CERI

Receipt No. 837-06-T-5301

STUDY CODE: K06-1194

FINAL REPORT

CHROMOSOMAL ABERRATION TEST OF 13F-AcOH USING CULTURED MAMMALIAN CELLS

May 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-AcOH Using Cultured Mammalian Cells
Study Code: K06-1194

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)).

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

Study Director: Signed in original May 2, 2007

QUALITY ASSURANCE STATEMENT

CERI Hita
Chemicals Evaluation and Research Institute, Japan

Sponsor: DAIKIN INDUSTRIES, Ltd.
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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	Dates of Inspection	Date Reported to study Director and Management
Protocol	February 2, 2007	February 3, 2007
Reinspection of protocol	February 6, 2007	February 6, 2007
Protocol Amendment	February 13, 2007	February 13, 2007
Preparation of Test Substance	February 19, 2007	February 19, 2007
Treatment of Cells	February 19, 2007	February 19, 2007
Protocol Amendment (No. 2)	February 23, 2007	February 23, 2007
Protocol Amendment (No. 3)	March 2, 2007	March 3, 2007
Raw Data and Draft Final Report	April 27, 2007	April 27, 2007
Reinspection of Raw Data and Draft Final Report	May 1, 2007	May 1, 2007
Draft Final Report (Second Time)	May 2, 2007	May 2, 2007
Reinspection of Draft Final Report (Second Time)	May 2, 2007	May 2, 2007
Final Report	May 2, 2007	May 2, 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	May 2, 2007
Preparation of Medium and Reagent	December 6 and 7, 2006	May 2, 2007
Cell Pre-culture	November 27, 2006	May 2, 2007
Collection of Cells and Preparation of Specimens	December 5, 2006	May 2, 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 May 2, 2007

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Study Code: K06-1194
Test Substance Code: HR6898
Sponsor Code: D-0060

TITLE

Chromosomal Aberration Test of 13F-AcOH 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
3-822 Ishii-machi, Hita-shi, 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/IU 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)).

PERIOD OF STUDY

Commencement of Study:	February 1, 2007
Initiation of Experiment (Initiation of Cell Growth Inhibition Test):	February 5, 2007
Completion of Experiment (Completion of Observation of Specimens):	April 5, 2007
Completion of Study:	May 2, 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 this 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 was recognized to be accurate copy were sent to the sponsor.

STUDY DIRECTOR AND PERSONS CONCERNED WITH THE STUDY AND THE OPERATION

Study Director:

Section 3, CERI Hita

Persons Concerned with The Study and Their Operation:

(Preparation of test substance solution, cell treatment and microscopic observation of specimens)

(Microscopic observation of specimens)

APPROVAL BY AUTHOR

Study Director: Signed in original May 2, 2007

SUMMARY

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

Chromosomal aberration test was conducted in the short-term treatments without and with S9 mix at the following doses of the test substance, 279, 335, 402, 482, 579, 694, 883 and 1000 μg/mL, which were set based on the result of cell growth inhibition test.

The doses for observation of specimens were selected at 482, 579 and 694 μg/mL in the short-term treatment without S9 mix and at 402, 482 and 579 μg/mL in the short-term treatment with S9 mix. In the observation, the frequencies of cells with structural aberrations and numerical aberration cells were scored.

As a result of observation of specimens, the frequencies of cells with structural aberration were over 10% in the short-term treatments without and with S9 mix and the frequencies increased in a dose-related manner. Therefore, structural aberration was judged to be positive. Furthermore, the maximum frequency of numerical aberration cells was 8% in the short-term treatment without S9 mix, and the frequencies of numerical aberration cells were over 10% in the short-term treatment with S9 mix and the frequencies increased in a dose-related manner. Therefore, the numerical aberration was also judged to be positive.

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

It was concluded that 13F-AcOH induced 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-tridecafluorooctanoic acid

Other name: 13F-AcOH

CAS No.: —

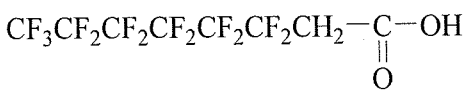
2) Lot No.

S6X01

3) Supplier

DAIKIN INDUSTRIES, Ltd.

4) Structural formula



(Molecular formula: $C_8H_3F_{13}O_2$)

5) Purity

99.4%

6) Names and concentrations of impurities

Unknown composition	0.6%

7) Physicochemical properties

Appearance at ordinary temperature:	white solid
Molecular weight:	378.09
Stability:	—
Melting point:	—
Boiling point:	—
Vapor pressure:	—
Partition coefficient (1-octanol/water):	—
Hydrolysis:	—
Solubility:	—

Degree of solubility

Water:	<100 mg/mL (measured at the testing facility)
DMSO:	≥378 mg/mL (measured at the testing facility)
Acetone:	soluble
Others:	—

8) Storage conditions

Stored in light-shading 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 frequencies of cells with structural aberration and the numerical aberration cells were below 5%.

CHL/IU cells have been recommended the availability on *in vitro* chromosome aberration test "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% dimethyl sulfoxide (DMSO) and were frozen in liquid nitrogen.

2.3 Culture Condition

Cells were cultured in a CO_2 incubator (MCO-345, SANYO Electric Co., Ltd.), which was set at 37°C and 5% CO_2 under humid condition.

2.4 Subculture

Cells were subcultured in 90-mm diameter plastic Petri dishes (Nunc A/S) twice a week. Passage number of cells was at 4 for the cell growth inhibition test and 8 for the 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. 06112409, manufactured on November 24, 2006, protein content: 22.8 mg/mL, Oriental Yeast Co., Ltd.), which was prepared from livers of 7-week-old male SD rats (body weight of rats: 211.1±9.7 g) administered 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 6 months after the day of manufacturing.

