**CA4920**

# CA4920 – Micronucleus Assay in Bone Marrow Cells of the Rat

**Final Report Amendment 2**

**DATA REQUIREMENTS:** OECD 474 (1997)
EPA OPPTS 870.5395 (1998)
EC 440/2008 B. 12 (2008)

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**STUDY COMPLETION DATE:** 17 April 2014

**REPORT AMENDMENT 2 COMPLETION DATE:** 11 June 2014

**PERFORMING LABORATORY:** Harlan Cytotest Cell Research GmbH
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**LABORATORY PROJECT ID:**
Report Number: 1601400
Study Number: 1601400
Task Number: TK0206007

**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 Harlan Cytotest Cell Research GmbH (Harlan CCR), 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 [C(97)186/Final].

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).

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

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## FLAGGING STATEMENT

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# QUALITY ASSURANCE STATEMENT

Harlan CCR Study: 1601400

Test substance: CA4920

Study Director: Dr. Eva Dony

Title: CA4920 – Micronucleus Assay in Bone Marrow Cells of the Rat

The general facilities and activities of Harlan CCR are inspected periodically and the results are reported to the responsible person and the management.

Study procedures were inspected periodically. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

| Phases and Dates of QAU Inspections/ Audits | | Dates of Reports to the Study Director and to Management |
|---|---|---|
| Study Plan: | 09 January 2014 | 09 January 2014 |
| 1st Amendment to Study Plan: | 22 January 2014 | 22 January 2014 |
| Process Inspection<br>Test Performance:<br>Test Item preparation & Test System preparation and application: | 16 + 17 January 2014<br><br>11 February 2014 | 17 January 2014<br><br>11 February 2014 |
| Report: | 24 March 2014 | 24 March 2014 |
| Revised Report: | 08 May 2014 | 08 May 2014 |
| 2nd Amendment to Report: | 05 June 2014 | 05 June 2014 |

This statement is to confirm that the present report reflects the raw data.

Head of Quality Assurance Unit for Frauke Hermann

Marina Hahn

.....

Date*: 11 June 2014

* This statement was originally signed by the Quality Assurance on 17 April 2014.

## PROJECT STAFF SIGNATURE

Study Director — Dr. Eva Dony

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Date*: 11 June 2014

* This statement was originally signed by the Study Director on 17 April 2014.

# GENERAL INFORMATION

## Contributors

The following contributed to this report in the capacities indicated:

| Name | Title |
|---|---|
| Dr. Eva Dony | Study Director |
| Dr. Susanne Bohnenberger | Deputy Study Director |
| Dr. Wolfgang Völkner | Management |
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## Study dates

| | |
|---|---|
| Study initiation date: | 09 January 2014 |
| Experimental start date: | 14 January 2014 |
| Experimental termination date: | 20 February 2014 |

## Deviations from the study plan

During the experimental part of the animals used for the main experiment the relative humidity was between 23 – 65 % for a maximum of 7 hours.

An ultraturrax was used to formulate the test substance for the high dose level.

The initial age of the animals at start of the main experiment was 6 – 7 weeks instead of 8 – 12 weeks.

The historical control data were updated.

These deviations did not affect the validity of the study.

**Retention of samples**

Raw data, slides, and a sample of the test substance.

**Performing laboratory test substance reference number**

S 1540811

**Other**

Harlan CCR will archive:

Raw data, study plan, report, and specimens (if any) for at least 3 years at the test facility's archive. Thereafter, the material will be transferred to the GLP archive of Harlan Laboratories Ltd. in Füllinsdorf, Switzerland for archiving the remaining time up to a total archiving period of 15 years. No data will be discarded without the Sponsor's written consent.

A sample of the test substance will be archived two years after the expiration date provided by the Sponsor. If no expiration date is given, the archiving period will be the required 15 years. Thereafter the samples will be discarded without further notice.

**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

This study was performed in order to investigate the potential of CA4920 to induce micronuclei in polychromatic erythrocytes (PCEs) in the bone marrow of the rat.

The test substance was suspended in 1% carboxymethylcellulose (CMC), which was also used as the vehicle control. The volume administered orally was 10 mL/kg body weight (b.w.). A correction factor of 1.02 was applied. At 24 h and 48 h after a single administration of the test substance, the bone marrow cells were collected for micronuclei analysis.

Seven males per test group (except the negative and positive control groups with five males only) were evaluated for the occurrence of micronuclei. Per animal 2000 PCEs were scored for micronuclei.

To describe a cytotoxic effect due to the treatment with the test substance the ratio between polychromatic and normochromatic erythrocytes was determined per slide and reported as the number of PCEs per 2000 erythrocytes.

The following dose levels of the test substance were investigated:
24 h preparation interval: 500, 1000, and 2000 mg/kg b.w.
48 h preparation interval: 2000 mg/kg b.w.

## 1.2 Results

The highest dose was estimated to be a suitable maximum tolerated dose based on a pre-experiment.

