SYN530561

SYN530561 - Chromosome Aberration Test in Human Lymphocytes *In Vitro*

Final Report

DATA REQUIREMENTS:	OECD 473 (1997) EPA OPPTS 870.5375 (1998) EC 440/2008 B. 10 (2008)
AUTHOR(S):	Dr. Susanne Bohnenberger
STUDY COMPLETION DATE:	September 07, 2009
PERFORMING LABORATORY:	Harlan Cytotest Cell Research GmbH (Harlan CCR) In den Leppsteinswiesen 19 64380 Rossdorf, Germany
LABORATORY PROJECT ID:	Report Number: 1266802 Study Number: 1266802 Task Number: T009049-08
SPONSOR:	Syngenta Ltd Jealott's Hill International Research Centre Bracknell, Berkshire RG42 6EY, United Kingdom

STATEMENTS 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), dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision dated June 27, 2002, and amended version dated July 02, 2008 ("BGBl.", p. 1146).

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final]

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

Study Director

Harlan CCR
Dr. Susanne Bohnenberger

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Date: September 07, 2009

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Number:	1266802
Test Item:	SYN530561
Study Director:	Dr. Susanne Bohnenberger
Title:	SYN530561 - Chromosome Aberration Test in Human Lymphocytes *in vitro*

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:	May 12, 2009	May 12, 2009
Study Inspection Preparation for application:	June 08, 2009	June 08, 2009
Report:	August 04, 2009	August 04, 2009

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

Head of Quality Assurance Unit for Frauke Hermann

S Ebert Sabine Ebert

Date: September 07, 2009

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
Dr. Susanne Bohnenberger	Study Director
Dr. Caroline Hall	Deputy Study Director
Dr. Wolfgang Völkner	Management
Frauke Hermann	Head of Quality Assurance Unit
Dr. Ewan D. Booth	Syngenta Study Manager

Study dates

Study initiation date:	12 May, 2009
Experimental start date:	18 May, 2009
Experimental termination date:	19 June, 2009

Deviations from the study plan

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision dated June 27, 2002, **and amended version dated July 02, 2008 ("BGBl.", p. 1146)**.

Reason for the deviation (printed in bold letters): updating.

Historical control data

Column: 'Cells scored' was replaced by **'Number of studies'**.

Reason for the deviation (printed in bold letters): updating as requested by the sponsor.

These deviations have no detrimental impact on the outcome of the study.

Retention of samples

Raw data, microscopic slides, and a sample of the test item.

Performing laboratory test substance reference number

S 1017411

Other

Harlan CCR will archive the following data for 15 years:

Raw data, study plan, original final report, and a sample of the test item.

Microscopic slides will be archived for at least 12 years.

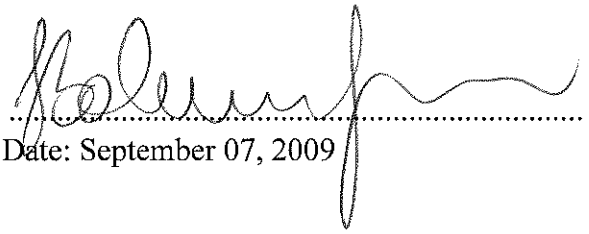
No data will be discarded without the Sponsor's consent.

Distribution of the report

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

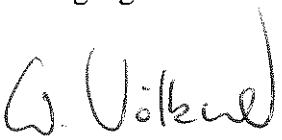
Project staff signatures

Study Director — Dr. Susanne Bohnenberger



Date: September 07, 2009

Management — Dr. Wolfgang Völkner



Date: September 07, 2009

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

This *in vitro* assay was performed to assess the potential of SYN530561 to induce structural chromosomal aberrations in the absence and presence of an exogenous metabolic activation system (liver S9 mix from phenobarbital/β-naphthoflavone treated male rats).

1.2 Results

In each experimental group two parallel cultures were analysed. Per culture 100 metaphase plates were scored for structural chromosomal aberrations, except for the positive control in Experiment II, in the absence of S9 mix, where 50 metaphase plates were scored.

The highest applied concentration in this study (2700.0 µg/mL of the test item, approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the current OECD Guideline 473.

Dose selection of the cytogenetic experiments was performed considering the toxicity data and in accordance with OECD Guideline 473.

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

In both independent experiments, no statistically significant and biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.

Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations.

1.3 Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce structural chromosomal aberrations in human lymphocytes *in vitro*.

Therefore, SYN530561 is considered to be non-clastogenic in this chromosome aberration test in the absence and presence of metabolic activation.

2.0 INTRODUCTION

2.1 Purpose

According to legal requirements agrochemicals have to be tested before use upon the possible danger to humans and environment. Genotoxicity studies provide important information for the assessment of the mutagenic potential of these substances (1,2). The *In vitro* Chromosome Aberration Test performed in this study is an essential part for genotoxicity studies of substances.

This *in vitro* test is an assay for the detection of structural chromosomal aberrations (3,4). Short-term cultures of human lymphocytes were stimulated to divide by the addition of a mitogen (e.g. phytohaemagglutinin, PHA) to the culture medium (3). Mitotic activity began at about 40 hours after PHA stimulation and reached a maximum at around 3 days. The chromosome constitution remained diploid during short-term culture.

Treatments should commence at around 50 - 80 hours after culture initiation when the cells are actively proliferating (5,6) and should be sampled first at about 22 hours later (about 1.5 fold of the normal cell cycle time (7)).

At preparation time 22 hours, a minimum of three cultures treated with separated concentrations of the test item are evaluated for the potential to induce structural chromosomal aberrations. The highest concentration should cause a significant reduction of the mitotic index (at least 50 %), the intermediate concentration should give some degree of mitotic inhibition, and the lowest concentration should show no toxicity. In case of non-toxicity the maximum concentration should be 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest, if possible.

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

To validate the test, control mutagens were tested in parallel to the test item.

2.2 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 Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 473 "*In vitro* Mammalian Chromosome Aberration Test".

United States Environmental Protection Agency Health Effects Test Guidelines. OPPTS 870.5375 (1998). "In vitro mammalian chromosome aberration test", EPA 712-C-98-223, August 1998.

