



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

2-10-94

FEB 10 1994

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

Subject: EPA ID # 007969-00057: Vinclozolin, CIC DER for a
Mammalian Cells in Culture Assay in CHO Cells, HLA
10536-0-437, BASF 31M0375/889128 (MRID No. 414969-02).

Tox. Chem. No.: 323C. Case No.: 011409.
PC Code: 113201. Submission No.: S443004.
DP Barcode: D192484. MRID No.: 414969-02.

From: David G Anderson, PhD. *David G Anderson 1/19/94*
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Thru: Karen Hamernik, PhD. *K.H. 11/31/94* *KB 2/4/94*
Section 3 Head,
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BASF submitted for review a report of a mutagenicity with vinclozolin.

Murli, H (1989) Mutagenicity Test on Reg. NO. 83 258, Batch 183 (ZST No. 88/375) In an *In Vitro* Cytogenetic Assay Measuring Chromosomal Aberration Frequencies in Chinese Hamster Ovary Cell (CHO) Cells (84-2), HLA 10536-0-437, BASF Proj. No. 31M0375/889128. Conducted by Hazelton Laboratories America, Inc. for BASF Corp., Agricultural Chemicals, report issued March 7, 1989. (MRID# 414969-02)

CONCLUSIONS:

Vinclozolin was administered at a soluble level of 125 µg/ml, +/- S9, insoluble levels ≥ 250 µg/ml, +/- S9, and a cytotoxic high dose level of 500 µg/ml, +/- S9, in a test for induction of chromosomal aberrations in CHO cells (1989). The test was negative.



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Core Classification: Acceptable. The study satisfies the requirements for Guidelines Series 84-2 for structural chromosome aberrations, category II.

Cover memo for CIC DER for chromosomal aberration, cat. II, HLA
10536-0-437; BASF 31M0375/889128/414969-
02/A:\VINCLV33.23C\CMMCA.CHO/DANDERSON/1/19/94.*

FINAL

DATA EVALUATION REPORT

VINCLOZOLIN

Study Type: Mutagenicity: Mammalian Cells in Culture Cytogenetic Assay
in Chinese Hamster Ovary Cells

Prepared for:

Health Effects Division
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Contract Number: 68D10075
Work Assignment Number: 2-138
Clement Number: 473
Project Officer: Caroline Gordon

GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE CYTOGENETICS

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Signature: *Irving Mauer*
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Signature: *Marion Copley*
Date: 11/29/93

DATA EVALUATION REPORT

STUDY TYPE: Mammalian cells in culture cytogenetic assay in Chinese hamster ovary cells

CHEMICAL: Vinclozolin

TOX CHEM. NUMBER: 323 C

PC Code: 113201

MRID Number: 414969-02

SYNONYMS/CAS NO.: 3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione

SPONSOR: BASF Corporation, Research Triangle Park, NC

TESTING FACILITY: Hazleton Laboratories America, Inc., Kensington, MD

TITLE OF REPORT: Report on the Mutagenicity Test on Reg. No. 83 258 (Vinclozolin) in an In Vitro Cytogenetic Assay Measuring Chromosomal Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells

AUTHOR: H. Murli

STUDY NUMBERS: HLA Study No. 10536-0-437; Registration Document No. BASF: 89/0073; Sponsor Project No. 31M0375/889128

REPORT ISSUED: March 7, 1989

CONCLUSIONS-EXECUTIVE SUMMARY: Negative for inducing chromosomal aberrations in Chinese hamster ovary (CHO) cells at a soluble level of 125 $\mu\text{g}/\text{mL}$ +/- S9, insoluble levels ≥ 250 $\mu\text{g}/\text{mL}$ +/- S9, and a cytotoxic high dose (500 $\mu\text{g}/\text{mL}$ +/- S9).

CORE CLASSIFICATION: Acceptable. The study satisfies the requirements for Guideline Series 84-2 genetic effects Category II, Structural Chromosome Aberrations and is acceptable for regulatory purposes.