After treatment with the test substance the number of PCEs per 2000 erythrocytes was not decreased as compared to the mean value of PCEs per 2000 erythrocytes of the vehicle control, thus indicating that CA4920 did not exert any significant cytotoxic effects in the bone marrow.

In comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval after administration of the test substance with any dose level used. For all treatment groups the mean values of micronuclei observed after treatment with CA4920 were very well within the historical vehicle control range. Additionally, no dose dependency was observed.

A dose of 20 mg/kg b.w. cyclophosphamide administered orally was used as the positive control, which showed a substantial increase of induced micronucleus frequency. The volume of the positive control administered was 10 mL/kg b.w..

## 1.3 Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the rat. Therefore, CA4920 is considered to be non-mutagenic in this bone marrow micronucleus assay.

# 2.0 INTRODUCTION

This report was issued to replace the Revised Report and to correct formatting errors in the font of the original report.

The changes presented in this amended report do not affect the integrity of the study. This amended report replaces the original final report and revised report. The replaced original of the final report and revised report will be archived with the raw data.

## 2.1 Purpose

This *in vivo* experiment was performed to assess the mutagenic properties of the test substance by means of the micronucleus test in bone marrow cells of the rat.

## 2.2 Justification of Test System

The occurrence of micronuclei in interphase cells provides an indirect but easy and rapid measure of chromosomal damage. Micronuclei arise from acentric chromosomal fragments or whole chromosomes induced by clastogens or agents affecting the spindle apparatus (1,2,3,4,5).

PCEs in the bone marrow of the rat are the cell population of choice for mammalian cells *in vivo*. PCEs are newly formed red blood cells and are easily identifiable by their staining properties. These cells have the advantage that the micronuclei can be readily detected because the nucleus is extruded from the erythroblast after the last cell division.

The first appearance of micronuclei in PCEs is at least 10 - 12 hours after a clastogenic exposure. This lag is due to the time required for the affected erythroblast to differentiate into a polychromatic erythrocyte. This differentiation process includes:

1. The time required for the damaged erythroblast to proceed to mitosis.
2. The mitotic delay induced by the treatment.
3. The formation of micronuclei due to acentric fragments or chromosomes that are not included in the daughter nuclei.
4. The time required for the expulsion of the main nucleus after the last mitosis to become a micronucleated PCE.

This newly formed cell population persists for about 20 hours in the bone marrow of the rat. During this time micronucleated PCEs can accumulate in the bone marrow in response to a clastogenic exposure, as the production of micronuclei extends over a considerable period of time.

The time at which the micronucleus frequency is at a maximum varies from agent to agent (6). Due to mitotic delay or metabolic and pharmacokinetic effects the appearance of micronucleated PCEs can be considerably delayed. Therefore, a single sampling time is not optimal. Results obtained with model mutagens showed that samples taken at 24 h and 48 h after treatment cover the intervals in which maximum frequencies of micronuclei occur.

For the initial assessment of clastogenic activity a single dose level at the maximum tolerated dose or that producing some indication of cytotoxicity (change in the ratio of polychromatic to normochromatic erythrocytes) and sampling at 24 h and 48 h after treatment is recommended. For verification two additional dose levels are tested at a sampling time of 24 h after treatment to establish a dose response effect.

To validate the test, a reference mutagen is 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 guidelines and recommendations:

9th Addendum to the OECD Guideline for Testing of Chemicals, Section 4, No. 474, adopted July 21, 1997, “Mammalian Erythrocyte Micronucleus Test“.

Environmental Protection Agency, Health Effects Test Guidelines OPPTS 870.5395 “Mammalian Erythrocyte Micronucleus Test“, EPA 712-C-98-226, August 1998.

Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Part B: Methods for the determination of toxicity and other health effects: Mutagenicity – In vivo Mammalian Erythrocyte Micronucleus Test, No B.12; No L 142.

# 3.0 MATERIALS AND METHODS

## 3.1 Test Substance

Internal Test Substance Number: S 1540811

The test substance and the information concerning the test substance were provided by the Sponsor.

| | |
|---|---|
| Identity: | CA4920 |
| Other Product Code: | CA4920A tech. |
| IUPAC Name: | 9-dichloromethylene-2,3,4,6,7,8-hexahydro-1H-1,4-methano-naphthalen-5-one oxime |
| Batch No.: | SMU1DP008 |
| Active Ingredient Content: | 98.1 % w/w<br>(Dose calculation was adjusted to purity.) |
| Stability in Solvent: | Not indicated by the Sponsor |
| Storage: | Room temperature |
| Expiry: | End of April 2017 (Retest Date) |

On the day of the experiment, the test substance was suspended in 1% CMC. Grinding of the test substance in a mortar was used to formulate the test substance. An ultraturrax was used to formulate the test substance for the high dose level. The vehicle was chosen by the Sponsor and due to its relative non-toxicity for the animals and ability to formulate a suitable dosing preparation. All animals received a single standard volume once orally. The oral route was used as this is of relevance to human risk assessment. A correction factor of 1.02 was applied.

