



**CCR PROJECT 500811**

**Project ID of the contracting Institute: RCC 390497**

***IN VITRO***  
**CHROMOSOME ABERRATION ASSAY**  
**IN CHINESE HAMSTER V79 CELLS**  
**WITH**

**FAT 40'515/A**

**REPORT**

**Study Completion Date:**

**August 03, 1995**

**RCC**

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**Group**

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HESSISCHES MINISTERIUM  
FÜR UMWELT, ENERGIE  
UND BUNDESANGELEGENHEITEN

## GLP-Beschneigung

### Beschneigung

Hiermit wird bestätigt, daß die Prüfungseinrichtung(en)

Cytotest Cell Research GmbH & Co KG

In den Leppsteinswiesen 19

in 6101 Roßdorf

(Ort, Anschrift)

der RCC Holding Verwaltung GmbH

(Firma)

am 03.08., 04.08., 05.08. und 06.08.92

(Datum)

von der für die Überwachung zuständigen Behörde über die 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.

### Toxikologische Eigenschaften

Im Auftrag

*Dr. Hecker*  
(Dr. Hecker)

Wiesbaden, den 28.09.1992



### Certificate

It is hereby certified that the test facility(ies)

Cytotest Cell Research GmbH & Co KG

In den Leppsteinswiesen 19

in 6101 Roßdorf

(location, address)

of RCC Holding Verwaltung GmbH

(company name)

on 03.08., 04.08., 05.08. and 06.08.92

(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.

### Toxicological properties

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## PREFACE

### General

Sponsor: CIBA-GEIGY AG  
Produktesicherheit TF 5.31  
CH-4002 Basel  
Dr. B. Bruttel

Study Monitor: Mr. E. Rüdin,  
RCC Registration & Consulting Co

Testing Facility: C C R  
CYTOTEST CELL RESEARCH GMBH & Co. KG  
In den Leppsteinswiesen 19  
D-64380 Roßdorf

CCR Project No.: 500811

Contracting Institute: R C C  
REGISTRATION AND CONSULTING  
COMPANY LTD.  
CH-4452 Itingen; Switzerland

RCC Project No.: 390497

Test Article: FAT 40'515/A

Title: *In vitro* Chromosome Aberration Assay in  
Chinese Hamster  
V79 Cells with FAT 40'515/A

### Project Staff

Study Director: Dr. Andreas Czich

Management: Markus Arenz

Quality Assurance Unit: Frauke Hermann

### Schedule

Date of Protocol  
and German Translation: February 06, 1995

Start of Pre-Experiments: February 23, 1995

Start of Experiments: May 05, 1995

End of Experiments: July 20, 1995

Date of Draft: July 26, 1995

Date of Final Report: August 03, 1995

**Project Staff Signatures**

**Study Director**

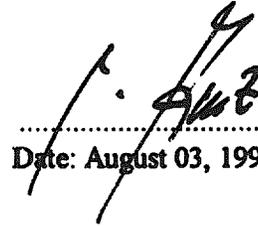
**Dr. Andreas Czich**



.....  
**Date: August 03, 1995**

**Management**

**Markus Arenz**



.....  
**Date: August 03, 1995**

## Quality Assurance

The study was performed in compliance with:

"Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated July 25, 1994 (BGBL. I 1994, S. 1703)."

"The OECD Principles of Good Laboratory Practice", Paris 1981.

## Guidelines

This study was conducted according to the procedures indicated by the following internationally accepted guidelines and recommendations:

First Addendum to the OECD Guideline for Testing of Chemicals, Section 4, No. 473, adopted May 26, 1983, "*In vitro* Mammalian Cytogenetic Test"

EEC Directive 92/69, L 383 A, Annex V, B 10, dated December 29, 1992.

Revised Chemical Substance Law (1987) according to the notification of December 9, 1986 by EA, Environmental Agency (no. 700); \*MHW, Ministry of Health and Welfare (No. 1039) and MITI, Ministry of International Trade and Industry (No. 1014), Japan.

## Archiving

C C R, D-64380 Roßdorf will archive the following data for 30 years:  
Raw data, protocol and copy of report.

A Sample of the test article will be archived for at least 2 years following the date on which the report is audited by the Quality Assurance Unit.

Microscopic slides will be archived for at least 12 years

No raw data or material relating to the study will be discarded without the sponsor's prior consent

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\* does not include dose selection

## Deviation to the Protocol

### 1) Positive Control

Name:	CPA; Cyclophosphamide
Supplier:	Aldrich Chemie, D-89555 Steinheim
Catalogue no.:	21870-7 (purity 98 %)
Dissolved in:	nutrient medium
Final Concentration:	0.47 µg/ml = 1.7 µM

Reason for the alteration: updating

### 2) The Test Article

CAS. No. was deleted as requested by the sponsor

## STATEMENT OF COMPLIANCE

Project Number: 500811  
Test Material : FAT 40'515/A  
Study Director: Dr. Andreas Czich  
Title: *In vitro* Chromosome Aberration Assay in Chinese Hamster  
V79 Cells with FAT 40'515/A

This study performed in the testing facility of C C R was conducted in compliance with Good Laboratory Practice Regulations.

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated July 25, 1994 (BGBL. I 1994 S. 1703)."

"The OECD Principles of Good Laboratory Practice", Paris 1981."

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

Study Director C C R  
Dr. Andreas Czich



Date: August 08, 1995

## QUALITY ASSURANCE UNIT

C C R, Cytotest Cell Research GmbH & Co. KG,  
In den Leppsteinswiesen 19  
D-64380 Roßdorf

### Statement

Project Number: 500811  
Test Material: FAT 40'515/A  
Study Director: Dr. Andreas Czich  
Title: *In vitro* Chromosome Aberration Assay in Chinese Hamster V79 Cells with FAT 40'515/A

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

Phases and Dates of QAU Inspections/ Audits	Dates of Reports to the Study Director and to Management
Protocol Audit: February 10, 1995	February 10, 1995
Process Inspection: May 09, 1995	May 09, 1995
Draft Audit: July 31, 1995 August 01, 1995	August 01, 1995

Quality Assurance Unit

Frauke Hermann

*F. Hermann*  
Date: August 08, 1995

## SUMMARY OF RESULTS

FAT 40'515/A, dissolved in DMSO, was assessed for its potential to induce structural chromosomal aberrations in V79 cells of the Chinese hamster *in vitro* in two independent experiments. The chromosomes were prepared 18 h and 28 h after start of treatment with the test article. The treatment interval was 4 h with metabolic activation, 18 h and 28 h without metabolic activation. In each experimental group two parallel cultures were set up. Per culture 100 metaphases were scored for structural chromosomal aberrations.

The concentration range for evaluation of the potential to induce structural aberrations had been determined in a pre-test using the XTT-assay and a qualitative evaluation of cell density and morphology as indicator for toxicity response. Dose selection was influenced by solubility of the test article. In the pre-test, toxic effects were observed in a concentration range from 30.0 - 100.0 µg/ml (without S9 mix) and 60.0 - 200.0 µg/ml with S9 mix. Precipitation was observed starting from 60 µg/ml in the absence of S9 mix and 100.0 µg/ml in the presence of S9 mix.

Table 1: Summary of results.

Experiment	S9 mix	Concentration µg/ml	Polyploid Cells	Mitotic index in %	Aberrant cells in % of control		
					incl gaps	excl. gaps	exchanges
I 18 h	-	5.0	1.5	81.8	14.5	13.5*	8.5
	-	30.0	3.0	25.9	24.0	20.5*	13.5
	-	50.0	1.5	17.0	30.0	27.5*	23.5
II 18 h	-	5.0	1.5	78.7	13.5	12.5*	8.0
	-	30.0	1.5	39.5	23.5	21.5*	13.5
	-	50.0	2.5	33.7	15.5	14.0*	8.0
I 28 h	-	10.0	2.0	80.3	16.0	12.0*	6.5
II 28 h	-	10.0	2.0	59.8	25.0	22.5*	16.0
I 18 h	+	5.0	1.0	120.9	2.0	2.0*	0.5
	+	30.0	2.5	114.9	2.5	2.5*	0.0
	+	50.0	1.0	106.0	5.0	3.5*	3.0
II 18 h	+	5.0	1.0	93.5	3.0	1.5	0.5
	+	30.0	1.0	116.8	8.0	5.5*	1.5
	+	50.0	2.0	39.1	18.5	16.0*	8.0
I 28 h	+	50.0	0.5	21.2	24.5	23.5*	17.5
II 28 h	+	30.0	0.0	125.7	2.0	0.5	0.0

\* statistically significant increases in aberration frequencies

In the absence of S9 mix, in both experiments the mitotic index was reduced after treatment with 30.0 and 50.0 µg/ml at the 18 h interval and after treatment with 10 µg/ml in experiment II at fixation interval 28 h. In the presence of S9 mix in both experiments the mitotic index was only reduced after treatment with 50.0 µg/ml in experiment II at the 18 h interval and in experiment I at the 28 h interval.

In both independent experiments, in most of the experimental groups, there were biologically relevant and statistically significant increases in cells with structural aberrations after treatment with the test article at both fixation intervals (with and without S9 mix).

In both experiments, no biologically relevant increase in the frequencies of polyploid metaphases was found after treatment with the test article compared to the frequencies of the controls.

Appropriate reference mutagens were used as positive controls and showed distinct increases in-cells with structural chromosomal aberrations.

### **Conclusion**

In conclusion, it can be stated that in the study described and under the experimental conditions reported, the test article induced structural chromosomal aberrations as determined by the chromosomal aberration test in the V79 cells (Chinese hamster cell line). Therefore, FAT 40'515/A is considered to be mutagenic in this chromosome aberration test.

