BIOSERVICE
SCIENTIFIC LABORATORIES GmbH

Mammalian Micronucleus Test of Murine Bone Marrow Cells

with

23081

Final Report

BSL BIOSERVICE Project No.: 010633

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Copy of GLP certificate

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FÜR ARBEITSSCHUTZ,
ARBEITSMEDIZIN UND SICHERHEITSTECHNIK**
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LfAS

GLP - B E S C H E I N I G U N G

Bescheinigung

Hiermit wird bestätigt, daß die Prüfeinrichtung(en)

in BSL Bioservice Scientific Laboratories GmbH
82152 Planegg (Ort, Anschrift)
Behringstraße 6
der Firma BSL Bioservice Scientific Laboratories GmbH (Firma)
am 29./30. November 1999 (Datum)

von der für die Überwachung zuständigen Behörde über Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist (sind).

Es wird hiermit bestätigt, daß folgende Prüfungen in dieser Prüfeinrichtung nach den Grundsätzen der Guten Laborpraxis durchgeführt werden.

Certificate

It is hereby certified that the test facility(les)

BSL Bioservice Scientific Laboratories GmbH
in 82152 Planegg (location, address)
Behringstraße 6
of Firma BSL Bioservice Scientific Laboratories GmbH (company name)
on 29./30. November 1999 (date)

was (were) inspected by the competent authority regarding compliance with the Principles of Good Laboratory Practice.

It is hereby certified that studies in this test facility are conducted in compliance with the Principles of Good Laboratory Practice.

Die Prüfungen von Stoffen und Zubereitungen betreffen folgende OECD-Prüfkategorie

Prüfkategorie 2: Prüfungen auf toxikologische Eigenschaften

Prüfkategorie 3: Prüfungen auf mutagene Eigenschaften (in vitro, in vivo)

Prüfkategorie 9: Sonstige Prüfungen; a) Mikrobiologische Sicherheitsprüfungen
b) Wirksamkeitsprüfungen an Zellkulturen

München, 04.08.2000
I.V.

Ritter
Leitender Gewerbedirektor

Contents

Preface

General

Sponsor:	
Monitor:	–
Testing Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstrasse 6 D-82152 Planegg/München
BSL BIOSERVICE - Project No.:	010633
Test Item:	23081
Title:	Mammalian Micronucleus Test of Murine Bone Marrow Cells with 23081

Project Staff

Study Director:	Dipl.-Biol. Uwe Hamann
Deputy Director of the Testing Facility:	Dr. Angela Lutterbach
Quality Assurance Unit:	Dr. Margarete Hoechst Dipl.-Biol. Birgit Schmegner Dipl.-Biol. Heidi Hübner

Schedule

Arrival of the Test Item:	April 09, 2001
Date of Draft Project Protocol:	April 23, 2001
Date of Project Protocol:	May 17, 2001
Start of Experiments:	August 06, 2001
End of Experiments:	November 08, 2001
Date of 1st Draft Report:	July 23, 2002
Date of 2nd Draft Report:	September 20, 2002
Date of 3rd Draft Report:	November 21, 2002
Date of Final Report:	December 17, 2002

Project Staff Signatures

Study Director: Dipl. Biol. Uwe Hamann

Date: 17.12.2002

Deputy Director of
the Testing Facility: Dr. Angela Lutterbach

Date: 17. Dez. 2002

Quality Assurance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended on May 08, 2001. Published May 14, 2001 in Bundesgesetzblatt 2001 part I no 21, pp. 844 – 854.

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study is assessed in compliance with the project protocol, the study plan and the Standard Operation Procedures of BSL BIOSERVICE. The study and/or the testing facility are periodically inspected by the Quality Assurance Unit and the dates and phases of the inspections are included in the report. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. The final report of the study is audited. A Quality Assurance Statement, signed by the Quality Assurance, is included in the report.

Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 474 "Mammalian Erythrocyte Micronucleus Test", adopted July 21, 1997.

EEC Directive 2000/32, L 136, Annex 4C, B 12, dated June 08, 2000.

Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP-regulations:

A copy of the Final Report, the Project Protocol, the Study Plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the project.

A sample of the test item will be stored according to the period fixed by the GLP-regulations. Also the microscopic slides will be archived according to the GLP-regulations. Samples that are unstable may be disposed of before that time. Remaining test material will be sent back to the sponsor on request. No raw data or material relating to the study will be discarded without the sponsor's prior consent.

Statement of Compliance

BSL BIOSERVICE

Project-No.: 010633

Test Item: 23081

Study Director: Dipl. Biol. Uwe Hamann

Title: Mammalian Micronucleus Test of Murine Bone Marrow Cells with 23081

This study performed in the testing facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended on May 08, 2001, published May 14, 2001.

OECD Principles of Good Laboratory Practice (as revised in 1997); Paris 1998.

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

Study Director: Dipl. Biol. Uwe Hamann

.....

Date: 16.12.2002

Quality Assurance Unit

BSL BIOSERVICE Scientific Laboratories GmbH
Behringstr. 6, D-82152 Planegg

Statement

BSL BIOSERVICE

Project-No.:	010633
Test Item:	23081
Study Director:	Dipl. Biol. Uwe Hamann
Title:	Mammalian Micronucleus Test of Murine Bone Marrow Cells with 23081

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates:

Phases of QAU Inspections	**Dates of QAU Inspections**	**Dates of Reports to the Study Director and Management**
Audit Project Protocol / Study Plan:	June 05, 2001	June 05, 2001
Experimental Phase Audit (Method Audit):	November 06, 2001	November 06, 2001
1st Draft Audit:	August 14, 2002	August 14, 2002
2nd Draft Audit:	September 30, 2002	September 30, 2002
3rd Draft Audit:	December 09, 2002	December 09, 2002
Final Audit:	December 18, 2002	December 18, 2002

This report reflects the raw data.

Member of the
Quality Assurance Unit: Margarete Haeckt

Date: 18. 12. 2002

Summary

This study was performed to investigate the potential of 23081 to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the mouse.

