


**STATEMENT**

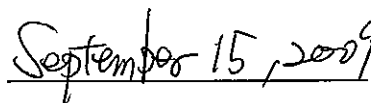
**TITLE OF STUDY**

Chromosomal Aberration Test of 13F-SFMA Using Cultured Mammalian Cells (Study Code: K06-1190)

I, the undersigned, hereby declare that this report provides a correct English translation of the final report (Study Code: K06-1190, issued on March 4, 2007) audited by Quality Assurance Unit of CERI Hita, Chemicals Evaluation and Research Institute, Japan.



Makoto Nakai  
CERI Hita



Date

Chemicals Evaluation and Research Institute, Japan



Receipt No.837-06-T-5257

STUDY CODE: K06-1190

## **FINAL REPORT**

### **CHROMOSOMAL ABERRATION TEST OF 13F-SFMA USING CULTURED MAMMALIAN CELLS**

March 2007

CERI Hita  
Chemicals Evaluation and Research Institute, Japan

**GLP STATEMENT**

CERI Hita  
Chemicals Evaluation and Research Institute, Japan

Sponsor: DAIKIN INDUSTRIES, LTD

Title: Chromosomal Aberration Test of 13F-SFMA Using Cultured Mammalian  
Cells

Study Code: K06-1190

I, the undersigned, hereby declare that this study was conducted in compliance with “Concerning Standard of the Testing Facilities Conducting the Test Relating to the New Chemical Substances” on Japanese GLP (Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau, MHLW, No. 3 (November 17, 2003) of the Manufacturing Industries Bureau, METI & No. 031121004 of the Environmental Health Department, MOE (November 21, 2003)) and “OECD Principles of Good Laboratory Practice” (November 26, 1997).

I also confirmed that this report accurately reflected the raw data and the test data were valid.

Study Director: Signed in original March 14, 2007  
Syozo Ajimi

**QUALITY ASSURANCE STATEMENT**

CERI Hita

Chemicals Evaluation and Research Institute, Japan

Sponsor: DAIKIN INDUSTRIES, LTD.Title: Chromosomal Aberration Test of 13F-SFMA Using Cultured Mammalian CellsStudy Code: K06-1190

This study was inspected by Quality Assurance Unit of CERI Hita, Chemicals Evaluation and Research Institute, Japan. The dates inspected and the dates reported these results to the study director and management are as follows.

Phase	Date of Inspection	Date Reported to Study Director and Management
Protocol	December 12, 2006	December 12, 2006
Preparation of Test Substance	December 13, 2006	December 13, 2006
Treatment of Cells	December 13, 2006	December 13, 2006
Reinspection of Protocol	December 22, 2006	December 22, 2006
Protocol Amendment	December 22, 2006	December 22, 2006
Protocol Amendment (No. 2)	January 19, 2007	January 19, 2007
Reinspection of Protocol Amendment (No. 2)	January 26, 2007	January 26, 2007
Protocol Amendment (No. 3)	January 26, 2007	January 26, 2007
Reinspection of Protocol Amendment (No. 3)	February 1, 2007	February 1, 2007
Protocol Amendment (No. 4)	February 1, 2007	February 1, 2007
Protocol Amendment (No. 5)	February 15, 2007	February 15, 2007
Raw Data and Draft Final Report	March 11, 2007	March 12, 2007
Reinspection of Raw Data and Draft Final Report	March 12, 2007	March 12, 2007
Draft Final Report (Second Time)	March 12, 2007	March 12, 2007
Reinspection of Draft Final Report (Second Time)	March 13, 2007	March 13, 2007
Final Report	March 14, 2007	March 14, 2007

The inspection result of following phase was reported to the study director and management based on the report of process-based inspection relevant to this study type and timeframe.

Phase	Date of Inspection	Date Reported to Study Director and Management
Preparation and Management of Positive Control Substance	November 24, 2006	March 13, 2007
Preparation of Medium and Reagent	December 6 and 7, 2006	March 13, 2007
Cell Pre-culture	November 27, 2006	March 13, 2007
Collection of Cells and Preparation of Specimens	December 5, 2006	March 13, 2007

I, the undersigned, hereby declare that this report provides an accurate description of the methods and procedures used in this study and that the reported results accurately reflect obtained raw data.

Head, Quality Assurance Unit: Signed in original March 14, 2007  
Ryuichiro Mizuguchi

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Study Code: K06-1190

Test Substance Code: HR6852

Sponsor Code: D-0060

**TITLE**

Chromosomal Aberration Test of 13F-SFMA Using Cultured Mammalian Cells

**SPONSOR**

DAIKIN INDUSTRIES, LTD.

1-1, Nishi-Hitotsuya, Settsu, Osaka 566-8585, Japan

**TESTING FACILITY**

CERI Hita

Chemicals Evaluation and Research Institute, Japan

822, 3-chome, Ishii-machi, Hita, Oita 877-0061, Japan

**PURPOSE OF STUDY**

The ability of the test substance to induce chromosomal aberrations was examined by using Chinese hamster lung fibroblasts (CHL/TU cells).

**TESTING METHOD**

This study was conducted in accordance with “III Mutagenicity Test: Chromosomal Aberration Test Using Cultured Mammalian Cells” prescribed in “Concerning Testing Methods Relating to the New Chemical Substances” on Japanese Test Guideline (Notification No. 1121002 of the Pharmaceutical and Food Safety Bureau, MHLW, No. 2 (November 13, 2003) of the Manufacturing Industries Bureau, METI & No. 031121002 of the Environmental Health Department, MOE (November 21, 2003)).

**GLP COMPLIANCE**

This study was conducted in compliance with “Concerning Standard of the Testing Facilities Conducting the Test Relating to the New Chemical Substances” on Japanese GLP (Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau, MHLW, No. 3 (November 17, 2003) of the Manufacturing Industries Bureau, METI & No. 031121004 of the Environmental Health Department, MOE (November 21, 2003)) and “OECD Principles of Good Laboratory Practice” (November 26, 1997).



## PERIOD OF STUDY

Commencement of Study:	December 8, 2006
Initiation of Experiment (Initiation of Cell Growth Inhibition Test):	December 13, 2006
Completion of Experiment (Completion of Observation of Specimens):	February 27, 2007
Completion of Study:	March 14, 2007

## STORAGE AND RETENTION PERIOD OF DATA

The raw data, protocol, protocol amendment, letter of test request, table of test substance information, final report, other record documents and specimens will be stored in the archive of this testing facility for 10 years after the date of receipt of the notification that they are applicable to Article 4, Paragraphs 1 or 2, Article 4-2, Paragraphs 2, 3 or 8, Article 5-4, Paragraph 2, Article 24, Paragraph 2 or Article 25-3, Paragraph 2 of the Japanese Chemical Substances Control Law No. 117 (1973). The sponsor will inform the date of receipt of the notification to the testing facility. After termination of the retention period, any measures taken will be done so with the approval of the sponsor.

The specimens to which the quality will be deteriorated will be retained only for the period when the quality can be secured. The sponsor's consent will be obtained before abandonment.

## RETENTION OF ORIGINAL DOCUMENTS

An original protocol, original protocol amendments and an original final report will be retained at the testing facility. The copies of their original that the study director will be recognized to be accurate copy will be sent to the sponsor.

## STUDY DIRECTOR AND PERSONS CONCERNED WITH THE STUDY AND THE OPERATION

Study Director: Syozo Ajimi  
Section 3, CERI Hita

## Persons Concerned with the Study and Their Operation:

Syozo Ajimi  
(Preparation of test substance solution, cell treatment and  
microscopic observation of specimens)  
Junko Kawaguchi  
(Microscopic observation of specimens)

APPROVAL BY AUTHOR

Study Director: Signed in original March 14, 2007  
Syozo Ajimi

## SUMMARY

The ability of 13F-SFMA to induce chromosomal aberrations was investigated by using Chinese hamster lung fibroblasts (CHL/IU cells).

Based on the result of cell growth inhibition test, the doses in the chromosomal aberration test were set at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320  $\mu\text{g/mL}$  in the short-term treatments without and with S9 mix and the 24 hours continuous treatment.

In chromosomal aberration test, three observation doses for evaluation were selected at 1080, 2160 and 4320  $\mu\text{g/mL}$  in all treatment methods. Then, the frequencies of cells with structural aberrations and numerical aberration cells were examined.

As a result of observation of specimens, the frequencies of cells with structural aberration and numerical aberration cells were below 5% at all observation doses of the test substance in the short-term treatments without and with S9 mix and the 24 hours continuous treatment, therefore, structural aberration and numerical aberration were judged to be negative.

On the other hand, the frequencies of cells with structural aberrations or numerical aberration cells in the negative control treated with dehydrated acetone were below 5%, and the frequencies of cells with structural aberrations in the positive controls treated with mitomycin C or cyclophosphamide monohydrate were above 20%, indicating the proper performance of the present study.

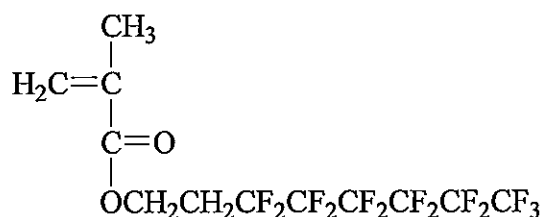
It was concluded that 13F-SFMA did not induce the chromosomal aberration under the present test conditions.

## MATERIALS AND METHODS

### 1. TEST SUBSTANCE AND POSITIVE CONTROL SUBSTANCES

#### 1.1 Test Substance (Information Provided by the Sponsor)

- 1) Name  
3,3,4,4,5,5,6,6,7,7,8,8,8-Tridecafluorooctyl methacrylate  
Other name: 13F-SFMA  
CAS No.: 2144-53-8
- 2) Lot No.  
6Y002
- 3) Supplier  
DAIKIN INDUSTRIES, LTD.
- 4) Structural formula



(Molecular formula: C<sub>12</sub>H<sub>9</sub>F<sub>13</sub>O<sub>2</sub>)

- 5) Purity  
99.8%
- 6) Names and concentrations of impurities  
Unknown 0.2%
- 7) Physicochemical properties
 

Appearance at ordinary temperature:	Colorless transparent liquid
Molecular weight:	432.18
Stability:	—
Melting point:	—
Boiling point:	92°C at 8 mmHg
Vapor pressure:	—
Partition coefficient (1-octanol/water):	—
Hydrolyzability:	Unknown
$(\text{CH}_2=\text{C}(\text{CH}_3)\text{COOCH}_2\text{CH}_2\text{C}_6\text{F}_{13}) \rightarrow \text{C}_6\text{F}_{13}\text{CH}_2\text{CH}_2\text{OH} + \text{CH}_2=\text{C}(\text{CH}_3)\text{COOH}$	
Solubility	—
Degree of solubility	
Water:	Insoluble

Dimethyl sulfoxide (DMSO):	<50.0 mg/mL (measured at the testing facility using dehydrated DMSO)
Acetone:	≥432 mg/mL (measured at the testing facility using dehydrated acetone)
Others:	—

## 8) Storage conditions

Stored in a dark place at room temperature (cabinet No. 1 in the test substance storage room, permissible range: 10-30°C).

