STATEMENT

TITLE OF STUDY

Chromosomal Aberration Test of 13F-EtOH Using Cultured Mammalian Cells (Study Code: K06-1192)

I, the undersigned, hereby declare that this report provides a correct English translation of the final report (Study Code: K06-1192, issued on March 26, 2007) audited by Quality Assurance Unit of CERI Hita, Chemicals Evaluation and Research Institute, Japan.

October 1, 2007

Date

CERI Hita

Chemicals Evaluation and Research Institute, Japan

CERI

Receipt No.837-06-T-5259

STUDY CODE: K06-1192

FINAL REPORT

CHROMOSOMAL ABERRATION TEST OF 13F-EtOH 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-EtOH Using Cultured Mammalian Cells
Study Code: K06-1192

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 26, 2007

QUALITY ASSURANCE STATEMENT

CERI Hita
Chemicals Evaluation and Research Institute, Japan

Sponsor: DAIKIN INDUSTRIES, LTD.
Title: Chromosomal Aberration Test of 13F-EtOH Using Cultured Mammalian Cells
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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	Date of Inspection	Date Reported to Study Director and Management
Protocol	December 13, 2006	December 13, 2006
Preparation of Test Substance	December 14, 2006	December 16, 2006
Treatment of Cells	December 14, 2006	December 16, 2006
Reinspection of Protocol	December 15, 2006	December 16, 2006
Protocol Amendment	December 20, 2006	December 20, 2006
Protocol Amendment (No. 2)	January 12, 2007	January 12, 2007
Protocol Amendment (No. 3)	January 17, 2007	January 17, 2007
Protocol Amendment (No. 4)	January 27, 2007	January 27, 2007
Protocol Amendment (No. 5)	March 6, 2007	March 6, 2007
Protocol Amendment (No. 6)	March 13, 2007	March 13, 2007
Raw Data and Draft Final Report	March 21, 2007	March 21, 2007
Reinspection of Raw Data and Draft Final Report	March 23, 2007	March 23, 2007
Draft Final Report (Second Time)	March 25, 2007	March 25, 2007
Reinspection of Draft Final Report (Second Time)	March 26, 2007	March 26, 2007
Final Report	March 26, 2007	March 26, 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 26, 2007
Preparation of Medium and Reagent	December 6 and 7, 2006	March 26, 2007
Cell Pre-culture	November 27, 2006	March 26, 2007
Collection of Cells and Preparation of Specimens	December 5, 2006	March 26, 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 26, 2007

TABLE OF CONTENTS

Study Code: K06-1192
Test Substance Code: HR6854
Sponsor Code: D-0060

TITLE

Chromosomal Aberration Test of 13F-EtOH 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/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)) 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 14, 2006
Completion of Experiment (Completion of Observation of Specimens):	March 12, 2007
Completion of Study:	March 26, 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:

Section 3, CERI Hita

Persons Concerned with the Study and Their Operation:

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

(Preparation of test substance solution and cell treatment)

(Microscopic observation of specimens)

APPROVAL BY AUTHOR

Study Director: Signed in original March 26, 2007

SUMMARY

The ability of 13F-EtOH 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 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL in the short-term treatments without and with S9 mix and at 67.0, 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL in the 24 hours continuous treatment.

In the chromosomal aberration test, the observation doses for evaluation were selected three doses in each treatment, *i.e.*, 116, 139 and 167 μg/mL in the short-term treatments without and with S9 mix and 96.5, 116 and 139 μg/mL in the 24 hours continuous treatment. Then, the frequencies of cells with structural aberrations and numerical aberration cells were examined.

As a result of observation of specimens, the frequencies of numerical aberration cells were below 5% at all observation doses of the test substance in all treatment methods, therefore, numerical aberration was judged to be negative. Although the frequencies of cells with structural aberrations were below 5% in the short-term treatment without S9 mix and the 24 hours continuous treatment, it was over 5% in the short-term treatment with S9 mix. Furthermore, as the result of the confirmation test, the frequencies were over 10% and increased in dose-related manner in the short-term treatment with S9 mix, therefore, structural aberration was 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 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-EtOH did not induce numerical aberration but induced structural 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

2-(Perfluorohexyl)ethanol

Other name: 13F-EtOH

CAS No.: 647-42-7

2) Lot No.

180804

3) Supplier

DAIKIN INDUSTRIES, LTD.

4) Structural formula

$HOCH_2CH_2CF_2CF_2CF_2CF_2CF_2CF_3$

(Molecular formula: $C_8H_5F_{13}O$)

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:	364.10
Stability:	–
Melting point:	–
Boiling point:	78°C at 14 mmHg
Vapor pressure:	–
Density:	1.678 g/cm^3 at 20°C
Partition coefficient (1-octanol/water):	–
Hydrolyzability:	Unknown
Solubility	–
Degree of solubility	
Water:	Insoluble
Dimethyl sulfoxide (DMSO):	≥364 mg/mL (measured at the testing facility)
Acetone:	Soluble (miscible in all proportions)
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_2 incubator (MCO-345, SANYO Electric Co., Ltd. and Model 530, Wakenyaku 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 Petri dishes (Nunc A/S) twice a week. Passage number of cells was at 7 for the first, 9 for the second and 15 for the third cell growth inhibition test, 17 for the first chromosomal aberration test and 12 for the confirmation 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, used for the cell growth inhibition test and the chromosomal aberration test, manufactured on September 1, 2006, protein content: 20.2 mg/mL and Lot No. 06112409, used for the the confirmation test, 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 rat (body weight of rats: 215.1±10.7 g (Lot No. 06090106) and 211.1±9.7 g (Lot No. 06112409)) 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 $MgCl_2$, 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 5.0×10^3 cells/mL were seeded into a dish and were cultured continuously for 3 days in the first and the second cell growth inhibition tests and the confirmation test. Five milliliter of a cell suspension of 1.5×10^4 cells/mL were seeded into a dish and were cultured continuously for 2 days in the third cell growth inhibition test and the chromosomal aberration test.

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 not soluble in water as preinformed by the sponsor. The test substance was soluble in DMSO at 364 mg/mL. The test substance solution at 364 mg/mL in 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 in this study.

3) Preparation method

After the test substance was weighed, DMSO 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. It was prepared without correcting purity of the test substance because the purity of the test substance was above 95%. Preparation was conducted under the yellow light.

4) Preparation time

The test substance solutions were prepared immediately before use. It was stored in a dark place at room temperature 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°C).

2) Preparation time and expiry in use

The positive control substance solutions were thawed at the time of use and used within 1.5 hours. 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.

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 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 3640 μg/mL equivalent to 10 mmol/L, as the maximum dose in the case of no cytotoxicity on the guideline, and 14.2, 28.4, 56.9, 114, 228, 445, 910, 1820 and 3640 μg/mL were set based on a geometric progression of 2, respectively.

In the first cell growth inhibition test, because the cell growth rates were fluctuated between dishes and/or clear dose-related decrease of cell growth rate was not obtained in the short-term treatment without S9 mix and the 24 hours continuous treatment, the second cell growth inhibition test were carried out in these treatments. The doses set in the second test were identical with the first test. In the short-term treatment with S9 mix, there was not fluctuation among cell growth rates. Therefore, the second test was not carried out for this treatment.

The fluctuation of the cell growth rate was also observed in the second test as with the first test in both treatments and the curve of cell growth rate were different to the first test in the short-term treatment without S9 mix. Therefore, the third test was carried out. In the short-term treatment with S9 mix, to confirm the reproducibility of the test result, the cell growth test was carried out employing same doses as the first test at the same time as the third test.

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 in the first and the third test 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 third cell growth inhibition test, the highest dose of the test substance was selected at the dose that was over IC_{50} because a cytotoxicity that the cell growth rate was below 50% was obtained for all treatment methods. IC_{50} was calculated at 160, 140 and 110 μg/mL in the short-term treatments without and with S9 mix and the 24 hours continuous treatment, respectively. Therefore, the highest dose was selected at 240 μg/mL in all treatment methods and the following seven or eight doses were set based on a geometric progression of 1.2.

Treatment method		Setting doses of test substance
Short-term treatment	Without S9 mix	80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL
	With S9 mix	80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL
24 hours continuous treatment		67.0, 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL

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 observation doses and the reason for selection are shown below.

In all treatment methods, 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%.

The lowest dose that the cell growth rate was below 50% was at 167 μg/mL in all treatment methods, therefore, the highest dose was selected at 167 μg/mL and 116, 139 and 167 μg/mL were selected as the doses for observation of specimens in the short-term treatments without and with S9 mix.

However, in the 24 hours continuous treatment, the index of metaphase cells was extremely low at 167 μg/mL and it was difficult to observe sufficient number of cells to evaluate chromosomal aberration. Therefore, 139 μg/mL that sufficient number of metaphase cells was existed was set as the highest dose and 96.5, 116 and 139 μg/mL were selected as the doses for observation of specimens.

