DSM R&D-archives Delft

P.O. Box 1,
2600 MA Delft
The Netherlands

DSM

Report number: 16.336

Date: 24-11-2003

CHROMOSOMAL ABERRATION TEST WITH ENZYME PREPARATION OF *ASPERGILLUS NIGER* GEP 44 IN CULTURED HUMAN LYMPHOCYTES

Report number V5002/08

Author(s): N. de Vogel

Department: TNO Nutrition and Food Research, Zeist

Experimental work:

Keywords:
Aspergillus niger
Chromosome aberration test
GLP
GEP44
Endoprotease
In vitro
Toxicology
Mutagenicity

Mailing list

Archiefrapportage M.E.M. Kuilman	

After use, please return to R&D-archives.

Signature author: Date manuscript 24 November 2003 Author N. de Vogel TNO Nutrition and Food Research, Zeist, The Netherlands	Signature responsible manager:

All rights strictly reserved. No part of this report may be reproduced in any form, by print Photoprint, microfilm of any other means without written permission from DSM Gist, P.O. Box 1, 2600 MA Delft, The Netherlands.

TNO Nutrition and Food Research

Nederlandse Organisatie voor
toegepast-natuurwetenschappelijk
onderzoek / Netherlands Organisation
for Applied Scientific Research

Location Zeist
Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

www.tno.nl

T +31 30 694 41 44
F +31 30 695 72 24

TNO report

V 5002/08

Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in cultured human lymphocytes

Date	24 November 2003
Author	N. de Vogel
At request of	DSM Food Specialties A. Fleminglaan 1 P.O. Box 1 2600 MA Delft The Netherlands
TNO Project number	010.45264/01.42
TNO Study number	5002/08
Sponsor Study code	-
Status report	Final report
Previous version	Draft report; 30 October 2003
Number of pages	32
Number of tables	11
Number of figures	-
Number of annexes	4
Number of appendices	-

All rights reserved.
No part of this publication may be reproduced and/or published by print, photoprint, microfilm or any other means without the previous written consent of TNO.

In case this report was drafted on instructions, the rights and obligations of contracting parties are subject to either the Standard Conditions for Research Instructions given to TNO, or the relevant agreement concluded between the contracting parties. Submitting the report for inspection to parties who have a direct interest is permitted.

© 2003 TNO

Summary

1. The test substance **Enzyme preparation from Aspergillus niger GEP 44** was examined for its potential to induce structural chromosomal aberrations in cultured human lymphocytes, in both the absence and presence of a metabolic activation system (S9-mix).

2. Two independent chromosomal aberration tests were conducted. The test substance was dissolved in RPMI-1640 medium prior to testing.

   In the first chromosomal aberration test, in the presence of S9-mix, the treatment/harvest times were 4/24 hours (pulse treatment). In the absence of S9-mix, the treatment/harvest times were 4/24 hours (pulse treatment) and 24/24 hours (continuous treatment).

   In the second chromosomal aberration test, in the presence of S9-mix, the treatment/harvest times were 4/24 hours (pulse treatment) and 4/48 hours (pulse treatment). In the absence of S9-mix, the treatment/harvest times were 24/24 hours (continuous treatment) and 48/48 hours (continuous treatment).

3. In both chromosomal aberration tests, the highest concentration of Enzyme preparation from Aspergillus niger GEP 44, analysed for the induction of chromosomal aberrations, was the maximum required concentration in the final culture medium (5000 μg/ml).

4. In two (independent) chromosomal aberration tests, in both the absence and presence of S9-mix, the test substance Enzyme preparation from Aspergillus niger GEP 44 did not induce a statistically significant increase in the number of cells with structural chromosomal aberrations at any of the dose levels and time points analysed, compared to the negative control values.

5. The positive control substances mitomycin C (in the absence of the S9-mix) and cyclophosphamide (in the presence of the S9-mix) induced the expected increases in the incidence of structural chromosomal aberrations.

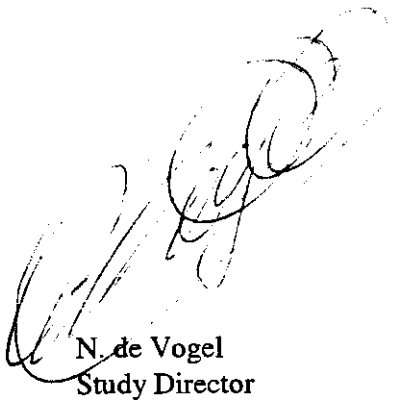
6. These data support the conclusion that, under the conditions used in this study, the test substance Enzyme preparation from Aspergillus niger GEP 44 was **not clastogenic** for cultured human lymphocytes.

Contents

Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results. All study activities performed by TNO Nutrition and Food Research were carried out in compliance with the current OECD Principles of Good Laboratory Practice (Organisation for Economic Co-operation and Development, Paris; ENV/MC/CHEM (98) 17.

The OECD Principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan.



N. de Vogel
Study Director

24 November 2003
Date

Approved by:

Dr. J.P. Groten
(Management;
Head, Department of Biomolecular Sciences)

24 November 2003
Date

Quality Assurance Statement

On	:Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in cultured human lymphocytes
TNO report number	:V5002/08
TNO project number	:010.45264/01.42
TNO study code	:5002/08
Status and date	:Final report; 24 November 2003

The protocol was inspected as follows:

Date of inspection:
28 July 2003

Date of report:
28 July 2003

The experimental phase of this study was inspected as follows:

Date of inspection:
23 July 2003

Date of report:
23 July 2003

This report was audited as follows:

Date of audit:
19 November 2003

Date of report:
19 November 2003

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all inspections were reported to the study director and the management on the dates indicated.