## 9) Precautions

Gloves, a mask, a head cap and a lab coat were worn.

## 1.2 Positive Control Substances

## 1) Mitomycin C (MMC)

Manufacturer: Kyowa Hakko Kogyo Co., Ltd.  
 Lot No.: 480AEL  
 Appearance: royal purple powder  
 Content: 99%  
 Grade: for injection

## 2) Cyclophosphamide monohydrate (CPA)

Manufacturer: Wako Pure Chemical Industries, Ltd.  
 Lot No.: PKQ7031  
 Appearance: white crystals or crystalline powder  
 Content: 99.0%  
 Grade: for biochemistry

## 3) Storage conditions

MMC was stored at room temperature (cabinet No. 2 in the test substance storage room, permissible range: 10-30°C) and CPA in a cold dark place (refrigerator No.13 in the test substance storage room, permissible range: 1-10°C).

## 4) Precautions

Gloves, a mask, a head cap and a lab coat were worn.

## 2. CELLS

## 2.1 Cell Line and Reason for Selection

Chinese hamster lung fibroblasts (CHL/IU cells) were supplied by Health Science Research Resources Bank, Japan Health Sciences Foundation on April 17, 2002. The modal number of chromosomes was 25 per cell. The doubling time was about 15 hours. It was confirmed in the testing facility that the cells were mycoplasma free and the spontaneous frequencies of cells with structural aberrations and the numerical

aberration cells were below 5%.

CHL/IU cells have been recommended the availability on *in vitro* chromosome aberration test prescribed in "Concerning Testing Methods Relating to the New Chemical Substances" on Japanese Test Guideline.

## 2.2 Storage

Cells were suspended in medium [Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd.) and 10 vol% heat-inactivated newborn calf serum (NBCS, Sanko Junyaku Co., Ltd.)] including 10 vol% DMSO and were frozen in liquid nitrogen.

## 2.3 Culture Condition

Cells were cultured in a CO<sub>2</sub> incubator (MCO-345, SANYO Electric Co., Ltd. and Model 530, Wakenyaku Co., Ltd.), which was set at 37°C and 5% CO<sub>2</sub> under humid condition.

## 2.4 Subculture

Cells were subcultured in 90-mm diameter Petri dishes (Nunc A/S) twice a week. Passage number of cells was at 7 for the cell growth inhibition test, 9 for the first, 16 for the second, 18 for the third and 3 for the fourth chromosomal aberration test after the receipt.

## 3. MEDIUM AND S9 MIX

### 3.1 Medium

L-Glutamine (final concentration: 0.292 g/L) and sodium hydrogen carbonate (final concentration: approximately 1.85 g/L) were added to Eagle's minimum essential medium (Lot No. 54860611, Nissui Pharmaceutical Co., Ltd.) and basal medium (MEM) was prepared. This medium was then supplemented with 10 vol% heat-inactivated NBCS (Lot No. 27020859, Sanko Junyaku Co., Ltd.).

### 3.2 S9 Mix

#### 1) Rat liver S9

S9 (Lot No. 06090106, manufactured on September 1, 2006, S9 protein content: 20.2 mg/mL, Oriental Yeast Co., Ltd.), which was prepared from livers of 7-week-old male SD rats (body weight: 215.1±10.7 g) administered intraperitoneally a combination of phenobarbital and 5,6-benzoflavone was used. S9 was frozen and preserved in an ultra-deep freezer (MDF-U481ATR, SANYO Electric Co., Ltd., the tolerance temperature: below -80°C) until use. S9 was used within six months after the day of manufacturing.

#### 2) Composition of S9 mix

One milliliter of S9 mix consisted of 0.3 mL S9, 5 µmol MgCl<sub>2</sub>, 33 µmol KCl, 5 µmol G-6-P, 4 µmol NADP and 4 µmol HEPES (pH 7.2) and S9 mix was prepared

just prior to use and was stored in ice until use.

#### 4. CELL PRE-CULTURE

A 60-mm diameter plastic Petri dish (Asahi Techno Glass Corporation) was used for cell culture.

Five milliliter of a cell suspension of  $1.5 \times 10^4$  cells/mL were seeded into a dish and were cultured continuously for 2 days in the cell growth inhibition test and the first chromosomal aberration test. Five milliliter of a cell suspension of  $5.0 \times 10^3$  cells/mL were seeded into a dish and were cultured continuously for 3 days in the second, third and fourth chromosomal aberration tests.

#### 5. PREPARATION OF TEST SUBSTANCE SOLUTION AND POSITIVE CONTROL SUBSTANCE SOLUTIONS

##### 5.1 Preparation of Test Substance Solution

###### 1) Solvent

Acetone (Lot No. EWJ5080, 100.0% in purity, special grade, Wako Pure Chemical Industries, Ltd.) dehydrated with Molecular Sieves 5A 1/8 (Lot No. CEQ7072, Wako Pure Chemical Industries, Ltd.) was used.

###### 2) Reason for selection of solvent

Because the test substance was possibly hydrolyzable as preinformed by the sponsor, water solubility was not examined. The test substance was not soluble in dehydrated DMSO at 50.0 mg/mL, but dissolved in dehydrated acetone at 432 mg/mL. The test substance solution at 432 mg/mL in dehydrated acetone was not indicated any change in color nor exothermic at room temperature within 2 hours after preparation. Therefore, dehydrated acetone was selected as a solvent in this study.

###### 3) Preparation method

After the test substance was weighed, dehydrated acetone was added to the test substance to make an original solution using a laboratory mixer. The test substance solutions of 100 times concentrations of the test substance in the medium were prepared with the solvent. The test substance solutions were prepared under the yellow light. It was prepared without correcting purity of the test substance because the purity of the test substance was above 95%.

###### 4) Preparation time

The test substance solutions were prepared immediately before use. It was stored in ice and used within 1 hour after preparation under the yellow light.

## 5.2 Preparation of Positive Control Substance Solutions

### 1) Preparation method and storage

MMC and CPA were dissolved in distilled water at 0.01 mg/mL and 1 mg/mL, respectively. The positive control substance solutions were frozen in an ultra-deep freezer (MDF-U481ATR, SANYO Electric Co., Ltd., the tolerance temperature: below  $-80^{\circ}\text{C}$ ).

### 2) Preparation time and expiry in use

The positive control substance solutions were thawed at the time of use and used within 1 hour. The stock solutions were used within 6 months after preparation.

## 6. TEST PROCEDURE

### 6.1 Cell Growth Inhibition Test

#### 1) Procedure

For the short-term treatment without S9 mix, the medium was removed from a pre-culture, and the cells were treated for 6 hours in well-mixed medium containing 30  $\mu\text{L}$  of the test substance solution or the solvent and 3 mL of the fresh medium. For the short-term treatment with S9 mix, the medium was removed from a pre-culture, and the cells were treated for 6 hours in well-mixed medium consisting of 0.5 mL of S9 mix and 30  $\mu\text{L}$  of the test substance solution or the solvent and 2.5 mL of the fresh medium. After treatment, the medium was removed, and the cells were rinsed 3 times with 2 mL of Dulbecco's physiological phosphate buffered solution without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Cells were then cultured for another 18 hours in 5 mL of fresh medium.

For the continuous treatment, the medium was removed from a pre-culture, and the cells were treated for the 24 hours with well-mixed medium containing 50  $\mu\text{L}$  of the test substance solution or the solvent and 5 mL of the fresh medium.

In the short-term and the continuous treatments, 50  $\mu\text{L}$  of a 10  $\mu\text{g}/\text{mL}$  demecolcine solution was added to each dish at 2 hours before the end of the culture.

At the start and the end of the treatment and at the end of the culture, precipitation of the test substance, the color change of the medium and the corrosion of the dish were observed macroscopically.

At the end of the culture, a cell suspension was prepared to collect from each dish by a treatment with 2 mL of 0.25 w/v% trypsin. After 200  $\mu\text{L}$  of the cell suspension was diluted with 10 mL of Cell Pack (Sysmex Corporation), the number of the cells was measured using a Microcell counter (CDA-500, Sysmex Corporation), and the cell growth rate and the 50% cell growth inhibition concentration ( $\text{IC}_{50}$ ) was calculated. The  $\text{IC}_{50}$  was obtained from a linear line drawn between 2 plots; the one being greater and the other lower than, and both closest to 50% of the cell



growth rate.

Remained cells were collected by a centrifugation at 1000 rpm (185×g) for 5 minutes and were treated hypotonically with 3 mL of 0.075 mol/L KCl at 37°C for 15 minutes. Following the hypotonic treatment, the cells were pre-fixed once with approximately 0.3 mL of a fixative solution (methanol : acetic acid = 3 : 1), and were completely fixed twice with 3 mL of fixative solution. Then, the cell suspension was prepared with a fixative solution, two drops of the suspension were placed on a glass slide, and stained about 15 minutes with 2 vol% Giemsa solution in 1/15 mol/L phosphate buffer solution (pH6.8). One specimen was prepared per dose.

## 2) Dose levels

In each treatment method, the highest dose was set at 4320 µg/mL equivalent to 10 mmol/L, as the maximum dose in the case of no cytotoxicity on the guideline, and 16.9, 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 µg/mL were set based on a geometric progression of 2, respectively. Duplicate dishes were used for each dose.

## 3) Observation and scoring

Specimens were observed to check the presence or absence of mitotic metaphase cells, and the frequency of the cells with chromosomal aberrations was calculated by observed 50 metaphase cells per dose at which the dose setting of chromosomal aberration test was considered to be referred.

### (1) Structural aberration

The number of cells with structural aberrations excluding gaps was recorded. Gaps were defined as an achromatic region smaller than the width of one chromatid.

### (2) Numerical aberration

The number of cells showing triploid or more was scored.

## 6.2 Chromosomal Aberration Test

### 1) Procedure

Chromosomal aberration test was carried out using the same procedure as that of the cell growth inhibition test, with the following positive controls. Four specimens per dose (two specimens per dish) were prepared.

Treatment method		Substance	Dose
Short-term treatment	Without S9 mix	MMC	0.1 µg/mL
	With S9 mix	CPA	6 µg/mL
24 hours continuous treatment		MMC	0.05 µg/mL

In the positive control, each dish was added with 30 µL of a 0.01 mg/mL MMC solution and 18 µL of a 1 mg/mL CPA solution for the short-term treatments without and with S9 mix, respectively, and 25 µL of a 0.01 mg/mL MMC solution for the continuous treatment.