2) Composition of S9 mix

One milliliter of S9 mix consisted of 0.3 mL S9, 5 μmol $MgCl_2$, 33 μmol KCl, 5 μmol glucose-6-phosphate, 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 milliliters of a cell suspension of 5.0×10^3 cells/mL were seeded into a dish and were cultured continuously for 3 days.

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

5.1 Preparation of Test Substance Solution

1) Solvent

DMSO (Lot No. SF074, 99.9% in purity, pure solvent for measuring ultraviolet absorption spectrum, DOJINDO Laboratories)

2) Reason for selection of solvent

The test substance was insoluble at 100 mg/mL in distilled water. The test substance was dissolved at 378 mg/mL in DMSO. The solution at 378 mg/mL prepared with DMSO was not indicated any change in color nor exothermic at room temperature within 2 hours after preparation. Therefore, DMSO was selected as a solvent.

3) Preparation method

After the test substance was weighed, DMSO was added to the test substance to make an original solution using a tube mixer. A required concentration series of the test substance solutions was prepared by dilution with the same solvent and prepared to 100 times concentrations of the test substance in the medium. The test substance solutions were prepared under the yellow light (weighing of the test substance: sodium lamp, preparation: yellow fluorescent lamp). It was prepared without correcting purity of the test substance because the purity of the test substance was above 95%.

4) Preparation time and storage

The test substance solutions were prepared immediately before use, stored at room temperature under the yellow lamp and used within 1 hour after preparation.

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 and stored in an ultra-deep freezer (MDF-U481ATR, SANYO Electric Co., Ltd., the tolerance temperature: below -80°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 μL 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 μL 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 Ca^{2+} and 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 24 hours with well-mixed medium containing 50 μL of the test substance solution or the solvent and 5 mL of the fresh medium.

In the short-term and the continuous treatments, 50 μL of a 10 μg/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. In each treatment method, the color of the medium was changed to yellow at the start of treatment at 473 μg/mL or more, therefore, pH of the medium was measured using a pH meter (D-13, HORIBA, Ltd.) just after adding the medium to the test substance solution. The medium without S9 mix was used for pH measurement.

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 μL 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 (IC_{50}) was calculated. The IC_{50} was obtained from a linear line drawn between 2 plots; the one was the lowest dose with the cell growth rate of less than 50% and the other was the next lower dose of this dose. Remaining cells were then 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 3780 μg/mL equivalent to 10 mmol/L, as the maximum dose in the case of no cytotoxicity on the guideline, and 8 lower doses, 14.8, 29.5, 59.1, 118, 236, 473, 945 and 1890 μg/mL were set based on a geometric progression of 2. Duplicate dishes were used for each dose.

3) Observation and scoring

Specimens were observed to check the presence or absence of mitotic metaphases, and the frequency of the cells with chromosomal aberration was calculated by observed 50 metaphases 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 polyploid 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 (2 specimens per dish) were prepared. In the cell growth inhibition test for the short-term treatment with S9 mix, the frequencies of cells with structural aberration were over 10% and the positive result was predicted in the short-term treatment, therefore, the chromosomal aberration test for the continuous treatment was not carried out.

Treatment method		Substance	Dose
Short-term treatment	without S9 mix	MMC	0.1 μg/mL
	with S9 mix	CPA	6 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.

2) Dose levels

In the cell growth inhibition test, because a cytotoxicity that the cell growth rate was below 50% was obtained in the short-term treatments without and with S9 mix, the highest dose of the test substance was selected at the dose that was over IC_{50}. IC_{50} were calculated at 630 μg/mL and 570 μg/mL in the short-term treatments without and with S9 mix, respectively, then the highest dose was selected at 1000 μg/mL and the 7 lower doses, 279, 335, 402, 482, 579, 694 and 833 μg/mL were set based on a geometric progression of 1.2 in each treatment. Duplicate dishes were used for each dose.

3) Observation

(1) Dose for observation

All specimens of the negative and the positive controls set as the control groups were observed.

The observation doses of the test substance were selected the consecutive doses of three stages. The selection doses and the selection reason are shown below.

In each treatment, because a cytotoxicity that the cell growth rate was below 50% was obtained, the highest dose for observation was selected at the lowest dose that the cell growth rate was below 50%. In the short-term treatment without S9 mix, the lowest dose that the cell growth rate was below 50% was 694 μg/mL, therefore, the doses for observation were selected at 482, 579 and 694 μg/mL. In the short-term treatment with S9 mix, the lowest dose that the cell growth rate was below 50% was 579 μg/mL, therefore, the doses for observation were selected at 402, 482 and 579 μg/mL.

After the selection of the observation doses, randomly slide numbers were allocated 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±2 chromosomes were scored 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 numbers of polyploid (cell with 38 or more chromosomes) cells among 200 metaphase cells per dose (50 cells per specimen) observed were recorded.

6.3 Confirmation Test

Because the obvious positive result was obtained in the short–term treatments in chromosomal aberaration test, 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 showed 10% or more with a dose-related increase or the frequencies of cells with structural aberrations showed 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. The frequency of numerical aberration cells was judged according to the same criteria as that of the structural aberration. In the treatment method that chromosomal aberrations were shown 5% or more, D_{20} value indicating a concentration which will induce chromosomal aberration of 20% was calculated.

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 in the negative control showed below 5%, and 3) the frequencies of cells with structural aberrations excluding gaps in the positive controls showed 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} were calculated at 630 μg/mL in the short-term treatment without S9 mix, at 570 μg/mL in the short-term treatment with S9 mix and at 400 μg/mL in 24 hours continuous treatment.

At the start and the end of the treatment, precipitation of the test substance was observed at 1890 μg/mL or more in each treatment. The corrosion of the culture

dish was not observed in any treatment method. In each treatment, the color of the medium was changed to yellow at 473 μg/mL or more at the start of the treatment. Therefore, pH of the medium was measured just after adding the medium to the test substance solution. The result was shown as follows.