MAMMALIAN CELLS IN CULTURE CYTOGENETICS

A. MATERIALS:

1. Test Material: Vinclozolin

Description: White powder

Identification numbers: 88/375 Reg. No. 83 258; Batch No. 183
(= ZST No. 88/375)

Purity: Assumed to be at least 99% (Based on analysis conducted by the performing laboratory-- see study report p. 43)

Receipt date: August 29, 1988

Stability: Not reported

Contaminants: None listed

Solvent used: Dimethyl sulfoxide (DMSO)

Other comments: Information included in the analytical report indicated that the test material was stored at room temperature. Analytical determinations were performed on dosing solutions to verify achieved concentrations.

2. Control Materials:

Negative: Untreated cells in McCoy's 5a medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and antibiotics.

Solvent/final concentration: DMSO/10 μ L/mL

Positive:

Nonactivation: Mitomycin C (MMC) was prepared in an unspecified solvent to yield final concentrations of 0.25 and 0.5 μ g/mL for the preliminary cytotoxicity assay and 0.04 or 0.08 μ g/mL for the cytogenetic assay. Only one dose was scored for chromosomal aberrations.

Activation: Cyclophosphamide (CP) was prepared in an unspecified solvent to yield final concentrations of 12.5 or 20 μ g/mL for the preliminary cytotoxicity assay and 12.5 or 25 μ g/mL for the cytogenetic assay. Only one dose was scored for chromosomal aberrations.

3. Activation: S9 derived from male Sprague Dawley (weight or age not specified)

<input checked="" type="checkbox"/> Aroclor 1254	<input checked="" type="checkbox"/> induced	<input checked="" type="checkbox"/> rat	<input checked="" type="checkbox"/> liver
<input type="checkbox"/> phenobarbital	<input type="checkbox"/> noninduced	<input type="checkbox"/> mouse	<input type="checkbox"/> lung
<input type="checkbox"/> none		<input type="checkbox"/> hamster	<input type="checkbox"/> other
<input type="checkbox"/> other:		<input type="checkbox"/> other	

The report did not indicate whether the S9 fraction was prepared in house or was purchased from a commercial source.

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The components of the S9 mix were as follows:

<u>Component</u>	<u>Final Concentration</u>
NADP	1.5 mg/mL
Isocitric acid	2.7 mg/mL
S9 homogenate	15 μ L/culture

4. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Concentrations ranging from 0.167 to 500 μ g/mL were evaluated with and without S9 activation.

(b) Cytogenetic assay:

(1) Nonactivated conditions: Five doses (50, 125, 250, 375, and 500 μ g/mL) were assayed with a 20-hour cell harvest.

(2) S9-activated conditions: Five doses (50.1, 125, 250, 375, and 501 μ g/mL) were assayed with a 10-hour cell harvest.

5. Test Cells: Chinese hamster ovary cells (CHO) were obtained from the laboratory of Dr. S. Wolf, University of California, San Francisco. CHO cell cultures were initiated in complete McCoy's 5a medium. Cultures were incubated for 24 hours at 37°C prior to treatment.

Properly maintained? Yes.

Cell line or strain periodically checked for mycoplasma contamination?
Not reported.

Cell line or strain periodically check for karyotype stability? Yes.

B. TEST PERFORMANCE:

1. Cell Treatment: Cells were exposed to the test material, solvent or positive control for ≈ 17.25 hours (nonactivated) 2 hours (activated).

2. Protocol:

(a) Preliminary cytotoxicity assay: Cell cultures, seeded at 0.3×10^6 cells/flask, were exposed to half-log dilutions of the test material ranging from 0.0167 to 500 μ g/mL, the negative control (culture medium), the solvent control (DMSO), or the positive controls (MMC -S9; CP +S9).

