstained).

Experimental procedures---UDS assay: In this portion of the assay, procedures were similar to those for the toxicity assay. The four concentrations of the test substance used were 16, 80, 400, and 2000 ug/ml, and 2 uM 4NQO, 100 mM DMN, 10 ug/ml DMSO, and untreated cells were tested as the positive, vehicle,

3. MATERIALS AND METHODS (continued)

and negative control groups, respectively. After adding the test solutions, 4 ul (4 uCi) of ^{3}H-thymidine was added, and the cultures were incubated for 5 hours. At the end of the incubation period, the cells were washed two times with BSS and fixed with ethanol/acetic acid (3:1). The report stated that the cover-slips were mounted on microscope slides and prepared for autoradiography. After a 6 day exposure, the slides were stained with hematoxylin-eosin.

Observations: The report described the observations made as follows:

> The background in the autoradiographs was determined in cell-free areas and found to be neglibly low. From each of the treatment groups and from the positive and the negative controls, 150 nuclei in altogether three slides (50 cells/slide) were scored, the number of silver grains were counted, and the mean values (5/treatment) calculated.
>
> Cells which were in the DNA-synthesis phase showed more than 120 silver grains/nucleus...These were excluded from the determiation of silver grain/nucleus count.

The authors stated that counts from treated groups at least three times greater than those in the negative control group were considered indicative of a positive response.

4. REPORTED RESULTS

According to the report, there was 60% viability in cells exposed to DMSO without the test substance. Viability was 71, 72, 81, 58, 54, 34, and 35% at the 31.25, 62.5, 125, 250, 500, 1000, and 2000 ug/ml concentrations, respectively. Based on these results, the authors stated that the highest concentration chosen for the main study was 2000 ug/ml.

The mean number of silver grains (with standard deviation) per nuclei are shown in Table 1 below. Table 2 shows grain counts in the cytoplasm and cell-free areas of the cultures examined, and Table 3 provides the historical data included in the report.

4. REPORTED RESULTS (continued)

Table 1

Mean silver grains/nucleus (and standard deviation) as reported for hepatocytes exposed to metalaxyl

Test group/ concentration (ug/ml)	Silver grains per nucleus*	Silver grains per nucleus (automated count)
Negative control	3.71 (5.02)	1.94 (2.57)
Vehicle control	3.23 (2.87)	2.53 (3.58)
CGA 48 988		
16	4.69 (5.38)	2.24 (3.11)
80	4.36 (7.20)	2.34 (2.14)
400	4.04 (3.96)	2.45 (2.29)
2000	3.98 (3.70)	1.02 (1.49)
4-NQO (2 uM)	48.0 (20.33)	33.5 (15.70)
DMN (100 mM)	16.6 (8.47)	15.7 (7.71)

*Made by microscopic examination.

Table 2

Mean silver grains/nucleus (and standard deviation) as reported for hepatocytes exposed to metalaxyl

Test group/ concentration (ug/ml)	Cytoplasmic silver grains per nucleus- (automated count)	Cell-free silver grains per nucleus- (automated count)
Negative control	1.28	0.33
Vehicle control	1.44	0.13
CGA 48 988		
16	0.93	0.33
80	1.11	0.33
400	1.14	0.20
2000	0.47	0.20
4-NQO (2 uM)	2.51	0.33
DMN (100 mM)	2.80	0.33

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4. REPORTED RESULTS (continued)

Table 3

Historical control data (grains/nucleus)

Experiment	Medium control	Vehicle control	DMN
1	3.06	3.41	24.3
2	3.62	4.67	30.0
3	4.22	--	37.5
4	4.40	4.99	30.9
5	4.14	4.60	33.7
6	5.03	5.51	39.4
7	7.12	7.79	41.1
8	5.31	4.88	53.2
9	3.47	4.08	27.0
10	5.53	5.76	52.5

005372

ATA EVALUATION RECORD

Metalaxyl (Tox. Chem. No. 375AA)

Guideline §84-2 Mutagenicity

MRID: Unassigned

Puri, E.; Müller, D. (1985) Autoradiographic DNA Repair Test on Rat Hepatocytes with CGA 48 988. Experiment No. 851004. (Unpublished study received June 2, 1986 under 100-601; prepared by Experimental Pathology Laboratories, CIBA-GEIGY Limited, Basle, Switzerland, submitted by CIBA-GEIGY Corp., Greensboro, NC; CDL:263117-A)

REVIEW RESULTS:

VALID X INVALID _____ INCOMPLETE _____

GUIDELINE: SATISFIED ___ PARTIALLY SATISFIED X NOT SATISFIED ___

DIRECT RVW TIME = START DATE: END DATE:

REVIEWED BY: Roger Gardner

TITLE: Toxicologist

ORG: TOX/Section 6

LOC/TEL: 557-7403

SIGNATURE: Roger Gardner DATE: 8-11-86

APPROVED BY: Jane Harris, Ph. D.

TITLE: Section Head

ORG: TOX/Section 6

LOC/TEL:

SIGNATURE: Jane E Harris DATE: 8/13/86

76

1. DISCUSSION AND CONCLUSION: There was adequate information presented in the report to support the conclusion of the investigators. Under the conditions of the experiment, metalaxyl did not induce unscheduled DNA synthesis in rat primary hepatocytes at concentrations of 0 to 2000 ug/ml.

   Core classification: Acceptable

2. TEST SUBSTANCE: Metalaxyl (CGA 48 988; Batch P. 503119; 95.7% purity)

   Reference mutagens: Dimethylnitrosamine (DMN 100 mM) was used as positive control substances.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for the test substance.

