CA5021

CA5021 - Micronucleus Test in Human Lymphocytes
In Vitro

Final Report

DATA REQUIREMENT(S): OECD 487 (2016)

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PERFORMING LABORATORY: Envigo CRS GmbH
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SPONSOR(S): Syngenta Ltd
Jealott's Hill International Research Centre
Bracknell, Berkshire RG42 6EY, United Kingdom

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study performed in the test facility of Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version.

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17
EC Commission Directive 2004/10/EC

These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

There were no circumstances that may have affected the quality or integrity of the study.

02 May 2018

Dr. Steffen Naumann
Genetic Toxicology *in vitro*

Date

Performing Laboratory:
Envigo CRS GmbH
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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Number:	1878100
Test substance:	CA5021
Study Director:	Dr. Steffen Naumann
Title:	CA5021 - Micronucleus Test in Human Lymphocytes *In Vitro*

Study based activities at the Test Facility Envigo CRS GmbH were audited and inspected. The details of these audits and inspections are given below.

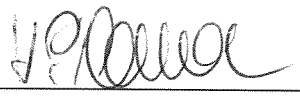
Type of Inspection	**Date(s) of Inspection**	**Date Reporting to Study Director, Test Facility Management**
Study Plan Verification	28 November 2017	28 November 2017
Process – based Assessment of response Test performance	 14 December 2017 23 January 2018	 14 December 2017 30 January 2018
Report Audit	04 April 2018	04 April 2018

In addition, process based inspections were conducted of other routine and repetitive procedures employed on this type of study at or about the time the study was in progress.

General facilities and activities where this study was conducted were inspected on an annual basis and results are reported to the relevant responsible person and Management.

The statement is to confirm, that this report reflects the raw data.

Quality Assurance



02 May 2018

H. Pilawa

Date

Quality Assurance Auditor
Envigo CRS GmbH

PROJECT STAFF SIGNATURE

Study Director — Dr. Steffen Naumann

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Date: 02 May 2018

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
Dr. Steffen Naumann	Study Director
Dipl. Biol. Andrea Sokolowski	Deputy Study Director
Dr. Wolfgang Völkner	Management
Frauke Hermann	Head of Quality Assurance Unit
Laura Brierley	Syngenta Study Manager

Study dates

Study initiation date:	30 November 2017
Experimental start date:	13 December 2017
Experimental termination date:	02 February 2018

Deviations from the guidelines

None

Retention of samples

None

Performing laboratory test substance number

S 1940811

Other

Records and documentation relating to this study will be maintained in the archives of Envigo CRS GmbH for a period of 4 years from the date on which the Study Director signs the final report. This will include but may not be limited to the Study Plan, any amendments, raw data, Report and specimens generated during the course of this study.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant archive of Envigo CRS (Switzerland) Ltd. at Füllinsdorf, Switzerland, for further archiving up to a total archiving period of 15 years.

A sample of the test item will not be archived.

Envigo will retain in its archive a copy of the study plan and final report, and any amendments indefinitely.

Deviations from the study plan

The following deviation from study plan occurred:

Preparation of cells
Due to an IT disruption the slides of Experiment II were labelled with a manual-generated random code instead of a computer-generated code.

This deviation was considered to have not affected the integrity or validity of the study.

Distribution of the report

Sponsor	2 × electronic copy (1 × pdf-file, 1 × word-file)
Study Director	1 × (original)

TABLE OF CONTENTS

1.0 EXECUTIVE SUMMARY

1.1 Study Design

The test substance CA5021, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments.

In each experimental group two parallel cultures were analyzed. Per culture at least 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (1851 µg/mL of the test substance, approx. 10 mM) was chosen with regard to the molecular weight and the purity (96.3%) of the test substance and with respect to the current OECD Guideline 487.

Concentration selection of the cytogenetic experiment was performed considering the toxicity data in accordance with OECD Guideline 487.