Dose (μg/mL)	pH of the medium just after adding the medium to the test substance solution
0	7.91
473	7.23
945	7.12
1890	6.87
3780	6.69

Although the frequencies of cells with structural aberration and the numerical aberration cells were below 5% at the observation doses of the test substance in the short-term treatment without S9 mix and 24 hours continuous treatment, the maximum frequencies of cells with structural aberration and the numerical aberration cells were 14.0% and 8.0%, respectively, in the short-term treatment with S9 mix and the increase of chromosomal aberration was observed.

2. CHROMOSOMAL ABERRATION TEST

2.1 Short-term Treatment (Tables 2, 3, 4 and Figs. 2, 3)

1) Without S9 mix

(1) Cell growth rate and IC_{50}

The cell growth rates at 279, 335, 402, 482, 579, 694, 833 and 1000 μg/mL of the test substance were 96.4, 88.6, 83.3, 70.0, 65.8, 49.3, 22.7 and 7.8%, respectively. The IC_{50} was calculated at 690 μg/mL.

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

Precipitation of the test substance and the corrosion of the culture dish were not observed at the start and the end of the treatment and the end of the culture at all doses. The color of the medium was changed to yellow at 482 μg/mL or more just after adding the medium to the test substance solution, however, the color of the medium returned to the ordinary color when adding the medium to the culture dish.

(3) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 2.0% in the negative control and 56.0% in the positive control. The frequencies of cells with structural aberrations at 482, 579 and 694 μg/mL were 0.5, 5.0 and 21.0%,

respectively. The freaquency was more than 10% and the freaquency increased in dose-relates manner, therefore, the results were judged to be positive.

(4) Frequency of numerical aberration cells

The frequencies of numerical aberration cells were 1.0% in the negative control and 0.5% in the positive control. The frequencies of numerical aberration cells at 482, 579 and 694 μg/mL were 1.5, 2.0 and 8.0%, respectively. The induction of numerical aberration was suspected because the frequency of the aberration cells showed 5% or more but less than 10%.

(5) D_{20} value

It was calculated at 0.77 mg/mL for structural aberration and at 2.2 mg/mL for numerical aberration.

2) With S9 mix

(1) Cell growth rate and IC_{50}

The cell growth rates at 279, 335, 402, 482, 579, 694, 833 and 1000 μg/mL of the test substance were 86.2, 75.3, 63.8, 59.4, 40.6, 20.1, 3.7 and 1.6%, respectively. The IC_{50} was calculated with 530 μg/mL.

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

Precipitation of the test substance and the corrosion of the culture dish were not observed at the start and the end of the treatment and the end of the culture. The color of the medium was changed to yellow at 482 μg/mL or more just after adding the medium to the test substance solution, however, the color of the medium returned to the ordinary color when adding the medium to the culture dish.

(3) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 1.5% in the negative control and 22.5% in the positive control. The frequencies of cells with structural aberrations at 402, 482 and 579 μg/mL were 0.5, 7.5 and 20.0%, respectively. The freaquency was more than 10% and the freaquency increased in dose-relates manner, therefore, the results were judged to be positive.

(4) Frequency of numerical aberration cells

The frequencies of numerical aberration cells were 0.5% in the negative control and 2.5% in the positive control. The frequencies of cells with structural aberrations at 402, 482 and 579 μg/mL were 3.5, 4.5 and 12.5%, respectively. The freaquency was more than 10% and the freaquency

increased in dose-relates manner, therefore, the results were judged to be positive.

(5) D_{20} value

It was calculated at 0.64 mg/mL for structural aberration and at 0.91 mg/mL for numerical aberration.

3. TYPICAL PHOTOS

The normal cell was shown in photo 1, and cell with structural aberration induced by the test substance was shown in photo 2 and numerical aberration cell induced by the test substance was shown in photo 3.

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.

As a result of chromosomal aberration test, for the short-term treatments without and with S9 mix, the frequencies of cells with structural aberration were over 10% and increased in a dose-related manner. Therefore, the structural aberration was judged to be positive. The frequencies of numerical aberration cells were over 10% in the short-term treatment with S9 mix and the frequencies increased in a dose-related manner. Therefore, numerical aberration was also judged to be positive.

In spite of pH decrease in the medium, it was presumed that the pH of the medium at 945 μg/mL or less was 7 or more because the pH of the medium is 7.12 at 945 μg/mL. Therefore, it was judged that induced structural aberrations was not caused by unphysiological condition derived from pH decrease in culture medium but caused by the test substance.

Based on the above results, it was considered that 13F-AcOH induced 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-AcOH