## OBJECTIVE

### Aims of the Study

This *in vitro* assay was performed to assess the potential of the test article to induce structural chromosomal aberrations by means of two independent chromosomal aberration experiments in the Chinese hamster cell line V79.

### Reasons for the Study

*In vitro* methods are valuable when it is desirable to accurately control the concentration and exposure time of cells to the test article under study. However, due to the limited capacity for metabolic activation of potential mutagens an exogenous metabolic activation system is necessary.

This *in vitro* test is an assay for the detection of structural chromosomal aberrations. These aberrations are frequently lethal to the damaged cells. However, cytogenetic damage in somatic cells is an indicator of a potential to induce more subtle chromosomal damage that is compatible with cell division. Similar damage induced in germinal cells may lead to heritable cytogenetic abnormalities. Heritable cytogenetic abnormalities are known to have deleterious effects in man, e.g. induction of neoplastic events or birth defects.

The V79 cells were exposed to the test article both with and without exogenous metabolic activation. The cells were then harvested at sequential intervals and chromosome preparations were made. The stained preparations were examined and metaphase cells were scored for chromosomal aberrations.

Chromosomal aberrations are generally evaluated in first post treatment mitoses. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome type aberrations also occur.

The time at which the aberration frequency is at the maximum varies from agent to agent. Because different chemicals have effects at different parts of the cell cycle and V79 cultures are asynchronous, multiple post-treatment sample times are necessary to precisely define the response. Due to mitotic delay or metabolic and pharmacokinetic effects the appearance of the first post-treatment mitosis can be considerably delayed. Therefore samples taken at 18 h and 28 h after beginning of treatment cover the intervals in which maximum aberration frequency is expected.

For the assessment of clastogenic activity three concentrations were evaluated at the central sampling time of 18 h and one concentration at 28 h. The highest concentration should exhibit a cytotoxic effect, if possible.

To validate the test, reference mutagens were tested in parallel to the test article.

## MATERIALS AND METHODS

### Test Article

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

Name:	FAT 40'515/A
Batch No.:	94MN100
Aggregate State at RT:	solid powder
Colour:	red
Purity:	ca. 90 %
Stability Pure:	stable under storage conditions
Stability In solvent:	2 hrs in water, PEG CMC
Storage:	room temperature
Expiration Date:	February 1999

On the day of the experiment (immediately before treatment), the test article was dissolved in DMSO (E. MERCK, D-64293 Darmstadt; purity 99 % ). The solvent was chosen according to its solubility properties and its non-toxicity to the cells. The final concentration of DMSO in the culture medium did not exceed 1 % v/v.

## Controls

### Negative Controls

Concurrent negative (culture medium) and solvent controls (DMSO) were performed.

### Positive Control Substances

#### Without metabolic activation

Name: EMS; Ethylmethanesulfonate  
Supplier: Merck-Schuchardt, D-85662 Hohenbrunn  
Catalogue no.: 820774 (purity: > 98 %)  
Dissolved in: nutrient medium  
Final Concentration: 600 µg/ml = 4.8 mM

Solution prepared on day of experiment.

The stability of the positive control substance in solution was proven by the mutagenic response in the expected range.

#### With metabolic activation

Name: CPA; Cyclophosphamide  
Supplier: Aldrich Chemie, D-89555 Steinheim  
Catalogue no.: 21870-7 (purity 98 %)  
Dissolved in: nutrient medium  
Final Concentration: 0.47 µg/ml = 1.7 µM

The stability of CPA at room temperature is good. At 20 °C only 1 % of CPA is hydrolysed per day in aqueous solution.

## Test System

### Reasons for the Choice of the Cell Line V79

The V79 cell line has been used successfully for many years in *in vitro* experiments. Especially the high proliferation rate (doubling time of clone V79/T5 in stock cultures: 12 h, determined on April 17, 1994) and a high plating efficiency of untreated cells (as a rule more than 50 %) both necessary for the appropriate performance of the study, recommend the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22.

Lacking metabolic activities of cells under *in vitro* conditions are a disadvantage of assays with cell cultures as many chemicals only develop mutagenic potential when they are metabolized by the mammalian organism. However, metabolic activation of chemicals can be achieved at least partially by supplementing the cell cultures with liver microsome preparations (S9 mix).

### Cell Cultures

Large stocks of the V79 cell line (supplied by LMP, Technical University Darmstadt, D-64287 Darmstadt) were stored in liquid nitrogen in the cell bank of C C R allowing the repeated use of the same cell culture batch in experiments. Before freezing, each batch was screened for mycoplasma contamination and checked for karyotype stability. Consequently, the parameters of the experiments remained similar because of the reproducible characteristics of the cells.

Thawed stock cultures were propagated at 37 °C in 80 cm<sup>2</sup> plastic flasks (GREINER, D-72632 Frickenhausen). About 5 x 10<sup>5</sup> cells per flask were seeded in 15 ml of MEM (minimal essential medium; SEROMED; D-12247 Berlin) supplemented with 10 % fetal calf serum (FCS; Boehringer Mannheim, D-68305 Mannheim). The cells were subcultured twice weekly. The cell cultures were incubated at 37 °C in an atmosphere with 4.5 % carbon dioxide (95.5 % air).

### Mammalian Microsomal Fraction S9 Mix

The S9 liver microsomal fraction was obtained from the livers of 8 - 12 weeks old male rats, strain Wistar-HanIbm (BRL, CH-4414 Füllinsdorf; weight approx. 220 - 320 g) which received a single i.p. injection of 500 mg/kg b.w. Aroclor 1254 (Antechnika, D-76275 Karlsruhe) in olive oil 5 days previously.

After cervical dislocation the livers of the animals were removed, washed in 150 mM KCl and homogenized. The homogenate, diluted 1+3 with KCl was centrifuged twice at 9.000 g for 10 minutes (4 °C). A stock of the supernatant containing the microsomes was frozen in ampoules of 2 or 3 ml and stored at -70 °C. Small numbers of the ampoules were kept at -20 °C for only several weeks before use. The protein content was determined using the analysis kit of Bio-Rad Laboratories, D-80939 München: Bio-Rad protein assay, Catalogue No. 500 000 6.

The protein concentration in the S9 preparation is usually between 20 and 45 mg/ml. In both experiments the protein concentration was 26.2 mg/ml (Lot. No.: 191294).

### S9 Mix

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

8 mM MgCl<sub>2</sub>  
33 mM KCl  
5 mM glucose-6-phosphate  
4 mM NADP

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

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

## **Pre-Test for Toxicity**

A pre-test was performed in order to determine the toxicity of the test article. The general culturing and experimental conditions in this pre-test were the same as described below for the mutagenicity experiment.

The following two methods were used:

### **Qualitative assesment:**

In a qualitative assessment, high density cultures (approx. 200000 cells/slide) were treated with the test article for simulating the conditions of the main experiment. A qualitative evaluation of cell number and cell morphology was made 4 h and 18 - 20 h after start of treatment.

### **XTT-Assay:**

The XTT-assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by hydrogenase activity in active mitochondria. 18 - 20 h after treatment with the test article the XTT-assay was initiated by adding a mixture of XTT-labeling reagent with an electron coupling reagent (PMS). After 4 h of incubation the absorption was be read at 450 nm (690 nm reference) using an ELISA reader (SLT, Labinstruments Austria, A-5082 Grödig). The viabilities of the cells were calculated as percentages of the solvent controls and reported tabularly.

## Dose Selection

The highest concentration used in the pre-test (200.0 µg/ml) was limited by the solubility of the test article. Precipitation of the test article in the culture medium occurred at concentrations higher than 30.0 µg/ml (without S9 mix) and 60.0 µg/ml (with S9 mix). Toxic effects were observed in a concentration range from 30 - 200 µg/ml in the absence and 60.0 - 200.0 µg/ml in the presence of S9 mix.

As qualitative assessment of the cultures in the presence of S9 mix exhibited only slight toxic effects up to highest tested concentration, 200.0 µg/ml were chosen as top concentration in experiment I (with S9 mix). Due to the strong test article induced cytotoxicity in the absence of S9 mix, 80 µg/ml were chosen as top concentration in experiment I

Table 2: Investigated concentrations

Fixation interval	Experiment	Concentrations µg/ml					
		<b>without S9 mix</b>					
18 h	I	3.0	5.0	10.0	30.0	50.0*	80.0*
18h	II	5.0	10.0	30.0	50.0*	60.0*	70.0*
28h	I			10.0	30.0	50.0*	80.0*
28h	II			5.0	10.0	30.0	50.0*
		<b>with S9 mix</b>					
18 h	I	5.0	10.0	30.0	50.0	100.0*	200.0*
18 h	II	5.0	10.0	30.0	50.0	70.0*	90.0*
28 h	I			30.0	50.0	100.0*	200.0*
28 h	II			30.0	50.0	70.0*	90.0*

\* Precipitation of the test article

In cytogenetic experiment I and II, in the absence of S9 mix cultures after treatment with 50.0 µg/ml (18 h) and 10.0 µg/ml (28 h) as highest concentrations were evaluated for cytogenetic damage.

In the presence of S9 mix, in experiment I at both fixation intervals and in experiment II at fixation interval 18 h cultures treated with 50.0 µg/ml (18 and 28 h) as top concentrations could be evaluated. At the 28 h fixation interval in experiment II 30 µg/ml could be evaluated as top concentration.

The cytogenetic evaluation of higher concentrations in the respective intervals (with and without S9 mix) was impossible due to strong toxic effects (reduced cell density and/or low metaphase number, partially combined with poor metaphase quality).

