2-7-97

MEMORANDUM

SUBJECT: 4-OH METABOLITE (SDS-3701) OF CHLOROTHALONIL - A Review of Three Mutagenic Studies

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and

Tom Myers, Risk Characterization and Analysis Branch
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THRU: Jess Rowland, M.S., Acting Section Head
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and

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Health Effects Division (7509C)

EPA ID: **DP Barcode:** D229654 **Submission Code:** S509030
PC Code: 081901
MRID Nos.: 00127846, 44022201 and 44022202

Registrant: ISK Biosciences Corporation, Mentor, OH

REQUEST: Review three mutagenic studies with the 4-OH metabolite of CHLOROTHALONIL.

RESPONSE:

This memorandum contains Executive Summaries for the three mutagenic studies which have been reviewed. Attached to this memorandum are the Data Evaluation Reports. These studies were reviewed in response to ISK comments on the draft RED Chapter.

EXECUTIVE SUMMARIES

CITATION: Killeen, Jr., J.C., (1978) Cell Transformation Assay with DS-3701. Microbiological Associates, La Jolla, CA/Bethesda, MD. Document No. 041-5TX-80-0015-003; Laboratory Report Number TPRC 193, T1117, October 6, 1978. **MRID No. 00127846.** Unpublished

EXECUTIVE SUMMARY:

In a mammalian cell transformation assay (MRID 00127846), Fischer rat embryo cells (F1706 P95 and H4536 P+2 cell lines) were exposed to DS-3701 (Chlorothalonil metabolite, 99+% a.i.) at concentrations of 0.1, 1.0 or 10.0 µg/mL. Following a 7-day exposure, cells were subcultured a total of 12 times. At each passage, cultures were evaluated microscopically for transformed foci and subcultures 3, 6, 10, and 12 were plated in semi-solid agar and screened for the transformed phenotype. Newborn Fischer rats were inoculated subcutaneously with cells from the third passage of each cell line treated with the test substance at 10 µg/mL, or the negative or positive controls.

The F1706 and H4536 cell lines were not transformed on exposure to DS-3701 at any of the assayed concentrations; no consistent phenotypic changes were noted in either cell line (no macroscopic colonies appeared in the semi-solid agar). Findings with the positive controls confirmed the sensitivity of the test system to detect cell transformation and the acetone negative control did not transform either cell line.

F1706 cells treated with DS-3701 (10 µg/mL) induced late tumors in the newborn rats. The H4536 cells treated with the test substance were non-tumorigenic. F1706 and H4536 cells treated with the MCA positive induced tumors and the acetone treated cells were non-tumorigenic. This study is **unacceptable** and cannot be upgraded because results with the F1706 cell line were inconclusive.

CITATION: Mizens, M. and Laveglia, J. (1994) In vitro Mammalian Cytogenetic Test with SDS-3701. Department of Toxicology and Animal Metabolism, Ricerca, Inc., 7528 Auburn Road, Painesville, Ohio. Project No. 94-0048. October 17, 1994. **MRID No. 44022201.** Unpublished.

Putman, D., Curry, P., and Schadly, E. (1994) In vitro Mammalian Cytogenetic Test with SDS-3701. Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland. Ricerca Project No. 94-0048. September 23, 1994. **MRID No. 44022201.** Unpublished.

EXECUTIVE SUMMARY:

In a mammalian cell chromosome aberration assay (MRID 44022201), Chinese Hamster ovary (CHO) cells were exposed to SDS-3701 (Chlorothalonil metabolite, 99.2% a.i.) in dimethyl sulfoxide at concentrations ranging from 8.2 to 2,080 μg/mL with and without S9 activation. Cultures without metabolic activation were exposed for 20 and 44 hours prior to harvesting. Cultures with metabolic activation were exposed for 4 hours and harvested 16 and 40 hours following the termination of treatment. The high doses used for evaluation of clastogenic effects (260 μg/mL, -S9 with 20 hour harvest; 130 μg/mL, -S9 with 44 hour harvest; or 520 μg/mL, +S9 with 20 and 44 hour harvests) were cytotoxic concentrations that provided sufficient cells for analysis.

In the absence of metabolic activation, statistically significant increases in the percentage of cells with structural chromosome aberrations were obtained at 130 μg/mL after 20 and 44 hours of treatment. A dose response in one trial was not reproducible. In the presence of metabolic activation, statistically significant increases in the percentage of cells with structural chromosome aberrations were obtained at 400 and 520 μg/mL in cells harvested 16 after treatment and at 260, 300, 400, and 520 μg/mL in cells harvested 40 hours after treatment. A reproducible dose response was also observed in the presence of metabolic activation. Positive controls induced the appropriate response.

This study is classified as **acceptable** and satisfies the guideline requirement **(§84-2)** for *in vitro* cytogenetic mutagenicity studies.

3

CITATION: Mizens, M. and Laveglia, J. (1995) In vivo Bone Marrow Chromosomal Analysis in Chinese Hamsters with SDS-3701. Department of Toxicology and Animal Metabolism, Ricerca, Inc., 7528 Auburn Road, Painesville, Ohio. Project No. 94-0049. June 2, 1995. **MRID No. 44022202.** Unpublished study.

Prodlock, R., Taylor, K., and Elmore, E. (1995) SDS-3701 In vivo Bone Marrow Chromosomal Analysis in Chinese Hamsters. Huntingdon Research Centre Ltd., P.O. Box 2, Huntingdon, Cambridgeshire, England. Ricerca Project No. 94-0049. May 26, 1995. **MRID No. 44022202.** Unpublished study.

EXECUTIVE SUMMARY:

In an *in vivo* bone marrow chromosome aberration assay (MRID 44022202), male and female Chinese hamsters were given a single oral dose of SDS-3701 (99.2% purity) in methylcellulose at levels of 125, 250, and 500 mg/kg bodyweight. Bone marrow was sampled 6, 24, and 48 hours after treatment. Results from a preliminary toxicity test at levels ranging from 432 to 2,000 mg/kg indicated that 500 mg/kg was the estimated maximum tolerated dose (MTD).