## 3.2 Controls

### 3.2.1 Negative control

The test substance vehicle was used as negative control.

| | |
|---|---|
| Name: | 1% CMC |
| Batch no.: | BCBD7651V |
| Expiry Date: | January 2017 |
| Suspended in: | sterile water |
| Batch no.: | 134618061 |
| Expiry Date: | October 2016 |
| Route and Frequency of Administration: | orally, once |
| Volume Administered: | 10 mL/kg b.w. |

### 3.2.2 Positive control

| | |
|---|---|
| Name: | Cyclophosphamide, CPA |
| Batch: | A0302605 |
| Expiry Date: | October 2014 |
| Dissolved in: | sterile water |
| Batch no.: | 134618061 |
| Expiry Date: | October 2016 |
| Dosing: | 20 mg/kg b.w. |
| Route and frequency of administration: | orally, once |
| Volume administered: | 10 mL/kg b.w. |

Solution prepared on day of administration.

The stability of CPA at room temperature was sufficient. At 25 °C only 3.5 % of its potency was lost after 24 hours (7).

## 3.3 Test System

### 3.3.1 Reasons for the choice of the experimental animal species

The rat is an animal that has been used for many years as a suitable experimental animal in cytogenetic investigations. There are many data available from such investigations, which may be helpful in the interpretation of results from the micronucleus test. In addition, the rat is an experimental animal in many physiological, pharmacological and toxicological studies. Data from such experiments also may be useful for the design and the performance of the micronucleus test (1,2,3,4,5,6).

| | |
|---|---|
| Strain: | Rat (Wistar) |
| Source | Harlan Laboratories B.V.<br>Postbus 6174<br>5960 AD Horst / The Netherlands |
| Number of Animals in the pre-test: | 2 males and 2 females for the pre-test |
| Number of Animals in the main study: | 43 males |
| Initial Age at Start of Experiment: | 6 - 7 weeks |
| Acclimation: | minimum 5 days |
| Initial Body Weight at Start of Treatment: | mean value 219.9 g (*SD ± 9.3 g); range 203.8 – 238.5 g |

According to the suppliers assurance the animals were in healthy condition. The animals were under acclimatisation in the animal house of Harlan CCR for a minimum of five days after their arrival. During this period the animals did not show any signs of illness or altered behaviour.

The animals were distributed into the test groups at random and identified by cage number.

*SD: Standard Deviation

### 3.3.2 Husbandry

The animals were kept conventionally. The experiment was conducted under standard laboratory conditions. The diet and water were routinely analysed to ensure the absence of any contaminant that could reasonably be expected to affect the purpose or integrity of the study. Certificates of analysis are retained at Harlan CCR.

| | |
|---|---|
| Housing: | group |
| Cage type: | Makrolon Type II (pre-test) / III (main study), with wire mesh top |
| Bedding: | Granulated soft wood bedding |
| Feed: | Pelleted standard diet, *ad libitum* |
| Water: | Tap water, *ad libitum* |
| Environment: | Temperature 22 ± 2°C<br>Relative humidity 45 - 65 % (for a short period 7 h the relative humidity was decreased to 23%)<br>Artificial light 6.00 a.m. - 6.00 p.m. |

## 3.4 Experimental Performance

### 3.4.1 Pre-Experiment

A preliminary study of acute toxicity was performed in both male and female mice (two animals per sex and dose level) under identical conditions as in the mutagenicity study concerning: animal strain, vehicle, route, frequency, and volume of administration.

The animals were treated once orally with the test substance and examined for acute toxic symptoms at intervals of around 0-1 h, 2-4 h, 5-6 h, 24 h, 30 h, and 48 h after administration of the test substance.

The test dose levels were chosen using doses from the following scheme starting at 2000 mg/kg:

5 – 8 – 12.5 – 20 – 32 – 50 – 80 – 125 – 200 – 320 – 500 – 800 – 1250 – 2000 mg/kg b.w..

No substantial sex specific differences on toxic symptoms were observed, therefore, the main experiment was performed using male animals only.

### 3.4.2 Main-Experiment

It is generally recommended to use the maximum tolerated dose or the highest dose that can be dissolved and administered reproducibly or 2000 mg/kg as the upper limit for non-toxic test substances.

The maximum tolerated dose level is determined to be the dose that causes signs of toxicity without having major effects on survival within 48 hours.

The administered volume was 10 mL/kg b.w..

Three adequately spaced dose levels spaced by a factor of 2 were applied, and bone marrow samples were collected at the central sampling interval of 24 h after treatment. For the highest dose level an additional bone marrow sample was taken at 48 h after treatment.

### 3.4.3 Study procedure

Seven males were assigned to each test group (except the negative and positive control groups with five animals each). The animals were identified by their cage number as shown in Table 1.