Commission Regulation (EC) No. 440/2008 B.10: "Mutagenicity – In vitro Mammalian Chromosome Aberration Test", dated May 30, 2008.

3.0 MATERIALS AND METHODS

3.1 Test substance

Internal Test Item Number: S 1017411

The test item and the information concerning the test item were provided by the sponsor.

Identity:	CSCC235899
Other product code:	SYN530561
Batch No.:	KI 7010/5
Molecular Weight:	267.3 g/mol
Purity:	99 %
Stability in Solvent:	Not indicated by the Sponsor
Storage:	In the refrigerator at +2 to +8 °C
Expiration Date:	March 31, 2014

On the day of the experiment (immediately before treatment), the test item was dissolved in DMSO (E. MERCK, 64293 Darmstadt, Germany; purity 99.5 %). The final concentration of DMSO in the culture medium was 0.5 % (v/v). The solvent was chosen due to its solubility properties and its relative non-toxicity to the cell cultures.

In both experiments, in a range of 503.8 to 2700.0 µg/mL the pH was adjusted to physiological values using small amounts of 2 N NaOH.

3.2 Controls

3.2.1 Solvent controls

Concurrent solvent controls (DMSO) were performed.

Name:	DMSO; Dimethyl sulfoxide
Supplier:	E. MERCK, 64293 Darmstadt, Germany
Purity:	99.5 %
Lot no.:	K39250731847

3.2.2 Positive control substances

Without metabolic activation

Name:	EMS; ethylmethane sulfonate
Supplier:	ACROS ORGANICS, 2440 Geel, Belgium
Purity:	≥ 98 %
Lot no.:	A0259466
Expiration Date:	March 2010
Dissolved in:	Nutrient medium
Final concentration:	825.0 µg/mL (Exp. I) 770.0 µg/mL (Exp. II)

Solutions were prepared on the day of experiment. The stability of the positive control substance in solution was proven by the mutagenic response in the expected range.

With metabolic activation

Name:	CPA; cyclophosphamide
Supplier:	Aldrich Chemie, 89555 Steinheim, Germany
Purity:	≥ 98 %
Lot no.:	097K1311
Expiration Date:	March, 2011
Dissolved in:	Nutrient medium
Final concentration:	15.0 µg/mL

The dilutions of the stock solutions were prepared on the day of experiment. The stability of CPA in solution at room temperature is good. At 25 °C only 3.5 % of its potency is lost after 24 hours (11).

3.3 Experimental design

3.3.1 Blood collection and delivery

Blood samples were obtained from healthy donors not receiving medication. For this study, blood was collected from a female donor (32 years old) for the first experiment and from a male donor (25 years old) for Experiment II.

Blood samples were drawn by venous puncture and collected in heparinized tubes by Dr. V. Theodor (64380 Rossdorf, Germany). The tubes were sent to Harlan CCR to initiate cell cultures within 24 hrs after blood collection. If necessary, the blood was stored before use at 4 °C.

3.4 Mammalian microsomal fraction S9 mix

3.4.1 S9 (Preparation by Harlan CCR)

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from 8 - 12 weeks old male Wistar rats (HsdCpb:WU, Harlan Laboratories GmbH, 33178 Borchen, Germany), weight approx. 220 - 320 g induced by applications of 80 mg/kg b.w. phenobarbital i.p. (Desitin; 22335 Hamburg, Germany) and β-naphthoflavone p.o. (Aldrich, 89555 Steinheim, Germany) each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1 part plus 3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at -80 °C. Small numbers of the ampoules can be kept at –20 °C for up to one week.

The protein concentration of the S9 preparation was 35.6 mg/mL (Lot no. 270309).

3.4.2 S9 Mix

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM	$MgCl_2$
33 mM	KCl
5 mM	Glucose-6-phosphate
4 mM	NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment, the S9 mix was stored in an ice bath. The S9 mix preparation was performed according to Ames et al. (1).

3.5 Range-finder

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the mutagenicity assay (8). Cytotoxicity is characterized by the percentages of mitotic suppression in comparison to the controls by counting 1000 cells per culture in duplicate. The experimental conditions in this pre-test phase were identical to those required and described below for the mutagenicity assay.

The pre-test phase was performed with 10 concentrations of the test item and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9 mix). The preparation interval was 22 hrs after start of the exposure.

3.6 Dose selection

The highest concentration used in the pre-test was chosen with regard to the current OECD Guideline for in vitro mammalian cytogenetic tests requesting for the top concentration clear toxicity with reduced mitotic indices below 50 % of control, and/or the occurrence of precipitation. In case of nontoxicity the maximum concentration should be 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest, if formulation in an appropriate solvent is possible.

With respect to the molecular weight of the test item, 2700.0 µg/mL of SYN530561 (approx. 10 mM) were applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations between 17.5 and 2700.0 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test on toxicity, no precipitation of the test item was observed. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.

Using reduced mitotic indices as an indicator for toxicity in Experiment I, no toxic effects were observed after 4 hrs treatment in the absence and presence of S9 mix. Considering the toxicity data of Experiment I, 2700.0 µg/mL (with and without S9 mix) was chosen as top concentration in Experiment II. The applied concentrations in the cytogenetic experiments are presented in Table 1.

3.7 Experimental performance cytogenetic experiment

3.7.1 Schedule

	Without S9 mix		With S9 mix	
	Exp. I	Exp. II	Exp. I	Exp. II
Exposure period	4 hrs	22 hrs	4 hrs	4 hrs
Recovery	18 hrs	—	18 hrs	18 hrs
Preparation interval	22 hrs	22 hrs	22 hrs	22 hrs

3.7.2 Culture initiation

Blood cultures were set up in bulk within 24 hrs after collection in 75 cm² cell culture flasks (Greiner, 72632 Frickenhausen, Germany). The culture medium was DMEM:F12 (Dulbecco's modified eagle medium/ Ham's F12 medium; mixture 1:1; Life Technologies GmbH, 76339 Eggenstein, Germany) containing 10 % FCS (fetal calf serum) provided by PAA Laboratories GmbH (35091 Cölbe, Germany). The antibiotic solution contains 10,000 U/mL penicillin and 10,000 µg/mL streptomycin (SEROMED, 12247 Berlin, Germany). Additionally, the medium was supplemented with Phytohemagglutinin (PHA, final concentration 3 µg/mL, SEROMED), the anticoagulant heparin (25,000 U.S.P.-U/mL, NATTERMANN, 50829 Köln, Germany), and HEPES (final concentration 10 mM, Serva, 69115 Heidelberg, Germany).

