DATA EVALUATION RECORD

R417888 (METABOLITE OF CHLOROTHALONIL)

Study Type: OCSPP 870.5375 [§84-2]; *In Vitro* Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes

EPA Contract No. EP-W-16-018
Task Assignment No. 35-23-018 (MRID 51485519)

Prepared for
Health Effects Division
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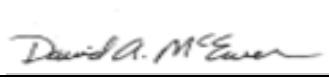
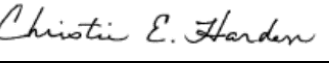
Primary Reviewer: David A. McEwen, B.S.	Signature: Date:	 02/24/2022
Secondary Reviewer: Christie E. Harden, B.S.	Signature: Date:	 04/11/2022
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Disclaimer

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Risk Assessment Branch I, HED (7509P) **Date:** 8/15/2023
Template version 02/06

DATA EVALUATION RECORD

STUDY TYPE: *In Vitro* Mammalian Cytogenetics (Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes); OCSPP 870.5375 [§84-2]; OECD 473.

PC CODE: 081901 **DP BARCODE:** D468198
TXR #: 0058619

TEST MATERIAL (PURITY): R417888 (metabolite of chlorothalonil, 95% a.i.)

SYNONYMS: 2-carbamoyl-3,5,6-trichloro-4-cyanobenzene-1-sulfonic acid, sodium salt

CITATION: Sokolowski, A. (2015) SYN548764[1] - Chromosome aberration test in human lymphocytes *in vitro*. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Laboratory Study No.: 1677402, July 30, 2015. MRID 51485519. Unpublished.

SPONSOR: Syngenta Ltd, Jealott's Hill International Research Center, Bracknell, Berkshire, UK

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: In a mammalian cell cytogenetics assay (chromosome aberration; MRID 51485519), lymphocyte cultures were prepared from human peripheral blood and exposed to R417888 (metabolite of chlorothalonil, 95% a.i., Batch No. MES 377/1) in deionized water at concentrations of **0**, 24.0, 42.1, 73.6, 128.8, 225.4, 394.5, 690.4, **1208.2, 2114.3,** and **3700.0** µg/mL (4-hour exposure, ±S9 in Exp. I and +S9 in Exp. II) and **0**, 24.0, 42.1, 73.6, 128.8, 225.4, 394.5, 690.4, **1208.2, 2114.3,** and **3700.0** µg/mL (22-hour exposure, −S9 in Exp. II)[2]. The S9 fraction was derived from the livers of male rats induced with phenobarbital and

[1] Actual structure synthesized and used in this study was the alternative isomer, R417888 (another chlorothalonil metabolite).
[2] Concentrations in **bold** were evaluated for aberrations.

β-naphthoflavone. Cyclophosphamide (CPA) and ethylmethane sulfonate (EMS) served as the positive controls in the presence and absence of S9, respectively.

No precipitation of the test material was observed at any concentration with or without S9 in either experiment.

In Exp. I, no cytotoxicity, or significant increases in aberration rates (excluding gaps) were observed at any concentration (±S9) after 4 hours of treatment.

In Exp. II, cytotoxicity (mitotic index = 39.7%) was observed at 3700 µg/mL (−S9) after 22 hours of continuous treatment. Also at this concentration, an increased aberration rate (6.0%, excluding gaps) was observed compared to the negative control (1.3%). This increase exceeded the historical control range (0.0-3.0%) and was two-fold greater than the 95% control limit (2.3%).

There was no increase in polyploid metaphases observed at any concentration (±S9) in either experiment. The positive controls (CPA and EMS) induced the appropriate responses in the presence and absence of S9, respectively. **There was evidence of chromosome aberrations induced over background in the absence of S9 activation at the highest concentration tested.** However, this occurred at one high cytotoxic concentration (3700 µg/mL) following extended exposure (22 hours) to the test substance. Therefore, the response was considered to provide limited biological evidence given the high concentration/cytotoxicity and the shape of the dose-response.

This study was meant to be conducted with the SYN548764 metabolite, but after re-examination of the analytical verification data, it was revealed that the structure was not correctly synthesized and was instead the alternative isomer, R417888. Therefore, the Ames, *in vitro* cytogenetics, Mouse lymphoma and *in vivo* micronucleus study results originally conducted on what was thought to be SYN548764 should be regarded as additional genotoxicity data generated on R417888. The *in vitro* gene mutation assays both gave negative results. This *in vitro* chromosome aberration assay showed an equivocal clastogenic response in the absence of metabolic activation at one high cytotoxic concentration following extended exposure to the test substance. In order to provide further clarity and to address this questionable finding, an *in vivo* mammalian erythrocyte micronucleus test was conducted which gave a negative response. Hence, these additional genotoxicity studies on R417888 confirm R417888 is non-genotoxic.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.5375, OECD 473) for *in vitro* mutagenicity (chromosome aberration) data.