## 2) Dose levels of the test substance

As the results of the cell growth inhibition test, in the short-term treatment without S9 mix and the continuous treatment, because a cytotoxicity that the cell growth rate was below 50% was not obtained, the highest dose was selected at 4320  $\mu\text{g/mL}$  equivalent to 10 mmol/L and the following 8 doses were set based on a geometric progression of 2. In the short-term treatment with S9 mix, although a cytotoxicity that the cell growth rate was below 50% was obtained and  $\text{IC}_{50}$  was calculated at 120  $\mu\text{g/mL}$ , the cell growth rate was over 50% at 1080  $\mu\text{g/mL}$  or more. Therefore, the highest dose was selected at 4320  $\mu\text{g/mL}$  and the following 8 doses were set based on a geometric progression of 2.

Treatment method		Setting doses of test substance
Short-term treatment	Without S9 mix	33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 $\mu\text{g/mL}$
	With S9 mix	33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 $\mu\text{g/mL}$
24 hours continuous treatment		33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 $\mu\text{g/mL}$

As the result of the chromosomal aberration test employed above doses, the dose that the lowest cell growth rate was observed was 135  $\mu\text{g/mL}$  in the short-term treatment without S9 mix and reproducible results were obtained between the cell growth inhibition test and chromosomal aberration test.

The most suppressed cell growth was observed from 270 to 540  $\mu\text{g/mL}$  in the short-term treatment with S9 mix and reproducible results were also obtained between the cell growth inhibition test and chromosomal aberration test.

However,  $\text{IC}_{50}$  values in the short-term treatments without and with S9 mix were 130 and 4320  $\mu\text{g/mL}$ , respectively, and variation from the cell growth inhibition test was observed in the chromosomal aberration test. Furthermore, decrease of cell growth rate at around 135  $\mu\text{g/mL}$  was not observed in the 24 hours continuous treatment which the treatment period was longer than the short-term treatments. Then, the result obtained from the short-term treatment without S9 mix was questioned. It was possibly thought that the water-shedding property of the test substance caused cytotoxicity in this treatment because the test substance shed water when medium was discarded after the treatment. Therefore, the second chromosomal aberration test was carried out in the short-term treatments with an operation to prevent shedding of water when exchanging medium. Same eight doses were set as the first chromosomal aberration test.

In the second chromosomal aberration test, it was unable to prevent water-shedding in the short-term treatment without S9 mix. As a result, dose-related decrease of cell growth rate was not obtained at around 540  $\mu\text{g/mL}$  and indexes of metaphase cells were different between dishes at 540 and 1080  $\mu\text{g/mL}$ . Therefore, the third test was carried out in the short-term treatment without S9 mix. Same eight doses

were set as the first and second tests.

In the third chromosomal aberration test, shedding of water was successfully prevented and decrease of cell growth rate from 67.5 to 2160  $\mu\text{g/mL}$  was reduced. However, it was unable to improve the fluctuation of indexes of metaphase cells between dishes at 1080 and 2160  $\mu\text{g/mL}$ . Therefore, the fourth test, employed same doses as previous tests, was carried out in the short-term treatment without S9 mix. Additionally, in the second test in the short-term treatment with S9 mix, the cell growth rate decreased from 135 and 270  $\mu\text{g/mL}$ , the chromosomal aberration test in this treatment was also carried out employing same doses as the first and second tests at the same time as the fourth test.

Duplicate dishes were used for each dose.

### 3) Observation

#### (1) Dose for observation

Specimens for observation were selected from the fourth test in the short-term treatments without and with S9 mix and from the first test in the 24 hours continuous treatment. All specimens of the negative and the positive controls set as the control groups in above tests were observed.

The observation doses of the test substance were selected the consecutive doses of three stages. The highest observation dose in each treatment and the reason for selection are shown below.

In the short-term treatments without and with S9 mix and the continuous treatment, because a cytotoxicity that the cell growth rate was below 50% was not obtained, and it was considered that chromosomal aberrations were observable at 4320  $\mu\text{g/mL}$  set as the highest dose, the observation doses of the test substance were selected at 1080, 2160 and 4320  $\mu\text{g/mL}$  in all treatment methods.

After the selection of the observation doses, slide numbers were allocated randomly to all observed specimens. All specimens were observed in a blinded manner.

#### (2) Structural Aberration

Two hundred metaphase cells per dose (50 cells per specimen) containing  $25 \pm 2$  chromosomes were observed using a microscope. The total number of cells with structural aberrations and the number of aberrant cells in each aberration category were recorded. Gaps were defined as an achromatic region smaller than the width of one chromatid and were recorded separately from the structural aberrations.

#### (3) Numerical Aberration

The number of polyploid (cell with 38 or more chromosomes) cells among 200 metaphase cells per dose (50 cells per specimen) observed was recorded.

### 6.3 Confirmation Test

In the chromosomal aberration test for the short-term treatments without and with S9

mix and the 24 hours continuous treatment, the frequencies of cells with structural aberration and numerical aberration cells were below 5% and the result was judged to be negative, therefore, a confirmation test was not conducted.

#### 7. JUDGEMENT CRITERIA OF RESULTS

The findings were judged to be positive when the frequencies of cells with structural aberrations or numerical aberrations were 10% or more with a dose-related increase, or the frequencies of aberrant cells were 5% or more both in the chromosomal aberration test and the confirmation test. The other cases were judged to be negative. No statistical analyses were used.

#### 8. VALIDITY OF TEST

This study was regarded as valid as follows: 1) the frequencies of cells with chromosomal aberrations did not fluctuate markedly between two culture dishes, 2) the frequencies of aberrant cells in the negative control were below 5%, and 3) the frequencies of cells with structural aberrations in the positive controls were 20% or more.

### FACTORS AFFECTED RELIABILITY OF TEST

There were no factors, which might affect the reliability of the test.

### TEST RESULTS

#### 1. CELL GROWTH INHIBITION TEST (Table 1 and Fig. 1)

The  $IC_{50}$ s were calculated more than 4320  $\mu\text{g/mL}$  in the short-term treatment without S9 mix and the 24 hours continuous treatment and at 120  $\mu\text{g/mL}$  in the short-term treatment with S9 mix.

In all treatment methods, precipitation of the test substance was observed at 2160  $\mu\text{g/mL}$  or more at the start and end of the treatment. The color change of the medium and the corrosion of the culture dish were not observed at any doses.

Shedding of water in the culture dishes was observed at 135  $\mu\text{g/mL}$  or more when exchanging treatment medium in the short-term treatments and when collecting cells in the continuous treatment.

The frequencies of numerical aberration cells were below 5% at all observation doses of the test substance in each treatment, but the maximum frequency of cells with structural aberrations was 8.0%.

## 2. CHROMOSOMAL ABERRATION TEST

### 2.1 Short-term Treatment

#### 1) The first test (Table 2 and Fig. 2)

##### (1) Without S9 mix

###### a) Cell growth rate and IC<sub>50</sub>

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 µg/mL of the test substance were 91.0, 80.7, 45.2, 64.8, 65.5, 65.3, 76.4 and 74.1%, respectively. The IC<sub>50</sub> was calculated at 130 µg/mL.

###### b) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 1080 µg/mL or more at the start and the end of the treatment and at 2160 µg/mL or more at end of the culture. The color change of the medium and the corrosion of the culture dish were not observed at any doses. Shedding of water in the culture dishes was observed at 135 µg/mL or more when exchanging treatment medium.

##### (2) With S9 mix

###### a) Cell growth rate and IC<sub>50</sub>

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 µg/mL of the test substance were 94.6, 91.3, 68.1, 57.2, 53.2, 66.4, 72.0 and 64.7%, respectively. The IC<sub>50</sub> was calculated more than 4320 µg/mL.

###### b) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 1080 µg/mL or more at the start and the end of the treatment and at 2160 µg/mL or more at the end of the culture. The color change of the medium and the corrosion of the culture dish were not observed at any doses. Shedding of water in the culture dishes was observed at 135 µg/mL or more when exchanging treatment medium.

#### 2) The second test (Table 3 and Fig. 3)

##### (1) Without S9 mix

###### a) Cell growth rate and IC<sub>50</sub>

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 µg/mL of the test substance were 98.6, 64.1, 62.8, 57.9, 80.4, 67.6, 67.9 and 81.5%, respectively. The IC<sub>50</sub> was calculated more than 4320 µg/mL.

###### b) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 1080 µg/mL or more at the start and the end of the treatment. The color change of the medium and the corrosion of the culture dish were not observed at any doses. Shedding of

water in the culture dishes was observed at 135 µg/mL or more when exchanging treatment medium.

(2) With S9 mix

a) Cell growth rate and IC<sub>50</sub>

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 µg/mL of the test substance were 95.2, 79.3, 43.3, 35.8, 53.8, 60.2, 68.9 and 59.9%, respectively. The IC<sub>50</sub> was calculated at 120 µg/mL.

b) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 1080 µg/mL or more at the start and the end of the treatment. The color change of the medium and the corrosion of the culture dish were not observed at any doses. Shedding of water in the culture dishes was observed at 135 µg/mL or more when exchanging treatment medium.

3) The third test (Table 4 and Fig. 4)

(1) Without S9 mix

a) Cell growth rate and IC<sub>50</sub>

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 µg/mL of the test substance were 120.4, 99.3, 133.8, 123.9, 108.0, 115.9, 101.3 and 106.4%, respectively. The IC<sub>50</sub> was calculated more than 4320 µg/mL.

b) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 1080 µg/mL or more at the start and the end of the treatment and at 2160 µg/mL or more at the end of the culture. The color change of the medium and the corrosion of the culture dish were not observed at any doses.

4) The fourth test (Tables 5, 6, 7 and Figs. 5, 6)

(1) Without S9 mix

a) Cell growth rate and IC<sub>50</sub>

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320 µg/mL of the test substance were 94.0, 70.4, 82.4, 74.4, 87.9, 85.3, 79.7 and 75.7%, respectively. The IC<sub>50</sub> was calculated more than 4320 µg/mL.

b) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 2160 µg/mL or more at the start and the end of the treatment. The color change of the medium and the corrosion of the culture dish were not observed at any doses.

c) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 1.0% in the negative control and 66.5% in the positive control.

The frequencies of cells with structural aberrations at 1080, 2160 and 4320  $\mu\text{g/mL}$  of the test substance were 0.5, 1.5 and 0.0%, respectively, therefore, the results were judged to be negative.

d) Frequency of numerical aberration cells

The frequencies of numerical aberration cells were below 5% at all doses including the negative and positive controls, therefore, the results were judged to be negative.

