EPA Reviewer: John E. Whalan
Registration Action Branch 2 (7509C) John Whalan 4-14-99

EPA Work Assignment Manager: Sanjivani Diwan, PhD
Toxicology Branch 1 (7509C)

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in Chinese hamster ovary cells
OPPTS NUMBER: 870.5375 OPP Guideline Number: §84-2

DP BARCODE: D241232 SUBMISSION CODE: S534142
P.C. CODE: 070705 TOX. CHEM. NO.: None

TEST MATERIAL (PURITY): KBR 3023 Technical (97.1% a.i.)

SYNONYMS: 1-(1-methyl-propoxycarbonyl)-2-(2-hydroxyethyl)- piperidine

CITATION: Gudi, R. and Schadly, E. (1997) Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells. Microbiological Associates, Inc., 9630 Medical Center Drive, Rockville, Maryland. Laboratory Study No. G97AD67.330. August 4, 1997. MRID 44408732. Unpublished.

SPONSOR: Bayer AG, Business Group Consumer Products, Research and Development, D-51368 Leverkusen, Bayerwerk, Bldg. 6210, Germany.

EXECUTIVE SUMMARY:

In an *in vitro* mammalian cell chromosome aberration assay (MRID 44408732), Chinese hamster ovary (CHO) cell cultures were exposed to dimethylsulfoxide (DMSO) alone or to KBR 3023 (97.1% a.i.) in DMSO at concentrations of 63, 125, 250, 500, 1000, 2000, and 4000 µg/mL continuously without metabolic activation (-S9) until harvest at 20 hours and for 4 hours with metabolic activation (+S9) with harvest at 12 hours. Untreated cultures were included, as were cultures treated with Mitomycin C or cyclophosphamide, respectively, to serve as positive controls for the nonactivated and activated series.

KBR 3023 was extremely toxic at ≥2000 µg/mL, without S9 activation (≤3% cell viability) and at 4000 µg/mL with S9 activation (≤1% cell viability). Without S9 activation, clastogenic activity ($p \leq 0.01$) was observed at 500 and 1000 µg/mL, with an accompanying dose response ($p \leq 0.05$). With S9 activation, no clastogenic activity was observed at any dose level. Untreated and vehicle controls were within historical control ranges and positive controls induced the appropriate responses. Thus, KBR 3023 is considered a clastogen in Chinese hamster ovary cells

in the absence of metabolic activation.

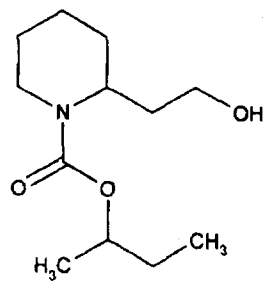
This positive study is classified as **Acceptable/Guideline** and satisfies the requirement for FIFRA Test Guideline **§870.5375** for *in vitro* cytogenetic mutagenicity data.

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

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: KBR 3023 Technical
   Description: Clear, colorless liquid
   Lot/Batch #: 030693
   Purity: 97.1% a.i.
   Stability of compound: Stable frozen. The sponsor recommended storage at cool temperatures; the test substance was stored at room temperature, protected from light.
   CAS #: 119515-38-7
   Structure:



   Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:
   Negative: Untreated and vehicle controls
   Solvent (final concentration): DMSO (1% in culture medium)
   Positive:
   Activation: Cyclophosphamide (10 and 20 µg/mL in distilled water)
   Nonactivation: Mitomycin C (0.08 and 0.15 µg/mL in distilled water)

3. Activation: Rat liver microsomal fraction (S9) was prepared from Aroclor 1254 induced male Sprague-Dawley rats. Immediately prior to use, frozen aliquots were thawed and added to a cofactor solution. The S9-cofactor mix contained 2 mM $MgCl_2$, 6 mM KCl, 1 mM glucose-6-phosphate, 1 mM NADP, and 20 µL S9 per mL serum-free McCoy's 5A

medium supplemented with streptomycin, penicillin, and L-glutamine. The final concentration of S9 in the culture medium was 2%.