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

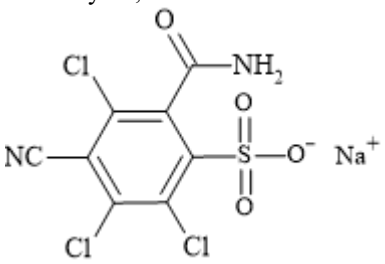
I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: R417888 (metabolite of chlorothalonil)

Description: White solid
Batch #: MES 377/1
Purity: 95% (w/w) a.i.

CAS # of parent: 1897-45-6
Expiration/Storage: February 28, 2017/2-8°C
Structure:



2. Control materials:

Negative control: The solvent alone served as the negative control
Solvent control: Deionized water (10.0% v/v)
Positive control: Non-activated: Ethylmethane sulfonate (EMS, 825 µg/mL, Exp. I and 550.0 µg/mL, Exp. II)
Activated: Cyclophosphamide (CPA, 15 µg/mL)

3. Activation: The S9 was derived from rat livers according to a Harlan CCR SOP.

X	Induced		Aroclor™ 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
		X	β-naphthoflavone		Hamster		Other
			Other		Other		

The S9 fraction (Lot No. 250914B) was prepared in house and was stored frozen (temperature not reported) until required. The batch was tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test; the protein content was 33.0 mg/mL. The S9 mix contained the following components: S9 fraction (0.75 mg/mL), 33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose-6-phosphate, and 4 mM NADP in 100 mM sodium-ortho-phosphate buffer (pH 7.4).

4. Test cells: Human blood samples were obtained from healthy non-smoking male (33 years old, Exp. I) and female (38 years old, Exp. II) donors not receiving medication. The samples were drawn via venous puncture and collected in heparinized tubes.

	Mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells	X	Human lymphocytes

Media: Dulbecco's Modified Eagle medium/Ham's F12 (1:1), supplemented with 200 mM GlutaMAX™, penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10% fetal bovine serum (FBS), 10 mM HEPES (buffer), and heparin (125 U.S.P.-U/mL).

Properly maintained?	X	Yes		No
Periodically checked for Mycoplasma contamination? **N/A**		Yes		No
Periodically checked for karyotype stability? **N/A**		Yes		No

Whole blood cultures were established within 30 hours after collection in cell culture flasks. The following volumes were added to the flasks (per 10 mL): 7.60 mL culture medium, 1.00 mL FBS, 0.10 mL antibiotic solution, 0.05 mL phytohemagglutinin, 0.05 mL heparin, 0.10 mL HEPES, and 1.10 mL whole blood. All cultures were incubated at 37°C with 5.5% CO_2 in humidified air for 48 hours.

5. Test compound concentrations used:

Non-activated conditions: Preliminary toxicity: A preliminary test was performed and as all the acceptability criteria were met, the data were presented as Experiment I.
Mutagenicity Assay: **0**, 24.0, 42.1, 73.6, 128.8, 225.4, 394.5, 690.4, **1208.2, 2114.3,** and **3700.0** µg/mL (4-hour exposure, Exp. I)
0, 24.0, 42.1, 73.6, 128.8, 225.4, 394.5, 690.4, **1208.2, 2114.3,** and **3700.0** µg/mL (22-hour exposure, Exp. II)

Activated conditions: Preliminary toxicity: A preliminary test was performed and as all the acceptability criteria were met, the data were presented as Experiment I.
Mutagenicity Assay: **0**, 24.0, 42.1, 73.6, 128.8, 225.4, 394.5, 690.4, **1208.2, 2114.3,** and **3700.0** µg/mL (4-hour exposure, Exp. I)
0, 225.4, 394.5, 690.4, **1208.2, 2114.3,** and **3700.0** µg/mL (4-hour exposure, Exp.II)

Concentrations in **bold** were evaluated for chromosome aberrations.

B. TEST PERFORMANCE

1. Preliminary cytotoxicity assay: A preliminary test was performed and as all the acceptability criteria were met, the data were presented as Experiment I of the cytogenetic assay (methods below).