## Experimental Performance

### Seeding of the Cultures

Exponentially growing stock cultures more than 50 % confluent were trypsinized at 37 °C for approximately 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium and a single cell suspension was prepared. The trypsin concentration was 0.2 % in Ca-Mg-free salt solution (Trypsin: Difco Laboratories, Detroit, USA).

The Ca-Mg-free salt solution was composed as follows (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose	1000 mg
NaHCO <sub>3</sub>	350 mg

Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution containing 200 mg/l EDTA (Ethylene diamine tetraacetic acid).

The cells were seeded into Quadriperm dishes (Heraeus, D-63450 Hanau) which contained microscopic slides (at least 2 chambers per dish and test group). In each chamber  $1 \times 10^4$  -  $6 \times 10^4$  cells were seeded with regard to preparation time. The medium was MEM + 10 % FCS (complete medium).

### Treatment

Exposure time 4 hours (with S9 mix):

In both independent experiments, after 48 h (28 h preparation interval) and 55 h (18 h preparation interval) the culture medium was replaced with serum-free medium containing different concentrations of the test article and 50 µl/ml S9 mix.

After 4 h the cultures were washed twice with "Saline G" and then the cells were cultured in complete medium for the remaining culture time.

The "Saline G" solution is composed as follows (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose	1100 mg
Na <sub>2</sub> HPO <sub>4</sub> · 7H <sub>2</sub> O	290 mg
KH <sub>2</sub> PO <sub>4</sub>	150 mg

pH is adjusted to 7.2

**Exposure time 18 and 28 hours (without S9 mix):**

In both independent experiments, after 48 h (28 h preparation interval) and 55 h (18 h preparation interval) the culture medium was replaced with complete medium (10 % FCS) containing different concentrations of the test article without S9 mix. This medium was not changed until preparation of the cells.

All cultures were incubated at 37 °C in a humidified atmosphere with 4.5 % CO<sub>2</sub> (95.5 % air).

**Preparation of the Cultures**

15.5 and 25.5 h after the start of the treatment colcemid was added (0.2 µg/ml culture medium) to the cultures. 2.5 h later, the cells were treated on the slides in the chambers with hypotonic solution (0.4 % KCl) for 20 min at 37 °C. After incubation in the hypotonic solution the cells were fixed with 3 + 1 methanol + glacial acetic acid. Per experiment both slides per group were prepared. After fixation the cells were stained with Giemsa (E. Merck, D-64293 Darmstadt).

**Analysis of Metaphase Cells**

Evaluation of the cultures was performed (according to standard protocol of the "Arbeitsgruppe der Industrie, Cytogenetik" (4)) using NIKON microscopes with 100x oil immersion objectives. Breaks, fragments, deletions, exchanges and chromosomal disintegrations were recorded as structural chromosomal aberrations. Gaps were recorded as well but not included in the calculation of the aberration rates. At least 100 well spread metaphases per culture were scored for cytogenetic damage on coded slides. Only metaphases with characteristic chromosome numbers of  $22 \pm 1$  were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined. In addition, the number of polyploid cells was scored (% polyploid metaphases; in the case of this aneuploid cell line polyploid means a near tetraploid karyotype).

**Data Recording**

The data generated were recorded in the raw data file. The results are presented in tabular form, including experimental groups with the test article, negative and positive controls.

**Acceptability of the Assay**

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

- a) The number of aberrations found in the negative and/or solvent controls falls within the range of historical laboratory control data: 0.00 % - 4.00 %.
- b) The positive control substances should produce significant increases of the number of cells with structural chromosomal aberrations.

## **Evaluation of Results**

A test article is classified as mutagenic if it induces reproducibly either a significant concentration-related increase in the number of structural chromosomal aberrations or a significant and reproducible positive response for at least one of the test points.

A test article producing reproducibly neither a significant concentration-related increase in the number of structural chromosomal aberrations nor a significant and reproducibly positive response at any one of the test points is considered non-mutagenic in this system.

This can be confirmed by means of the chi-square test. However, both biological and statistical significance should be considered together.

## DISCUSSION OF RESULTS

The test article FAT 40'515/A was assessed for its potential to induce structural chromosomal aberrations in V79 cells of the Chinese hamster *in vitro* in the absence and presence of metabolic activation by S9 mix. Two independent experiments were performed.

The chromosomes were prepared 18 h and 28 h after start of treatment with the test article, which was dissolved in DMSO. The treatment interval was 4 h with metabolic activation, 18 h and 28 h without metabolic activation.

In each experimental group two parallel cultures were set up. Per culture 100 metaphases were scored for structural chromosomal aberrations.

The highest concentration used in the pre-test was limited by the solubility of the test article. 200.0 µg/ml was chosen as highest concentration in the pre-test. In the absence of S9 mix strong toxic effects were observed in a concentration range from 30.0 - 200.0 µg/ml (see page 24 whereas in the presence of S9 mix toxic effects were observed from 60.0 - 100.0 µg/ml. At 200.0 µg/ml in the presence of S9 mix the relative value of surviving cell was increased due to strong precipitation of the red coloured test article.

Due to the strong cytotoxic effects of the test article in the absence of S9 mix, 80.0 µg/ml was chosen as top concentration in experiment I. As qualitative assessment of treated cultures in the presence of S9 mix revealed only slight toxic effects up to the highest tested concentration, 200.0 µg/ml was chosen as top concentration in experiment I. Following concentrations were evaluated:

Table 3: Evaluated concentrations

Fixation interval	Experiment	without S9 mix µg/ml			with S9 mix µg/ml		
18 h	I	5.0	30.0	50.0*	5.0	30.0	50.0
18 h	II	5.0	30.0	50.0*	5.0	30.0	50.0
28 h	I		10.0			50.0	
28 h	II		10.0			30.0	

\* precipitation was observed

In the absence of S9 mix, in both experiments the mitotic indices were reduced at the 18 h interval after treatment with 30.0 µg/ml (exp. I: 25.9 %; exp. II: 39.7 %) and 50.0 µg/ml (exp. I: 17.0 %; exp. II: 33.7 %) and in experiment II at fixation interval 28 h after treatment with 10 µg/ml (59.8 %). In the presence of S9 mix in both experiments the mitotic index was reduced only after treatment with 50.0 µg/ml at the 18 h interval in experiment II (39.1 %) and at the 28 h interval in experiment I (21.2 %).

At fixation intervals 18 and 28 h in the absence of S9 mix a reproducible significant and dose dependent increase in aberration frequencies was observed. At the 18 h interval cultures after treatment with 5.0, 30.0 and 50.0 µg revealed increased aberration frequencies in experiment I (13.5 %, 20.5 % and 27.5 %) as well as in experiment II (12.5 %, 21.5 % and 14.0 %). The slight decrease in the aberration frequency in experiment II after treatment with 50.0 µg/ml could be explained by precipitation of the test article at this experimental point. At the 28 h interval cultures after treatment with 10 µg/ml showed reproducible significantly increased aberration frequencies (exp. I 12.0 % and exp. II 22.5 %).

%). Additionally, at all experimental points in the absence of S9 mix number of cells carrying exchanges were distinctly increased in comparison to the solvent controls. The test article induced structural chromosome aberrations not only at concentrations which revealed strong cytotoxicity (30.0 µg/ml) and/or precipitation (50.0 µg/ml), but also at concentrations (5 µg/ml) which induced only slight reductions of the mitotic index.

In the presence of S9 mix in experiment I a statistically significant dose dependent increase in the aberration frequency was observed. At the 18 h interval test article concentrations of 5.0, 30.0 and 50.0 µg/ml induced aberration frequencies of 2.0, 2.5 and 3.5 %. These values are within our historical control data range (0.0 % - 4.0 %). Additionally, at 50 µg/ml an increase in cells carrying exchanges was observed (3.0 %). In conjunction with the results of experiment II, the results of experiment I at the 18 h interval must be regarded as biologically relevant.

In experiment II at the 18 h interval a dose dependent and statistically significant increase in aberration frequencies were observed. Test article concentrations of 5.0, 30.0 and 50.0 µg/ml induced aberration frequencies of 1.5, 5.5 and 16.0 %.

In experiment I at the 28 h interval in the presence of S9 mix 50 µg/ml induced 23.5 % aberrant cells. In experiment II only cultures after treatment with 30.0 µg/ml could be evaluated and exhibited no increase in the aberration frequency. Evaluation of higher concentrations within all experiments was impossible due to strong cytotoxic effects induced by the test article.

Tables 5 and 8 show the occurrence of polyploid metaphases. In both experiments, no biologically relevant increase in the rate of polyploid metaphases (exp. I: 1.0 % - 3.0 %; exp. II: 1.0 % - 2.5 %) as compared to the rates of the controls (exp. I: 1.0 % - 3.0 %; exp. II: 0.0 % - 2.5 %) were found after treatment with the test article.

In both experiments, EMS (0.6 mg/ml) and CPA (0.47 µg/ml) were used as positive controls and showed distinct increases in cells with structural chromosomal aberrations.

In conclusion, it can be stated that in the study described and under the experimental conditions reported, the test article FAT 40'515/A induced reproducibly structural chromosomal aberrations in the V79 cells (Chinese hamster cell line).

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## ANNEX: TABLES OF RESULTS

### Pre-Test for Toxicity

In the pre-test the toxicity of the test article was examined using the XTT-assay which is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by hydrogenase activity in active mitochondria.

Table 4: Cytotoxicity of FAT 40'515/A to cultures of Chinese hamster cell line V79.