M. Th. A. Wolters, BSc.
(Quality Assurance Auditor)

Date: 25 november 2003

GLP compliance monitoring unit statement

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 88/320/EEC the conformity with the OECD Principles of GLP was assessed on 4-7 and 17-21 February and19-20 June 2003 at

TNO Nutrition and Food Research
Utrechtseweg 48, P.O. Box 360
3700 AJ ZEIST

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity studies, mutagenicity studies, studies on behaviour in water, soil and air, bio-accumulation, residue studies, and analytical and clinical chemistry.

The Hague, 01 July 2003

R. Jaspers, PhD
GLP Compliance Monitoring Department

Inspectorate for Health Protection and Veterinary Public Health
Ministry of Health, Welfare and Sport

Testing facility

This chromosomal aberration test was conducted at:

TNO Nutrition and Food Research
P.O. Box 360, 3700 AJ ZEIST, the Netherlands
Telephone +31 30 69 44 144
Telefax +31 30 69 57 224
Visitors address: Utrechtseweg 48, Zeist, the Netherlands

Contributors

Department of Biomolecular Sciences:

Study director	: N. de Vogel
Deputy study director	: Dr. C.A.M. Krul
Chromosomal aberration analysis	: L.A.E. Schenk
Management (Head, Department of Biomolecular Sciences)	: Dr. J.P. Groten

1. Introduction

At the request of DSM Food Specialties, P.O. Box 1, 2600 MA Delft, The Netherlands, Enzyme preparation from Aspergillus niger GEP 44 was examined for its potential to induce chromosomal aberrations in cultured human lymphocytes, in both the absence and presence of a metabolic activation system (S9-mix).

The study was conducted according to a protocol entitled: "Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in cultured human lymphocytes", which was approved by the study director on 2 July 2003.

The protocol had been drafted in accordance with:
- the OECD guideline 473, Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test, adopted 21 July 1997.

2. Experimental procedures

2.1 Test substance

Name	:	Enzyme preparation from Aspergillus niger GEP 44
Chemical name	:	Prolyl oligopeptidase
Active component	:	endoprotease
CAS Reg. No.	:	72162-84-6
Batch number	:	JLL 03 006 IDF
Production date	:	March 2003
Date of received	:	27 June 2003
Labelling on container	:	Endopro
Package	:	a plastic bottle
Colour/appearance	:	a transparent brown liquid
Dry matter content	:	25.9 %
Quantity	:	100 g net weight (determined by the Sponsor) 154.38 g gross weight (determined by TNO)
Storage conditions	:	< 18°C
Supplier	:	DSM Food Specialties BV
Expiry date	:	March 2004
TNO internal reference no.	:	030087

These data were provided by the sponsor unless otherwise indicated. Characterization and verification of the test substance identity and properties are, however, the responsibility of the sponsor. A Test Material Information Sheet (TMIS), a Material Safety Data Sheet (MSDS) and a Certificate of Analysis concerning the test substance were provided by the sponsor. The Certificate of Analysis, concerning the test substance, is included as Annex 4 of this report.

2.2 Test system

2.2.1 Tissue culture media and other chemicals

RPMI-1640 medium (with Glutamax-I), foetal calf serum and penicillin-streptomycin were purchased from Life Technologies (Gibco) BV, Breda, The Netherlands; nicotinamide-adenine dinucleotide phosphate disodium salt (NADP) and D-glucose-6-phosphate disodium salt (G-6-P) from Roche Diagnostics, Almere, The Netherlands; Aroclor 1254 from Monsanto Chemical Company, St. Louis, USA; colcemid from Fluka AG, Buchs, Switzerland; methanol, glacial acetic acid and Giemsa stain from Merck-Darmstadt, Darmstadt, FRG; Phytohaemagglutinin (PHA) from Murex Biotech Ltd., Kent, England; Mitomycin C from Sigma Chemical Company, St. Louis, USA; Cyclophosphamide from Asta Werke, Bielefeld, FRG.

2.2.2 Blood samples

Blood samples, used in both chromosomal aberration tests, were taken by venapuncture from two healthy, non-smoking males, not currently taking any medication, and collected in sterile, heparinized tubes. The specimen was then gently mixed on a "rock-n-roller" to prevent clotting. The cultures were set up within one hour of withdrawal of the blood. For both chromosomal aberration tests, blood samples were taken from two different blood donors.

2.2.3 Culture medium for human lymphocytes

The medium for culturing the human peripheral blood lymphocytes consisted of RPMI-1640 medium (with Glutamax-I), supplemented with heat-inactivated (45 min, 56 °C) foetal calf serum (20 %), penicillin (100 IU/ml medium), streptomycin (100 µg/ml medium) and phytohaemagglutinin (25 µg/ml medium). Small inocula of whole blood (0.5 ml) were added to tubes containing 4.5 ml of culture medium. The blood cultures were incubated in sterile screw-capped (loose) tubes for 48 h at c. 37 °C in humidified air containing 5% CO_2. Thereafter, different concentrations of the test solutions were added to the cultures.

2.2.4 Metabolic activation system

The S9-mix consists of a liver homogenate fraction (S9) and cofactors as described by Ames et al. (1975) and Maron and Ames (1983). The S9, used in this study, was part of a batch prepared on 25 September 2002. The preparation and characterization of the batch is described in Annex 1. Immediately before use, a S9-mix was prepared by mixing the thawed S9 with a NADPH-generating system. The final concentrations of the various ingredients in the S9-mix were: $MgCl_2$ 8 mM; KCl 33 mM; G-6-P 5 mM; NADP 4 mM; sodium phosphate 100 mM (pH 7.4) and S9 40 % (v/v).

