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

1988
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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: MGK 264 (N-octyl bicycloheptene dicarboximide) - Submission of Studies Under Accession Nos. 403991-01, -02, -03, and -04
EPA ID No. 1021-88

TB Project No.: 8-0318
Caswell No.: 613

FROM: Irving Mauer, Ph.D. Irving Mauer 4-11-88
Toxicology Branch
Hazard Evaluation Division (TS-769C)

TO: Phil Hutton/Mendelsohn, PM Team 17
Insecticide-Rodenticide Branch
Registration Division (TS-767C)

THRU: Judith W. Hauswirth, Ph.D., Head Judith W. Hauswirth 4/12/88
Section VI, Toxicology Branch
Hazard Evaluation Division (TS-769C)

and

Theodore M. Farber, Ph.D. 4/19/88
Chief, Toxicology Branch
Hazard Evaluation Division (TS-769C)

Registrant: McLaughlin Gormley King Company
Minneapolis, MN

Request:

Under cover letter of October 12, 1987, the registrant submitted the following four mutagenicity studies for review and evaluation by Toxicology Branch (TB). All four were performed at Microbiological Associates (MBA), Inc., Bethesda, MD.

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1. Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test), Study No. MBA-T5205.501014, dated September 16, 1986 (EPA Accession No. 403991-01).

2. Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells with a Confirmatory Assay, Study No. MBA-T5205.337001, dated January 14, 1986 (EPA Accession No. 403991-02).

3. Unscheduled DNA Synthesis in Rat Primary Hepatocytes (UDS), Study No. MBA-T5205.380009, dated April 20, 1987 (EPA Accession No. 403991-03).

4. L5178Y TK +/- Mouse Lymphoma Mutagenesis Assay, Study No. MBA-T5205.701020, dated December 15, 1986 (EPA Accession No. 403991-04)).

TB Conclusions:

TB has reviewed and evaluated these studies, as summarized below (full DATA REVIEWS are attached).

	Study	Reported Results	TB Evaluation
1.	Ames	Negative for reversion w/without activation (MA) up to toxic levels (333 to 1000 ug, -MA; 3333 to 10,000 ug, +MA).	ACCEPTABLE
2.	CHO	Negative for structural chromosome aberrations w/without mammalian activation up to purportedly toxic doses (0.04 to 0.07 uL/mL).	UNACCEPTABLE, due to major deficiencies in conduct of this study.
3.	UDS	Negative in a single assay at the HDT of 0.03 uL/mL.	UNACCEPTABLE, because HDT produced no toxicity.
4.	L5178Y	"Weakly" Positive at toxic doses in the absence of activation. Negative in the presence of activation.	ACCEPTABLE

Attachments

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TOXICOLOGY BRANCH: DATA REVIEW

Reviewed by: Irving Mauer, Ph.D.
Toxicology Branch
Hazard Evaluation Division

TB Project: 8-0318

Date: 4/11/88

Through: Judith W. Hauswirth, Ph.D., Head
Section VI, Toxicology Branch
Hazard Evaluation Division

Judith W Hauswirth 4/12/88

Chemical: MGK 264 Insecticide Synergist (N-octyl bicycloheptene dicarboximide)

Caswell: 613
EPA Chem: 057001

Study Type: Mutagenicity - Reverse Gene Mutation in Bacteria (Salmonella/Microsome-Ames)

Citation: Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test) with a Confirmatory Assay.

Accession No.: 403991-01

MRID: N/A

Sponsor: McLaughlin Gormley King Company (MGK)
Minneapolis, MN

Testing Lab: Microbiological Associates (MBA), Inc.
Bethesda, MD

Study No.: T5205-501014

Study Date: September 16, 1986

TB Conclusions/Evaluation:

ACCEPTABLE, demonstrating negative results (no increase in revertents) in any of the Ames battery of Salmonella tester strains, with or without mammalian metabolic activation (Aroclor 1254-stimulated rat liver S9 microsomes), up to toxic dose levels (333 to 1000 ug/plate without activation; 3333 to 10,000 ug/plate with S9).

DETAILED REVIEW

Test Article: MGK 264 Insecticide Synergist (N-octyl bicycloheptenedicarboximide), Lot No. 3843 (percent purity not stated), a clear liquid diluted with acetone for testing.

Procedures:

These assays were conducted with experimental materials and media/reagents by methods described in published articles by Bruce Ames, using tester strains received directly from his laboratory at UCLA-Berkeley. Just prior to use, all tester strains were checked for the integrity of their genetic markers by conventional procedures.

A preliminary dose range-finding study was carried out by exposing single plates of TA100 cells overnight to 10 dose levels of test article from 10 to 10,000 ug/plate, in the absence and presence of an exogenous mammalian activation system consisting of the S9 homogenate of rat liver microsomal enzymes stimulated by Aroclor 1254 plus NADP-based generating cofactors (= S9 Mix). The maximum tolerated dose is sought by three criteria: The condition of the background bacterial lawn (reduced to absent), the appearance of compound precipitation (slight to heavy), and reduction in revertents at increasing toxic doses.

The test article was assayed in the battery of five Salmonella tester strains for induced reversion in triplicate plates at five dose levels in three experiments (B1, B2, and B3), both in the presence and absence of S9 mix as mammalian metabolic activation. Appropriate vehicle and positive controls were run concurrently, also with and without activation. All plates were incubated for 48 hours at 37 °C, following which revertent colonies were counted. Colonies within given dilution series were counted either entirely by automated counter, or entirely by hand. Where precipitation was present of sufficient severity as to interfere with automated counting, colonies were counted manually. The condition of the background lawn was assessed for evidence of test article toxicity, which was scored relative to vehicle controls, by a numerical code system (1 to 6) describing increasing severity (1, normal; 2, slightly reduced; 3, moderately reduced; 4, extremely reduced; 5, absent; 6, obscured by precipitate).