1.2 Results

In the absence and presence of S9 mix, no cytotoxicity or precipitation was observed up to the highest applied concentration.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test item. In Experiment I (4 hour exposure) in the absence of S9 mix, a value of 1.45% micronucleated cells after treatment with 1851 µg/mL exceeded the 95% control limit of the historical control data (0.06 – 1.19%). Since the value is not statistically significant increased and no dose-dependency was observed, the finding is considered to be biologically irrelevant. Additionally, the long exposure experiment in the absence of S9 mix was clearly negative. Therefore the criteria for a negative response were met in the presence and absence of S9 in this assay.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

1.3 Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the CA5021did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.
Therefore, CA5021 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

2.0 INTRODUCTION

2.1 Purpose

The occurrence of micronuclei in interphase cells provides an indirect but easy and rapid measure of structural chromosomal damage and aneugenicity in cells that have undergone cell division during or after exposure to the test substance. Micronuclei arise from chromosomal fragments or whole chromosomes and rarely occur spontaneously but are inducible by clastogens or agents affecting the spindle apparatus (Countryman and Heddle, 1976; Obe and Beek, 1982, Rosefort *et al*, 2004).

2.2 Justification of Test System

The induction of cytogenetic damage in human lymphocytes was assessed in two independent experiments with one preparation interval (40 hours). Human lymphocytes have been widely used for this assay type as described in the OECD test guideline 487 (2016).

Micronuclei should only be evaluated in cells that have completed mitosis during exposure to the test substance or during the post-exposure period and thus a cytokinesis blocker, cytochalasin B, is added to the cell culture to ensure that there are binucleated cells to be evaluated for micronuclei (Rosefort et al, 2004).

Treatments started after a 48 hour stimulation period with phytohaemagglutinine (PHA) when cells were actively proliferating and the cells were prepared at approx. 2 – 2.5 fold of the normal cell cycle time.

For validation of the test, control mutagens were tested in parallel to the test substance.

2.3 Regulatory Guidelines

This study was conducted according to the procedures indicated by the following internationally accepted guideline and recommendations:

- OECD Guideline for the Testing of Chemicals No. 487 "*In vitro* Mammalian Cell Micronucleus Test", adopted 29 July 2016.

The following alterations from the guidelines were performed:

- A series of in-house non-GLP validation experiments was performed to get distinct responses of statistical significance when using the specified positive controls (Bohnenberger et al, 2011). To achieve such response the test design, specifically for the treatment, the recovery phase and harvest time, was slightly modified comparing the current proposal given in the OECD Guideline 487. The optimum positive control micronuclei responses were found with the time schedule stated in section 3.7.1.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance and the information concerning the test substance were provided by Syngenta:

Identification:	CA5021
Alternative test item name:	SYN549110
Batch:	81306EI100
Purity:	96.3 % w/w*
Molecular weight:	178.2 g/mol
Physical state / Appearance:	Light brown solid
Retest Date:	End of October 2019
Storage Conditions:	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

*Correction for purity was made.

3.2 Test Substance Preparation

On the day of the experiment (immediately before treatment), the test substance was dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.5 % (v/v). The solvent was chosen due to its solubilisation properties and its relative non-toxicity to the cell cultures (Easterbrook *et al*, 2001).

The osmolarity and pH of the test substance dissolved in DMSO and diluted in culture medium were determined by using an osmometer or a pH meter in the pre-experiment without metabolic activation in the solvent control and in the highest three concentrations.

3.3 Controls

3.3.1 Solvent controls

Concurrent solvent controls (culture medium with 0.5 % DMSO) were performed.

Name:	DMSO
Supplier:	Fisher Chemical, 58239 Schwerte, Germany
Purity:	99.99 %
Lot No. / Expiry Date:	1684307 / October 2022

3.3.2 Positive control substances

Without metabolic activation

Name:	Mitomycin C (MMC) (pulse treatment)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	106 M 4106 V
Expiry Date:	January 2018
Purity:	98 %
Dissolved in:	Deionised water
Concentration:	0.8 µg/mL

Name:	Demecolcine (continuous treatment)*
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	BCBP 6056 V
Expiry Date:	June 2018
Purity:	≥ 98 %
Dissolved in:	Deionised water
Concentration:	125 ng/mL

* OECD 487, paragraph 33 permits the use of an alternative positive control agent if a sufficient laboratory historical data base has been established and is scientifically justified.