The test item could be administered in aqueous solution with technically applicable precipitation at a maximum concentration of 20 mg/ml. The test item was not soluble in cotton seed oil.

24 and 48 h after a single application of the test item the bone marrow cells were collected for micronuclei analysis. Ten animals (5 males, 5 females) per test group were evaluated for the occurrence of micronuclei. 2000 polychromatic erythrocytes (PCE) per animal were scored for micronuclei.

In a pre-experiment 3 female and 3 male mice received a single dose of 400 mg/kg b.w. intraperitoneally. The volume administered was 20 ml/kg b.w.. After administration all animals showed signs of toxicity (reduced spontaneous activity, lethargy, palpebral closure, prone position at 1, 1.5 and 6 h post administration and died within 24 h after start of treatment. After administration of 200 mg/kg b.w. (10 ml/kg b.w.) the animals survived and signs of toxicity were observed (lethargy 1 h post administration, no symptoms of systemic toxicity were found 6 h, 24 h and 48 h post application). Due to this result 200 mg/kg b.w. was tested as the maximum dose in the main experiment.

To describe a cytotoxic effect due to the treatment with the test item the relative PCE (rel. PCE = proportion of polychromatic (immature) erythrocytes among total erythrocytes) was determined. In comparison to the historical controls, the rel. PCE was decreased in some dose groups of treatment with 23081, but also in the negative controls of female mice. As these effects are not dose related, they cannot be used as an unequivocal indicator for the test item having reached the bone marrow.

The following dose levels of the test item were investigated:

24 h preparation interval: 20, 100 and 200 mg/kg b.w.

48 h preparation interval: 200 mg/kg b.w.

In comparison to the corresponding negative controls there was no substantial enhancement in the percentage of cells with micronuclei at 24 h and 48 h preparation interval or at any dose level of the test item.

To confirm the validity of the assay the reference mutagen Cyclophosphamide (40 mg/kg b.w.) was used as positive control and showed a distinct increase of induced micronucleus frequency.

Conclusion

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the mouse.

Therefore, 23081 is considered to be non-mutagenic in the micronucleus assay.

Objective

The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test item to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow of animals, usually rodents. The purpose of the micronucleus test is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. When a bone marrow erythroblast develops into a polychromatic erythrocyte (PCE), the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise enucleated cytoplasm.

The bone marrow of rodents is routinely used in this test. Micronuclei can be distinguished by a number of criteria. The frequency of micronucleated immature (polychromatic) erythrocytes is the biological end point. This mammalian in vivo micronucleus test is especially relevant for assessing mutagenic hazard. The test system allows consideration of factors of in vivo metabolism, pharmacokinetics and DNA repair processes although these may vary among species, among tissues and among genetic endpoints.

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. Four different major steps are included in the differentiation process:

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

The newly formed cell population persists for about 20 hours in the bone marrow of the mouse. During this time micronucleated PCEs accumulate in the bone marrow as a production of micronuclei extends over a considerable period of time.

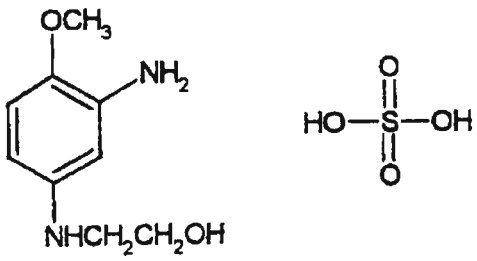
The assessment of clastogenic activity is carried out with a dose level at the maximum tolerable dose (MTD) or that producing some indication of cytotoxicity (changes in rel. PCE). Two additional doses (a middle and a low dose) are usually assayed 24 h after the treatment. If the highest applicable dose without toxic effects is 2000 mg/kg b.w., a limit test using this concentration is sufficient to estimate clastogenic potential of the test item. Sampling times are at 24 h and 48 h after treatment.

As validity criterion for the test, a reference mutagen is tested in parallel to the test item.

Materials and Methods

Characterisation of the Test Item

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

Code:	23081
Chemical name:	5-((2-Hydroxyethyl)amino)-2-methoxy-Anilin-sulfat (1:1)
Batch No.:	101
Sample-No.:	R00056178
Aggregate State at RT:	solid, powder
Colour:	grey-blue
Structural Formula:	
Molecular Formula:	$C_9H_{14}N_2O_2.H_2O_4S$
Molecular Weight:	280.30
Certificate of Analysis:	12.07.2000
Stability:	warranted for 3 years
Storage:	room temperature
Safety precautions	Routine hygienic procedures were sufficient to assure personnel health and safety.

The test item was prepared and diluted with *Aqua dest.* All animals received a single standard volume i.p. of 10 ml per kg b.w.. The vehicle was chosen due to its non-toxicity for the animals.

The Controls

Positive and negative controls were included.

The Negative Control

The vehicle of the test item *Aqua dest* was used as negative control. All control animals were handled in an identical manner to the test group subjects.

The Positive Control

Name	CPA; Cyclophosphamide
Supplier	SIGMA
Catalogue no.	C0768 (purity: at least 98 %)
Lot no.	87H0207
Dissolved in	0.9% NaCl
Final concentration	40 mg/kg b.w.
Route and frequency of administration	i.p., single
Volume administered	10 ml/kg b.w.

The solution was prepared on day of administration. The stability of CPA at room temperature is quite good (1% is hydrolysed per day in aqueous solution).

The sampling time for the controls was 24 hours.

The Test System

The animals

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

Strain:	NMRI, young healthy adult
Source:	HARLAN WINKELMANN
Number of animals:	5 of each sex per dose group
Initial age at start of acclimatisation:	7 - 12 weeks
Age at start of treatment:	minimum 8 weeks

According to the suppliers assurance the animals were in a healthy condition. The animals were under quarantine in the animal facilities of BSL BIOSERVICE for a minimum of 5 days after arrival. During this period the animals did not show any signs of illness or altered behaviour.

Husbandry

The animals were kept conventionally. The experiment was conducted under standard laboratory conditions.