The following volumes are added to the flasks (per 10 mL):

7.70 mL culture medium
1.00 mL fetal calf serum
0.10 mL L-glutamine
0.10 mL antibiotic solution
0.10 mL HEPES
0.05 mL phytohemagglutinin
0.05 mL heparin
0.90 mL whole blood

All incubations were done at 37 °C in a humidified atmosphere with 5.5 % CO_2 (94.5 % air).

3.7.3 Treatment

Exposure time 4 hours
About 70 hrs after seeding for each test group 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks (Nunc GmbH & Co. KG, 65203 Wiesbaden, Germany). The culture medium was replaced with serum-free medium, containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL medium were used. Concurrent solvent, and positive controls were performed. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant with the dissolved test item was discarded and the cells were re-suspended in "saline G". The washing procedure was repeated once as described.

The "saline G" solution was composed as follows (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose x H_2O	1100 mg
$Na_2HPO_4 \times 7H_2O$	290 mg
KH_2PO_4	150 mg

pH was adjusted to 7.2

After washing the cells were re-suspended in complete culture medium and cultured until preparation.

Exposure time 22 hours (without S9 mix)
About 70 hrs after seeding for each test group 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks (Nunc GmbH & Co. KG, 65203 Wiesbaden, Germany). The culture medium was replaced with complete medium (with 10 % FCS) containing the test item without S9 mix. The culture medium at continuous treatment was not changed until preparation of the cells. Concurrent solvent and positive controls were performed.

All cultures were incubated at 37 °C in a humidified atmosphere with 5.5 % CO_2 (94.5 % air).

3.7.4 Preparation of the cultures

Three hours before harvesting, colcemid (Fluka, 89203 Neu-Ulm, Germany) was added to the cultures (final concentration 0.2 µg/mL). The cultures were harvested by centrifugation 22 hrs after beginning of treatment. The supernatant was discarded and the cells were re-suspended in approximately 5 mL hypotonic solution (0.0375 M KCl). The cell suspension was then allowed to stand at 37 °C for 20 to 25 minutes. After removal of the hypotonic solution by centrifugation the cells were fixed with a mixture of methanol and glacial acetic acid (3 parts plus 1 part). At least two slides per experimental group were prepared by dropping the cell suspension onto a clean microscope slide. The cells for evaluation of cytogenetic damage were stained with Giemsa (MERCK, 64293 Darmstadt, Germany).

3.7.5 Analysis of metaphase cells

The slides were evaluated (according to standard protocol of the "Arbeitsgruppe der Industrie, Cytogenetik" (9)) using NIKON microscopes with 100 x oil immersion objectives. Breaks, fragments, deletions, exchanges and chromosomal disintegrations were recorded as structural chromosome aberrations. Gaps were recorded as well, but they were not included in the calculation of the aberration rates (4). 100 well spread metaphase plates per culture were scored for cytogenetic damage on coded slides, except for the positive control in Experiment II, in the absence of S9 mix, where 50 metaphase plates were scored. Only metaphases with 46 ± 1 centromer regions were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined.

3.8 Data recording

The generated data were recorded in the laboratory protocol. The results were presented in tabular form, including experimental groups with the test item, solvent controls, and positive controls, respectively.

3.9 Acceptability of the assay

The chromosomal aberration assay is considered acceptable if it meets the following criteria:

a) The number of aberrations found in the solvent controls falls within the range of historical laboratory control data range (Appendix I).

b) The positive control substances should produce significant increases in the number of cells with structural chromosome aberrations, which are within the range of the laboratory's historical control data:.

Test group Final concentration	Aberrant cells in % (excl. gaps) Range	Test group Final concentration	Aberrant cells in % (excl. gaps) Range
Without S9 mix		With S9 mix	
EMS 330 – 880 µg/mL	4.0 - 47.0 %	CPA 15.0 – 45.0 µg/mL	7.5 - 40.0 %

3.10 Evaluation of results

A test item is classified as non-mutagenic if:
- the number of induced structural chromosome aberrations in all evaluated dose groups is in the range of our historical control data (Appendix I).
- no significant increase of the number of structural chromosome aberrations is observed.

A test item is classified as mutagenic if:
- the number of induced structural chromosome aberrations is not in the range of our historical control data (Appendix I).

and
- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Statistical significance was confirmed by means of the Fisher´s exact test (10) ($p < 0.05$). However, both biological and statistical significance should be considered together (12). If the above mentioned criteria for the test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed.

Although the inclusion of the structural chromosome aberrations is the purpose of this study, it is important to include the polyploids and endoreduplications. The following criteria is valid:

A test item can be classified as aneugenic if:
- the number of induced numerical aberrations is not in the range of our historical control data (Appendix I).

4.0 RESULTS AND DISCUSSION

The test item SYN530561, dissolved in DMSO, was assessed for its potential to induce chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure periods was 4 hours with S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test item.

In each experimental group two parallel cultures were analysed. 100 metaphase plates per culture were scored for structural chromosomal aberrations, except for the positive control in Experiment II, in the absence of S9 mix, where 50 metaphase plates were scored. 1000 cells per culture were counted for determination of mitotic index.

The highest treatment concentration in this study, 2700.0 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight and with respect to the OECD Guideline for in vitro mammalian cytogenetic tests.

In both experiments, in the absence and presence of S9 mix, no precipitation of the test item in the culture medium was observed. No relevant increase in the osmolarity was observed (e.g. Exp. I: solvent control: 345 mOsm, pH 7.2 versus 367 mOsm and pH 7.1 at 2700.0 µg/mL). The pH value had to be adjusted to physiological values using small amounts of 2 N NaOH in a range of 503.8 to 2700.0 µg/mL.