No clinical signs of toxicity were noted up to the highest dose tested (500 mg/kg). The positive control induced the expected high yield of cells with abnormal chromosome morphology. There was, however, no convincing evidence that SDS-3701 induced a clastogenic response. There was no significant increase in the incidence of chromosome damage at either the 24- or 48- hour sampling times. A significant increase in the incidence of aberrant cells was obtained at the 6-hour sampling time. However, since the increase was not dose related and was not reproduced at later sampling times, it was not considered to be related to the test substance.

This study is classified as **acceptable** and satisfies the guideline requirement **(§84-2)** for *in vivo* cytogenetic mutagenicity studies.

Sign-off date: 02/07/97
DP Barcode: D229654
HED DOC Number: "NONE"
Toxicology Branch: TB2

5

DATA EVALUATION RECORD

(DS-3701) CHLOROTHALONIL METABOLITE

Study Type: §84-2; Mutagenicity - Cell Transformation Assay with DS-3701

Work Assignment No. 2-35A (MRID 00127846)

Prepared for

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

Prepared by

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

Primary Reviewer:
Sandra Daussin

Signature: _____
Date: _____

Secondary Reviewer:
William Spangler, Ph.D.

Signature: _____
Date: _____

Project Manager:
William Spangler, Ph.D.

Signature: _____
Date: _____

Quality Assurance:
Michael Norvell, Ph.D.

Signature: _____
Date: _____

Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent

to signing by Dynamac Corporation personnel.

EPA Reviewer: A. Levy, PhD
Review Section II, Toxicology Branch II (7509C)

EPA Secondary Reviewer: J. Rowland, MS
Review Section II, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Mammalian cells in transformation assay in rat embryo cells (F1706 P95 and H4536 P+2 cell lines)
OPPTS Number: 870.5300 OPP Guideline Number: (§84-2)

DP BARCODE: D229654 SUBMISSION CODE: S509030
P.C. CODE: 081901 TOX. CHEM. NO.: 215B

TEST MATERIAL (PURITY): DS-3701 (Chlorothalonil metabolite, 99.2% active ingredient, a.i.)

SYNONYMS: 4-Hydroxy-2,5,6-trichloroisophthalonitrile

CITATION: Killeen, Jr., J.C., (1978) Cell Transformation Assay with DS-3701. Microbiological Associates, La Jolla, CA/Bethesda, MD. Document No. 041-5TX-80-0015-003; Laboratory Report Number TPRC 193, T1117, October 6, 1978. MRID 00127846. Unpublished

SPONSOR: Diamond Shamrock Corporation, PO Box 348, Painesville, OH

EXECUTIVE SUMMARY: In a mammalian cell transformation assay (MRID 00127846), Fischer rat embryo cells (F1706 P95 and H4536 P+2 cell lines) were exposed to DS-3701 (Chlorothalonil metabolite, 99+% a.i.) at concentrations of 0.1, 1.0 or 10.0 μg/mL. Following a 7-day exposure, cells were subcultured a total of 12 times. At each passage, cultures were evaluated microscopically for transformed foci and subcultures 3, 6, 10, and 12 were plated in semi-solid agar and screened for the transformed phenotype. Newborn Fischer rats were inoculated subcutaneously with cells from the third passage of each cell line treated with the test substance at 10 μg/mL, or the negative or positive controls.

The F1706 and H4536 cell lines were not transformed on exposure to DS-3701 at any of the assayed concentrations; no consistent phenotypic changes were noted in either cell line (no macroscopic colonies appeared in the semi-solid agar). Findings with the positive controls confirmed the sensitivity of the test system to detect cell transformation and the acetone negative control did not transform either cell line.

8

F1706 cells treated with DS-3701 (10 μg/mL) induced late tumors in the newborn rats. The H4536 cells treated with the test substance were non-tumorigenic. F1706 and H4536 cells treated with the MCA positive induced tumors and the acetone treated cells were non-tumorigenic. This study is **unacceptable** and cannot be upgraded because results with the F1706 cell line were inconclusive.

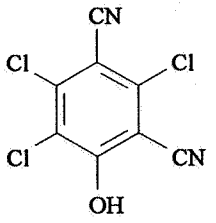
COMPLIANCE: A signed and dated GLP statement was provided. Quality assurance, data confidentially, and flagging statements were not provided.

9

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: DS-3701 (Chlorothalonil metabolite)
   Description: white powder
   Lot/Batch #: 8307-26-A-1
   Purity: 99+% a.i.
   Stability of compound: Stable in culture for at 129 days at -20 C
   CAS #: 28343-61-5
   Structure:



   Solvent used: acetone and saline

   Other comments: The test article was stored at -10 C in the dark. Immediately prior to use the test article was diluted with acetone to 4000 µg/mL then with saline to 200 µg/mL. Further serial dilutions of this stock solution were made with culture medium. Final dosing solutions were stored in the dark and used within 3 hours of preparation.

2. Control Materials:
   Negative: acetone at 1:1000 (v/v).
   Positive: methylcholanthrene (MCA) at 0.1 µg/mL

3. Activation: Cells treated with the positive control, negative control, and at the highest concentration of the test compound (10 µg/ml) were subcultured and inoculated subcutaneously into newborn Fischer rats. Rats were killed after three months and examined for tumors.

4. Test Cells: mammalian cells in culture
   ___ mouse lymphoma L5178Y cells
   ___ Chinese hamster ovary (CHO) cells
   ___ V79 cells (Chinese hamster lung fibroblasts)
   ___ other (list):
   _X_ rat embryo F1706 P95 and H4536 P+2 (clone of F1706 chronically infected with murine leukemia virus, RLV) cells

   Properly maintained? **Yes**
   Periodically checked for Mycoplasma contamination? Not specified

Periodically checked for karyotype stability? Not specified
Periodically "cleansed" against high spontaneous background?
Yes

Media: Eagle's Minimum Essential Medium (EMEM) supplemented with fetal bovine serum and antibiotics

5. End Points: Morphologically altered cells and macroscopic foci of increased cell density in semisolid agar.

6. Test compound concentrations used:

Preliminary cytotoxicity assay: 0.01, 0.1, 1.0, 10, and 100 μg/mL for five days.