Housing:	5 animals of identical sex per cage
Cage type:	Macrolon Type III (Hereto)
Bedding:	granulated soft wood bedding (ALTROMIN)
Feed:	pelleted standard diet (ALTROMIN)
Water:	tap water, ad libitum (Zweckverband Würmtal, Planegg)
Environment:	temperature 19-25° C relative humidity 55 ± 10 % artificial light 6:00 - 18:00

The animals were distributed into the test groups at random and individually marked for identification by tail drawing.

Experimental Performance

Pre-experiment for Toxicity

The test item could be administered in aqueous solution with technically applicable precipitation at a maximum concentration of 20 mg/ml. This corresponds to a dose of 200 mg/kg b.w. after application of 10 ml/kg b.w.. Additionally cotton seed oil was tested as solvent. The test item was not soluble in cotton seed oil, only suspensions with fast sedimenting precipitate were obtained. Due to this result the test item was applied in an aqueous vehicle.

In a pre-experiment 3 female and 3 male mice received a single dose of 400 mg/kg b.w. intraperitoneally. The volume administered was 20 ml/kg b.w.. After administration all animals showed signs of toxicity (reduced spontaneous activity, lethargy, palpebral closure, prone position at 1, 1.5 and 6 h post administration and died within 24 h after start of treatment. After administration of 200 mg/kg b.w. (10 ml/kg b.w.) the animals survived up to 48 h and signs of toxicity were observed (lethargy 1 h post administration, no symptoms of systemic toxicity were found 6 h, 24 h and 48 h post application). Due to this result 200 mg/kg b.w. was tested as the maximum dose in the main experiment.

Dose Selection

Dose selection was based on the data obtained from the pre-test and the highest applied dose (200 mg/kg b.w. for 24 and 48 h) was the maximum applicable level. Two other dose groups were chosen (100 and 20 mg/kg b.w.) at the 24 h treatment interval.

Main Experiment

For each test group five male and five female mice were assigned and randomly tail tagged. At the beginning of the experiment the animals were individually weighed and the administered volume (10 ml/kg b.w.) adjusted to the animal's body weight. The animals received the test item once intraperitoneally. Sampling of the bone marrow was carried out on single animals 24 and 48 hours after treatment.

Bone Marrow Preparation

Bone marrow was obtained from the femurs immediately following sacrifice by cervical dislocation of the animals. Cells were removed from the femurs by cutting off the epiphyses and by flushing the marrow out with fetal calf serum using a 5 ml syringe. The cell suspension was centrifuged at 200 x g for 10 minutes and the supernatant was discarded. A drop of the resuspended cell pellet was spread on a slide as a smear. This was air-dried and stained with May-Grünwald/Giemsa (MERCK). At least one slide was made from each bone marrow sample.

Analysis

All slides, including those of positive and negative controls, were coded before microscopic analysis. Evaluation of the slides was performed using microscopes with 100 x oil immersion objectives. 2000 immature erythrocytes per animal were scored for the incidence of micronucleated immature erythrocytes. To detect an eventually occurring cytotoxic effect of the test item the ratio between immature and mature erythrocytes was determined. At least 200 immature erythrocytes which were counted per slide and the result was expressed as relative PCE (rel. PCE = proportion of polychromatic (immature) erythrocytes among total erythrocytes).

Evaluation of Results

There are several criteria for determining a positive result:

- dose-related increase in the number of micronucleated cells and/or
- biologically relevant increase in the number of micronucleated cells for at least one of the dose groups.

According to the OECD guideline, the biological relevance as well as the statistical significance of the results are the criterion for the interpretation.

A test item is considered to be negative if there is no biological relevant and/or statistical significant increase in the number of micronucleated cells at any dose level.

For the statistics the nonparametric Mann-Whitney test was performed. However, both biological relevance and statistical significance will be considered together.

Deviation to Project Protocol

Concerning: Project Staff

Before:

Quality Assurance Unit:	Dr. Margarete Hoechst Dipl. Biol. Maike Führböter

New:

Quality Assurance Unit:	Dr. Margarete Hoechst Dipl. Biol. Birgit Schmegner Dipl. Biol. Heidi Hübner

Reason for alteration:

Personnel change.

Concerning: Quality Assurance

Before:

This study will be conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated August 01, 1994 (BGBL. I, 1994, S. 1703).

New:

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended on May 08, 2001. Published May 14, 2001 in Bundesgesetzblatt 2001 part I no 21, pp. 844 – 854.

Reason for alteration:

Updating the guidelines.

Concerning: Analysis

Before:

To describe an eventually occurring cytotoxic effect of the test item the ratio between immature and mature erythrocytes will be determined with at least 200 immature erythrocytes which will be carried out in the same sample and will be expressed in mature erythrocytes per 1000 immature erythrocytes.

New:

To detect an eventually occurring cytotoxic effect of the test item the ratio between immature and mature erythrocytes was determined. At least 200 immature erythrocytes were counted per slide and the result was expressed as relative PCE (rel. PCE = proportion of polychromatic (immature) erythrocytes among total erythrocytes).

Reasons for alteration:

Updating presentation of the data.

Concerning: Evaluation of Results

Before:

A test item is considered as mutagenic if it:

- induces a statistically significant increase in the number of micronucleated immature erythrocytes for at least on of the test points.

A test item is considered as non-mutagenic if it:

- does not produce a statistically significant increase in the number of micronucleated immature erythrocytes at any of the test points.

For the statistics the nonparametric Mann-Whitney test will be used. However, both biological and statistical significance will be considered together.

New:

There are several criteria for determining a positive result:

- dose-related increase in the number of micronucleated cells and/or
- biologically relevant increase in the number of micronucleated cells for at least one of the dose groups.

According to the OECD guideline, the biological relevance as well as the statistical significance of the results are the criterion for the interpretation.

A test item is considered to be negative if there is no biological relevant and/or statistical significant increase in the number of micronucleated cells at any dose level.

For the statistics the nonparametric Mann-Whitney test was used. However, both biological relevance and statistical significance will be considered together.

Reasons for alteration:

More detailed description of the evaluation criteria.

These deviations did not affect the quality and integrity of the study.

