SYN549522

SYN549522 SC (A22011B) – Micronucleus Assay in Bone Marrow Cells of the Rat

Final Report

DATA REQUIREMENT(S): OECD 474 (2016)

AUTHOR(S): Dr. Eva Dony

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PERFORMING LABORATORY: Envigo CRS GmbH
In den Leppsteinswiesen 19
64380 Rossdorf, Germany

LABORATORY PROJECT ID: Report Number: 1913400
Study Number: 1913400
Task Number: TK0317164

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

VOLUME 1 OF 1 OF STUDY

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 [C(97)186/Final]

These procedures are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHW, MAFF, and METI).

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

Dr. Eva Dony
Study Director *in vivo* Genotoxicity

E. D
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Date: 26 August 2019

Performing Laboratory:
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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Envigo Study Number: 1913400

Test Substance: SYN549522 SC (A22011B)

Study Director: Dr. Eva Dony

Study Title: SYN549522 SC (A22011B) – Micronucleus Assay in Bone Marrow Cells of the Rat

Study-related procedures conducted at the test facility were audited and inspected. The details of these audits and inspections are given below.

Dates and Types of QA Inspections			**Reported to the relevant Study Director and Test Facility Management**
Date of Inspection	Type of Inspection	Phase Inspected	Report Date
25 July 2018	Study Plan Verification	N/A	25 July 2018
27 August 2018	Study – based	Test Item Preparation Test System Preparation and Application	27 August 2018
28 August 2018	Study – based	Test Performance	28 August 2018
02 January 2019	Report Audit	N/A	02 January 2019

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.

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

Quality Assurance:

H. Pilawa

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Date:

PROJECT STAFF SIGNATURE

Study Director Dr. Eva Dony

E. D

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Date: 26 August 2019

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
Dr. Eva Dony	Study Director
Frauke Hermann	Head of Quality Assurance Unit
Eva Lessmann	Syngenta Study Manager

Study Dates

Study initiation date:	25 July 2018
Experimental start date:	31 July 2018
Experimental completion date:	12 November 2018

Deviations from the Guidelines

None

Retention of Samples

Raw data and microscopic slides.

Performing Laboratory Test Substance Reference Number

[S 1983911]

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, and Report.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant Archive of Covance CRS (Switzerland) Ltd.[1] at Füllinsdorf, Switzerland, for further archiving up to a total archiving period of 15 years.

Samples and specimens that no longer afford evaluation will be discarded in accordance with Standard Operating Procedures and without further notice.

A sample of the test item will not be archived.

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

[1] On 04 June 2019 the legal entity Envigo CRS (Switzerland) was renamed as Covance CRS (Switzerland) Ltd.. No other changes to the legal entity have occurred.

Deviations from the study plan

The following deviations from study plan occurred:

> The relative humidity in the animal room was between approximately 28-65 % instead of 45–65% and the temperature was between 20 to 29°C instead of 20 to 24°C for several hours.

These deviations to the study plan, however, did not affect the validity of the study.

Distribution of the report

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

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

The study was performed in order to investigate the potential of SYN549522 SC (A22011B) to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the rat.

The test substance was suspended in sterile water, which was also used as the vehicle control. The volume administered orally was 10 mL/kg body weight (b.w.). At 24 and 48 hours 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 a total number of 6000 polychromatic erythrocytes (PCEs) were scored for micronuclei.

To describe a cytototxic 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 total erythrocytes.

The following dose levels of the 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 one pre-experiment.

After treatment with the test substance the number of PCEs per total erythrocytes was not substantially decreased as compared to the mean value of PCEs per total erythrocytes of the vehicle control, indicating that SYN549522 SC (A22011B) did not exert any significant cytotoxic effects in the bone marrow. Exposure to the bone marrow by the active ingredients in the formulation tested in this study is supported by previous toxicological studies

In comparison to the corresponding vehicle controls, there was a minimal increase in the frequency of the detected micronuclei at the 24 h preparation interval after administration of the test substance at all three dose levels used. For all treatment 24h groups the mean values of micronuclei observed after treatment with SYN549522 SC (A22011B) were slightly higher than the corresponding vehicle control and slightly outside of the upper 95% confidence interval of the historical vehicle control. These increases in micronucleus frequency in the 24 h preparation groups, however, were not considered to be of biological relevance since they were not statistically significant, and no clear dose dependency was observed in the linear regression analysis.