With metabolic activation

Name:	Cyclophosphamide (CPA)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	MKBX 1822 V
Expiry Date:	January 2019
Purity:	97 – 103 %
Dissolved in:	Saline (0.9 % NaCl [w/v])
Concentration:	15.0 µg/mL

The dilutions of the stock solutions were prepared on the day of the experiment. The stability of the positive control substance in solution is unknown but a mutagenic response in the expected range is sufficient biological evidence for chemical stability.

3.4 Experimental Design

3.4.1 Reason for the choice of human lymphocytes

Human lymphocytes are commonly used in the *in vitro* micronucleus test and have been used successfully for a long time in *in vitro* experiments. They show stable spontaneous micronucleus frequencies at a low level (Countryman and Heddle, 1976; Evans and O'Riordan, 1975).

3.4.2 Blood collection and delivery

Blood samples were obtained from healthy non-smoking donors not receiving medication. For this study, blood was collected from a male donor (32 years old) for Experiment I and from a male donor (25 years old) for Experiment II.

Blood samples were drawn by venous puncture and collected in heparinized tubes by Dr. V. Theodor (64380 Rossdorf, Germany). The tubes were sent to Envigo CRS GmbH to initiate cell cultures within 24 h after blood collection.

3.5 Mammalian Microsomal Fraction S9 Mix

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Wistar rats (RjHan:WI; weight approx. 200 – 320 g, Janvier Labs, 53941 Saint-Berthevin Cedex, France) induced by peroral administration of 80 mg/kg b.w. phenobarbital (Sigma-Aldrich Chemie GmbH, 82024 Taufkirchen, Germany) and by peroral administrations of ß-naphthoflavone (Acros Organics, 2440 Geel, Belgium) each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1+3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at –80 °C. Small numbers of the ampoules can be kept at –20 °C for up to one week.

Each batch of S9 is routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test (Ames et al, 1975).

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation was 32.7 mg/mL (Lot no. 270717).

3.6 Concentration Selection

Concentration selection was performed according to the current OECD Guideline 487 for the *in vitro* micronucleus test (2016). The highest test substance concentration should be 10 mM, 2 mg/mL, or 2 µL/mL, whichever is the lowest. Four test substance concentrations were evaluated for cytogenetic damage.

In case of test item induced cytotoxicity, measured by a reduced cytokinesis-block proliferation index (CBPI) and expressed as cytostasis, or precipitation / phase separation (observed at the end of test item exposure by the unaided eye) the concentration selection

should reflect these properties of the test substance. Where cytotoxicity occurs, the applied concentrations should cover a range from no to approximately 55 ± 5 % cytostasis. For poorly soluble test substances, which are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed, should produce turbidity or visible precipitation / phase separation.

3.7 Experimental Performance Cytogenetic Experiment

3.7.1 Schedule

	Without S9 mix		With S9 mix
	Exp. I	Exp. II	Exp. I
Stimulation period (h)	48	48	48
Exposure period (h)	4	20	4
Recovery (h)	16	—	16
Cytochalasin B exposure (h)	20	20	20
Total culture period (h)	88	88	88

3.7.2 Culture conditions

Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 h after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (phytohemagglutinin) (3 µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).
The following volumes were added to the flasks (per 10 mL):

7.60 mL culture medium
1.00 mL fetal bovine serum
0.10 mL antibiotic solution
0.05 mL phytohemagglutinin (stock solution: 0.6 mg/mL)
0.05 mL heparin
0.10 mL HEPES
1.10 mL whole blood

All incubations were done at 37 °C with 5.5 % CO_2 in humidified air.

3.7.3 Pre-experiment

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the main assay.

The pre-test was performed with 10 concentrations of the test substance separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

3.7.4 Cytogenetic experiment

Pulse exposure
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with serum-free medium containing the test substance. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in 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 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test substance. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

3.7.5 Preparation of cells

The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in saline G (approximately 5 mL) and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in KCl solution (5 mL, 0.0375 M) and incubated at 37 °C for 20 minutes. Ice-cold fixative mixture of methanol and glacial acetic acid (1 mL, 19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The mounted cells were Giemsa-stained and, after drying, covered with coverslips. All slides were labeled with a computer-generated random code to prevent scorer bias.

