CERI

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Study Number	K06-1564

FINAL REPORT

CHROMOSOMAL ABERRATION TEST OF APFHx(C-1500N)
USING CULTURED MAMMALIAN CELLS

July, 2017

Chemicals Evaluation and Research Institute, Japan, Hita

GLP STATEMENT

Chemicals Evaluation and Research Institute, Japan, Hita

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: Chromosomal aberration test of APFHx(C-1500N) using cultured mammalian cells

Study Number: K06-1564

The study described in this report was conducted in compliance with the following GLP principle.

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: July 3, 2017
Date

TABLE OF CONTENTS

1. TITLE

Chromosomal aberration test of APFHx(C-1500N) using cultured mammalian cells

2. SPONSOR

Name DAIKIN INDUSTRIES, LTD.

Address 1-1, Nishi Hitotsuya, Settsu-shi, Osaka 566-8585, Japan.

3. TESTING FACILITY

Name Chemicals Evaluation and Research Institute, Japan, Hita (CERI Hita)

Address 3-822, Ishii-machi, Hita-shi, Oita, 877-0061, Japan

4. OBJECTIVE

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

5. TEST METHODS

"OECD Guideline for the Testing of Chemicals, No. 473, *In Vitro* Mammalian Chromosomal Aberration Test" (Adopted: July 29, 2016)

6. GLP PRINCIPLES

OECD Principles of Good Laboratory Practice, November 26, 1997

7. DATES

Study initiation date	March 3, 2017
Experimental starting date	March 13, 2017
Experimental completion date	June 19, 2017
Study completion date	July 3, 2017

8. PERSONNEL CONCERNED WITH STUDY

Study Director:

Section 3, CERI Hita

Study Staff:

(Preparation of test substance solution and cell treatment)

(Microscopic observation of specimens)

9. STORAGE OF RAW DATA, ETC.

The original study plan, the original final report, the raw data, documents concerning the study presented by the sponsor, other records and specimens are stored in the archives of the testing facility. The storage period is 10 years after the study completion date.

The management of these items after the storage period (continuation of storage, disposal or return) will be discussed with the sponsor.

10. APPROVAL BY AUTHOR

Study Director: July 3, 2017
Date

11. SUMMARY

The ability of APFHx(C-1500N) to induce chromosomal aberrations was investigated by using Chinese hamster lung fibroblasts (CHL/IU cells).

The frequencies of cells with structural aberrations and numerically aberrant cells at all observation doses of the test substance in all treatment methods were within the range of the historical data of the negative control. Therefore, structural aberration and numerical aberration were judged to be negative.

Consequently, it was concluded that APFHx(C-1500N) did not induce chromosomal aberrations under the present test conditions.

12. MATERIALS

12.1 Test Substance and Control Substance

a) Test substance (information provided by the sponsor)

1) Chemical name, etc.

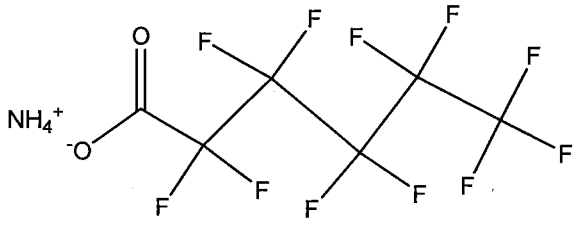
Chemical name 2,2,3,3,4,4,5,5,6,6,6-Undecafluorohexanoic acid, ammonium salt

Other name APFHx(C-1500N)

CAS number 21615-47-4

2) Structural formula, etc.

Structural formula



Molecular formula $C_6H_4F_{11}NO_2$

Molecular weight 331.08

3) Purity, etc.

Purity 50%

Impurity Water: 50%

Supplier DAIKIN INDUSTRIES, LTD.

Lot number C150E62004

The concentration of the test substance was corrected by purity.

4) Physicochemical properties

Appearance at ordinary temperature Colorless transparent liquid

Stability Stable in storage condition

Solubility to vehicle, etc.