In this study no cytotoxicity was observed up to the highest applied concentration.

In both experiments, in the absence and presence of S9 mix, no statistically significant and biologically relevant increase in the number of cells carrying structural chromosome aberrations was observed. The aberration rates of the cells after treatment with the test item (0.0 – 3.0 % aberrant cells, excluding gaps) were close to the solvent control values (0.5 – 2.5 % aberrant cells, excluding gaps) and were within the range of the laboratory's historical solvent control data.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.

In both experiments, either EMS (770 and 825 µg/mL) or CPA (15 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

In conclusion, it can be stated that under the experimental conditions reported, the test item SYN530561 did not induce structural chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of metabolic activation.

5.0 REFERENCES

1. Ames, B.N., McCann, J. and Yamasaki, E. (1975)
   Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test.
   Mutation Res., 31, 347-363
2. Obe, G. and Beek, B. (1982)
   The human leukocyte test system, in: Chemical mutagens, principles and methods for their detection, Vol. 7 (De Serres, F.J., Hollander, A., eds.) Plenum Press, N.Y., London, 337-400
3. Evans, H.J. and O'Riordan, M.L. (1975)
   Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mutation Res., 31, 135-148
4. Preston, R.J.J, San Sebastian, J.R. and Mc Fee, A.F. (1987)
   The in vitro human lymphocyte assay for assessing the clastogenicity of chemical agents
   Mutation Res., 189, 175-183
5. Swierenga, S.H.H., Heddle, J.A., Sigal, E.A., Gilman, J.P.W., Brillinger, R.L., Douglas G.R. and Nestmann, E.R. (1991)
   Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV: Chromosome aberration and sister chromatid exchange in Chinese hamster ovary, V79 Chinese hamster lung and human lymphocyte cultures.
   Mutation Res., 246, 301-322
6. Bean, C.L., Armstrong, M.J. and Galloway, S.M. (1992)
   Effect of sampling time on chromosome aberration yield for 7 chemicals in Chinese hamster ovary cells. Mutation Res., 265, 31-44
7. Morimoto, K., Sato, M. and Doizumi, A. (1983).
   Proliferative kinetics of human lymphocytes in culture measured by auto-radiography and sister chromatid differential staining. Experimental Cell Res., 145, 349-356
8. Armstrong, M.J., Bean, C.L. and Galloway, S.M. (1992)
   A quantitative assessment of the cytotoxicity associated with chromosomal aberration detection in Chinese hamster ovary cells. Mutation Res., 265, 45-60
9. Engelhardt, G. (1987)
   "Arbeitsgruppe der Industrie, Cytogenetik". Standard-Protokoll zur cytogenetischen Auswertung von Mitose- und Meiosechromosomen bei der Routineuntersuchung.
10. Richardson, C., et al (1989)
    Analysis of data from *in vitro* cytogenetic assays
    Kirkland, D.J. (ed.). "Statistical evaluation of mutagenicity test data“, Cambridge University Press, Cambridge, 141-154
11. Gallelli, J.F. (1967)
    Stability studies of drugs used in intraveneous solutions.
    Am. J. Hosp. Pharm., 24, 425-433
12. Kirkland, D.J. (1992)
    Chromosomal aberration tests *in vitro*: problems with protocol design and interpretation of results.
    Mutagenesis, 2, 95-106

TABLES SECTION

TABLE 1 Doses Applied in the Chromosome Aberration Assay with SYN530561

Exp.	Prep. interval	Exposure period	Concentrations in µg/mL									
Without S9 mix												
I	22 hrs	4 hrs	17.5	30.7	53.7	94.0	164.5	287.9	503.8	**881.6**	**1542.9**	**2700.0**
II	22 hrs	22 hrs			53.7	94.0	164.5	287.9	503.8	**881.6**	**1542.9**	**2700.0**
With S9 mix												
I	22 hrs	4 hrs	17.5	30.7	53.7	94.0	164.5	287.9	503.8	**881.6**	**1542.9**	**2700.0**
II	22 hrs	4 hrs						287.9	503.8	**881.6**	**1542.9**	**2700.0**

Evaluated experimental points are shown in bold characters

TABLE 2 Summary of Results

Summary of results of the chromosomal aberration study with SYN530561

Exp.	Preparation interval	Test item concentration in µg/mL	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	Aberrant cells in % excl. gaps*	Aberrant cells in % with exchanges
Exposure period 4 hrs without S9 mix						
I	22 hrs	Solvent control[1]	100.0	2.0	1.5	0.0
		Positive control[2]	74.4	10.5	**10.0**[S]	1.5
		881.6	98.4	1.5	1.5	0.0
		1542.9	99.2	3.0	3.0	0.0
		2700.0	104.4	3.0	2.0	0.0
Exposure period 22 hrs without S9 mix						
II	22 hrs	Solvent control[1]	100.0	0.5	0.5	0.0
		Positive control[#2]	43.0	36.0	**34.0**[S]	9.0
		881.6	93.8	2.0	2.0	0.0
		1542.9	107.0	0.0	0.0	0.0
		2700.0	92.1	0.0	0.0	0.0
Exposure period 4 hrs with S9 mix						
I	22 hrs	Solvent control[1]	100.0	3.0	2.5	0.0
		Positive control[3]	31.8	24.5	**24.0**[S]	1.5
		881.6	94.6	1.0	1.0	0.0
		1542.9	88.0	1.0	0.5	0.0
		2700.0	84.0	3.5	3.0	0.0
II	22 hrs	Solvent control[1]	100.0	1.5	1.5	0.0
		Positive control[3]	36.2	14.0	**13.5**[S]	1.5
		881.6	89.6	0.0	0.0	0.0
		1542.9	85.8	1.0	1.0	0.0
		2700.0	86.6	1.5	1.0	0.0

* Inclusive cells carrying exchanges
\# Evaluation of 50 metaphases per culture
S Aberration frequency statistically significant higher than corresponding control values
1 DMSO 0.5 % (v/v)
2 EMS: Exp. I 825.0 µg/mL; Exp. II 770 µg/mL
3 CPA 15.0 µg/mL

TABLE 3 Toxicity - Experiment I (Cytotoxicity of SYN530561 to the Cultures of Human Lymphocytes)

In Experiment I the mitotic index in two cultures (1000 cells per culture) was determined.