2.3 Chromosomal aberration tests

2.3.1 Solubility and preparation of the test substance

As stated in the Certificate of Analysis (see Annex 4), provided by the sponsor, the dry matter content of the test substance is 25.9 % (259 mg/ml). The test substance was thawed prior to preparation of the stock solutions.

For the first test, 2 ml of the test substance was diluted with 8.36 ml RPMI-1640 culture medium without serum. For the second test, 3 ml of the test substance was diluted with 12.54 ml RPMI-1640 culture medium without serum. This resulted in brownish acqueous stock solutions of 50 mg/ml. These stock solutions were obtained to yield the maximum final concentration in the culture medium of 5000 μg/ml, as stipulated by the protocol. Before use, the stock solutions of 50 mg/ml were sterilized by passing through a low protein binding 0.45 μm filter to guaranty the sterility during the tests. After filtration, brownish stock solutions were obtained. Serial dilutions were prepared in RPMI-1640 culture medium without serum. For both the first and second chromosomal aberration test, it was decided to use 5000 μg/ml as highest final concentration of the test substance in the culture medium. For the first test, seven additional concentrations of the test substance, ranging from 2500 to 39 μg/ml, at a two-fold interval, were included. For the second test, five additional concentrations of the test substance, ranging from 4000 to 1000 μg/ml (more narrow spaced), were included.

2.3.2 The first chromosomal aberration test

For the first chromosomal aberration test, 0.5 ml of whole blood was incubated for 48 h at 37° C in humidified air containing 5 % CO_2. The incubation was carried out in sterile screw-capped (loose) centrifuge tubes containing 4.5 ml culture medium (see 2.2.3). After this incubation period, the cells were exposed to different concentrations of the test substance in both the absence and presence of a metabolic activation system (S9-mix).

Just before use, the test substance was thawed and a sterile stock solution of the test substance of 50 mg/ml was prepared in culture medium without serum. Thereafter, serial dilutions of the test substance were prepared in culture medium without serum. Culture medium without serum was used as negative control. Mitomycin C and cyclophosphamide were used as positive control substance in the absence and the presence of S9-mix, respectively. In all instances duplicate cultures were used. In the absence of S9-mix, the length of the treatment period was 4 hours (pulse treatment) and 24 hours (continuous treatment). In the presence of S9-mix the length of the treatment period was 4 hours (pulse treatment). In both the absence and presence of S9-mix, the harvesting time was 24 hours after onset of the treatment.

In the absence of S9-mix, 0.5 ml of each dilution of the test substance and 50 μl of the positive control substance were added directly to the cells. The total volume of each culture was 5 ml. Thereafter, the blood cultures were incubated at 37° C in humidified air containing 5 % CO_2 and treated for 4 hours (pulse treatment group) and 24 hours (continuous treatment group). After the treatment period of 4 hours (pulse treatment group only), the medium with the test substance was removed and the cells were washed twice with phosphate-buffered saline (PBS) and subsequently supplied with freshly prepared culture medium (see 2.2.3) and incubated for an additional 20 h at 37° C in humidified air containing 5 % CO_2.

In the presence of S9-mix, the culture medium was replaced by culture medium without serum. To each culture, 0.5 ml of each dilution of the the test substance and 50 μl of the positive control substance were then added to the blood cultures. Thereafter, 0.5 ml of the S9-mix (see 2.2.4) was added to each culture. The blood

cultures were incubated for 4 hours (pulse treatment, because of the toxicity of the S9-mix for the cells) at 37° C in humidified air containing 5 % CO_2. The total volume of each culture was 5 ml. After the incubation period of 4 hours, the medium with the test substance was removed.

The cells were washed twice with phosphate-buffered saline (PBS) and supplied with freshly prepared culture medium (see 2.2.3). The cells were incubated for an additional 20 h at 37 ° C in humidified air containing 5 % CO_2. The dose levels of the test substance and positive controls, used in the first chromosomal aberration test, are given in the Tables 1-3.

2.3.3 The second chromosomal aberration test

The second (independent) chromosomal aberration test was carried out essentially as described in paragraph 2.3.2. In the absence of S9-mix, one group of cultures was treated continuously for 24 hours and and another group of cultures was treated continuously for 48 hours. In the presence of S9-mix, one group of cultures was pulse-treated for 4 hours and harvested 24 hours after onset of the treatment. A second group of cultures was pulse-treated for 4 hours and harvested 48 hours after onset of the treatment.

The dose levels of the test substance and positive controls, used in the second chromosomal aberration test, were based on the results of the first chromosomal aberration test and are given in the Tables 4-7.