(2) With S9 mix

a) Cell growth rate and  $\text{IC}_{50}$

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320  $\mu\text{g/mL}$  of the test substance were 93.8, 85.0, 55.5, 67.4, 71.2, 64.2, 61.2 and 61.6%, respectively. The  $\text{IC}_{50}$  was calculated more than 4320  $\mu\text{g/mL}$ .

b) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 2160  $\mu\text{g/mL}$  or more at the start and the end of the treatment. The color change of the medium and the corrosion of the culture dish were not observed at any doses. Slight shedding of water in the culture dishes was observed at 135  $\mu\text{g/mL}$  when exchanging treatment medium.

c) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 1.5% in the negative control and 58.5% in the positive control.

The frequencies of cells with structural aberrations at 1080, 2160 and 4320  $\mu\text{g/mL}$  of the test substance were 2.5, 2.0 and 4.0%, respectively, therefore, the results were judged to be negative.

d) Frequency of numerical aberration cells

The frequencies of numerical aberration cells were below 5% at all doses including the negative and positive controls, therefore, the results were judged to be negative.

2.2 Twenty Four Hours Continuous Treatment (Tables 2, 8 and Figs. 2, 7)

1) Cell growth rate and  $\text{IC}_{50}$

The cell growth rates at 33.8, 67.5, 135, 270, 540, 1080, 2160 and 4320  $\mu\text{g/mL}$  of the test substance were 104.8, 83.8, 88.3, 75.7, 71.1, 70.6, 70.6 and 60.1%, respectively. The  $\text{IC}_{50}$  was calculated more than 4320  $\mu\text{g/mL}$ .

- 2) Precipitation of the test substance, color change of medium and corrosion of culture dish

Precipitation of the test substance was observed at 1080  $\mu\text{g/mL}$  or more at the start of the treatment and at 2160  $\mu\text{g/mL}$  or more at end of the treatment. The color change of the medium and the corrosion of the culture dish were not observed at any doses.

- 3) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 2.0% in the negative control and 83.5% in the positive control.

The frequencies of cells with structural aberrations were 1.0% at all three doses, 1080, 2160 and 4320  $\mu\text{g/mL}$ , therefore, the results were judged to be negative.

- 4) Frequency of numerical aberration cells

The frequencies of numerical aberration cells were below 5% at all doses including the negative and positive controls, therefore, the results were judged to be negative.

## DISCUSSION AND CONCLUSION

In each treatment method in the chromosomal aberration test, the frequencies of cells with chromosomal aberrations did not fluctuate markedly between two culture dishes, and the frequencies of cells with aberrations were below 5% in the negative controls, and the frequencies of cells with structural aberrations excluding gaps were 20% or more in the positive controls, indicating that the present study was appropriately performed.

In the exchange of medium, when medium or phosphate buffered salt solution were discarded, water was shed in culture dishes at several doses and fluctuations in both cell growth rates and indexes of metaphase cells between dishes were observed in these doses.

It was considered that these fluctuations were resulted from the cytotoxicity including dehydration by the contact of the test substance with cells because the test substance was classified to fluorine compound. Therefore, the chromosomal aberration tests were carried out with an operation to prevent shedding of water when exchanging medium and specimens obtained from these treatments were observed.

As a result of observation of specimens, the frequencies of cells with structural aberration and numerical aberration cells were below 5% at all observation doses of the test substance in the short-term treatments without and with S9 mix and the 24 hours continuous treatment, therefore, both structural aberration and numerical aberration were judged to be negative.

In the cell growth inhibition test, the frequency of cells with structural aberration was found to be up to 8.0% and induction of chromosomal aberration was suspected in the short-term treatments without and with S9 mix. However, it was considered that the frequency of aberrant cells increased in the inappropriate test condition because shedding of water by the



test substance was found at the doses that the frequency of aberrant cells was 5% or more. Although the frequency of cells with structural aberration was also found to be up to 8.0% and induction of chromosomal aberration was suspected in the continuous treatment, increase of the frequency was not verified in the chromosomal aberration test. Therefore, it was thought that the result was obtained accidentally because the number of cells for observation was fifty and too small to evaluate.

Based on the above results, it was considered that 13F-SFMA did not induce the chromosomal aberration under the present test conditions.

#### REFERENCES

1. Toshio Sofuni (ed.) (1999) Data book of chromosomal aberration test *in vitro*. Revised edition, 1998 (in Japanese). Life-science Information Center.
2. Japan Environmental Mutagenicity Society/Mammalian Mutagenicity Study Group (ed.) (1988) Atlas of chromosomal aberration induced with chemical substance (in Japanese). Asakura Publishing Co., Tokyo.

Table 1 Results of cell growth inhibition test of 13F-SFMA

Substance	Dose ( $\mu\text{g/mL}$ )	Treatment- recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium <sup>a)</sup>			Frequency of cells with aberrations (%) <sup>b)</sup>	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
dehydrated acetone	0	6-18	-	100	-	-	-	0.0	0.0
13F-SFMA	16.9	6-18	-	112.2	-	-	-	n.o.	n.o.
	33.8	6-18	-	106.3	-	-	-	n.o.	n.o.
	67.5	6-18	-	76.3	-	-	-	0.0	4.0
	135	6-18	-	54.3	-	-	-	2.0	0.0
	270	6-18	-	64.2	-	-	-	4.0	2.0
	540	6-18	-	70.4	-	-	-	8.0	0.0
	1080	6-18	-	78.4	-	-	-	0.0	0.0
	2160	6-18	-	73.4	+	+	-	2.0	0.0
	4320	6-18	-	76.7	+	+	-	4.0	0.0
IC <sub>50</sub> : >4320 $\mu\text{g/mL}$									
dehydrated acetone	0	6-18	+	100	-	-	-	0.0	0.0
13F-SFMA	16.9	6-18	+	99.0	-	-	-	n.o.	n.o.
	33.8	6-18	+	96.4	-	-	-	n.o.	n.o.
	67.5	6-18	+	82.6	-	-	-	2.0	2.0
	135	6-18	+	42.6	-	-	-	6.0	0.0
	270	6-18	+	33.4	-	-	-	8.0	0.0
	540	6-18	+	37.7	-	-	-	4.0	2.0
	1080	6-18	+	67.5	-	-	-	2.0	2.0
	2160	6-18	+	65.4	+	+	-	2.0	0.0
	4320	6-18	+	67.5	+	+	-	2.0	0.0
IC <sub>50</sub> : 120 $\mu\text{g/mL}$									
dehydrated acetone	0	24-0	-	100	-	-	/	0.0	0.0
13F-SFMA	16.9	24-0	-	93.6	-	-	/	n.o.	n.o.
	33.8	24-0	-	90.2	-	-	/	n.o.	n.o.
	67.5	24-0	-	81.5	-	-	/	n.o.	n.o.
	135	24-0	-	74.0	-	-	/	0.0	0.0
	270	24-0	-	73.4	-	-	/	4.0	2.0
	540	24-0	-	70.9	-	-	/	2.0	0.0
	1080	24-0	-	74.4	-	-	/	2.0	0.0
	2160	24-0	-	61.1	+	+	/	6.0	0.0
	4320	24-0	-	57.9	+	+	/	8.0	0.0
IC <sub>50</sub> : >4320 $\mu\text{g/mL}$									

Acetone was dehydrated with molecular sieves 5A 1/8.

n.o.: not observed

a) Precipitation of the test substance: -, absence; +, presence.

b) The frequency of cells with chromosomal aberrations was calculated by observing 50 metaphases per dose.

The dose was set at 4320  $\mu\text{g/mL}$  equivalent to 10 mmol/L of the test substance, which was the maximum dose in the guidelines, the dose levels based on a geometric progression of 2 were selected.

Table 2 Results of the first chromosomal aberration test of 13F-SFMA

Substance	Dose ( $\mu\text{g}/\text{mL}$ )	Treatment- recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium <sup>a)</sup>			Frequency of cells with aberrations (%) <sup>b)</sup>	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
dehydrated acetone	0	6-18	-	100	-	-	-	n.o.	n.o.
13F-SFMA	33.8	6-18	-	91.0	-	-	-	n.o.	n.o.
	67.5	6-18	-	80.7	-	-	-	n.o.	n.o.
	135	6-18	-	45.2	-	-	-	n.o.	n.o.
	270	6-18	-	64.8	-	-	-	n.o.	n.o.
	540	6-18	-	65.5	-	-	-	n.o.	n.o.
	1080	6-18	-	65.3	+	+	-	n.o.	n.o.
	2160	6-18	-	76.4	+	+	+	n.o.	n.o.
	4320	6-18	-	74.1	+	+	+	n.o.	n.o.
MMC	0.1	6-18	-	ND	-	-	-	n.o.	n.o.
IC <sub>50</sub> : 130 $\mu\text{g}/\text{mL}$									
dehydrated acetone	0	6-18	+	100	-	-	-	n.o.	n.o.
13F-SFMA	33.8	6-18	+	94.6	-	-	-	n.o.	n.o.
	67.5	6-18	+	91.3	-	-	-	n.o.	n.o.
	135	6-18	+	68.1	-	-	-	n.o.	n.o.
	270	6-18	+	57.2	-	-	-	n.o.	n.o.
	540	6-18	+	53.2	-	-	-	n.o.	n.o.
	1080	6-18	+	66.4	+	+	-	n.o.	n.o.
	2160	6-18	+	72.0	+	+	+	n.o.	n.o.
	4320	6-18	+	64.7	+	+	+	n.o.	n.o.
CPA	6	6-18	+	ND	-	-	-	n.o.	n.o.
IC <sub>50</sub> : >4320 $\mu\text{g}/\text{mL}$									
dehydrated acetone	0	24-0	-	100	-	-	-	2.0	0.5
13F-SFMA	33.8	24-0	-	104.8	-	-	-	n.o.	n.o.
	67.5	24-0	-	83.8	-	-	-	n.o.	n.o.
	135	24-0	-	88.3	-	-	-	n.o.	n.o.
	270	24-0	-	75.7	-	-	-	n.o.	n.o.
	540	24-0	-	71.1	-	-	-	n.o.	n.o.
	1080	24-0	-	70.6	+	-	-	1.0	1.0
	2160	24-0	-	70.6	+	+	-	1.0	0.5
	4320	24-0	-	60.1	+	+	-	1.0	2.0
MMC	0.05	24-0	-	ND	-	-	-	83.5	0.5
IC <sub>50</sub> : >4320 $\mu\text{g}/\text{mL}$									

Acetone was dehydrated with molecular sieves 5A 1/8.

MMC: mitomycin C, CPA: cyclophosphamide monohydrate

ND: not detected, n.o.: not observed

a) Precipitation of the test substance: -, absence; +, presence.

b) The frequency of cells with chromosomal aberrations was calculated by observing 200 metaphases per dose.