### 3.4.4 Treatment

At the beginning of the treatment the animals (including the controls) were weighed and the individual volume to be administered was adjusted to the animal's body weight. The animals received the test substance, the negative or the positive control substance once orally. Seven males were treated per dose group and sampling time. Five males each were treated for the negative and positive control groups. The animals of all dose groups, except the positive control group were examined for acute toxic symptoms at intervals of around 0-1 h, 2-4 h, 5-6 h, 24 h, and 48 h after administration of the test substance or the vehicle controls.

Sampling of the bone marrow was done 24 and 48 hours after treatment, respectively.

## 3.5 *Post mortem* Investigations

### 3.5.1 Preparation of the animals

The animals were sacrificed using $CO_2$ followed by bleeding. The femora were removed, the epiphyses were cut off and the marrow was flushed out with fetal calf serum using a syringe. The nucleated cells were separated from the erythrocytes using the method of Romagna (8). The cell suspensions were passed through a column consisting of α-Cellulose and Cellulose. The columns will then be washed with Hank´s buffered saline. The cell suspension was centrifuged at 1500 rpm (390 × g) for 10 minutes and the supernatant was discarded. A small drop of the re-suspended cell pellet was spread on a slide. The smear was air-dried and then stained with May-Grünwald /Giemsa. Cover slips were mounted with EUKITT. At least one slide was made from each bone marrow sample.

## 3.6 Data Evaluation

### 3.6.1 Slide analysis

Evaluation of the slides was performed using NIKON microscopes with 100× oil immersion objectives. Per animal 2000 PCEs were analysed for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes was determined from the same slide and expressed in polychromatic erythrocytes per 2000 erythrocytes. The analysis was performed with coded slides. Immature and mature erythrocytes were identified by their pale and blue to green colour, respectively. Micronuclei are distinguished by being small nuclei separate from and additional to the main nuclei of the cells.

All animals per test group were evaluated as described.

### 3.6.2 Data recording

The data generated are recorded in the laboratory records. The results are presented in tabular form, including experimental groups, negative, and positive control. The micro-nucleated cells per 2000 PCEs and the ratio of polychromatic erythrocytes to total erythrocytes are presented for each animal.

### 3.6.3 Acceptance criteria

The study was considered valid as the following criteria were met:

- at least 5 animals per group could be evaluated.
- PCE to erythrocyte ratio was not less than 20 % of the negative control.
- the positive control showed a statistically significant and biologically relevant increase of micronucleated PCEs compared to the negative control.

### 3.6.4 Evaluation of results

A test substance is classified as mutagenic if it induces either a dose-related increase or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group. Statistical methods (nonparametric Mann-Whitney test (9)) were used as an aid in evaluating the results. However, the primary point of consideration was the biological relevance of the results.

A test substance that fails to produce a biologically relevant increase in the number of micronucleated polychromatic erythrocytes is considered non-mutagenic in this system.

A test substance failing to meet the criteria for a positive or negative response may be judged equivocal in this assay and may be considered for further investigation.

# 4.0 RESULTS AND DISCUSSION

## 4.1 Pre-experiment

In the pre-experiment 2 male and 2 female animals received a single oral dose of CA4920 (2000 mg/kg b.w.) suspended in 1% CMC (10 mL/kg b.w.). The animals treated with 2000 mg/kg b.w. displayed signs of toxicity as shown in Table 2.

On the basis of these data 2000 mg/kg b.w., the maximum OECD Guideline recommended dose for this assay was considered suitable. No substantial gender specific differences in toxicity were observed, thus, the main study was performed using male animals only, as permitted by the Guideline.

## 4.2 Signs of Toxicity in the Main Experiment

In the main experiment for the high dose groups 14 males (2 × 7 males per group) received orally a single dose of 2000 mg/kg b.w. CA4920 suspended in 1% CMC. The volume administered was 10 mL/kg b.w.. The animals treated with 2000 mg/kg b.w. displayed signs of toxicity as shown in Table 3, which included reduced spontaneous activity only. These signs of systemic toxicity are indicative of systemic exposure to the test material.

For the mid dose group 7 males received orally a single dose of 1000 mg/kg b.w. CA4920 suspended in 1% CMC. The volume administered was 10 mL/kg b.w.. The animals treated with 1000 mg/kg b.w. did not show any clinical symptoms.

For the low dose group 7 males received orally a single dose of 500 mg/kg b.w. CA4920 suspended in 1% CMC. The volume administered was 10 mL/kg b.w.. The animals treated with 500 mg/kg b.w. did not show any clinical symptoms.

The animals of the vehicle control groups (1% CMC) for both sampling times also did not show any clinical symptoms.

### 4.2.1 Micronucleus test results

The mean number of polychromatic erythrocytes was not decreased after treatment with the test substance as compared to the mean value of PCEs of the vehicle control, indicating that CA4920 did not have any significant cytotoxic properties on the bone marrow (Table 4).