2.3.4 Cell harvesting, preparation of slides and scoring

Two hours before the end of the total incubation period of 24 or 48 h the cultured lymphocytes were arrested in the metaphase stage of their mitosis by the addition of colcemid (final concentration: 0.1 μg/ml medium). The cells were harvested by low speed centrifugation, treated for 15 min in a waterbath at 37° C with a hypotonic solution (0.075 M KCl), fixed three times with a freshly prepared 3:1 (v/v) mixture of methanol and acetic acid and processed for chromosomal preparations. Slides were stained for 15 min in a 2 % solution of Giemsa, rinsed in distilled water, air-dried and embedded with a Tissue-TEK cover slipper. The slides were coded by a qualified person not involved in scoring of the slides to enable "blind" scoring. At least 1000 stimulated lymphocytes (500 on each slide) were examined in each culture to determine the percentage of cells in mitosis (mitotic index). On the basis of the results of the mitotic index scoring and the observations with respect to the quality of the metaphases, at least three concentrations of the test substance together with the negative (solvent) and positive controls were selected for the analysis of chromosomal aberrations. The selection criteria were:

- for relatively insoluble substances the highest dose tested should be the lowest insoluble concentration in the final culture medium,
- at least 3 dose levels analysable for chromosomal aberrations should be obtained, usually at a 2-fold or smaller interval,
- if possible, the highest concentration should reduce the mitotic index with at least 50 % (but not more than 70 %) when compared to the negative control value,
- the lowest concentration should be on the borderline of mitotic inhibition.

Subsequently, the selected concentrations, together with the negative and positive control cultures, were analysed for the induction of chromosomal aberrations (see Tables 8-11).

In both the absence and the presence of the S9-mix, 200 well-spread metaphases (100 metaphases per culture and 50 metaphases per slide) per concentration of the test substance together with the negative (vehicle) control and the positive control, each containing 46 centromeres, were analysed by microscopic examination for structural chromatid-type and chromosome-type aberrations (gaps, breaks and exchanges) and for numerical chromosomal aberrations (polyploid and endoreduplication) and other anomalies (see Annex 2 for definition of aberrations). Cells with polyploid and endoreduplication or heavily damaged cells (cells with multiple aberrations) were not counted and included in the 200 analysed cells and in the total aberration frequency, because this test is not designed to measure numerical aberrations and is not routinely used for that purpose. The Vernier readings of all aberrant metaphase scored were recorded.

2.4 Statistical analysis

Data were analysed statistically by the Fisher's exact probability test (two-sided) to determine significant differences between treated and control cultures.

2.5 Evaluation of results

The study is considered valid if the positive controls give a statistically significant increase and if the negative controls are within the historical range (see Annex 3), and if at least 160 cells of the intended cells are analysable at each dose level.

A response is considered to be positive if the percentage of cells with structural chromosomal aberrations is statistically significant higher ($p<0.05$) compared to the vehicle control.

A test substance is considered to be clastogenic if a dose-related increase in the percentage of cells with structural chromosomal aberrations over the concurrent control frequencies is observed, or if a single positive test point is observed in both assays.

A test substance is considered to be equivocal in the chromosomal aberration test if it produces a positive response at a single test point in only the second test, and if the positive response is higher than the historical range for negative controls.

A test substance is considered to be negative in the chromosomal aberration test if it produces neither a dose-related increase in the percentage of cells with structural chromosomal aberrations nor a positive response at a single test point in both tests or in the second test only.

Cells with only gaps, heavily damaged cells (cells with multiple aberrations) and cells with polyploid and endoreduplication are recorded separately and not included in the final assessment of clastogenic activity.

Both statistical significance and biological relevance are considered together in the evaluation of the results.

2.6 Time schedule

The study was conducted between 21 July 2003 (start of the first chromosomal aberration test) and 14 October 2003 (last day of slide analysis of the second chromosomal aberration test).

2.7 Retention of records, samples and specimens

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study, including microscopic slides, will be retained in the archives of the TNO Nutrition and Food Research for a period of at least 15 years after submission of the final report.

2.8 Deviations from the protocol

The TNO project number is 010.45264/01.42, instead of 010.45264/01.41.

3. Results and discussion

Two independent chromosomal aberration tests were conducted with Enzyme preparation from Aspergillus niger GEP 44. The results are summarized in the Tables 1-7 (mitotic index scoring) and in the Tables 8-11 (chromosomal analysis of the selected cultures). In both chromosomal aberration tests, the highest concentration analysed for chromosomal aberrations, was the maximum required concentration.

In the first chromosomal aberration test, in the continuous treatment group without metabolic activation (S9-mix), the mitotic indices of the analysed concentrations (1250, 2500 and 5000 μg/ml) were reduced to 91%, 59% and 45% respectively, of that of the concurrent control value. In this treatment group, the test substance did not induce a statistically significant increase in the number of aberrant cells at any of the concentrations analysed (see Table 1 and 9).

In the first chromosomal aberration test, in the pulse treatment group with metabolic activation (S9-mix), the mitotic indices of the analysed concentrations (1250, 2500 and 5000 μg/ml) were reduced to 60%, 74% and 82% respectively, of that of the concurrent control value. In this treatment group, the test substance did not induce a statistically significant increase in the number of aberrant cells at any of the concentrations analysed (see Table 2 and 8).

In the first chromosomal aberration test, in the pulse treatment group without metabolic activation (S9-mix), the mitotic indices of the analysed concentrations (1250, 2500 and 5000 μg/ml) were 101%, 85% and 101% respectively, of that of the concurrent control value. In this treatment group, the test substance did not induce a statistically significant increase in the number of aberrant cells at any of the concentrations analysed (see Table 3 and 8).

In the second chromosomal aberration test, in the continuous treatment group of 24 hours, without metabolic activation (S9-mix), the mitotic indices of the analysed concentrations (2000, 4000 and 5000 μg/ml) were reduced to 72%, 43% and 49% respectively, of that of the concurrent control value. The mitotic indices of the not selected concentrations for chromosomal aberration analysis (3000 and 1500 μg/ml) were reduced to 39% and 53% respectively, of that of the concurrent control value. In this treatment group, the test substance did not induce a statistically significant increase in the number of aberrant cells at any of the concentrations analysed (see Table 4 and 10).