Concentration per ml	S9 mix	XTT-absorption (OD 450/690 nm) (mean $\pm$ SD)	percentage of viable cells relative to the solvent control
negative control	-	1.503 $\pm$ 0.172	101.4
solvent control DMSO	-	1.485 $\pm$ 0.104	100.0
1.0 $\mu$ g	-	1.446 $\pm$ 0.103	96.9
3.0 $\mu$ g	-	1.414 $\pm$ 0.051	94.5
6.0 $\mu$ g	-	1.311 $\pm$ 0.079	86.6
10.0 $\mu$ g	-	1.362 $\pm$ 0.203	90.5
30.0 $\mu$ g	-	1.015 $\pm$ 0.114	63.8
60.0 $\mu$ g	-	0.974 $\pm$ 0.089	60.6
100.0 $\mu$ g	-	0.529 $\pm$ 0.056	26.4
200.0 $\mu$ g	-	0.549 $\pm$ 0.039	27.9
negative control	+	1.197 $\pm$ 0.054	109.2
solvent control DMSO	+	1.111 $\pm$ 0.101	100.0
1.0 $\mu$ g	+	1.121 $\pm$ 0.080	101.1
3.0 $\mu$ g	+	1.098 $\pm$ 0.065	98.7
6.0 $\mu$ g	+	1.147 $\pm$ 0.061	103.9
10.0 $\mu$ g	+	1.068 $\pm$ 0.065	95.5
30.0 $\mu$ g	+	1.082 $\pm$ 0.098	97.0
60.0 $\mu$ g	+	0.854 $\pm$ 0.101	72.8
100.0 $\mu$ g	+	0.710 $\pm$ 0.055	57.5
200.0 $\mu$ g	+	0.956 $\pm$ 0.040	83.6

## Experiment I

Table 5: Number of polyploid cells and mitotic index; fixation intervals 18 h and 28 h; with and without metabolic activation

	conc. per ml	S9 mix	fixation interval	polyploid cells*				mitotic index**			
				culture		total	mean	absolute		mean	%***
				1	2			1	2		
Neg. control	0 µg	-	18 h	3	3	6	3.0	11.1	15.8	13.5	100.0
Solv. control <sup>#</sup>	1 %	-	18 h	3	1	4	2.0	15.1	9.6	12.4	100.0
Pos. control <sup>###</sup>	600.0 µg	-	18 h	2	1	3	1.5	13.0	9.2	11.1	82.5
Test article	5.0 µg	-	18 h	2	1	3	1.5	11.4	8.8	10.1	81.8
"	30.0 µg	-	18 h	4	2	6	3.0	3.6	2.8	3.2	25.9
"	50.0 µg	-	18 h	1	2	3	1.5	2.6	1.6	2.1	17.0
Neg. control		+	18 h	1	1	2	1.0	16.5	17.6	17.1	100.0
Solv. control <sup>#</sup>	1 %	+	18 h	3	0	3	1.5	11.7	11.8	11.8	100.0
Pos. control <sup>###</sup>	0.47 µg	+	18 h	3	1	4	2.0	11.5	11.7	11.6	68.0
Test article	5.0 µg	+	18 h	1	1	2	1.0	15.6	12.8	14.2	120.9
"	30.0 µg	+	18 h	1	4	5	2.5	13.4	13.6	13.5	114.9
"	50.0 µg	+	18 h	2	0	2	1.0	15.5	9.4	12.5	106.0
Solv. control <sup>#</sup>	1 %	-	28 h	2	2	4	2.0	8.3	13.5	10.9	100.0
Test article	10.0 µg	-	28 h	3	1	4	2.0	9.0	8.5	8.8	80.3
Solv. control <sup>#</sup>	1 %	+	28 h	3	1	4	2.0	11.9	14.1	13.0	100.0
Test article	50.0 µg	+	28 h	0	1	1	0.5	2.8	2.7	2.8	21.2

\* The number of polyploid cells was determined in a sample of 100 cells per culture of each test group

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

\*\*\* For the positive control groups, the relative values of the mitotic index are related to the negative controls; for the test article treatment groups the values are related to negative controls or to the solvent controls in case the test article could not be dissolved in culture medium

# DMSO

## EMS

### CPA

## Abbreviations

g = gap, ig = iso-gap, gaps are acentromeric lesions of chromatid or chromosome type where no dislocation of chromosomal material is visible (independent of the size of the acentromeric region).

b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps] only exchanges are recorded additionally in these cells), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

Table 6: Structural chromosome aberrations Experiment I; fixation interval 18 h

slide no.	cells scored	% aberrant cells			aberrations													
		incl. gaps	excl. gaps	with ex-changes	gaps		chromatid type				chromosome type				other			
					g	ig	b	f	d	ex	ib	if	id	ex	ma	cd		
					without S9 mix													
Negative control					0	0	2	0	0	0	0	0	0	0	0	0	0	0
1	100				0	0	2	0	0	0	0	0	0	0	0	0		
2	100				0	0	0	1	0	0	0	0	0	0	0	0		
1+2	200	1.5	1.5	0.0	0	0	2	1	0	0	0	0	0	0	0	0		
Solvent control: DMSO 1 %					1	0	0	0	0	0	0	0	0	0	0	0	0	
1	100				1	0	0	0	0	0	0	0	0	0	0	0		
2	100				0	0	1	1	0	0	0	0	0	0	0	0		
1+2	200	1.5	1.0	0.0	1	0	1	1	0	0	0	0	0	0	0	0		
Positive control: EMS 600.0 µg / ml					2	0	0	2	0	17	1	0	0	3	0	0		
1	100				2	0	0	2	0	17	1	0	0	3	0	0		
2	100				3	0	5	0	0	14	0	0	0	0	0	0		
1+2	200	16.5	15.0	13.5	5	0	5	2	0	31	1	0	0	3	0	0		
Test article: 5.0 µg / ml					2	0	2	6	0	10	4	0	0	1	0	0		
1	100				2	0	2	6	0	10	4	0	0	1	0	0		
2	100				1	0	1	3	0	9	0	1	0	0	0	0		
1+2	200	14.5	13.5	8.5	3	0	3	9	0	19	4	1	0	1	0	0		
Test article: 30.0 µg / ml					9	2	7	2	0	14	2	0	0	0	0	0		
1	100				9	2	7	2	0	14	2	0	0	0	0	0		
2	100				4	2	3	5	0	17	1	1	0	0	1	0		
1+2	200	24.0	20.5	13.5	13	4	10	7	0	31	3	1	0	0	1	0		
Test article: 50.0 µg / ml					7	0	3	5	0	29	1	0	0	1	0	0		
1	100				7	0	3	5	0	29	1	0	0	1	0	0		
2	100				9	1	3	1	0	28	0	0	0	0	0	0		
1+2	200	30.0	27.5	23.5	16	1	6	6	0	57	1	0	0	1	0	0		
					with S9 mix													
Negative control					1	0	0	1	0	0	0	0	0	0	0	0	0	0
1	100				1	0	0	1	0	0	0	0	0	0	0	0		
2	100				0	0	1	0	0	1	0	0	0	0	0	0		
1+2	200	2.0	1.5	0.5	1	0	1	1	0	1	0	0	0	0	0	0		
Solvent control: DMSO 1 %					0	0	0	0	0	0	0	0	0	0	0	0	0	
1	100				0	0	0	0	0	0	0	0	0	0	0	0		
2	100				0	0	0	0	0	0	0	0	0	0	0	0		
1+2	200	0.0	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0		
Positive control: CPA 0.47 µg / ml					1	0	0	1	0	7	1	0	0	0	0	0		
1	100				1	0	0	1	0	7	1	0	0	0	0	0		
2	100				0	0	2	3	0	4	0	0	0	1	0	0		
1+2	200	8.5	8.0	6.0	1	0	2	4	0	11	1	0	0	1	0	0		
Test article: 5.0 µg / ml					0	0	2	1	0	0	0	0	0	0	0	0	0	
1	100				0	0	2	1	0	0	0	0	0	0	0	0		
2	100				0	0	1	0	0	1	0	0	0	0	0	0		
1+2	200	2.0	2.0	0.5	0	0	3	1	0	1	0	0	0	0	0	0		
Test article: 30.0 µg / ml					0	0	0	0	0	0	0	0	0	0	0	0	0	
1	100				0	0	0	0	0	0	0	0	0	0	0	0		
2	100				0	0	2	3	0	0	0	0	0	0	0	0		
1+2	200	2.5	2.5	0.0	0	0	2	3	0	0	0	0	0	0	0	0		
Test article: 50.0 µg / ml					2	0	0	0	1	3	0	0	0	0	0	0	0	
1	100				2	0	0	0	1	3	0	0	0	0	0	0		
2	100				1	0	0	0	0	3	0	0	0	0	0	0		
1+2	200	5.0	3.5	3.0	3	0	0	0	1	6	0	0	0	0	0	0		