In comparison to the corresponding vehicle controls there was no biologically relevant enhancement and no statistically significant increase in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test substance (Table 4 and 5).

For all treatment groups the mean values of micronuclei observed after treatment with CA4920 were very well within the historical vehicle control range.

## 4.3 Discussion

The test substance CA4920 was assessed in the micronucleus assay for its potential to induce micronuclei in PCEs in the bone marrow of the rat.

The test substance was suspended in 1% CMC, which was also used as the vehicle control. The volume administered orally was 10 mL/kg body weight (b.w.). A correction factor of 1.02 was applied. At 24 h and 48 h after a single administration of the test substance, the bone marrow cells were collected for micronuclei analysis.

Seven males per test group (except the negative and positive control groups with five males only) were evaluated for the occurrence of micronuclei. Per animal 2000 PCEs were scored for micronuclei.

To describe a cytotoxic effect due to the treatment with the test substance the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and reported as the number of PCEs per 2000 erythrocytes. The following dose levels of the test substance were investigated:

24 h preparation interval: 500, 1000, and 2000 mg/kg b.w.

48 h preparation interval: 2000 mg/kg b.w.

As estimated by a pre-experiment in male and female mice, 2000 mg CA4920 per kg b.w. was suitable as the highest dose for male mice. Since no obvious substantial gender-specific differences in the sensitivity to the test substance were observed and as requested by the sponsor, the main experiment was performed using male animals only.

The mean number of PCEs was not decreased after treatment with the test substance as compared to the mean value of PCEs of the vehicle control, indicating that CA4920 did not have any significant cytotoxic properties in the bone marrow.

In comparison to the corresponding vehicle control values there was no biologically relevant enhancement and no statistically significant increase in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test substance. The mean values of micronuclei observed after treatment with CA4920 were for all dose groups very well within the historical vehicle control range. Additionally no dose dependence was observed.

A dose of 20 mg/kg b.w. CPA administered orally was used as positive control which showed a statistically significant increase of induced micronucleus frequency. The volume of the positive control administered was 10 mL/kg b.w.

## 5.0 CONCLUSIONS

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test substance did not induce micronuclei as determined by the micronucleus test in the bone marrow cells of the rat. Therefore, CA4920 is considered to be non-mutagenic in this bone marrow micronucleus assay.

## 6.0 REFERENCES


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# TABLES SECTION

**TABLE 1 Identification of the Animals by their Cage Number**

| Test group | hours post-treatment 24 | 48 |
|---|---|---|
| Negative control | 1 – 5 | 32 – 36 |
| Low dose | 6 – 12 | |
| Medium dose | 13 – 19 | |
| High dose | 20 – 26 | 37 – 43 |
| Positive control | 27 – 31 | |

**TABLE 2 Pre-Experiment for Toxicity: 2000 mg/kg b.w. CA4920**

| Signs of Toxicity | hours post-treatment male / female | | | | | |
|---|---|---|---|---|---|---|
| | 0-1 h | 2-4 h | 5-6 h | 24 h | 30 h | 48 h |
| Reduction of spontaneous activity | 0/0 | 2/1 | 1/0 | 0/0 | 0/0 | 0/0 |

**TABLE 3 Signs of Toxicity in the Main Experiment: 2000 mg/kg b.w. CA4920**

| Signs of Toxicity | hours post-treatment males | | | | |
|---|---|---|---|---|---|
| | 0-1 h | 2-4 h | 5-6 h | 24 h | 48 h* |
| Reduction of spontaneous activity | 0 | 6 | 6 | 0 | 0 |

*: data from 7 males only.

**TABLE 4 Summary of Micronucleus Test Results**

| test group | dose mg/kg b.w. | sampling time (h) | PCEs with micronuclei (%) | range | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| negative control | 0 | 24 | 0.120 | 0 - 7 | 1064 |
| test substance | 500 | 24 | 0.207 | 2 - 8 | 1112 |
| test substance | 1000 | 24 | 0.179 | 1 - 6 | 1123 |
| test substance | 2000 | 24 | 0.257 | 2 - 9 | 1129 |
| positive control | 20 | 24 | 2.470 | 26 -71 | 1061 |
| negative control | 0 | 48 | 0.270 | 1 - 8 | 1042 |
| test substance | 2000 | 48 | 0.336 | 3 -12 | 1106 |

**TABLE 5 Biometry**

Statistical significance at the five per cent level ($p < 0.05$) for the incidence of micronuclei was evaluated by means of the non-parametric Mann-Whitney test.

| Negative control versus test group | Significance | p |
|---|---|---|
| 500 mg CA4920 /kg b.w.; 24 h | - | 0.1174 |
| 1000 mg CA4920 /kg b.w.; 24 h | - | 0.2083 |
| 2000 mg CA4920 /kg b.w.; 24 h | - | 0.0694 |
| 20 mg CPA/kg b.w.; 24 h | + | 0.0040 |
| 2000 mg CA4920 /kg b.w.; 48 h | - | 0.4394 |