Results

Pre-experiment for Toxicity

A preliminary study on acute toxicity was performed with the same strain and under identical conditions as in the mutagenic study.

The test item could be administered in aqueous solution with technically applicable precipitation at a maximum concentration of 20 mg/ml.

In a pre-experiment 3 female and 3 male mice received a single dose of 400 mg/kg b.w. intraperitoneally. The volume administered was 20 ml/kg b.w.. After administration all animals showed signs of toxicity (reduced spontaneous activity, lethargy, palpebral closure, prone position at 1, 1.5 and 6 h post administration and died within 24 h after start of treatment. After administration of 200 mg/kg b.w. (10 ml/kg b.w.) the animals survived and signs of toxicity were observed (lethargy 1 h post administration, no symptoms of systemic toxicity were found 6 h, 24 h and 48 h post application). Due to this result 200 mg/kg b.w. was tested as the maximum dose in the main experiment.

Table 1: Toxic reactions during the pre-experiment for toxicity

400 mg/kg b.w.

toxic reactions	hours post-application (males/females)				
	1 h	1.5 h	6 h	24 h	48 h
reduction of spontaneous activity	2/3	2/1	2/1	-	-
lethargy	2/3	2/1	2/1	-	-
palpebral closure	2/3	2/1	2/1	-	-
prone position	2/3	2/1	2/1	-	-
ruffled fur	-	-	-	-	-
death	1/0	0/2	-	2/1	-

- = no observed effect

Table 2: Toxic reactions during the pre-experiment for toxicity

200 mg/kg b.w.

toxic reactions	hours post-application (males/females)				
	1 h	6 h	24 h	48 h	72 h
reduction of spontaneous activity	-	-	-	-	-
lethargy	3/3	-	-	-	-
palpebral closure	3/3	-	-	-	-
prone position	-	-	-	-	-
ruffled fur	-	-	-	-	-
death	-	-	-	-	-

- = no observed effect

Toxicity in the Main Experiment

Ten males and ten females were treated with the highest applicable dose of 200 mg/kg b.w.. The animals of both sexes expressed no toxic reactions after start of treatment with 200 mg/kg b.w..

Body Weight Range

Table 3: Body weight range of all animals differentiated per sex

male			
b.w. range [g]:	**25.0**	**-**	**28.0**
b.w. mean [g] ± S.D.:	**26.6**	**±**	**1.0**
b.w. variation [%]:		**±**	**5.6**

female			
b.w. range [g]:	**21.0**	**-**	**27.0**
b.w. mean [g] ± S.D.:	**23.6**	**±**	**1.4**
b.w. variation [%]:		**±**	**12.7**

Summary of Results

Table 3: *Aqua dest.*, 24 hours preparation time

Animal (male)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	25.0	0	**1**	0.41
2	26.0	0	**9**	0.57
3	28.0	0	**5**	0.46
4	28.0	0	**0**	0.53
5	26.0	0	**0**	0.50
b.w. range [g]:	25.0 - 28.0			
b.w. mean [g] ± S.D.:	26.6 ± 1.3			
b.w. variation [%]:	± 5.6			
MN total:			**15**	
MN mean ± S.D.:			**3 ± 3.9**	
MN [%]:			**0.15**	
rel. PCE mean:				0.49

Animal (female)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	25.0	0	**3**	0.24
2	24.0	0	**5**	0.24
3	23.0	0	**1**	0.28
4	24.0	0	**2**	0.37
5	23.0	0	**0**	0.32
b.w. range [g]:	23.0 - 25.0			
b.w. mean [g] ± S.D.:	23.8 ± 0.8			
b.w. variation [%]:	± 4.2			
MN total:			**11**	
MN mean ± S.D.:			**2.2 ± 1.9**	
MN [%]:			**0.11**	
rel. PCE mean:				0.29

b.w.: body weight at the beginning of the experiment
MN: micronuclei in 2000 PCE per animal
rel. PCE: quotient of polychromatic (immature) erythrocytes to total erythrocytes
S.D.: standard deviation

Table 4: Positive Control; 40 mg/kg b.w. Cyclophosphamide (CPA), 24 hours preparation time

Animal (male)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	27.0	40	**31**	0.46
2	25.0	40	**36**	0.41
3	26.0	40	**40**	0.36
4	28.0	40	**23**	0.44
5	27.0	40	**44**	0.44
b.w. range [g]:	25.0 - 28.0			
b.w. mean [g] ± S.D.:	26.6 ± 1.1			
b.w. variation [%]:	± 5.6			
MN total:			**174**	
MN mean ± S.D.:			**34.8 ± 8.2**	
MN [%]:			**1.74**	
rel. PCE mean:				0.42

Animal (female)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	25.0	40	**45**	0.66
2	25.0	40	**43**	0.52
3	24.0	40	**39**	0.51
4	25.0	40	**32**	0.56
5	25.0	40	**41**	0.61
b.w. range [g]:	24.0 - 25.0			
b.w. mean [g] ± S.D.:	24.8 ± 0.4			
b.w. variation [%]:	± 2.0			
MN total:			**200**	
MN mean ± S.D.:			**40 ± 5.0**	
MN [%]:			**2.00**	
rel. PCE mean:				0.57

b.w.: body weight at the beginning of the experiment
MN: micronuclei in 2000 PCE per animal
rel. PCE: quotient of polychromatic (immature) erythrocytes to total erythrocytes
S.D.: standard deviation

Table 5: 23081; 200 mg/kg b.w., 24 hours preparation time

Animal (male)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	26.0	200	**4**	0.43
2	27.0	200	**3**	0.36
3	27.0	200	**10**	0.41
4	28.0	200	**0**	0.36
5	27.0	200	**3**	0.65
b.w. range [g]:	26.0 - 28.0			
b.w. mean [g] ± S.D.:	27.0 ± 0.7			
b.w. variation [%]:	± 3.7			
MN total:			**20**	
MN mean ± S.D.:			**4 ± 3.7**	
MN [%]:			**0.20**	
rel. PCE mean:				0.44