Substance	Dose (μg/mL)	Treatment-recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium [a]			Frequency of cells with aberrations (%)[b]	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
DMSO	0	6-18	-	100	-	-	-	2.0	0.0
13F-AcOH	14.8	6-18	-	93.2	-	-	-	n.o.	n.o.
	29.5	6-18	-	90.0	-	-	-	n.o.	n.o.
	59.1	6-18	-	98.9	-	-	-	n.o.	n.o.
	118	6-18	-	94.1	-	-	-	n.o.	n.o.
	236	6-18	-	85.8	-	-	-	2.0	0.0
	473	6-18	-	71.6	-,Y	-	-	0.0	0.0
	945	6-18	-	5.3	-,Y	-	-	no meta	
	1890	6-18	-	0.2	+,Y	+	-	no meta	
	3780	6-18	-	0.4	+,Y	+	-	no meta	
				IC_{50} : 630 μg/mL					
DMSO	0	6-18	+	100	-	-	-	2.0	0.0
13F-AcOH	14.8	6-18	+	102.5	-	-	-	n.o.	n.o.
	29.5	6-18	+	102.2	-	-	-	n.o.	n.o.
	59.1	6-18	+	100.6	-	-	-	n.o.	n.o.
	118	6-18	+	98.1	-	-	-	n.o.	n.o.
	236	6-18	+	96.9	-	-	-	0.0	2.0
	473	6-18	+	61.9	-,Y	-	-	14.0	8.0
	945	6-18	+	2.8	-,Y	-	-	no meta	
	1890	6-18	+	0.4	+,Y	+	-	no meta	
	3780	6-18	+	1.1	+,Y	+	-	no meta	
				IC_{50} : 570 μg/mL					
DMSO	0	24-0	-	100	-	-		0.0	0.0
13F-AcOH	14.8	24-0	-	108.8	-	-		n.o.	n.o.
	29.5	24-0	-	104.3	-	-		n.o.	n.o.
	59.1	24-0	-	97.9	-	-		n.o.	n.o.
	118	24-0	-	95.5	-	-		2.0	2.0
	236	24-0	-	70.6	-	-		2.0	0.0
	473	24-0	-	41.4	-,Y	-		few meta	
	945	24-0	-	0.4	-,Y	-		no meta	
	1890	24-0	-	0.8	+,Y	+		no meta	
	3780	24-0	-	0.8	+,Y	+		no meta	
				IC_{50} : 400 μg/mL					

DMSO: dimethylsulfoxide

n.o.: not observed, few meta: the frequency of metaphases was extremely few, no meta: metaphases were not observed

a) Precipitation of the test substance: -, absence; +, presence. Color change of medium to yellow: Y

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

The highest dose was set at 3780 μg/mL equivalent to 10 mmol/L of the test substance, which was the maximum dose in the case of no cytotoxicity according to the guidelines, and above dose levels based on a geometric progression of 2 were selected.

Table 2 Results of chromosomal aberration test of 13F-AcOH

Substance	Dose (μg/mL)	Treatment-recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium [a]			Frequency of cells with aberrations (%)[b]	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
DMSO	0	6-18	-	100	-	-	-	2.0	1.0
13F-AcOH	279	6-18	-	96.4	-	-	-	n.o.	n.o.
	335	6-18	-	88.6	-	-	-	n.o.	n.o.
	402	6-18	-	83.3	-	-	-	n.o.	n.o.
	482	6-18	-	70.0	-,Y	-	-	0.5	1.5
	579	6-18	-	65.8	-,Y	-	-	5.0	2.0
	694	6-18	-	49.3	-,Y	-	-	21.0	8.0
	833	6-18	-	22.7	-,Y	-	-	n.o.	n.o.
	1000	6-18	-	7.8	-,Y	-	-	few meta	
MMC	0.1	6-18	-	ND	-	-	-	56.0	0.5
			IC_{50} :	690 μg/mL		D_{20} (mg/mL):		0.77	2.2
DMSO	0	6-18	+	100	-	-	-	1.5	0.5
13F-AcOH	279	6-18	+	86.2	-	-	-	n.o.	n.o.
	335	6-18	+	75.3	-	-	-	n.o.	n.o.
	402	6-18	+	63.8	-	-	-	0.5	3.5
	482	6-18	+	59.4	-,Y	-	-	7.5	4.5
	579	6-18	+	40.6	-,Y	-	-	20.0	12.5
	694	6-18	+	20.1	-,Y	-	-	n.o.	n.o.
	833	6-18	+	3.7	-,Y	-	-	no meta	
	1000	6-18	+	1.6	-,Y	-	-	no meta	
CPA	6	6-18	+	ND	-	-	-	22.5	2.5
			IC_{50} :	530 μg/mL		D_{20} (mg/mL):		0.64	0.91

DMSO: dimethylsulfoxide, MMC: mitomycin C, CPA: cyclophosphamide monohydrate

ND: not detected

n.o.: not observed, few meta: the frequency of metaphases was extremely few, no meta: metaphases were not observed

a) Precipitation of the test substance: -, absence; +, presence. Color change of medium to yellow: Y

The color of the medium was changed to yellow just after adding the medium to the test substance solution. However, the color of the medium returned to the ordinary color when adding the medium to the culture dish.

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

Table 3 Results of chromosomal aberration test (short-term treatment without S9 mix)

Name of test substance: 13F-AcOH

K06-1194

Treatment time (h)	S9 mix	Dose (μg/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 break	Chromatid exchange	Chromosome break	Chromosome exchange	Others	Total number of cells with aberrations			Number of cells observed	Polyploids	Others	Total number of cells with aberrations
6 - 18	-	Negative control (DMSO)	100	1	2	0	1	0	4	0	100	100	2	0	2
			100	0	0	0	0	0	0	0		100	0	0	0
		0	200	1 (0.5)	2 (1.0)	0 (0.0)	1 (0.5)	0 (0.0)	4 (2.0)	0 (0.0)		200	2 (1.0)	0 (0.0)	2 (1.0)
6 - 18	-	279	0								88.5	0			
			0								104.3	0			
			0								(96.4)	0			
6 - 18	-	335	0								87.6	0			
			0								89.5	0			
			0								(88.6)	0			
6 - 18	-	402	0								87.9	0			
			0								78.7	0			
			0								(83.3)	0			
6 - 18	-	482	100	1	0	0	0	0	1	0	68.3	100	3	0	3
			100	0	0	0	0	0	0	1	71.7	100	0	0	0
			200	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	1 (0.5)	(70.0)	200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	-	579	100	2	3	0	0	0	5	0	69.8	100	2	0	2
			100	3	2	0	0	0	5	0	61.7	100	2	0	2
			200	5 (2.5)	5 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	10 (5.0)	0 (0.0)	(65.8)	200	4 (2.0)	0 (0.0)	4 (2.0)
6 - 18	-	694	100	8	19	0	0	0	21	0	47.4	100	7	3	10
			100	11	15	0	0	0	21	0	51.1	100	5	1	6
			200	19 (9.5)	34 (17.0)	0 (0.0)	0 (0.0)	0 (0.0)	42 (21.0)	0 (0.0)	(49.3)	200	12 (6.0)	4 (2.0)	16 (8.0)
6 - 18	-	833	0								23.1	0			
			0								22.2	0			
			0								(22.7)	0			
6 - 18	-	1000	0	few metaphases							7.4	0	few metaphases		
			0								8.1	0			
			0								(7.8)	0			
6 - 18	-	Positive control (MMC)	100	28	40	0	0	0	53	0		100	1	0	1
			100	31	45	0	1	0	59	1		100	0	0	0
		0.1	200	59 (29.5)	85 (42.5)	0 (0.0)	1 (0.5)	0 (0.0)	112 (56.0)	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 were shown at the first and the 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 the second lines. The average of them was shown at the third line.