Furthermore, at the 48 h preparation interval, no increase in micronucleus frequency was observed in the treated animals.

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, SYN549522 SC (A22011B) is considered to be negative, i.e. non-genotoxic, in this bone marrow micronucleus assay.

2.0 INTRODUCTION

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).

Polychromatic erythrocytes (PCE) 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 PCE. 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 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:

Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, Section 4, No. 474, adopted July 29, 2016, “Mammalian Erythrocyte Micronucleus Test”.

3.0 MATERIALS AND METHODS

3.1 Test Substance

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

Identification:	SYN549522 SC (A22011B)
Other product codes:	SYN549522 SC (450)
Batch:	SMU7JP001
Identity of the Active Ingredients	confirmed
Active Ingredients Content	Content of SYN549522: 38.1% w/w (corresponding to 448 g/l) Content of SYN547386: 34.3% w/w (corresponding to 403 g/l) Content of SYN548941: 3.83% w/w (corresponding to 45.0 g/l)
Recertification Date:	30 November 2020
Physical State / Appearance:	Beige liquid
Storage Conditions: (provided by the Sponsor)	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

Dose calculation was not adjusted to content of the active ingredients.

On the day of the experiment, the test substance was suspended in sterile water. Vortexing was used to formulate the test substance in the vehicle. Homogeneity of the test substance in vehicle was maintained during treatment using a magnetic stirrer. All animals received a single standard volume once orally. The vehicle was chosen due to its relative non-toxicity for the animals and its ability to form a suitable dosing suspension. The oral route was used as this is of relevance to human risk assessment.

The preparations were made freshly before the dosing occasion.

3.2 Controls

3.2.1 Negative controls

The test substance vehicle was used as a negative control.

Name:	sterile water
Batch No.:	19KD10GA
Expiry Date:	March 2019
Route and Frequency of Administration	orally, once
Volume Administered:	10 mL/kg b.w.

3.2.2 Positive control

Name:	Cyclophosphamide (CPA)
Batch:	A0395065
Expiry Date:	01 August 2019
Dissolved in:	sterile water
Batch No.:	19KD10GA
Expiry Date:	March 2019
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 CPA has been shown to lose only 3.5% of its potency 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:	Charles River Laboratories Research Models and Services Germany GmbH Sandhofer Weg 7, 97633 Sulzfeld, Germany
Number of Animals in the pre-test:	2 males and 2 females
Number of Animals in the main study:	43 males
Initial Age at Start of Experiment:	6 – 7 weeks (main study)
Acclimation:	minimum 5 days
Body Weight at Start of Treatment:	mean value 203.2 g (SD ± 8.2 g); range 189.8 – 223.8 g
Body Weight at End of Treatment:	mean value 211.8 g (SD ± 9.9 g); range 191.1 – 240.6 g

According to the supplier's assurance, the animals were in healthy condition. The animals were under acclimatisation in the animal house of Envigo CRS GmbH 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

3.4 Husbandry

The animals were kept as described below. 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 Envigo CRS GmbH.

Housing:	Group
Cage Type:	Macrolon Type IV, 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, except for deviations
	Relative humidity 45 – 65%, except for deviations (with the aim of 50 – 60%)
	Artificial light 6.00 a.m. – 6.00 p.m.

3.5 Experimental Performance

3.5.1 Pre-experiment

A preliminary study of acute toxicity was performed in both male and female rats (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 b.w.:

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

3.5.2 Main experiment

It is generally recommended to use the maximum tolerated dose or the highest dose that can be formulated and administered reproducibly or 2000 mg/kg b.w. 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.. Animals were dosed once with the test substance and termination for bone marrow collection performed at 24 or 48 h post dosing as described in the OECD 474 test guideline.

Three adequately spaced dose levels spaced by a factor of 2 were applied and bone marrow samples were collected 24 h after treatment from each treatment group. For the highest dose level an additional bone marrow sample was taken at 48 h after treatment from a further group of animals.

3.5.3 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.

3.5.4 Treatment

At the beginning of the treatment and at the end of the in-life phase the animals (including the negative and positive control treated animals) were weighed and the individual dose 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 by oral gavage, using a stainless steel feeding needle with rounded tip (1.2 Gauge) and disposable syringe at a dose volume of 10 mL/kg b.w.. 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 and the negative control 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 h and 48 h after treatment, respectively.