Animal (female)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	23.0	200	**1**	0.52
2	23.0	200	**2**	0.46
3	24.0	200	**5**	0.42
4	23.0	200	**1**	0.34
5	23.0	200	**5**	0.53
b.w. range [g]:	23.0 - 24.0			
b.w. mean [g] ± S.D.:	23.2 ± 0.4			
b.w. variation [%]:	± 2.2			
MN total:			**14**	
MN mean ± S.D.:			**2.8 ± 2.0**	
MN [%]:			**0.14**	
rel. PCE mean:				0.46

b.w.: body weight at the beginning of the experiment
MN: micronuclei in 2000 PCE per animal
rel. PCE: quotient of polychromatic (immature) erythrocytes to total erythrocytes
S.D.: standard deviation

Table 6: 23081; 200 mg/kg b.w., 48 hours preparation time

Animal (male)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	26.0	200	**3**	0.31
2	28.0	200	**0**	0.24
3	27.0	200	**5**	0.43
4	26.0	200	**2**	0.28
5	26.0	200	**2**	0.35
b.w. range [g]:	26.0 - 28.0			
b.w. mean [g] ± S.D.:	26.6 ± 0.9			
b.w. variation [%]:	± 3.8			
MN total:			**12**	
MN mean ± S.D.:			**2.4 ± 1.8**	
MN [%]:			**0.12**	
rel. PCE mean:				0.32

Animal (female)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	26.0	200	**0**	0.31
2	27.0	200	**1**	0.50
3	23.0	200	**1**	0.22
4	23.0	200	**2**	0.28
5	21.0	200	**0**	0.48
b.w. range [g]:	21.0 - 27.0			
b.w. mean [g] ± S.D.:	24.0 ± 2.4			
b.w. variation [%]:	± 12.5			
MN total:			**4**	
MN mean ± S.D.:			**0.8 ± 0.8**	
MN [%]:			**0.04**	
rel. PCE mean:				0.36

b.w.: body weight at the beginning of the experiment
MN: micronuclei in 2000 PCE per animal
rel. PCE: quotient of polychromatic (immature) erythrocytes to total erythrocytes
S.D.: standard deviation

Table 7: 23081; 100 mg/kg b.w., 24 hours preparation time

Animal (male)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	25.0	100	**5**	0.29
2	25.0	100	**0**	0.30
3	28.0	100	**0**	0.18
4	26.0	100	**2**	0.18
5	27.0	100	**5**	0.26
b.w. range [g]:	25.0 - 28.0			
b.w. mean [g] ± S.D.:	26.2 ± 1.3			
b.w. variation [%]:	± 5.7			
MN total:			**12**	
MN mean ± S.D.:			**2.4 ± 2.5**	
MN [%]:			**0.12**	
rel. PCE mean:				0.24

Animal (female)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	23.0	100	**7**	0.39
2	22.0	100	**0**	0.51
3	24.0	100	**3**	0.42
4	21.0	100	**2**	0.42
5	25.0	100	**5**	0.43
b.w. range [g]:	21.0 - 25.0			
b.w. mean [g] ± S.D.:	23.0 ± 1.6			
b.w. variation [%]:	± 8.7			
MN total:			**17**	
MN mean ± S.D.:			**3.4 ± 2.7**	
MN [%]:			**0.17**	
rel. PCE mean:				0.44

b.w.: body weight at the beginning of the experiment
MN: micronuclei in 2000 PCE per animal
rel. PCE: quotient of polychromatic (immature) erythrocytes to total erythrocytes
S.D.: standard deviation

Table 8: 23081; 20 mg/kg b.w., 24 hours preparation time

Animal (male)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	26.0	20	**0**	0.28
2	26.0	20	**2**	0.41
3	26.0	20	**10**	0.28
4	27.0	20	**0**	0.31
5	28.0	20	**2**	0.44
b.w. range [g]:	26.0 - 28.0			
b.w. mean [g] ± S.D.:	26.6 ± 0.9			
b.w. variation [%]:	± 3.8			
MN total:			**14**	
MN mean ± S.D.:			**2.8 ± 4.1**	
MN [%]:			**0.14**	
rel. PCE mean:				0.35

Animal (female)	b.w. [g]	Dose [mg/kg b.w.]	MN	rel. PCE
1	23.0	20	**4**	0.42
2	23.0	20	**2**	0.22
3	22.0	20	**4**	0.33
4	23.0	20	**2**	0.22
5	24.0	20	**3**	0.26
b.w. range [g]:	22.0 - 24.0			
b.w. mean [g] ± S.D.:	23.0 ± 0.7			
b.w. variation [%]:	± 4.3			
MN total:			**15**	
MN mean ± S.D.:			**3 ± 1.0**	
MN [%]:			**0.15**	
rel. PCE mean:				0.29

b.w.: body weight at the beginning of the experiment
MN: micronuclei in 2000 PCE per animal
rel. PCE: quotient of polychromatic (immature) erythrocytes to total erythrocytes
S.D.: standard deviation

Table 9: Percentage of Cells with Micronuclei

Dose Group	concentration [mg/kg b.w.]	preparation time [hours]	male [%]	female [%]
NC	0	24	0.15	0.11
test item	20	24	0.14	0.15
test item	100	24	0.12	0.17
test item	200	24	0.20	0.14
test item	200	48	0.12	0.04
CPA	40.0	24	1.74	2.00

NC: negative control
CPA: Cyclophosphamide

Biometry

Table 10:

Statistical significance at the 5% level ($p < 0.05$) was evaluated by means of the non-parametric Mann-Whitney test.

Negative Control versus Test Group	preparation time [hours]	Significance		p-value	
		male	female	male	female
20 mg/kg b.w.	24	-	-	1.0000	0.4206
100 mg/kg b.w.	24	-	-	1.0000	0.5476
200 mg/kg b.w.	24	-	-	0.6905	0.6905
200 mg/kg b.w.	48	-	-	0.8413	0.2222
CPA 40 mg/kg b.w.	24	+	+	0.0079	0.0079

+: significant
-: not significant

Discussion

The test item 23081 was assessed in the micronucleus assay for its potential to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the mouse.