3.7.6 Evaluation of cytotoxicity damage

The cytotoxicity was judged in the course of a microscopically pre-check of the specimen slides for guideline requested quality and quantity criteria in a first step.
Subsequently the CBPI was used as the preferred method for quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity by the OECD Guideline 487. To describe cytotoxic effects the CBPI was determined in 500 cells per culture. Evaluation of the slides was performed using microscopes with 40 x objectives. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criteria. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Under some circumstances test the CBPI does not reflect the cytotoxicity accurately and concentrations may be excluded from the evaluation during the microscopic pre-check. CBPI measures proliferation and may not detect cytotoxic events like necrosis, oncosis and apoptosis. In particular mononuclear cells without cytoplasm (representing cells which undergo cell death in the treatment cell cycle) are not represented in the CBPI because those cells do not fulfil the quality criteria for evaluation (see section 3.7.7). This can result in too few cells available for scoring.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mononucleate cells
BINC	Binucleate cells
MUNC	Multinucleate cells

$$\text{Cytostasis \%} = 100 - 100\,[(CBPI_T - 1) / (CBPI_C - 1)]$$

T	Test substance
C	Solvent control

3.7.7 Evaluation of cytogenetic damage

Evaluation of the slides was performed using microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not be more than one third of the area of the main nucleus. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells.

3.8 Data Recording

The data were recorded in the laboratory documentation. The results are presented in tabular form, including experimental groups with the test substance, solvent controls, and positive controls, respectively.

3.9 Acceptability Criteria

The micronucleus assay will be considered acceptable if it meets the following criteria:

- The concurrent solvent control will normally be within the 95% control limits of the laboratory's historical solvent control data.
- The concurrent positive controls should induce responses that are compatible with the laboratory historical positive control data and produce a statistically significant increase.
- Cell proliferation criteria in the solvent control are considered to be acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides must allow the evaluation of an adequate number of cells and concentrations.

The criteria for the selection of top concentration are consistent with those described in section 'Concentration selection'.

3.10 Interpretation of Results

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test substance concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test substance concentrations should be within the 95% control limits of the laboratory's historical solvent control data

The test substance is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test substance concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition
- The results are outside the range of the 95% control limit of the laboratory historical solvent control data

When all of the criteria are met, the test substance is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

However, results may remain questionable regardless of the number of times the experiment is repeated. If the data set will not allow a conclusion of positive or negative, the test substance will therefore be concluded as equivocal.

3.11 Statistical Analysis

Statistical significance was confirmed by the Chi-squared test ($p < 0.05$) using a validated "R" Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells, obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

4.0 RESULTS AND DISCUSSION

The test substance CA5021, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analyzed. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides making a total of 2000 binucleated cells per test substance concentration. To assess cytotoxicity the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration the pre-test for toxicity, 1851 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight and the purity (96.3%) of the test substance and with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

Test substance concentrations ranging from 14.0 µg/mL to 1851 µg/mL (with and without S9 mix) were chosen for evaluation of cytotoxicity. Since the cultures fulfilled the requirements for cytogenetic evaluation, this test was designated Experiment I.

For Experiment II, 1851 µg/mL (without S9 mix) were chosen as top treatment concentration.

The applied concentrations for each experiment are presented in Table 1.

No relevant influence on the osmolarity and pH was observed.

		Concentration [µg/mL]	Osmolarity [mOsm]	pH
Exp. I	Solvent control	-	398	7.4
	CA5021	705	n.d.	7.1
	CA5021	1234	n.d.	6.9
	CA5021	1851	388	6.9

n.d. Not determined

In the absence and presence of S9 mix, no cytotoxicity or precipitation was observed up to the highest applied concentration.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test item. In Experiment I (4 hour exposure) in the absence of S9 mix, a value of 1.45% micronucleated cells after treatment with 1851 µg/mL exceeded the 95% control limit of the historical control data (0.06 – 1.19%). Since the value is not statistically significantly increased versus the concurrent solvent control and no dose-dependency was observed, the finding is considered to be biologically irrelevant. Additionally, this was not repeatable in the long exposure experiment. Therefore, the criteria for a negative response were met in the presence and absence of S9 in this assay.

Demecolcine (125 ng/mL), MMC (0.8 µg/mL) or CPA (15.0 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei.

5.0 CONCLUSIONS

In conclusion, it can be stated that under the experimental conditions reported, CA5021 did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.