To be considered a valid assay, the author established the following criteria:

1. The tester strains must maintain integrity of genetic markers, especially those making them more sensitive, such as: (i) the deep rough wall mutation in all strains (rfa), demonstrated by sensitivity to crystal violet; and (ii) the pKM101 plasmid (R factor) in strains TA98 and TA100, demonstrated by resistance to ampicillin.

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2. Acceptable ranges of background (spontaneous) revertents, as determined by expert panels in publications (e.g., de Serres and Shelby, Science 203:583-568, 1979):

TA98	10-50
TA100	80-240
TA1535	5-45
TA1537	3-21
TA1538	5-35

3. The minimum number of bacteria per plate (titer) per culture must be 0.3×10^9.

4. Positive control values must show at least threefold increases over mean vehicle control for the respective strain.

5. At least three dose levels must be nontoxic in order to satisfactorily evaluate assay data.

For a test article to be considered positive, the authors suggest that at least a doubling in mean revertents per plate in at least one strain must be found, accompanied by a dose response at increasing test concentrations. If the observed dose-responsive increase in TA1537 or TA1538 revertents per plate is less than threefold, the response must be reproducible.

Results:

In the range-finding study, toxic effects of test article treatment were observed in nonactivated and S9-activated TA100 cells at dose levels of 333 ug/plate and above, and 3333 ug/plate and above, respectively. At these doses, the first appearance of reduced background bacterial lawn (Code 2-3), test article precipitation and a decrease in revertents (47% and 59%) were observed (Report Table 1, attached to this review). The results of this preliminary test indicated to the authors that the "appropriate maximum dose" for plating in the mutagenicity assays was 333 ug/nonactivated plate and 3333 ug/activated.

Individual data on revertents and background lawn from triplicate plates per test article concentration and controls are displayed for each tester strain and activation status of the initial assay (Experiment B1) as Report Tables 2 to 9, of the repeat assay (Experiment B2) as Report Table 10 to 19, and of the confirmatory assay (Experiment B3) as Report Tables 20 to 29. Results from the individual data tabulations are summarized for each experiment as Report Tables 30 (B1), 31 (B2) and 32 (B3), which are attached to this review.

The initial assay (B1) employed test article doses of 3.3, 10, 33, 100, and 333 ug/plate for the nonactivated series, and 33, 100, 333, 1000, and 3333 ug/plate under activation conditions. These schedules were derived from the maximum dose considered "appropriate" in the preliminary dose-selection test The results of this assay were considered unsatisfactory because:

1. The number of vehicle revertents per plate in the TA1537 assay was outside the acceptable background range, and hence no TA1537 plates were evaluated.

2. There was no apparent toxicity in testing strains TA98 (Report Tables 2 and 3) and TA1538 (Report Tables 8 and 9) under both nonactivation and activation conditions, and in strain TA1535 in the presence of metabolic activation (Report Table 7).

3. TA100 with/without activation (Report Tables 4 and 5) and TA1535 in the absence of S9 (Report Table 6) manifested less toxicity than anticipated from the range-finding study (Table 1).

Therefore, a repeat study (B2) was performed with all five tester strains exposed to five dose levels under both nonactivation and activation conditions, but the highest doses tested were increased to 1000 and 10,000 ug/plate, respectively. The other four doses tested were: 10, 33, 100, and 333 ug/plate in the absence of mammalian activation; and 100, 333, 1000, and 3333 ug/plate in the presence of the activation system. In reporting the results of Experiment B2, the authors recorded that strains TA98 and TA1538 without activation (Report Tables 10 and 18) once again showed no apparent toxicity. Therefore, in a third, confirmatory assay (B3), two additional higher doses were tested on TA98 and TA1538 cultures without activation, namely 3333 and 10,000 ug/plate. Evidence of toxicity (reduced background lawn and revertent count) and precipitation were finally achieved, as indicated in Report Tables 20 (TA98) and 28 (TA1538), and all other experimental parameters were considered as acceptable by the authors.

In no tester strains with or without metabolic activation in the three experiments was there any increase in revertent colony counts over solvent control. Hence the authors concluded that MGK 264 Insecticide Synergist was negative in Ames testing under conditions established for this study.

TB Evaluation:

Both a protocol, as well as a Quality Assurance Statement (dated and signed September 17, 1986) were included in the report of this study.

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The procedures employed in this study were adequate and the study as a whole well-conducted such that the results generated may be considered valid. The test article appears to have no mutagenic effect in this test system. The study is judged ACCEPTABLE.

Attachments

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SECTION D

DOWN RANGE-FINDING

STUDY RESULTS

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MGK Scientific Review - Toxicology Review Number 006674

Pages 9 through 14 are not included. The pages contain detailed test methods and results submitted by the pesticide registrant.

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TOXICOLOGY BRANCH: DATA REVIEW

Reviewed by: Irving Mauer, Ph.D.
Toxicology Branch
Hazard Evaluation Division

TB Project: 8-0318

Date: 4-11-88

Through: Judith W. Hauswirth, Ph.D., Head
Section VI, Toxicology Branch
Hazard Evaluation Division

Judith W Hauswirth
4/12/88

Chemical: MGK 264 Insecticide Synergist (N-octyl bicycloheptene dicarboximide)

Caswell: 613
EPA Chem: 057001

Study Type: Mutagenicity - chromosome aberrations in vitro (CHO)

Citation: Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells with a Confirmatory Assay

Accession No.: 403991-02

MRID: N/A

Sponsor: McLaughlin Gormley King (MGK)
Minneapolis, MN

Testing Lab: Microbiological Associates (MBA)
Bethesda, MD

Study No.: MBA-T5205.337001

Study Date: January 14, 1987

TB Conclusions/Evaluation:

UNACCEPTABLE. A number of major and minor procedural deficiencies compromise the reported negative results generated in this study.