The test item could be administered in aqueous solution with technically applicable precipitation at a maximum concentration of 20 mg/ml. The test item was not soluble in cotton seed oil, only suspensions with fast sedimenting precipitate were obtained.

24 and 48 h after a single application of the test item the bone marrow cells were collected for micronuclei analysis. Ten animals (5 males, 5 females) per test group were evaluated for the occurrence of micronuclei. 2000 polychromatic erythrocytes (PCE) per animal were scored for micronuclei.

In a pre-experiment 3 female and 3 male mice received a single dose of 400 mg/kg b.w. intraperitoneally. The volume administered was 20 ml/kg b.w.. After administration all animals showed signs of toxicity (reduced spontaneous activity, lethargy, palpebral closure, prone position at 1, 1.5 and 6 h post administration and died within 24 h after start of treatment. After administration of 200 mg/kg b.w. (10 ml/kg b.w.) the animals survived and signs of toxicity were observed (lethargy 1 h post administration, no symptoms of systemic toxicity were found 6 h, 24 h and 48 h post application). Due to this result 200 mg/kg b.w. was tested as the maximum dose in the main experiment.

To describe a cytotoxic effect due to the treatment with the test item the relative PCE (rel. PCE = proportion of polychromatic (immature) erythrocytes among total erythrocytes) was determined. In comparison to the historical controls, the rel. PCE was decreased in some dose groups of treatment with 23081, but also in the negative controls of female mice. As these effects are not dose related, they cannot be used as an unequivocal indicator for the test item having reached the bone marrow.

The following dose levels of the test item were investigated in the main experiment:

24 h preparation interval: 20; 100 and 200 mg/kg b.w.

48 h preparation interval: 200 mg/kg b.w.

The values of micronuclei observed in the negative control groups were 0.15 % for male and 0.11 % for female mice. The values of micronuclei observed after treatment with 23081 were 0.14% for male and 0.15% for female mice (20 mg/kg b.w. 24 h) and 0.12% or 0.17% (100 mg/kg b.w. 24 h) respectively. At 200 mg/kg b.w. (MTD) the values of micronuclei observed after treatment with 23081 were 0.20% for male and 0.14% for female mice (24 h) and 0.12% or 0.04% (48 h), respectively.

In comparison to the corresponding negative controls there was no statistically significant enhancement ($p<0.05$) in the percentage of cells with micronuclei at any preparation interval or dose level of the test item.

Cyclophosphamide (40 mg/kg bw) administered i.p. was used as positive control which showed a significant increase of induced micronucleus frequency (percentage of cells with micronuclei was 1.74 % for male and 2.00 % for female mice).

Conclusion

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item did not induce micronuclei as determined by the micronucleus test in the bone marrow cells of the mouse.

Distribution of the Report

Sponsor	1x (original)
Study Director	1x (copy)

References

(1). Krauth, J. (1971)
Locally most powerful tied rank test in a Wilcoxon situation
Annals of Mathematical Statistics, 42, 1949-1956

(2). Heddle, J.A. (1973)
A rapid in vivo test for chromosomal damage
Mutation Res., 18, 187-190

(3). Schmid, W. (1976)
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In: A. Hollaender (Ed.), Chemical Mutagens, Vol.4, Plenum Press, New York, pp. 31-33

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Sex differences in micronucleus induction with hycanthone methanesulfonate in bone marrow cells of mice
Mutation Res. 283, 249-253

(5). Van Hummelen, P., A. Delener, Ph. Vanparys and M. Kirsch-Volders (1992)
Discrimination of aneuploidogens from clastogens by C-banding, DNA and area measurements of micronuclei from mouse bone marrow
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(6). Vanparys, P., G. Deknudt, F. Vermeiren, M. Sysmanns and R. Marsboom (1992)
Sampling times in micronucleus testing
Mutation Res. 282, 191-196

(7). Hayashi M., R. R. Tice, J. T. MacGregor, D. Anderson, D. H. Blakey, M. Kirsh-Volders, F.B. Oleson Jr. F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou, B. Vannier (1994)
In vivo rodent erythrocyte micronucleus assay.
Mutation Res. 312, 293-304

Annex

Table 11: Historical Laboratory Control Data (rel. PCE = quotient of polychromatic (immature) erythrocytes to total erythrocytes, 1995 - 6/2001).

	rel. PCE			
	negative control		positive control	
	male	femal	male	femal
mean	0.51	0.55	0.55	0.55
SD	0.08	0.06	0.07	0.06
RSD [%]	16.52	11.80	12.57	10.06
min.	0.29	0.34	0.47	0.46
max.	0.64	0.64	0.68	0.63
n =	15	15	14	14

mean: mean PCE
SD: Standard Deviation
RSD: relativ Standard Deviation
min.: minimum PCE
max.: maximum PCE
n: Number of assays

Table 12: Historical Laboratory Control Data (micronuclei [%], 1995 - 6/2001)

	Negative Control micronuclei		Positive Control micronuclei	
	male	femal	male	femal
mean	0.17	0.17	2.19	1.57
SD	0.08	0.07	1.00	0.41
RSD [%]	47.1	40.2	45.80	26.24
min.	0.10	0.05	1.01	0.76
max.	0.38	0.30	4.52	2.22
n =	15	15	14	14

mean: mean PCE
SD: Standard Deviation
RSD: relativ Standard Deviation
min.: minimum MN
max.: maximum MN
n: Number of assays

BIOSERVICE
SCIENTIFIC LABORATORIES GmbH

Mammalian Micronucleus Test of Murine Bone Marrow Cells

with

23081

Project Protocol

BSL BIOSERVICE Project No.: 010633

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SwiftCode: BYLADEMM (Bayer. Landesbank München)
Deutsche Bank Planegg, BLZ 700 700 24, Kto. 9 407 750

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Zentralstelle der Länder für Gesundheitsschutz bei Medizinprodukten
vertreten im
Deutscher Akkreditierungs Rat
DAR