Transformation assay: 0.1, 1.0. and 10.0 μg/mL.

B. TEST PERFORMANCE

1. In vitro cell treatment:

a. For the cytotoxicity assay, F1706 cells (500 cells/60 mm dish) were exposed to the test substance, negative control (acetone 1:1000) or positive control (0.1 μg/mL MCA) for 5 days. Triplicate dishes were assayed for each dose level and control. Dishes were stained with carbolfuchsin and the relative plating efficiency (RPE) was determined as follows:

RPE = [(avg. # of colonies per 3 test dishes)/
(avg. # of colonies per 3 media control dishes)] x 100.

b. For the transformation assay, confluent cultures of the F1706 and H4536 cells were diluted 1:4 on day 1 and fed on day 2 with medium containing the test substance at various concentrations. [Note that from the data given in the MRID, the exact cell densities used were illegible.] On day 4 cultures were subdivided 1:2 in medium containing the graded doses of the test substance on days 2 and 7. On day 9, cells were washed twice and re-fed with media no longer containing the test substance. After the 7-day exposure period, cells were subcultured a total of 12 times. At each passage, cultures were subdivided and one portion was continued through subculturing. Cells in the second portion were held for an additional 2 weeks, stained, and examined for transformed foci. Subcultures 3, 6, 10, and 12 of each cell line were screened for the transformed phenotype as indicated by the formation of macroscopic colonies in semisolid agar. Negative and positive controls were included in the assay.

11

2. Tumor Induction:

To test for tumor induction, separate litters of newborn Fischer rats (6-12 rats/litter) were inoculated subcutaneously with third passage (see above) F1706 and H4536 cells treated with the highest assayed concentration of the test substance (10 μg/mL), the positive control, or the negative control. For each rat, $5x10^5$ cells in a volume of in 0.05 mL of growth medium were injected. Animals were sacrificed at 3 months; tumors were removed and fixed in 10% neutral buffered formalin. Two animals/litter were subjected to a complete necropsy of the abdominal and thoracic cavities.

3. Statistical Methods: Not performed.

4. Evaluation Criteria: An assay was considered valid if (i) cell lines treated with the negative control showed no evidence of phenotypic transformation in the stained flask, did not grow in the soft agar, and did not induce tumors in newborn rats, (ii) cell lines treated with the positive control showed evidence of phenotypic transformation in the stained flask, grew in the semi-solid agar, and induced tumors in newborn rats, and (iii) there was no bacterial contamination in any of the cell lines.

II. RESULTS

A. Analytical Determination Analyses of the 200-μg/mL stock solution were performed initially and after 129 days of frozen (-20° C) storage. Both analyses indicated that the stock solution was 108% of nominal.

B. Preliminary cytotoxicity assay - Data from the study report are presented in Table 1. The cytotoxicity assay was conducted with F1706 cells exposed to DS-3701 at concentrations of 0.01, 0.1, 1.0, 10, or 100 μg/mL for five days. A 17% reduction in the RPE was noted at 10.0 μg/mL indicating only slight toxicity at this dose level. At 100 μg/mL the RPE was reduced by 96%. Based on these results, the cell transformation assay was conducted using DS-3701 dose levels of 0.1, 1.0, and 10.0 μg/mL.

Table 1. Preliminary cytotoxicity assay[a]

	Media	MCA µg/mL	Acetone	DS-3701 µg/mL				
		0.1	1:1,000	100	10	1.0	0.1	0.01
No. of macro-scopic colonies per dish	66 82 96 80 91 74	91 82 82	93 88 86	3 3 3	86 68 48	94 83 90	88 92 90	86 88 94
RPE	100	104	109	4	83	109	110	110

a Data extracted from the study report p.480

C. Mutagenicity assay - Data from the *in vitro* portion of the assay are presented in Tables 2 and 3. The F1706 and H4536 cell lines were not transformed upon exposure to DS-3701 at any of the assayed concentrations; no consistent phenotypic changes were noted in either cell line and macroscopic colonies did not grow on the semi-solid agar. By contrast, the positive control (0.1 µg/mL MCA) induced neoplastic transformation in both cell lines, however, macroscopic colonies were formed on the semi-solid agar for H4536 cells only. The acetone negative control did not transform either cell line.

13

Table 2. Transformation effects of DS-3701 on F1706 and H4536 cell lines[a].

Cell Line and Description	Passage Number	P+2*	P+3	P+5	P+7	P+8	P+9	P+10	P+12
F1706 P95 + Acetone 1:1,000		-	-	-	-	-	-	-	-
F1706 P95 + 0.1 µg/mL MCA		-	-	-	-	-	+**	±	+
F1706 P95 + 0.1 µg/mL DS3701		-	-	-	-	-	-	±	-
F1706 P95 + 1.0 µg/mL DS3701		-	-	lost	-	-	-	-	-
F1706 P95 + 10.0 µg/mL DS3701		-	-	-	-	-	-	-	-
H4536 P97 + Acetone 1:1,000		-	lost	-	-	-	±	-	-
H4536 P97 + 0.1 µg/mL MCA		-	-	-	-	±	±	+	+
H4536 P97 + 0.1 µg/mL DS3701		-	-	-	-	-	-	-	-
H4536 P97 + 1.0 µg/mL DS3701		-	-	-	-	-	-	-	-
H4536 P97 + 10.0 µg/mL DS3701		-	-	-	-	±	-	-	-

a Data were extracted from the study report pages 481 & 482.
* P+2 means 2 subcultures after removal of test compound.
** + mean phenotypic change from oriented, smooth, contact inhibited cells to fuel of cell lasking contact inhibition and orientation.