3.6 *Post Mortem* Investigations

3.6.1 Preparation of the animals

The animals were sacrificed using CO_2 followed by cervical dislocation. The femora were removed, the epiphyses were cut off and the marrow was flushed out with fetal calf serum using a disposable syringe. The nucleated cells were separated from the erythrocytes using the method of Romagna *et. al.*. Briefly, the cell suspensions was passed through a column consisting of α-Cellulose and Cellulose. The columns were then washed with Hank's buffered saline. The cell suspension was centrifuged at 1500 rpm (3900 x 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.

3.7 Data Evaluation

3.7.1 Slide analysis

Evaluation of the slides was performed using NIKON microscopes with 100x oil immersion objectives. Per animal at least 4000 polychromatic erythrocytes (PCE) were analysed for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes was determined from the same slide by counting until 500 PCEs had been determined among total erythrocytes and expressed as polychromatic erythrocytes per total erythrocytes counted. 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.

3.7.2 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 micronucleated cells per 4000 PCEs and the ratio of polychromatic erythrocytes to total erythrocytes are presented for each animal.

3.7.3 Acceptance criteria

The study is considered valid as the following criteria are met:

- The concurrent negative control is considered acceptable for addition to the laboratory historical control database (should ideally be within the 95% control limits of the distribution of the historical negative control database).
- At least 5 animals per group can be evaluated.
- The appropriate number of doses and cells have been analysed.
- PCE to total erythrocyte ratio should not be less than 20% of the negative control.
- The positive control shows a statistically significant increase of micronucleated PCEs compared to the negative control and is comparable to those in the historical positive control database.

3.7.4 Evaluation of results

A test substance is classified as positive in the assay if

a) At least one of the treatment groups exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes compared with the concurrent negative control,

b) This increase is dose-related at least at one sampling time when evaluated with an appropriate trend test, and

c) Any of these results are outside the distribution of the historical negative control data (e.g., Poisson-based 95% control limits).

There is no requirement for verification of a clearly positive or negative response. In case the response is neither clearly negative nor clearly positive as described above or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgment and/or further investigations.

A test item that fails to produce a biologically relevant increase in the number of micronucleated polychromatic erythrocytes, applying the above mentioned criteria, is considered negative in this system, given that there is evidence for bone marrow exposure.

Statistical methods (nonparametric Mann-Whitney test (7) and a linear regression analysis) were used as an aid in evaluating the results.

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 2000 mg/kg b.w. of SYN549522 SC (A22011B). The animals treated with 2000 mg/kg b.w. displayed signs of toxicity as shown in Table 2 which included salivation.

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

4.2 Signs of Toxicity in the Main Experiment

In the main experiment for the high dose groups 14 males (2 x 7 males per test group) received orally a single dose of 2000 mg/kg b.w. SYN549522 SC (A22011B).

For the mid dose group 7 males received orally a dose of 1000 mg/kg b.w. SYN549522 SC (A22011B).

For the low dose group 7 males received a dose of 500 mg/kg b.w. SYN549522 SC (A22011B).

The animals of all dose groups did not show any clinical signs or urine discolouration.

The animals of the vehicle control groups (sterile water) for both sampling times also did not show any clinical symptoms or urine discolouration.

4.3 Micronucleus Test Results

The test substance was administered at 500, 1000, and 2000 mg/kg b.w. for the 24 h treatment interval and 2000 mg/kg b.w. for the 48 h interval. At the end of the treatment phase, 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 a total number of 6000 PCEs were scored for micronuclei.

To determine whether there was a cytotoxic effect due to the treatment with the test substance the ratio between polychromatic and normochromatic erythrocytes was determined in the same samples and reported as the number of PCEs per total erythrocytes.

The ratio of polychromatic erythrocytes to total erythrocytes was not substantially decreased after treatment with the test substance as compared to the man value of PCEs of the vehicle control, indicating that SYN549522 SC (A22011B) did not have any significant cytotoxic properties in the bone marrow.

In comparison to the corresponding vehicle controls, there was a minimal increase in the frequency of the detected micronuclei at the 24 h preparation interval after administration of the test substance at all three dose levels used. For all 24h treatment groups the mean values of micronuclei observed after treatment with SYN549522 SC (A22011B) were slightly higher than the corresponding vehicle control and slightly outside of the upper 95% confidence interval of the historical vehicle control. These increases in micronucleus frequency in the 24 h preparation groups, however, were not considered to be of biological relevance since they were not statistically significant, and no clear dose dependency was observed.