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±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 in the short-term treatment with S9 mix, frequencies of cells with structural aberrations increased slightly, 6.0% and 5.0% for 116 and 167 μg/mL, respectively, and induction of chromosomal aberration was suspected. Therefore, confirmation test was carried out in the short-term treatment with S9 mix. In confirmation test, 8 doses, 67.0, 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL, were set based on a geometric progression of 1.2.

In the confirmation test, because a cytotoxicity that the cell growth rate was below 50% was also obtained, the highest dose for observation was set at the lowest dose that the cell growth rate was below 50% and consecutive three doses were selected. The lowest dose that the cell growth rate was below 50% was 167 μg/mL, therefore, 116, 139 and 167 μg/mL were selected. Experimental procedures and observation of specimens in the confirmation test were conducted according to the chromosomal aberration test.

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. D_{20} value indicating a concentration that chromosomal aberration was observed in 20% of cells was calculated in the treatment method that the frequencies of cells with chromosomal aberrations were 5% or more.

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

1.1 The first test (Table 1 and Fig. 1)

The IC_{50}s were calculated at 580 μg/mL in the short-term treatment without S9 mix, at 140 μg/mL in the short-term treatment with S9 mix and at 160 μg/mL in the 24 hours continuous treatment.

At the start and the end of the treatment, precipitation of the test substance was observed at 228 μg/mL or more in the short-term treatment without S9 mix and the 24 hours continuous treatment and at 455 μg/mL or more in the short-term treatment with S9 mix. The color change of the medium and the corrosion of the culture dish were not observed at any doses.

The frequencies of cells with structural aberrations and numerical aberration cells were below 5% at all observation doses of the test substance in each treatment.

1.2 The second test (Table 2 and Fig. 2)

The IC_{50}s were calculated at 190 μg/mL in the short-term treatment without S9 mix and at 160 μg/mL in the 24 hours continuous treatment.

Precipitation of the test substance was observed at 228 μg/mL or more in all treatment methods at the start and the end of the treatment and at 1820 μg/mL or more in the short-term treatment without S9 mix 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. The specimens were not observed.

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

The IC_{50}s were calculated at 160 μg/mL in the short-term treatment without S9 mix, at 140 μg/mL in the short-term treatment with S9 mix and at 110 μg/mL in the 24 hours continuous treatment.

Precipitation of the test substance was observed at 455 μg/mL or more in all treatment methods at the start and the end of the treatment and at 910 μg/mL or more in the short-term treatments without and with S9 mix 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.

The frequencies of cells with structural aberrations and numerical aberration cells were below 5% at all observation doses of the test substance in each treatment.

2. CHROMOSOMAL ABERRATION TEST

2.1 Short-term Treatment (Tables 4, 5, 6 and Figs. 4, 5)

1) Without S9 mix

(1) Cell growth rate and IC_{50}

The cell growth rates at 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL of the test substance were 99.9, 99.2, 85.8, 62.5, 29.0, 0.8 and 0.4%, respectively. The IC_{50} was calculated at 150 μg/mL.

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

Precipitation of the test substance, color change of the medium and the corrosion of the culture dish were not observed at any doses at the start and the end of the treatment and the end of the culture.

(3) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 3.0% in the negative control and 68.5% in the positive control.

The frequencies at 116, 139 and 167 μg/mL of the test substance were 3.5, 2.0 and 2.5%, respectively. 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.

2) With S9 mix

(1) Cell growth rate and IC_{50}

The cell growth rates at 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL of the test substance were 93.5, 92.1, 80.5, 76.2, 44.9, 23.2 and 46.4%, respectively. The IC_{50} was calculated at 160 μg/mL.

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

Precipitation of the test substance, color change of the medium and the corrosion of the culture dish were not observed at any doses at the start and the end of the treatment and the end of the culture.

(3) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 0.5% in the negative control and 56.0% in the positive control.

The frequencies at 116, 139 and 167 μg/mL of the test substance were 6.0, 4.0 and 5.0%, respectively, and were more than 5%.

(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.

(5) D_{20} value

It was calculated at 0.68 mg/mL for structural aberration.

2.2 Twenty Four Hours Continuous Treatment (Tables 4, 7 and Figs. 4, 6)

1) Cell growth rate and IC_{50}

The cell growth rates at 67.0, 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL of the test substance were 99.4, 87.2, 85.3, 85.1, 56.5, 8.7, 1.8 and 7.1%, respectively. The IC_{50} was calculated at 140 μg/mL.

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

Precipitation of the test substance, color change of the medium and the corrosion of the culture dish were not observed at any doses at the start and the end of the treatment and the end of the culture.

3) Frequency of cells with structural aberrations

The frequencies of cells with structural aberrations were 1.5% in the negative control and 77.0% in the positive control.

The frequencies at 96.5, 116 and 139 μg/mL were 1.5, 1.5 and 0.5%, respectively, 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.

3. CONFIRMATION TEST IN SHORT-TERM TREATMENT WITH S9 MIX (Tables 8, 9 and Figs. 7, 8)

3.1 Cell Growth Rate and IC_{50}

The cell growth rates at 67.0, 80.4, 96.5, 116, 139, 167, 200 and 240 μg/mL of the test substance were 92.9, 83.7, 76.0, 67.6, 52.1, 33.4, 3.8 and 1.7%, respectively. The IC_{50} was calculated at 140 μg/mL.

3.2 Precipitation of the Test Substance, Color of Medium and Corrosion of Culture Dish

Precipitation of the test substance, color change of the medium and the corrosion of the culture dish were not observed at any doses at the start and the end of the treatment and the end of the culture.

3.3 Frequency of Cells with Structural Aberrations

The frequencies of cells with structural aberrations were 2.0% in the negative control and 38.5% in the positive control.

The frequencies at 116, 139 and 167 μg/mL were 1.5, 7.5 and 11.5%, respectively. It was confirmed that the frequencies were over 10% and increased in dose-related manner. Therefore, the results were judged to be positive.

3.4 Frequency of Numerical Aberration Cells

The frequencies of numerical aberration cells were below 5.0% at all doses including the negative and positive controls, therefore, the results were judged to be negative.

3.5 D_{20} Value

It was calculated at 0.27 mg/mL for structural aberration.

4. TYPICAL PHOTOS

The normal cell was shown in photo 1 and cells with structural aberration induced by 13F-EtOH were shown in photos 2 and 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 observation of specimens, the frequencies of 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, numerical aberration were judged to be negative. Although the frequencies of cells with structural aberrations were below 5% in the short-term treatment without S9 mix and the 24 hours continuous treatment, and was over 5% in the short-term treatment with S9 mix. The frequencies of cells with structural aberrations in the short-term treatment with S9 mix were over 10% and increased in dose-related manner in the confirmation test, therefore, structural aberration was judged to be positive.

It was considered that fluctuation of cell growth rate between the first and second cell growth inhibition test was caused by insufficient mixing of medium and test substance solution when exposing cultured cells because density of the test substance was high, 1.678 g/cm^3, and sedimentation rate in the medium was fast.

The fluctuation was successfully reduced in the third cell growth inhibition test, the chromosomal aberration test and the confirmation test by well-mixing medium and test substance solution when exposing cultured cells. Therefore, these tests were employed for evaluation in this study.

Based on the above results, it was concluded that 13F-EtOH did not induce numerical aberration but induced structural 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 the first cell growth inhibition test of 13F-EtOH

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	-	-	-	4.0	0.0
13F-EtOH	14.2	6-18	-	96.1	-	-	-	n.o.	n.o.
	28.4	6-18	-	94.4	-	-	-	n.o.	n.o.
	56.9	6-18	-	96.5	-	-	-	n.o.	n.o.
	114	6-18	-	87.4	-	-	-	n.o.	n.o.
	228	6-18	-	84.7	+	+	-	2.0	0.0
	455	6-18	-	57.6	+	+	-	4.0	0.0
	910	6-18	-	30.2	+	+	-	4.0	2.0
	1820	6-18	-	28.4	+	+	-	2.0	0.0
	3640	6-18	-	42.3	+	+	-	2.0	0.0
				IC_{50} : 580 μg/mL					
DMSO	0	6-18	+	100	-	-	-	0.0	2.0
13F-EtOH	14.2	6-18	+	97.4	-	-	-	n.o.	n.o.
	28.4	6-18	+	91.2	-	-	-	n.o.	n.o.
	56.9	6-18	+	85.9	-	-	-	0.0	0.0
	114	6-18	+	59.2	-	-	-	2.0	4.0
	228	6-18	+	14.2	-	-	-	few meta	
	455	6-18	+	12.8	+	+	-	few meta	
	910	6-18	+	16.0	+	+	-	few meta	
	1820	6-18	+	9.9	+	+	-	few meta	
	3640	6-18	+	9.8	+	+	-	few meta	
				IC_{50} : 140 μg/mL					
DMSO	0	24-0	-	100	-	-		0.0	0.0
13F-EtOH	14.2	24-0	-	95.9	-	-		n.o.	n.o.
	28.4	24-0	-	110.3	-	-		n.o.	n.o.
	56.9	24-0	-	97.1	-	-		0.0	0.0
	114	24-0	-	86.9	-	-		0.0	0.0
	228	24-0	-	1.8	+	+		no meta	
	455	24-0	-	30.1	+	+		0.0	2.0
	910	24-0	-	7.0	+	+		no meta	
	1820	24-0	-	41.4	+	+		few meta	
	3640	24-0	-	30.0	+	+		few meta	
				IC_{50} : 160 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.

b) The frequency of cells with chromosomal aberrations was calculated by observing 50 metaphases per dose. The highest dose was set at 3640 μg/mL equivalent to 10 mmol/L, as the maximum dose in case of no cytotoxicity on the guidelines, the dose levels based on a geometric progression of 2 were selected.