14

Table 3. Semi-solid agar assay[a].

Cell Line and Description	Passage Number	P+3*	P+6	P+10	P+12
F1706 P95 + Acetone 1:1,000		-	-	-	-
F1706 P95 + 0.1 µg/mL MCA		-	-	-	±
F1706 P95 + 0.1 µg/mL DS3701		-	-	-	-
F1706 P95 + 1.0 µg/mL DS3701		-	-	-	-
F1706 P95 + 10.0 µg/mL DS3701		±	-	-	-
H4536 P97 + Acetone 1:1,000		-	-	-	-
H4536 P97 + 0.1 µg/mL MCA		-	±	-	+**
H4536 P97 + 0.1 µg/mL DS3701		-	-	-	-
H4536 P97 + 1.0 µg/mL DS3701		-	-	-	-
H4536 P97 + 10.0 µg/mL DS3701		-	-	-	-

a Data extracted from the study report p.483.
* P+3 means 3 subcultures after removal of test compound.
** + means formation of macroscopic colonies by 3 weeks incubation in semi-solid agar.

Data from the *in vivo* portion of the assay are presented in Table 4. The F1706 cells treated with 10 µg/mL of the test substance induced late tumors in 6 of the 6 newborn rats. Tumors were first noted at 92 days post-inoculation and were growing progressively at termination (99 days post-inoculation). The H4536 cells treated with the test substance were non-tumorigenic with all 12 inoculated rats tumor-free at sacrifice (3 months). Positive controls (F1706 and H4536 cells treated with the MCA) induced tumors in 12 of 12 rats and 9 of 10 rats, respectively with tumors first noted at 67 and 70 days post-inoculation. Negative controls (acetone) exhibited no induced tumors.

15

Table 4. Animal tumor results[a,b].

Cell Line and Description	# of Animals Inoculated[c]		Date Inoculated	# Positive		Date of 1st tumor	# of Days	# Days to Highest % Tumors	% Positive
	Male	Female		Male	Female				
F1706 P95 + 1:1,000 Acetone	5	5	4-1-78	0	0	-	-	-	0.00
F1706 P95 + 0.1 µg/mL MCA	10	2	4-4-78	10	2	6-9-78	67	67	100.00
F1706 F95 + 10 µg/mL DS3701	1[d]	5	4-1-78	1	5	7-2-78	92	92	100.00
H4536 P97 + 1:1,000 Acetone	5	7	4-3-78	0	0	-	-	-	0.00
H4536 P97 + 0.1 µg/mL MCA	6[e]	4	4-1-78	5	4	6-9-78	70	70	90.00
H4536 P97 + µg/mL DS3701	4	8	4-2-78	0	0	-	-	-	0.00

a Data extracted from the study report p.484.
b Tumor-free animals held 97 to 99 days
c F344 newborn Fischer rats inoculated subcutaneously with 5 X 10^5 cells (0.5 mL) which had been treated 9 subcultures earlier with either test or control chemicals.
d Tumor excised and preserved (92 days post-inoculation)
e 2 tumors excised and preserved. One tumor was given to Diamond Shamrock (70 days post-inoculation) and the second sent to Bethesda for pathology (92 days)

Based on these results, the study author concluded that DS-3701 did not transform the H4536 cell line and that the results with the F1706 cell line were inconclusive.

III. **DISCUSSION:**

A. Reviewer's discussion - The reviewer agrees with the study author's conclusion that the test substance, DS-3701 did not induce transformations in the H4536 cell line and that the results of the F1706 assay are inconclusive. *In vitro*, no consistent phenotypic changes were noted in either cell line and macroscopic colonies did not grow on the semi-solid agar. Results *in vivo*, however, were not consistent. Treated F1706 cells (10 µg/mL of DS-3701) induced late tumors in all 6 of the inoculated newborn rats, while treated H4536 cells (10 µg/mL of DS-3701) were non-tumorigenic in all 12 inoculated newborn

16

rats.

B. Study deficiencies - No exogenous metabolic activation system was included in the assay and the stability of the test article was not determined. Signed and dated quality assurance, data confidentiality and flagging statements were not provided. In addition, results with the F1706 cell line were inconclusive. This study is **unacceptable** and cannot be upgraded.

Sign-off date: 02/07/97
DP Barcode: D229654
HED DOC Number: "NONE"
Toxicology Branch: TB2

18

DATA EVALUATION RECORD

SDS-3701 (CHLOROTHALONIL METABOLITE)

Study Type: §84-2; *In vitro* Chromosome Aberration Assay in Chinese Hamster Ovary (CHO) Cells

Work Assignment No. 2-35C (MRID 44022201)

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:
Steven Brecher, Ph.D. Signature: _____
Date: _____

Secondary Reviewer:
William J. Spangler, Ph.D. Signature: _____
Date: _____

Program Manager:
Mary L. Menetrez, Ph.D. Signature: _____
Date: _____

Quality Assurance:
Michael Norvell, Ph.D. Signature: _____
Date: _____

Disclaimer

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

EPA Reviewer: Alan Levy, PhD _____
Review Section I, Toxicology Branch II (7509C)

EPA Secondary Reviewer: Nancy McCarrol _____
Review Section III, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in Chinese hamster ovary (CHO) cells

OPPTS Number: 870.5375 OPP Guideline Number: (§84-2)

DP BARCODE: D229654 SUBMISSION CODE: S509030
P.C. CODE: 081901 TOX. CHEM. NO.: None

TEST MATERIAL (PURITY): SDS-3701 (Chlorothalonil metabolite, 99.2% active ingredient, a.i.)