+ = significant;
- = not significant;

**TABLE 6 Micronuclei in Polychromatic Erythrocytes (PCE) and Relationship PCE/Total Erythrocytes Scoring 24 Hours after Treatment**

A. Negative control:

| animal no. | sex | test group | dose mg/kg b.w. | micronucleated cells per 2000 PCEs per animal | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| 1 | m | 1% CMC | 0 | 2 | 1012 |
| 2 | m | | | 0 | 981 |
| 3 | m | | | 1 | 1149 |
| 4 | m | | | 2 | 1156 |
| 5 | m | | | 7 | 1020 |
| | | | sum | 12 | 5318 |
| | | | mean (± SD) | 2.4 (± 2.7) | 1064 |
| | | **percent cells with micronuclei** | | **0.120** | |

B. 500 mg/kg b.w. test substance:

| animal no. | sex | test group | dose mg/kg b.w. | micronucleated cells per 2000 PCEs per animal | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| 6 | m | CA4920 | 500 | 2 | 1104 |
| 7 | m | | | 8 | 1208 |
| 8 | m | | | 4 | 1052 |
| 9 | m | | | 6 | 1112 |
| 10 | m | | | 2 | 1175 |
| 11 | m | | | 3 | 1103 |
| 12 | m | | | 4 | 1029 |
| | | | sum | 29 | 7783 |
| | | | mean (± SD) | 4.1 (± 2.2) | 1112 |
| | | **percent cells with micronuclei** | | **0.207** | |

**TABLE 6 Micronuclei in Polychromatic Erythrocytes (PCE) and Relationship PCE/Total Erythrocytes Scoring 24 Hours after Treatment (Continued)**

C. 1000 mg/kg b.w. test substance:

| animal no. | sex | test group | dose mg/kg b.w. | micronucleated cells per 2000 PCEs per animal | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| 13 | m | CA4920 | 1000 | 3 | 934 |
| 14 | m | | | 1 | 1340 |
| 15 | m | | | 3 | 1318 |
| 16 | m | | | 2 | 1008 |
| 17 | m | | | 4 | 1141 |
| 18 | m | | | 6 | 1040 |
| 19 | m | | | 6 | 1080 |
| | | | sum | 25 | 7861 |
| | | | mean (± SD) | 3.6 (± 1.9) | 1123 |
| | | **percent cells with micronuclei** | | **0.179** | |

D. 2000 mg/kg b.w. test substance:

| animal no. | sex | test group | dose mg/kg b.w. | micronucleated cells per 2000 PCEs per animal | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| 20 | m | CA4920 | 2000 | 2 | 991 |
| 21 | m | | | 2 | 1142 |
| 22 | m | | | 7 | 1123 |
| 23 | m | | | 7 | 976 |
| 24 | m | | | 7 | 1267 |
| 25 | m | | | 9 | 1078 |
| 26 | m | | | 2 | 1329 |
| | | | sum | 36 | 7906 |
| | | | mean (± SD) | 5.1 (± 3.0) | 1129 |
| | | **percent cells with micronuclei** | | **0.257** | |

**TABLE 6 Micronuclei in Polychromatic Erythrocytes (PCE) and Relationship PCE/Total Erythrocytes Scoring 24 Hours after Treatment (Continued)**

E. Positive control:

| animal no. | sex | test group | dose mg/kg b.w. | micronucleated cells per 2000 PCEs per animal | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| 27 | m | Cyclophosphamide | 20 | 44 | 1189 |
| 28 | m | | | 48 | 1074 |
| 29 | m | | | 26 | 877 |
| 30 | m | | | 58 | 1133 |
| 31 | m | | | 71 | 1032 |
| | | | sum | 247 | 5305 |
| | | | mean (± SD) | 49.4 (± 16.7) | 1061 |
| | | **percent cells with micronuclei** | | **2.470** | |

**TABLE 7 Micronuclei in Polychromatic Erythrocytes (PCE) and Relationship PCE/Total Erythrocytes Scoring 48 Hours after Treatment**

A. Negative control:

| animal no. | sex | test group | dose mg/kg b.w. | Micronucleated cells per 2000 PCEs per animal | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| 32 | m | 1% CMC | 0 | 1 | 966 |
| 33 | m | | | 8 | 1034 |
| 34 | m | | | 8 | 976 |
| 35 | m | | | 5 | 1192 |
| 36 | m | | | 5 | 1041 |
| | | | sum | 27 | 5209 |
| | | | mean (± SD) | 5.4 (± 2.9) | 1042 |
| | | **percent cells with micronuclei** | | **0.270** | |