Table 7: Structural chromosome aberrations Experiment I  
with and without S9 mix; fixation interval 28 h

slide no.	cells scored	% aberrant cells			aberrations												
		incl. gaps	excl. gaps	with ex-changes	gaps		chromatid type				chromosome type				other		
					g	ig	b	f	d	ex	ib	if	id	ex	ma	cd	
					without S9 mix												
Solvent control: DMSO 1 %					2	0	0	1	0	0	0	0	0	0	0	0	0
1	100				1	0	1	0	0	0	0	0	0	0	0	0	
2	100				3	0	1	1	0	0	0	0	0	0	0	0	
1+2	200	2.5	1.0	0.0													
Test article: 10.0 µg / ml					4	0	1	0	0	16	1	0	0	0	0	0	
1	100				6	0	1	8	0	7	0	1	0	0	0	0	
2	100				10	0	2	8	0	23	1	1	0	0	0	0	
1+2	200	16.0	12.0	6.5													
					with S9 mix												
Solvent control: DMSO 1 %					0	0	1	1	0	0	0	0	0	0	0	0	0
1	100				0	0	0	2	0	0	0	0	0	0	0	0	
2	100				0	0	1	3	0	0	0	0	0	0	0	0	
1+2	200	2.0	2.0	0.0													
Test article: 50.0 µg / ml					4	0	6	7	1	18	0	1	0	0	0	0	
1	100				0	0	3	2	0	25	1	0	0	0	1	0	
2	100				4	0	9	9	1	43	1	1	0	0	1	0	
1+2	200	24.5	23.5	17.5													

Abbreviations: g = gap; ig = iso-gap; b = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion;  
ma = multiple aberrations (= more than 5 events in one cell excluding gaps)  
ex = exchange; cd = chromosomal disintegration (= pulverization)

## Experiment II

Table 8: Number of polyploid cells and mitotic index; fixation intervals 18 h and 28 h; with and without metabolic activation

	conc. per ml	S9 mix	fixation interval	polyploid cells*				mitotic index**			
				culture		total	mean	absolute		mean	%***
				1	2			1	2		
Neg. control	0 µg	-	18 h	2	3	5	2.5	18.0	17.9	18.0	100.0
Solv. control <sup>#</sup>	1 %	-	18 h	1	3	4	2.0	15.9	14.1	15.0	100.0
Pos. control <sup>##</sup>	600.0 µg	-	18 h	2	3	5	2.5	13.6	10.0	11.8	65.7
Test article	5.0 µg	-	18 h	1	2	3	1.5	12.7	10.9	11.8	78.7
"	30.0 µg	-	18 h	0	3	3	1.5	6.2	5.7	6.0	39.7
"	50.0 µg	-	18 h	2	3	5	2.5	3.9	6.2	5.1	33.7
Neg. control		+	18 h	0	2	2	1.0	18.5	15.2	16.9	100.0
Solv. control <sup>#</sup>	1 %	+	18 h	1	0	1	0.5	13.7	14.2	14.0	100.0
Pos. control <sup>###</sup>	0.47 µg	+	18 h	3	1	4	2.0	10.8	9.2	10.0	59.3
Test article	5.0 µg	+	18 h	1	1	2	1.0	13.5	12.6	13.1	93.5
"	30.0 µg	+	18 h	0	2	2	1.0	16.4	16.2	16.3	116.8
"	50.0 µg	+	18 h	0	4	4	2.0	5.4	5.5	5.5	39.1
Solv. control <sup>#</sup>	1 %	-	28 h	1	2	3	1.5	13.3	12.1	12.7	100.0
Test article	10.0 µg	-	28 h	1	3	4	2.0	8.4	6.8	7.6	59.8
Solv. control <sup>#</sup>	1 %	+	28 h	0	1	1	0.5	11.0	13.9	12.5	100.0
Test article	30.0 µg	+	28 h	0	0	0	0.0	15.1	16.2	15.7	125.7

\* The number of polyploid cells was determined in a sample of 100 cells per culture of each test group

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

\*\*\* For the positive control groups, the relative values of the mitotic index are related to the negative controls; for the test article treatment groups the values are related to negative controls or to the solvent controls in case the test article could not be dissolved in culture medium

# DMSO

## EMS

### CPA

## Abbreviations

g = gap, ig = iso-gap, gaps are achromatic lesions of chromatid or chromosome type where no dislocation of chromosomal material is visible (independent of the size of the achromatic region).

b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps] only exchanges are recorded additionally in these cells), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

Table 9: Structural chromosome aberrations Experiment II; fixation interval 18 h

slide no.	cells scored	% aberrant cells			aberrations													
		incl. gaps	excl. gaps	with ex-changes	gaps		chromatid type				chromosome type				other			
					g	ig	b	f	d	ex	ib	if	id	ex	ma	cd		
without S9 mix																		
Negative control					2	0	1	0	0	0	0	0	0	0	0	0	0	0
1	100				0	0	0	0	0	0	0	0	0	0	0	0		
2	100				2	0	1	0	0	0	0	0	0	0	0	0		
1+2	200	1.5	0.5	0.0	2	0	1	0	0	0	0	0	0	0	0	0		
Solvent control: DMSO 1 %					0	0	0	0	0	0	0	0	0	0	0	0	0	
1	100				2	0	1	0	0	0	0	0	0	0	0	0		
2	100				2	0	1	0	0	0	0	0	0	0	0	0		
1+2	200	1.5	0.5	0.0	2	0	1	0	0	0	0	0	0	0	0	0		
Positive control: EMS 600.0 µg / ml					3	0	7	6	0	9	0	1	0	0	0	0	0	
1	100				2	0	1	4	0	15	1	0	0	0	0	1		
2	100				5	0	8	10	0	24	1	1	0	0	0	1		
1+2	200	19.0	17.0	9.5														
Test article: 5.0 µg / ml					1	0	0	3	0	11	0	0	0	0	0	0	0	
1	100				2	0	3	3	0	5	0	0	0	0	0	0		
2	100				3	0	3	6	0	16	0	0	0	0	0	0		
1+2	200	13.5	12.5	8.0														
Test article: 30.0 µg / ml					1	0	6	2	0	12	0	0	0	0	0	0	0	
1	100				4	0	5	7	0	15	3	0	0	0	2	0		
2	100				5	0	11	9	0	27	3	0	0	0	2	0		
1+2	200	23.5	21.5	12.5														
Test article: 50.0 µg / ml					3	0	3	5	0	12	1	0	0	0	0	0	0	
1	100				1	0	2	3	0	5	2	0	0	0	0	0		
2	100				4	0	5	8	0	17	3	0	0	0	0	0		
1+2	200	15.5	14.0	8.0														
with S9 mix																		
Negative control					4	0	2	1	0	0	0	1	0	0	0	0	0	
1	100				3	0	0	0	0	1	0	0	0	0	0	0		
2	100				7	0	2	1	0	1	0	1	0	0	0	0		
1+2	200	5.0	2.0	0.5														
Solvent control: DMSO 1 %					2	0	2	1	0	1	0	0	0	0	0	0	0	
1	100				1	0	0	0	0	0	0	0	0	0	0	0		
2	100				3	0	2	1	0	1	0	0	0	0	0	0		
1+2	200	3.5	2.0	0.5														
Positive control: CPA 0.47 µg / ml					4	0	2	2	0	3	0	0	0	0	0	0	0	
1	100				2	0	7	1	1	8	0	1	0	0	0	0		
2	100				6	0	9	3	1	11	0	1	0	0	0	0		
1+2	200	11.5	9.5	4.5														
Test article: 5.0 µg / ml					0	0	1	0	0	1	0	0	0	0	0	0	0	
1	100				3	0	1	0	0	0	0	0	0	0	0	0		
2	100				3	0	2	0	0	1	0	0	0	0	0	0		
1+2	200	3.0	1.5	0.5														
Test article: 30.0 µg / ml					2	0	3	0	0	1	0	0	0	0	0	0	0	
1	100				3	0	4	0	0	2	0	0	0	0	1	0		
2	100				5	0	7	0	0	3	0	0	0	0	1	0		
1+2	200	8.0	5.5	1.5														
Test article: 50.0 µg / ml					6	0	3	4	0	7	0	0	0	2	1	0		
1	100				1	0	4	6	0	8	2	0	0	0	1	0		
2	100				7	0	7	10	0	15	2	0	0	2	2	0		
1+2	200	18.5	16.0	8.0														

Table 10: Structural chromosome aberrations Experiment II  
with and without S9 mix; fixation interval 28 h

slide no.	cells scored	% aberrant cells			aberrations											
		incl. gaps	excl. gaps	with ex-changes	gaps		chromatid type				chromosome type				other	
					g	ig	b	f	d	ex	ib	if	id	ex	ma	cd
					without S9 mix											
Solvent control: DMSO 1 %																
1	100				2	0	0	0	0	0	0	0	0	0	0	0
2	100				1	0	0	1	0	0	0	0	0	0	0	0
1+2	200	2.0	0.5	0.0	3	0	0	1	0	0	0	0	0	0	0	0
Test article: 10.0 µg / ml																
1	100				1	0	1	2	0	20	1	0	0	1	4	0
2	100				4	0	0	3	0	22	4	0	0	0	0	0
1+2	200	25.0	22.5	16.0	5	0	1	5	0	42	5	0	0	1	4	0
					with S9 mix											
Solvent control: DMSO 1 %																
1	100				0	0	0	0	0	0	0	0	0	0	0	0
2	100				2	0	0	0	0	0	0	0	0	0	0	0
1+2	200	1.0	0.0	0.0	2	0	0	0	0	0	0	0	0	0	0	0
Test article: 30.0 µg / ml																
1	100				0	0	1	0	0	0	0	0	0	0	0	0
2	100				3	1	0	0	0	0	0	0	0	0	0	0
1+2	200	2.0	0.5	0.0	3	1	1	0	0	0	0	0	0	0	0	0

Abbreviations: g = gap; ig = iso-gap; b = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion;  
ma = multiple aberrations (= more than 5 events in one cell excluding gaps)  
ex = exchange; cd = chromosomal disintegration (= pulverization)

**BIOMETRY**

Statistical significance at the five per cent level ( $p < 0.05$ ) was evaluated by means of the chi-square test. Evaluation was performed only for cells carrying aberrations exclusive gaps.