A linear regression (least squares, calculated using the validated statistical program RScript LM_v02.Rnw) was performed to assess a possible dose dependent increase of mean micronuclei values. The mean number of micronuclei obtained for the groups treated with the test item was compared to the vehicle control group. A trend is judged as significant whenever the p-value (probability value) is below 0.05. A p-value of 0.3458 was obtained, demonstrating that there was no dose dependent increase of mean micronuclei values.

Furthermore, at the 48 h preparation interval, no increase in micronucleus frequency was observed in the treated animals.

A dose of 20 mg/kg b.w. cyclophosphamide administered orally was used as positive control which showed a statistically significant increase of induced micronucleus frequency indicating the correct performance of the assay.

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, SYN549522 SC (A22011B) is considered to be negative, i.e. non-genotoxic, 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. SYN549522 SC (A22011B)

Signs of Toxicity	hours post-treatment males / females					
	0-1 h	2-4 h	5-6 h	24 h	30 h	48 h
Salivation	2/0	0/0	0/0	0/0	0/0	0/0

TABLE 3 Summary of Micronucleus Test Results

Test Group	Dose mg/kg b.w.	Sampling time	Mean MN/4000 PCE	SD MN/4000 PCE	Mean % MN	Range min	Range max	Ratio PCE/ total Ery	% ratio Vehicle
negative control	0	24	14.7	8.9	0.37	8	30	0.624	100.00
A22011B	500	24	20.8	9.9	0.52	11	40	0.628	100.64
A22011B	1000	24	21.5	6.7	0.54	11	28	0.638	102.24
A22011B	2000	24	20.7	5.7	0.52	12	29	0.631	101.12
positive control	20	24	53.6	13.3	1.34	35	73	0.527	84.46
negative control	0	48	12.6	4.8	0.31	7	19	0.627	100.00
A22011B	2000	48	12.3	3.4	0.31	8	19	0.584	93.14

MN: Micronuclei
PCE: Polychromatic erythrocytes

TABLE 4 Biometry (Mann-Whitney test)

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 using the validated statistical program RScript Wilcoxon_2.Rnw.

Negative control versus test group	Significance	p
500 mg SYN549522 SC (A22011B) /kg b.w.; 24 h	-	0.164
1000 mg SYN549522 SC (A22011B) /kg b.w.; 24 h	-	0.202
2000 mg SYN549522 SC (A22011B) /kg b.w.; 24 h	-	0.149
20 mg CPA/kg b.w.; 24 h	+	0.012
2000 mg SYN549522 SC (A22011B) /kg b.w.; 48 h	-	1.0

+ = significant
- = not significant

TABLE 5 Micronuclei in Polychromatic Erythrocytes (PCE) and Relationship PCE/Total Erythrocytes Scoring 24 h after Treatment

A. Negative control (sterile water):

Test Group	Dose mg/kg b.w.	Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)				Evaluation 500 PCE in total Erythrocytes		
			No. PCE	No. MN	No. MN/4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
Vehicle	0	1	6000	14	9.3	0.23	750	250	0.667
		2	6000	12	8.0	0.20	821	321	0.609
		3	6000	17	11.3	0.28	807	307	0.620
		4	6000	22	14.7	0.37	873	373	0.573
		5	6000	45	30.0	0.75	768	268	0.651
			Mean	22.0	14.7	0.37	803.8	303.8	0.624
			SD	13.4	8.9	0.22	48.1	48.1	0.037

B. 500 mg/kg b.w. SYN549522 SC (A22011B):

Test Group	Dose mg/kg b.w.	Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)				Evaluation 500 PCE in total Erythrocytes		
			No. PCE	No. MN	No. MN/4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
Dose 1	500	6	6000	35	23.3	0.58	726	226	0.689
		7	6000	38	25.3	0.63	807	307	0.620
		8	6000	24	16.0	0.40	807	307	0.620
		9	6000	22	14.7	0.37	795	295	0.629
		10	6000	17	11.3	0.28	844	344	0.592
		11	6000	22	14.7	0.37	829	329	0.603
		12	6000	60	40.0	1.00	779	279	0.642
			Mean	31.1	20.8	0.52	798.1	298.1	0.628
			SD	14.8	9.9	0.25	38.3	38.3	0.032

