DATA EVALUATION RECORD

5/9/2000

MESOTRIONE (ZA 1296)

Study Type: §84-2; *In vitro* Chromosome Aberration Assay in Human Lymphocytes

Work Assignment No. 2-01-52GG (MRID 44373524)

Prepared for

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

Prepared by

Pesticide Health Effects Group
Sciences Division
Dynamac Corporation
2275 Research Boulevard
Rockville, MD 20850-3268

Primary Reviewer:
Joan L. Harlin, M.S.

Signature: Joan L. Harlin
Date: 3/17/00

Secondary Reviewer:
Steven Brecher, Ph.D.

Signature: Steven Brecher
Date: 3/28/00

Program Manager:
Mary L. Menetrez, Ph.D.

Signature: Mary L Menetrez
Date: 3/28/00

Quality Assurance:
Sharon Meyer, Ph.D.

Signature: Sharon A. Meyer
Date: 03/20/00

Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

1

124

EPA Reviewer: Irving Mauer, Ph.D. Date: 8/13/00
Registration Action Branch 3, HED (7509C)
EPA Work Assignment Manager: Marion Copley, DVM, DABT Date: 8/9/00
Registration Action Branch 1, HED (7509C)

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in human lymphocytes
OPPTS NUMBER: 870.5375 OPP Guideline Number: §84-2

DP BARCODE: D259369 SUBMISSION CODE: S541375
P.C. CODE: 122990 TOX. CHEM. NO.: None

TEST MATERIAL (PURITY): ZA 1296 (mesotrione, 98.1%)

SYNONYMS: 2-[4-(Methylsulphonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione

CITATION: Griffiths, K., Mackay, J.M. (1994) ZA 1296: An Evaluation in the In Vitro Cytogenetic Assay in Human Lymphocytes. Zeneca Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK. Laboratory Project Report No. CTL/P/4187. Laboratory Project Study No. SV0700. February 21, 1994. MRID 44373524. Unpublished.

SPONSOR: Zeneca Inc., Agricultural Products, Wilmington, DE

EXECUTIVE SUMMARY:

In two independent *in vitro* mammalian cell chromosome aberration assays (MRID 44353724), primary human lymphocyte cell cultures from one male and one female donor were exposed to culture medium or dimethylsulfoxide (DMSO) alone or to ZA 1296 (98.1% a.i.) in dimethylsulfoxide (DMSO) at concentrations of 250, 1000, and 1500 or 2000 µg/mL without metabolic activation (-S9) and 250, 1000, and 2000 µg/mL with metabolic activation (+S9) provided by phenobarbitol/beta naphthaflavone-mix. Cultures were treated for 20 hours without S9 activation and harvested immediately or 24 hours after cessation of treatment, or 3 hours with S9 activation and harvested 17 hours after cessation of treatment. Microscopic slides of metaphase-arrested lymphocytes were prepared and 150-200 metaphases per donor per dose level ±S9 were examined for chromosomal aberrations.

ZA 1296 was tested to cytotoxic levels. The dose levels selected for the chromosome aberration assay were based on pH of the culture medium, the mitotic index, and cytotoxic effects on chromosome structure. With cell harvest immediately after a 20-hour treatment period in the absence of metabolic activation, dose-related, statistically significant increases ($p<0.05$ or $p<0.01$) in the percent of metaphases with aberrations excluding gaps were observed in male

2

125

donor lymphocyte cultures treated with ZA 1296 at 1000 or 1500 µg/mL. However, the percent of metaphases with aberrations in the concurrent negative control was lower than expected. A dose-related increase in percent metaphases with aberrations was also observed in corresponding female donor lymphocyte cultures; however, the increases were not statistically significant. There were no increases in percent metaphases with aberrations in S9-activated cultures or in non-activated cultures sampled 24 hours after cessation of treatment. Under the given experimental conditions, ZA 1296 was not clastogenic with S9 activation and was equivocal for clastogenic activity without S9 activation. The sensitivity of the assay system to detect structural chromosome alterations was adequately demonstrated by the results obtained with the positive controls (cyclophosphamide, +S9; mitomycin C, -S9).

