7-25-96

DATA EVALUATION REPORT

AZOXYSTROBIN

STUDY TYPE: Other Genotoxicity: UNSCHEDULED DNA SYNTHESIS IN RAT HEPATOCYTES/MAMMALIAN CELLS - *IN VIVO/IN VITRO* PROCEDURE (84-2)

Prepared for

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Office of Pesticide Programs
U.S. Environmental Protection Agency
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Toxicology Branch I (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Other Genotoxicity: Unscheduled DNA Synthesis in Rat Hepatocytes/Mammalian Cells - *in vivo/in vitro* Procedure
OPPTS 870.5550 [§84-2]

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

TEST MATERIAL (PURITY): E5504 (Azoxystrobin) (97.2% w/w)

SYNONYMS: ICIA5504

CITATION: Kennelly, J. (1992) E5504: Assessment for the induction of unscheduled DNA synthesis in rat hepatocytes *in vivo*. ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK. Report No CTL/P/3682, May 28, 1992. MRID 43678149. Unpublished.

SPONSOR: ICI Americas Inc., Agricultural Products, Wilmington, Delaware 19897

EXECUTIVE SUMMARY: In an *in vivo/in vitro* unscheduled DNA synthesis (UDS) assay in rat hepatocytes (MRID 43678149), E5504 (97.2% w/w) , at doses of 1250 and 2000 mg/kg, was administered to 5 male Alderley Park (Alpk:APfSD) rats per test group by oral gavage. The test material was delivered once in corn oil at 10 ml/kg. Hepatocytes from 5 rats per test group were isolated at 2 or 16 hours post-treatment and cultured for determination of tritiated thymidine incorporation into DNA using the autoradiographic technique.

A preliminary toxicity test using doses ranging from 500 to 2000 mg/kg showed no signs of acute toxicity at any dose although diarrhea and urinary incontinence were seen at each dose level. An acute oral MLD value of greater than 5000 mg/kg E5504 had been previous demonstrated at this laboratory. Because E5504 was virtually non-toxic, it was tested to the limit dose of 2000 mg/kg for the UDS assay. No signs of cytotoxicity were seen in hepatocytes isolated from the treated rats. The net nuclear grain count was determined for 60 hepatocytes per animal and the percent of cells in repair recorded. A second independent assay was conducted. **There was no evidence that E5504 at either 1250 or 2000 mg/kg increased the incidence of UDS over solvent control values in hepatocytes isolated from rats 2 or 16 hours post-treatment,** but without any evidence presented that the test material (or its

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active metabolites) as administered (once orally, and up to a so-called "limit dose" of 2000 mg/kg) reached the target tissue (hepatocytes) in concentrations sufficient to register any effect (cytotoxicity, and/or genotoxicity). In contrast, hepatocytes from animals given the reference mutagens responded appropriately, with the vast majority of cells in repair (i.e., with net nuclear grain counts significantly in excess of +5).

Since signs of clinical toxicity (diarrhea, urinary incontinence) were observed in an initial range-finding study, at doses up to 2000 mg/kg (but not in additional rats given the same dosage, nor in the main study), we may consider the data requirements for this type of in vivo study to be satisfied, according to current FIFRA Test Guidelines.

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

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: E5504
   Description : light brown solid
   Lot/Batch #: P39/D7534/22
   Purity: % a.i. 97.2% w/w
   Stability of compound: responsibility of sponsor
   CAS #: not provided
   Structure: not provided
   Solvent used: dried corn oil

2. Control materials

   Solvent/final volume/Route of administration:
   dried corn oil/10 mL/kg/oral gavage

   Positive/Final dose(s)/Route of administration:
   16 hour treatment:
   2-acetylaminofluorene/25 mg/kg/oral gavage
   16 hour treatment:
   1,2-dimethylhydrazine-2HCl/20 mg/kg/oral gavage
   2 hour treatment:
   N-nitrosodimethylamine/10 mg/kg/oral gavage
   2 hour treatment:
   1,2-dimethylhydrazine-2HCl/20 mg/kg/oral gavage

3. Test compound concentrations used

   1250 and 2000 mg/kg / 10 mL/kg / oral gavage

4. Test animal and cells

   Hepatocytes from male Alderley Park (Alpk:APfSD) rats

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5. Cell preparation

a. Perfusion technique

Hepatocytes were isolated from the rats by a two-stage collagenase perfusion technique. Livers were perfused *in situ* with Buffer 1 (see Appendix for buffer and media composition) at 40 mL/min (total of approximately 450 mL) to remove blood from the liver. Perfusion then continued with approximately 200 mL of Buffer 2 at 40 ml/min at which time a calcium and collagenase solution was added and perfusion continued. Perfusion rate was reduced to 20 mL/min and when the reticular pattern of the liver began to break up and the liver became "spongy" the perfusion was stopped (10-15 min). The liver was removed to a glass beaker and minced with scissors. The crude homogenate was diluted with WE-complete medium, filtered through 150 μM nylon bolting cloth and the cell suspension centrifuged at 40 g for 2 min. The cell pellet was resuspended in WE-complete medium and the centrifugation step repeated twice more. The final cell pellet was resuspended in 30 ml of WE-complete medium and cell viability determined by trypan blue exclusion. The cell suspension was diluted with WE-complete medium to obtain a final viable cell count of 1.5×10^5/mL.

b. Culture preparation

Coverslips, etched side up, were placed in six-well plates and each cell suspension plated out as six 3 mL replicates. The cultures were incubated at 37°C in 95% air: 5% CO_2 (v/v) for 1.5 - 2.0 hours to permit the cells to attach. The medium was then removed, the hepatocytes washed with 3 mL of WE-incomplete medium and 2 mL of WE-incomplete medium containing ^{3}H-thymidine added to each culture. Cultures were incubated for 4 hours at 37°C in 95% air: 5% CO_2 (v/v). Following incubation, the cultures were washed three times with 2 mL WE + thymidine solution (unlabeled) to remove unincorporated radiolabel. Cultures were then incubated at least 12 hours with 3 mL of the same medium.