4. Test Compound Concentrations Used:

a. Preliminary Cytotoxicity Assays:

Nine dose levels of KBR 3023 (0.5, 1.5, 5, 15, 50, 150, 500, 1500, and 5000 µg/mL) were tested by exposing cell monolayers for 6 hours in the absence of S9 activation or 4 hours in the presence of S9 activation and harvesting cells after 26 hours. Vehicle controls were included. Cell viability, growth index and growth inhibition, and average generation time were determined.

b. Mutagenicity Assays:

(1) Nonactivated conditions: Seven dose levels of KBR 3023 (63, 125, 250, 500, 1000, 2000, and 4000 µg/mL) were tested by exposing cell monolayers continuously until harvest after 20 hours of incubation.

(2) Activated conditions: Seven dose levels of KBR 3023 (63, 125, 250, 500, 1000, 2000, and 4000 µg/mL) were tested by exposing cell monolayers for 4 hours and harvesting after 12 hours of incubation.

Vehicle and untreated controls and the appropriate positive controls were included in the assays.

5. Test Cells: The Chinese hamster ovary CHO-K_1 cell line was the test system used. The cells were grown in 25 cm^2 tissue culture flasks containing 5 mL of McCoy's 5A medium with 10% fetal calf serum (FCS) and supplemented with L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were preincubated at 37°C, in a CO_2 incubator (5% CO_2) for 16-24 hours prior to treatment.

Properly maintained? **Yes**

Cell line or strain periodically checked for Mycoplasma contamination? **Yes**

Cell line or strain periodically checked for karyotype stability? **Only early passage (≤20) cells were used to assure stability**

B. TEST PERFORMANCE

1. Preliminary Cytotoxicity Assay: Single culture flasks were seeded with approximately $5x10^5$ CHO cells on the day prior to treatment with KBR 3023. The culture medium was drained off at the start of treatment and replaced with 5 mL of fresh medium, to which

was added 50 µL DMSO alone or test substance in DMSO. For cultures requiring metabolic activation, 1.0 mL of 5X S9 mix was added to 4 mL of culture medium. Treatment times of 6 hours without S9 or 4 hours with S9 were selected. A 50 µL aliquot of 1 mM 5-bromo-2-deoxyuridine (BrdU) was added to each flask two hours after initiation of treatment. At completion of the treatment period the cells were washed, refed with medium containing 0.01 mM BrdU, and returned to the incubator for a total of 26 hours from the initiation of treatment. Two hours prior to cell harvest, Colcemid (0.1 µg/mL) was added to each flask. The cells were harvested by trypsinization, counted using a Coulter counter, and viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the vehicle control. Cells in metaphase were prepared for sister chromatid differentiation using a modified fluorescence-Giemsa technique. Slides were evaluated for the percentage of first (M_1), second (M_2), and third (M_3) division metaphase cells for estimation of the test substance effect on cell cycle kinetics. The average generation time (AGT) was calculated for each treatment condition[a]. The dose levels of KBR 3023 selected for the main study were based on cell growth inhibition and cell cycle delay observed in the preliminary cytotoxicity assay.

[a] AGT = (24 hrs x 100)/[(no. M_1 cells x 1) + (no. M_2 cells x 2) + (no. M_3 cells x 3)]

2. Cytogenetic Assay: The highest concentration selected for the evaluation of chromosome aberrations was based on ≤50% cell growth inhibition compared to the vehicle control in the preliminary cytotoxicity assay.

   a. Cell treatment: CHO cell monolayers were grown as described in section A.5 and treated as described in section A.4.b. Duplicate culture flasks were seeded with approximately $5x10^5$ cells on the day prior to treatment with KBR 3023. On the day of testing, the culture medium was drained off at the start of treatment and replaced with 5 mL of fresh medium, to which was added 50 µL of DMSO containing the appropriate concentration of KBR 3023. For cultures requiring metabolic activation, 1.0 mL of 5X S9 mix was added to 4 mL of culture medium. The appropriate untreated (medium alone or medium containing S9 mix), vehicle, and positive control cultures in duplicate were included. S9-activated cultures were exposed for 4 hours, the cells were rinsed, refed with fresh medium, and returned to the incubator for an additional 8 hours prior to harvesting. Non-activated cultures were exposed continuously up to cell harvest at 20 hours. A concurrent cytotoxicity test was conducted with both activated and non-activated studies. At cell harvest, aliquots of cell suspensions were counted using a Coulter counter, and cell viability was determined by trypan blue dye exclusion. The counts obtained were used to determine cell growth inhibition relative to the vehicle control.