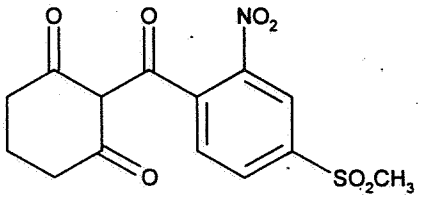
This study is classified as **acceptable (§84-2)**. It satisfies the requirement for FIFRA Test Guideline for *in vitro* cytogenetic mutagenicity data.

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

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: ZA 1296
   Description: Light beige solid
   Lot/Batch #: P6
   Purity: 98.1% a.i.
   Stability of compound: The test material was reported to have been used within the stated expiration date (not reported), based on information from the Sponsor. No additional information was provided. The test material was stored at room temperature in the dark.
   CAS #: 104206-82-8
   Structure: (Not present in report; presumably copied from independent source):



   Vehicle used: Dimethylsulfoxide (DMSO)
   Other comments: Serial dilutions with DMSO were made from a stock solution.

2. Control Materials:
   Negative: Vehicle and untreated controls
   Vehicle/final concentration: DMSO, 10 µL/mL
   Positive:
   Nonactivation (concentrations, solvent): Mitomycin C at 0.2 µg/mL in sterile double deionized water
   Activation (concentrations, solvent): Cyclophosphamide at 50 µg/mL in sterile double deionized water

3. Activation: S9 derived from Alderley Park (Alpk:APfSD) male albino rats:

___ Aroclor 1254 ___ phenobarbital ___ none _x_ phenobarbital/ ß-naphthoflavone	_x_ induced ___ non-induced	_x_ rat ___ mouse ___ hamster	_x_ liver ___ lung

If other, describe below

Describe S9 mix composition: Both the S9 and S9-cofactor mix were prepared in-house. The S9 mix contained S9 fraction:cofactor solution (1:1, v:v). The cofactor solution contained $MgCl_2$ (6 mM), KCl (25 mM), glucose-6-phosphate (4 mM). NADP (3 mM), and sodium phosphate buffer at pH 7.5 (75 mM). The final S9 culture concentration was approximately 1%.

4. Test compound concentrations used:

The doses selected for evaluation of chromosome aberrations were based on the results obtained for effects of the test article on pH, solubility, and mitotic index. Independent assays were performed using lymphocytes from human venous blood (Donors 1, male and 2, female)

Cytotoxicity Test

Donor 1: Nine dose levels of ZA 1296 (10, 50, 100, 250, 500, 750, 1000, 1500, and 2000 µg/mL; ±S9) were used. Cultures were evaluated 68 hours after culture initiation for effects of ZA 1296 on pH, osmolality, and mitotic index.

Donor 2: Five dose levels of ZA 1296 (250, 750, 1000, 1500, and 2000 µg/mL; ±S9) were used. Cultures were evaluated 68 hours after culture initiation for effects of ZA 1296 on mitotic index. In addition, three dose levels of ZA 1296 (1000, 1500, and 2000 µg/mL; ±S9) were used to determine the mitotic index 92 hours after culture initiation.

Duplicate solvent and untreated controls were included in each study. Donor 1 untreated controls were analyzed instead of the solvent controls because the latter were unsuitable for chromosome aberration analysis (large proportion of burst cells). Assays for the Donor 2 cell cultures sampled at 92 hours did not include positive controls. The appropriate positive controls were included in all other assays.

Chromosome aberration assay

Donor 1: Three dose levels of ZA 1296 (250, 1000, and 1500 µg/mL, ±S9) were evaluated 68 hours after culture initiation.

Donor 2: Three dose levels of ZA 1296 (250, 1000, and 2000 µg/mL, ±S9) were evaluated 68 hours after culture initiation. Additional dose levels of ZA 1296 (1500 µg/mL, -S9 and 2000 µg/mL, +S9) were evaluated 92 hours after the culture initiation.

5. Test cells: Whole blood from one male and one female donor and phytohemagglutinin to stimulate cell division were added as separate cultures to RPMI-1640 tissue culture medium containing approximately 10% fetal bovine serum (FBS), heparin (1 IU/mL), penicillin (100 IU/mL), streptomycin (100 µg/mL), and incubated at 37°C for 48 hours

with gentle daily mixing where possible prior to treatment. Two cultures were prepared per test group..

