ICCR ROßDORF

Isopyrazam/Difenoconazole

Isopyrazam/Difenoconazole SC (A21295D) – Micronucleus Assay in Bone Marrow Cells of the Rat

Final Report

TEST GUIDELINE(S): OECD 474 (2016)

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PERFORMING LABORATORY: ICCR-Roßdorf GmbH
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SPONSOR(S): Syngenta Ltd
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study performed in the test facility of ICCR-Roßdorf 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]

EC Commission Directive 2004/10/EC (Official Journal No L 50/44)

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), 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.

E.D

15 July 2021

Dr. Eva Dony
Study Director
Genetic Toxicology *in vivo*

Date

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Date

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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

ICCR Study Number: 2165300
Test Substance: Isopyrazam/Difenoconazole SC (A21295D)
Study Director: Dr. Eva Dony
Study Title: Isopyrazam/Difenoconazole SC (A21295D) – Micronucleus Assay in Bone Marrow Cells of the Rat

Study based activities at the Test Facility ICCR-Roßdorf 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	19 April 2021	19 April 2021
1st Amendment to Study Plan Verification	10 May 2021	10 May 2021
Study – based Test System Preparation & Application	19 May 2021	19 May 2021
Report Audit	12 July 2021	13 July 2021

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

Manuella Thomsen

Quality Assurance Auditor
ICCR-Roßdorf GmbH

15 July 2021
Date

GENERAL INFORMATION

Contributors
The following contributed to this report in the capacities indicated:

Name	Title
Dr. Eva Dony	Study Director
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Study Dates
Study initiation date: 19 April 2021
Experimental start date: 27 April 2021
Experimental completion date: 23 June 2021

Deviations from the Guidelines
None

Retention of Samples
Raw data and microscopic slides.

Performing Laboratory Test Substance Reference Number
[S 2126421]

Other
Records and documentation relating to this study (including electronic records) will be maintained in the archives of ICCR-Roßdorf 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 cause of the study.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant archive Rhenus Archiv Services GmbH, Frankfurt am Main 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.

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

Deviations from the study plan
None

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 isopyrazam/difenoconazole SC (A21295D) 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.

Six females per test group (except the negative and positive control groups with five females only) were evaluated for the occurrence of micronuclei. Per animal 4000 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 a pre-experiment. Clinical signs observed, which included exophthalmos, piloerection, closed eyes, sunken flanks, hunched posture, decreased activity, lethargy/apathy, ataxia, and purring respiratory sounds, were indicative of systemic exposure to the test substance.

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 isopyrazam/difenoconazole SC (A21295D) 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 at any dose level used. For all treatment groups the group mean values of micronuclei observed after treatment with isopyrazam/difenoconazole SC (A21295D) were below or near the corresponding vehicle control value and well within the the 95% control limit of the historical vehicle control data. Additionally, no dose dependency was observed. Except for one individual value in the low

dose group (% micronuclei in PCE determined for animal 9) all single animal values were also well within historical vehicle control values.

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, isopyrazam/difenoconazole SC (A21295D) is considered to be negative, i.e. non- genotoxic (non- clastogenic, non-aneugenic) 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 and recommendations:

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.

Information as provided by the Sponsor.

Identification:	Isopyrazam/Difenoconazole SC (A21295D)
Batch:	SG40038
Content of difenoconazole (CGA169374):	11.93% w/w corresponding to 130 g/L
Content of isopyrazam (SYN520453):	12.105% w/w corresponding to 132 g/L
Recertification Date:	29 August 2023
Physical State / Appearance:	Liquid, off-white
Storage Conditions:	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.

3.2 Controls

3.2.1 Negative controls

The test substance vehicle was used as a negative control.

Name:	sterile water
Batch No.:	19PDA110
Expiry Date:	March 2023
Route and Frequency of Administration	orally, once
Volume Administered:	10 mL/kg b.w.

3.2.2 Positive control

Name:	Cyclophosphamide (CPA)
Batch:	A0412722
Purity:	97%
Expiry Date:	24 July 2021
Dissolved in:	sterile water
Batch No.:	19PDA110
Expiry Date:	March 2023
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:	39 females
Initial Age at Start of Experiment:	6 – 10 weeks (main study)
Acclimation:	minimum 5 days

Body Weight at Start of Treatment:	mean value 145.6 g (SD ± 7.8 g); range 127.9 – 163.0 g
Body Weight at End of Treatment:	mean value 149.2 g (SD ± 7.2 g); range 133.3 – 164.2 g
	SD: Standard Deviation

According to the supplier's assurance, the animals were in healthy condition. The animals were under acclimatisation in the animal house of ICCR-Roßdorf 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.