Study Director — Dipl.-Biol. Uwe Hamann

Date: 17.05.2001

Deputy Director of
the Testing Facility — Dr. Angela Lutterbach

Date: 17.05.2001

Quality Assurance Unit — Dr. Margarete Hoechst (or)
Dipl. Biol. Maike Führböter

Date: 05.06.2001

Sponsor

Date: 19.06.01

Contents

Preface

General

Sponsor:	
Monitor:	Dr. M. Bracher
Testing Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6 D-82152 Planegg/Munich Germany
BSL BIOSERVICE-Project No.:	010633
Test Item:	23081
Title:	Mammalian Micronucleus Test of Murine Bone Marrow Cells with 23081

Project Staff

Study Director:	Dipl.-Biol. Uwe Hamann
Deputy Director of the Testing Facility:	Dr. Angela Lutterbach
Quality Assurance Unit:	Dr. Margarete Hoechst Dipl. Biol. Maike Führböter

Schedule

Arrival of the Test Item:	April 09, 2001
Date of Draft Project Protocol:	April 23, 2001
Date of Project Protocol:	May 17, 2001
Proposed Start of Experiments:	June, 2001
Proposed End of Experiments:	August, 2001
Proposed Date of Final Report:	September, 2001

Quality Assurance

This study will be conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated August 01, 1994 (BGBL. I, 1994, S. 1703).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1.

Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study will be assessed in compliance with the project protocol, the study plan and the Standard Operation Procedures of BSL Bioservice. The study and/or the testing facility will be periodically inspected by the Quality Assurance Unit and the dates and phases of the inspections will be included in the report. These inspections and audits will be carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. The final report of the study will be audited. A Quality Assurance Statement, signed by the Quality Assurance, will be included in the report.

Guidelines

This study will follow the procedures indicated by the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 474 adopted 21st. July 1997 "Mammalian Erythrocyte Micronucleus Test"

EEC Directive 2000/32, L 136, Annex 4C, B 12, dated June 08, 2000.

Amendment Procedures

This project protocol can be amended at the discretion of the study director and/or in consultation with the sponsor. Changes or revisions of this project protocol will be documented for stated reasons, signed and dated by the study director. All such amendments will be sent to the sponsor for approval and will be retained with the original project protocol.

Deviation Procedures

Unplanned changes to the project protocol and/or SOP's will be documented in the raw data. These deviations will be evaluated by the study director and an amendment will be issued if necessary. The chapter "Deviations to Project Protocol" in the report will reflect every deviation and its anticipated effects on the outcome of the study.

Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP-regulations:

A copy of the final report, the project protocol, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondance with the sponsor concerning the project.

If test item is left over a sample will be stored according to the period fixed by the GLP-regulations. Also the microscopic slides will be archived according to the GLP-regulations. Samples that are unstable may be disposed of before that time. No raw data or material relating to the study will be discarded without the sponsor's prior consent. Unless otherwise agreed upon, remaining test item will be discarded three months after release of the report.

Introduction

The micronucleus test is a mammalian *in vivo* mutagenicity test for the detection of damage to the chromosomes or the mitotic apparatus induced by various products (1,2,3,4). The purpose of the micronucleus test is to identify those agents that cause structural or numerical chromosome aberrations. When an erythroblast develops into a polychromatic erythrocyte (PCE), the main nucleus is extruded, whereas any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Micronuclei are easily detected in these cells because they lack a main nucleus. An increase in the micronucleus frequencies of polychromatic erythrocytes of treated animals is an indication of genetic toxicity. The bone marrow of rodents is routinely used in this assay as polychromatic erythrocytes are produced in this tissue. This assay is especially relevant to assessing mutagenicity hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair process.

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.

Four different major steps are included in the differentiation process which consist of the time required for the damaged erythroblast to proceed to mitosis, the mitotic delay due to treatment, the formation of micronuclei due to acentric fragments or chromosomes that are not included in the daughter nuclei and the time required for the expulsion of the main nucleus after the last mitosis to become a micronucleated PCE.

The newly formed cell population persists for about 20 hours in the bone marrow of the mouse. During this time micronucleated PCEs accumulate in the bone marrow as a production of micronuclei extends over a considerable period of time.

The assessment of clastogenic activity will be carried out with a single dose level at the maximum tolerable dose (MTD) or that producing some indication of cytotoxicity (ratio of polychromatic to normochromatic erythrocytes changes) and sampling times at 24h and 48h after treatment. Two additional doses (a middle and a low dose) are also assayed 24h after the treatment. If at a dose-level of 2000 mg/kg b.w. no toxic effects were seen, 2000 mg/kg b.w. will be the highest investigated dose.

To validate the test, a reference mutagen will be tested in parallel to the test item.

Materials and Methods

Characterisation of the Test Item

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

Code:	23081
Chemical name:	5-((2-Hydroxyethyl)amino)-2-methoxy-Anilin-sulfat (1:1)
Batch No.:	101
Sample-No.:	R00056178
Aggregate State at RT:	solid, powder
Colour:	grey-blue
Structural Formula:	will be added in the report if provided
Molecular Formula:	$C_9H_{14}N_2O_2.H_2O_4S$
Molecular Weight:	280.30
Certificate of Analysis:	12.07.2000
Stability:	warranted for 3 years
Storage:	room temperature
Safety Precautions:	Routine hygienic procedures will be sufficient to assure personnel health and safety.

The test item will be dissolved or suspended in an appropriate solvent or vehicle. Freshly prepared solutions of the test item will be employed unless stability data demonstrate the acceptability of storage. The solvent/vehicle will be chosen to its relatively nontoxicity for the animals.

Controls

Positive and negative controls will be included in each experiment.

The Negative Control

The vehicle of the test item will be used as negative control. All control animals will be handled in an identical manner to the test group subjects.

The Positive Control Substance

Name	CPA; Cyclophosphamide
Supplier	SIGMA, D-82024 Taufkirchen
Catalogue No.	C0768 (purity: at least 98%)
Dissolved in	physiological saline
Dosing	40 mg/kg b.w.
Route and frequency of administration	i.p., single
Volume administered	10 ml/kg b.w.

The solution will be prepared on day of administration. The stability of CPA at room temperature is quite good (1% is hydrolysed per day in aqueous solution).