Properly maintained? **Yes**

Cell line or strain periodically checked for Mycoplasma contamination? **Not applicable**

Cell line or strain periodically checked for karyotype stability? **Not applicable**

B. TEST PERFORMANCE

1. Concurrent Cytotoxicity Assay: Prepared lymphocyte cells were preincubated for 48 hours prior to treatment with ZA 1296. The test substance in DMSO was added (in a volume of 10 µL/mL) to the cells in the culture medium and incubated for an additional 20 hours. For cultures requiring metabolic activation, S9-mix was added to the culture medium simultaneously with the treatment, incubated for 3 hours, washed free of treatment medium, fresh medium without S9 was added, and the cultures were incubated for an additional 17 hours. Additional cultures from the female donor were treated and incubated as described, the culture medium was replaced with fresh medium 68 hours after culture initiation and the cultures were re-incubated for an additional 24 hours. The appropriate vehicle and positive controls were included. For the male donor, untreated controls were used because of technical problems with the vehicle control. A positive control was not included with the 92-hour sampling.

   The mitotic index (percentage of cells in metaphase) was determined by scoring cells per concentration in the treatment groups and positive and negative controls and comparing the percentage of cells in mitosis with the corresponding value for the vehicle or untreated control.

2. Cytogenetic Assay:
   a. Cell treatment:
      Cells exposed to test compound, vehicle, or positive control for 20 hours (nonactivated) or 3 hours (activated)

   b. Spindle inhibition
      Inhibition used/concentration: Colcemid at 0.4 µg/mL
      Administration time: 2 hours (before cell harvest)

   c. Cell harvest
      Cells exposed to test material, solvent or positive control were harvested immediately or 24 hours after 20-hour exposure (nonactivated) and 17 hours after the 3-hour exposure (activated)

      After incubation with colcemid at 37°C, the cultures were centrifuged, the supernatant

removed, and the cells resuspended in hypotonic 0.075 M KCl solution at room temperature for approximately 10 minutes, followed by fixation of the cells with methanol/glacial acetic acid (3:1, v:v).

d. Details of slide preparation: Following fixation, the cells were centrifuged and resuspended in fresh fixative. Single drops of the cell suspension were dropped onto moist microscope slides, air-dried, stained with a filtered 10% Giesma solution, and mounted in DPX when dry. Four slides were prepared for each culture.

e. Metaphase analysis
No. of cells examined per dose: **200 for males; 150-200 for females**

Scored for structural: **Yes**

Scored for numerical: **No**

Coded prior to analysis: **Yes**

f. Evaluation criteria: The criteria for a valid test were not reported.

The mitotic index was determined by scoring 1000 lymphocytes per culture, where appropriate, for each treated, solvent , untreated, and positive control culture, and calculating the percentage of cells in metaphase. The highest concentration of ZA 1296 selected for chromosomal aberration analysis in the presence of metabolic activation for both donors was based on a reduction in pH of 0.4 units, the limit of solubility of ZA 1296 in the solvent, and the suitability of the metaphase preparations. The highest concentration of ZA 1296 selected for chromosomal aberration analysis in the absence of metabolic activation in the female donor cultures was based on a reduction in pH of 0.4 units, the limit of solubility of ZA 1296 in the solvent, and the suitability of the metaphase preparations. Mean mitotic index was significantly reduced compared to the controls, and cytotoxic effects on the chromosome structure were observed. The highest concentration of ZA 1296 selected for chromosomal aberration analysis in the absence of metabolic activation in the male donor cultures was based on a reduction in pH of 0.3 units, a significant reduction in mean mitotic index compared to the control values, and some cytotoxic effects on chromosome structure.

For the chromosome aberration assays, 150-200 lymphocyte metaphases per dose level per sex were scored for gaps, breaks, fragments-minutes, multiple damage, interchanges, and other rearrangements. A positive response was a statistically significant difference in the number/percent of metaphases with structurally aberrant chromosomes (excluding gaps) between treated and negative control cultures ($p<0.05$ and $p<0.01$, Fisher exact test).