Therefore, CA5021 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

6.0 REFERENCES

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TABLES SECTION

TABLE 1 Concentrations Applied in the Micronucleus Assay with CA5021

Exp.	Prep. interval (h)	Exposure period (h)	Concentrations (µg/mL)								
Without S9 mix											
I	40	4	14.0	24.5	43.0	75.2	132	230	**403**	**705**	**1234**
											1851
II	40	20				132	230	**403**	**705**	**1234**	**1851**
With S9 mix											
I	40	4	14.0	24.5	43.0	75.2	132	230	**403**	**705**	**1234**
											1851

Evaluated experimental points are shown in bold characters

TABLE 2 Summary of Results of the Micronucleus Assay with CA5021

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**
Exposure period 4 hrs without S9 mix					
I	40 hrs	Solvent control[1#]	1.99		0.95
		Positive control[2]	1.70	29.8	**15.70[S]**
		403	2.04	n.c.	1.05
		705	2.00	n.c.	0.80
		1234	2.07	n.c.	0.80
		1851	2.07	n.c.	1.45
Exposure period 20 hrs without S9 mix					
II	40 hrs	Solvent control[1]	2.04		0.35
		Positive control[3]	1.82	20.7	**3.05[S]**
		403	1.94	9.5	0.10
		705	2.01	2.3	0.25
		1234	2.02	1.5	0.20
		1851	1.94	9.2	0.35
Exposure period 4 hrs with S9 mix					
I	40 hrs	Solvent control[1]	2.13		0.85
		Positive control[4]	1.96	15.1	**5.30[S]**
		403	2.16	n.c.	0.50
		705	2.13	0.2	0.60
		1234	2.04	8.0	0.45
		1851	2.06	6.3	0.90

* For the positive control groups and the test item treatment groups the values are related to the solvent controls

** The number of micronucleated cells was determined in a sample of 2000 binucleated cells

The number of micronucleated cells was determined in a sample of 4000 binucleated cells

s The number of micronucleated cells is statistically significantly higher than corresponding control values

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

1 DMSO 0.5 % (v/v)

2 MMC 0.8 µg/mL

3 Demecolcine 125 ng/mL

4 CPA 15.0 µg/mL

TABLE 3 Toxicity - Experiment I (Cytotoxicity of CA5021 to the Cultures of Human Lymphocytes)

Concentration (µg/mL)	Exposure time (h)	Preparation interval (h)	CBPI per 500 cells*	Cytostasis (%)
Without S9 mix				
Solvent control	4	40	1.99	-
14.0	4	40	n.d.	n.d.
24.5	4	40	n.d.	n.d.
43.0	4	40	n.d.	n.d.
75.2	4	40	2.03	n.c.
132	4	40	2.01	n.c.
230	4	40	2.01	n.c.
403	4	40	2.04	n.c.
705	4	40	2.00	n.c.
1234	4	40	2.07	n.c.
1851	4	40	2.07	n.c.
With S9 mix				
Solvent control	4	40	2.13	-
14.0	4	40	n.d.	n.d.
24.5	4	40	n.d.	n.d.
43.0	4	40	2.11	1.5
75.2	4	40	2.12	1.1
132	4	40	2.10	2.9
230	4	40	2.13	0.5
403	4	40	2.16	n.c.
705	4	40	2.13	0.2
1234	4	40	2.04	8.0
1851	4	40	2.06	6.3

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures

n.d. Not determined

n.c. Not calculated as the CBPI was equal or higher than solvent control value

TABLE 4 Toxicity - Experiment II (Cytotoxicity of CA5021 to the Cultures of Human Lymphocytes)

Concentration (µg/mL)	Exposure time (h)	Preparation interval (h)	CBPI per 500 cells*	Cytostasis (%)
Without S9 mix				
Solvent control	20	40	2.04	-
132	20	40	2.05	n.c.
230	20	40	2.01	2.3
403	20	40	1.94	9.5
705	20	40	2.01	2.3
1234	20	40	2.02	1.5
1851	20	40	1.94	9.2

Experimental groups evaluated for cytogenetic damage are shown in bold characters
* Mean value of two cultures
n.c. Not calculated as the CBPI was equal or higher than solvent control value