Concentration (µg/mL)	Exposure time	Preparation interval	Mitotic cells per 1000 cells*	% of solvent control
Without S9 mix				
Solvent control	4 hrs	22 hrs	18.4	100.0
17.5	4 hrs	22 hrs	n.d.	n.d.
30.7	4 hrs	22 hrs	n.d.	n.d.
53.7	4 hrs	22 hrs	n.d.	n.d.
94.0	4 hrs	22 hrs	n.d.	n.d.
164.5	4 hrs	22 hrs	n.d.	n.d.
287.9	4 hrs	22 hrs	18.3	99.7
503.8	4 hrs	22 hrs	19.2	104.6
881.6	4 hrs	22 hrs	18.1	98.4
1542.9	4 hrs	22 hrs	18.2	99.2
2700.0	4 hrs	22 hrs	19.2	104.4
With S9 mix				
Solvent control	4 hrs	22 hrs	17.5	100.0
17.5	4 hrs	22 hrs	n.d.	n.d.
30.7	4 hrs	22 hrs	n.d.	n.d.
53.7	4 hrs	22 hrs	n.d.	n.d.
94.0	4 hrs	22 hrs	n.d.	n.d.
164.5	4 hrs	22 hrs	n.d.	n.d.
287.9	4 hrs	22 hrs	17.7	101.4
503.8	4 hrs	22 hrs	17.1	97.7
881.6	4 hrs	22 hrs	16.5	94.6
1542.9	4 hrs	22 hrs	15.4	88.0
2700.0	4 hrs	22 hrs	14.7	84.0

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures in %

n.d. Not determined

TABLE 4 Toxicity - Experiment II (Cytotoxicity of SYN530561 to the Cultures of Human Lymphocytes)

In Experiment II the mitotic index in two cultures (1000 cells per culture) was determined.

Concentration (µg/mL)	Exposure time	Preparation interval	Mitotic cells per 1000 cells*	% of solvent control
Without S9 mix				
Solvent control	22 hrs	22 hrs	17.8	100.0
53.7	22 hrs	22 hrs	17.0	95.5
94.0	22 hrs	22 hrs	16.2	90.7
164.5	22 hrs	22 hrs	17.8	100.0
287.9	22 hrs	22 hrs	18.6	104.5
503.8	22 hrs	22 hrs	16.7	93.5
881.6	22 hrs	22 hrs	16.7	93.8
1542.9	22 hrs	22 hrs	19.1	107.0
2700.0	22 hrs	22 hrs	16.4	92.1
With S9 mix				
Solvent control	4 hrs	22 hrs	18.3	100.0
287.9	4 hrs	22 hrs	17.2	94.2
503.8	4 hrs	22 hrs	16.3	89.3
881.6	4 hrs	22 hrs	16.4	89.6
1542.9	4 hrs	22 hrs	15.7	85.8
2700.0	4 hrs	22 hrs	15.8	86.6

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures in %

TABLE 5 Experiment I - Mitotic Index; (Preparation Interval 22 hrs with and without S9 Mix)

Treatment group	Conc. per mL	S9 mix	Exposure period/ Recovery	Mitotic indices* Absolute 1	Absolute 2	Mean	%**
Solv. control[#]	0.5 %	-	4 / 18 hrs	18.4	18.3	18.4	100.0
Pos. control[##]	825.0 µg	-	4 / 18 hrs	14.6	12.7	13.7	74.4
Test item	881.6 µg	-	4 / 18 hrs	17.7	18.4	18.1	98.4
″	1542.9 µg	-	4 / 18 hrs	18.9	17.5	18.2	99.2
″	2700.0 µg	-	4 / 18 hrs	19.2	19.1	19.2	104.4
Solv. control[#]	0.5 %	+	4 / 18 hrs	18.1	16.8	17.5	100.0
Pos. control[###]	15.0 µg	+	4 / 18 hrs	6.1	5.0	5.6	31.8
Test item	881.6 µg	+	4 / 18 hrs	15.8	17.2	16.5	94.6
″	1542.9 µg	+	4 / 18 hrs	13.1	17.6	15.4	88.0
″	2700.0 µg	+	4 / 18 hrs	16.7	12.6	14.7	84.0

* The mitotic index was determined in a sample of 1000 cells per culture of each test group in %

** For the positive control groups and the test item groups, the relative values of the mitotic index are related to the solvent controls

[#] DMSO

[##] EMS

[###] CPA

TABLE 6 Structural Chromosome Aberrations Experiment I; (Preparation Interval 22 hrs without S9 Mix: Exposure Period 4 hrs)

Slide no.	Cells scored	% Aberrant cells incl. gaps*	excl. gaps*	with ex-changes	Aberrations: Gaps g	ig	Chromatid type b	f	d	ex	Chromosome type ib	if	id	cx	Other ma	cd
					Without S9 mix											
Solvent control: DMSO 0.5 %																
1	100				1	0	1	1	0	0	1	0	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	2.0	1.5	0.0	1	0	1	1	0	0	1	0	0	0	0	0
Positive control: EMS 825.0 µg / mL																
1	100				2	0	3	1	0	2	2	1	0	0	1	0
2	100				0	0	9	1	0	1	2	0	0	0	0	0
1 + 2	200	10.5	10.0	1.5	2	0	12	2	0	3	4	1	0	0	1	0
Test item: 881.6 µg / mL																
1	100				0	0	1	0	0	0	0	1	0	0	0	0
2	100				0	0	0	1	0	0	0	0	0	0	0	0
1 + 2	200	1.5	1.5	0.0	0	0	1	1	0	0	0	1	0	0	0	0
Test item: 1542.9 µg / mL																
1	100				0	0	3	0	0	0	0	1	0	0	0	0
2	100				0	0	2	0	0	0	0	0	0	0	0	0
1 + 2	200	3.0	3.0	0.0	0	0	5	0	0	0	0	1	0	0	0	0
Test item: 2700.0 µg / mL																
1	100				1	0	2	0	0	0	0	0	0	0	0	0
2	100				1	0	1	0	0	0	1	1	0	0	0	0
1 + 2	200	3.0	2.0	0.0	2	0	3	0	0	0	1	1	0	0	0	0