Table 2 Results of the second cell growth inhibition test of 13F-EtOH

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	-	-	-	n.o.	n.o.
13F-EtOH	14.2	6-18	-	109.9	-	-	-	n.o.	n.o.
	28.4	6-18	-	114.2	-	-	-	n.o.	n.o.
	56.9	6-18	-	125.3	-	-	-	n.o.	n.o.
	114	6-18	-	110.5	-	-	-	n.o.	n.o.
	228	6-18	-	14.3	+	+	-	n.o.	n.o.
	455	6-18	-	4.0	+	+	-	few meta	
	910	6-18	-	4.5	+	+	-	few meta	
	1820	6-18	-	52.3	+	+	+	n.o.	n.o.
	3640	6-18	-	22.1	+	+	+	n.o.	n.o.
				IC_{50} : 190 μg/mL					
DMSO	0	24-0	-	100	-	-		n.o.	n.o.
13F-EtOH	14.2	24-0	-	93.7	-	-		n.o.	n.o.
	28.4	24-0	-	88.6	-	-		n.o.	n.o.
	56.9	24-0	-	90.2	-	-		n.o.	n.o.
	114	24-0	-	84.8	-	-		n.o.	n.o.
	228	24-0	-	2.3	+	+		no meta	
	455	24-0	-	1.5	+	+		no meta	
	910	24-0	-	4.7	+	+		no meta	
	1820	24-0	-	23.2	+	+		n.o.	n.o.
	3640	24-0	-	7.5	+	+		few meta	
				IC_{50} : 160 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.

In the first cell growth inhibition test, because the cell growth rates were fluctuated between dishes, the doses that were identical with the first test were set.

Table 3 Results of the third cell growth inhibition test of 13F-EtOH

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	-	-	-	0.0	0.0
13F-EtOH	14.2	6-18	-	97.0	-	-	-	n.o.	n.o.
	28.4	6-18	-	92.7	-	-	-	n.o.	n.o.
	56.9	6-18	-	97.6	-	-	-	0.0	0.0
	114	6-18	-	85.7	-	-	-	0.0	0.0
	228	6-18	-	2.5	-	-	-	few meta	
	455	6-18	-	1.1	+	+	-	few meta	
	910	6-18	-	1.5	+	+	+	no meta	
	1820	6-18	-	0.5	+	+	+	no meta	
	3640	6-18	-	0.4	+	+	+	no meta	
				IC_{50} : 160 μg/mL					
DMSO	0	6-18	+	100	-	-	-	0.0	0.0
13F-EtOH	14.2	6-18	+	94.7	-	-	-	n.o.	n.o.
	28.4	6-18	+	85.0	-	-	-	n.o.	n.o.
	56.9	6-18	+	80.5	-	-	-	0.0	0.0
	114	6-18	+	65.8	-	-	-	2.0	2.0
	228	6-18	+	7.1	-	-	-	few meta	
	455	6-18	+	1.0	+	+	-	no meta	
	910	6-18	+	0.8	+	+	+	no meta	
	1820	6-18	+	0.3	+	+	+	no meta	
	3640	6-18	+	0.1	+	+	+	no meta	
				IC_{50} : 140 μg/mL					
DMSO	0	24-0	-	100	-	-		0.0	0.0
13F-EtOH	14.2	24-0	-	92.4	-	-		n.o.	n.o.
	28.4	24-0	-	89.3	-	-		n.o.	n.o.
	56.9	24-0	-	97.0	-	-		2.0	0.0
	114	24-0	-	47.1	-	-		0.0	0.0
	228	24-0	-	0.3	-	-		no meta	
	455	24-0	-	0.2	+	+		no meta	
	910	24-0	-	0.2	+	+		no meta	
	1820	24-0	-	0.2	+	+		no meta	
	3640	24-0	-	0.2	+	+		no meta	
				IC_{50} : 110 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.

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

The doses were identical with the first test.

Table 4 Results of chromosomal aberration test of 13F-EtOH

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	-	-	-	3.0	0.5
13F-EtOH	80.4	6-18	-	99.9	-	-	-	n.o.	n.o.
	96.5	6-18	-	99.2	-	-	-	n.o.	n.o.
	116	6-18	-	85.8	-	-	-	3.5	0.0
	139	6-18	-	62.5	-	-	-	2.0	0.5
	167	6-18	-	29.0	-	-	-	2.5	0.5
	200	6-18	-	0.8	-	-	-	no meta	
	240	6-18	-	0.4	-	-	-	no meta	
MMC	0.1	6-18	-	ND	-	-	-	68.5	0.0
			IC_{50} :	150 μg/mL					
DMSO	0	6-18	+	100	-	-	-	0.5	0.5
13F-EtOH	80.4	6-18	+	93.5	-	-	-	n.o.	n.o.
	96.5	6-18	+	92.1	-	-	-	n.o.	n.o.
	116	6-18	+	80.5	-	-	-	6.0	0.0
	139	6-18	+	76.2	-	-	-	4.0	1.5
	167	6-18	+	44.9	-	-	-	5.0	1.5
	200	6-18	+	23.2	-	-	-	n.o.	n.o.
	240	6-18	+	46.4	-	-	-	n.o.	n.o.
CPA	6	6-18	+	ND	-	-	-	56.0	0.0
			IC_{50} :	160 μg/mL			D_{20} :	0.68 mg/mL	-
DMSO	0	24-0	-	100	-	-		1.5	0.0
13F-EtOH	67.0	24-0	-	99.4	-	-		n.o.	n.o.
	80.4	24-0	-	87.2	-	-		n.o.	n.o.
	96.5	24-0	-	85.3	-	-		1.5	1.0
	116	24-0	-	85.1	-	-		1.5	0.5
	139	24-0	-	56.5	-	-		0.5	0.5
	167	24-0	-	8.7	-	-		few meta	
	200	24-0	-	1.8	-	-		no meta	
	240	24-0	-	7.1	-	-		no meta	
MMC	0.05	24-0	-	ND	-	-		77.0	0.5
			IC_{50} :	140 μg/mL					

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.

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

Table 5 Results of chromosomal aberration test (short-term treatment without S9 mix)

Name of test substance : 13F-EtOH

K06-1192

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	4	0	1	0	0	4	0	100	100	1	0	1
			100	1	1	0	0	0	2	0		100	0	0	0
		0	200	5 (2.5)	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	6 (3.0)	0 (0.0)		200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	-	80.4	0								103.8	0			
			0								96.0	0			
			0								(99.9)	0			
6 - 18	-	96.5	0								105.9	0			
			0								92.4	0			
			0								(99.2)	0			
6 - 18	-	116	100	4	0	0	0	0	4	1	84.7	100	0	0	0
			100	2	1	0	0	0	3	0	86.9	100	0	0	0
			200	6 (3.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	7 (3.5)	1 (0.5)	(85.8)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	-	139	100	3	0	0	0	0	3	0	58.3	100	0	0	0
			100	0	0	1	0	0	1	0	66.7	100	1	0	1
			200	3 (1.5)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	4 (2.0)	0 (0.0)	(62.5)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	-	167	100	4	0	0	0	0	4	0	29.4	100	1	0	1
			100	0	1	0	0	0	1	1	28.6	100	0	0	0
			200	4 (2.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	5 (2.5)	1 (0.5)	(29.0)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	-	200	0	no metaphases							1.1	0	no metaphases		
			0								0.4	0			
			0								(0.8)	0			
6 - 18	-	240	0	no metaphases							0.4	0	no metaphases		
			0								0.4	0			
			0								(0.4)	0			
6 - 18	-	Positive control (MMC)	100	46	60	0	0	0	75	1		100	0	0	0
			100	34	58	0	0	0	62	1		100	0	0	0
		0.1	200	80 (40.0)	118 (59.0)	0 (0.0)	0 (0.0)	0 (0.0)	137 (68.5)	2 (1.0)		200	0 (0.0)	0 (0.0)	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.

DMSO: Dimethylsulfoxide

MMC: Mitomycin C

no metaphases: metaphases were not observed

The specimens at 80.4, 96.5, 200 and 240 μg/mL were not observed.