Table 3 Results of the second chromosomal aberration test of 13F-SFMA

Substance	Dose ( $\mu\text{g/mL}$ )	Treatment- recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium <sup>a)</sup>			Frequency of cells with aberrations (%) <sup>b)</sup>	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
dehydrated acetone	0	6-18	-	100	-	-	-	n.o.	n.o.
13F-SFMA	33.8	6-18	-	98.6	-	-	-	n.o.	n.o.
	67.5	6-18	-	64.1	-	-	-	n.o.	n.o.
	135	6-18	-	62.8	-	-	-	n.o.	n.o.
	270	6-18	-	57.9	-	-	-	n.o.	n.o.
	540	6-18	-	80.4	-	-	-	few meta	
	1080	6-18	-	67.6	+	+	-	few meta	
	2160	6-18	-	67.9	+	+	-	n.o.	n.o.
	4320	6-18	-	81.5	+	+	-	n.o.	n.o.
MMC	0.1	6-18	-	ND	-	-	-	n.o.	n.o.
IC <sub>50</sub> : >4320 $\mu\text{g/mL}$									
dehydrated acetone	0	6-18	+	100	-	-	-	n.o.	n.o.
13F-SFMA	33.8	6-18	+	95.2	-	-	-	n.o.	n.o.
	67.5	6-18	+	79.3	-	-	-	n.o.	n.o.
	135	6-18	+	43.3	-	-	-	n.o.	n.o.
	270	6-18	+	35.8	-	-	-	n.o.	n.o.
	540	6-18	+	53.8	-	-	-	n.o.	n.o.
	1080	6-18	+	60.2	+	+	-	n.o.	n.o.
	2160	6-18	+	68.9	+	+	-	n.o.	n.o.
	4320	6-18	+	59.9	+	+	-	n.o.	n.o.
CPA	6	6-18	+	ND	-	-	-	n.o.	n.o.
IC <sub>50</sub> : 120 $\mu\text{g/mL}$									

Acetone was dehydrated with molecular sieves 5A 1/8.

MMC: mitomycin C, CPA: cyclophosphamide monohydrate

ND: not detected

n.o.: not observed, few meta: the frequency of metaphases was extremely few

a) Precipitation of the test substance: -, absence; +, presence.

Table 4 Results of the third chromosomal aberration test of 13F-SFMA

Substance	Dose ( $\mu\text{g/mL}$ )	Treatment- recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium <sup>a)</sup>			Frequency of cells with aberrations (%)	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
dehydrated acetone	0	6-18	-	100	-	-	-	n.o.	n.o.
13F-SFMA	33.8	6-18	-	120.4	-	-	-	n.o.	n.o.
	67.5	6-18	-	99.3	-	-	-	n.o.	n.o.
	135	6-18	-	133.8	-	-	-	n.o.	n.o.
	270	6-18	-	123.9	-	-	-	n.o.	n.o.
	540	6-18	-	108.0	-	-	-	n.o.	n.o.
	1080	6-18	-	115.9	+	+	-	n.o.	n.o.
	2160	6-18	-	101.3	+	+	+	few meta	
	4320	6-18	-	106.4	+	+	+	n.o.	n.o.
MMC	0.1	6-18	-	ND	-	-	-	n.o.	n.o.
IC <sub>50</sub> : >4320 $\mu\text{g/mL}$									

Aceton was dehydrated with molecular sieves 5A 1/8.

MMC: mitomycin C, CPA: cyclophosphamide monohydrate

ND: not detected

n.o.: not observed, few meta: the frequency of metaphases was extremely few

a) Precipitation of the test substance: -, absence; +, presence.

Table 5 Results of the fourth chromosomal aberration test of 13F-SFMA

Substance	Dose ( $\mu\text{g/mL}$ )	Treatment- recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium <sup>a)</sup>			Frequency of cells with aberrations (%)	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
dehydrated acetone	0	6-18	-	100	-	-	-	1.0	1.5
13F-SFMA	33.8	6-18	-	94.0	-	-	-	n.o.	n.o.
	67.5	6-18	-	70.4	-	-	-	n.o.	n.o.
	135	6-18	-	82.4	-	-	-	n.o.	n.o.
	270	6-18	-	74.4	-	-	-	n.o.	n.o.
	540	6-18	-	87.9	-	-	-	n.o.	n.o.
	1080	6-18	-	85.3	-	-	-	0.5	1.0
	2160	6-18	-	79.7	+	+	-	1.5	0.0
	4320	6-18	-	75.7	+	+	-	0.0	2.5
MMC	0.1	6-18	-	ND	-	-	-	66.5	0.5
IC <sub>50</sub> : >4320 $\mu\text{g/mL}$									
dehydrated acetone	0	6-18	+	100	-	-	-	1.5	0.5
13F-SFMA	33.8	6-18	+	93.8	-	-	-	n.o.	n.o.
	67.5	6-18	+	85.0	-	-	-	n.o.	n.o.
	135	6-18	+	55.5	-	-	-	n.o.	n.o.
	270	6-18	+	67.4	-	-	-	n.o.	n.o.
	540	6-18	+	71.2	-	-	-	n.o.	n.o.
	1080	6-18	+	64.2	-	-	-	2.5	0.0
	2160	6-18	+	61.2	+	+	-	2.0	0.0
	4320	6-18	+	61.6	+	+	-	4.0	0.5
CPA	6	6-18	+	ND	-	-	-	58.5	0.0
IC <sub>50</sub> : >4320 $\mu\text{g/mL}$									

Acetone was dehydrated with molecular sieves 5A 1/8.

MMC: mitomycin C, CPA: cyclophosphamide monohydrate

ND: not detected, n.o.: not observed

a) Precipitation of the test substance: -, absence; +, presence.

b) The frequency of cells with chromosomal aberrations was calculated by observing 200 metaphases per dose.

Table 6 Results of the fourth chromosomal aberration test (short-term treatment without S9 mix)

Treatment time (h)	S9 mix	Dose (µg/mL)	Number of cells with structural chromosomal aberrations (frequency%)										Number of cells with numerical chromosomal aberrations (frequency%)		
			Number of cells observed	Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange	Others	Total number of cells with aberrations	Number of gaps (frequency%)	Cell growth rate (%)	Number of cells observed	Polyploids	Others	Total number of cells with aberrations
6 - 18	-	Negative control (Acetone) 0	100	0	0	0	1	0	0	1	1	100	0	0	0
			100	1	0	0	0	0	1	1	100	3	0	3	
			200	1 ( 0.5)	0 ( 0.0)	0 ( 0.0)	1 ( 0.5)	0 ( 0.0)	2 ( 1.0)	2 ( 1.0)	92.8	3 ( 1.5)	0 ( 0.0)	3 ( 1.5)	
6 - 18	-	33.8	0	0	0	0	0	0	0	0	95.1	0	0	0	
			0	0	0	0	0	0	0	0	( 94.0)	0	0	0	
			0	0	0	0	0	0	0	0	65.7	0	0	0	
6 - 18	-	67.5	0	0	0	0	0	0	0	0	75.1	0	0	0	
			0	0	0	0	0	0	0	0	( 70.4)	0	0	0	
			0	0	0	0	0	0	0	0	81.4	0	0	0	
6 - 18	-	135	0	0	0	0	0	0	0	0	83.3	0	0	0	
			0	0	0	0	0	0	0	0	( 82.4)	0	0	0	
			0	0	0	0	0	0	0	0	76.2	0	0	0	
6 - 18	-	270	0	0	0	0	0	0	0	0	72.6	0	0	0	
			0	0	0	0	0	0	0	0	( 74.4)	0	0	0	
			0	0	0	0	0	0	0	0	90.0	0	0	0	
6 - 18	-	540	0	0	0	0	0	0	0	0	85.7	0	0	0	
			0	0	0	0	0	0	0	0	( 87.9)	0	0	0	
			0	0	0	0	0	0	0	0	82.5	1	0	1	
6 - 18	-	1080	100	0	0	0	1	0	0	0	88.0	1	0	1	
			100	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	1 ( 0.5)	0 ( 0.0)	1 ( 0.5)	0 ( 0.0)	( 85.3)	2 ( 1.0)	0 ( 0.0)	2 ( 1.0)	
			200	2	0	0	0	0	2	2	75.8	0	0	0	
6 - 18	-	2160 †	100	1	0	0	0	0	0	1	83.5	0	0	0	
			100	3 ( 1.5)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	3 ( 1.5)	2 ( 1.0)	( 79.7)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	
			200	0	0	0	0	0	0	0	78.1	2	0	2	
6 - 18	-	4320 †	100	0	0	0	0	0	0	0	73.2	3	0	3	
			100	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	( 75.7)	5 ( 2.5)	0 ( 0.0)	5 ( 2.5)	
			200	35	55	0	2	0	65	0	100	0	0	0	
6 - 18	-	Positive control (MMC) 0.1	100	40	47	0	0	0	68	2	100	1	0	1	
			100	75 ( 37.5)	102 ( 51.0)	0 ( 0.0)	2 ( 1.0)	0 ( 0.0)	133 ( 66.5)	2 ( 1.0)	200	1 ( 0.5)	0 ( 0.0)	1 ( 0.5)	
			200	0	0	0	0	0	0	0	100	0	0	0	

Treatment time comprised treatment-time and recovery-time.

The number of aberrant cells at each dish was shown at the first and second lines. The total number of them was shown at the third line.

Cell growth rate at each dish was shown at the first and second lines. The average of them was shown at the third line.

Acetone was dehydrated with molecular sieves 5A 1/8.

MMC: Mitomycin C

†:Precipitation of the test substance was observed at the start and the end of the treatment.

The specimens at 33.8, 67.5, 135, 270 and 540 µg/mL were not observed.