In the second chromosomal aberration test, in the continuous treatment group of 48 hours, without metabolic activation (S9-mix), the mitotic indices of the analysed concentrations (2000, 4000 and 5000 μg/ml) were reduced to 75%, 47% and 55% respectively, of that of the concurrent control value. The mitotic indices of the not selected concentrations for chromosomal aberration analysis (3000, 1500 and 1000 μg/ml) were reduced to 68%, 81% and 62% respectively, of that of the concurrent control value. In this treatment group, the test substance did not induce a

statistically significant increase in the number of aberrant cells at any of the concentrations analysed (see Table 5 and 10).

In the second chromosomal aberration test, in the pulse treatment group with metabolic activation (S9-mix), at the early sampling time of 24 hours, the mitotic indices of the analysed concentrations (3000, 4000 and 5000 μg/ml) were reduced to 79%, 81% and 91% respectively, of that of the concurrent control value. In this treatment group, the test substance did not induce a statistically significant increase in the number of aberrant cells at any of the concentrations analysed (see Table 6 and 11).

In the second chromosomal aberration test, in the pulse treatment group with metabolic activation (S9-mix), at the later sampling time of 48 hours, the mitotic index of two concentrations analysed (3000 and 5000 μg/ml) were not reduced, when compared to the concurrent control value. The mitotic index of the moderate concentration analysed (4000 μg/ml) was reduced to 93% of that of the concurrent control value. The mitotic indices of the not selected concentrations for chromosomal aberration analysis (2000, 1500 and 1000 μg/ml) were not reduced, when compared to the concurrent control value. In this treatment group, the test substance did not induce a statistically significant increase in the number of aberrant cells at any of the concentrations analysed (see Table 7 and 11).

As required, the positive control substances, mitomycin C (in the absence of the S9-mix) and cyclophosphamide (in the presence of the S9-mix), gave the expected statistically significant increases in the number of cells with structural chromosomal aberrations, which demonstrates the validity of the test system.

4. Conclusion

These data of two performed chromosomal aberration tests support the conclusion that, under the conditions used in this study, the test substance Enzyme preparation from Aspergillus niger GEP 44 was **not clastogenic** for cultured human lymphocytes.

5. References

- Ames, B.N., J. McCann and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mut. Res. 31 (1975) on *in vitro* tests for chromosomal aberrations. Mutation Res., 312 (1994) 241-261.
- Galloway, S.M., Aardema, M.J., Ishidate, M.Jr., Ivett, J.L., Kirkland, D.J., Morita, T., Mosesso, P. And Sofuni, T.: International workshop on standardisation of genotoxicity test procedures. Report from working group on *in vitro* tests for chromosomal aberrations. Mutation Res., 312 (1994) 241-261.
- Kao, F.T. and T.T. Puck. Genetics of somatic mammalian cells. VII. Proc. Nat. Acad. Sci. (USA) 60 (1968) 1275-1281.
- Maron, D.M. and B.N. Ames. Revised methods for the Salmonella mutagenicity test. Mutation Res. 113 (1983) 173-215.
- Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test, OECD Guidelines for the Testing of Chemicals, no. 473. Organisation for Economic Co-Operation and Development, Paris, 21 July 1997.
- OECD Principles of Good Laboratory Practice (as revised in 1997) by the Organisation for Economic Co-operation and Development (OECD), Paris; ENV/MC/CHEM(98)17.

Tables

Table 1: Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in the absence of S9-mix: mitotic index (Test 1)

treatment time:			24 h		
harvest time:			24 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
serum free cult. medium	0	1000	8.9	100	+
		1000	6.7		+
test substance	5000	1000	3.7	45	+
		1000	3.2		+
	2500	1000	4.0	59	+
		1000	5.1		+
	1250	1000	7.5	91	+
		1000	6.7		+
mitomycin C	0.2	1000	3.4	46	+
		1000	3.7		+

Table 2: Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in the presence of S9-mix: mitotic index (Test 1)

treatment time: / harvest time:			4 h / 24 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
serum free cult. medium	0	1000	9.4	100	+
		1000	8.7		+
test substance	5000	1000	7.7	82	+
		1000	7.2		+
	2500	1000	5.9	74	+
		1000	7.5		+
	1250	1000	5.7	60	+
		1000	5.2		+
cyclophospha mide	25	1000	2.9	32	+
		1000	2.8		+

Table 3: Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in the absence of S9-mix: mitotic index (Test 1)

treatment time: / harvest time:			4 h / 24 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
serum free cult. medium	0	1000	7.0	100	+
		1000	6.6		+
test substance	5000	1000	5.8	101	+
		1000	8.0		+
	2500	1000	5.1	85	+
		1000	6.4		+
	1250	1000	6.8	101	+
		1000	7.0		+
mitomycin C	0.4	1000	5.0	73	+
		1000	4.9		+

Table 4: Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in the absence of S9-mix: mitotic index (Test 2)

treatment time:			24 h		
harvest time:			24 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
serum free cult. medium	0	1000	9.3	100	+
		1000	8.3		+
test substance	5000	1000	4.6	49	+
		1000	3.9		+
	4000	1000	3.6	43	+
		1000	4.0		+
	3000	1000	3.2	39	-
		1000	3.5		-
	2000	1000	7.6	72	+
		1000	4.9		+
	1500	1000	3.7	53	-
		1000	5.7		-
	1000	@			
		@			
mitomycin C	0.2	1000	2.9	32	+
		1000	2.7		+