K06-1192

Table 6 Results of chromosomal aberration test (short-term treatment with S9 mix)

Name of test substance : 13F-EtOH

K06-1192

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	0	0	0	0	0	0	0	100	100	0	0	0
			100	1	0	0	0	0	1	0		100	1	0	1
		0	200	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)		200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	+	80.4	0								91.2	0			
			0								95.8	0			
			0								(93.5)	0			
6 - 18	+	96.5	0								90.5	0			
			0								93.7	0			
			0								(92.1)	0			
6 - 18	+	116	100	2	3	0	0	0	4	0	84.4	100	0	0	0
			100	4	5	0	0	0	8	0	76.5	100	0	0	0
			200	6 (3.0)	8 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)	12 (6.0)	0 (0.0)	(80.5)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	139	100	3	2	0	0	0	5	0	69.8	100	1	0	1
			100	3	0	0	0	0	3	0	82.6	100	2	0	2
			200	6 (3.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (4.0)	0 (0.0)	(76.2)	200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	+	167	100	2	0	0	0	0	2	0	47.9	100	1	0	1
			100	6	4	0	0	0	8	0	41.9	100	2	0	2
			200	8 (4.0)	4 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (5.0)	0 (0.0)	(44.9)	200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	+	200	0								20.7	0			
			0								25.6	0			
			0								(23.2)	0			
6 - 18	+	240	0								49.1	0			
			0								43.7	0			
			0								(46.4)	0			
6 - 18	+	Positive control (CPA)	100	18	42	1	0	0	51	0		100	0	0	0
			100	25	47	0	1	0	61	0		100	0	0	0
		6	200	43 (21.5)	89 (44.5)	1 (0.5)	1 (0.5)	0 (0.0)	112 (56.0)	0 (0.0)		200	0 (0.0)	0 (0.0)	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.

DMSO: Dimethylsulfoxide

CPA: Cyclophosphamide monohydrate

The specimens at 80.4, 96.5, 200 and 240 μg/mL were not observed.

K06-1192

Table 7 Results of chromosomal aberration test (continuous treatment)

Name of test substance : 13F-EtOH

K06-1192

Treatment time (h)	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
24 - 0	Negative control (DMSO)	100	0	0	0	0	0	0	0	100	100	0	0	0
		100	1	1	0	1	0	3	0		100	0	0	0
	0	200	1 (0.5)	1 (0.5)	0 (0.0)	1 (0.5)	0 (0.0)	3 (1.5)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
24 - 0	67.0	0								106.6	0			
		0								92.2	0			
		0								(99.4)	0			
24 - 0	80.4	0								86.6	0			
		0								87.8	0			
		0								(87.2)	0			
24 - 0	96.5	100	1	0	0	2	0	3	0	84.7	100	2	0	2
		100	0	0	0	0	0	0	0	85.9	100	0	0	0
		200	1 (0.5)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	3 (1.5)	0 (0.0)	(85.3)	200	2 (1.0)	0 (0.0)	2 (1.0)
24 - 0	116	100	0	1	0	0	0	1	0	81.0	100	1	0	1
		100	1	1	0	0	0	2	0	89.2	100	0	0	0
		200	1 (0.5)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	0 (0.0)	(85.1)	200	1 (0.5)	0 (0.0)	1 (0.5)
24 - 0	139	100	1	0	0	0	0	1	0	55.9	100	1	0	1
		100	0	0	0	0	0	0	0	57.1	100	0	0	0
		200	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	(56.5)	200	1 (0.5)	0 (0.0)	1 (0.5)
24 - 0	167	0	few metaphases							8.6	0	few metaphases		
		0								8.7	0			
		0								(8.7)	0			
24 - 0	200	0	no metaphases							3.1	0	no metaphases		
		0								0.5	0			
		0								(1.8)	0			
24 - 0	240	0	no metaphases							9.7	0	no metaphases		
		0								4.5	0			
		0								(7.1)	0			
24 - 0	Positive control (MMC)	100	36	65	0	0	0	75	0		100	0	0	0
		100	43	70	0	0	0	79	0		100	1	0	1
	0.05	200	79 (39.5)	135 (67.5)	0 (0.0)	0 (0.0)	0 (0.0)	154 (77.0)	0 (0.0)		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.

DMSO: Dimethylsulfoxide

MMC: Mitomycin C

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

no metaphases: metaphases were not observed

The specimens at 67.0, 80.4, 167, 200 and 240 μg/mL were not observed.

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Table 8 Results of confirmation test of 13F-EtOH

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	0.5
13F-EtOH	67.0	6-18	+	92.9	-	-	-	n.o.	n.o.
	80.4	6-18	+	83.7	-	-	-	n.o.	n.o.
	96.5	6-18	+	76.0	-	-	-	n.o.	n.o.
	116	6-18	+	67.6	-	-	-	1.5	0.5
	139	6-18	+	52.1	-	-	-	7.5	2.0
	167	6-18	+	33.4	-	-	-	11.5	0.0
	200	6-18	+	3.8	-	-	-	no meta	
	240	6-18	+	1.7	-	-	-	no meta	
CPA	6	6-18	+	ND	-	-	-	38.5	0.0
			IC_{50} :	140 μg/mL			D_{20} :	0.27 mg/mL	-

DMSO: dimethylsulfoxide, CPA: cyclophosphamide monohydrate

ND: not detected

n.o.: not observed, no meta: metaphases were 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 9 Results of confirmation test (short-term treatment with S9 mix)

Name of test substance : 13F-EtOH

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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	0	1	1	1	0	3	0	100	100	1	0	1
			100	0	1	0	0	0	1	0		100	0	0	0
		0	200	0 (0.0)	2 (1.0)	1 (0.5)	1 (0.5)	0 (0.0)	4 (2.0)	0 (0.0)		200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	+	67.0	0								95.2	0			
			0								90.5	0			
			0								(92.9)	0			
6 - 18	+	80.4	0								83.4	0			
			0								83.9	0			
			0								(83.7)	0			
6 - 18	+	96.5	0								75.0	0			
			0								76.9	0			
			0								(76.0)	0			
6 - 18	+	116	100	0	1	0	0	0	1	1	71.4	100	1	0	1
			100	0	1	1	0	0	2	0	63.7	100	0	0	0
			200	0 (0.0)	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	3 (1.5)	1 (0.5)	(67.6)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	+	139	100	4	6	0	0	0	9	0	51.3	100	1	0	1
			100	3	3	0	0	0	6	0	52.8	100	3	0	3
			200	7 (3.5)	9 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	15 (7.5)	0 (0.0)	(52.1)	200	4 (2.0)	0 (0.0)	4 (2.0)
6 - 18	+	167	100	8	6	0	1	0	14	0	33.1	100	0	0	0
			100	7	5	1	0	0	9	0	33.6	100	0	0	0
			200	15 (7.5)	11 (5.5)	1 (0.5)	1 (0.5)	0 (0.0)	23 (11.5)	0 (0.0)	(33.4)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	200	0								6.3	0			
			0								1.3	0			
			0								(3.8)	0			
6 - 18	+	240	0								1.8	0			
			0								1.5	0			
			0								(1.7)	0			
6 - 18	+	Positive control (CPA)	100	17	30	0	0	0	40	1		100	0	0	0
			100	16	31	0	0	0	37	0		100	0	0	0
		6	200	33 (16.5)	61 (30.5)	0 (0.0)	0 (0.0)	0 (0.0)	77 (38.5)	1 (0.5)		200	0 (0.0)	0 (0.0)	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.