It is acceptable that the positive control can be administered by a route different from the test agent and sampled at only a single time.

The sampling time for the controls is 24 h after treatment.

The Animals

Any commonly used rodent species is acceptable but mice are preferred because of their common use in toxicology and the availability of extensive data on micronucleus induction by a wide variety of agents (1,2,3,4,5). In addition, the mouse is an experimental animal in many physiological, pharmacological studies.

Strain:	NMRI, young healthy adult
Source:	HARLAN WINKELMANN D-33178 Borchen
Number of animals:	5 of each sex per dose group
Initial age at start of acclimatization:	7 - 12 weeks
Age at start of treatment:	minimum 8 weeks

The animals are in healthy condition according to the suppliers assurance and undergo a quarantine for a minimum of five days after their arrival. The animals will be distributed into the test groups at random.

Identification will be ensured by cage number and individual marking (e.g. tail).

Housing and Feeding Conditions

Appropriate diet and drinking water will be supplied ad libitum. Husbandry, temperature, humidity and light cycles will be controlled as dictated by good animal husbandry practice.

Pre-Experiment for Toxicity

If necessary, a preliminary study on acute toxicity will be performed with the same strain and under identical conditions as in the mutagenicity study. Three animals of each sex were treated for detection of the maximum tolerable dose. The limit dose which will be administered is 2000 mg/kg b.w..

The use of the maximum tolerable dose or the highest dose which can be formulated and administered reproducibly is generally recommended. The volume to be administered will be compatible with physiological space available.

The maximum tolerable dose is defined as the dose producing signs of toxicity such as body tremor, partial paralysis, sunken/bulging eyes, apathy and etc.. Higher dose levels, based on the same dosing regimen can be expected to produce lethality within 48 hours.

Dose levels for the main experiment will be chosen based on the results from the preliminary test.

Exposure Concentrations

The test item will be applied with at least three different concentrations at the 24 h sampling time based on the data acquired from the pre-experiment. The highest administered dose should induce toxic effects if not applied at the maximum dose. The lowest dose should not induce any toxic effects in the animals.

Study Procedure

Each treated and control group will include five males and five females. Identification will be ensured by cage number and individual marking (e.g. tail).

Administration of the Test Item

The test item will be administered intraperitoneally.

The animals will be weighed at beginning of treatment and the individual volume to be administered is adjusted to the animal´s body weight. The animals will receive the test item once. Ten animals will be treated per dose group.

Bone Marrow Preparation

Bone marrow will be obtained from the femurs immediately following sacrifice by cervical dislocation of the animals. Cells will be removed from the femurs by cutting off the epiphyses and by flushing the marrow out with fetal calf serum using a 5 ml syringe. The cell suspension is centrifuged at 1,500 rpm for 10 minutes and the supernatant is discarded. A drop of the resuspended cell pellet will be spread on a slide as a smear. This will be air-dryed and stained with May-Grünwald/Giemsa (MERCK, D-64293 Darmstadt). At least one slide will be made from each bone marrow sample.

Analysis

All slides, including those of positive and negative controls, will be independently coded before microscopic analysis. Evaluation of the slides will be performed using OLYMPUS microscopes with 100x oil immersion objectives. 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. To describe an eventually occuring cytotoxic effect of the test item the ratio between immature and mature erythrocytes will be determined with at least 200 erythrocytes which will be carried out in the same sample and will be expressed in mature erythrocytes per 1000 immature erythrocytes.

Data Recording

The data generated will be recorded in the laboratory protocol. The results will be presented in tabular form, including positive and negative controls and experimental groups. The experimental unit is the animal. The number of immature erythrocytes scored, the number of micronucleated immature erythrocytes, the percentage of micronucleated cells and the percentage of immature among total erythrocytes will be listed seperately for each animal analysed.

Evaluation of Results

A test item is considered as mutagenic if it:

- induces a statistically significant increase in the number of micronucleated immature erythrocytes for at least one of the test points.

A test item is considered as non-mutagenic if it:

- does not produce a statistically significant increase in the number of micronucleated immature erythrocytes at any of the test points.

For the statistics the nonparametric Mann-Whitney test will be used. However, both biological and statistical significance will be considered together.

Reporting

The results of the study will be reported in a detailed test report. The test report will include the following:

- a copy of the GLP-certificate of the testing facility,
- the name and address of the sponsor, the testing facility and the study schedule,
- the names of the study director and other scientists and supervisory personnel involved in the study,
- the name and address of the study monitor,
- the signatures of the study director and the management,
- the signed and dated reports of each of the individual scientists or other professionals involved in the study,
- all dates of all project protocol amendments,
- a list of all deviations to project protocol,
- the quality assurance statement, signed by the quality assurance,
- the statement of compliance, signed by the study director,
- the storage locations of all archived data, specimens and samples ,
- the test item identification, either by name or code number,
- the concentration, purity, stability, composition and other appropriate characteristics of the test item, if data will be provided by the sponsor,
- a description of the test system, including the biological materials used, a detailed description of application and treatment, dose levels of the test item, toxicity data, negative and positive controls, historical laboratory data,
- a description of the methods with references,
- a description, discussion, and interpretation of all results, if necessary a statistical evaluation.

Distribution of the Project Protocol

Sponsor	2x (original, copy)
Study Director	1x (copy)
QAU	1x (copy)

References

(1) Krauth, J. (1971)
Locally most powerful tied rank test in a Wilcoxon situation
Annals of Mathematical Statistics, 42, 1949-1956

(2) Heddle, J.A. (1973)
A rapid in vivo test for chromosomal damage
Mutation Res., 18, 187-190

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Mutation Res. 283, 249-253

(5)Van Hummelen, P., A. Delener, Ph. Vanparys and M. Kirsch-Volders (1992)
Discrimination of aneuploidogens from clastogens by C-banding, DNA and area meaurements of micronuclei from mouse bone marrow
Mutation Res. 271, 13-28

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(7) Hayashi M., R. R. Tice, J. T. MacGregor, D. Anderson, D. H. Blakey, M. Kirsh-Volders, F.B. Oleson Jr. F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou, B. Vannier (1994)
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