In the nonactivated system, cells were exposed for 2 hours to the test material; BrdU (final concentration: 10 μ M) was added to the cultures, and incubation was continued for 23 hours. Approximately 27.5 hours before the cell harvest, monolayers were washed, refed with fresh complete medium containing BrdU and reincubated in the presence of 0.1 μ g/mL colcemid.

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In the S9-activated system, cultures were exposed for 2 hours without FCS. After exposure, cells were washed twice, refed with complete medium containing BrdU (final concentration: 10 μ M) and reincubated for 23 hours. Colcemid was added, and cultures were incubated for an additional 2.5 hours.

After incubation, monolayers were visually evaluated for confluency and mitotic or dead cells. Surviving cells from three of the highest nonactivated and S9-activated doses were evaluated for cell-cycle kinetics.

Metaphase cells were collected by mitotic shake-off, swollen in a hypotonic 0.075 M solution of KCl and fixed in absolute methanol:glacial acetic acid (3:1). Estimation of cell-cycle delay was accomplished by staining the cells with the modified fluorescence-plus-Giemsa techniques of Perry and Wolff¹ and Goto et al.² One hundred metaphase cells per culture were examined for the percentage of first (M_1), between first and second (M_{1+}), and second or beyond second ($\geq M_2$) division metaphases. Based on the findings, doses and harvest times were selected for the cytogenetic assay.

(b) Cytogenetic assay:

- (1) Treatment: Cultures (in duplicate), seeded at 1.2 to 1.5×10^6 cells, were exposed to the selected test material doses, the negative, solvent, or positive controls in both the presence and absence of S9 activation.

In the nonactivated system, cells were dosed for 17.25 hours. Cultures were washed, refed medium containing colcemid and reincubated for approximately 2.5 hours. Under S9-activated conditions, cells were exposed for 2 hours, washed, refed culture medium and incubated for 7.5 hours. Colcemid was added 2.5 hours before the cultures were harvested.

Cells were collected, swollen and fixed as described for the preliminary cytotoxicity assay. Slides were stained with 5% Giemsa and coded.

- (2) Metaphase analysis: One hundred morphologically normal cells (containing 19-23 centromeres) per culture from four dose levels of the test material and the negative, solvent and positive controls were scored for chromosome aberrations.

¹Perry, P. and Wolff, S. (1974). New Giemsa method for the differential staining of sister chromatids. Nature 251:156-158.

²Goto, K., Maeda, S., Kano, Y., and Sugiyama, T. (1978). Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma 66:351-359.

- (3) Statistical methods: The data were evaluated for statistical significance at $p \leq 0.05$ by Fisher's exact test with an adjustment for multiple comparisons.³ The negative and solvent (DMSO) controls were pooled if no statistical differences were found.
- (4) Evaluation criteria: The biological significance of the results was evaluated relative to the overall chromosome aberration frequencies, percentage of cells with aberrations, percentage of cells with >1 aberration, dose-response, and the types of aberrations observed.

C. REPORTED RESULTS:

1. Test Material Solubility: Stock solutions of the test material prepared in DMSO to contain ≥ 147 mg/mL were not soluble when added to culture medium. At an applied concentration of 488 $\mu\text{g/mL}$, an opaque suspension was obtained in the culture medium. Accordingly, 500 $\mu\text{g/mL}$ was selected as the starting concentration for the preliminary cytotoxicity assay.
2. Preliminary Cytotoxicity Assay: Compound precipitation was reported at the two highest doses (167 and 500 $\mu\text{g/mL}$) under both nonactivated and S9-activated conditions. Both levels without S9 activation were cytotoxic to the monolayers and caused severe mitotic suppression (Table 1). Cell-cycle delay was also apparent at 50 $\mu\text{g/mL}$ -S9. In the presence of S9 activation, 500 $\mu\text{g/mL}$ caused a slight reduction in visible mitotic cells and monolayer confluence. Cytotoxicity was not observed at lower levels and there was no clear evidence of interference with cell-cycle progression. Based on these observations, the study author selected a nonactivated dose range of 50-500 $\mu\text{g/mL}$ with a 20-hour delayed harvest and a dose range of 50-500 $\mu\text{g/mL}$ with a 10-hour harvest for the S9-activated test.
3. Cytogenetic Assays: The study author indicated that compound precipitation was observed at the three highest nonactivated and S9-activated test material levels. Results for both phases of testing were as follows:
 - (a) Nonactivated assay: Floating debris and a reduction in mitotic cells were reported at 500 $\mu\text{g/mL}$. No signs of cytotoxicity were observed at doses ≤ 375 $\mu\text{g/mL}$. As the data presented in Table 2 show, there were no significant increases in the percentage of cells with chromosomal aberrations in the treatment groups.