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 ICCR-Roßdorf 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, Relative humidity 45 – 65%, (with the aim of 50 – 60%) Artificial light 6.00 a.m. – 6.00 p.m. ventilation: at least eight air changes per hour

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 at a dose level of 2000 mg/kg b.w. since excessive toxic symptoms were not expected at this dose level, 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.

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

Six females 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 and individual tail markings 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. Six females were treated per dose group and sampling time. Five females 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 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), 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 isopyrazam/difenoconazole SC (A21295D). The animals treated with 2000 mg/kg b.w. displayed signs of toxicity as shown in Table 2 which included exophthalmos, piloerection, closed eyes, sunken flanks, hunched posture, decreased activity, lethargy/apathy, ataxia, and purring respiratory sounds. Recovery of all animals was complete approximately 30 h after application.

On the basis of these data 2000 mg/kg b.w. was considered suitable for this study. As females were shown to be slightly more sensitive based on the results of the range finder, the main study was performed with females only, as permitted by the OECD Guideline.

4.2 Signs of Toxicity in the Main Experiment

In the main experiment 12 females (2 x 6 females per test group) received orally a single dose of 2000 mg/kg b.w. isopyrazam/difenoconazole SC (A21295D).

The animals treated with 2000 mg/kg b.w. displayed signs of toxicity as shown in Table 3, which included exophthalmos, piloerection, partially closed eyes, sunken flanks, hunched posture, decreased activity, prostation, lethargy/apathy, and ataxia.

For the mid dose group 6 females received orally a dose of 1000 mg/kg b.w. isopyrazam/difenoconazole SC (A21295D).

The animals treated with 1000 mg/kg b.w. displayed signs of toxicity as shown in Table 4, which included exophthalmos, piloerection, decreased activity, and ataxia.

For the low dose group 6 females received a dose of 500 mg/kg b.w. isopyrazam/difenoconazole SC (A21295D).

The animals treated with the low dose and of the vehicle control groups (sterile water) for both sampling times did not show any clinical symptoms.

4.2.1 Micronucleus test results

The mean number of polychromatic erythrocytes was not substantially decreased after treatment with the test substance as compared to the mean value of PCEs of the vehicle control, indicating that isopyrazam/difenoconazole SC (A21295D) did not have any significant cytotoxic properties on the bone marrow (Table 5).

In comparison to the corresponding vehicle controls there was no biologically relevant enhancement or statistically significant increase in the frequency of the detected micronuclei

at any preparation interval and dose level after administration of the test substance (Table 5 and 6).

For all treatment groups the mean values of micronuclei observed after treatment with isopyrazam/difenoconazole SC (A21295D) were well within the 95% control limit of the historical vehicle control data (Appendix 1).

4.3 Discussion

The test substance isopyrazam/difenoconazole SC (A21295D) 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 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.

Six females per test group (except the negative and positive control groups with five females only) were evaluated for the occurrence of micronuclei. Per animal 4000 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.

As predicted by a the pre-experiment in male and female mice rats, 2000 mg/kg b.w. of isopyrazam/difenoconazole SC (A21295D) were suitable as the highest (maximum tolerated/guideline recommended) dose. As females were shown to be slightly more sensitive based on the results of the range finder, the main study was performed with females only. Clinical signs observed from test substance treated animals were indicative of systemic exposure to the test substance (10).

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 isopyrazam/difenoconazole SC (A21295D) 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 or statistically significant increase in the frequency of the detected micronuclei at any preparation interval or dose level after administration of the test substance. The group mean values of micronuclei observed after treatment with isopyrazam/difenoconazole SC (A21295D) were below or near the respective vehicle control group for all dose groups and well within the 95% control limit of the historical vehicle control data. Additionally, no dose dependency was observed.

Except for one single value in the low dose group (% micronuclei in PCE determined for animal 9) all individual animal values were also well within historical vehicle control values.

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 0.8147 was obtained, demonstrating that there was no dose dependent increase of mean micronuclei values.