**Experiment I**

	Solvent control versus	fixation interval	S9 mix	p-value
Test group	5.0 µg/ml	18 h	-	0.001 > p > 0.0*
"	30.0 µg/ml	18 h	-	0.001 > p > 0.0*
"	50.0 µg/ml	18 h	-	0.001 > p > 0.0*
"	5.0 µg/ml	18 h	+	0.5 > p > 0.25*
"	30.0 µg/ml	18 h	+	0.25 > p > 0.01*
"	50.0 µg/ml	18 h	+	0.01 > p > 0.001*
"	10.0 µg/ml	28 h	-	0.001 > p > 0.0*
"	50.0 µg/ml	28 h	+	0.001 > p > 0.0*
<b>Negative control versus Positive Control</b>				
EMS	600 µg/ml	18 h	-	0.001 > p > 0*
CPA	0.47 µg/ml	18 h	+	0.01 > p > 0*

**Experiment II**

	Solvent control versus	fixation interval	S9 mix	p-value
Test group	5.0 µg/ml	18 h	-	0.001 > p > 0.0*
"	30.0 µg/ml	18 h	-	0.001 > p > 0.0*
"	50.0 µg/ml	18 h	-	0.001 > p > 0.0*
"	5.0 µg/ml	18 h	+	n.c.
"	30.0 µg/ml	18 h	+	0.1 > p > 0.05
"	50.0 µg/ml	18 h	+	0.001 > p > 0.0*
"	10.0 µg/ml	28 h	-	0.001 > p > 0.0*
"	30.0 µg/ml	28 h	+	0.9 > p > 0.1
<b>Negative control versus Positive Control</b>				
EMS	600 µg/ml	18 h	-	0.001 > p > 0*
CPA	0.47 µg/ml	18 h	+	0.01 > p > 0.001*

n.c. = not calculated as the aberration rate is equal or lower than the control rate  
 \* aberration rate is statistically significantly higher than the control rate

Attached form 1: Result of Chromosomal Aberration Test ( Experiment I; fixation interval 16 h)

Treatment	Time (h)	S9 Mix	Conc.* µg/ml	** Number of cells observed	Number of Polyploidy	Number of percentage (%) of cells with aberrations**										
						Assessment	Chromatid aberration			Chromosome aberration		others	Total		Assessment	
							gap	ctb	cte	csb	cse		-g	+g		
Negative control	18	-	0	100	3		0	2	0	0	0	0	0	2	2	
				100	3		0	0	0	0	0	1	1	1		
				200	6	(3.0)	0	2	0	0	0	1	3	3		
Solvent vehicle DMSO ***	18	-	1%	100	3		1	0	0	0	0	0	0	1	1	
				100	1		0	1	0	0	0	1	2	2		
				200	4	(2.0)	1	1	0	0	0	1	2	3		
Positive control EMS ***	18	-	600.0	100	2		2	0	14	1	3	2	16	18		
				100	1		3	5	10	0	0	0	14	15		
				200	3	(1.5)	5	5	24	1	3	2	30	33	+	
Test article	18	-	5.0	100	2		2	1	7	4	1	6	15	16		
				100	1		1	1	9	0	0	4	12	13		
				200	3	(1.5)	3	2	16	4	1	10	27	29	+	
Test article	18	-	30.0	100	4		8	7	13	2	0	2	20	23		
				100	2		6	3	14	1	0	6	21	25		
				200	6	(3.0)	14	10	27	3	0	8	41	48	++	
Test article	18	-	50.0	100	1		7	3	21	1	1	3	27	29		
				100	2		9	3	25	0	0	1	27	31		
				200	3	(1.5)	16	6	46	1	1	4	54	60	++	
Negative control	18	+	0	100	1		1	0	0	0	0	1	1	2		
				100	1		0	1	1	0	0	0	2	2		
				200	2	(1.0)	1	1	1	0	0	1	3	4		
Solvent vehicle DMSO ***	18	+	1%	100	3		0	0	0	0	0	0	0	0		
				100	0		0	0	0	0	0	0	0	0		
				200	3	(1.5)	0	0	0	0	0	0	0	0		
Positive control CPA ***	18	+	0.47	100	3		1	0	7	1	0	1	8	9		
				100	1		0	2	4	0	1	2	8	8		
				200	4	(2.0)	1	2	11	1	1	3	16	17	+	
Test article	18	+	5.0	100	1		0	2	0	0	0	1	3	3		
				100	1		0	1	1	0	0	0	1	1		
				200	2	(1.0)	0	3	1	0	0	1	4	4		
Test article	18	+	30.0	100	1		0	0	0	0	0	0	0	0		
				100	4		0	2	0	0	0	3	5	5		
				200	5	(2.5)	0	2	0	0	0	3	3	5		
Test article	18	+	50.0	100	2		2	0	3	0	0	1	4	6		
				100	0		1	0	3	0	0	0	3	4		
				200	2	(1.0)	3	0	6	0	0	1	7	10	+	

Amount of S9 mix (5%); treatment time of the test substance: 4 h with S9 mix; 18 and 26 h without S9 mix; recovery time of cells after treatment with the test substance: 14 h and 24 h (with S9 mix); no recovery without S9 mix

\* Concentration of treatment - Fill in the value in order to beginning with low concentrations

\*\* Fill in the number of cells observed, polyploids and cells showing structural chromosomal aberrations per each plate in line 1+2, and the total in line 3, and the percentage (%) of cells showing aberrations in parenthesis with every concentration of treatment. Gap(g) - Fill in the total value of chromatid-type and chromosome-type. Sum total (-g) - Fill in the total number of cells except those which has only gaps. If there are many aberrations in one cell, calculate that cell as one cell showing aberrations. For example, if there are two breaks and two exchanges in one cell, calculate that cell as one showing aberrations, one showing breaks and one cell showing exchanges.

ctb: chromatid break cte: chromatid exchange csb: chromosome break cse: chromosome exchange others: fragmentation etc. (except pulverisation)

\*\*\* [-] - Fill in name of the solvent or the positive control

Attached form 2: Results of chromosomal Aberration Test ( Experiment I; fixation interval 20 h)

Treatment	Time (h)	SP Min	Conc.* µg/ml	**		Number of percentage (%) of cells with aberrations**											
				Number of cells observed	Number of Polyploidy	Assessment		gap		Chromatid aberration		Chromosome aberration		others	Total		Assessment
						g	Assessment	g	ctb	cte	cab	cae	-g		+g		
Solvent vehicle DMSO ***	28	-	1%	100	2		2	0	0	0	0	0	1	1	3		
				100	2		1	1	0	0	0	0	1	2			
				200	4	-	3	1	0	0	0	1	2	5			
				(2.0)	-	(1.5)	(0.5)	(0.0)	(0.0)	(0.0)	(0.5)	(1.0)	(2.5)	-			
Test article	28	-	10.0	100	3		4	1	8	1	0	0	10	13			
				100	1		5	1	5	0	0	9	14	19			
				200	4	-	9	2	13	1	0	9	24	32			
				(2.0)	-	(4.5)	(1.0)	(6.5)	(0.5)	(0.0)	(4.5)	(12.0)	(16.0)	+			
Solvent vehicle DMSO ***	28	+	1%	100	3		0	1	0	0	0	1	2	2			
				100	1		0	0	0	0	0	2	2	2			
				200	4	-	0	1	0	0	0	3	4	4			
				(2.0)	-	(0.0)	(0.5)	(0.0)	(0.0)	(0.0)	(1.5)	(2.0)	(2.0)	-			
Test article	28	+	50.0	100	0		4	6	15	0	0	7	25	27			
				100	1		0	3	20	1	0	3	22	22			
				200	1	-	4	9	35	1	0	10	47	49			
				(0.5)	-	(2.0)	(4.5)	(17.5)	(0.5)	(0.0)	(5.0)	(23.5)	(24.5)	++			

Attached form 3: Result of Chromosomal Aberration Test ( Experiment II; fixation interval 18 h)

Treatment	Time (h)	S9 Mix	Conc. * µg/ml	** Number of cells observed	Number of Polyploidy		Number of percentage (%) of cells with aberrations**								Assessment	
					Assessment	gap			Chromatid aberration		Chromosome aberration		others	Total		
						g	ctb	cte	csb	csc	-g	+g				
Negative control	18	-	0	100	2		2	1	0	0	0	0	1	3		
				100	3		0	0	0	0	0	0	0	0		
				200	5	(2.5)	2	1	0	0	0	0	0	1	3	
Solvent vehicle DMSO ***	18	-	1%	100	1		0	0	0	0	0	0	0	0		
				100	3		2	1	0	0	0	0	1	3		
				200	4	(2.0)	2	1	0	0	0	0	1	3		
Positive control EMS ***	18	-	600.0	100	2		3	7	7	0	0	5	16	18		
				100	3		2	1	12	1	0	4	18	20		
				200	5	(2.5)	5	8	19	1	0	9	34	38	+	
Test article	18	-	5.0	100	1		1	0	11	0	0	3	14	15		
				100	2		2	3	5	0	0	3	11	12		
				200	3	(1.5)	3	3	16	0	0	6	25	27	+	
Test article	18	-	30.0	100	0		1	6	11	0	0	2	18	18		
				100	3		4	4	14	3	0	9	25	29		
				200	3	(1.5)	5	10	25	3	0	11	43	47	++	
Test article	18	-	50.0	100	2		3	3	11	1	0	4	17	19		
				100	3		1	1	5	2	0	3	11	12		
				200	5	(2.5)	4	4	16	3	0	7	28	31	+	
Negative control	18	+	0	100	0		4	2	0	0	0	1	3	6		
				100	2		3	0	1	0	0	0	1	4		
				200	2	(1.0)	7	2	1	0	0	1	4	10	+	
Solvent vehicle DMSO ***	18	+	1%	100	1		2	2	1	0	0	1	4	6		
				100	0		1	0	0	0	0	0	0	1		
				200	1	(0.5)	3	2	1	0	0	1	4	7	-	
Positive control CPA ***	18	+	0.47	100	3		4	2	3	0	0	2	7	10		
				100	1		2	6	6	0	0	3	12	13		
				200	4	(2.0)	6	8	9	0	0	5	19	23	+	
Test article	18	+	5.0	100	1		0	1	1	0	0	0	2	2		
				100	1		3	1	0	0	0	0	1	4		
				200	2	(1.0)	3	2	1	0	0	0	3	6	-	
Test article	18	+	30.0	100	0		2	3	1	0	0	0	4	6		
				100	2		3	4	2	0	0	1	7	10		
				200	2	(1.0)	5	7	3	0	0	1	11	16	+	
Test article	18	+	50.0	100	0		6	3	6	0	2	5	13	17		
				100	4		1	3	8	2	0	7	19	20		
				200	4	(2.0)	7	6	14	2	2	12	32	37	+	