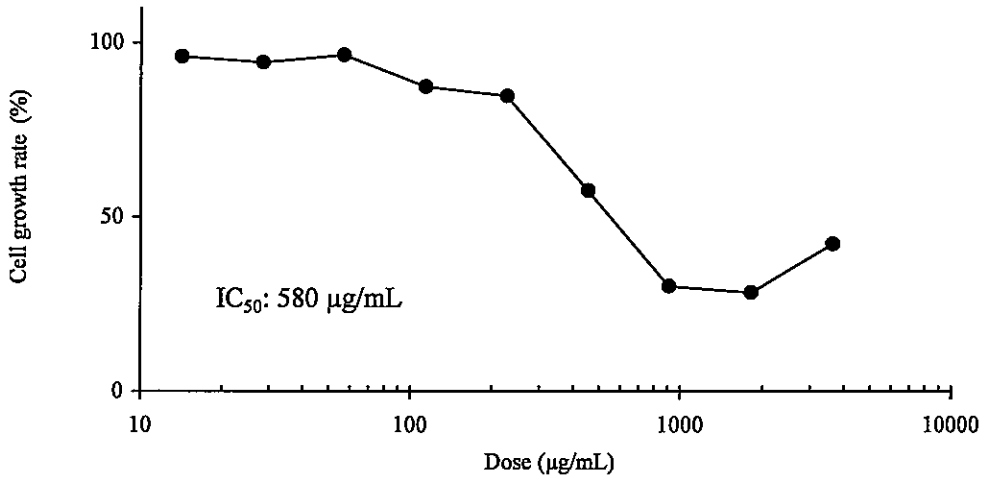
DMSO: Dimethylsulfoxide

CPA: Cyclophosphamide monohydrate

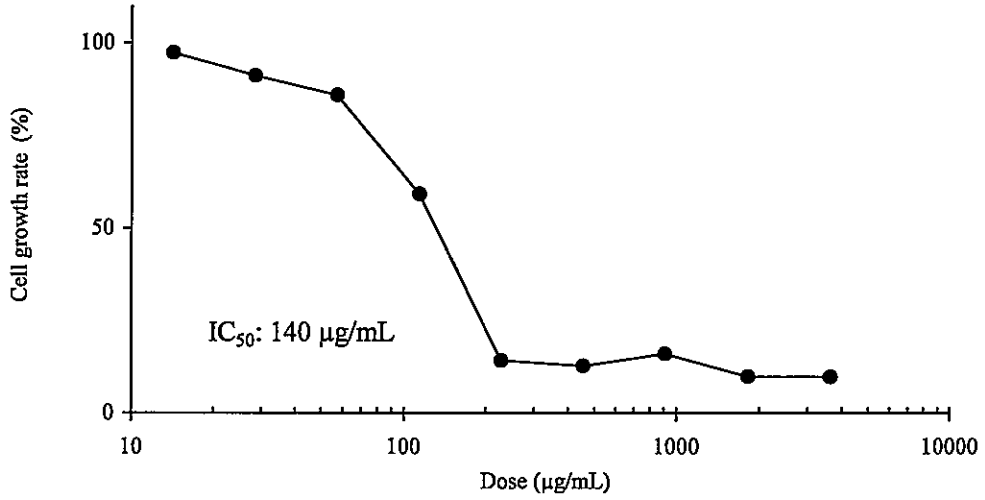
no metaphases: metaphases were not observed

The specimens at 67.0, 80.4, 96.5, 200 and 240 μg/mL were not observed.

K06-1192



Short-term treatment without S9 mix



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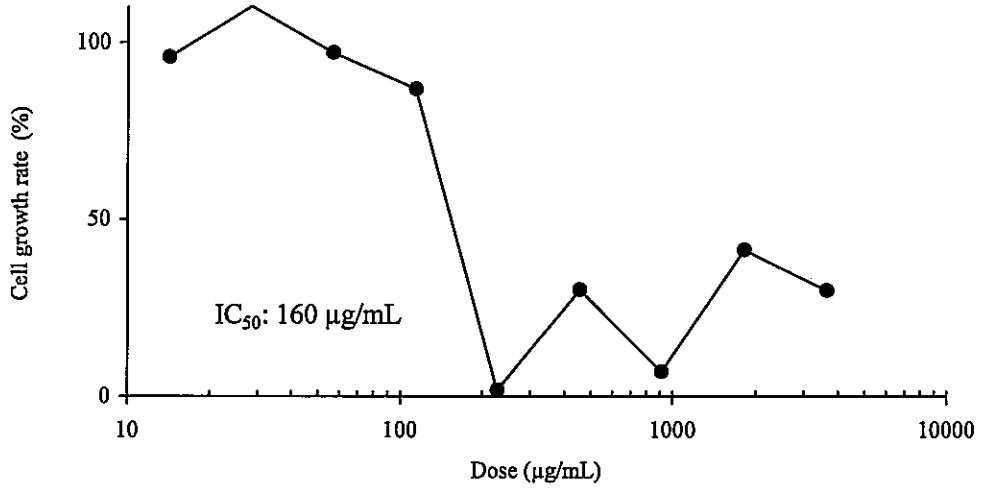
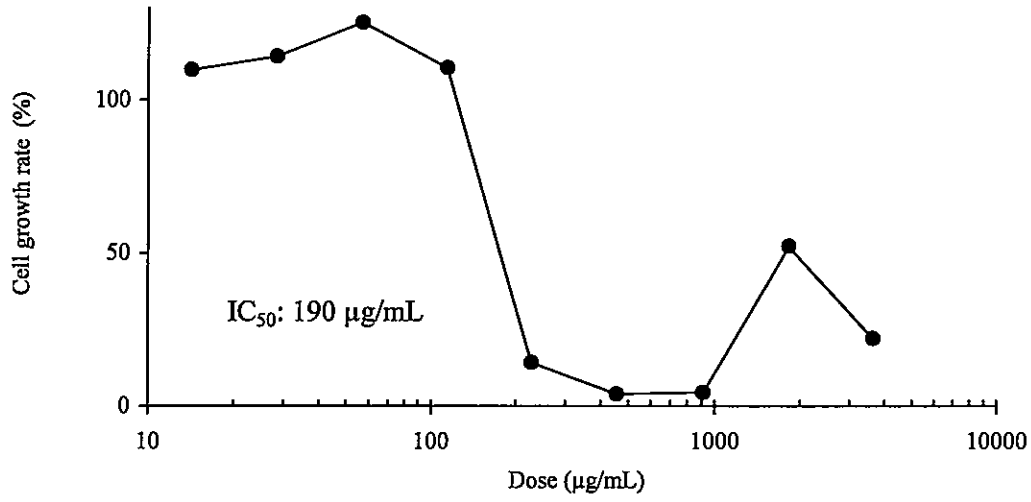
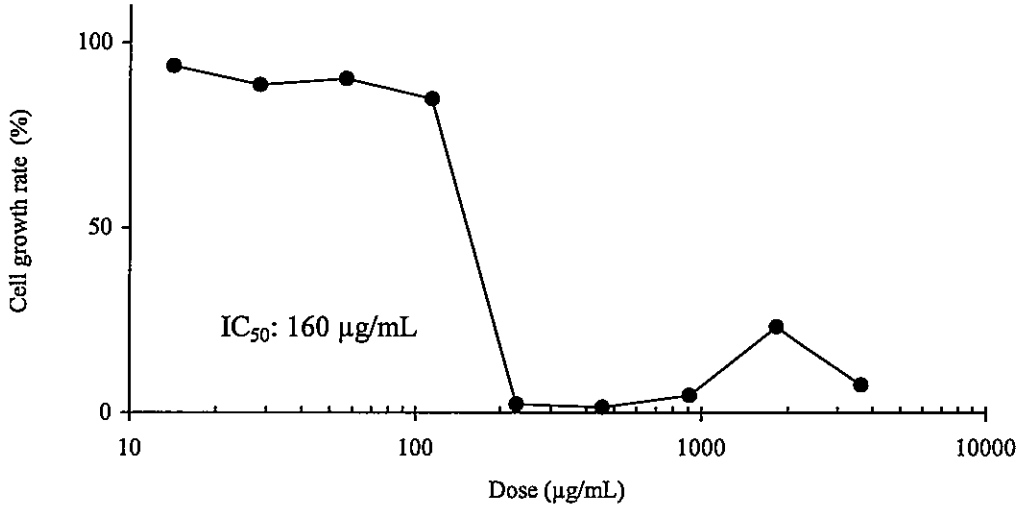