Endoreduplication was shown at others in numerical chromosomal aberrations.

DMSO: dimethylsulfoxide

MMC: mitomycin C

few metaphases: the frequency of metaphases was extremely few and it was unable to analyze chromosomes

The specimens at 279, 335, 402, 833 and 1000 μg/mL were not observed.

K06-1194

Table 4 Results of chromosomal aberration test (short-term treatment with S9 mix)

Name of test substance: 13F-AcOH

K06-1194

Treatment time (h)	S9 mix	Dose (μg/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 break	Chromatid exchange	Chromosome break	Chromosome exchange	Others	Total number of cells with aberrations			Number of cells observed	Polyploids	Others	Total number of cells with aberrations
6 - 18	+	Negative control (DMSO)	100	1	0	0	0	0	1	0	100	100	1	0	1
			100	0	0	1	1	0	2	2		100	0	0	0
		0	200	1 (0.5)	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	3 (1.5)	2 (1.0)		200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	+	279	0								82.3	0			
			0								90.1	0			
			0								(86.2)	0			
6 - 18	+	335	0								73.8	0			
			0								76.7	0			
			0								(75.3)	0			
6 - 18	+	402	100	1	0	0	0	0	1	0	61.0	100	4	0	4
			100	0	0	0	0	0	0	0	66.5	100	3	0	3
			200	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	(63.8)	200	7 (3.5)	0 (0.0)	7 (3.5)
6 - 18	+	482	100	5	3	0	1	0	8	0	57.5	100	4	0	4
			100	5	3	0	0	0	7	1	61.2	100	4	1	5
			200	10 (5.0)	6 (3.0)	0 (0.0)	1 (0.5)	0 (0.0)	15 (7.5)	1 (0.5)	(59.4)	200	8 (4.0)	1 (0.5)	9 (4.5)
6 - 18	+	579	100	13	14	0	0	0	18	0	37.8	100	5	9	14
			100	13	19	0	0	0	22	0	43.3	100	3	8	11
			200	26 (13.0)	33 (16.5)	0 (0.0)	0 (0.0)	0 (0.0)	40 (20.0)	0 (0.0)	(40.6)	200	8 (4.0)	17 (8.5)	25 (12.5)
6 - 18	+	694	0								20.0	0			
			0								20.2	0			
			0								(20.1)	0			
6 - 18	+	833	0								3.7	0			
			0	no metaphases							3.7	0	no metaphases		
			0								(3.7)	0			
6 - 18	+	1000	0								1.4	0			
			0	no metaphases							1.8	0	no metaphases		
			0								(1.6)	0			
6 - 18	+	Positive control (CPA)	100	15	6	1	0	0	22	1		100	3	0	3
			100	7	15	2	0	0	23	1		100	2	0	2
		6	200	22 (11.0)	21 (10.5)	3 (1.5)	0 (0.0)	0 (0.0)	45 (22.5)	2 (1.0)		200	5 (2.5)	0 (0.0)	5 (2.5)

Treatment time comprised treatment-time and recovery-time.

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

Endoreduplication was shown at others in numerical chromosomal aberrations.