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DETAILED REVIEW

Test Material: MGK-264 Insecticide Synergist (N-octyl bicycloheptene dicarboximide), Lot No. 3843 (% purity no stated), a clear liquid, diluted in acetone for testing.

Test Organism: Chinese hamster ovary (CHO-K_1) cells, from American Type Culture Collection, Rockville, MD, CCL No. 61.

Control Articles: Triethylenemelamine (TEM) from Polysciences, Inc., Warrenton, PA, dissolved in distilled water. Cyclophosphamide (CP), from Sigma Chemical, St. Louis, MO, dissolved in distilled water.

Procedures:

In a preliminary toxicity test to select dose levels for the main assays, cells were exposed to solvent alone (acetone), or nine concentrations of test article ranging from 0.0001 to 1.0 uL/mL, for 6 hours in the absence of activation and for 2 hours in the presence of metabolic activation consisting of Aroclor 1254-stimulated rat liver microsomes (S9) plus generating NADP cofactors (S9 MIX). Two hours after initiation of treatment, 50 uL of 1 mM bromodeoxyuridine (BrdU) were added to each nonactivation culture, and incubation "continued as needed" (as stated by the author) to determine cell growth potential and cell cycle delay. After their 2-hour treatment, activated cultures were washed free of treatment medium, and fresh medium containing 0.01 mM BrdU was added for an additional 24-hour incubation.

Cells were harvested by trypsinization, counted to estimate relative cell growth (compared to solvent control), and prepared for sister-chromatid exchange differentiation by a modification of the published fluorescence plus Giemsa technique (FPG) of Perry and Wolf (1974). Metaphase preparations were scored for the percentages of first, second, and third divisions (M1, M2, and M3, respectively), which estimate the test article's effect on cell cycle kinetics ("delay").

[NB: The addition of the mitotic-arresting agent, Colcemid, 2 hours before harvest is not mentioned here (toxicity test), although it is included in the preparation of metaphase cells in both the protocol and the procedures of the main chromosome aberration assays.]

Two separate aberration assays were conducted both at the same test article concentrations selected from the toxicity test: 0.005, 0.01, 0.02, and 0.4 uL/mL (each dose in duplicate) for nonactivation cultures; 0.01, 0.02, 0.04, 0.06, and 0.08 uL/mL under activation conditions. In the nonactivation assay, cells

were exposed for 14 hours, the treatment medium removed and replaced by fresh medium containing 0.1 ug/mL Colcemid for the final 2 hours of incubation. In the S9-activated study, cells were exposed for only 2 hours, the treatment medium removed, replaced with fresh medium, and returned to the incubator for an additional 6 hours. Colcemid (0.1 ug/mL) was added for a further 2 hours.

Cells in metaphase from both activation and nonactivation cultures were collected by "mitotic shake-off," pelleted by centrifugation at low speed (800 rpm for 5 minutes), resuspended in 0.075 M KCl (to expand cells), fixed in Carnoy's Fluid, and stored overnight or longer in the fixative at 4 °C. Microscope preparations were made by having one to two drops of cell suspension fall onto the center of a moist glass slide and allowed to dry overnight. Dried slides were stained with 5% Giemsa, air dried and made permanent with mounting medium. Slides were coded with random numbers and scored without regard to treatment group.

Whenever possible, 100 metaphases from each dose level and/or treatment (50 per duplicate flask) were scored for structural chromosome aberrations (gaps, breaks, acentric fragments, deletions, dicentrics, rings, triradials, quadriradials, other complex rearrangements, pulverization, severe multiple damage with > 10 aberrations per cell) and numerical abnormalities (polyploidy, endoreduplication).

In each of the assays (initial and confirmatory), acetone and TEM (final concentration, 1 ug/mL) served, respectively, as solvent and positive controls in the absence of activation, while acetone plus the S9 mix and CP (100 ug/mL) were solvent and positive controls, respectively, under S9 activation conditions. In each assay, growth medium alone served as the untreated (background) control.

Although chromosome and chromatid gaps were counted, they were not included in the percentage of aberrations. Statistical analysis of the frequency of structural aberrations per cell was conducted with Student's t-test. To be considered valid, the authors proposed that the number of aberrations in the negative control be no greater than 6 percent. The positive control values must be statistically increased (by at least $p \leq 0.05$, t-test) over negative or solvent control.

Results:

In the preliminary toxicity test, some precipitation was observed at the two highest test article concentrations (0.3 and 1.0 uL/mL) in both nonactivation and activation cultures. The authors recorded severe inhibition of relative cell growth at these two concentrations as well as at the one immediately lower, 0.1 uL/mL, and no scorable metaphases could be located at these

three top doses (Report Tables 1 and 2, attached to this review). A moderate degree of inhibition was observed at the next lower dose, 0.03 uL/mL, but virtually none at the five lower doses; enough cells were available at these concentrations to estimate cell cycle kinetics (percentages of cells in M1, M2, M3).

Based upon the results of the toxicity test, the test concentrations selected for the mutagenicity assays were 0.005, 0.01, 0.02, and 0.04 uL/mL for nonactivation cultures, and 0.01, 0.02, 0.04, 0.06, and 0.08 uL/mL for S9 activation cultures. The test article was reported to produce a moderate level of cell cycle delay in the absence of S9.

[NB: However, no data to support this reported delay was included in the Final Report of this study.]

To overcome this delay in order to assure that all cells evaluated were in first-division metaphase (M1), the harvest of nonactivated cultures was established at 16 hours, in contrast to 10 hours for activated cultures.