@ cultures were superfluous and no slided prepared

Table 5: Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in the absence of S9-mix: mitotic index (Test 2)

treatment time:			48 h		
harvest time:			48 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
serum free cult. medium	0	1000	4.7	100	+
		1000	5.9		+
test substance	5000	1000	3.4	55	+
		1000	2.4		+
	4000	1000	1.7	47	+
		1000	3.3		+
	3000	1000	3.5	68	-
		1000	3.7		-
	2000	1000	4.6	75	+
		1000	3.3		+
	1500	1000	4.1	81	-
		1000	4.4		-
	1000	1000	3.4	62	-
		1000	3.2		-
mitomycin C	0.2	1000	1.7	26	+
		1000	1.0		+

Table 6: Chromosomal aberration test with Enzyme preparation from Aspergillus niger GEP 44 in the presence of S9-mix: mitotic index (Test 2)

treatment time: / harvest time:			4 h / 24 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
serum free cult. medium	0	1000	7.4	100	+
		1000	7.6		+
test substance	5000	1000	7.8	91	+
		1000	5.8		+
	4000	1000	5.6	81	+
		1000	6.6		+
	3000	1000	5.5	79	+
		1000	6.2		+
	2000	@			
		@			
	1500	@			
		@			
	1000	@			
		@			
cyclophospha mide	25	1000	1.5	23	+
		1000	1.9		+

@ cultures were superfluous and no slided prepared

Table 7: Chromosomal aberration test with enzyme preparation of Bacillus subtilis (XAS-1) in the presence of S9-mix: mitotic index (Test 2)

treatment time:			4 h		
harvest time:			48 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
serum free cult. medium	0	1000	4.7	100	+
		1000	3.4		+
test substance	5000	1000	4.8	105	+
		1000	3.8		+
	4000	1000	3.4	93	+
		1000	4.2		+
	3000	1000	5.1	117	+
		1000	4.5		+
	2000	1000	4.0	115	-
		1000	5.4		-
	1500	1000	5.0	115	-
		1000	4.3		-
	1000	1000	3.0	98	-
		1000	4.9		-
cyclophospha mide	25	1000	5.0	112	+
		1000	4.2		+

Table 8 Results of Chromosomal Aberration Test 1 (Short time treatment in the absence and presence of S9-mix)

Test Substance: Enzyme preparation from Aspergillus niger GEP 44

Treatment / harvest (h)	S9-mix	Dose level of Test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing chromosome numerical aberrations			
			Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics[2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
4/24	-	Neg. control (serum free cult. medium)	100	0	0	0	0	0	0		0	100	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
		1250	100	0	0	0	0	0	0	-	0	101	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
		2500	100	0	0	0	0	0	0	-	0	85	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
		5000	100	0	0	0	0	0	0	-	0	101	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
		Pos. control (mitomycin C) 0.4	100	12	10	2	0	0	23	***	0	73	100	0	0	0
			100	9	15	4	0	0	24		0		100	0	0	0
			200	21	25	6	0	0	47 (23.5)		0		200	0	0	0 (0.0)
4/24	+	Neg. control (serum free cult. medium)	100	0	0	0	0	0	0		0	100	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
		1250	100	1	1	0	0	0	2	-	0	60	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	1	1	0	0	0	2 (1.0)		0		200	0	0	0 (0.0)
		2500	100	0	0	0	0	0	0	-	0	74	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
		5000	100	0	0	0	0	0	0	-	0	82	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
		Pos. control (cyclophos-phamide; 25.0)	100	15	6	3	0	0	20	***	0	32	100	0	0	0
			100	17	9	0	0	0	24		0		100	0	0	0
			200	32	15	3	0	0	44 (22.0)		0		200	0	0	0 (0.0)

1) Gap(g) - total number of cells showing only gaps (chromatid-type and chromosome-type gaps).

2) Fisher's exact probability test (two-sided), - P>0.05, * P<0.05, ** P<0.01, *** P<0.001

Table 9 Results of Chromosomal Aberration Test 1 (Continuous treatment in the absence of S9-mix)

Test Substance: Enzyme preparation from Aspergillus niger GEP 44

Treatment / harvest (h)	Dose level of Test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing numerical chromosome aberrations			
		Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics[2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
24/24	neg.contr. (serum free cult.med.)	100	0	0	0	0	0	0		0	100	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	1250	100	0	0	0	0	0	0	-	0	91	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	2500	100	1	0	0	0	0	1	-	0	59	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	1	0	0	0	0	1 (0.5)		0		200	0	0	0 (0.0)
	5000	100	0	0	0	0	0	0	-	0	45	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	Pos. control (mitomycin C) 0.2	100	10	4	3	0	0	13	***	0	46	100	0	0	0
		100	10	6	3	0	0	18		0		100	0	0	0
		200	20	10	6	0	0	31 (15.5)		0		200	0	0	0 (0.0)

1) Gap(g) - total number of cells showing only gaps (chromatid-type and chromosome-type gaps).
2) Fisher's exact probability test (two-sided), - P>0.05, * P<0.05, ** P<0.01, *** P<0.001

Table 10 Results of Chromosomal Aberration Test 2 (Continous treatment in the absence of S9-mix)