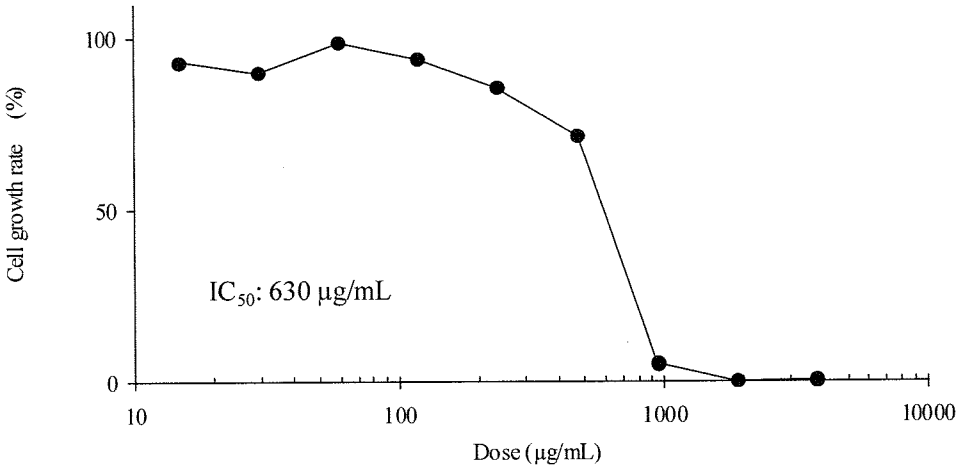
DMSO: dimethylsulfoxide

CPA: cyclophosphamide monohydrate

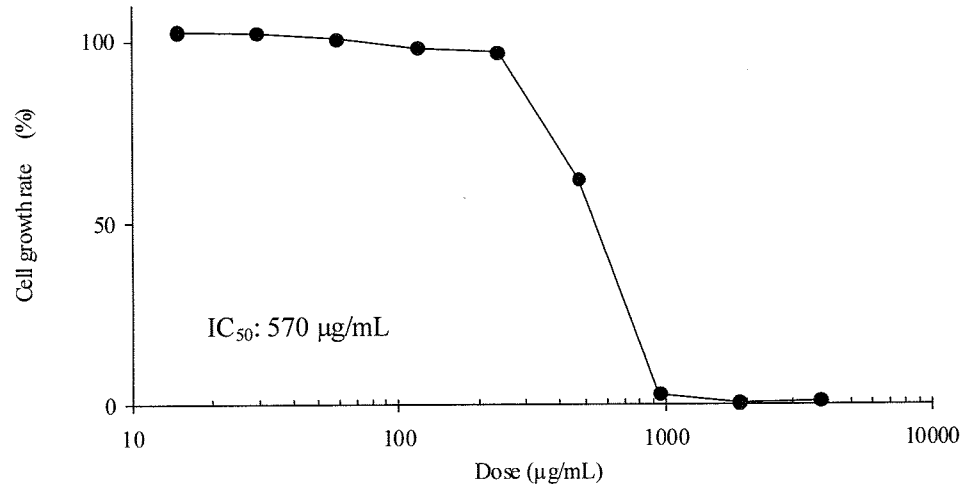
no metaphases: metaphases were not observed.

The specimens at 279, 335, 694, 833 and 1000 μg/mL were not observed.

K06-1194



Short-term treatment without S9 mix



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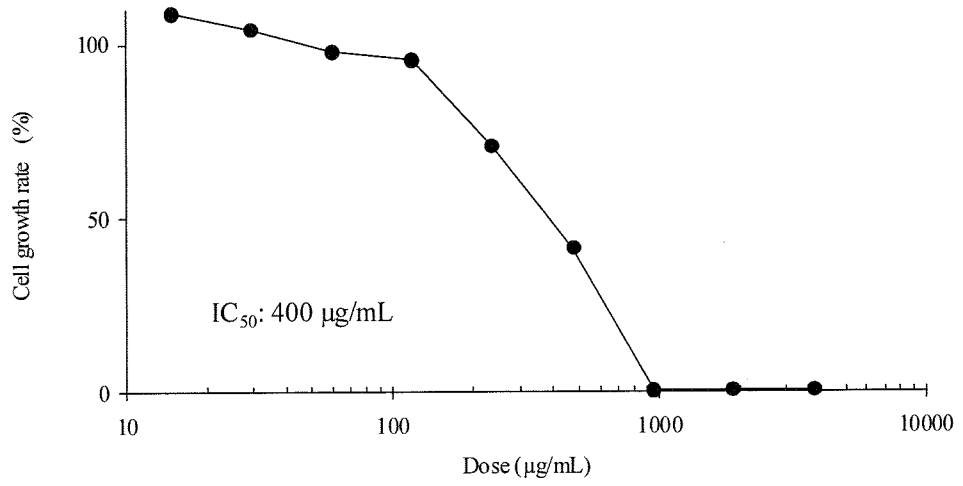
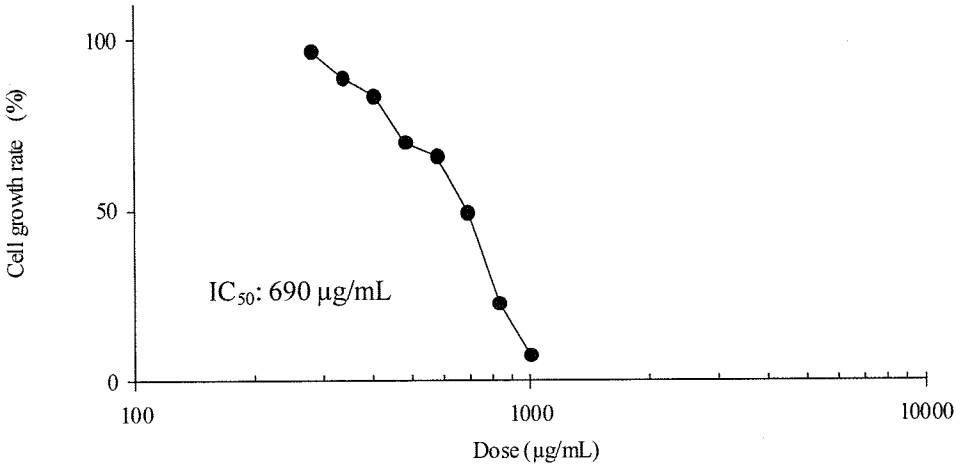
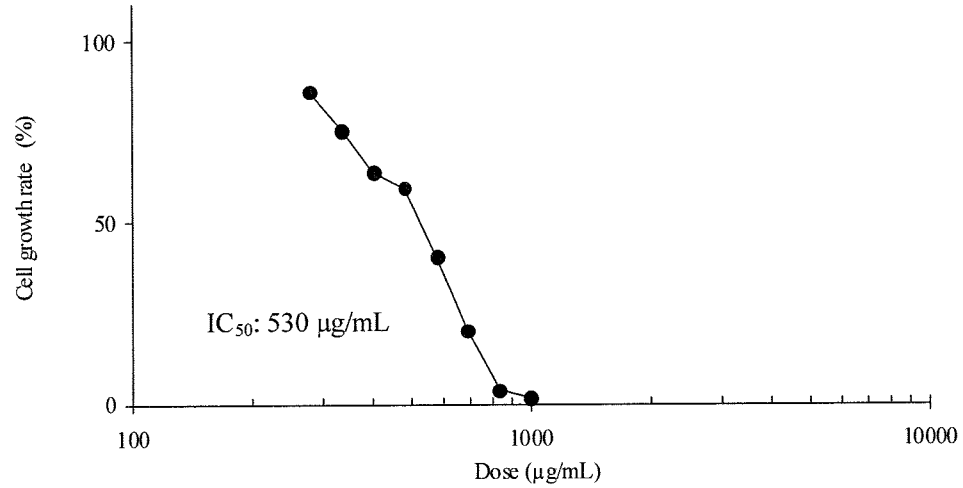