7
TBC

g. Statistical analysis: Data were evaluated using the Fisher exact test with confidence intervals of 5% and 1%.II.

II REPORTED RESULTS

A. Analytical Determinations

ZA 1296 was reported to be soluble in vehicle (DMSO) at up to 200 mg/mL. Analytical determinations of the dose formulations were not performed.

The effects on pH and mitotic index were used to determine the highest concentrations to be used in the chromosome aberration assays. Treatment of culture medium with ZA 1296 at 1500 and 2000 µg/mL reduced pH by 0.3 and 0.4 units, respectively. Osmolality of the culture medium was not affected by ZA 1296 at any concentration. Data for pH and osmolality determinations are presented in Study Report Tables 8 and 9, pages 29 and 30.

B. Concurrent Cytotoxicity Assay

Human lymphocytes in culture were exposed to ZA 1296 with or without S9 activation at nine concentrations ranging from 10 to 2000 µg/mL (male donor) or five concentrations ranging from 250 to 2000 µg/mL. The cells were harvested 20 or 44 hours after the start of treatment. Tabulated results of the cytotoxicity assay were reported in Study Report Tables 1-3, pages 22-24. On the basis of mitotic index and reduction of pH of the culture medium, three dose levels were selected for analysis of chromosome aberrations ranging from 250 to 1500 µg/mL for the male donor (-S9), and 250 to 2000 µg/mL for the male donor (+S9) and the female donor (±S9) at the 68-hour sampling time. In addition, dose levels of 1500 µg/mL (-S9) and 2000 µg/mL (+S9) were selected for metaphase analysis at the 92-hour sampling time.

The mitotic index was determined for each culture. At the 68-hour sampling time, the mean mitotic index was reduced relative to the control values in the male donor at 1500 µg/mL (31%) and in the female donor at 2000 µg/mL (42%) in the absence of metabolic activation. No significant reductions in mitotic index were observed in any of the other treatment groups with or without metabolic activation or in cultures sampled at 92 hours.

C. Cytogenetic Assay

With S9 activation, no statistically or biologically significant increases in aberration frequency were observed at any dose level. Without S9 activation, there were statistically significant increases in percent metaphases with aberrations excluding gaps in male donor cultures treated at 1000 µg/mL (4.00% treated vs. 0.50% for untreated control, $p<0.05$) and 1500 µg/mL (5.50%, $p<0.01$) and sampled at 68 hours. In both assays, the positive controls (used in the 68-hour sampling only) induced statistically significant ($p<0.01$) increases in percent aberrant metaphases. Results of the cytogenetic assays were summarized in Study Report Tables 4-7, pages 25-28. The results from individual cultures were reported in Study

8

Report Appendices C and D, pages 39-44. The cytogenetic assay results were presented as mean values in Study Report Table 4-7, pages 25-28, and are summarized in Tables 1 and 2 below.

Table 1. Summary of Clastogenic Effects of ZA 1296 in Male Donor Lymphocytes (Donor 1) Harvested at 68 Hours.[a]

Treatment (µg/mL)	No. of Cells Analyzed	Mean % Mitotic Index	Mean % Aberrant Cells Excluding Gaps	Aberrations/Cell Excluding Gaps
With metabolic activation (+S9)				
Untreated Control	200	5.1	3.00	0.030
ZA 1296 (250)	200	5.7	2.00	0.025
ZA 1296 (1000)	200	4.6	0.00	0.000
ZA 1296 (2000)	200	4.5	1.50	0.015
Cyclophosphamide (50)	100	2.9[b]	34.00**	0.430
Without metabolic activation (-S9)				
Untreated Control	200	7.7	0.50	0.005
ZA 1296 (250)	200	6.0	1.50	0.015
ZA 1296 (1000)	200	5.7	4.00*	0.045
ZA 1296 (1500)	200	5.3	5.50**	0.060
Mytomycin C (0.2)	100	3.6[b]	40.00**	0.580

a Data taken from Study Report Tables 1, 2, 4, and 6, pages 22, 23, 25, 27 and Appendices C and D, pages 39-44.
b Positive control mitotic index and % aberrant cells were determined from a single culture.
* $p<0.05$, ** $p<0.01$.