In the initial assay without exogenous metabolic activation, the two highest doses, 0.02 and 0.04 uL/mL, were slightly to moderately toxic (by monolayer examination), and only 53 and 22 cells, respectively, suitable for scoring could be found (Table 3, attached). The untreated and positive controls also had low cell yields since, respectively, only 68 and 73 cells were scorable.

The percent of cells with structural chromosome aberrations in cultures treated at 0.02 uL/mL was higher than solvent control (15% vs. 2%), and the number of aberrations per cell increased significantly ($p < 0.05$) over acetone cultures (1.00 vs. 0.04). At no other test concentration was there a significant increased clastogenic effect. The positive control, TEM, produced 2.11 aberrations per cell, highly statistically significant over untreated control (0.00 aberrations/cell), and distributed among the 52 affected cells of the 73 cells that could be scored.

In the S9 activation portion of the initial assay (Table 4, attached), the three highest doses tested (0.04, 0.06, and 0.08 uL/mL) were found to be moderately to severely toxic, such that no scorable metaphases were located at 0.06 and 0.08 uL/mL; and only three at 0.04 uL/mL (too few to analyze). No increase in aberrations over control was recorded in the remaining two test article treated groups (0.01, 0.02 uL/mL), whereas CP induced 1.58 aberrations per cell ($p < 0.01$), in 24/100 affected cells.

The cell yield in the nonactivated confirmatory assay was much improved, since the full complement of scorable metaphases were located at all treatments (Table 5 attached). At harvest, the authors reported slight to moderate toxicity (by examination

of monolayers) at the two highest concentrations (0.02, 0.04 uL/mL), but recorded no significant increases in aberrations above that of the solvent control (0.06) at any test article dose. TEM produced 0.73 aberrations per cell, a highly significant increase over the untreated control (0.04) in the 37/100 affected cells analyzed.

The activated portion of the confirmatory assay had to be run twice, the authors report, because of "technical difficulties in slide preparation" (unspecified); only results of the second were presented (Table 6, attached). As in the initial assay, dose levels of 0.06 and 0.08 uL/mL test article were reportedly toxic at harvest examination of the cell monolayers, to the extent that no scorable metaphases could be found. No increases in aberrations over acetone control (0.02), however, were counted in the remaining test article groups (0.01, 0.08, and 0.02 structural aberrations per cell at, respectively, 0.01, 0.02, and 0.02 uL/mL). CP produced 0.61 aberrations per cell in 22/100 affected cells.

The author concluded that, since:

1. Both positive and negative controls fulfilled the criteria for a valid assay; and

2. The test article, MGK 264 Insecticide Synergist, did not reproducibly produce structural chromosome aberrations when tested with and without a mammalian metabolic activation system under the conditions of this assay plus a confirmatory study, therefore, it was considered to be negative in this test system.

TB Evaluation:

The test article has indeed been tested in CHO cells in independent (repeat) assays, with nominally negative results. Certain procedural inadequacies, however, create great suspicion in the validity of the data generated and their interpretation.

1. Most importantly, according to the cell cycle kinetic data in Tables 1 and 2, except for treatment at 0.03 uL/mL in nonactivated cultures, the majority of cells were in second-division metaphase (M2) at all other treatments. In the development of this test system, authorities have made it eminently clear that cells for this test must be analyzed in the first division following treatment (i.e., a majority of cells should be in M1) in order to capture the total amount of structural chromosome aberrations. A certain proportion of aberrations (mitoses which are so damaged as to be incapable of surviving to next cell division) is lost between M1 and M2. [The decision for harvest times failing to assure M1 cells

also contradicts the author's own assertion that harvest times be set so that all cells evaluated be in M1 (p. 11 of report).]

2. No data have been provided in the Report on the stated cell cycle delay in treated nonactivation cultures, which prompted extending harvest time.

3. As a corollary to our concern in 1. and 2., the author has provided no information on cell generation time of the CHO-K_1 cell line. It has been published that cell reduplication of various subclones of this line can vary between 8 and 14 hours.

4. In the toxicity test, the total incubation-plus-treatment durations with test article were not mentioned. As given in the Report (according to the methods section on page 9 of the Report), nonactivated cells were treated with test compound for 6 hours (with 4-hour exposure to BrdU running concurrently), and then it is stated: ". . . incubation was continued as required." On the other hand, activated cells were treated for 2 hours, following which treatment medium was replaced by fresh medium containing BrdU for 24 hours. Again, this last procedure would guarantee obtaining metaphases in M2 (sometimes in M3), with only a minority in M1.

5. It is not clear why the dose-selection for activated cultures included levels of 0.06 and 0.08 uL/mL, since these are fairly close to the 0.1 uL/mL dose in the toxicity test which produced severe cell growth inhibition and no scorable metaphases. And, as demonstrated in the two assays, both doses were toxic and resulted in no scorable metaphases (Tables 4 and 6).

6. No data (nor any mention) were presented in the Results section on the scoring for numerical aberrations (polyploidy and endoreduplication) proposed in the procedures section.

Therefore, until these deficiencies are satisfied by explanation and/or data, this study must be judged UNACCEPTABLE.

Attachments

MGK Scientific Review - Toxicology Review Number 006674

Pages 21 through 26 are not included. The pages contain detailed test methods and results submitted by the pesticide registrant.