* Inclusive cells carrying exchanges
Cells may contain more than one aberration

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

TABLE 7 Structural Chromosome Aberrations Experiment I; (Preparation Interval 22 hrs with S9 Mix: Exposure Period 4 hrs)

Slide no.	Cells scored	% Aberrant cells incl. gaps*	excl. gaps*	with ex-changes	Aberrations: Gaps g	ig	Chromatid type b	f	d	ex	Chromosome type ib	if	id	cx	Other ma	cd
					With S9 mix											
Solvent control: DMSO 0.5 %																
1	100				1	0	2	0	0	0	0	1	0	0	0	0
2	100				0	0	1	0	0	0	0	1	0	0	0	0
1 + 2	200	3.0	2.5	0.0	1	0	3	0	0	0	0	2	0	0	0	0
Positive control: CPA 15.0 µg / mL																
1	100				3	0	30	0	0	1	3	0	0	0	1	0
2	100				0	0	19	0	0	2	1	1	0	0	0	0
1 + 2	200	24.5	24.0	1.5	3	0	49	0	0	3	4	1	0	0	1	0
Test item: 881.6 µg / mL																
1	100				0	0	0	1	0	0	0	0	0	0	0	0
2	100				0	0	1	0	0	0	0	0	0	0	0	0
1 + 2	200	1.0	1.0	0.0	0	0	1	1	0	0	0	0	0	0	0	0
Test item: 1542.9 µg / mL																
1	100				1	0	1	0	0	0	0	0	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	1.0	0.5	0.0	1	0	1	0	0	0	0	0	0	0	0	0
Test item: 2700.0 µg / mL																
1	100				0	0	0	1	0	0	0	0	0	0	0	0
2	100				1	0	4	0	0	0	1	0	0	0	0	0
1 + 2	200	3.5	3.0	0.0	1	0	4	1	0	0	1	0	0	0	0	0

* Inclusive cells carrying exchanges
Cells may contain more than one aberration

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

TABLE 8 Experiment II - Mitotic Index; (Preparation Interval 22 hrs with and without S9 Mix)

Treatment group	Conc. per mL	S9 mix	Exposure period/ Recovery	Mitotic indices* Absolute 1	Absolute 2	Mean	%**
Solv. control[#]	0.5 %	-	22 / 0 hrs	18.6	17.0	17.8	100.0
Pos. control[##]	770.0 µg	-	22 / 0 hrs	7.9	7.4	7.7	43.0
Test item	881.6 µg	-	22 / 0 hrs	17.1	16.3	16.7	93.8
″	1542.9 µg	-	22 / 0 hrs	19.3	18.8	19.1	107.0
″	2700.0 µg	-	22 / 0 hrs	16.1	16.7	16.4	92.1
Solv. control[#]	0.5 %	+	4 / 18 hrs	18.7	17.8	18.3	100.0
Pos. control[###]	15.0 µg	+	4 / 18 hrs	6.2	7.0	6.6	36.2
Test item	881.6 µg	+	4 / 18 hrs	15.6	17.1	16.4	89.6
″	1542.9 µg	+	4 / 18 hrs	12.8	18.5	15.7	85.8
″	2700.0 µg	+	4 / 18 hrs	13.7	17.9	15.8	86.6

* The mitotic index was determined in a sample of 1000 cells per culture of each test group in %

** For the positive control groups and the test item groups, the relative values of the mitotic index are related to the solvent controls

[#] DMSO

[##] EMS

[###] CPA

TABLE 9 Structural Chromosome Aberrations Experiment II; (Preparation Interval 22 hrs without S9 Mix: Exposure Period 22 hrs)

Slide no.	Cells scored	% Aberrant cells incl. gaps*	excl. gaps*	with ex-changes	Aberrations: Gaps g	ig	Chromatid type b	f	d	ex	Chromosome type ib	if	id	cx	Other ma	cd
					Without S9 mix											
Solvent control: DMSO 0.5 %																
1	100				0	0	0	0	0	0	0	0	0	0	0	0
2	100				0	0	1	0	0	0	0	0	0	0	0	0
1 + 2	200	0.5	0.5	0.0	0	0	1	0	0	0	0	0	0	0	0	0
Positive control: EMS 770 µg / mL**																
1	50				0	0	19	0	0	7	0	1	0	0	1	0
2	50				6	1	16	0	0	3	2	0	0	0	0	0
1 + 2	100	36.0	34.0	9.0	6	1	35	0	0	10	2	1	0	0	1	0
Test item: 881.6 µg / mL																
1	100				0	0	2	0	0	0	0	0	0	0	0	0
2	100				0	0	2	0	0	0	0	0	0	0	0	0
1 + 2	200	2.0	2.0	0.0	0	0	4	0	0	0	0	0	0	0	0	0
Test item: 1542.9 µg / mL																
1	100				0	0	0	0	0	0	0	0	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	0.0	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0
Test item: 2700.0 µg / mL																
1	100				0	0	0	0	0	0	0	0	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	0.0	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0

* Inclusive cells carrying exchanges
** 50 metaphase plates per culture were evaluated due to strong clastogenic effects
Cells may contain more than one aberration

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

TABLE 10 Structural Chromosome Aberrations Experiment II; (Preparation Interval 22 hrs with S9 Mix: Exposure Period 4 hrs)