³Sokal, R.R. and Rohlf, F.J. 1981. Biometry, Ed. W.H. Freeman and Company, New York.

TABLE 1. Representative Results of the Preliminary Test for Delay of Cell-Cycle Progression with Vinclozolin

Substance	Dose/mL	S9 Activation	% Cells ^a			Monolayer Confluence (%)
			M ₁	M ₁ +	≥M ₂	
<u>Negative Control</u>						
Culture medium	--	-	6	11	83	100
	--	+	3	8	89	100
<u>Solvent Control</u>						
Dimethyl sulfoxide	10 μL	-	9	14	77	100
	10 μL	+	6	20	74	100
<u>Test Material</u>						
Vinclozolin	50 μg ^b	-	85	13	2	100
	167 μg ^c	-	100	0	0	71
	500 μg ^c	-	100	0	0	71
	50 μg ^b	+	5	13	82	100
	167 μg ^c	+	6	30	64	100
	500 μg ^c	+	14	34	52	86

^aPercent cells in first (M₁), between first and second (M₁+), and in second or beyond (≥M₂) division
^bCultures exposed to 16.7 μg/mL +/- S9 were not evaluated.
^cCompound precipitation

Note: Data were extracted from the study report p. 24.

Table 2. Representative Results from the In Vitro Cytogenetic Assay in Chinese Hamster Ovary Cells Treated with Vinclozolin in the Absence of S9 Activation

Substance	Dose/mL	Harvest Time (hours)	No. of Cells Scored	Number of Aberrations Per Cell ^a	Percent of Cells With Aberrations ^a	Biologically Significant Aberrations (No/Type) ^b
<u>Negative Control</u>						
McCoy's 5a medium	--	20	200	0.02	1.5	30
<u>Solvent Control</u>						
Dimethyl sulfoxide	10 µL	20	200	0.0	0.0	--
<u>Positive Control</u>						
Mitomycin C	0.08 µg	20	200	0.22	15.5*	6TB; 7SB; 16TR; 6QR 4ID; 4D; 1R
<u>Test Material</u>						
Vinclozolin	125 µg	20	200	0.01	1.0	2D
	250 µg ^c	20	200	0.01	0.5	1D
	375 µg ^c	20	200	0.01	1.0	1SB; 1D
	500 µg ^{c,d}	20	200	0.00	1.0	--

^aGaps excluded
^bAbbreviations used:

TB = Chromatid break; SB = Chromosome break; ID = interstitial deletion; TR = Triradial; QR = Quadriradial; D = Dicentric; R = Ring; Compound precipitation

^cCytotoxic effects on the monolayers were reported at this dose.

^dSignificantly (p<0.01) greater than pooled negative and solvent controls by Fisher's Exact test with multiple comparisons

Note: Data were extracted from the study report, p. 25 and 26.