24 hours continuous treatment

Fig. 1 Results of the first cell growth inhibition test of 13F-EtOH

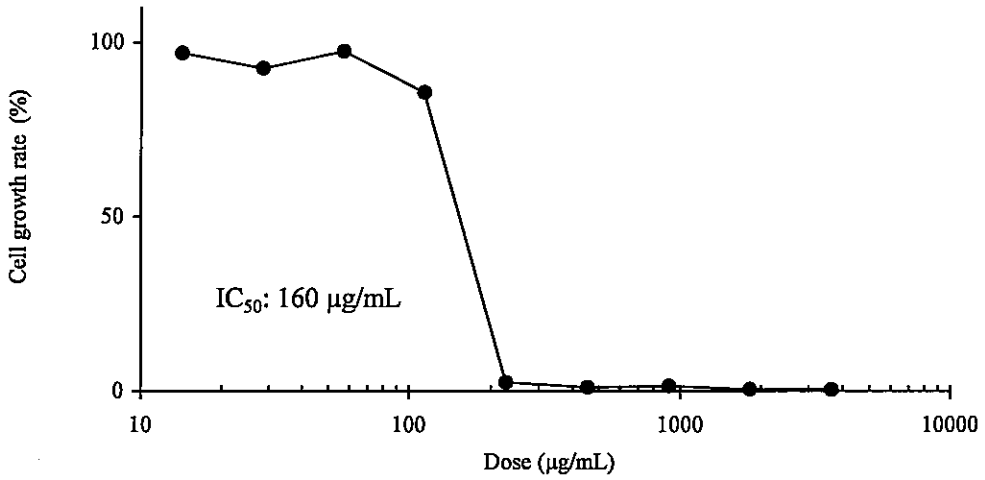


Short-term treatment without S9 mix

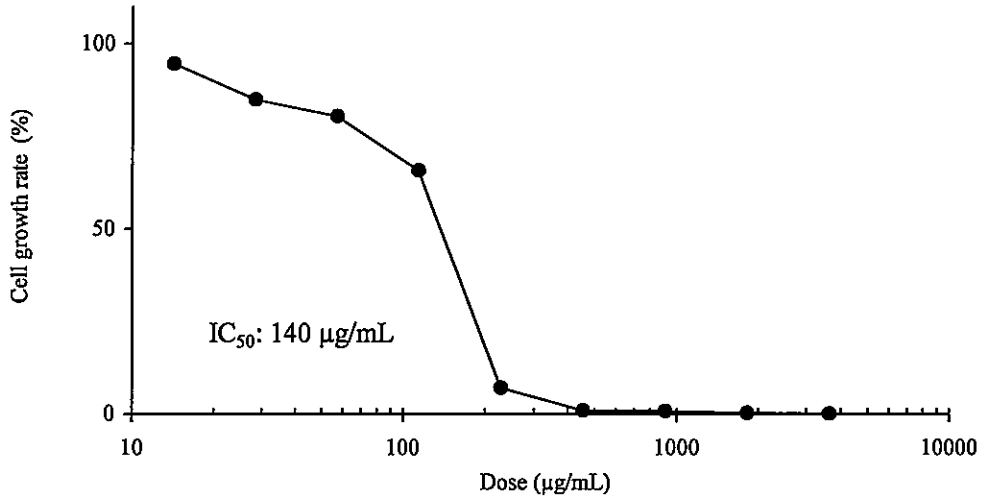


24 hours continuous treatment

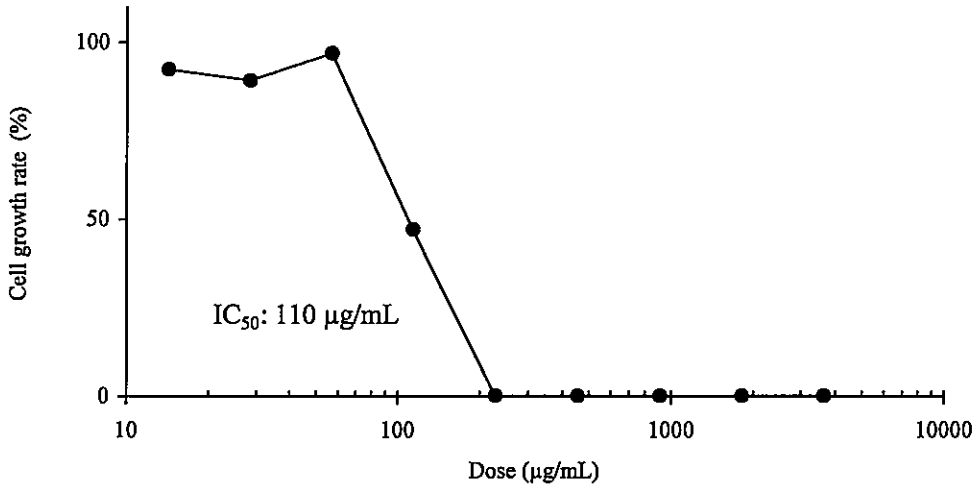
Fig. 2 Results of the second cell growth inhibition test of 13F-EtOH



Short-term treatment without S9 mix

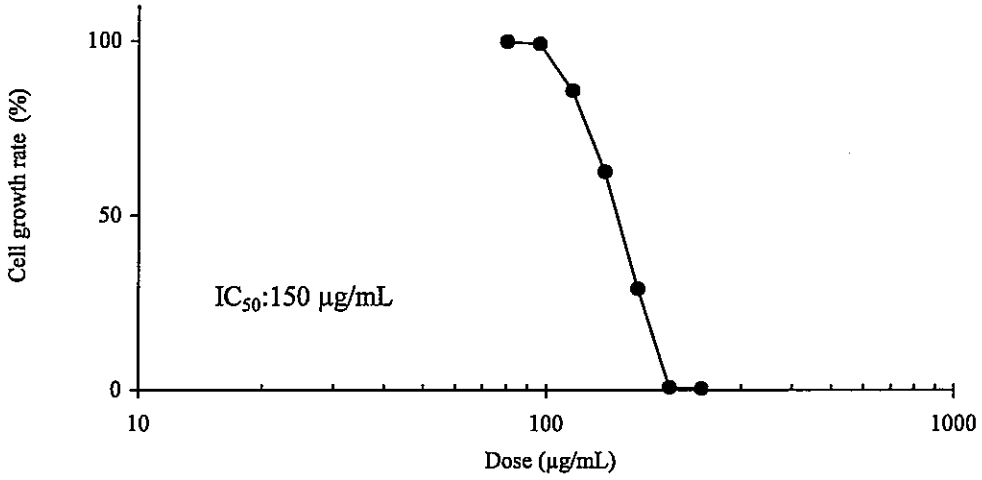


Short-term treatment with S9 mix

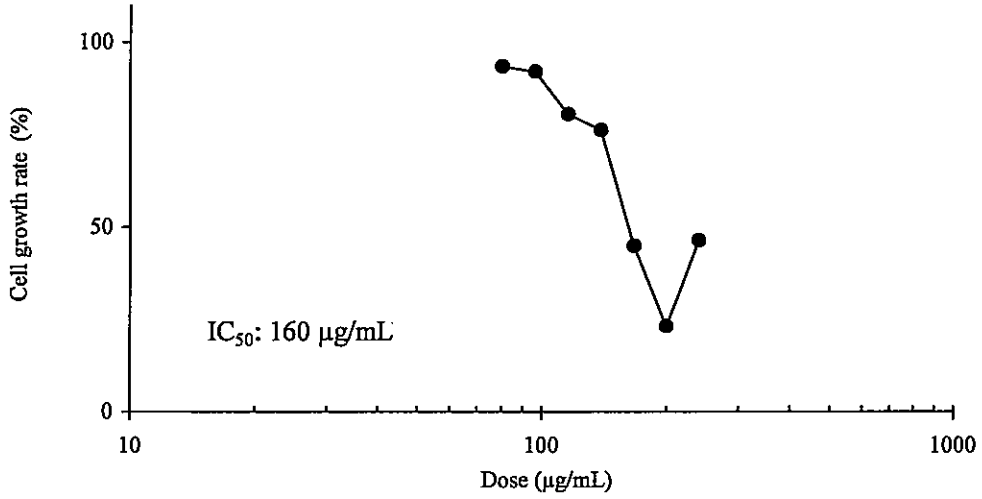


24 hours continuous treatment

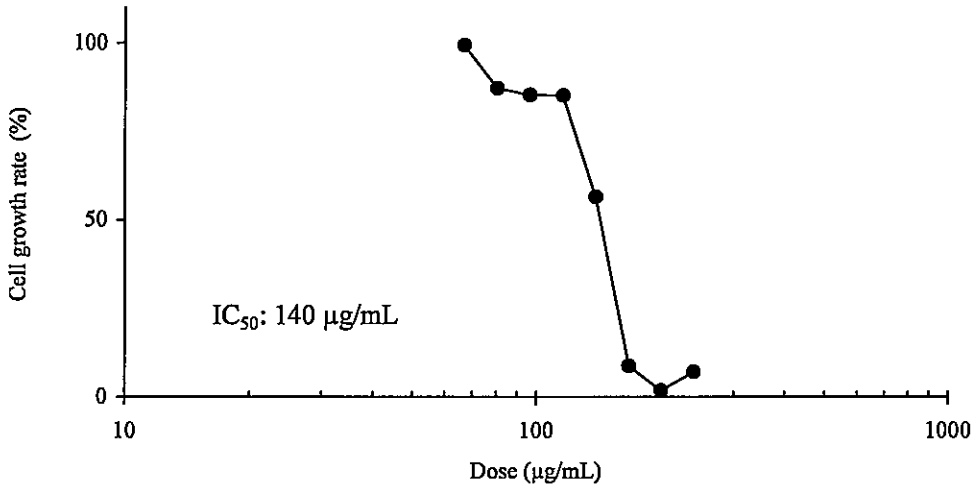
Fig. 3 Results of the third cell growth inhibition test of 13F-EtOH