9

Table 2. Summary of Clastogenic Effects of ZA 1296 in Female Donor Lymphocytes (Donor 2) Sampled at 68 or 92 Hours.[a]

Treatment (µg/mL or µL/mL)	No. of Cells Analyzed	Mean % Mitotic Index	Mean % Aberrant Cells Excluding Gaps	Aberrations/Cell Excluding Gaps
68-Hour Sampling Interval				
With Metabolic Activation (+S9)				
DMSO (10)	200	5.9	0.00	0.000
ZA 1296 (250)	150	6.5	0.00	0.000
ZA 1296 (1000)	200	6.4	1.00	0.010
ZA 1296 (2000)	200	10.1	0.00	0.000
Cyclophosphamide (50)	35	2.4[b]	22.86**	0.229
Without Metabolic Activation (-S9)				
DMSO (10)	200	8.8	1.00	0.010
ZA 1296 (250)	200	4.9	1.00	0.010
ZA 1296 (1000)	200	8.4	2.50	0.025
ZA 1296 (2000)	193	5.1	3.63	0.036
Mitomycin C (0.2)	50	4.4[b]	24.00**	0.380
92-Hour Sampling Interval				
With Metabolic Activation (+S9)				
DMSO (10)	200	5.9	1.00	0.015
ZA 1296 (2000)	200	7.6	0.00	0.000

(Table 2 continues)

10

133

Treatment (µg/mL or µL/mL)	No. of Cells Analyzed	Mean % Mitotic Index	Mean % Aberrant Cells Excluding Gaps	Aberrations/Cell Excluding Gaps
Without Metabolic Activation (-S9)				
DMSO (10)	200	6.4	3.50	0.040
ZA 1296 (1500)	200	5.9	4.00	0.040

a Data taken from Study Report Tables 1-7, pages 22-28 and Appendices C and D, pages 39-44.
b Positive control mitotic index and % aberrant cells were determined from a single culture.
* $p<0.05$, ** $p<0.01$.

III. DISCUSSION/CONCLUSIONS:

A. Investigator's Conclusions

The study authors concluded that ZA 1296, in this *in vitro* cytogenetic test, was not clastogenic to human lymphocytes both with and without metabolic activation.

B. Reviewer's Discussion/Conclusions

ZA 1296 was tested to cytotoxic levels. The dose levels selected for the chromosome aberration assay were based on pH of the culture medium, the mitotic index, and cytotoxic effects on chromosome structure. With a 20-hour treatment period in the absence of metabolic activation, dose-related, statistically significant differences ($p<0.05$ or $p<0.01$) in metaphases with aberrations excluding gaps were observed at the 68-hour sampling interval in male donor lymphocyte cultures treated with ZA 1296 at 1000 or 1500 µg/mL. The study authors concluded that the increases were due to the low control value, and therefore, are not considered biologically significant. The reviewers do not agree with this conclusion for the following reasons: (i) a dose-related increase was observed for the male donor, (ii) although statistical significance was not obtained, a dose-related increase was also observed for the female donor (-S9) at the 68-hour sampling interval, and (iii) a repeat test (-S9) with a 68-hour sampling interval was not performed. Therefore, the reviewers conclude that under the given experimental conditions, ZA 1296 was not clastogenic with S9 activation and was equivocal for clastogenic activity without S9 activation. The sensitivity of the assay system to detect structural chromosome alterations was adequately demonstrated by the results obtained with the positive controls (cyclophosphamide, +S9; Mitomycin C, -S9).

C. Study Deficiencies -

Several minor deficiencies noted in the study that are not considered to affect the validity of the study results are:

11

134

(1) Criteria for a valid test were not provided.

(2) Dose preparations were not analyzed for actual concentrations of test compound.

(3) Historical negative (vehicle, untreated) control data were not provided

These deficiencies would not be expected to alter the study conclusions, because ZA 1296 was tested to cytotoxic concentrations (based on reduction of mitotic index and suitability of metaphases for analysis).