MAMMALIAN CELLS IN CULTURE CYTOGENETICS

- (b) S9-activated assay: As in the nonactivated assay, signs of cytotoxicity were apparent at 501 $\mu\text{g}/\text{ml}$. At this dose, cellular debris, floating dead cells, an unhealthy monolayer, a 25% reduction of cell monolayer confluence, and a slight reduction in visible mitotic cells were observed. Examination of cell cultures for cytogenetic effects revealed that there were no significant increases in the percentage of cells with aberrations at any treatment dose (Table 3). Our reviewers noted the presences of rare complex aberrations at 250 and 375 $\mu\text{g}/\text{mL}$; however, similar aberrations at comparable frequencies were also seen in the negative and solvent control groups. The finding is, therefore, not indicative of a clastogenic effect of the test material.

By contrast to the negative results with the treatment material, the positive controls for both the nonactivated and S9-activated assays (MMC -S9; CP +S9) induced significant ($p < 0.01$) increases in the percentage of cells with aberrations.

4. Analytical Determinations: Although the purity of the test material was not specifically listed, our reviewers assume that vinclozolin (88/375 Reg. No. 83258) was at least 99% pure. This assumption was based on the reported method validation procedures used to prepare the standard and working standard solutions of the test material and the high correlation between actual and theoretical values (see study report, pp. 33-44). Dosing solutions prepared for the nonactivated and S9-activated phases of testing were analyzed and found to contain $\pm 6\%$ of the target concentrations.

Based on the overall results, the study author concluded that the test material was not clastogenic in this mammalian cell cytogenetic assay.

- D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that the study was properly conducted and that the study author interpreted the data correctly. Vinclozolin was assayed over a concentration range that included a cytotoxic dose ($\approx 500 \mu\text{g}/\text{mL} +/- \text{S9}$), insoluble levels ($\geq 250 \mu\text{g}/\text{mL} +/- \text{S9}$) and a soluble concentration ($125 \mu\text{g}/\text{mL} +/- \text{S9}$) but failed to induce a clastogenic response in cultured CHO cells. The sensitivity of the test system to detect a positive response was also adequately demonstrated by the significant ($p < 0.01$) clastogenic effects observed in cultures treated with the nonactivated ($0.08 \mu\text{g}/\text{mL}$ MMC) or the S9-activated ($25 \mu\text{g}/\text{mL}$ CP) positive controls.
- E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement was signed and dated March 2, 1989.)
- F. CORE CLASSIFICATION: Acceptable. The study satisfies the requirements for FIFRA Test Guideline Series 84-2 for genetic effects Category II, Structural Chromosome Aberrations and is acceptable for regulatory purposes.

Table 3. Representative Results from the In Vitro Cytogenetic Assay in Chinese Hamster Ovary Cells Treated with Vinclozolin in the Presence of S9 Activation

Substance	Dose/ml	Harvest Time (hours)	No. of Cells Scored	Number of Aberrations Per Cell	Percent of Cells with Aberrations ^a	Number of Cells with Biologically Significant Aberrations (No/Type) ^b
<u>Negative control</u>						
McCoy's 5a medium	--	10	200	0.01	1.0	11R; 1D
<u>Solvent Control</u>						
Dimethyl sulfoxide	10 µL	10	200	0.02	1.5	2D; 1R
<u>Positive Control</u>						
Cyclophosphamide	25 µg	10	200	0.28	17.5*	12TB; 14SB; 31D; 18TR 6QR; 2CR; 1R
<u>Test Material</u>						
Vinclozolin	125 µg	10	200	0.00	0.0	--
	250 µg ^c	10	200	0.01	0.5	1CR
	375 µg ^c	10	200	0.01	1.0	1TB; 1TR
	501 µg ^{c,d}	10	200	0.01	0.5	1SB

^aGaps excluded
^bAbbreviations used:

TB = Chromatid break; SB = Chromosome break; ID = Interstitial deletion; TR = Triradial; QR = Quadridial
D = Dicentric; R = Ring; CR = Complex Rearrangement

^cCompound precipitation

^dCytotoxic effects on the monolayers were reported at this dose level.

*Significantly (p<0.01) greater than pooled negative and solvent controls by Fisher's Exact test with multiple comparisons

Note: Data were extracted from the study report, p. 27 and 28.