2. Cytogenetic assay: After a culture establishment period of 48 hours, the test material, solvent control, or positive control (volume not reported) were added to duplicate blood cultures in serum-free medium. For the cultures requiring activation, 50 µL of S9 mix was added. The total volume of the cultures was 10 mL. The cultures were exposed for 4 hours, centrifuged for 5 minutes, and the supernatant was discarded. For Exp. II, an additional culture was exposed for 22 hours without S9. After the exposure period, the cells were resuspended and washed with saline G (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose•H_2O, 192 mg/L Na_2HPO_4•$2H_2O$, and 150 mg/L KH_2PO_4). After washing, the cells were resuspended in complete culture medium (with 10% FBS) and incubated for an additional 18 hours. Approximately 3 hours prior to collection, the cultures were treated with Colcemid® (0.2 µg/mL).

a.	**Cell exposure time:**	Test material	Solvent control	Positive control
	Non-activated:	4 hours (Exp. I) 22 hours (Exp. II)	4 hours (Exp. I) 22 hours (Exp. II)	4 hours (Exp. I) 22 hours (Exp. II)
	Activated:	4 hours (Exp. I & II)	4 hours (Exp. I & II)	4 hours (Exp. I & II)
b.	**Spindle inhibition:**			
	Inhibitor used/concentration:	Colcemid® (0.2 µg/mL)		
	Administration time:	3 hours (before cell harvest)		
c.	**Cell harvest time after initiation of treatment:**	Test material	Solvent control	Positive control
	Non-activated:	22 hours (Exp. I & II)	22 hours (Exp. I & II)	22 hours (Exp. I & II)
	Activated:	22 hours (Exp. I & II)	22 hours (Exp. I & II)	22 hours (Exp. I & II)

d. Details of slide preparation: After cell division was arrested, the cultures were centrifuged, the supernatant was removed, and the cells were resuspended in a hypotonic solution (0.0375 M KCl) and allowed to stand for 20 minutes at 37°C. The cultures were centrifuged, the supernatant discarded, and the cells were fixed with methanol/glacial acetic acid (3:1, v:v). At least two slides per group were prepared by dropping a small amount of the lymphocyte suspension onto clean, wet microscope slides and allowing to dry. The slides were stained with Giemsa, mounted with coverslips after drying, and coded prior to analysis.

e. Metaphase analysis:

No. of cells examined per dose: 300 cells (150/replicate) in the treatment, positive control, and solvent control groups were scored for structural and numerical aberrations. The mitotic index was recorded as the % of cells in mitosis per 1000 cells counted.

Scored for structural?	[X]	Yes	[ ]	No
Scored for numerical?	[X]	Yes (polyploidy)	[ ]	No
Coded prior to analysis?	[X]	Yes	[ ]	No

f. Evaluation criteria:

Assay validity: The assay was considered valid if the following criteria were met:

- The frequency of cells with structural chromosome aberrations in the solvent control was within the provided laboratory historical control range; and
- The frequency of cells with structural chromosome aberrations in the positive controls were statistically significantly increased relative to the solvent control.

Positive result: The test article was deemed positive for mutagenicity if the following criteria were met:

- At least one of the test concentrations exhibited a statistically significant increase compared to the concurrent negative control,
- The increase was dose-related when evaluated with an appropriate trend test, and
- Any of the results were outside the distribution of the historical negative control data.

g. Statistical analysis: The frequencies of aberrant cells were statistically analyzed with the Fisher's exact test ($p<0.05$). However, both biological and statistical significance were

considered together.

II. REPORTED RESULTS: The dose formulations were not analyzed for actual concentrations. Addition of the test material did not have any significant effect on the osmolality or pH of the culture media.

A. PRELIMINARY CYTOTOXICITY ASSAY: A preliminary test was performed, and the data were reported as Exp. I.

B. CYTOGENETIC ASSAY: The results of the cytogenetic assay are summarized in Tables 1a and 1b.

No precipitation of the test material was observed at any concentration with or without S9 in either experiment.

In Exp. I, no cytotoxicity (indicated by a mitotic index less than 50%) or significant increases in aberration rates (excluding gaps) were observed at any concentration (±S9).

In Exp. II, cytotoxicity (mitotic index = 39.7%) was observed at 3700 µg/mL (−S9) after 22 hours of continuous treatment. An increased ($p \leq 0.01$) aberration rate (6.0%, excluding gaps) also was observed at this concentration compared to the negative control (1.3%). This increase exceeded the historical control range (0.0-3.0%) and was two-fold greater than the 95% control limit (2.3%).

There was no increase in polyploid metaphases observed at any concentration (±S9) in either experiment. The positive controls (EMS and CPA) induced increases ($p<0.001$) in the mean percentage of cells with structural aberrations (excluding gaps) in both experiments.