Test Substance: Enzyme preparation from Aspergillus niger GEP 44

Treatment / harvest (h)	Dose level of Test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing numerical chromosome aberrations			
		Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics[2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
24/24	Neg. contr. (serum free cult.med.)	100	0	0	0	0	0	0		0	100	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	2000	100	0	0	0	0	0	0	-	0	72	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	4000	100	0	0	1	0	0	1	-	0	43	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	1	0	0	1 (0.5)		0		200	0	0	0 (0.0)
	5000	100	1	0	0	0	0	1	-	0	49	100	0	0	0
		100	0	0	0	0	0	0		1		100	0	0	0
		200	1	0	0	0	0	1 (0.5)		1		200	0	0	0 (0.0)
	Pos. contr. (mitomycin C) 0.2	100	18	12	4	0	0	29	***	0	32	100	0	0	0
		100	17	11	3	0	0	27		0		100	0	0	0
		200	35	23	7	0	0	56 (28.0)		0		200	0	0	0 (0.0)
48/48	Neg. contr. (serum free cult.med.)	100	0	0	0	0	0	0		0	100	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	2000	100	0	0	0	0	0	0	-	0	75	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	4000	100	0	0	0	0	0	0	-	0	47	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	5000	100	0	0	0	0	0	0	-	0	55	100	0	0	0
		100	1	0	0	0	0	1		0		100	0	0	0
		200	1	0	0	0	0	1 (0.5)		0		200	0	0	0 (0.0)
	Pos. contr. (mitomycin C) 0.2	100	35	31	1	0	0	54	***	0	26	100	0	0	0
		100	34	21	3	0	0	49		1		100	0	0	0
		200	69	52	4	0	0	103 (51.5)		1		200	0	0	0 (0.0)

1) Gap(g) - total number of cells showing only gaps (chromatid-type and chromosome-type gaps).

2) Fisher's exact probability test (two-sided), - P>0.05, * P<0.05, ** P<0.01, *** P<0.001

Table 11 Results of Chromosomal Aberration Test 2 (Short and long time post treatment in the presence of S9-mix)

Test Substance: Enzyme preparation from Aspergillus niger GEP 44

Treatment / harvest (h)	Dose level of Test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing numerical chromosome aberrations			
		Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics[2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
4/24	Neg. contr. (serumfree cult. med.)	100	0	0	0	0	0	0		0	100	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	3000	100	0	0	0	0	0	0	-	0	79	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	4000	100	0	0	0	0	0	0	-	0	81	100	0	0	0
		100	0	1	0	0	0	1		0		100	0	0	0
		200	0	1	0	0	0	1 (0.5)		0		200	0	0	0 (0.0)
	5000	100	0	0	0	0	0	0	-	0	91	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	Positive control (Cyclophos-phamide; 25)	100	19	11	3	0	0	29	***	0	23	100	0	0	0
		100	21	7	6	0	0	31		0		100	0	0	0
		200	40	18	9	0	0	60 (30.0)		0		200	0	0	0 (0.0)
4/48	Neg. contr. (serumfree cult. med.)	100	0	0	0	0	0	0		0	100	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	3000	100	0	0	1	0	0	1	-	0	117	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	1	0	0	1 (0.5)		0		200	0	0	0 (0.0)
	4000	100	0	0	0	0	0	0	-	0	93	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	5000	100	0	0	0	0	0	0	-	0	105	100	0	0	0
		100	0	0	0	0	0	0		0		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		0		200	0	0	0 (0.0)
	Positive c. (Cyclophos-phamide; 25)	100	7	1	0	0	0	8	***	0	112	100	0	0	0
		100	8	2	4	0	0	12		0		100	0	0	0
		200	15	3	4	0	0	20(10.0)		0		200	0	0	0 (0.0)

1) Gap(g) - total number of cells showing only gaps (chromatid-type and chromosome-type gaps).

2) Fisher's exact probability test (two-sided), - P>0.05, * P<0.05, ** P<0.01, *** P<0.001

Annexes

Annex 1 Preparation and characterization of Aroclor 1254-induced rat liver homogenate (batch of 25 September 2002)

The batch of S9 dated 25 September 2002, was prepared according to Ames et al. (1975) and Maron and Ames (1983) as follows.

Methods

Male Wistar rats, obtained from Charles River Deutschland, Sulzfeld, Germany were injected intraperitoneally with a single dose of Aroclor 1254 (nominal dose of 500 mg/kg body weight) in soya bean oil (20% w/v). The rats were provided with tap water and the Institute's stock diet ad libitum. Five days after the injection of Aroclor 1254 the rats were killed by CO_2 asphyxiation. The livers were removed aseptically and immediately put into a cold, sterile 0.15 M KCl solution. After washing in the KCl solution, the livers were weighed, cut into pieces and homogenized in 3 volumes of 0.15 M KCl solution in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for 10 minutes at 9,000 g. The supernatant, which is called S9, was collected and divided into small aliquots in sterile polypropylene vials. The vials were quickly frozen on dry ice and subsequently stored in a freezer at <-60 °C. The S9 was checked for sterility.
The protein and cytochrome P-450 content of the S9 fraction were determined according to the method published by Rutten et al. (1987).

Results

The protein content of the batch of S9 was 34.9 g/litre.
The cytochrome P-450 content of the batch of S9 was 35.7 μmol/litre.
The batch of S9 contained 1.02 μmol cytochrome P-450 per gram protein.
The sterility check of the batch of S9 resulted in 2 colonies per 100 μl S9.

Conclusion

The batch of S9 of 25 September 2002 meets all of the in-house quality criteria.