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TOXICOLOGY BRANCH: DATA REVIEW

Reviewed by: Irving Mauer, Ph.D.
Toxicology Branch
Hazard Evaluation Division

TB Project: 8-0318

Date: 4-11-88

Through: Judith W. Hauswirth, Ph.D., Head
Section VI, Toxicology Branch
Hazard Evaluation Division

Judith W Hauswirth 4/12/88

Chemical: MGK 264 Insecticide Synergist (N-octyl bicycloheptene dicarboximide)

Caswell: 613
EPA Chem: 057001

Study Type: Mutagenicity - DNA Damage/Repair in Mammalian Cells in vitro (HPC/UDS)

Citation: Unscheduled DNA Synthesis in Rat Primary Hepatocytes

Accession No.: 403991-03

MRID: N/A

Sponsor: McLaughlin Gormley King (MGK)
Minneapolis, MN

Testing Lab: Microbiological Associates (MBA)
Bethesda, MD

Study No.: T5205.380009

Study Date: April 20, 1987

TB Conclusions/Evaluation:

UNACCEPTABLE. The reported negative result in a single assay is not supported because the highest dose was insufficient to produce any toxicity.

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DETAILED REVIEW

Test Article: MGK 264, Lot No. 3843 (% ai not stated), a clear liquid, diluted with acetone for testing.

Procedures:

Hepatocytes obtained from the livers of adult male rats* by the Williams method were established as primary cultures (HPC) in 35 mm dishes containing glass coverslips. A preliminary cytotoxicity test was conducted to find the appropriate high dose (MTD) as well as dose range for the main assay. For this test, replicate HPCs were exposed for 18 to 20 hours to 10 doses of MGK 264, ranging from 0.0003 to 10 uL/mL, and culture fluids free of cells (by centrifugation) assayed for lactic acid dehydrogenase (LDH) activity. According to the author, the measurement of LDH released by cells into the culture medium has been shown to be a "good measure of decreasing cell viability."

[However, no bibliographic reference(s) nor other documentation for this assertion were provided in the Final Report, nor indeed any comparison with the standard trypan blue dye exclusion method for cell viability, mentioned on page 22 of the protocol included as an APPENDIX (Volume 3 submission pp. 17-29).]

A second cytotoxicity test was performed prior to the main unscheduled DNA synthesis (UDS) assays at six MGK 264 doses, ranging from 0.000003 to 0.3 uL/mL. [This test, however was not described in the procedures section of the Final Report.]

According to the protocol, two UDS assays were to be conducted, an initial and confirmatory test, but the latter was deleted on request of the sponsor, MGK (Protocol Amendment, dated March 20, 1987; p. 29 APPENDIX). Based, however, on the results of the two preliminary toxicity tests, six replicate plates were set up per test and control treatment, and exposed to 10 test article concentrations ranging from 0.0001 to 0.3 uL/mL for 18 to 20 hours; concurrently, three test per dose and three control dishes received tritiated-thymidine (3H-TdR) at a final concentration of 10 uCi/mL. At the end of the exposure period, cells of these UDS coverslip cultures were washed free of treatment media, swelled in 1% sodium citrate, fixed in 3:1 (Carnoy's ethanol:acetic acid), and

*Both Fischer-344 and Sprague-Dawley are mentioned as being used in this study, but no rationale is given for using two strains, nor which strain was employed for which portions of the study, nor how many animals (one or more?) donated hepatocytes for this study.

allowed to dry. The coverslips were then mounted cell side out on standard (3 x 1 inch) microscope glass slides, coated with Kodak NTB photographic emulsion, dried for 1.5 hours, and stored at 4 °C in light-tight boxes for 10 days. The preparations were then developed with standard Kodak chemicals (D19, fixer) to visualize silver grains, stained in hemotoxylin-eosin-sodium acetate and permanently mounted.

The remaining three cultures per treatment were harvested as "parallel toxicity plates," by removal of a portion of the cell-free culture medium for LDH determination of relative survival and relative toxicities. Dimethylbenzanthracene (DMBA, 3 and 10 ug/mL) in dimethylsulfoxide (DMSO) served as the positive control for the UDS and parallel toxicity assays; DMSO was used as the solvent control for DMBA cultures.

All slides were coded and read "blind" on an Artek Colony Counter. Nuclear grains, representing unscheduled DNA synthesis (UDS) due to compound damage and repair, were counted in 25 cells on each of the three coverslips per treatment (75 cells in all), where possible, and net nuclear counts determined by subtracting from the nuclear count the average cytoplasmic count of three nuclear-sized areas adjacent to each nucleus. Cells in replicative ("scheduled") DNA synthesis, identified by nuclei completely blackened by grains, and nuclei exhibiting toxic effects (dark staining, disrupted membranes, irregular shape), were not counted.

Mean net nuclear counts were calculated and standard deviations (SD) determined for each treatment slide, as well as the grand mean and SD for each dose level and the percent of cells in repair (i.e., cells with five or more net nuclear grains). These grain counts and their SDs, as well as percent cell survival, were computed by a Lotus 1-2-3 program on an IBM PC computer.

According to the criteria described in the Report, a mean net nuclear grain count equal to five or more over the control at a particular dose level signified a positive result. The test article was also considered positive if it induced a dose-related response with a significant increase in mean net nuclear grains compared to control at at least one dose. Absent a dose response, a test article was considered positive if there were significant increases in mean net nuclear grain counts for at least two successive doses. However, if the test article was positive at only one dose level, with no dose-relation, it was considered "marginally positive." Finally, it was proposed that the test compound be considered negative if no significant increase in net nuclear grain counts was found at any dose.

Results:

Based on measuring the release of cellular LDH into the medium, the first toxicity test resulted in relative toxicities (RT) at the dose range chosen which were characterized by the

investigator as "biphasic." As depicted in Table 1 (attached to this review) no toxicity was found at doses up to 0.01 uL/mL, then RT increased steeply to 59 percent at the next higher concentration (0.03 uL/mL) and remained at moderately high levels up to 77 percent at the next three higher concentrations (0.1, 0.3, and 1.0 uL/mL), but then decreased to 49 percent at 3 uL/mL, and finally to 17 percent at the HDT, 10 uL/mL. The results of this test prompted the investigator to consider 0.3 uL/mL as the highest dose for the UDS assay.