Short-term treatment without S9 mix

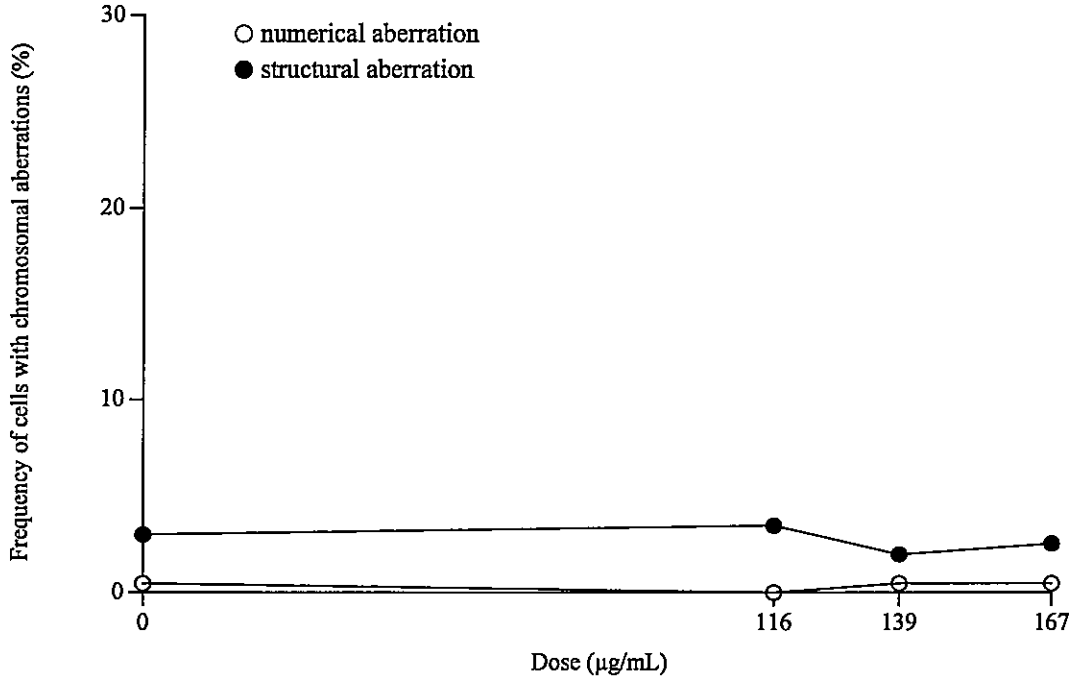


Short-term treatment with S9 mix

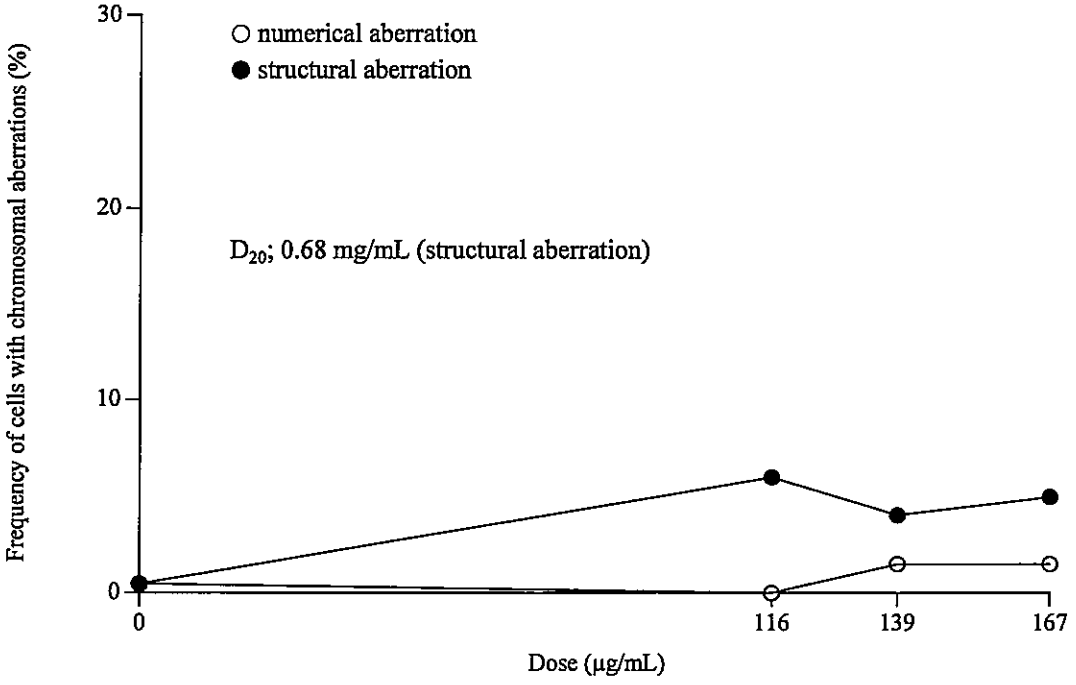


24 hours continuous treatment

Fig. 4 Cell growth rate in chromosomal aberration test of 13F-EtOH



Without S9 mix



With S9 mix

Fig. 5 Results of chromosomal aberration test in short-term treatments of 13F-EtOH

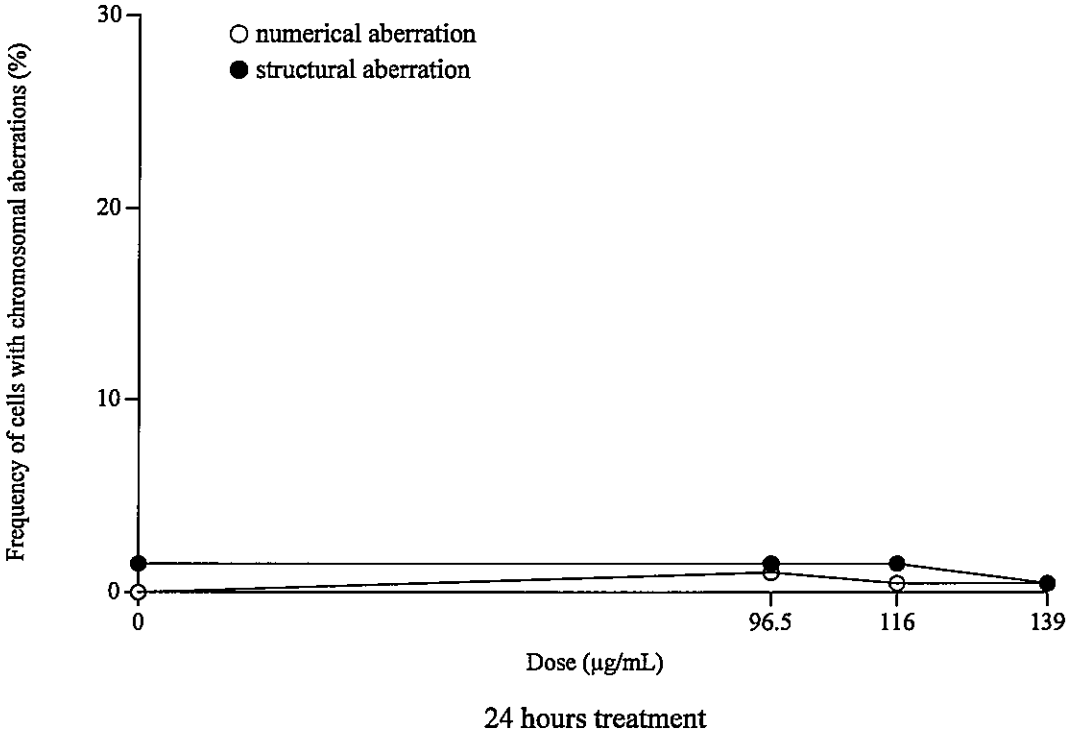


Fig. 6 Results of chromosomal aberration test in continuous treatment of 13F-EtOH