SYNONYMS: 4-Hydroxy-2,5,6-trichloroisophthalonitrile

CITATION: Mizens, M. and Laveglia, J. (1994) In Vitro Mammalian Cytogenetic Test with SDS-3701. Department of Toxicology and Animal Metabolism, Ricerca, Inc., 7528 Auburn Road, Painesville, Ohio. Project No. 94-0048. October 17, 1994. MRID 44022201. Unpublished.

Putman, D., Curry, P., and Schadly, E. (1994) *In Vitro* Mammalian Cytogenetic Test with SDS-3701. Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland. Ricerca Project No. 94-0048. September 23, 1994. MRID 44022201. Unpublished.

SPONSOR: ISK Biosciences Corporation, 5966 Heisley Road, P.O. Box 8000, Mentor, OH

EXECUTIVE SUMMARY:

In a mammalian cell chromosome aberration assay (MRID 44022201), Chinese Hamster ovary (CHO) cells were exposed to SDS-3701 (Chlorothalonil metabolite, 99.2% a.i.) in dimethyl sulfoxide at concentrations ranging from 8.2 to 2,080 µg/mL with and without S9 activation. Cultures without metabolic activation were exposed for 20 and 44 hours prior to harvesting. Cultures with metabolic activation were exposed for 4 hours and harvested 16 and 40 hours following the termination of treatment. The high doses used for evaluation of clastogenic effects (260 µg/mL, -S9 with 20 hour harvest; 130 µg/mL, -S9 with 44 hour harvest; or 520 µg/mL, +S9 with 20 and 44 hour harvests) were cytotoxic concentrations that provided sufficient cells for analysis.

In the absence of metabolic activation, statistically significant increases in the percentage of cells with structural chromosome aberrations were obtained at 130 µg/mL after 20 and 44 hours of treatment. A dose response in one trial was not reproducible. In the presence of metabolic activation, statistically significant increases in the percentage of cells with structural chromosome aberrations were obtained at 400 and 520 µg/mL in cells harvested 16 after treatment and at 260, 300, 400, and 520 µg/mL in cells harvested 40 hours after treatment. A reproducible dose response was also observed in the presence of metabolic activation. Positive controls induced the appropriate response.

This study is classified as **acceptable** and satisfies the guideline requirement **(§84-2)** for *in vitro* cytogenetic mutagenicity studies .

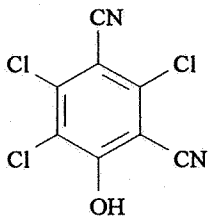
COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

21

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: SDS-3701 (Chlorothalonil metabolite)
Description: White powder
Lot/Batch #: 0301
Purity: 99.2% a.i.
Stability of compound: Reported to be stable for at least three years; expiration date (April 1999) obtained from MRID 44022202
CAS #: 28343-61-5
Structure:



Solvent used: Dimethyl sulfoxide (DMSO)
Other comments: The test article was stored at room temperature, protected from light. The test article, at the stock concentration of 208 mg/mL, formed a suspension in DMSO that went into solution when warmed to 37° C for 15 minutes.

2. Control Materials:
Negative: DMSO
Solvent/final concentration: DMSO/1%
Positive:
Activation: Cyclophosphamide (6.25, 12.5, 25, and 50 μg/mL)
Nonactivation: Triethylenemelamine (0.25, 0.5 μg/mL) and Mitomycin C (0.08, 0.15 μg/mL).

Note: Triethylenemelamine was used in the first assay and mitomycin C was used in the repeat assay. Distilled water was the solvent for the positive controls. The highest dose level of each positive control with sufficient scorable metaphases was selected for analysis and is the only one reported in the tables.

3. Activation - The S9 was derived from Aroclor 1254 induced liver from male Sprague-Dawley rats. It was prepared in advance and stored at approximately -70 C until use. The S9 fraction was thawed just prior to use, and 20 μL of S9 mixed with 1 mM NADP, 2 mM $MgCl_2$, 6 mM KCl, and 1 mM glucose-6-phosphate was added per

mL of culture medium to give a final S9 concentration of 2%.

4. Test compound concentrations used

a. Cytotoxicity Assays: Cytotoxicity was evaluated in parallel with the cytogenetic assays.

b. Cytogenetic Assays:

1) Nonactivated conditions: Nine dose levels of SDS-3701 (8.2, 16.3, 32.5, 65, 130, 260, 520, 1,040, and 2,080 µg/mL - 20 hour treatment) were tested in the initial assay; five dose levels (130, 160, 200, 230, and 260 µg/mL - 20 hour treatment) and six dose levels (8.2, 16.3, 32.5, 65, 130, and 260 µg/mL - 44 hour treatment) were tested in the repeat assays.

2) Activated conditions: Nine dose levels of SDS-3701 (8.2, 16.3, 32.5, 65, 130, 260, 520, 1,040, and 2,080 µg/mL - 4 hour treatment/16 hour recovery) were tested in the initial assay; five dose levels (260, 300, 400, 520, and 600 µg/mL - 4 hour treatment/16 and 40 hour recovery) were tested in the repeat assays.

For both the activated and nonactivated conditions, duplicate cultures were used at each dose level, solvent and untreated (negative) controls were included, and the cytotoxicity assays were performed as integral parts of the mutagenicity assays.

5. Test cells - Chinese hamster ovary (CHO) cell line K_1 was the test system used. The cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Cultures were incubated at 37 C° in a humidified atmosphere containing 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? **Yes**

B. TEST PERFORMANCE

1. Treatment - Cultures were seeded with CHO cells at a density of $5x10^5$ cells/flask (20 hour studies) or $2.5x10^5$ cells/flask (44 hour studies) in 5 mL of nutrient medium and incubated for 16-24 hours prior to

23

treatment. A 50 µL aliquot of the test or control article in solvent, or solvent alone was added to the cells in culture medium. For cultures requiring metabolic activation, the 5 mL of culture medium included the S9-cofactor mix. An untreated control containing cells in complete medium or S9-containing medium was included. A sampling time of 20 hours (1.5x the cell cycle) was selected and, for the repeat assay, a second sampling at 44 hours (24 after the first one) was selected.