In order to verify the results of the initial toxicity test, however, a second test was performed, but at six doses separated by a factor of 10, ranging from 0.000003 to 0.3 uL/mL (Table 2, attached). In contrast to the investigator's assertion in the text (Results and Discussion, page 11 of the Report) that the results of this second preliminary assay "gave results very similar to the first," Table 2 presents data on RT which indicate no toxicity by LDH measurements at doses up to 0.003 uL/mL, and an apparent dose-related increase in RT at the two highest doses tested (31 and 110 percent at 0.003 and 0.3 uL/mL, respectively).

A third cytotoxicity test was conducted concurrently with the main UDS assay* at ten closely spaced concentrations from 0.00001 to 0.3 uL/mL (Table 3, attached). No toxicity was recorded at doses up to 0.03 uL/mL (in contrast to positive results at this concentration recorded in two prior toxicity tests; see Tables 1 and 2).

The four higher doses produced essentially a flat dose-response. The cultures were examined "microscopically" [presumably to assess cell viability by trypan blue exclusion as given in the protocol, although this is not stated here], and "extreme" toxicity was found at 0.06 uL/mL and above, but only a "moderate" degree of toxicity at 0.03 uL/mL [the actual data, however, were not included in the study report]. Therefore, the highest dose evaluated for UDS was stated to be 0.03 uL/mL.

> [With reference to the LDH method for assessing toxicity, the investigator stated: "It has been our experience, when using LDH measurements to estimate the toxicity of a chemical to hepatocytes, that toxicity shifts often occur between experiments. Each preparation of hepatocytes is slightly different, therefore, toxicity changes within one-half log doses are not considered significant." ("Results and Discussion" page 11 of Final Report).]

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*Referred to in the Results and Discussion section of the Report as a "second UDS assay"; based on the Protocol Amendment, however, only one UDS assay was run.

The single assay for UDS at five test article concentrations ranging from 0.0003 to 0.03 uL/mL revealed cell survivals no different than the solvent (acetone) control at all doses tested, and no significant increase in mean net nuclear silver grain counts at any dose (Table 4, attached). In contrast, both 3 and 10 ug/mL DMBA produced highly significant increases in grain counts (with 100% of the cells examined having five or more net nuclear grains); cell survival relative to the solvent control (DMSO) was 80 percent.

The investigator concluded that the test article, MGK 264, did not cause a significant increase in UDS as measured by mean net nuclear silver grain counts in primary rat hepatocyte cultures, and therefore was considered negative under the conditions of this study.

TB Evaluation:

Both a protocol (APPENDIX pages 17 to 29), and a Quality Assurance statement (signed and dated May 29, 1987) attesting to periodic inspections of the study and data, were included in the Final Report.

A number of major problems and deficiencies in both procedures and reporting persuade this reviewer to conclude that the test article has been inadequately tested, and therefore little confidence can be placed in the data generated, or in the author's interpretation.

For example:

1. It is not clear why--because it is not stated--two strains of rat were apparently used. How many of each strain were used, in what portions of the study?

2. The use of LDH release from cells as a measure of viability (or toxicity) requires documentation, including comparisons with the standard trypan blue dye exclusion method. The LDH method did not appear to be consistent between tests in this study, despite the author's rationalization about discounting "toxicity shifts" between experiments ("Results and Discussion," page 11 of Final Report). In addition, the sequence of toxicity testing as written in the Report is quite unclear, and the necessity of having to run three cytotoxicity tests to select doses for a single main (UDS) assay only reinforces the suspicion that LDH measurement is not a reliable method for toxicity screening.

3. "Microscopic examination" of the cultures in the "parallel" cytotoxicity test (Table 3) is not explained. It could have meant determination of toxicity by trypan blue exclusion (as indicated in the Protocol), but this is not so stated.

4. Although such microscopic examination of the cultures in the parallel toxicity test (Table 3) indicated "extreme" toxicity at 0.06 uL/mL but only "moderate" at 0.03 uL/mL, no data were presented to define these qualitative terms. More important, however, is the fact that no toxicity at all was elicited in the main assay, even at a dose (0.03 uL/mL) that presumably should have shown some toxicity, one criterion of adequacy required by the Agency's Health Effects Testing Guidelines.

5. While not absolutely required, a confirmatory assay should be performed to support the results of the initial assay.

Since the compound has been inadequately tested and the study inadequately reported, the results obtained do not support the author's conclusion that MGK 264 was negative in this UDS assay. The study is judged UNACCEPTABLE.

Attachments

MGK Scientific Review - Toxicology Review Number 006674

Pages 33 through 36 are not included. The pages contain detailed test methods and results submitted by the pesticide registrant.

006674 -C4

TOXICOLOGY BRANCH: DATA REVIEW

Reviewed by: Irving Mauer, Ph.D. TB Project: 8-0318
Toxicology Branch
Hazard Evaluation Division Date: 04-11-88

Through: Judith W. Hauswirth, Ph.D., Head
Section VI, Toxicology Branch
Hazard Evaluation Division 4/12/88

Chemical: MGK 264 Insecticide Synergist (N-octyl bicycloheptene dicarboximide)

Caswell: 613
EPA Chem: 057001

Study Type: Mutagenicity - Forward Mutation in Mammalian Cells in vitro (L5178Y/TK)

Citation: L5178Y TK +/- Mouse Lymphoma Mutagenesis Assay

Accession No.: 403991-04

MRID: N/A

Sponsor: McLaughlin Gormley King (MGK)
Minneapolis, MN

Testing Lab: Microbiological Associates (MBA)
Bethesda, MD

Study No.: T5205.701020

Study Date: December 15, 1986

TB Conclusions/Evaluation:

ACCEPTABLE in demonstrating positive results in repeat assays of nonactivated cultures exposed to toxic concentrations of MGK 264, but negative results in the presence of S9 metabolic activation (i.e., a "weak" mutagen).