Table 7 Results of the fourth chromosomal aberration test (short-term treatment with S9 mix)

Treatment time (h)	S9 mix	Dose (µg/mL)	Number of cells with structural chromosomal aberrations (frequency%)										Cell growth rate (%)	Number of cells with numerical chromosomal aberrations (frequency%)				
			Number of cells with structural chromosomal aberrations (frequency%)					Number of cells with numerical chromosomal aberrations (frequency%)						Number of cells observed	Polyploids	Others	Total number of cells with aberrations	
			Number of cells observed	Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange	Others	Total number of cells with aberrations	Number of gaps (frequency%)	Number of cells observed	Polyploids						Others
6-18	+	Negative control (Acetone)	100	0	0	1	0	0	0	0	0	1	0	0	100	1	0	1
6-18	+	33.8	100	2	0	0	0	0	0	0	2	1	0	100	0	0	0	
6-18	+	67.5	200	2	1.0	0	0.0	1	0.5	0	0.0	3	1.5	92.3	1	0.0	1	
6-18	+	135	0	0	0	0	0	0	0	0	0	0	0	95.2	0	0	0	
6-18	+	270	0	0	0	0	0	0	0	0	0	0	0	(93.8)	0	0	0	
6-18	+	540	0	0	0	0	0	0	0	0	0	0	0	88.4	0	0	0	
6-18	+	1080	0	0	0	0	0	0	0	0	0	0	0	81.6	0	0	0	
6-18	+	2160 †	0	0	0	0	0	0	0	0	0	0	0	(85.0)	0	0	0	
6-18	+	4520 †	0	0	0	0	0	0	0	0	0	0	0	60.5	0	0	0	
6-18	+	Positive control (CPA)	100	2	0	1	1	0	0	0	4	0	0	100	0	0	0	
6-18	+	6	100	0	0	1	0	0	0	1	1	0	0	66.7	0	0	0	
6-18	+	6	200	2	1.0	2	1.0	1	0.5	0	0.0	5	2.5	200	0	0.0	0	
6-18	+	6	100	1	1	0	0	0	0	0	2	1	0	(64.2)	0	0.0	0	
6-18	+	6	100	0	2	0	0	0	0	0	2	0	0	57.7	0	0	0	
6-18	+	6	100	1	0.5	3	1.5	0	0.0	0	0.0	4	2.0	100	0	0.0	0	
6-18	+	6	200	5	0	1	1	0	0	0	5	1	0	(61.2)	0	0.0	0	
6-18	+	6	100	0	2	2	1.0	2	1.0	0	0	3	0	56.8	1	0	1	
6-18	+	6	200	5	2.5	2	1.0	2	1.0	0	0.0	8	4.0	200	1	0.5	1	
6-18	+	6	100	19	55	0	0	1	0	0	58	2	0	(61.6)	0	0.0	0	
6-18	+	6	100	28	43	0	0	0	0	0	59	2	0	100	0	0	0	
6-18	+	6	200	47	23.5	98	49.0	0	0.0	1	0.5	117	58.5	200	0	0.0	0	

Treatment time comprised treatment-time and recovery-time.

The number of aberrant cells at each dish was shown at the first and second lines. The total number of them was shown at the third line.

Cell growth rate at each dish was shown at the first and second lines. The average of them was shown at the third line.

Acetone was dehydrated with molecular sieves 5A 1/8.

CPA: Cyclophosphamide monohydrate

†: Precipitation of the test substance was observed at the start and the end of the treatment.

The specimens at 33.8, 67.5, 135, 270 and 540 µg/mL were not observed.



Table 8 Results of the fourth chromosomal aberration test (continuous treatment)

Treatment time (h)	Dose ( $\mu\text{g/mL}$ )	Number of cells with structural chromosomal aberrations (frequency%)						Number of gaps (frequency%)	Cell growth rate (%)	Number of cells with numerical chromosomal aberrations (frequency%)			
		Number of cells observed	Chromatid exchange			Total number of cells with aberrations	Number of cells observed			Polyploids	Others	Total number of cells with aberrations	
			Chromatid break	Chromatid exchange	Chromosome break								Chromosome exchange
24 - 0	Negative control (Acetone)	100	1	0	0	0	0	0	100	0	0	0	0
		100	3	0	0	0	0	0	100	1	0	0	1
		200	4 ( 2.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	200	1 ( 0.5)	0 ( 0.0)	0 ( 0.0)
24 - 0	33.8	0						99.6	0				
		0							109.9 (104.8)	0			
		0							86.7 ( 83.8)	0			
24 - 0	67.5	0						80.8 ( 83.8)	0				
		0							85.7 ( 88.3)	0			
		0							90.9 ( 88.3)	0			
24 - 0	135	0						75.2 ( 75.7)	0				
		0							76.2 ( 73.3)	0			
		0							68.8 ( 71.1)	0			
24 - 0	270	0						58.4 ( 72.8)	0				
		0							72.8 ( 70.6)	0			
		0							65.1 ( 65.1)	0			
24 - 0	540	100	0	0	0	0	0	100	2	0	0	2	
		100	1	0	0	1	0	0	100	0	0	0	
		200	1 ( 0.5)	0 ( 0.0)	0 ( 0.0)	1 ( 0.5)	0 ( 0.0)	2 ( 1.0)	0 ( 0.0)	200	2 ( 1.0)	0 ( 0.0)	2 ( 1.0)
24 - 0	2160 †	100	1	0	0	0	0	100	1	0	0	1	
		100	1	0	0	0	0	0	100	0	0	0	
		200	2 ( 1.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	2 ( 1.0)	0 ( 0.0)	200	1 ( 0.5)	0 ( 0.0)	1 ( 0.5)
24 - 0	4320 †	100	0	2	0	0	0	100	0	0	0	0	
		100	0	0	0	0	0	0	100	0	0	0	
		200	0 ( 0.0)	2 ( 1.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	2 ( 1.0)	0 ( 0.0)	200	4 ( 2.0)	0 ( 0.0)	4 ( 2.0)
24 - 0	Positive control (MMC) 0.05	100	48	63	0	0	0	100	0	0	0	0	
		100	39	77	0	0	0	0	100	1	0	1	
		200	87 ( 43.5)	140 ( 70.0)	0 ( 0.0)	0 ( 0.0)	0 ( 0.0)	167 ( 83.5)	1 ( 0.5)	200	1 ( 0.5)	0 ( 0.0)	1 ( 0.5)

Treatment time comprised treatment-time and recovery-time.

The number of aberrant cells at each dish was shown at the first and second lines. The total number of them was shown at the third line.

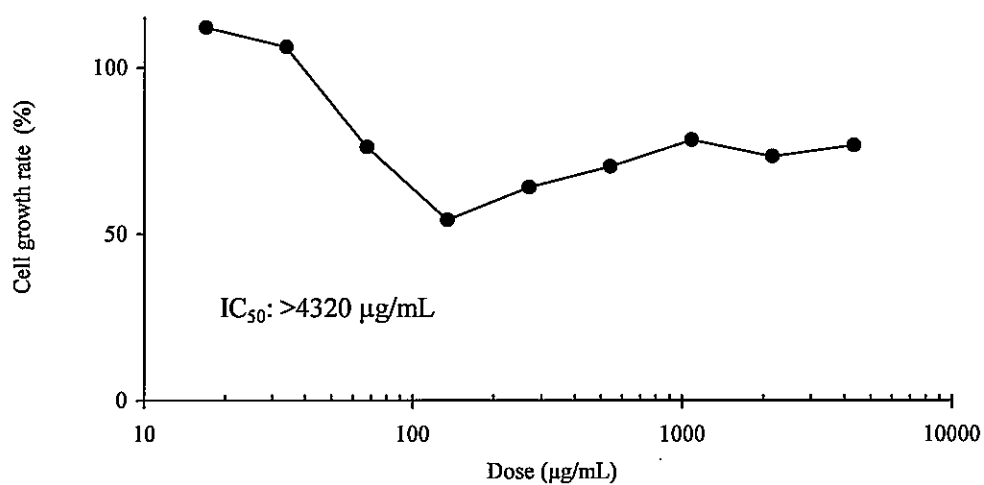
Cell growth rate at each dish was shown at the first and second lines. The average of them was shown at the third line.

Acetone was dehydrated with molecular sieves 5A 1/8.

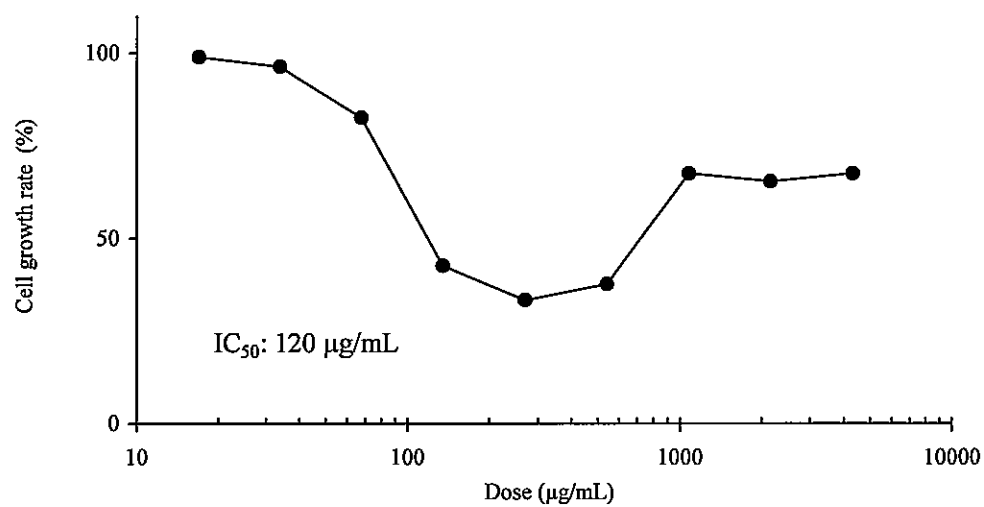
MMC: Mitomycin C

†:Precipitation of the test substance was observed at 1080  $\mu\text{g/mL}$  or more at the start of the treatment, at 2160  $\mu\text{g/mL}$  or more at the end of the treatment.

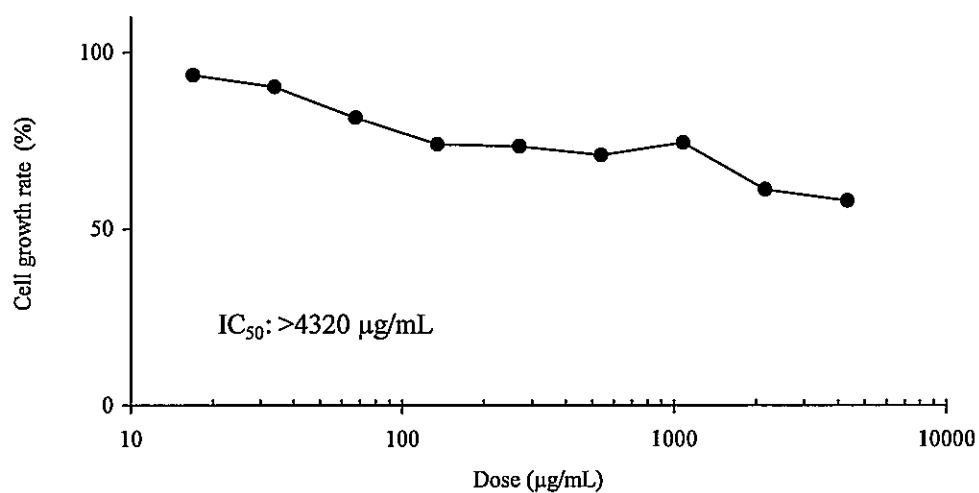
The specimens at 33.8, 67.5, 135, 270 and 540  $\mu\text{g/mL}$  were not observed.