   b. Spindle inhibition:
   Inhibitor used/concentration: Colcemid at 0.1 µg/mL of culture medium
   Administration time: 2 hours before cell harvest

c. Cell harvest: In cultures with or without metabolic activation, cells were harvested immediately after the two hour Colcemid treatment. The medium was poured off and cells were recovered from monolayers by trypsinization. Cells were centrifuged and the supernatant was removed. Hypotonic treatment with 0.075 M KCl solution at room temperature for 4-8 minutes was followed by centrifugation and fixation of the cells with two changes of Carnoy's fixative (methanol/glacial acetic acid, 3:1, v/v) and storage overnight in fixative at 2-6°C.

d. Details of slide preparation: Following fixation, the cells were centrifuged and resuspended in a small amount of fixative, then dropped onto microscope slides, air dried overnight, and stained with 5% Giemsa solution. Stained slides were air dried and coverslips were added.

e. Metaphase analysis:
No. of metaphases examined per dose: **200**
No. of metaphases examined in the vehicle control: **200**
No. of centromeres per metaphase figure: **20±2**
No. of metaphases examined in the cyclophosphamide positive control: **200**
No. of metaphases examined in the mitomycin C positive control: **200**
Scored for structural aberrations: **Yes**
Scored for numerical polyploidy: **No**
Coded prior to analysis: **Yes**

f. Evaluation criteria: The assay was considered acceptable if there was a significant increase ($p \leq 0.05$, Fisher's exact test) in the percentage of cells with chromosome aberrations induced by the positive control(s) and if the chromosome aberration rate for the untreated or vehicle controls was ≤6%.

A clastogenic response was claimed if the percentages of cells with aberrations were increased in a dose-responsive manner with one or more dose levels being statistically elevated relative to the vehicle control group ($p \leq 0.05$). A significant increase at the high dose only with no dose response was considered suspect. A significant increase at one dose level other than the high dose with no dose response was considered equivocal.

g. Statistical analysis: Statistical analysis of the percent aberrant cells was performed by pair-wise comparison of treated and positive control groups to respective vehicle controls using the Fisher's exact test. In the event of a positive Fisher's test at any test substance dose level, the Cochran-Armitage test was used to measure dose-responsiveness. Differences were considered significant at $p \leq 0.05$.

II. REPORTED RESULTS

KBR 3023 was soluble in DMSO at a concentration of 500 mg/mL, the maximum concentration tested. The dose preparations were not analyzed for actual concentration. It was reported that concentrations of KBR 3023 up to 5000 µg/mL did not alter the osmolality or significantly affect the pH in culture medium.

A. Preliminary Cytotoxicity Assay

In the preliminary cytotoxicity assay, CHO cells in culture were exposed to 9 KBR 3023 concentrations ranging of 0.5 to 5000 µg/mL for 6 hours in the absence of metabolic activation and for 4 hours in the presence of metabolic activation. Cultures were harvested 26 hours after initiation of treatment to determine the inhibition of growth and generation time. Cell growth inhibition was 100% at 5000 µg/mL ±S9. At the next highest dose, 1500 µg/mL, cell growth inhibition was 35% (-S9) and 28% (+S9). Lower dose levels were essentially non-toxic (±S9). At 1500 µg/mL, the highest scorable dose level, the average generation time determined by sister chromatid differentiation with BrdU was 12-13 hours (±S9). The results of the preliminary cytotoxicity assay were reported in Tables 1-4 of the Study Report. On the basis of this preliminary cytotoxicity assay, 7 dose levels ranging from 63 to 4000 µg/mL (±S9) were selected for the cytogenetic assay.