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DETAILED REVIEW

Test Article: MGK 264, Lot No. 3843 (% ai not stated), a clear liquid with a mild odor, diluted in acetone for testing.

Procedures:

In a preliminary dose-finding toxicity test, single tubes of L5178Y mouse lymphoma cells (clone 3.7.2C, obtained from D. Clive, Burroughs-Wellcome, RTP, NC) were exposed to six concentrations of test article (in multiples of 10) ranging from 0.001 to 100 uL/mL, in both the absence and the presence of exogenous metabolic activation provided by Aroclor 1254-induced rat liver microsomes (S9), plus the generating cofactors, NADP plus isocitric acid (S9 mix). After 4 hours' incubation, cells were centrifuged and washed free of treatment media, resuspended in fresh medium, gassed with 5% CO_2 in air, and reincubated at 37 °C for 24 to 48 hours, at which time cell population densities were determined with an electronic cell counter.

Based on the results of the toxicity test, activated and nonactivated duplicate cultures of L5178Y cells (cleansed of spontaneous TK-/- mutants by 24 hours' treatment with a stock combination of thymidine + hypoxanthine + methotrexate + glycine, THMG solution, followed by 72 incubation in THG, i.e., without methotrexate) were exposed for 4 hours to test article at concentrations ranging from that producing 100 percent toxicity plus 15 serial eighthlog dilutions, giving 16 dose levels decreasing hundredfold from highest to lowest.

Ethylmethanesulfonate (EMS, 1.0 and 0.5 uL/mL) and 7,12-dimethylbenz[α]anthrace (DMBA, 7.5 and 5.0 ug/mL) served as positive controls for the nonactivated and activated series, respectively. To minimize possible photoinactivation, the preparation and addition of test article was carried out under amber light, and incubation was in total darkness during the exposure period.

Following treatment, cultures were incubated for 2 days (expression period) with cell population adjustments made at 24 and 48 hours, then cloned in triplicate in a medium containing 0.23 percent granulated agar, plus triflurothymidine (TFT, 3 ug/mL) as selecting agent, which restricts the growth of nonmutant (TK +/-) cells. Another three plates were established for viable counts (VC) and contained only fresh medium (without TFT). Both TFT and VC plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 10 to 12 days (selection and growth period).

Following the selection and growth period, both TFT and VC plates were scored for total number of colonies per plate. The mutation frequency (MF) was determined as:

$$MF = \frac{\text{Average Number of Colonies in TFT}}{\text{Average Number of Colonies} \times 10^4 \text{ in VC}} \times 2$$

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The following criteria for assessing the genetic activity of a test article in this experimental test system were presented by the author (machine-copied from pages 15 and 16 of the Final Report):

> Positive - if there is a positive dose response and one or more of the three highest doses in the 10% or greater Total Growth range exhibit a mutant frequency which is two-fold greater than the background level. All data including that from cultures with less than 10% Total Growth will be used to establish the dose response relationship. The first assay and the confirmatory assay must both demonstrate a positive response to call a test article a (positive) mutagen.
>
> Equivocal - if there is no dose response but any one or more of the three highest doses with 10% or greater Total Growth exhibit a two-fold increase in mutant frequency over background, or if there is a dose response but no culture exhibits a two-fold increase in mutant frequency over background. If an assay produces a positive response and the confirmatory assay produces an equivocal or negative response, then the results for the test article will be classed as having produced an equivocal response.
>
> Negative - if there is no dose response in cultures with 10% or greater Total Growth and none of these test cultures exhibit a two-fold or greater increase in mutant frequency over background. Both assays must demonstrate a negative response for the test article to be classed as negative.

Results:

The results of the initial cytotoxicity test and the two mutagenicity assays (initial and confirmatory) have been summarized in 13 tabulations and 4 graphical illustrations (figures).

In the range-finding test, toxicity was complete (100%) at 0.1 uL/mL with and without activation, and moderate at the next lower dose, 0.01 uL/mL (Table 1, attached to this review).

[NB: In the absence of activation, there was also no growth at the two higher doses, 1 and 10 uL/mL, but an anomalous increased cell growth (169% of control) at

100 ul/ml, while in S9-activated cultures, 1.0 uL/mL was also lethal but apparent dose-responsive increases were recorded at 10 and 100 uL/mL, respectively, 13 percent and 71 percent of control values (Table 1). Neither of these anomalies were mentioned, nor explained by the author.]

Therefore, the dose levels chosen for the initial mutagenicity assay were stated to range from the lethal 0.1 uL/mL down to 0.0013 uL/mL for both activated and nonactivated test cultures (Tables 3 and 5, attached).

[NB: However, the highest dose apparently tested was 0.032 in nonactivated cultures (Table 3, attached), whereas 0.0042 uL/mL was the LDT in the activated series (Table 5, attached).]

After the expression period of 2 days, 9 nonactivated concentrations ranging from 0.018 to 0.0013 uL/mL and 10 S9-activated dose levels were selected for cloning (Tables 2 and 4, attached). These doses produced a range of relative suspension growths (RSG, relative to control) from 16 percent to near the control value in nonactivated cultures (Table 3), and RSGs ranging from 23 percent to 116 percent under S9 activation (Table 5). In addition to the tabulations, both the cloning and total compound toxicity data of the initial assay were presented graphically, as Figure 1 (for nonactivation) and Figure 2 (with S9 activation).