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

C. 1000 mg/kg b.w. SYN549522 SC (A22011B):

Test Group	Dose mg/kg b.w.	Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)				Evaluation 500 PCE in total Erythrocytes		
			No. PCE	No. MN	No. MN/4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
Dose 2	1000	13	6000	39	26.0	0.65	895	395	0.559
		14	6000	42	28.0	0.70	756	256	0.661
		15	6000	16	10.7	0.27	750	250	0.667
		16	6000	41	27.3	0.68	814	314	0.614
		17	6000	28	18.7	0.47	752	252	0.665
		18	6000	23	15.3	0.38	750	250	0.667
		19	6000	37	24.7	0.62	794	294	0.630
		Mean		32.3	21.5	0.54	787.3	287.3	0.638
		SD		10.1	6.7	0.17	53.8	53.8	0.040

D. 2000 mg/kg b.w. SYN549522 SC (A22011B):

Test Group	Dose mg/kg b.w.	Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)				Evaluation 500 PCE in total Erythrocytes		
			No. PCE	No. MN	No. MN/4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
Dose 3	2000	20	6000	31	20.7	0.52	827	327	0.605
		21	6000	18	12.0	0.30	834	334	0.600
		22	6000	28	18.7	0.47	743	243	0.673
		23	6000	24	16.0	0.40	796	296	0.628
		24	6000	35	23.3	0.58	727	227	0.688
		25	6000	43	28.7	0.72	834	334	0.600
		26	6000	38	25.3	0.63	801	301	0.624
		Mean		31.0	20.7	0.52	794.6	294.6	0.631
		SD		8.5	5.7	0.14	43.6	43.6	0.036

TABLE 7 Micronuclei in Polychromatic Erythrocytes (PCE) and Relationship PCE/Total Erythrocytes Scoring 24 h after Treatment (continued)

E. Positive control (Cyclophosphamide 20 mg/kg b.w.):

Test Group	Dose mg/kg b.w.	Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)				Evaluation 500 PCE in total Erythrocytes		
			No. PCE	No. MN	No. MN/4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
Positive	20	27	6000	53	35.3	0.88	928	428	0.539
		28	6000	78	52.0	1.30	962	462	0.520
		29	6000	109	72.7	1.82	1174	674	0.426
		30	6000	81	54.0	1.35	879	379	0.569
		31	6000	81	54.0	1.35	863	363	0.579
			Mean	80.4	53.6	1.34	961.2	461.2	0.527
			SD	19.8	13.3	0.33	125.3	125.3	0.061

TABLE 8 Micronuclei in Polychromatic Erythrocytes (PCE) and Relationship PCE/Total Erythrocytes Scoring 48 h after Treatment

A. Negative control (sterile water):

Test Group	Dose mg/kg b.w.	Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)				Evaluation 500 PCE in total Erythrocytes		
			No. PCE	No. MN	No. MN/4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
Vehicle		32	6000	14	9.3	0.23	737	237	0.678
		33	6000	18	12.0	0.30	930	430	0.538
		34	6000	23	15.3	0.38	788	288	0.635
		35	6000	11	7.3	0.18	805	305	0.621
		36	6000	29	19.3	0.48	754	254	0.663
			Mean	19.0	12.6	0.31	802.8	302.8	0.627
			SD	7.2	4.8	0.12	76.0	76.0	0.055

B. 2000 mg/kg b.w. SYN549522 SC (A22011B):

Test Group	Dose mg/kg b.w.	Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)				Evaluation 500 PCE in total Erythrocytes		
			No. PCE	No. MN	No. MN/4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
Dose 3	2000	37	6000	15	10.0	0.25	858	358	0.583
		38	6000	16	10.7	0.27	998	498	0.501
		39	6000	18	12.0	0.30	775	275	0.645
		40	6000	20	13.3	0.33	814	314	0.614
		41	6000	12	8.0	0.20	881	381	0.568
		42	6000	28	18.7	0.47	909	409	0.550
		43	6000	20	13.3	0.33	801	301	0.624
			Mean	18.4	12.3	0.31	862.3	362.3	0.584
			SD	5.1	3.4	0.09	76.0	76.0	0.049