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, isopyrazam/difenoconazole SC (A21295D) is considered to be negative, i.e. non-genotoxic (non-clastogenic and non-aneugenic) in this bone marrow micronucleus assay.

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TABLES SECTION

TABLE 1 Identification of the Animals by their Cage Number and Tail Markings

Test group	hours post-treatment	
	24	48
Negative control	1 – 5	29 – 33
Low dose	6 – 11	
Medium dose	12 – 17	
High dose	18 – 23	34 – 39
Positive control	24 – 28	

TABLE 2 Pre-Experiment for Toxicity: 2000 mg/kg b.w. Isopyrazam/Difenoconazole SC (A21295D)

Signs of Toxicity	hours post-treatment males / females					
	0-1 h	2-4 h	5-6 h	24 h	30 h	48 h
Exophthalmos	2/2	0/0	0/0	0/0	0/0	0/0
Piloerection	0/2	0/2	0/2	2/2	0/0	0/0
Closed eyes	0/0	0/2	0/0	0/0	0/0	0/0
Sunken flanks	0/0	0/2	0/0	0/0	0/0	0/0
Hunched posture	0/0	0/2	0/2	0/0	0/0	0/0
Decreased activity	2/2	2/0	2/2	2/0	0/0	0/0
Lethargy/apathy	0/0	0/2	0/0	0/0	0/0	0/0
Ataxia	2/2	2/2	0/2	0/0	0/0	0/0
Purring respiratory sounds	0/0	0/0	0/1	0/0	0/0	0/0

TABLE 3 Signs of Toxicity in the Main Experiment: 2000 mg/kg b.w. Isopyrazam/Difenoconazole SC (A21295D)

Signs of Toxicity	hours post-treatment females				
	0-1 h	2-4 h	5-6 h	24 h	48 h*
Exophthalmos	12	0	0	0	0
Piloerection	0	12	12	12	0
Partially Closed Eyes	0	12	12	0	0
Sunken Flanks	0	0	6	0	0
Hunched Posture	0	0	6	0	0
Decreased Activity	12	12	12	12	0
Prostration	12	2	1	0	0
Lethargy/Apathy	0	2	0	0	0
Ataxia	12	12	6	0	0

*data from 6 females only

TABLE 4 Signs of Toxicity in the Main Experiment: 1000 mg/kg b.w. Isopyrazam/Difenoconazole SC (A21295D)

Signs of Toxicity	hours post-treatment females			
	0-1 h*	2-4 h	5-6 h	24 h
Exophthalmos	4	0	0	0
Piloerection	0	6	0	0
Decreased Activity	4	6	6	6
Ataxia	4	0	0	0

TABLE 5 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		Ratio PCE/ total Ery	% ratio Vehicle
						min	max		
negative control	0	24	6.0	4.6	0.15	3	14	0.449	100.00
test substance	500	24	11.3	6.4	0.29	5	23	0.471	104.90
test substance	1000	24	8.3	4.8	0.21	1	14	0.519	115.59
test substance	2000	24	6.5	3.1	0.17	3	12	0.436	97.10
positive control	20	24	43.4	18.0	1.09	20	70	0.382	85.08
negative control	0	48	6.0	1.4	0.15	4	7	0.465	100.00
test substance	2000	48	3.8	1.6	0.10	1	5	0.495	106.45

TABLE 6 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 using the validated statistical program RScript Wilcoxon_2.Rnw. Furthermore, the Holm-Bonferroni Method was used to adjust for the familywise error rate of multiple comparisons.

Negative control versus test group	Significance	p	p adjusted
500 mg Isopyrazam/Difenoconazole SC (A21295D) /kg b.w.; 24 h	-	0.12	0.360
1000 mg Isopyrazam/Difenoconazole SC (A21295D) /kg b.w.; 24 h	-	0.519	1.0
2000 mg Isopyrazam/Difenoconazole SC (A21295D) /kg b.w.; 24 h	-	0.516	1.0
20 mg CPA/kg b.w.; 24 h	+	0.012	0.048
2000 mg Isopyrazam/Difenoconazole SC (A21295D) /kg b.w.; 48 h	-	0.072	0.072

+ = significant vs. concurrent vehicle control
- = not significant vs. concurrent vehicle control