24 hours continuous treatment

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

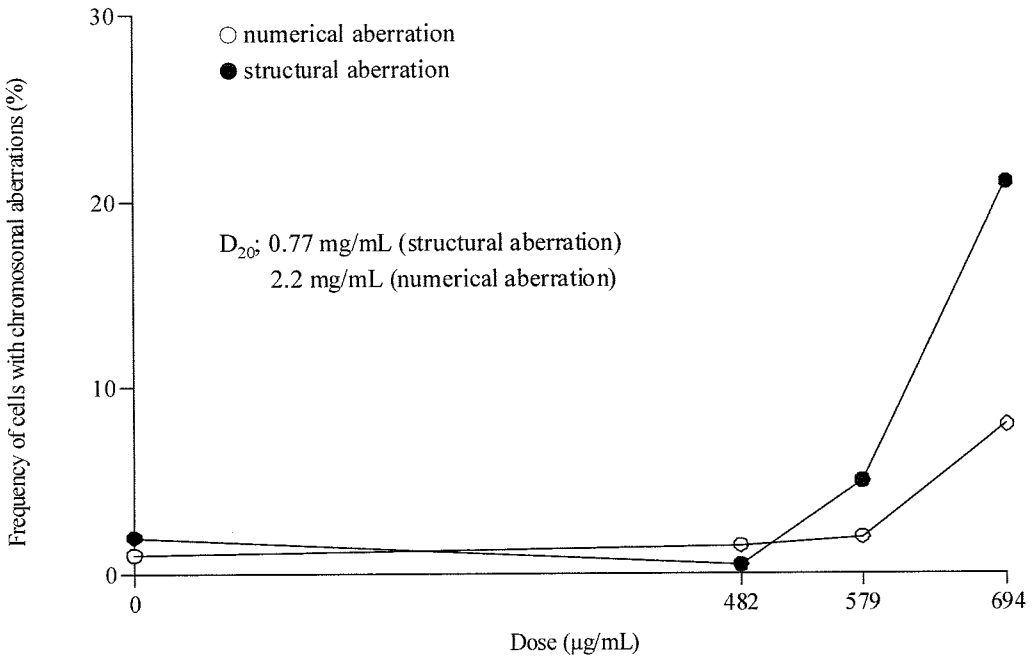


Short-term treatment without S9 mix

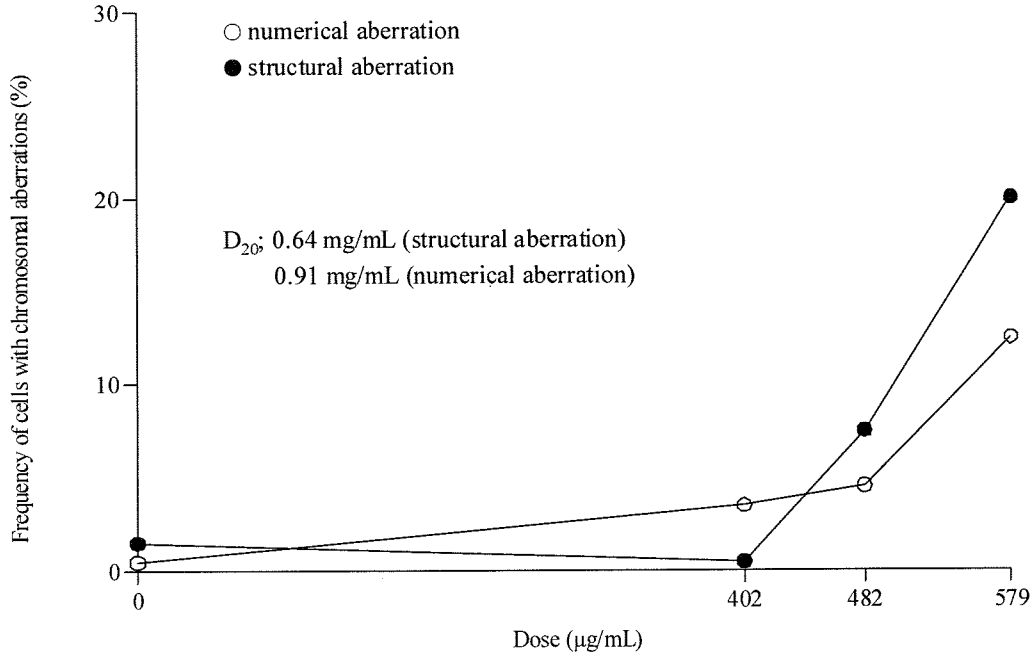


Short-term treatment with S9 mix

Fig. 2 Cell growth rate in chromosomal aberration test of 13F-AcOH



Without S9 mix



With S9 mix

Fig.3 Results of chromosomal aberration test in short-term treatments of 13F-AcOH