TABLE 5 Experiment I - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 4 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure / preparation	Cell proliferation culture 1*			Proliferation Index	Cell proliferation culture 2*			Proliferation Index		
				c1	c2	c4-c8	CBPI	c1	c2	c4-c8	CBPI	CBPI mean	Cytostasis [%]
Solv. control#	0.5 %	-	4 / 40 h	105	307	87	1.96	73	342	85	2.02	1.99	
Pos. control##	0.8 µg	-	4 / 40 h	197	258	45	1.70	191	268	41	1.70	1.70	29.8
Test item	403 µg	-	4 / 40 h	71	317	112	2.08	92	317	91	2.00	2.04	n.c.
″	705 µg	-	4 / 40 h	77	326	97	2.04	100	319	81	1.96	2.00	n.c.
″	1234 µg	-	4 / 40 h	72	325	103	2.06	60	341	99	2.08	2.07	n.c.
″	1851 µg	-	4 / 40 h	62	312	126	2.13	85	325	90	2.01	2.07	n.c.

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

DMSO

MMC

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

TABLE 6 Experiment I - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 4 h with S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure / preparation	Cell proliferation culture 1*			Proliferation Index	Cell proliferation culture 2*			Proliferation Index		
				c1	c2	c4-c8	CBPI	c1	c2	c4-c8	CBPI	CBPI mean	Cytostasis [%]
Solv. control#	0.5 %	+	4 / 40 h	22	394	84	2.12	13	405	82	2.14	2.13	
Pos. control##	15.0 µg	+	4 / 40 h	39	402	59	2.04	100	360	40	1.88	1.96	15.1
Test item	403 µg	+	4 / 40 h	25	389	86	2.12	16	374	110	2.19	2.16	n.c.
″	705 µg	+	4 / 40 h	18	402	80	2.12	20	393	87	2.13	2.13	0.2
″	1234 µg	+	4 / 40 h	57	394	49	1.98	15	421	64	2.10	2.04	8.0
″	1851 µg	+	4 / 40 h	22	420	58	2.07	19	438	43	2.05	2.06	6.3

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

DMSO

CPA

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

TABLE 7 Experiment I - Number of Micronucleated Cells; Exposure Period 4 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation	Micronucleated cells									
				Binucleate cells with *n* micronuclei culture 1			sum culture 1	Binucleate cells with *n* micronuclei culture 2			sum culture 2	sum in 2000 binucleate cells	[%]
				1	2	>2		1	2	>2			
Solv. control[#]	0.5 %	-	4 / 40 h	7	0	0	7	30	1	0	31	38*	0.95
Pos. control[##]	0.8 µg	-	4 / 40 h	124	6	0	130	165	17	2	184	314	15.70
Test item	403 µg	-	4 / 40 h	8	0	0	8	12	1	0	13	21	1.05
″	705 µg	-	4 / 40 h	11	2	0	13	3	0	0	3	16	0.80
″	1234 µg	-	4 / 40 h	5	1	1	7	7	2	0	9	16	0.80
″	1851 µg	-	4 / 40 h	12	3	3	18	9	2	0	11	29	1.45

[#] DMSO
[##] MMC
* Evaluation of 4000 binucleate cells

TABLE 8 Experiment I - Number of Micronucleated Cells; Exposure Period 4 h with S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation	Micronucleated cells									
				Binucleate cells with *n* micronuclei culture 1			sum culture 1	Binucleate cells with *n* micronuclei culture 2			sum culture 2	sum in 2000 binucleate cells	[%]
				1	2	>2		1	2	>2			
Solv. control[#]	0.5 %	+	4 / 40 h	10	0	0	10	7	0	0	7	17	0.85
Pos. control[##]	15.0 µg	+	4 / 40 h	56	3	1	60	45	1	0	46	106	5.30
Test item	403 µg	+	4 / 40 h	7	0	0	7	3	0	0	3	10	0.50
″	705 µg	+	4 / 40 h	6	0	0	6	5	1	0	6	12	0.60
″	1234 µg	+	4 / 40 h	3	0	0	3	6	0	0	6	9	0.45
″	1851 µg	+	4 / 40 h	11	0	0	11	6	1	0	7	18	0.90