The mitotic index for each culture was recorded as the percentage of cells in mitosis per 500 cells counted. Toxicity was determined by comparing the mitotic index with the corresponding value for the solvent control. The highest test article concentration demonstrating at least 50% mitotic inhibition compared to the solvent control was selected as the highest concentration for analysis of structural chromosome aberrations, together with three or four lower concentrations. In addition, at the 44-hour harvest, the percent polyploid cells was recorded per 100 metaphase cells.

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

3. Cell harvest - In cultures without metabolicactivation, cells were harvested immediately after termination of treatment. In cultures with metabolic activation, cells were harvested 16 and 40 hours after termination of treatment. The experiment was terminated by hypotonic treatment (0.075 M KCL), followed by fixation (methanol:acetic acid, 3:1) overnight at 2-6 C.

4. Details of slide preparation - Following fixation, cells were dropped onto glass slides and air dried. The slides were stained with 5% Giemsa for analysis.

5. Metaphase analysis
No. of cells examined per dose: **200**
No. of cells examined in the solvent control: **200**
No. of cells examined in the cyclophosphamide positive control: **200**
No. of cells examined in the trietylenemelamine/ mitomycin C positive control: **200**
Scored for structural aberrations: **Yes**
Scored for numerical polyploidy: **Yes**
Coded prior to analysis: **Yes**

24

6. Evaluation criteria - The assay was considered valid if the following criteria were met: (i) the frequency of cells with structural chromosome aberrations in either the untreated or solvent control was no greater than 6% and (ii) the percentage of cells with chromosome aberrations in the positive control was statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the untreated control.

   A positive response was claimed if one of the following conditions were met: (i) the percentage of metaphases with aberrations was increased in a dose responsive manner with one or more dose levels being statistically elevated relative to the solvent control group ($p \leq 0.05$) or (ii) there was a reproducible and significant increase in aberration frequency at a single dose level.

7. Statistical analysis - Fisher's exact test was used for pairwise comparison of the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

II. RESULTS

A. Analytical Determinations:
The concentrations of the stock dose preparations from the initial (208 mg/mL) and repeat (60 mg/mL) assays were analyzed by high performance liquid chromatography to confirm the intended concentrations of the test substance in the solvent. The concentrations found were 104% and 101% of the nominal concentrations, respectively.

B. Cytotoxicity assessment - In the initial assay without metabolic activation (20 hour treatment), and nine concentrations ranging from 8.2 to 2,080 µg/mL, the highest concentration of 260 µg/mL selected for chromosome analysis reduced the mitotic index by 94% relative to the solvent control. Higher concentrations resulted in complete mitotic inhibition. In the initial assay with metabolic activation (4 hour treatment/16 hour recovery), and nine concentrations ranging from 8.2 to 2,080 µg/mL, the highest concentration of 520 µg/mL selected for chromosome analysis reduced the relative mitotic index by 86%. Higher concentrations resulted in complete mitotic inhibition.

25

In the repeat assays without activation, the 20 hour treatment had five concentrations ranging from 130 to 260 μg/mL, and the highest concentration of 260 μg/mL selected for chromosome analysis reduced the relative mitotic index by 89%. The 44 hour treatment had six concentrations ranging from 8.2 to 260 μg/mL, and the highest concentration of 130 μg/mL selected for chromosome analysis reduced the relative mitotic index by 87%. Complete mitotic inhibition was observed at 260 μg/mL. In the repeat assays with activation, the 4 hour treatment/16 hour recovery had five concentrations ranging from 260 to 600 μg/mL, and the highest concentration of 520 μg/mL selected for chromosome analysis reduced the relative mitotic index by 62%. The 4 hour treatment/40 hour recovery had 5 concentrations ranging from 260 to 600 μg/mL, and the highest concentration of 520 μg/mL selected for chromosome analysis reduced the relative mitotic index by 91%. Complete mitotic inhibition was observed at 600 μg/mL at both the 20 and 44 hour harvests. The results are shown in Appendix 1 (study report Tables 7 and 8, pages 22 and 23).

C. Cytogenetic assay - The results are summarized in Table 1. In the initial assay without metabolic activation (20 hour treatment), the percentage of cells with structural aberrations was significantly increased at concentrations of 65, 130, and 260 μg/mL in a dose-related manner. In the initial assay with activation (4 hour treatment/16 hour recovery), the percentage of cells with structural aberrations was significantly increased at 520 μg/mL and a dose response was observed.

In the repeat assays without activation, the percentage of cells with structural aberrations in the 20-hour treatment was not significantly increased at any concentration. In the 44 hour treatment, the percentage of cells with structural aberrations was significantly increased at 130 μg/mL, but a dose response was not observed. In the repeat assays with activation, the percentage of cells with structural aberrations in the 4 hour treatment/16 hour recovery was significantly increased at 400 and 520 μg/mL and a dose response was observed. In the 4 hour treatment/40 hour recovery, the percentage of cells with structural aberrations was significantly increased at all concentrations tested (260, 300, 400, and 520 μg/mL), and a dose response was observed.

The percentage of polyploid cells was not significantly increased at 44 hours at any dose level with or without

26

metabolic activation. The positive and negative controls fulfilled the requirements for a valid test.