TABLE 9 Individual Animal Weights

Dose Group	Animal No.	Animal weights before treatment				Animal weights before sacrifice			
		Weight [g]	Mean [g]	SD [g]	Range [g]	Weight [g]	Mean [g]	SD [g]	Range [g]
Vehicle Control 24 h	1	191.8	203.3	9.4	191.8 - 214.0	199.1	210.7	9.9	199.1 - 221.2
	2	201.4				206.6			
	3	197.7				205.7			
	4	214.0				221.1			
	5	211.7				221.2			
500 mg/kg b.w. 24 h SYN549522 SC (A22011B)	6	211.9	200.8	9.4	189.8 - 211.9	213.9	205.6	9.1	195.3 - 218.7
	7	191.6				195.3			
	8	207.8				211.7			
	9	197.4				201.7			
	10	211.4				218.7			
	11	195.4				201.2			
	12	189.8				196.7			
1000 mg/kg b.w. 24 h SYN549522 SC (A22011B)	13	203.8	205.7	4.5	199.4 - 211.6	210.5	212.0	3.5	207.7 - 218.4
	14	200.8				209.0			
	15	207.1				212.7			
	16	211.6				218.4			
	17	210.0				214.0			
	18	199.4				207.7			
	19	206.9				211.9			
2000 mg/kg b.w. 24 h SYN549522 SC (A22011B)	20	193.2	202.6	8.0	193.2 - 213.2	191.1	207.0	10.0	191.1 - 221.0
	21	198.0				203.7			
	22	194.5				200.0			
	23	208.2				212.3			
	24	213.2				214.9			
	25	200.6				205.9			
	26	210.6				221.0			
Positive Control 24 h	27	197.0	199.8	7.2	190.7 - 208.3	207.9	208.3	6.8	197.9 - 215.3
	28	208.3				215.3			
	29	205.8				213.5			
	30	197.3				207.1			
	31	190.7				197.9			
Vehicle Control 48 h	32	201.5	207.0	12.0	194.1 - 223.8	218.1	222.2	12.7	208.7 - 240.6
	33	223.8				240.6			
	34	214.6				229.0			
	35	194.1				208.7			
	36	201.0				214.5			
2000 mg/kg b.w. 48 h SYN549522 SC (A22011B)	37	200.2	203.6	8.6	193.1 - 214.9	213.2	218.3	8.5	206.9 - 230.2
	38	193.1				206.9			
	39	204.7				220.6			
	40	193.8				210.4			
	41	205.3				220.4			
	42	214.9				230.2			
	43	213.2				226.1			
Summary			**203.2**	**8.2**	**189.8 - 223.8**		**211.8**	**9.9**	**191.1 - 240.6**

APPENDICES SECTION

APPENDIX 1 Historical Control Data

October 2014 – December 2018

Vehicle Control	
min	0.025
max	0.750
mean	0.248
95% Ctr. Limit	-0.006 | 0.501
SD	0.127
2x SD	0.253
Range	1 - 30
No° indiv. Values	129
Positive Control (Cyclophosphamide)	
min	0.450
max	4.375
mean	1.650
95% Ctr. Limit	-0.096 | 3.397
SD	0.873
2x SD	1.747
Range	18 - 175
No° indiv. Values	85

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 stamp inscription:
Hessian Ministry for Environment, Area for Agricultural Use and Consumer Protection

APPENDIX 3 Certificate of Analysis

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

Certificate of Analysis

A22011B
SYN549522 SC (450)
SMU7JP001

Batch Identification	**SMU7JP001**
Other Batch ID	1013163
Product Code	**A22011B**
Other Product Code(s)	SYN549522 SC (450)
Chemical Analysis (Active Ingredient content)	
– **Identity of the Active Ingredient(s)***	**confirmed**
– **Content of SYN549522***	**38.1 % w/w corresponding to 448 g/l**
– **Content of SYN547386***	**34.3 % w/w corresponding to 403 g/l**
– **Content of SYN548941***	**3.83 % w/w corresponding to 45.0 g/l**
	The Active Ingredient(s) content is within the FAO limits.
Methodology used for Characterization / Recertification	HPLC, chiral HPLC, oscillating density meter
Physical Analysis	
– **Appearance**	**beige liquid**
– **Density***	**1175 kg/m³**
Stability:	
– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of November 2020**

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: CHMU170748
Study number(s) of batch recertification: ---

Authorization: 30-November-2017

Dr. Christian Mink
Analytical Development & Product Chemistry