TABLE 1a. Summary data for Experiment I of the chromosome aberration study in human lymphocytes with R417888. [a]

Concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%)		
		Including gaps [b]	Excluding gaps [b]	Historical control range [c]
Exposure for 4 hours (−S9)				
0	100.0	2.0	1.7	0.0-3.0
1208.2	88.6	1.7	1.7	
2114.3	101.8	1.3	1.3	
3700.0	85.2	2.7	2.7	
EMS (825 µg/mL)	65.7	8.3	7.3***	6.5-43.0
Exposure for 4 hours (+S9)				
0	100.0	2.3	2.3	0.0-3.5
1208.2	97.9	2.0	1.3	
2114.3	106.8	1.0	1.0	
3700.0	112.0	1.7	1.0	
CPA (15 µg/mL)	67.5	15.7	15.3***	7.5-39.0

a Data obtained from Tables 2 & 11 on pages 32 & 41 and Appendix 1 on pages 43-45 of MRID 51485519.
b Including cells carrying exchanges.
c Historical control data (excluding gaps) from 88-142 studies. Historical control data can be found on pages 43-45 of MRID 51485519
*** Significantly different from control; $p<0.001$.

TABLE 1b. Summary data for Experiment II of the chromosome aberration study in human lymphocytes with R417888. [a]

Concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%)		
		Including gaps [b]	Excluding gaps [b]	Historical control range
Exposure for 22 hours (−S9)				
0	100.0	1.3	1.3	0.0-3.0
1208.2	70.8	2.3	2.0	
2114.3	66.3	2.7	2.7	
3700.0	39.7	6.7	6.0***	
EMS (550 µg/mL)	44.5	18.3	18.0***	8.5-64.0
Exposure for 4 hours (+S9)				
0	100.0	3.7	3.0	0.0-3.5
1208.2	97.4	3.0	2.7	
2114.3	94.0	4.5	3.8	
3700.0	99.6	3.0	2.3	
CPA (15 µg/mL)	65.3	16.3	15.7***	7.5-39.0

a Data obtained from Tables 2 & 11 on pages 32 & 41 and Appendix 1 on pages 43-45 of MRID 51485519.
b Including cells carrying exchanges.
c Historical control data (excluding gaps) from 88-142 studies. Historical control data can be found on pages 43-45 of MRID 51485519
*** Significantly different from control; $p<0.001$.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: Under the experimental conditions reported, the test substance induced structural chromosomal aberrations in human lymphocytes *in vitro* in the absence of a metabolic activation system at one high cytotoxic concentration only. SYN548764 was tested up to 3700 µg/plate in accordance with a limit concentration of 10 mM as required by the EPA (1998) and EU (2008) test guidelines, a higher concentration than required by the OECD 473 guideline (2014). Therefore, SYN548764 is considered to display equivocal clastogenic activity in this chromosome aberration test when tested up to the highest applied concentration.

B. REVIEWER COMMENTS: No precipitation of the test material was observed at any concentration with or without S9 in either experiment.

In Exp. I, no cytotoxicity, or significant increases in aberration rates (excluding gaps) were observed at any concentration (±S9) following 4 hours of treatment.

In Exp. II, cytotoxicity (mitotic index = 39.7%) was observed at 3700 µg/mL (−S9) after 22 hours of continuous treatment. Also at this concentration, an increased ($p<0.001$) aberration rate (6.0%, excluding gaps) was observed compared to the negative control (1.3%). This increase exceeded the historical control range (0.0-3.0%) and was two-fold greater than the 95% control limit (2.3%).

There was no increase in polyploid metaphases observed at any concentration (±S9) in either experiment. The positive controls (CPA and EMS) induced the appropriate responses in the presence and absence of S9, respectively. **There was evidence of chromosome aberrations induced over background in the absence of S9 activation at the highest concentration tested.**

This study was meant to be conducted with the SYN548764 metabolite, but after re-examination of the analytical verification data, it was revealed that the structure was not correctly synthesized and was instead the alternative isomer, R417888. Therefore, the Ames, *in vitro* cytogenetics, Mouse lymphoma and *in vivo* micronucleus study results originally conducted on what was thought to be SYN548764 should be regarded as additional genotoxicity data generated on R417888. The *in vitro* gene mutation assays both gave negative results. This *in vitro* chromosome aberration assay showed an equivocal clastogenic response in the absence of metabolic activation at one high cytotoxic concentration following extended exposure to the test substance. In order to provide further clarity and to address this questionable finding, an *in vivo* mammalian erythrocyte micronucleus test was conducted which gave a negative response. Hence, these additional genotoxicity studies on R417888 confirm R417888 is non-genotoxic.

C. STUDY DEFICIENCIES: The following deficiency was noted, but does not change the conclusions of this DER, as this data will only be used qualitatively:

- The dose formulations were not analyzed for actual concentrations.