Short-term treatment without S9 mix



Short-term treatment with S9 mix



24 hours continuous treatment

Fig. 1 Results of cell growth inhibition test of 13F-SFMA

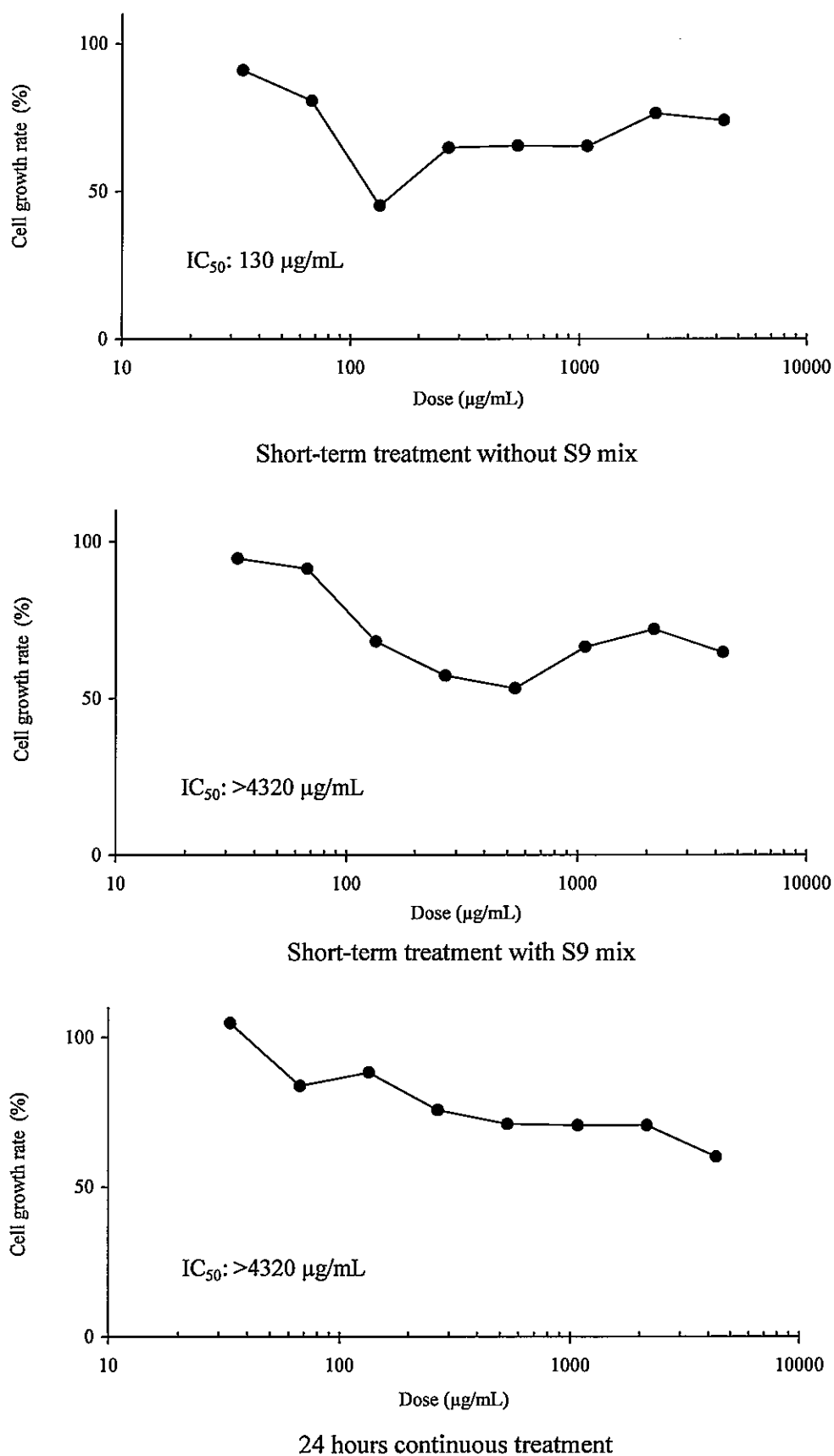


Fig. 2 Cell growth rate of the first chromosomal aberration test of 13F-SFMA

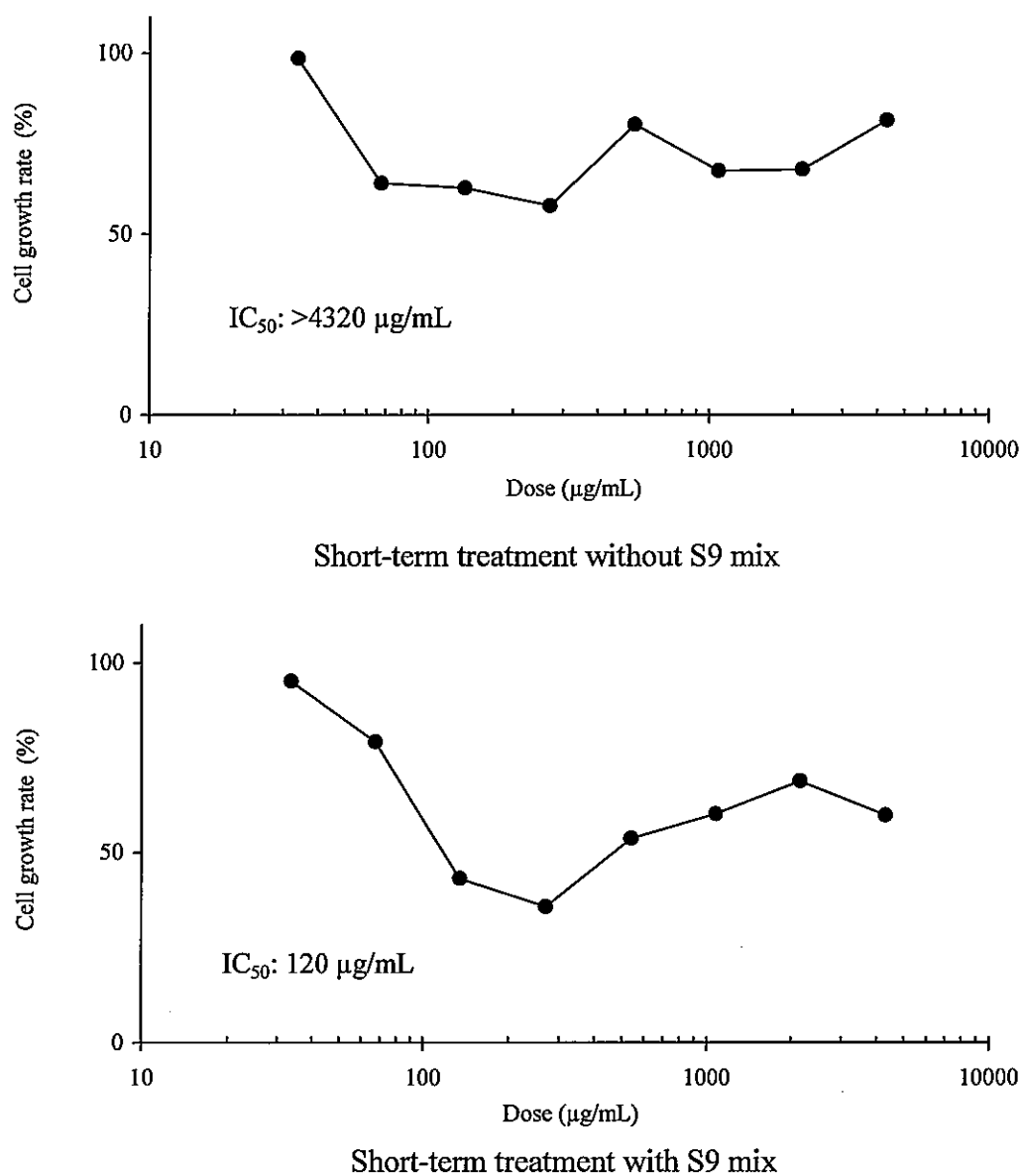


Fig. 3 Cell growth rate of the second chromosomal aberration test of 13F-SFMA

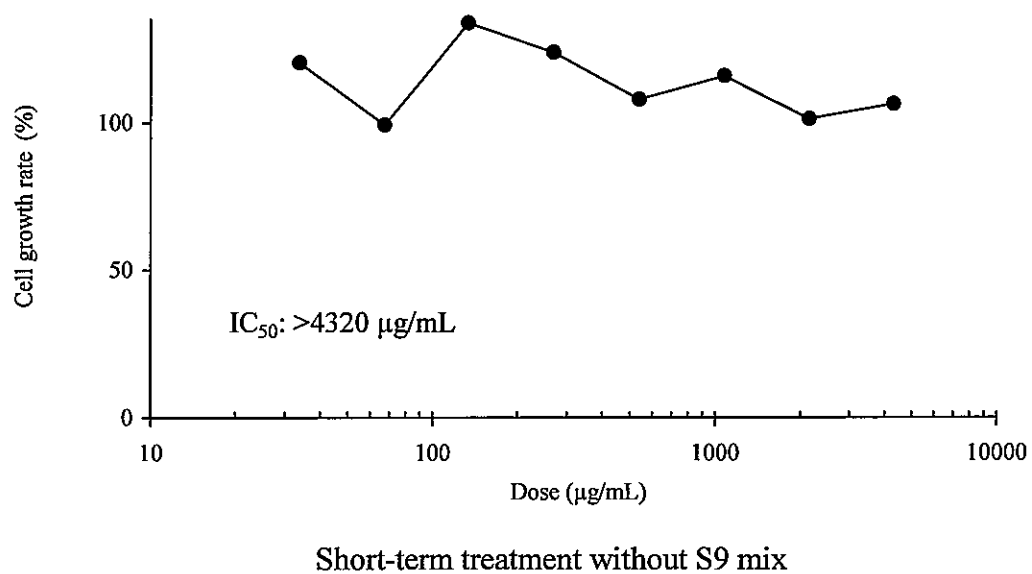