Table 1. Summary of Cytogenetic Results[a]

Treatment (μg/mL)	S9 Activation	Harvest Time (hrs)	Cells With Structural Aberrations (%)
Initial			
DMSO	-	20	0.5
32.5	-	20	0.0
65	-	20	3.5*
130	-	20	4.5**
260	-	20	7.4**
TEM	-	20	18.0**
DMSO	+	20	1.0
260	+	20	3.0
520	+	20	14.0**
CP	+	20	57.0**

27

Repeat			
DMSO	+	20	3.5
300	+	20	2.0
400	+	20	24.0**
520	+	20	42.5**
CP	+	20	70.5**
DMSO	-	44	0.5
65	-	44	1.0
130	-	44	23.6**
MMC	-	44	56.5**
DMSO	+	44	0.5
260	+	44	7.0**
300	+	44	4.0*
400	+	44	8.5**
520	+	44	23.5**
CP	+	44	100.0**

[a]extracted from study report Tables 7 and 8
TEM - Triethylenemelamine (0.5 μg/mL)
CP - Cyclophosphamide (25 μg/mL)
MMC - Mitomycin C (0.08 μg/mL)
* $p \leq 0.05$; ** $p \leq 0.01$; Fisher's exact test

III. DISCUSSION

A. Investigators' Conclusions - The test article was a weak positive for structural chromosome aberrations without metabolic activation and positive with metabolic activation. It was negative for numerical chromosome aberrations with and without metabolic activation.

B. Reviewer's Discussion - SDS-3701 was tested to cytotoxic concentrations (260 μg/mL, -S9 with 20 hour harvest; 130 μg/mL, -S9 with 44 hour harvest; or 520 μg/mL, +S9 with 20 and 44 hour harvests) and the limit of solubility. We agree with the authors' conclusions that the test article was positive for structural chromosome aberrations and negative for numerical chromosome aberrations with and without metabolic activation. We note that under all assay conditions,

28

damage to the chromosomes was primarily manifested as chromatid-type aberrations. The sensitivity of the assay system to detect damage to chromosomes was adequately demonstrated by the results obtained with the positive controls. We conclude, therefore, that the results of this study provide sufficient evidence to consider SDS-3701 positive in this *in vitro* test system.

IV. STUDY DEFICIENCIES

No deficiencies that would be expected to alter the conclusions of the study were identified.

Sign-off date: 02/07/97
DP Barcode: D229654
HED DOC Number: "NONE"
Toxicology Branch: TB2

30

DATA EVALUATION RECORD

SDS-3701 (CHLOROTHALONIL Metabolite)

Study Type: §84-2; *In vivo* Chromosome Aberration Assay in Chinese Hamster Bone Marrow Cells

Work Assignment No. 2-35B (MRID 44022202)

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: Steven Brecher, Ph.D.	Signature: _____ Date: _____
Secondary Reviewer: William J. Spangler, Ph.D.	Signature: _____ Date: _____
Program Manager: Mary L. Menetrez, Ph.D.	Signature: _____ Date: _____
Quality Assurance: Michael Norvell, Ph.D.	Signature: _____ Date: _____

Disclaimer

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

31

EPA Reviewer: Alan Levy, Ph.D. _____
Review Section I, Toxicology Branch II (7509C)

EPA Secondary Reviewer: Nancy McCarrol, B.S. _____
Review Section III, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: *In vivo* mammalian chromosome aberrations in chinese hamster bone marrow cells

OPPTS Number: 870.5385 OPP Guideline No: §84-2

DP BARCODE: D229654 SUBMISSION CODE: S509030
P.C. CODE: 081901 TOX. CHEM. NO.: None

TEST MATERIAL (PURITY): SDS-3701 (Chlorothalonil metabolite, 99.2% active ingredient, a.i.)

SYNONYMS: 4-Hydroxy-2,5,6-trichloroisophthalonitrile

CITATION: Mizens, M. and Laveglia, J. (1995) In Vivo Bone Marrow Chromosomal Analysis in Chinese Hamsters with SDS-3701. Department of Toxicology and Animal Metabolism, Ricerca, Inc., 7528 Auburn Road, Painesville, Ohio. Project No. 94-0049. June 2, 1995. MRID 44022202. Unpublished study.

Proudlock, R., Taylor, K., and Elmore, E. (1995) SDS-3701 In Vivo Bone Marrow Chromosomal Analysis in Chinese Hamsters. Huntingdon Research Centre Ltd., P.O. Box 2, Huntingdon, Cambridgeshire, England. Ricerca Project No. 94-0049. May 26, 1995. MRID 44022202. Unpublished study.

SPONSOR: ISK Biosciences Corporation, 5966 Heisley Road, P.O. Box 8000, Mentor, Ohio

EXECUTIVE SUMMARY:

In an *in vivo* bone marrow chromosome aberration assay (MRID 44022202), male and female Chinese hamsters were given a single oral dose of SDS-3701 (99.2% purity) in methylcellulose at levels of 125, 250, and 500 mg/kg bodyweight. Bone marrow was sampled 6, 24, and 48 hours after treatment. Results from a preliminary toxicity test at levels ranging from 432 to 2,000 mg/kg indicated that 500 mg/kg was the estimated maximum tolerated dose (MTD).

No clinical signs of toxicity were noted up to the highest dose tested (500 mg/kg). The positive control induced the expected high yield of cells with abnormal chromosome morphology. There

32

was, however, no convincing evidence that SDS-3701 induced a clastogenic response. There was no significant increase in the incidence of chromosome damage at either the 24- or 48- hour sampling times. A significant increase in the incidence of aberrant cells was obtained at the 6-hour sampling time. However, since the increase was not dose related and was not reproduced at later sampling times, it was not considered to be related to the test substance.

This study is classified as **acceptable** and satisfies the guideline requirement **(§84-2)** for *in vivo* cytogenetic mutagenicity studies.

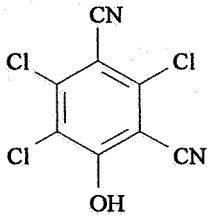
COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

33

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: SDS-3701 (Chlorothalonil metabolite)
Description: White powder
Lot/Batch #: 0301
Purity: 99.2% a.i.
Stability of compound: Reported to be stable for at least three years; expiration date - April 1999
CAS #: 28343-61-5
Structure:



Vehicle used: Methylcellulose (1% aqueous)
Other comments: The test substance was stored at room temperature protected from light.