[#] DMSO
[##] CPA

TABLE 9 Experiment II - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 20 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure / preparation (h)	Cell proliferation culture 1* c1	c2	c4-c8	Proliferation Index CBPI	Cell proliferation culture 2* c1	c2	c4-c8	Proliferation Index CBPI	CBPI mean	Cytostasis [%]
Solv. control[#]	0.5 %	-	20 / 40	15	450	35	2.04	29	425	46	2.03	2.04	
Pos. control[##]	125 ng	-	20 / 40	138	352	10	1.74	69	412	19	1.90	1.82	20.7
Test item	403 µg	-	20 / 40	97	375	28	1.86	23	446	31	2.02	1.94	9.5
″	705 µg	-	20 / 40	35	436	29	1.99	10	461	29	2.04	2.01	2.3
″	1234 µg	-	20 / 40	19	455	26	2.01	18	450	32	2.03	2.02	1.5
″	1851 µg	-	20 / 40	11	474	15	2.01	70	422	8	1.88	1.94	9.2

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

[#] DMSO

[##] Demecolcine

TABLE 10 Experiment II - Number of Micronucleated Cells; Exposure Period 20 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ Preparation (h)	Micronucleated cells									
				Binucleate cells with *n* micronuclei culture 1			sum culture 1	Binucleate cells with *n* micronuclei culture 2			sum culture 2	sum in 2000 binucleate cells	[%]
				1	2	>2		1	2	>2			
Solv. control[#]	0.5 %	-	20 / 40	4	0	0	4	3	0	0	3	7	0.35
Pos. control[##]	125 ng	-	20 / 40	25	3	4	32	23	3	3	29	61	3.05
Test item	403 µg	-	20 / 40	2	0	0	2	0	0	0	0	2	0.10
″	705 µg	-	20 / 40	3	0	1	4	1	0	0	1	5	0.25
″	1234 µg	-	20 / 40	0	0	0	0	4	0	0	4	4	0.20
″	1851 µg	-	20 / 40	5	0	0	5	2	0	0	2	7	0.35

[#] DMSO
[##] Demecolcine

TABLE 11 Biometry

Statistical significance was confirmed by using the Chi-squared test ($\alpha < 0.05$) using a validated R Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

Biometry of Experiment I (Chi-squared test)

Test substance versus solvent control (µg/mL)		Preparation interval (h)	Exposure period (h)	S9 mix	Chi^2
Test substance	403	40	4	-	0.137
"	705	40	4	-	n.c.
"	1234	40	4	-	n.c.
"	1851	40	4	-	3.019
"	403	40	4	+	n.c.
"	705	40	4	+	n.c.
"	1234	40	4	+	n.c.
"	1851	40	4	+	0.029
Positive control versus solvent control (µg/mL)					
MMC	0.8	40	4	-	525.276[s]
CPA	15.0	40	4	+	66.441[s]

n.c. Not calculated as the micronucleus rate is equal or lower than the control rate
s Micronucleus rate is statistically significantly higher than the control rate

Biometry of Experiment II (Chi-squared test)

Test substance versus solvent control (µg/mL)		Preparation interval (h)	Exposure period (h)	S9 mix	Chi^2
Test substance	403	40	20	-	n.c.
"	705	40	20	-	n.c.
"	1234	40	20	-	n.c.
"	1851	40	20	-	n.c.
Positive control versus solvent control per mL					
Demecolcine	125 ng	40	20	-	43.624[s]

n.c. Not calculated as the micronucleus rate is equal or lower than the control rate
s Micronucleus rate is statistically significantly higher than the control rate

TABLE 11 Biometry (continued)

A linear regression was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells, obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Linear regression (Trend test)

Experimental groups	p-value
Experiment I, exposure period 4 hrs without S9 mix	0.341
Experiment I, exposure period 4 hrs with S9 mix	0.832
Experiment II, exposure period 20 hrs without S9 mix	0.741

APPENDICES SECTION

APPENDIX 1 Historical Control Data

Percentage of micronucleated cells in human lymphocyte cultures with and without metabolic activation (2017)

Aqueous solvents: DMEM/Ham's F12, Deionised water (10 % v/v)
Organic solvents: DMSO (0.5 or 1.0 %), Acetone, Ethanol and THF (0.5 %)