B. TEST PERFORMANCE

1. Dose selection

Dose selection was based on a preliminary toxicity study in which two male rats per dose were given single 10 ml/kg bodyweight oral doses of E5504 in corn

oil at 500, 800, 1250 or 2000 mg/kg. Rats were observed for 4 days post-treatment. Five additional male rats were dosed at 2000 mg/kg to confirm the highest dose selected for the UDS assay. A previous study conducted in this laboratory showed an acute oral MLD of greater than 5000 mg/kg, essentially non-toxic.

2. UDS assay

a. Treatment

Each rat was given a single oral dose, by gavage, of the appropriate concentration of E5504, solvent or positive control at a volume of 10 mL/kg bodyweight. A duplicate, independent assay was conducted.

b. Slide preparation

Cultures were prepared for fixation by removing the medium and washing the coverslips with 2-3 mL of WE-incomplete medium of physiological saline. Cultures were then fixed with 2 ml of a freshly prepared mixture of 1:3 glacial acetic acid : absolute alcohol (v/v) for 10 min, repeated three times. Coverslips were washed four times with distilled water, dried and mounted cell side up to microscope slides with DPX.

c. Autoradiography

Coded slides were coated with Ilford K2 photographic emulsion and kept at 4°C in the dark for 14 days. The emulsion was developed in KODAK D19, fixed with Ilford HYPAM fixer and stained with Meyers Haemalum and eosin Y phloxine.

d. Grain counting

Silver grains were counted using a microscope-mounted image analyzer linked to a computer. Usually, 30 morphologically normal cells per slide, 60 cells per animal were scored. If necessary, a third slide was scored to obtain 60 cells per animal. Cells were picked at random from all quadrants of the slide. For each cell the number of grains over the nucleus was counted as was the number of grains over an equivalent area of the cytoplasm adjacent to the nucleus and most heavily labeled. The net grain count was calculated by subtracting the cytoplasmic count from the nuclear count.

3. Evaluation criteria

Mean net nuclear grain counts and the percent of cells in repair (a cell in repair was defined as one with a net nuclear grain count of five or more) were calculated for each rat and for each treatment group. To be acceptable, the negative (solvent) controls must have a cytoplasmic grain count of less than 40 and a mean net nuclear grain count less than zero. Historically, no negative control animal in the testing laboratory has had a net nuclear grain count greater than zero. Positive controls should produce a net nuclear grain count of at least five with at least 20% of the cells in repair. The results of a test are considered negative if the mean net nuclear grain count of all treated animals is less that zero. As just mentioned, a net nuclear grain count of at least five was required for an acceptable positive control and was the laboratory's defining value for a cell in repair; however, the author also states that the results are considered positive (they indicate a UDS response) if the mean net nuclear grain count is zero or higher in a treated animal. Presumably, the difference reflects the laboratory's historical experience and belief that "a net nuclear grain count of greater than zero represents a biologically significant departure from normal". The test material is considered an unequivocal genotoxic agent in the UDS assay if the response is reproduced in concurrently treated animals and repeated in an independent experiment.

II. REPORTED RESULTS

E5504 did not induce a UDS response in hepatocytes from rats treated with either 1250 or 2000 mg/kg when the cells were cultured at 2 or 16 hours post-treatment. The net nuclear grain counts were below zero in all treated animals and no different than solvent control values. There was likewise no significant difference between solvent controls and treated animals in the percent of cells in repair. The data are presented in Appendix Tables 1 - 3 (MRID #43678149, Tables 1, 2A,2B, pp 20-22).

Solvent and positive control values were appropriate.

III. REVIEWER'S DISCUSSION/CONCLUSIONS

A. Guidelines specific for the *in vivo/in vitro* method for conducting an unscheduled DNA synthesis assay are not published in the Federal Register; however, Butterworth et al (1987) published a protocol and guide for the *in vivo* rat hepatocyte UDS assay. The present study followed

acceptable guidelines. The highest dose tested (2000 mg/kg), while not the MTD, is an acceptable upper dose. Positive and solvent control values were appropriate. There were no study deficiencies that compromized the results.

B. STUDY DEFICIENCIES

There were no study deficiencies that compromised the acceptability of the study.

References

Butterworth, BE, Ashby, J., Bermudez, E. et al. A Protocol and Guide for the *in vivo* Rat Hepatocyte DNA-repair Assay. Mutation Research, 189: 123-133, 1987.

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APPENDIX

Azoxystrobin

Page _____ is not included in this copy.

Pages 9 through 11 are not included in this copy.

The material not included contains the following type of information:

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