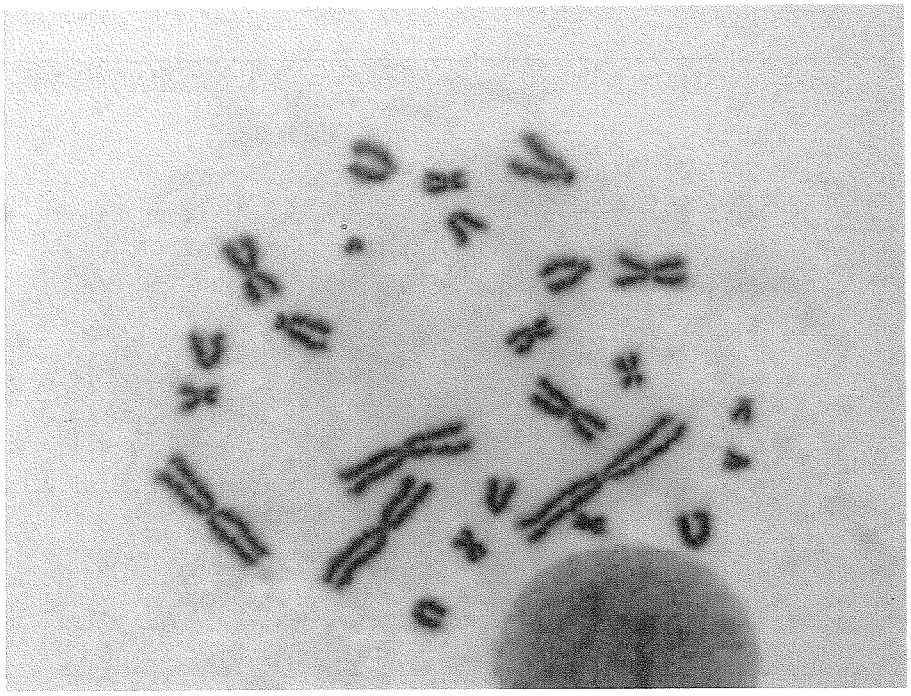


Photo 1 Normal cell
Negative control in short-term treatment with S9 mix

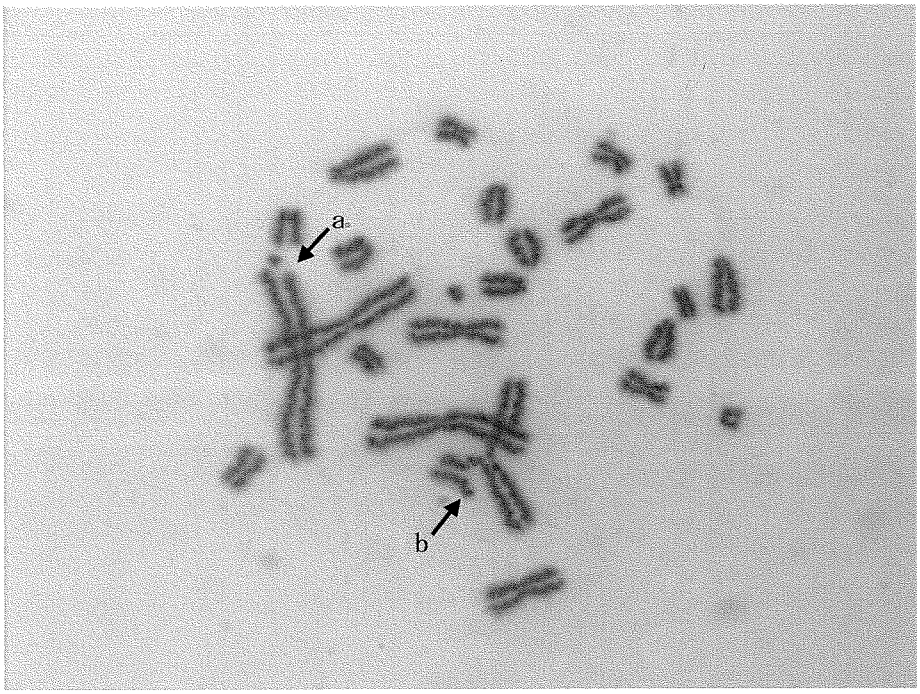


Photo 2 Structural aberration induced by 13F-AcOH
579 μg/mL in short-term treatment with S9 mix
a : chromatid break b : chromatid exchange

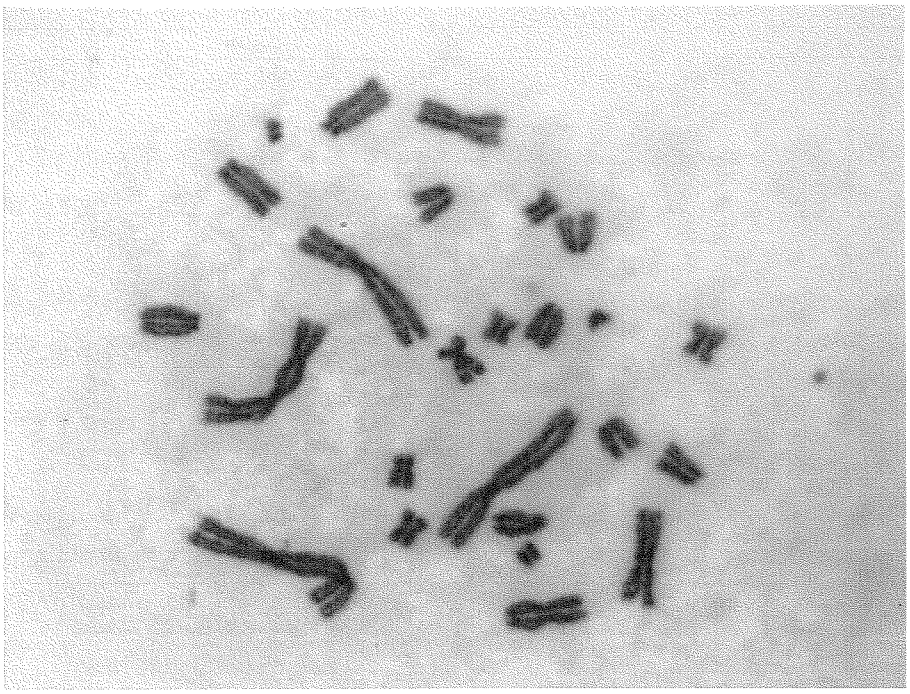


Photo 3 Numerical aberration induced by 13F-AcOH
579 μg/mL in short-term treatment with S9 mix
Polyploid (endoreduplication)