7/25/96

DATA EVALUATION REPORT

AZOXYSTROBIN

STUDY TYPE: MAMMILIAN CELLS IN CULTURE GENE MUTATION ASSAY IN L5178Y MOUSE LYMPHOMA CELLS

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Biomedical and Environmental Information Analysis Section
Health Sciences Research Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831
Task Order No. 95-19W

Primary Reviewer:
B. L. Whitfield, Ph.D.
Signature: B L Whitfield
Date: 6/10/96

Secondary Reviewers:
Cheryl B. Bast, Ph.D., D.A.B.T.
Signature: C B Bast
Date: 6-5-96

Robert H. Ross, M.S., Group Leader
Signature: R H R
Date: 6-5-96

Quality Assurance:
Susan Chang, M.S.
Signature: S S Chang
Date: 6-5-96

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory, managed by Lockheed Martin Energy Research Corp. for the U.S. Department of Energy under contract number DE-AC05-96OR22464.

EPA Reviewer: I. Mauer, Ph.D. _____, Date 7-18-96
Toxicology Branch I (7509C)
EPA Secondary Reviewer:
M. Copley, D.V.M., D.A.B.T _____, Date 7/22/96
Toxicology Branch I (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Mammalian cells in culture gene mutation assay in L5178Y mouse lymphoma cells
OPPTS 870.5375 [§84-2]

D.P. BARCODE: D218319
P.C.CODE: 128810
SUBMISSION CODE: S489692
TOX. CHEM. NO.: none

TEST MATERIAL (PURITY): ICIA5504 (Azoxystrobin)(96.2% w/w)

SYNONYMS: none provided

CITATION: Callander, R. and P. Clay (1993) ICIA5504: Assessment of mutagenic potential using L5178Y mouse lymphoma cells. Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK. Report number CTL/P/3963, May 20, 1993. MRID 43678145. Unpublished.

SPONSOR: Zeneca Inc., Agricultural Products, Wilmington, Delaware 19897

EXECUTIVE SUMMARY: In a forward mutation study at the TK locus in L5178Y mouse lymphoma cells in culture (MRID 43678145) cells were exposed to ICIA5504 (96.2% w/w) in the presence and absence of an exogenous metabolic activation system (S9 mix), at concentrations of: 1st assay - 8, 15, 30, 60 µg/mL; 2nd assay - 34, 45, 60, 80 µg/mL and 3rd assay - 26, 33, 41, 51, 64, 80 µg/mL. Preparations for metabolic activation were made from Phenobarbital plus β-Naphthoflavone induced rat liver. The test material was delivered in DMSO.

ICIA5504 was tested to an upper concentration limited by cytotoxicity. The positive controls were acceptable as were the solvent controls in all cases except the second experiment without S9 mix where the solvent control was said to be out of the acceptable range ($0.8 - 6.0 \times 10^{-4}$ mutants per survivor). Mean cell survival at the HDT (60 ug/mL) in the first experiment was 30% in the absence of S9 mix and 26% in the presence of S9 mix. The maximum dose of test material was raised to 80 µg/mL in the second and third experiments with mean survival values without and with S9 mix of 10% and 4% in experiment 2 and 12% and 8% in experiment 3, respectively. Small, but statistically significant, increases in mutation frequency were seen in treated cells in all three experiments when S9 mix was present and also in Experiments 1 and 3 when S9 mix was absent. Mutation data from cultures without S9 mix in Experiment 2 were not presented because of the unacceptable solvent control. The mutation frequencies seen, both with and without S9 mix, in experiments 1 - 3 are small and inconsistent

2 44C

within and between experiments. For example, in experiment 1 with S9 mix, the mutation frequency at 8 μg/mL (2.5×10^{-4}) is not significantly different than the solvent control value of 2.1×10^{-4} while the mutation frequencies at 15, 30 and 60 μg/mL (3.7, 3.9 and 3.5×10^{-4}) are virtually the same and significantly increased over the solvent control at the $p < 0.01$ level. Comparable doses tested in experiment 2 with S9 mix, 34 μg/mL and in experiment 3, 26 and 33 μg/mL, with or without S9 did not significantly increase the mutation frequency over solvent control values, but higher (moderately to severely cytotoxic) doses did.

This study is classified as acceptable. Although the study cannot be considered definitive, we agree with the investigator that the test material is positive for forward gene mutation at the TK-locus in L5178Y mouse lymphoma cells. The study satisfies the requirements for FIFRA Test Guideline 84-2 for *in vitro* mammalian cell gene mutation studies.

COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: ICIA5504

Description: light-brown solid
Lot/Batch No.: reference R215504, batch P49/D7534/46
Purity: % a.i. 96.2% w/w
Stability of compound: not provided
CAS No.: not provided
Structure: not provided
Solvent used: DMSO

2. Control materials

Solvent/final concentration: DMSO/10 μL/mL
Positive:
Non-activation: Ethyl methanesulfonate(EMS)/750 μg/mL
Activation: *N*-nitrosodimethylamine(DMN)/600 μg/mL

3. Activation

S9 derived from Phenobarbital plus β-Naphthoflavone induced rat liver (Male Alderley Park (Alpk:APfSD) albino rats were used)

S9 mix composition: (the following added to 20 mL cell culture)

Co-factor solution: 200 μL
0.75 mM NADP, disodium salt
12.0 mM glucose-6-phosphate, sodium salt

48C

Supernatant from liver homogenate 1.0 mL

4. Test compound concentrations used

Non-activated conditions:
1st assay - 8, 15, 30, 60 µg/mL
2nd assay - 34, 45, 60, 80 µg/mL
3rd assay - 26, 33, 41, 51, 64, 80 µg/mL
Activated conditions:
1st assay - 8, 15, 30, 60 µg/mL
2nd assay - 34, 45, 60, 80 µg/mL
3rd assay - 26, 33, 41, 51, 64, 80 µg/mL

5. Test cells

L5178Y mouse lymphoma cells in culture

Properly maintained? YES

Periodically checked for Mycoplasma contamination? YES

Periodically checked for karyotype stability? UNKNOWN

Periodically "cleansed" against high spontaneous background? UNKNOWN

6. Locus examined

TK locus
Selection agent: 4 µg/mL trifluorothymidine (TFT)

B. TEST PERFORMANCE

1. Cell treatment

a. Cells exposed to test compound, negative/solvent or positive controls for: 4 hours (non-activated) 4 hours (activated)

b. After washing, cells cultured for 3 days (expression period) before cell selection:

c. After expression, 2000 cells/well (2 microwell plates/ group) were cultured for 10-12 days in selection medium to determine numbers of mutants and 1.6 cells/well 2 microwell plates/group) were cultured for 10-12 days without selection medium to determine cloning efficiency.

2. Protocol

A culture of L5178Y (TK+/-) cells in exponential growth (1 - 1.2 x 10^6 cells/mL) was diluted 50:50 v/v with serum free medium. After 1 hour at 37°C, 20 mL aliquots of the cell culture (5 - 6 x 10^5 cells/mL) were

45

taken for treatment, test material or control solutions were added to the 20 mL treatment cultures at 1% v/v and the cultures incubated at 37°C for 4 hours. Culture flasks were rotated during the 4 hour treatment period. S9 mix was added immediately before treatment for studies with metabolic activation. The test material was removed by centrifuging the cultures at 250g for 5 minutes, removing the supernatant and resuspending the cell pellet in 50 mL of fresh medium. All treatments were conducted in duplicate.

Survival following treatment was determined by plating 200 µL per well of a diluted sample of each culture (8 cells per mL), into two 96 well microwell plates (1.6 cells per well) and incubating the plates at 37°C in 5% CO_2 and 98% relative humidity.

Mutation induction at the TK locus was determined after a 72 hour expression time. Cultures were diluted to 1 x 10^4 cells per mL (a sample from each culture was diluted to 8 cells per mL for cytotoxicity determination), TFT was added to a final concentration of 4 µg/mL and 200 µL aliquots per well were dispensed into four 96 well microwell plates (2000 cells per well). After 10-12 days, the plates were scored for cell survival and mutant colony induction (small or large colonies). A small colony was defined as one whose average diameter was less than 25% of the diameter of the well (typically about 15%) with a dense colonal morphology. A large colony was defined as one whose average diameter was greater than 25% of the diameter of the well with less densely packed cells, especially around the edges of the colony. A well containing both large and small colonies was scored as a large colony.

Results were expressed as mutation frequency per viable cell and analyzed for statistical significance as follows: "The data were considered by logit regression, using a complementary log-log link function. The dependent variable was the number of empty wells. This procedure provided maximum likelihood estimates of log mutant frequencies. Variancies were inflated by the heterogeneity factor, assuming departures from linearity to be random."

Tests for trend in log mutant frequency with dose level were done for each experiment, with and without S9 and an overall test for trend combining data across experiments was performed. Intergroup comparisons of log mutant frequency were also performed using the same heterogeneity factor as the tests for trend. All tests were one sided.