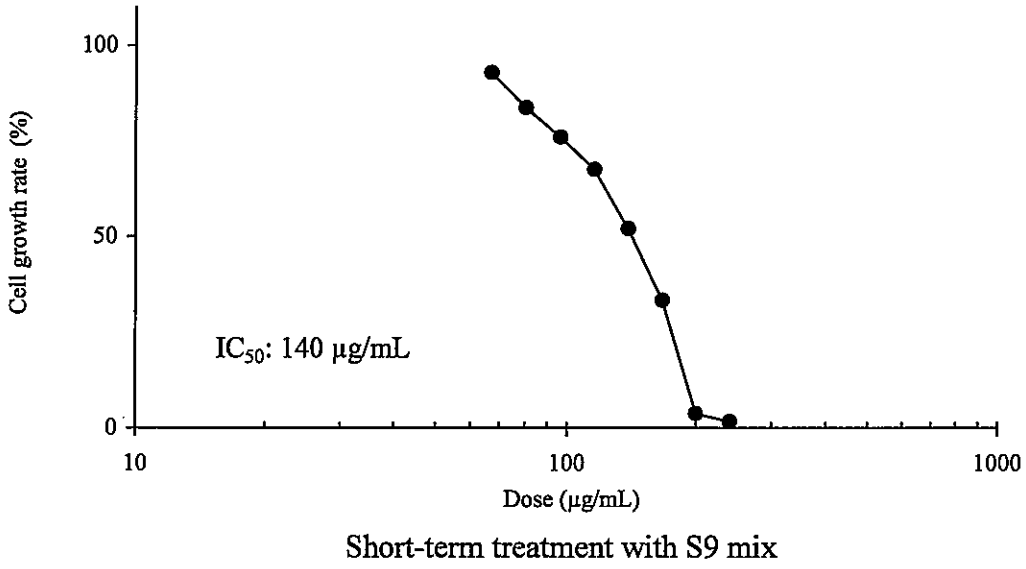


Fig. 7 Cell growth rate in confirmation test of 13F-EtOH

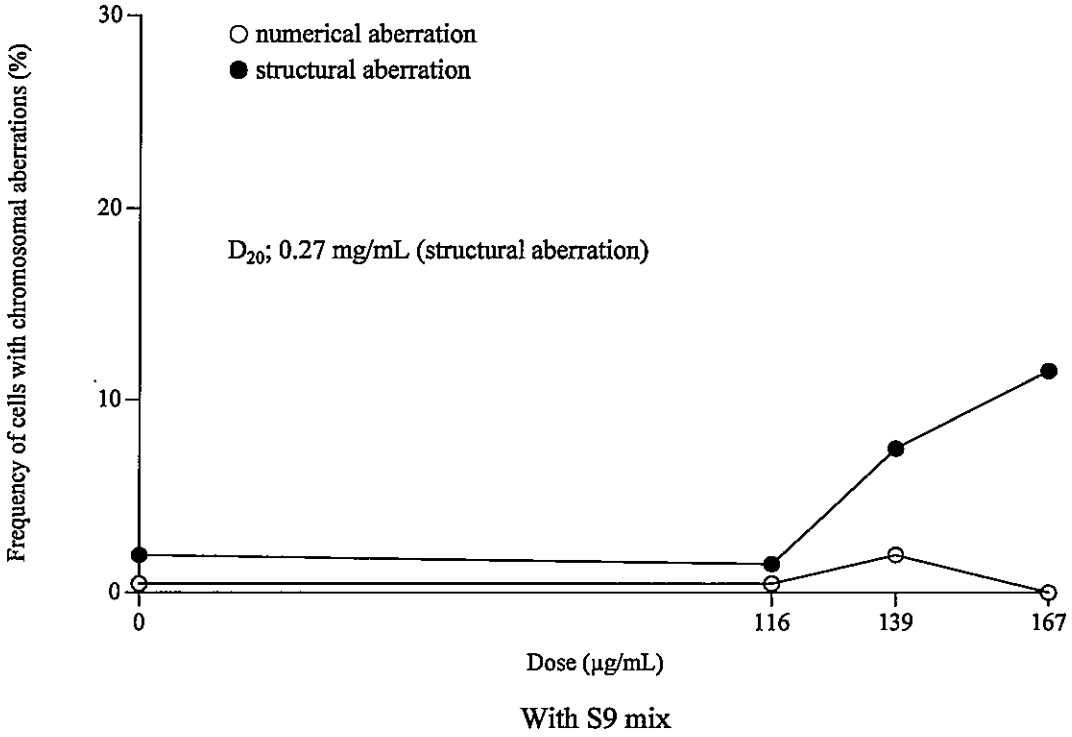


Fig. 8 Results of confirmation test in short-term treatment of 13F-EtOH

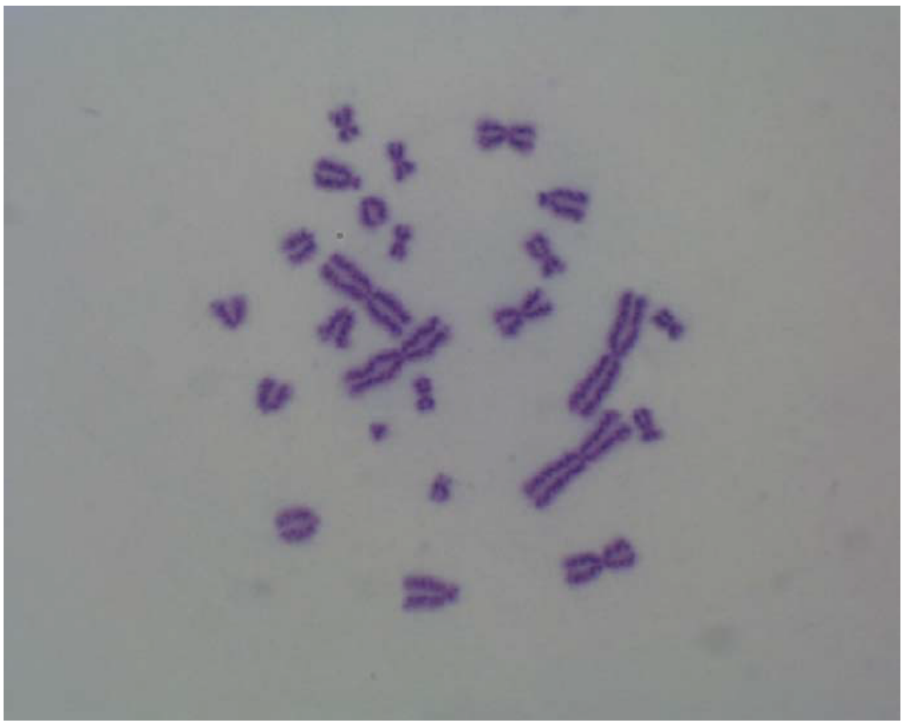


Photo 1　Normal cell
Negative control for short-term treatment with S9 mix in confirmation test

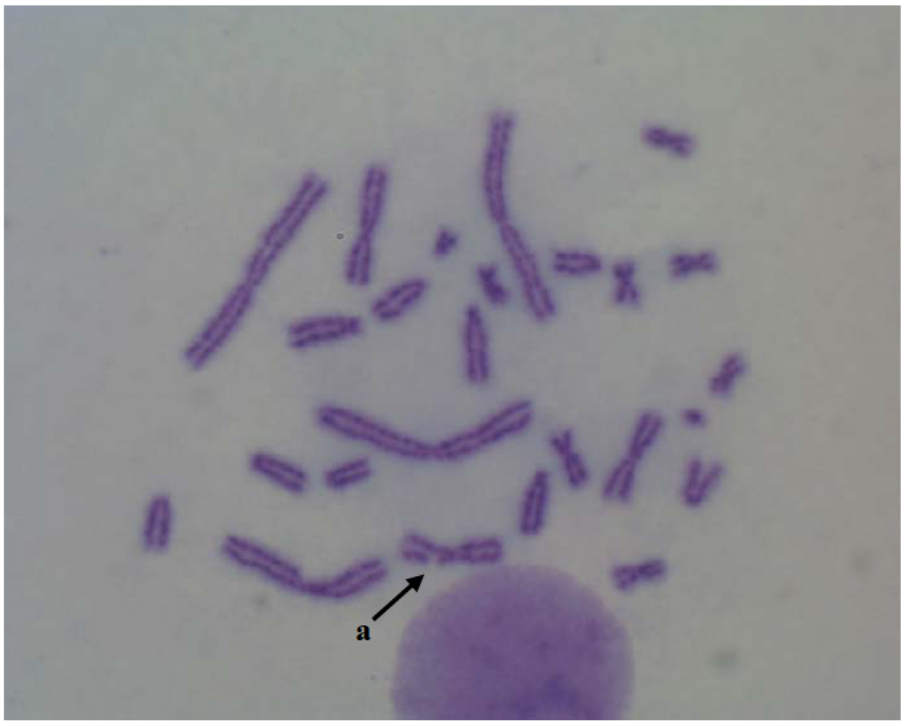


Photo 2　Structural aberration induced by 13F-EtOH
167 µg/mL for short-term treatment with S9 mix in confirmation test
a: chromatid break

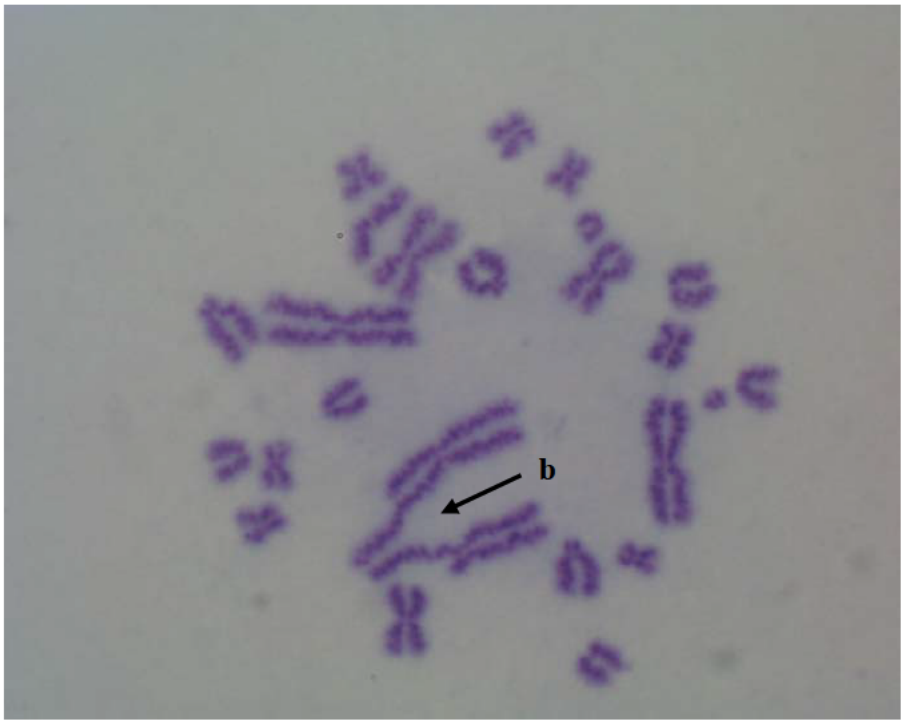


Photo 3 Structural aberration induced by 13F-EtOH
167 μg/mL for short-term treatment with S9 mix in confirmation test
b: chromatid exchange