2. Control Materials:

Vehicle/Final volume/Route of administration:
Methylcellulose; 20 mL/kg; oral gavage
Positive/Final dose(s)/Route of administration:
Cyclophosphamide 40 mg/kg in purified water; oral gavage

3. Test compound administration:
Volume of test substance administered: 20 mL/kg
Route of administration: Oral gavage
Dose levels used: Preliminary toxicity test - 432, 720, 1,200, and 2,000 mg/kg; Cytogenetic assay - 125, 250, and 500 mg/kg

Rationale for dose selection: Selection of doses for the preliminary toxicity test was based on the known toxicity of the test substance. Selection of doses for the cytogenetic assay was based on the MTD estimated from the toxicity test.

4. Test animals: Species: Chinese hamster

a. Age 7-16 weeks
Weight (initiation): Cytogenetic assay: male 25.1-39.1 g, female 22.3-36.5 g
Source: Wrights of Essex Breeding Centre Ltd., Chelmsford, Essex, England

34

b. No. animals used per dose: 5 males; 5 females/sacrifice
Note: An additional group of 6 males and 6 females received the high dose and were used as replacement animals in the event of unscheduled deaths in the primary group.

c. Properly maintained? Yes

B. TEST PERFORMANCE

1. Treatment and Sampling Times:
a. Test compound and vehicle control:
Dosing: single dose by oral gavage
Sampling (after last dose): 6, 24, and 48 hours

b. Positive control:
Dosing: single dose by oral gavage
Sampling (after last dose): 24 hours

Colchicine administered: 2 hours before sacrifice by intraperitoneal injection at 4 mg/kg

2. Tissues and Cells Examined: 50 bone marrow metaphase spreads were examined per animal.

3. Details of slide preparation: Animals from each dose group were sacrificed by cervical dislocation. Marrow was aspirated from both femurs, and suspended in Hank's balanced salt solution. Cells were swollen in hypotonic KCl, fixed overnight at 4 C in methanol: glacial acetic acid (3:1), dropped onto glass slides and air dried. The cells were then stained with Giemsa.

Scored for structural: **Yes**
Scored for numerical: **No**
Coded prior to analysis: **Yes**

4. Statistical methods: Significant relationships between the number of cells with aberrations in individual treated and positive control groups relative to the concurrent negative control were determined using the Wilcoxon sum of ranks test. Multiple group comparisons were made using the Kruskal-Wallis test. Jonckheere's test was used to analyze trend. Where no obvious differences were noted between males and females, the two sexes were combined for statistical analysis.

5. Evaluation Criteria: The criteria for a valid test were not reported. A positive response was indicated by a statistically significant ($p<0.01$), dose-related increase in the incidence of aberrant metaphases for the treatment groups compared with the concurrent negative control group.

35

II. REPORTED RESULTS

A. Solubility and Stability: Dose formulations of the test substance were prepared as suspensions in 1% methylcellulose. Analytical determination of the dose formulations from the preliminary toxicity test indicated that the concentrations were between 95% and 106% of nominal. The results from the cytogenetic assay were between 99% and 102% of nominal.

B. Preliminary Toxicity Test: Animals were examined regularly for toxic signs and mortality up to 48 hours after treatment when surviving animals were weighed and sacrificed. In the preliminary toxicity test, 2 of 4 animals receiving 720 mg/kg and 3 of 4 animals receiving 1,200 or 2,000 mg/kg died. Animals in the 432 mg/kg dose group exhibited increased respiratory rate and piloerection. Bodyweight depression was observed in the 2,000 mg/kg dose group. Based on these results, the MTD was estimated to be 500 mg/kg.

C. Cytogenetics assay:

1. Animal Observations: No clinical signs of toxicity were observed in any dose group in the cytogenetic assay, except that one animal in the 250 mg/kg dose group was found dead one hour after dosing. A necropsy indicated no sign of mis-dosing. No marked reduction in bodyweight was observed in any dose group.

2. Chromosome aberrations: Animals treated with SDS-3701 did not show any significant increase in the incidence of chromosome damage at either the 24- or 48- hour sampling times. A significant increase in the incidence of aberrant cells was obtained at the 6-hour sampling time ($p<0.01$ using the Kruskal-Wallis test). However, since the increase was not dose related and was not reproduced at later sampling times, it was not considered to be related to the test substance. In addition, there was a relatively low incidence of aberrations for the concurrent negative control group (0.2%) compared with the negative control group for the 48-hour sampling time (1.4%). The positive control showed a substantial and statistically significant increase in the incidence of cells with chromosome aberrations ($p<0.001$ using the Wilcoxon sum of ranks test). A summary of the results is presented as an attachment at the end of this DER (study report Table 1, page 19).

36

III. DISCUSSION/CONCLUSIONS:

A. Investigator's Conclusions
A single oral dose of SDS-3701, at levels up to the MTD, did not cause chromosome damage in bone marrow cells of Chinese hamsters.

B. Reviewer's Discussion
We concur with the study authors that SDS-3701 did not cause chromosome damage in this *in vivo* assay when tested to the estimated MTD. We assess that the lack of any sign of compound toxicity suggests that the test animals could have tolerated a higher dose. However, since there was no evidence of a genotoxic response, it is not likely that testing at a higher dose level would alter the outcome of the study. The sensitivity of this test to detect a genotoxic response was demonstrated by a significant increase in the number of cells with chromosome aberrations induced by the positive control. We conclude, therefore, that the study provided acceptable evidence that SDS-3701 was not clastogenic when tested to a dose near the MTD.

C. STUDY DEFICIENCIES - The lack of any sign of compound toxicity suggests that the test animals could have tolerated a higher dose. However, since there was no evidence of a genotoxic response, it is not likely that testing at a higher dose level would alter the outcome of the study.

ATTACHMENT

THE FOLLOWING ATTACHMENT IS NOT AVAILABLE ELECTRONICALLY
SEE THE FILE COPY

Sign-off date: 02/07/97
DP Barcode: D229654
HED DOC Number: "NONE"
Toxicology Branch: TB2