B. Cytogenetic Assay

In the cytogenetic assay, CHO cells in culture were exposed to 7 KBR 3023 concentrations ranging of 63 to 4000 µg/mL, continuously until harvest at 20 hours in the absence of metabolic activation, and for 4 hours in the presence of metabolic activation, with harvest at 12 hours. Without S9 activation, excessive toxicity was observed at dose levels of ≥2000 µg mL. At 1000 µg/mL, relative cell growth inhibition was 46%. Therefore, dose levels of 125, 250, 500, and 1000 µg/mL were analyzed for chromosome aberrations. With S9 activation, excessive toxicity was observed at 4000 µg/mL. At 2000 µg/mL, relative cell growth inhibition was 49%. Therefore, dose levels of 250, 500, 1000, and 2000 µg/mL were analyzed for chromosome aberrations. Results of the concurrent cytotoxicity assays were reported in study report Tables 5 and 7. At 500 and 1000 µg/mL (-S9), the mitotic indices were 74% of the vehicle control and relative cell growth inhibition was 32% and 46%, respectively. At these dose levels, the percentage of cells with structural aberrations was significantly increased ($p \leq 0.01$) and a dose response was evident ($p \leq 0.05$). In the S9-activated study, the percentage of cells with structural aberrations was not statistically increased above that of the vehicle control at any dose level. The accompanying positive controls (±S9) induced statistically significant increases in the percentages of aberrant metaphases. Results of the cytogenetic assays were reported in study report Tables 6 and 8 and summarized in Table 9. The summary results extracted from Table 9 are presented in Table 1 below.

Table 1. Summary of Clastogenic Effects of KBR 3023 in CHO Cells

Treatment	±S9	Treatment/Harvest Time (hrs)	Mitotic Index	Cells Scored	Aberrations Per Cell (Mean±SD)	Cells With Aberrations (%)
Untreated	-	20/20	5.7	200	0.050 ± 0.313	3.0
DMSO	-	20/20	5.7	200	0.020 ± 0.140	2.0
KBR 3023 (µg/mL)						
125	-	20/20	5.4	200	0.055 ± 0.350	3.0
250	-	20/20	4.7	200	0.045 ± 0.231	4.0
500	-	20/20	4.2	200	0.155 ± 0.492	11.5**
1000	-	20/20	4.2	200	0.205 ± 1.043	10.5**
Mitomycin C (0.08 µg/mL)	-	20/20	3.2	200	0.910 ± 1.404	48.0**
Untreated	+	4/12	6.4	200	0.015 ± 0.122	1.5
DMSO	+	4/12	6.0	200	0.045 ± 0.252	3.5
KBR 3023 (µg/mL)						
250	+	4/12	5.3	200	0.015 ± 0.122	1.5
500	+	4/12	5.3	200	0.025 ± 0.186	2.0
1000	+	4/12	6.0	200	0.040 ± 0.196	4.0
2000	+	4/12	0.2	31	0.290 ± 1.270	9.7
Cyclophosphamide (10 µg mL)	+	4/12	1.0	200	0.655 ± 1.294	36.5**

** $p \leq 0.01$

III. DISCUSSION/CONCLUSIONS

A. Investigator's Conclusions

The study authors concluded that, in this *in vitro* cytogenetic test, KBR 3023 showed evidence of clastogenic activity at toxic dose levels in the absence of metabolic activation and no evidence of clastogenic activity at any dose level in the presence of metabolic activation.

B. Reviewer's Discussion

KBR 3023 was tested to toxic concentrations. A positive clastogenic response ($p \leq 0.01$) was observed at the two highest dose levels evaluated (500 and 1000 µg/mL) without metabolic activation. These dose levels were moderately toxic, with mitotic indices that were 74% of the vehicle control value. A positive dose response ($p \leq 0.05$) was also reported. At the highest dose level evaluated (2000 µg/mL) with metabolic activation, and the only cytotoxic dose (mitotic index 3% of vehicle control value), a 2.8X increase in the percent of cells with structural aberrations above the vehicle control value was observed. However, the response was not statistically significant, and it occurred in only one of the two culture flasks. There were no significant increases in clastogenic response above vehicle control values for any other dose level tested with metabolic activation. The untreated and vehicle control results were within the ranges of the reported historical controls. The sensitivity of the assay system to detect damage to chromosomes was adequately demonstrated by the results obtained with the positive controls (mitomycin C, -S9 and cyclophosphamide, +S9), which were also within the historical ranges reported for the laboratory. The reviewers agree with the study authors' conclusion that under the given experimental conditions, KBR 3023 was clastogenic without metabolic activation and non-clastogenic with metabolic activation for Chinese hamster ovary cells cultured *in vitro*.

C. Study deficiencies

Dose preparations were not analyzed for actual concentrations of test compound. However, this deficiency would not be expected to alter the study conclusions because KBR 3023 was tested to cytotoxic concentrations and a clastogenic response was observed in the absence of metabolic activation.