Solvent Control without S9		
Micronucleated cells in %		
	Pulse treatment (4/40)	Continuous treatment (20/40)
No. of experiments	61*	65**
Mean	0.62	0.56
95 % Ctrl limit	**0.06 – 1.19**	**0.00 – 1.11**
1x SD (2x SD)	0.28 (0.56)	0.28 (0.56)
Min – Max	0.00 – 1.18	0.05 – 1.10

* Aqueous solvents – 21 Experiments; Organic solvents – 40 Experiments
** Aqueous solvents – 24 Experiments; Organic solvents – 41 Experiments

Solvent Control with S9	
Micronucleated cells in %	
	Pulse treatment (4/40)
No. of experiments	80*
Mean	0.73
95 % Ctrl limit	**0.08 – 1.38**
1x SD (2x SD)	0.33 (0.66)
Min – Max	0.10 – 1.85

* Aqueous solvents – 30 Experiments; Organic solvents – 50 Experiments

APPENDIX 1 Historical Control Data (Continued)

Percentage of micronucleated cells in human lymphocyte cultures with and without metabolic activation (2017)

Positive Control without S9		
Micronucleated cells in %		
	Pulse treatment (4/40)	Continuous treatment (20/40)
	MMC	Demecolcin
No. of experiments	62	65
Mean	14.63	3.68
95 % Ctrl limit	**3.92 – 25.34**	**1.47 – 5.89**
1x SD (2x SD)	5.35 (10.70)	1.11 (2.22)
Min – Max	2.60 – 28.50	2.10 – 8.80

Positive Control with S9	
Micronucleated cells in %	
	Pulse treatment (4/40)
	CPA
No. of experiments	86
Mean	5.45
95 % Ctrl limit	**0.70 – 10.20**
1x SD (2x SD)	2.37 (4.74)
Min – Max	2.25 – 13.30

APPENDIX 2 Copy of GLP Certificate

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

ENVIGO CRS GmbH
In den Leppsteinswiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according chemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical studies on biological materials

13. – 16. Juli 2015
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Th. Zimmermann, Referatsleiter, Wiesbaden, den **14. September 2015**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hess. Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

English name and address of the GLP Monitoring Authority:
Hessian Ministry for Environment, Energy, Agriculture and Consumer Protection;
Department II 10; P.O. Box 31 09; 65189 Wiesbaden
Translation of the seal inscription:
Hessian Ministry for Environment, Rural Regions and Consumer Protection

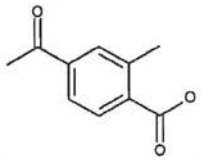
APPENDIX 3 Certificate of Analysis

syngenta

Syngenta Crop Protection AG
GLP Testing Facility WMU
Analytical Development & Product Chemistry
Breitenloh 5
4333 Münchwilen, Switzerland

Certificate of Analysis

CA5021 tech.



81306EI100 - Purity: 96.3 %

Batch Identification	**81306EI100**
Other Batch ID	1007619
Product Code	**CA5021 tech.**
Other Product Code(s)	CA5021A
ISO Common Name	---
CA Reg. No.	---
CA Index Name	---
IUPAC Name	4-acetyl-2-methylbenzoic acid
Molecular formula	$C_{10}H_{10}O_3$
Molecular mass	178.2
Chemical Analysis	
– **Identity of CA5021***	**confirmed**
– **Content of CA5021***	**96.3 % w/w** The detailed results of the characterization are listed in the final report
Methodology used for Characterization / Recertification	NMR, HPLC, Karl Fischer Titration
Physical Analysis	
– **Appearance***	**Light brown solid**
Stability:	
– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of October 2019**

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.
This Certificate of Analysis summarizes data which originates either from a single study or from several individual studies. Tests marked with an asterisk (*) have been conducted in compliance with GLP.
Raw data, documentation, study plans, any amendments to study plans and reports pertaining to this/these study/studies are stored under the study number(s) referenced below within the archives of the GLP Testing Facility WMU at Syngenta Crop Protection AG, Switzerland.

Study number of batch characterization: CHMU170609
Study number(s) of batch recertification:

Authorization: 16-October-2017

Dr. Karine Heintz
Analytical Development & Product Chemistry

Page 1 of 1