B. 2000 mg/kg b.w. test substance:

| animal no. | sex | test group | dose mg/kg b.w. | micronucleated cells per 2000 PCEs per animal | PCE per 2000 erythrocytes |
|---|---|---|---|---|---|
| 37 | m | CA4920 | 2000 | 4 | 1135 |
| 38 | m | | | 7 | 892 |
| 39 | m | | | 7 | 1176 |
| 40 | m | | | 12 | 1044 |
| 41 | m | | | 3 | 1375 |
| 42 | m | | | 11 | 1076 |
| 43 | m | | | 3 | 1044 |
| | | | sum | 47 | 7742 |
| | | | mean | 6.7 (± 3.7) | 1106 |
| | | **percent cells with micronuclei** | | **0.336** | |

## TABLE 8 Individual Animal Weights at the Start of the Experiment

| Dose Group | Animal No. | Initial Weight (g) | Mean Weight (g) | Standard Deviation | Range (g) |
|---|---|---|---|---|---|
| Negative control Group; 1% CMC; 24h Interval | 1 | 206.9 | | | |
| | 2 | 231.9 | | | |
| | 3 | 212.4 | | | |
| | 4 | 210.6 | | | |
| | 5 | 234.2 | 219.2 | ± 12.8 | 206.9 – 234.2 |
| Low Dose Group (500 mg/kg b.w.); 24h Interval | 6 | 227.2 | | | |
| | 7 | 215.9 | | | |
| | 8 | 223.4 | | | |
| | 9 | 238.5 | | | |
| | 10 | 220.0 | | | |
| | 11 | 220.6 | | | |
| | 12 | 213.6 | 222.7 | ± 8.3 | 213.6 – 238.5 |
| Medium Dose Group (1000 mg/kg b.w.); 24h Interval | 13 | 234.3 | | | |
| | 14 | 219.8 | | | |
| | 15 | 232.2 | | | |
| | 16 | 219.6 | | | |
| | 17 | 220.0 | | | |
| | 18 | 218.4 | | | |
| | 19 | 203.8 | 221.2 | ± 10.1 | 203.8 – 234.3 |
| High Dose Group (2000 mg/kg b.w.); 24h Interval | 20 | 224.9 | | | |
| | 21 | 220.4 | | | |
| | 22 | 215.2 | | | |
| | 23 | 206.7 | | | |
| | 24 | 207.3 | | | |
| | 25 | 234.8 | | | |
| | 26 | 220.6 | 218.6 | ± 9.9 | 206.7 – 234.8 |
| Positive Control (CPA, 20 mg/kg b.w.); 24h Interval | 27 | 213.5 | | | |
| | 28 | 211.9 | | | |
| | 29 | 230.8 | | | |
| | 30 | 231.1 | | | |
| | 31 | 213.9 | 220.2 | ± 9.8 | 211.9 – 231.1 |
| Negative Control Group;1% CMC; 48h Interval | 32 | 221.0 | | | |
| | 33 | 212.1 | | | |
| | 34 | 214.2 | | | |
| | 35 | 231.2 | | | |
| | 36 | 215.6 | 218.8 | ± 7.7 | 212.1 – 231.2 |
| High Dose Group (2000 mg/kg b.w.); 48h Interval | 37 | 208.4 | | | |
| | 38 | 204.7 | | | |
| | 39 | 230.1 | | | |
| | 40 | 231.2 | | | |
| | 41 | 223.8 | | | |
| | 42 | 219.6 | | | |
| | 43 | 211.4 | 218.5 | ± 10.6 | 204.7 – 231.2 |
| Summary | | | 219.9 | ± 9.3 | 203.8 – 238.5 |