In the nonactivated series of this first mutagenicity assay, a mutant frequency (MF) twice the mean of solvent controls (0.6) was registered after cloning at the second highest concentration of MGK 264 tested, 0.013 uL/mL (1.2), which displayed a total growth (TG) of 11 percent (Table 2). None of the other nonactivated cultures cloned, whose TGs ranged from 17 percent to 102 percent, exhibited MFs significantly greater than the solvent value. All S9-activated cultures cloned, with TGs ranging from 22 percent to 160 percent, exhibited MFs comparable to the solvent control, 0.4 to 0.6 vs. 0.5, respectively (Table 4).

Nonactivated cultures treated with the mutagen EMS (0.5 and 1.0 uL/mL) as positive control responded with MFs of 5.4 and 24.4, 10 and 40 times the mean solvent MF (= 0.6), at a TG of 34 percent for the lower dose, but only 1 percent at 1.0 uL/mL (Table 6). In S9-activated cultures, the positive control, DMBA (5.0 and 7.5 ug/mL), produced MFs of 1.8 and 3.3, only 2.25 and 4.125 times the mean solvent MF (= 0.8), and at a TG of near background (92%) at the lower dose, and about 30 percent at 7.5 ug/mL (Table 7).

In the repeat ("confirmatory") assay, duplicate nonactivated cultures were stated to have been exposed to concentrations of test article ranging from 0.0002 to 0.02 uL/mL. [NB: Table 9, however, records the LDT as 0.004 uL/mL.] After the expression

period, 10 nonactivated cultures representing five doses ranging from 0.004 to 0.015 uL/mL were chosen for cloning. These concentrations produced a range of RSGs from 5 to 26 percent of control (Table 9). On the other hand, S9-activated cultures were stated to have been treated at test doses ranging from 0.008 to 0.08 uL/mL. [NB: Table 11, however, records the LDT as 0.018 uL/mL.] Ten S9-activated cultures representing five doses (ranging from 0.018 to 0.059 uL/mL) were cloned, and produced RSGs ranging from 10 to 89 percent (Table 11).

MFs at least twice the control were recorded in six cloned nonactivated cultures; at the HDT, 0.015, as well as at 0.012, 0.009, 0.007, and the LDT, 0.004 uL/mL (Table 8). The increases ranged from twofold to 3.6-fold the mean solvent MF of 0.5, and represented TGs of 3 to 19 percent (the entire range of cell growth for this assay). The remaining four nonactivated cultures that were cloned had MFs no greater than the solvent value; their total growth ranged from 11 to 19 percent. The author maintained that no clear dose-response was evident at these purportedly "positive" concentrations.

In the activated series of the confirmatory assay, none of the cloned cultures manifested MFs "significantly" increased over the mean solvent MF, i.e., twofold or more (Table 10). The TG of these cultures ranged from 12 to 111 percent of control.

In addition to the data summaries of the second assay provided as Tables 8 to 11, the cloning and total compound toxicity data for the test article were prepared graphically, as Figure 3 (nonactivation) and Figure 4 (with S9 activation).

Nonactivated cultures treated with the same two dose levels of EMS (0.5, 1.0 uL/mL) responded after cloning with MFs 10 and 32 times the solvent value, at TGs of 33 and 5 percent. In the activation series, 5 and 7.5 ug/mL DMBA produced 5 and 11 times the solvent control value, at TGs of 57 and 9 percent, respectively (Table 13).

From the results of these repeat assays of MGK 264, the investigators concluded that:

1. The test article was negative (i.e., no increased MF, twofold or greater) in the presence of metabolic activation, when tested up to doses producing moderate toxicity (ca. 20% TG).

2. In the absence of activation, one culture that was cloned in the first assay and six in the confirmatory assay [not "three," as stated in the Report and Conclusion section] exhibited MFs twice or more the solvent values, at levels of toxicity ranging from moderately severe (TG = 11 to 19%), to very severe (TG = 3 to 8%).

3. No clear-cut dose-response was evident in these "positive" nonactivated cultures and no reproducibility in "mutagenic" response within concentrations. Further, the response for individual cultures was found only at highly "toxic" levels, i.e., at TGs less than 20 percent.

4. Although the results meet the (author's) criteria for an "equivocal," the author considered them unlikely to represent "significant biological responses," because they occurred at such toxic levels. These "responses" were considered more likely to represent cell toxicity (possibly associated with epigenetic phenomena) rather than a "true mutagenic response."

TB Evaluation:

Both a protocol, as well as a Quality Assurance Statement (signed December 15, 1986) attesting to periodic inspections of the procedures and data, were included in the Final Report.

The design, conduct, and reporting of this study are adequate, and therefore the data generated may be considered valid. While the test article produced "positive" results (a doubling or greater in MF compared to solvent value), in nonactivated cultures of both assays these occurred at toxic levels (TG < 20%). In contrast, consistent negative responses were found in MGK 264-treated S9-activated cultures at levels of total cell growth generally greater than 20 percent. We agree with the author that the positive responses in cultures treated in the absence of metabolic activation were not dose related nor reproducible within a single dose, and thus meet the author's criteria for an equivocal response.

Although there are a number of minor procedural deficiencies and concradictions, this study is judged ACCEPTABLE in reporting equivocal but positive results in repeat assays of MGK 264 in nonactivated cultures of L5178Y cells exposed to toxic concentrations (TG < 20%), but negative responses in S9-activated cultures (TG > 20%).

Attachments

006674

MGK Scientific Review - Toxicology Review Number 006674

Pages 43 through 60 are not included. The pages contain detailed test methods and results submitted by the pesticide registrant.