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

A. Negative control (sterile water):

Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)			Evaluation 500 PCE in total Erythrocytes		
	No. PCE	No. MN/ 4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE per total Ery
1	4000	3	0.08	1272	772	0.393
2	4000	6	0.15	1316	816	0.380
3	4000	3	0.08	1093	593	0.457
4	4000	4	0.10	961	461	0.520
5	4000	14	0.35	1011	511	0.495
	Mean	6.0	0.15	1130.6	630.6	0.449
	SD	4.6	0.11	157.2	157.2	0.061

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

Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)			Evaluation 500 PCE in total Erythrocytes		
	No. PCE	No. MN/ 4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE per total Ery
6	4000	9	0.23	1111	611	0.450
7	4000	5	0.13	1307	807	0.383
8	4000	13	0.33	1679	1179	0.298
9	4000	23	0.58	802	302	0.623
10	4000	7	0.18	870	370	0.575
11	4000	11	0.28	1008	508	0.496
	Mean	11.3	0.29	1129.5	629.5	0.471
	SD	6.4	0.16	323.5	323.5	0.121

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

Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)			Evaluation 500 PCE in total Erythrocytes		
	No. PCE	No. MN/ 4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE per total Ery
12	4000	10	0.25	905	405	0.552
13	4000	10	0.25	1161	661	0.431
14	4000	14	0.35	808	308	0.619
15	4000	11	0.28	1172	672	0.427
16	4000	4	0.10	988	488	0.506
17	4000	1	0.03	860	360	0.581
	Mean	8.3	0.21	982.3	482.3	0.519
	SD	4.8	0.12	154.4	154.4	0.079

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

Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)			Evaluation 500 PCE in total Erythrocytes		
	No. PCE	No. MN/ 4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE per total Ery
18	4000	6	0.15	1184	684	0.422
19	4000	7	0.18	1079	579	0.463
20	4000	7	0.18	1201	701	0.416
21	4000	4	0.10	1167	667	0.428
22	4000	12	0.30	1204	704	0.415
23	4000	3	0.08	1057	557	0.473
	Mean	6.5	0.17	1148.7	648.7	0.436
	SD	3.1	0.08	64.3	64.3	0.025

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

Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)			Evaluation 500 PCE in total Erythrocytes		
	No. PCE	No. MN/ 4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE per total Ery
24	4000	42	1.05	1485	985	0.337
25	4000	70	1.75	1280	780	0.391
26	4000	47	1.18	1253	753	0.399
27	4000	20	0.50	1373	873	0.364
28	4000	38	0.95	1198	698	0.417
	Mean	43.4	1.09	1317.8	817.8	0.382
	SD	18.0	0.45	112.9	112.9	0.031

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

A. Negative control (sterile water):

Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)			Evaluation 500 PCE in total Erythrocytes		
	No. PCE	No. MN/ 4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
29	4000	7	0.18	1175	675	0.426
30	4000	4	0.10	1502	1002	0.333
31	4000	7	0.18	1224	724	0.408
32	4000	5	0.13	910	410	0.549
33	4000	7	0.18	823	323	0.608
	Mean	6.0	0.15	1126.8	626.8	0.465
	SD	1.4	0.04	270.1	270.1	0.111

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

Animal No.	Micronuclei in Polychromatic Erythrocytes (PCE)			Evaluation 500 PCE in total Erythrocytes		
	No. PCE	No. MN/ 4000 PCE	% MN	Total No Ery	NCE per total Ery	Ratio PCE/Total Ery
34	4000	5	0.13	1182	682	0.423
35	4000	1	0.03	1088	588	0.460
36	4000	5	0.13	1166	666	0.429
37	4000	5	0.13	922	422	0.542
38	4000	3	0.08	1050	550	0.476
39	4000	4	0.10	780	280	0.641
	Mean	3.8	0.10	1031.3	531.3	0.495
	SD	1.6	0.04	154.6	154.6	0.083