Slide no.	Cells scored	% Aberrant cells			Aberrations											
		incl. gaps*	excl. gaps*	with ex-changes	Gaps		Chromatid type				Chromosome type				Other	
					g	ig	b	f	d	ex	ib	if	id	cx	ma	cd
					With S9 mix											
Solvent control: DMSO 0.5 %																
1	100				0	0	0	1	0	0	0	0	0	0	0	0
2	100				0	0	1	1	0	0	0	0	0	0	0	0
1 + 2	200	1.5	1.5	0.0	0	0	1	2	0	0	0	0	0	0	0	0
Positive control: CPA 15 µg / mL																
1	100				1	0	10	0	0	0	0	2	0	0	1	0
2	100				0	0	18	0	0	3	0	2	0	0	0	0
1 + 2	200	14.0	13.5	1.5	1	0	28	0	0	3	0	4	0	0	1	0
Test item: 881.6 µg / mL																
1	100				0	0	0	0	0	0	0	0	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	0.0	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0
Test item: 1542.9 µg / mL																
1	100				0	0	1	0	0	0	0	1	0	0	0	0
2	100				0	0	0	1	0	0	0	1	0	0	0	0
1 + 2	200	1.0	1.0	0.0	0	0	1	1	0	0	0	2	0	0	0	0
Test item: 2700.0 µg / mL																
1	100				0	0	1	0	0	0	0	0	0	0	0	0
2	100				1	0	1	0	0	0	0	0	0	0	0	0
1 + 2	200	1.5	1.0	0.0	1	0	2	0	0	0	0	0	0	0	0	0

* Inclusive cells carrying exchanges
Cells may contain more than one aberration

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

TABLE 11 Biometry

Statistical significance at the five per cent level ($p < 0.05$) for abberation frequency was evaluated by means of the Fisher's exact test. Evaluation was performed only for cells carrying aberrations excluding gaps.

Biometry of Experiment I

Test group versus solvent control		Preparation interval	Exposure period	S9 mix	p-value
Test group	881.6 µg/mL	22 hrs	4 hrs	-	n.c.
"	1542.9 µg/mL	22 hrs	4 hrs	-	0.169
"	2700.0 µg/mL	22 hrs	4 hrs	-	0.362
"	881.6 µg/mL	22 hrs	4 hrs	+	n.c.
"	1542.9 µg/mL	22 hrs	4 hrs	+	n.c.
"	2700.0 µg/mL	22 hrs	4 hrs	+	0.386
Positive control versus solvent control					
EMS	825.0 µg/mL	22 hrs	4 hrs	-	$< 0.001^{s}$
CPA	15.0 µg/mL	22 hrs	4 hrs	+	$< 0.001^{s}$

n.c. Not calculated as the aberration rate is equal or lower than the control rate
s Aberration rate is statistically significant higher than the control rate

Biometry of Experiment II

Test group versus solvent control		Preparation interval	Exposure period	S9 mix	p-value
Test group	881.6 µg/mL	22 hrs	22 hrs	-	0.108
"	1542.9 µg/mL	22 hrs	22 hrs	-	n.c.
	2700.0 µg/mL	22 hrs	22 hrs	-	n.c.
"	881.6 µg/mL	22 hrs	4 hrs	+	n.c.
"	1542.9 µg/mL	22 hrs	4 hrs	+	n.c.
"	2700.0 µg/mL	22 hrs	4 hrs	+	n.c.
Positive control versus solvent control					
EMS	770.0 µg/mL	22 hrs	22 hrs	-	$< 0.001^{s}$
CPA	15.0 µg/mL	22 hrs	4 hrs	+	$< 0.001^{s}$

n.c. Not calculated as the aberration rate is equal or lower than the control rate
s Aberration rate is statistically significant higher than the control rate

APPENDICES SECTION

APPENDIX 1 Historical Control Data

Percentage of aberrant cells in Human lymphocyte cultures (2006 to 2007)

Without S9 mix										
		Aberrant cells (%)								
Test group Concentration	No. of studies	Including gaps			Excluding gaps			With exchanges		
		Range	Mean	Calculated range*	Range	Mean	Calculated range*	Range	Mean	Calculated range*
Negative control										
Culture medium DMEM: F12	7	0.0-5.0	1.3	0.5-2.1	0.0-4.0	1.1	0.4-1.7	0.0-1.0	0.0	0.0-0.1
Aqueous solv. ** 10 % (v/v)	19	0.0-4.5	1.2	0.5-1.8	0.0-4.0	0.9	0.3-1.6	0.0-1.0	0.1	0.0-0.2
Organic solv. *** 0.5 % (v/v)	88	0.0-4.0	1.5	0.7-2.3	0.0-3.5	1.1	0.5-1.8	0.0-1.0	0.0	0.0-0.1
Total	**114**	**0.0-5.0**	**1.4**	**0.6-2.2**	**0.0-4.0**	**1.1**	**0.4-1.8**	**0.0-1.0**	**0.1**	**0.0-0.1**
Positive control										
EMS 330–880 µg/mL	113	5.0-47.0	14.4	9.3-19.4	4.0-47.0	13.6	8.8-18.4	0.0-17.0	3.1	1.0-5.2
With S9 mix										
		Aberrant cells (%)								
Test group Concentration	No. of studies	Including gaps			Excluding gaps			With exchanges		
		Range	Mean	Calculated range*	Range	Mean	Calculated range*	Range	Mean	Calculated range*
Negative control										
Culture medium DMEM: F12	7	1.0-3.0	2.1	1.5-2.6	0.5-3.0	1.7	1.1-2.3	0.0-0.5	0.1	0.0-0.3
Aqueous solv. ** 10 % (v/v)	17	0.0-4.0	1.4	0.6-2.2	0.0-3.0	1.0	0.4-1.6	0.0-0.5	0.0	0.0-0.0
Organic solv. *** 0.5 % (v/v)	90	0.0-3.5	1.5	0.8-2.2	0.0-3.5	1.2	0.6-1.8	0.0-0.5	0.0	0.0-0.1
Total	**114**	**0.0-4.0**	**1.5**	**0.8-2.2**	**0.0-3.5**	**1.2**	**0.6-1.8**	**0.0-0.5**	**0.0**	**0.0-0.1**
Positive control										
CPA 15.0–45.0 µg/mL	112	7.5-41.0	13.5	9.3-17.1	7.5-40.0	12.9	8.9-16.9	0.0-10.5	2.1	0.9-3.4

* Mean ± standard deviation

** Aqueous solvents: deionised water and 0.9 % (w/v) saline

*** Organic solvents: acetone, DMSO, ethanol and tetrahydrofurane

APPENDIX 1 Historical Control Data (Continued)