50C

II. REPORTED RESULTS

A. Preliminary cytotoxicity assay

Based on solubility and toxicity determinations from a preliminary range-finding study (no details were provided), a maximum dose level of ICIA5504 of 60 μg/mL, both with and without S9 mix, was selected for the main mutagenicity study. Toxicity seen in the first experiment in the main mutagenicity study was not as high as desired; therefore, the maximum dose used in the second and third experiments was increased to 80 μg/mL.

B. MUTAGENICITY ASSAY

Results of the three experiments in the main mutagenicity study are summarized in Appendix Tables 1 - 3 (MRID 43678145 Table 1[p18], Table 2 [p19], Table 3 [p20]). Mean cell survival in the first experiment was 30% in the absence of S9 mix and 26% in the presence of S9 mix. The maximum dose of test material was raised to 80 μg/mL in the second and third experiments with mean survival values without and with S9 mix of 10% and 4% in experiment 2 and 12% and 8% in experiment 3, respectively.

Small, but statistically significant, increases in mutation frequency were seen in treated cells in all three experiments when S9 mix was present and also in Experiments 1 and 3 when S9 mix was absence. Mutation data from cultures without S9 mix in Experiment 2 were not presented because they were considered invalid due to a spontaneous (solvent control) mutation frequency outside the acceptable range ($0.8 - 6.0 \times 10^{-4}$ mutants per survivor).

III. REVIEWER'S DISCUSSION/CONCLUSIONS

A. Although there were statistically significant increases in mutation frequency seen in this study, and a statistically significant test for linear trend with dose in most experiments, it is not clear that the increases are biologically significant. The mutation frequencies seen, both with and without S9 mix, in experiments 1 - 3 are small and inconsistent within and between experiments. In experiment 1 with S9 mix, the mutation frequency at 8 μg/mL (2.5×10^{-4}) is not significantly different than the solvent control value of 2.1×10^{-4} while the mutation frequencies at 15, 30 and 60 μg/mL (3.7, 3.9 and $3,5 \times 10^{-4}$) are virtually the same and significantly increased over the solvent control at the $p < 0.01$ level. Comparable doses tested in experiment 2 with S9 mix, 34 μg/mL and in experiment 3, 26 and 33 μg/mL, did not significantly increase the mutation frequency over solvent control values. These were the lowest doses tested in these experiments even though a statistically significant increase in mutation frequency was seen at 15 μg/mL in the

5A

first experiment. The mutation frequency with S9 mix in experiment 3 at 33 μg/mL (1.7×10^{-4}) was not significantly increased over the solvent control value of 1.3×10^{-4}; however, at 41 μg/mL the highest mutation frequency obtained in this experiment (4.9×10^{-4}, $p < 0.01$) was seen. The mutation frequency dropped by half at 51 and 64 μg/mL (2.5 and 2.4 $\times 10^{-4}$ respectively) although still significant at $p < 0.01$ then increased to 3.6×10^{-4} at 80 μg/mL. Cell survival was essentially the same at 41, 51 and 64 μg/mL.

In experiment 1 without S9 mix, the mutation frequency fluctuated, with no significant increase at 8 μg/mL, a significant increase at 15 μg/mL ($p < 0.01$), no significant increase at 30 μg/mL and finally a significant increase at 60 μg/mL ($p < 0.01$). The author did not show the data from experiment 2 without S9 mix because the data were considered invalid due to a spontaneous mutant frequency outside the acceptable range. The third experiment without S9 mix also showed variable results with fluctuations in mutation frequency at five concentrations between 26 and 64 μg/mL (1.7 and 1.9 $\times 10^{-4}$ respectively) with a jump to 6.1×10^{-4} at 80 μg/mL.

B. STUDY DEFICIENCIES

Because the increases in mutation frequency were quite small and no clear, linear increase in mutation frequency with dose was seen (in spite of the statistically significant test for linear trend with dose), it is difficult to accept the results of this study as a definitive test of the mutagenicity of ICIA5504 in mammalian cells in culture.

It is also difficult to understand the dose selection for the second and third experiments. Results of the first experiment showed that the mutation frequencies, both with and without S9 mix, were not significantly different than the control values at 8 μg/mL but were significantly increased ($p < 0.01$) at 15 μg/mL. The mutation frequencies were actually slightly higher at 15 μg/mL than at 30 or 60 μg/mL. Yet the lowest dose selected for the second experiment was 34 μg/mL and a higher dose of 80 μg/mL added to increase cell killing. 80 μg/mL was excessively toxic with S9 mix in the second experiment but was used again in the third experiment where toxicity was acceptable.

52C

APPENDIX

APPENDIX

Azoxystrobin

Page _____ is not included in this copy.

Pages 10 through 12 are not included in this copy.

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.

X FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

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.