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	135.7	146.3	10.1	135.7 - 163.0	145.2	151.4	7.6	145.2 - 164.2
	2	163.0				164.2			
	3	142.9				147.7			
	4	143.5				147.3			
	5	146.3				152.4			
500 mg/kg b.w. 24 h Isopyrazam/ Difenoconazole SC (A21295D)	6	144.9	147.1	3.9	140.8 - 150.6	150.1	150.9	3.3	145.6 - 155.1
	7	150.4				153.3			
	8	146.1				149.6			
	9	140.8				145.6			
	10	149.5				151.8			
	11	150.6				155.1			
1000 mg/kg b.w. 24 h Isopyrazam/ Difenoconazole SC (A21295D)	12	148.6	145.4	7.0	132.0 - 152.1	148.4	147.4	7.0	133.3 - 151.8
	13	132.0				133.3			
	14	144.6				148.6			
	15	152.1				151.3			
	16	147.5				151.8			
	17	147.6				150.9			
2000 mg/kg b.w. 24 h Isopyrazam/ Difenoconazole SC (A21295D)	18	150.7	151.4	2.0	148.7 - 153.7	146.8	151.8	2.8	146.8 - 154.0
	19	153.6				154.0			
	20	151.8				153.4			
	21	149.8				150.4			
	22	148.7				152.2			
	23	153.7				153.9			
Positive Control 24 h	24	148.9	142.6	7.5	131.0 - 148.9	152.3	145.0	7.2	135.4 - 152.3
	25	145.2				149.0			
	26	131.0				135.4			
	27	139.4				139.6			
	28	148.4				148.9			
Vehicle Control 48 h	29	151.1	143.9	12.0	127.9 - 155.7	155.3	151.3	11.4	135.6 - 164.2
	30	134.6				143.9			
	31	155.7				164.2			
	32	127.9				135.6			
	33	150.1				157.3			
2000 mg/kg b.w. 48 h Isopyrazam/ Difenoconazole SC (A21295D)	34	135.6	141.9	8.8	131.0 - 152.0	141.0	146.5	8.9	137.0 - 155.2
	35	131.0				137.4			
	36	150.0				154.8			
	37	135.9				137.0			
	38	152.0				155.2			
	39	146.9				153.3			

APPENDICES SECTION

APPENDIX 1 Historical Control Data (Oct 2014 - Dec 2020)

Vehicle Controls (%)	Female animals
min	0.025
max	0.425
mean	0.205
95% Ctr. Limit	-0.012 | 0.422
SD	0.109
2x SD	0.217
Range of individual animal micronuclei values*	1 - 17
No° indiv. values	28

Positive Controls (%)	Female animals
min	0.375
max	2.325
mean	1.017
95% Ctr. Limit	0.121 | 1.914
SD	0.448
2x SD	0.896
Range of individual animal micronuclei values*	15 - 93
No° indiv. values	23

*per 4000 Polychromatic Erythrocytes

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

ICCR-Roßdorf GmbH
Institute for Competent Contract Research
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 and clinical chemistry testing

22.11.2018, 21.02.2019, 12. bis 14.03.2019
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

Brandt

Dr. Astrid Brandt, Referentin, Wiesbaden, den **23. Oktober 2019**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hessisches Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz, Mainzer Straße 80, D 65189 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

GLP Testing Facility GOA
Analytical & Product Chemistry

Syngenta Biosciences Pvt. Ltd.
Santa Monica Works,
Corlim, Ilhas Goa 403 110
India

Certificate of Analysis

A21295D
isopyrazam/difenoconazole SC (125/125)
SG40038

Batch Identification	**SG40038**
Other Batch ID	1156078
Product Code	**A21295D**
Other Product Code(s)	CGA169374/SYN520453 SC (125/125)

Chemical Analysis
(Active Ingredient content)

– **Identity of the Active Ingredients***	**Confirmed**
– **Content of difenoconazole (CGA169374)***	**11.93 % w/w corresponding to 130 g/l**
– **Content of isopyrazam (SYN520453) ***	**12.105 % w/w corresponding to 132 g/l**

The Active Ingredient(s) content is within the FAO limits.

Methodology used for Characterization: HPLC, oscillating density meter

Physical Analysis

– **Appearance**	**Off-white liquid**
– **Density***	**1093 kg/m³**

Stability:

– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of August 2023**

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.

This Certificate of Analysis is summarizing data which originate 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 study(ies) are stored under the study number(s) referenced below within the archives of the GLP Testing Facility Goa at Syngenta Biosciences Pvt. Ltd., Santa Monica Works, Corlim, Ilhas, Goa 403110.

Study number of batch characterization: SMG16520
Study number(s) of batch recertification: ---

Authorization: 16-Sep-2020

Sunil B. Khot
Analytical and Product Chemistry, Goa

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