Fig. 4 Cell growth rate of the third chromosomal aberration test of 13F-SFMA

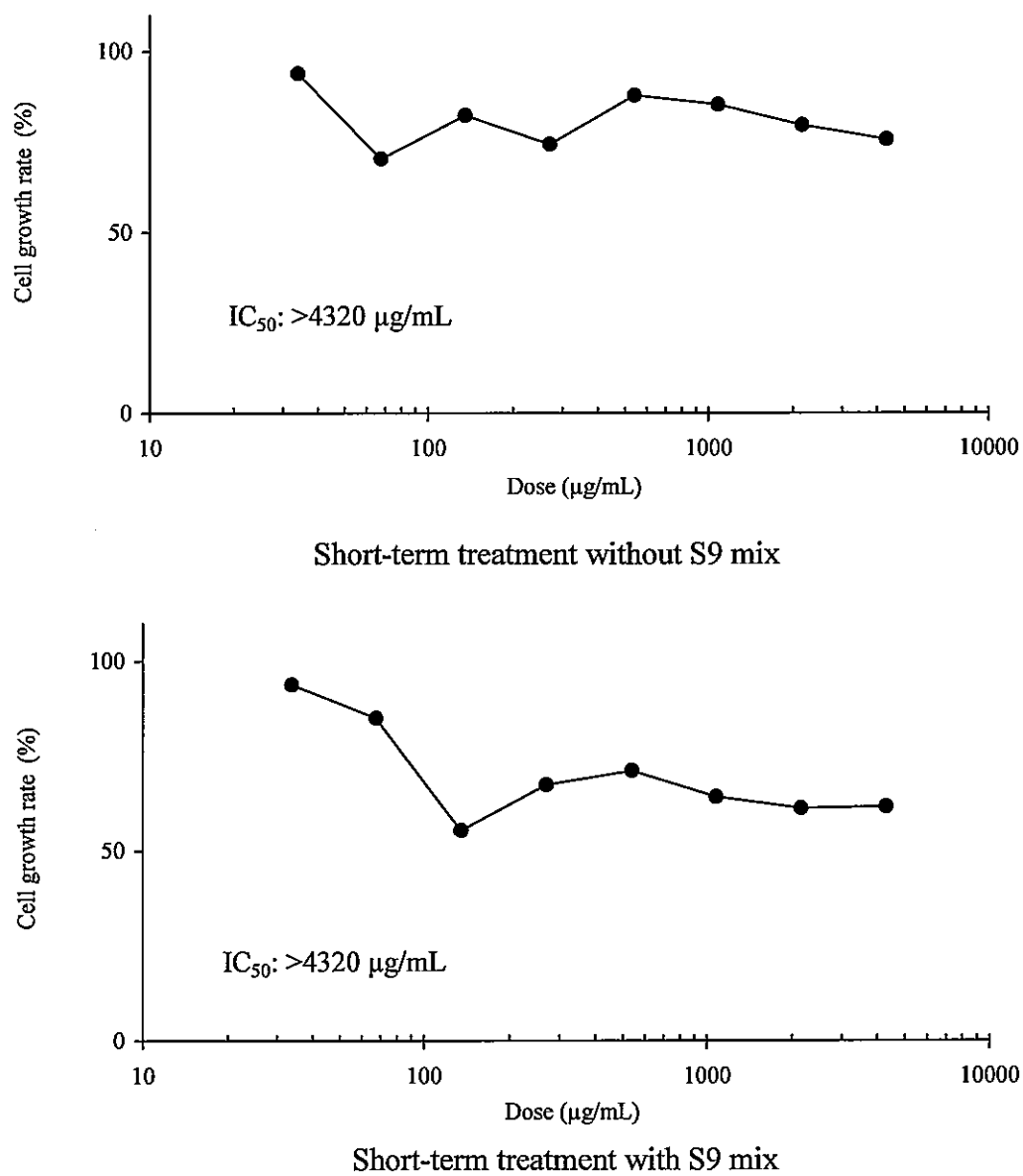


Fig. 5 Cell growth rate of the fourth chromosomal aberration test of 13F-SFMA

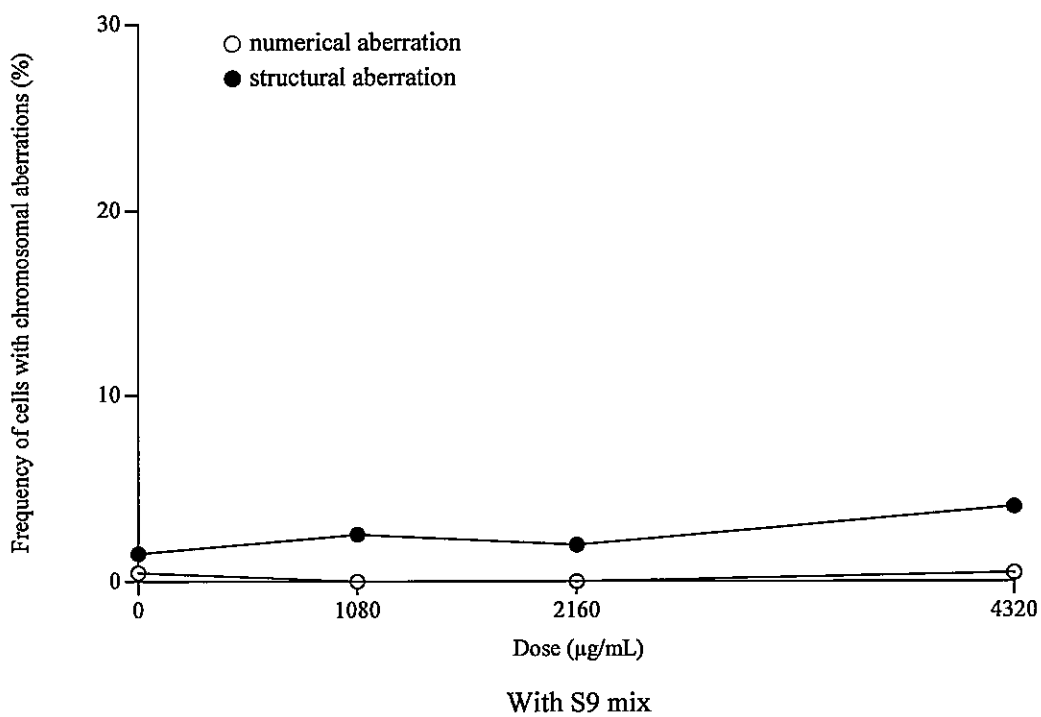
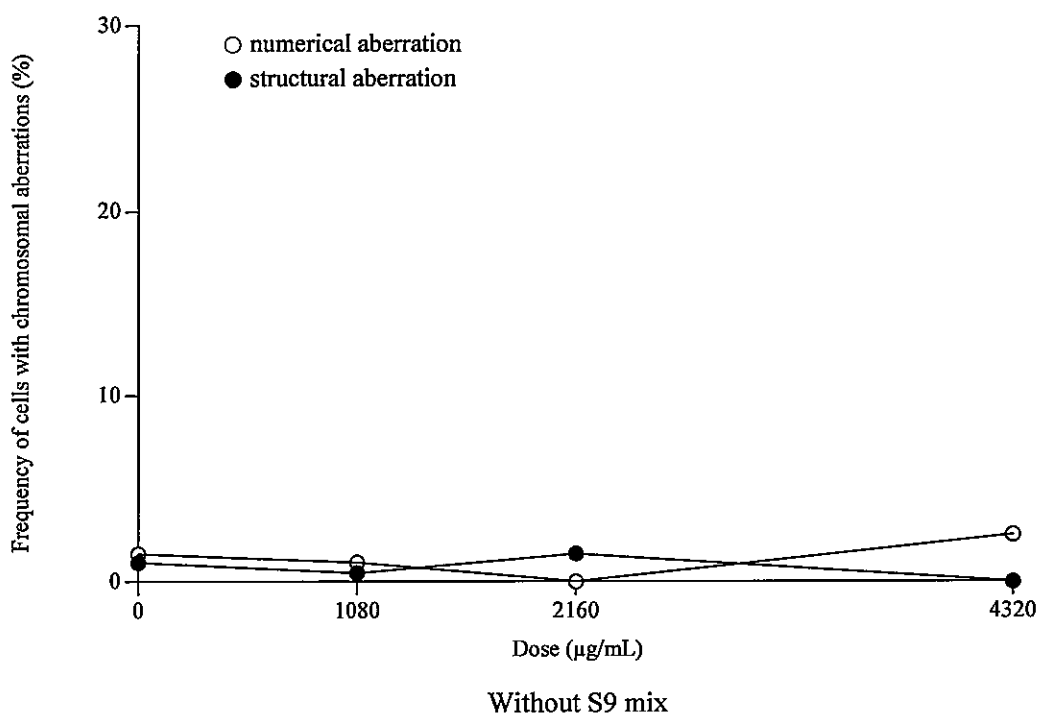


Fig. 6 Results of chromosomal aberration test in short-term treatments of 13F-SFMA

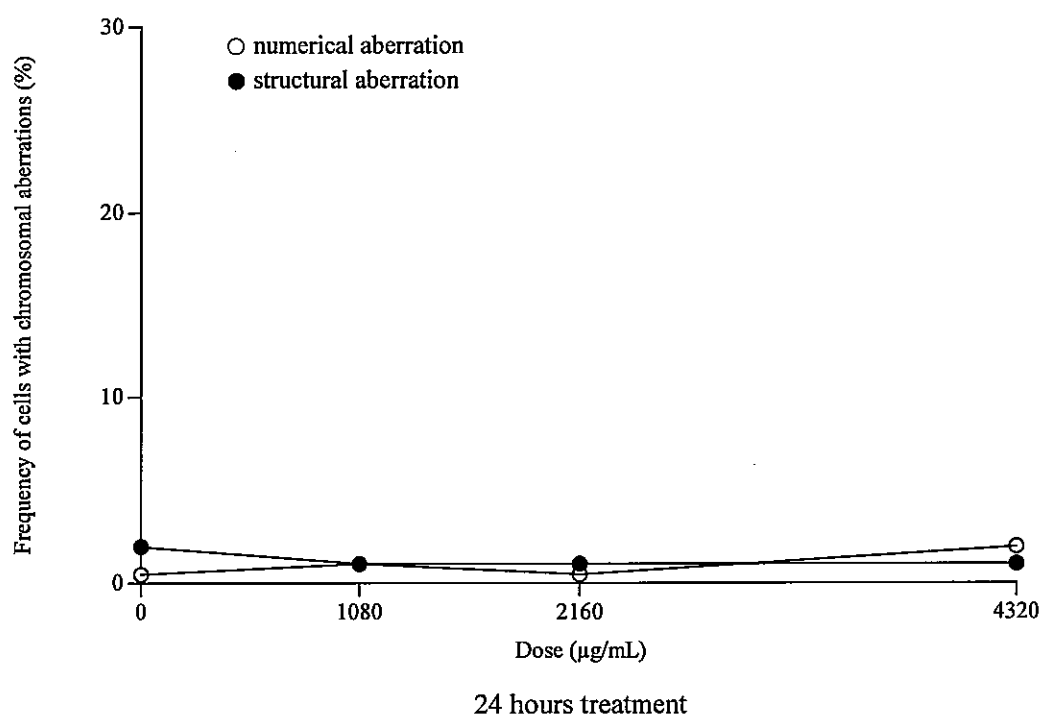


Fig. 7 Results of chromosomal aberration test in continuous treatment of 13F-SFMA