3. MATERIALS AND METHODS

Test species: Liver hepatocytes were freshly isolated from an adult male Tif: RAI f (SPF) strain rat. The procedure was described in the report as follows:

> ...the liver is perfused in situ through the protal vein for about eight minutes with the following medium: 121 mM NaCl, 6 mM KCl, 0.6 mM $MgSO_4$, 12 mM $NaCO_3$, 0.74 mM KH_2PO_4, 5 mM glucose. The medium is aerated with Carbogen (95% O_2, 5% CO_2), its temperature is 37° C, the pH about 7.4. After insertion of a canule in the thoracal part of the ven cava, the perfusion is continued for further 15-20 minutes by recirculation of the medium, which is supplemented with 0.05% collagenase and 2.5 mM $CaCl_2$.
>
> The liver is then carefully excised and placed into a dish containing calcium-free Hanks' solution (4° C). After opening the Glisson's capsule, the cells are dispersed by gently shaking of the liver in the solution.

After these cells were washed, they were suspended in Williams' medium E for use in the toxicity and UDS assays.

Cell culture conditions: Cells were grown in Williams' Medium E supplemented with 10% fetal bovine serum. They were incubated in an atmosphere described as humidified and containing 5% CO_2. Cultures were grown in Petri dishes with a series of compartments containing cover-slips. These compartments were seeded with 4 ml of medium having a cell density of 10^5/ml, and the seeded cultures were incubated for 1.5 to 2 hours to allow the cells to attach to the cover-slips. After the attachment period, the cells were washed and a fresh 2 ml aliquot of Williams' Medium E was added. These cultures were then incubated overnight before they were used in the experiment.

Experimental procedures---Preliminary toxicity assay: Concentrations of 0, 250, 500, 1000, 2000, 4000, 6000, and 8000 ug/ml were evaluated for their toxicity to the hepatocytes. Twenty ul of the test substance in DMSO was added to each compartment to attain these concentrations, and the treated cultures were incubated for five hours. At the end of that period, the medium was removed

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3. MATERIALS AND METHODS (continued)

from the cultures, and the cells were washed with BSS. They were then stained with Trypan-blue solution for five minutes, and fixed. One-hundred cells were counted, and the percentage of unstained cells was noted. The criterion for selection of doses for the main study was that 25% of the cells examined should be viable (unstained).

Experimental procedures---UDS assay: In this portion of the assay, procedures were similar to those for the toxicity assay. The four concentrations of the test substance used were 16, 80, 400, and 2000 ug/ml, and 100 mM DMN, 10 ug/ml DMSO, and untreated cells were tested as the positive, vehicle, and negative control groups, respectively. After adding the test solutions, 8 ul of ^{3}H-th - ymidine (25 Ci/mM) was added, and the cultures were incubated for 5 hours. At the end of the incubation period, the cells were washed two times with BSS and fixed with ethanol/acetic acid (3:1). The report stated that the cover-slips were mounted on microscope slides and prepared for autoradiography. After a 6 day exposure, the slides were stained with hematoxylin-eosin.

Observations: The report described the observations made as follows:

> The background in the autoradiographs was determined in cell-free areas and fo ıd to be negligibly low. From each of the treatment groups and from the positive and the negative controls, 150 nuclei in altogether three slides (50 cells/slide) were scored, the number of silver grains counted, and the mean values and standard deviations calculated...
>
> Cells which were in the DNA-synthesis phase showed more than 120 silver grains/nucleus...These were excluded from the determination of silver grain/nucleus count.

The authors stated that counts from treated groups at least two times greater than those in the negative control group and/or a statistically significant dose-related increase were considered indicative of a positive response.

4. REPORTED RESULTS

According to the report, there was 93% viability in cells exposed to DMSO without the test substance. Viability was 90, 94, 91, and 91% at th3 250, 500, 1000, and 2000 ug/ml concentrations, respectively. No viability was observed at the 4000, 6000, or 8000 ug/ml levels. Based on these results, the authors stated that the highest concentration chosen for the main study was 2000 ug/ml.

The mean number of silver grains (with standard deviation) per nuclei are shown in Table 1 below. Table 1 also shows grain counts in the cytoplasm and cell-free areas of the cultures examined. The historical control data for 26 other studies which were conducted during the same year as the experiment discussed herein indicated that negative control results ranged from 0.89 to 3.01 grains/nucleus, and the vehicle controls (DMSO) ranged from 0.94 to 4.23 grains/nucleus. The response to DMN during that time ranged from 11.9 to 22.8 grains/nucleus.

4. REPORTED RESULTS (continued)

Table 1

Mean silver grains/nucleus (and standard deviation), along with mean cytoplasmic, and background counts as reported for hepatocytes exposed to metalaxyl

Test group/ concentration (ug/ml)	Silver grains per nucleus (automated count)	Cytoplasmic silver grains per nucleus- (automated count)	Cell-free silver grains per nucleus- (automated count)
egative control	1.56 (1.37)	1.96	0.40
Vehicle control	2.87 (1.95)	2.55	0.47
CGA 48 988			
16	2.73 (1.90)	2.80	0.60
80	3.54 (2.25)	3.38	0.40
400	3.2[illegible] (2.16)	2.47	0.40
2000	1.9[illegible] (1.45)	1.26	0.13
DMN (100 mM)	34.7 (12 62)	5.52	0.67

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