Amount of S9 mix (5%); treatment time of the test substance: 4 h with S9 mix; 18 and 26 h without S9 mix; recovery time of cells after treatment with the test substance: 14 h and 24 h (with S9 mix); no recovery without S9 mix

- \* Concentration of treatment - Fill in the value in order to beginning with low concentrations
- \*\* Fill in the number of cells observed, polyploids and cells showing structural chromosomal aberrations per each plate in line 1+2, and the total in line 3, and the percentage (%) of cells showing aberrations in parenthesis with every concentration of treatment. Gap(g) - Fill in the total value of chromatid-type and chromosome-type. Sum total (-g) - Fill in the total number of cells except those which has only gaps. If there are many aberrations in one cell, calculate that cell as one cell showing aberrations. For example, if there are two breaks and two exchanges in one cell, calculate that cell as one showing aberrations, one showing breaks and one cell showing exchanges.  
 ctb: chromatid break cte: chromatid exchange csb: chromosome break cse: chromosome exchange others: fragmentation etc, (except pulverisation)
- \*\*\* [] - Fill in name of the solvent or the positive control

Attached form 4: Results of chromosomal Aberration Test ( Experiment II; fixation interval 28 h)

Treatment	Time (h)	S9 Mix	Conc. ° µg/ml	** Number of cells observed	Number of Polyploidy		Number of percentage (%) of cells with aberrations**								
					Assessment	Chromatid aberration			Chromosome aberration		others	Total		Assessment	
						gap	cb	ctc	cb	cc		%	%		
Solvent vehicle DMSO ***	28	-	1%	100	1		2	0	0	0	0	0	0	2	
				100	2		3	0	0	0	0	1	1	2	
				200	3		3	0	0	0	0	1	1	4	
				(1.5)	-	(1.5)	(0.0)	(0.0)	(0.0)	(0.0)	(0.5)	(0.5)	(2.0)	-	
Test article	28	-	10.0	100	1		1	1	14	1	1	6	22	23	
				100	3		4	0	17	4	0	3	23	27	
				200	4		5	1	31	5	1	9	45	50	
				(2.0)	-	(2.5)	(0.5)	(16.0)	(2.5)	(0.5)	(4.5)	(22.5)	(25.0)	-	
Solvent vehicle DMSO ***	28	+	1%	100	0		0	0	0	0	0	0	0	0	
				100	1		2	0	0	0	0	0	0	2	
				200	1		2	0	0	0	0	0	0	2	
				(0.5)	-	(1.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(1.0)	-	
Test article	28	+	30.0	100	0		0	1	0	0	0	0	1	1	
				100	0		3	0	0	0	0	0	0	3	
				200	0		3	1	0	0	0	0	1	4	
				(0.0)	-	(1.5)	(0.5)	(0.0)	(0.0)	(0.0)	(0.0)	(0.5)	(2.0)	-	

**Report of Results of Chromosomal Aberration Test Report in Cultured Mammalian Cells (V79)**

**1. General Items**

Name of the new chemical substance IUPAC nomenclature							
Other name	Physico-chemical properties	Molecular weight					
Structural formula or rational method, in case both are unknown) C <sub>15</sub> H <sub>17</sub> N <sub>6</sub> O <sub>5</sub> Cl		Appearance at ordinary room temperature					
		Stability					
		Melting point	°C				
Purity of the new chemical substance tested		Wt %	Boiling point	°C			
		Name and concentration of impurities	Wt %	Vapour pressure			
				Partition coefficient	°C		
				Solubility Degree of solubility	Water		
		DMSO					
		Ethanol					
Others ( )							

[Remarks] Because physicochemical properties are reference materials, fill in spaces to extent possible

1. "Stability" - Fill in the stability for water, other solvents, heat, light, etc.
2. "Vapor pressure" - Fill in the vapor pressure of the test substance at 25 °C
3. "Partition coefficient" Fill in the value, the temperature used and the name of the solvent used for the measurement
4. "Solubility" - Fill in such information as water-soluble, soluble in oil
5. "Degree of solubility" - Fill in the solubility at 25 °C for each solvent

**2. Kind of a Cell Line - Culture condition**

Name of cell line	V79 cells	Obtained from LMP Darmstadt Date obtained April 21, 1987		
Species	Chinese Hamster			
Culture medium	MEM	Manufacturer Seromed, D-12247 Berlin		
Serum	Fetal Calf Serum 10 %	Manufacturer (Lot No.) Boehringer Mannheim (147 18402) Date obtained December 12, 1994 D-68261 Mannheim.		
Doubling Time	12 - 16 h	Freezing condition at -196°C in liquid nitrogen		
Passage No.	for each experiment a freshly thawed cell batch will be used	Culture condition	Vessel	Plastic flask 80 cm <sup>2</sup>
Number of chromosomes (mode)	22		Temperature	37 °C
			CO <sub>2</sub>	4.5 %
Remarks				

3. S9 Mix

(1) Source of S9 (encircle the applicable number and fill in the relevant entries)

1. Made in house <input type="checkbox"/>	Prepared on	December 19, 1995	(date)
	Supplier		
2. Purchase <input type="checkbox"/>	Prepared on		(date)
	Purchased on		(date)
	Lot No.		

(2) Storage temperature, etc. of S9

Storage temperature	- 80 °C	Name and model of storage apparatus	GFL Type 6475
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(3) Preparation of S9 (if purchased material, fill in spaces to extent possible)

Animal used		Inducing substance	
Species, Strain	Wistar rats, Hanlbrn	Name	Aroclor 1254
Sex	male	Administration method	i. p. injection
Age (in weeks)	8-12 weeks	Administration period	single dose of 500 mg/kg b.w.
Weight	220 - 320 g	and amount (mg/kg-weight)	5 days prior to S9 preparation

(4) Composition of S9 Mix

Constituents	Amount in 1 ml S9 Mix	Constituents	Amount in 1 ml S9 Mix
S9	0.57 ml	NADPH	/ $\mu\text{mol}$
MgCl <sub>2</sub>	8 $\mu\text{mol}$	NADH	/ $\mu\text{mol}$
KCl	33 $\mu\text{mol}$	Na-phosphate buffer (pH 7.4)	100 $\mu\text{mol}$
Glucose-6-phosphate	5 $\mu\text{mol}$	Others: NADP	4 $\mu\text{mol}$

(5) Treatment condition of S9 Mix (encircle the applicable number and fill in the relevant entries)

	1. Plate method <input type="checkbox"/>	2. Floating cell method <input type="checkbox"/>
Amount of S9 mix (Concentration)	5 %	/
Amount of S9 protein (Final concentration)	0.75 mg/ml	/
Treatment time	4 h	/
Culture time after treatment with test substance	14 and 24 h, respectively	/
Remarks		

4. Cell Growth Inhibition Test / Cell Metabolism (XTT Assay)

(1) Test condition and preparation of the solution of the test substance

Period of experiment		from April 11, 1995	to April 12, 1995
		without metabolic activation	with metabolic activation
Cell	Number of seeded cells	approx. $2 \times 10^5$ cells/well	approx. $2 \times 10^5$ cells/well
	Days after initiation of cult.	1 day	1 day
Plate	Form	96 well plate	96 well plate
	Size	surface: $0.32 \text{ cm}^2$ ; volume: $380 \mu\text{l}$	surface: $0.32 \text{ cm}^2$ ; volume: $380 \mu\text{l}$
	Manufacturer	Greiner D-72632 Frickenhausen	Greiner D-72632 Frickenhausen
	Number of plates for each concentration	8	8
	Volume of culture medium	$100 \mu\text{l/well}$	$100 \mu\text{l/well}$
Preparation of solution of the test article	Kind of solvent	DMSO	DMSO
	Concentration of the original solution of the test article		
	Amount of the test article	20.0 mg/ml	20.0 mg/ml
	Volume of the solvent	12 ml	12 ml
	Condition of the solution of the test article (encircle)	dissolved <input type="checkbox"/> suspended <input type="checkbox"/> others: -	dissolved <input type="checkbox"/> suspended <input type="checkbox"/> others: -
	Time after preparation	10 - 15 minutes	10 - 15 minutes
	Method of preservation	not preserved	not preserved
	Method of sterilization	sterile filtration	sterile filtration
Treatment of the test article	Added volume of the prepared solution	DMSO: 1 % in culture medium	DMSO: 1 % in culture medium
	Period of treatment	20 h	4 h
	Added volume of S9 Mix		0.25 ml/chamber
Method of Cytotoxicity Estimation		The XTT-assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by hydrogenase activity in active mitochondria. 18 - 20 h after treatment with the test article the XTT-assay was initiated by adding a mixture of XTT-labeling reagent with an electron coupling reagent (PMS). After 4 h of incubation the absorption was read at 450 nm (690 nm reference) using an ELISA reader (SLT, Labinstruments Austria, A-5082 Grödig). The viabilities of the cells were calculated as percentages of the solvent controls and reported tabularly.	
Remarks			