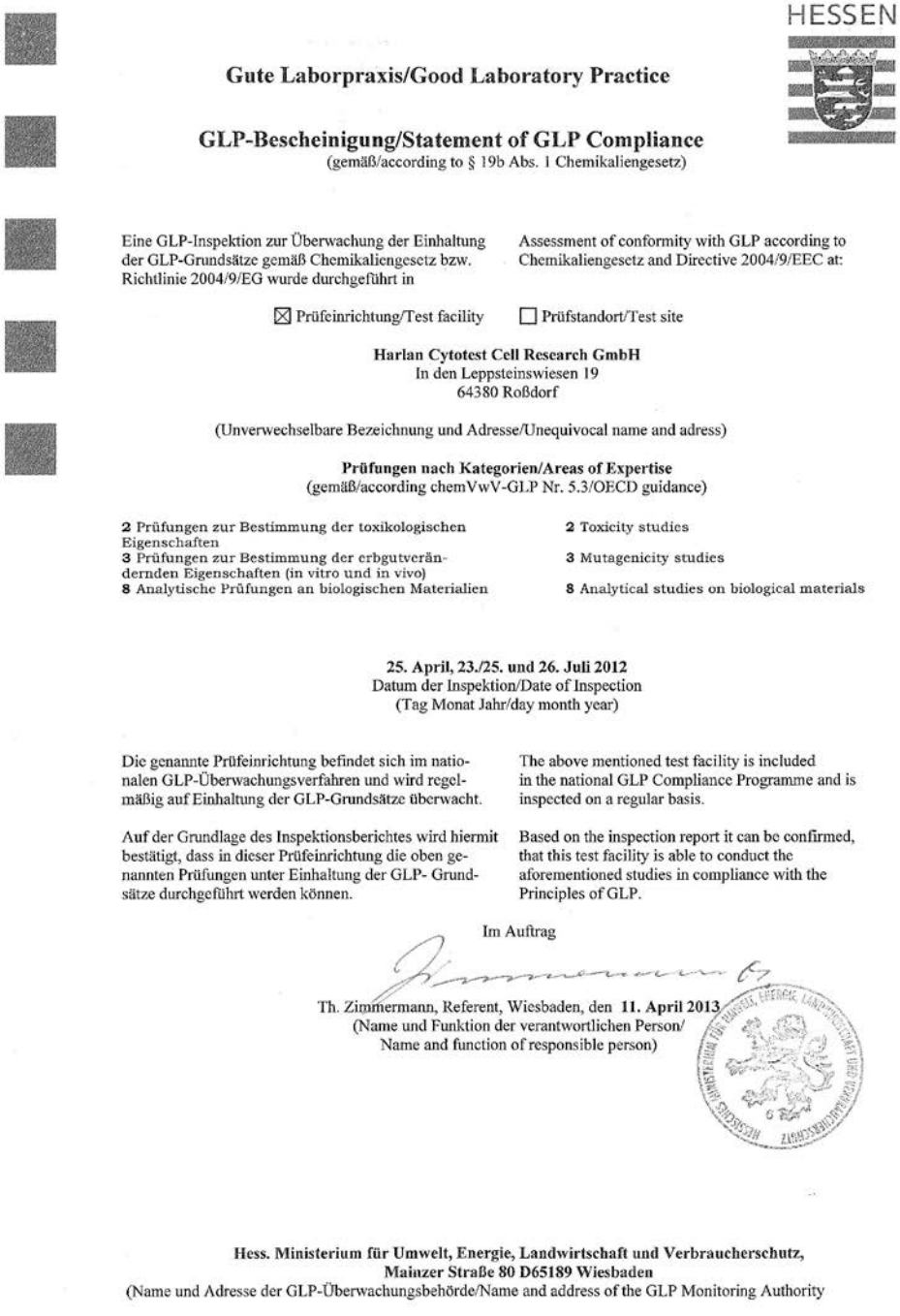
# APPENDICES SECTION

## APPENDIX 1 Historical Control Data

**2009 – 2013**

| Micronucleated cells | Negative Controls Males | Positive Controls (CPA) Males |
|---|---|---|
| Mean ± SD (%) | 0.196 ± 0.123 | 2.276 ± 1.061 |
| Range of mean group value (%) | 0.000 - 0.550 | 0.300 - 5.700 |
| Range (individual animal data) | 0 - 11 | 6 - 114 |
| No. of Experiments | 36 | 36 |

## 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

**Harlan Cytotest Cell Research 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 |

**25. April, 23./25. und 26. Juli 2012**
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, Referent, Wiesbaden, den **11. April 2013**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

**Hess. Ministerium für Umwelt, Energie, 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
English translation of the GLP Monitoring Authority Stamp: Hessian Ministry for Environment, Area for Agricultural Use and Consumer Protection

## APPENDIX 3 Copy of Certificate of Analysis

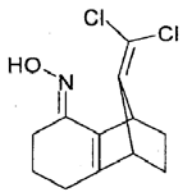
syngenta

**GLP Testing Facility WMU**
Analytical Development & Product Chemistry

**Syngenta Crop Protection Münchwilen AG**
Breitenloh 5
CH-4333 Münchwilen

# Certificate of Analysis

**CA4920**




**SMU1DP008**
**Purity 98.1 %**

| | |
|---|---|
| **Batch Identification** | **SMU1DP008** |
| **Product Code** | **CA4920 tech.** |
| Other Product Code(s) | CA4920A |
| ISO Common Name | --- |
| CA Reg. No. | --- |
| CA Index Name | --- |
| IUPAC Name | 9-dichloromethylene-2,3,4,6,7,8-hexahydro-1H-1,4-methano-naphthalen-5-one oxime |
| Molecular formula | $C_{12}H_{13}Cl_2NO$ |
| Molecular mass | 258.1 |
| **Chemical Analysis** | |
| – **Identity *** | **confirmed** |
| – **Content of CA4920 *** | **98.1 % w/w** |
| Methodology used for Characterization / Recertification | HPLC |
| **Physical Analysis** | |
| – **Appearance *** | **Pale yellow powder** |

**Stability:**

| | |
|---|---|
| – **Storage Temperature** | **< 30°C** |
| – **Recertification Date** | **End of April 2017** |

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 Muenchwilen AG.

Study number of batch characterization: 123266
Study number(s) of batch recertification: 125709

Authorization: 25 April 2013

Dr. R. Kettner
Analytical Development & Product Chemistry