Percentage of polyploid cells in Human lymphocyte cell cultures (2006 to 2007)

Without S9 mix				
Polyploid cells (%)				
Test group Concentration	No. of studies	Range	Mean	Calculated range*
Negative control				
Culture medium DMEM: F12	7	0.0-0.6	0.1	0.0-0.3
Aqueous solv. ** 10 % (v/v)	19	0.0-0.5	0.1	0.0-0.3
Organic solv. *** 0.5 % (v/v)	88	0.0-0.8	0.1	0.0-0.2
Total	**114**	**0.0-0.8**	**0.1**	**0.0-0.2**
Positive control**				
EMS 330–880 µg/mL	113	0.0-0.6	0.1	0.0-0.1
With S9 mix				
Polyploid cells (%)				
Test group Concentration	No. of studies	Range	Mean	Calculated range*
Negative control				
Culture medium DMEM: F12	7	0.0-0.4	0.1	0.0-0.2
Aqueous solv. ** 10 % (v/v)	17	0.0-0.4	0.1	0.0-0.2
Organic solv. *** 0.5 % (v/v)	90	0.0-0.8	0.1	0.0-0.2
Total	**114**	**0.0-0.8**	**0.1**	**0.0-0.2**
Positive control**				
CPA 15.0–45.0 µg/mL	112	0.0-0.6	0.0	0.0-0.1

* Mean ± standard deviation

** Aqueous solvents: deionised water and 0.9 % (w/v) saline

*** Organic solvents: acetone, ethanol, DMSO, and tetrahydrofurane

**** Positive control only for induction of chromosomal aberrations

APPENDIX 2 Chromosome Aberrations: Classification and Criteria

1. Gaps

Gaps are small areas of the chromosome, which are unstained. The chromatids remain aligned as normal and the gap does not extend along the chromatid for a distance greater than the width of a chromatid. If the gap occurs on one chromatid only it is a chromatid gap (g).

2. Chromatid breaks

Chromatid breaks (b) vary in appearance. The chromatid may remain aligned but show a gap which is too large to classify as a gap. Alternatively, the chromatid may be broken so that the broken fragment is displaced. In some cases, the fragment is not seen at all. A chromatid fragment (f) should be scored if the chromosome of origin cannot be identified. In addition, deletions can occur as a result of a break. The missing terminal end of a chromatid in the assessed metaphase is classified as deletion (d).

3. Chromosome breaks

Chromosome breaks (ib) are breaks in both chromatids of the chromosome. A fragment with two chromatids is formed and this may be displaced by varying degrees. Breaks are distinguished from gaps by the size of the unstained region. A chromosome break is scored if the fragment is associated with a chromosome from which it was probably derived. However, fragments are often seen in isolation and are then scored as chromatid fragments (if). In addition, isodeletions can occur as a result of a isobreak. The missing terminal end of a chromosome in the assessed metaphase is classified as isodeletion (id).

4. Exchanges

Exchanges are formed by faulty rejoining of broken chromosomes and may be of the chromosome or chromatid type. Chromatid exchanges (ex) have numerous different forms but are generally not further classified. Where multiple exchanges have occurred each exchange point is counted as one chromatid exchange. Chromosome exchanges (cx) generally appear as either a dicentric or a ring form, either of which can be associated with a fragment, which if possible should be scored as part of the exchange.

5. Multiple Aberrations

If many aberrations are present in one metaphase, the exact details may not be scorable. This is particularly the case when chromosome pulverisation (cd) occurs. If the number of aberrations is greater than 4 then the cell is classified as multiple aberrant (ma).

6. Chromosome Number

If the chromosome (centromere) number is 46±1 then it is classified as a diploid cell and scored for aberrations. If less than 46±1 chromosomes are counted then the cell is ignored under the assumption that some chromosomes may have been lost for technical reasons. If multiple copies of the haploid chromosome number (other than diploid) are scored then the count is recorded and the cell classified as polyploid. If the chromosomes are arranged in closely apposed pairs, i.e. 4 chromatids instead of 2, the cell is scored as endoreduplicated (e).

APPENDIX 3 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
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
6 Prüfungen zur Bestimmung von Rückständen	**6** Residues
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical studies on biological materials

15.08. und 27. – 29.10.2008
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 **30. März 2009**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, LÄNDLICHEN RAUM UND VERBRAUCHERSCHUTZ 2

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

APPENDIX 4 Certificate of Analysis

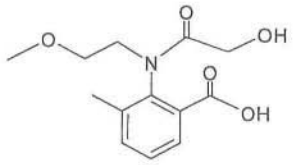
syngenta

GLP Testing Facility WMU
Analytical Development &
Product Chemistry GS2131

Syngenta Crop Protection
Münchwilen AG
Breitenloh 5
CH-4333 Münchwilen

Certificate of Analysis

SYN530561



KI 7010/5 - Purity 99 %

Batch Identification	**KI 7010/5**
Product Code	**SYN530561**
Parent	CGA17020
Other Product Code(s)	CSCC235899
ISO Common Name	---
CA Reg. No.	---
CA Index Name	---
IUPAC Name	2-[(2-hydroxy-acetyl)-(2-methoxy-ethyl)-amino]-3-methyl-benzoic acid
Molecular formula	$C_{13}H_{17}NO_5$
Molecular mass	267.3
Chemical Analysis	
– **Identity ***	**confirmed**
– **Content of SYN530561 ***	**99 % w/w** (estimated error: ± 2 %)
Methodology used for Characterization / Reanalysis	HPLC, NMR (qualitative and quantitative), Titration (K.Fischer)
Physical Analysis	
– **Appearance ***	**white solid**
Stability:	
– **Storage Temperature**	**< 10°C**
– **Reanalysis Date**	**End of March 2014**

The stability of this test substance will be controlled by reanalysis of material held in the inventory at Syngenta Crop Protection AG at the appropriate time.

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.

Characterization: 111826 Reanalysis: 115777, 119544

Authorization:

Feb 19, 2009

S. De Benedictis
Analytical Development & Product Chemistry