References

- Ames, B.N., J. McCann and E. Yamasaki "Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian microsome mutagenicity test." Mutation Res. 31 (1975) 347-365.
- Maron, D.M. and B.N. Ames "Revised methods for the Salmonella mutagenicity test." Mutation Res. 113 (1983) 173-215.
- Rutten, A.A.J.J.L., H.E. Falke, J.F. Catsburg, R. Topp, B.J. Blaauboer, I. van Holstein, L. Doorn and F.X.R. van Leeuwen "Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes. Reinvestigation of assay conditions." Arch. Toxicol. 61 (1987) 27-33.

Annex 2 Definition of chromosomal aberrations

Chromatid gap:	An achromatic lesion smaller than the width of one chromatid, and with minimal misalignment of the chromatid.
Chromatid break:	A breakage of one chromatid larger than the width of one chromatid, or a clear misalignment of a chromatid.
Chromatid exchange:	A breakage and reunion between chromatids from different chromosomes (interchange) or within a chromosome (intrachange; including interstitial deletion).
Chromosome gap:	An achromatic lesion at an identical site in both chromatids smaller than the width of one chromatid, and with minimal misalignment of the chromatids.
Chromosome break:	A breakage at an identical site of both chromatids larger than the width of one chromatid, or a clear misalignment of the chromatids (misalignment of the chromatids can result in cases where only the acentric fragment but not the shortened monocentric chromosome can be identified).
Chromosome exchange:	A breakage of both chromatids with a reunion between chromatids from different chromosomes (dicentric) or within a chromosome (ring).
Multiple aberrations:	A cell containing more than 10 chromosomal aberrations.
Polyploidy:	A cell containing a multiple of the haploid chromosome number (n) other than the diploid number (i.e., 3n, etc.).
Endoreduplication:	A cell in which after a S (synthesis) period of DNA replication, the nucleus did not go into mitosis but started another S period. The result is chromosomes with 4, 8, 16 or more chromatids.

References:

- Savage, Annotation: Classification and relationships of induced chromosome structural change. *J. Med. Gen.* 13, 103-122, 1975.
- Scott, D. Dean, B.J., Danford, N.D., and Kirkland, D.J. Metaphase chromosome aberration assays *in vitro*. In: Basic Mutagenicity Tests. UKEMS Recommended Procedures, editor D.J. Kirkland, Cambridge University Press, Report. Part 1 revised, pp. 62 - 86, 1990.

Annex 3 - Historical data of chromosomal aberration tests in cultured human lymphocytes

Historical negative controls

Studies started in 1989-2003: Overview from May, 2003.

Treatment/ harvest times	% of cells with aberrations (excl. the cells with only gaps) (number of assays)	
	medium or saline without S9-mix (range)	DMSO without S9-mix (range)
3-6/24 hours	0.0 - 1.5 (7)	0.5
24/24 hours	0.0 - 1.0 (11)	0.0 - 1.0 (9)
48/48 hours	0.0 - 0.5 (9)	0.0 - 0.5 (4)
all time points	0.0 - 1.5 (27)	0.0 - 1.0 (13)
	medium or saline with S9-mix (range)	DMSO with S9-mix (range)
3-6/24 hours	0.0 - 1.0 (11)	0.0-1.5 (9)
3-6/48 hours	0.0 - 0.0 (7)	0.0-1.0 (4)
all time points	0.0 - 1.0 (18)	0.0-1.5 (13)

Annex 3 - (continued) Historical data of chromosomal aberration tests in cultured human lymphocytes

Historical positive controls

Studies started in 1989-2003: Overview from May, 2003.

Treatment/ harvest times	Compound	% of cells with aberrations (excl. cells with only gaps) range (number of assays)	
		without S9-mix	
3-6/24 hours	mitomycin C: 0.1 μg/ml	27.0	(1)
24/24 hours	mitomycin C: 0.05 μg/ml	11.0 - 59.0	(12)
48/48 hours	mitomycin C: 0.05 μg/ml	13.5 - 30.0	(3)
4/24 hours	mitomycin C: 0.4 μg/ml	24.0 - 35.0	(5)
24/24 hours	mitomycin C: 0.2 μg/ml	14.0 - 40.5	(6)
4/24 hours	mitomycin C: 0.2 μg/ml	17.0 - 19.0	(2)
48/48 hours	mitomycin C: 0.2 μg/ml	37.5 - 63.0	(4)
48/48 hours	mitomycin C: 0.1 μg/ml	19.0	(1)
		with S9-mix	
3-6/24 hours	cyclophosphamide: 25 μg/ml	28.0 - 48.0	(8)
3-6/24 hours	cyclophosphamide: 20 μg/ml	30.0 - 36.0	(5)
3-6/48 hours	cyclophosphamide: 20 μg/ml	8.5 - 22.0	(2)
4/24 hours	cyclophosphamide: 15 μg/ml	12.5 - 27.5	(4)
4/48 hours	cyclophosphamide: 15 μg/ml	5.5 - 13.5	(2)
4/24 hours	cyclophosphamide: 30 μg/ml	18.5 - 28.0	(2)
4/48 hours	cyclophosphamide: 30 μg/ml	11.5 - 16.0	(3)
4/48 hours	cyclophosphamide: 30 μg/ml	5.5	(1)

Duplicate cultures

Statistically significant differences between duplicate cultures from negative and positive controls and from test substances in 3 different studies (studies 460040-001, 460040-002 and 470040/001) were evaluated by the Fisher's exact probability test (2-sided). In total 93 different duplicate treatments were evaluated, among which 3 were observed with statistically significant differences between duplicate cultures.

Reference

- TNO report V97.361 Historical data Genetic Toxicity tests, Revision 4, 2003.