(2) XTT-absorbtion, percentage of viable cells

	Concentration (µg/ml)	XTT-absorption (OD 450/690 nm mean ± SD)	percentage of viable cells* relative to the solvent control
without metabolic activation system	Blank	0.168 ± 0.010	
	Negative control	1.197 ± 0.054	109.20
	Solvent (MEM)	1.111 ± 0.101	100.00
	1.0	1.121 ± 0.080	101.12
	3.0	1.098 ± 0.065	98.66
	6.0	1.147 ± 0.061	103.86
	10.0	1.068 ± 0.065	95.46
	30.0	1.082 ± 0.098	96.92
	60.0	0.854 ± 0.101	72.80
	100.0	0.710 ± 0.055	57.46
	200.0	0.956 ± 0.040	83.60
without metabolic activation system	Blank	0.186 ± 0.040	
	Negative control	1.503 ± 0.172	101.37
	Solvent (MEM)	1.485 ± 0.104	100.00
	1.0	1.446 ± 0.103	96.94
	3.0	1.414 ± 0.051	94.49
	6.0	1.311 ± 0.079	86.62
	10.0	1.362 ± 0.203	90.51
	30.0	1.015 ± 0.114	63.76
	60.0	0.974 ± 0.089	60.62
	100.0	0.529 ± 0.056	26.41
	200.0	0.549 ± 0.039	27.90

\* = corrected for blank

5. Chromosomal aberration test

(1) Test condition and preparation of the solution of the test article in experiment I

Period of experiment		from May 10, 1995	to June 20, 1995
		without metabolic activation	with metabolic activation
Cell	Number of seeded cells	between 1.0 - 6.0 x 10 <sup>4</sup> cells/chamber	between 1.0 - 6.0 x 10 <sup>4</sup> cells/chamber
	Days after initiation of cult.	48 hours	48 hours
Plate	Form	Quadriperm dishes	Quadriperm dishes
	Size	4 chambers/dish: 76 x 26 mm each	4 chambers/dish: 76 x 26 mm each
	Manufacturer	Heraeus D-63450 Hanau	Heraeus D-63450 Hanau
	Number of chambers for each concentration	4	4
	Volume of culture medium	5 ml/chamber	5 ml/chamber
Preparation of solution of the test article	Kind of solvent	DMSO	DMSO
	Concentration of the original solution of the test subst	20.0 mg/ml	20.0 mg/ml
	Amount of the test article	240 mg	240 mg
	Volume of the solvent	12 ml	12 ml
	Condition of the solution of the test article (encircle)	dissolved <input type="checkbox"/> suspended <input type="checkbox"/> others: -	dissolved <input type="checkbox"/> suspended <input type="checkbox"/> others: -
	Time after preparation	10 - 15 minutes	10 - 15 minutes
	Method of preservation	not preserved	not preserved
	Method of sterilization	sterile filtration	sterile filtration
Treatment of the test article	Added volume of the prepared solution	DMSO: 1 % in culture medium 5 ml culture medium/flask	DMSO: 1 % in culture medium 5 ml culture medium/flask
	Period of treatment	18 h, 28 h	4 h
	Added volume of S9 Mix		0.25 ml/chamber
Mitotic inhibitor	Name	colcemide	colcemide
	Concentration	0.2 µg/ml	0.2 µg/ml
	Period of treatment	2.5 h	2.5 h
Method of cell preparation, fixation and staining		Preparation of cultures was done as follows: Colcemide: 2.5 h before preparation Treatment of the cells with hypotonic solution (0.4 % KCl) for 20 min. (37° C); fixation with methanol: glacial acetic acid 3+1; preparation of metaphases by flame spreading on the microscope slides; staining with giemsa; determination of the mitotic index by scoring of 1000 cells per slide	
Remarks	Cells were seeded on sterilized microscopic slides in Quadriperm dishes (4 chambers/dish). Preparation and spreading of the metaphases was done on the slides on which the cells were seeded.		

[Note] "Method of cell preparation" - Fill in the method of sample preparation (method of preparation, fixation, staining, determination).

**(2) Rate of mitotic activity**

The mitotic activity in negative control group is 100 %, and fill in the descending order of the test article; experiment I

	fixation interval (h)	Concentration of the test article ( 20.0 mg/ml )	mitotic activity ( % )
without metabolic activation system	18	solvent control DMSO	100.0
		5.0	81.8
		30.0	25.9
		50.0	17.0
with metabolic activation system	18	solvent control DMSO	100.0
		5.0	120.9
		30.0	114.9
		50.0	106.0
without metabolic activation system	28	solvent control DMSO	100.0
		10.0	80.3
with metabolic activation system	28	solvent control DMSO	100.0
		50.0	21.2

**(3) Test results of experiment I**

Test results are shown on the attached forms 1 and 2.

(4) Test condition and preparation of the solution of the test article in experiment II

Period of experiment		from May 31, 1995	to July 26, 1995
		without metabolic activation	with metabolic activation
Cell	Number of seeded cells	between $1.0 - 6.0 \times 10^4$ cells/chamber	between $1.0 - 6.0 \times 10^4$ cells/chamber
	Days after initiation of cult.	48 hours	48 hours
Plate	Form	Quadriperm dishes	Quadriperm dishes
	Size	4 chambers/dish: 76 x 26 mm each	4 chambers/dish: 76 x 26 mm each
	Manufacturer	Heraeus D-63450 Hanau	Heraeus D-63450 Hanau
	Number of chambers for each concentration	4	4
	Volume of culture medium	5 ml/chamber	5 ml/chamber
Preparation of solution of the test article	Kind of solvent	DMSO	DMSO
	Concentration of the original solution of the test subst		
	Amount of the test article	10.0 mg/ml	10.0 mg/ml
	Volume of the solvent	200 mg	200 mg
	Condition of the solution of the test article (encircle)	dissolved o suspended o others:	dissolved o suspended o others: -
	Time after preparation	10 - 15 minutes	10 - 15 minutes
	Method of preservation	not preserved	not preserved
	Method of sterilization	sterile filtration	sterile filtration
Treatment of the test article	Added volume of the prepared solution	DMSO: 1 % in culture medium ???? 5 ml culture medium/flask	DMSO: 1 % in culture medium ???? 5 ml culture medium/flask
	Period of treatment	18 h, 28 h	4 h
	Added volume of S9 Mix	/	0.25 ml/chamber
Mitotic inhibitor	Name	colcemide	colcemide
	Concentration	0.2 µg/ml	0.2 µg/ml
	Period of treatment	2.5 h	2.5 h
Method of cell preparation, fixation and straining	Preparation of cultures was done as follows: Colcemide: 2.5 h before preparation Treatment of the cells with hypotonic solution (0.4 % KCl) for 20 min. (37° C); fixation with methanol: glacial acetic acid 3+1; preparation of metaphases by flame spreading on the microscope slides; staining with giemsa; determination of the mitotic index by scoring of 1000 cells per slide		
Remarks	Cells were seeded on sterilized microscopic slides in Quadriperm dishes (4 chambers/dish). Preparation and spreading of the metaphases was done on the slides on which the cells were seeded.		

[Note] "Method of cell preparation" - Fill in the method of sample preparation (method of preparation, fixation, staining, determination).

**(5) Rate of mitotic activity**

(The mitotic activity in negative control group is 100 %, and fill in the descending order of the test article; experiment I

	fixation interval (h)	Concentration of the test article (10.0 mg/ml)	mitotic activity (%)
without metabolic activation system	18	solvent control DMSO	100.0
		5.0	78.7
		30.0	39.7
		50.0	33.7
with metabolic activation system	18	solvent control DMSO	100.0
		5.0	93.5
		30.0	116.8
		50.0	39.1
without metabolic activation system	28	solvent control DMSO	100.0
		10.0	59.8
with metabolic activation system	28	solvent control DMSO	100.0
		30.0	125.7

**(6) Test results of experiment II**

Test results are shown on the attached forms 3 and 4.

(7) Judgement of the results

Judgement (encircle one)	positive $\sigma$	negative $\sigma$
Reason for judgement		
D20 - value* 0.018 mg/ml (with S9 mix) 0.055 mg/ml (without S9 mix)	Metabolic activation with $\sigma$ without $\sigma$	Experiment I, 18 h: Experiment I, 28 h: Experiment II, 18 h: Experiment II, 28 h:

\* Concentration (mg/ml) of the test article where 20 % metaphases show structural chromosome aberrations.

(8) Referential matters

In conclusion, it can be stated that in the study described and under the experimental conditions reported, the test article FAT 40'515/A induced reproducibly structural chromosomal aberrations in the V79 cells (Chinese hamster cell line).

[Remark] "Referential matters" - Fill in the view etc. of the Study Director on the test results.

6. Others

Testing institution	Name	CCR Cytotest Cell Research GmbH & Co. KG
	Address	In den Leppsteinswiesen 19, D-64380 Roßdorf, F.R.G.; Tel. 06154 / 807-0
Study Director	Name / Title	Dr. Andreas Czich
Test dates	from: February 23, 1995	to: August 03, 1995