Vehicle	Solubility	Stability in vehicle
Water	≥ 200 mg/mL*1	Stable*1, *2

*1: Confirmed at the testing facility

*2: Confirmed from the facts on account of changes in color, exothermic reaction or gas generation at room temperature until 2 hours after preparation

5) Storage condition

The test substance was put into a light-shielded and an airtight container and stored at room temperature in the test substance storage room (permissible range: from 10°C to 30°C).

6) Safety

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, a mask, a head cap, safety glasses and a lab coat were worn.

b) Negative Control Substance (Vehicle)

1) Name

Distilled water

2) Manufacturer, lot number and grade

Manufacturer	Otsuka Pharmaceutical Factory
Lot number	K6G73
Grade	for injection

3) Reason for selection of vehicle

The test substance was dissolved in distilled water at 200 mg/mL. This solution was considered to be stable from the facts on account of neither change in color, exothermic reaction nor gas generation until 2 hours after preparation at room temperature. Therefore, distilled water was selected as a vehicle.

4) Storage condition

Negative control substance was stored at room temperature at the preparation room No. 2.

c) Positive Control Substances

1) Name, etc.

Name	Manufacturer	Lot number	Purity	Grade
Mitomycin C (MMC)	Kyowa Hakko Kirin	575AEA	102.9%	for injection
Cyclophosphamide monohydrate (CPA)	Wako Pure Chemical Industries	WEF3430	99.2%	for biochemistry

2) Storage condition

MMC was stored in the test substance storage room at room temperature (permissible range: from 10°C to 30°C) and CPA was put into a light-shielded container and stored in a cold place of the test substance storage room (permissible range: from 1°C to 10°C).

3) Safety

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, a mask, a head cap, safety glasses and a lab coat were worn.

12.2 Cells

a) Cell line and reason for selection, etc.

Cell line	Chinese hamster lung fibroblasts (CHL/IU cells)
Source of cells	Health Science Research Resources Bank, Japan Health Sciences Foundation
Received date	April 17, 2002
Modal number of chromosomes	25 per cell
Doubling time	about 15 hours

Mycoplasma	negative
Spontaneous frequencies of cells with structural aberrations and the numerically aberrant cells	< 5%
Reason for selection	CHL/IU cells have been recommended in the methods described in "5. TEST METHODS".

b) Storage

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

c) Culture condition

Incubator	CO_2 incubator (MCO-18AIC, SANYO Electric)
Temperature	37°C
Humidity	under humid condition
CO_2 concentration	5%

d) Subculture

Culture vessel	90-mm diameter Petri dish (Nunc)
Frequency of passage	twice or three times a week
Passage number of cells	7 for cell growth inhibition test 19 for chromosomal aberration test

12.3 Medium and S9 Mix

a) Medium

L-Glutamine (final concentration: 0.292 g/L) and sodium hydrogen carbonate (final concentration: approximately 1.95 g/L) were added to Eagle's minimum essential medium (Lot number 730609, Nissui Pharmaceutical) and basal medium (MEM) was prepared. This medium was then supplemented with 10 vol% heat-inactivated NBCS (NBCS of Lot number 1455368 (Life Technologies) and NBCS of Lot number S12160S0750 (BioWest) were mixed in a ratio of 4 : 1).

b) S9 mix

1) Name

S-9 MIX for chromosomal aberration test

2) Manufacturer, etc.

Lot number	Manufacturer	Manufactured date	Lot number of S9	Test
CAM201701A	Kikkoman Biochemifa Company	January 27, 2017	RAA201701A	Cell growth inhibition test
CAM201702A		February 24, 2017	RAA201702A	Chromosomal aberration test

3) Storage condition

S9 mix was stored in a frozen place in the cell experimental room No. 2 (permissible range: −80°C or below).

4) Manufactured method of S9

Lot number	Manufacturer	Manufactured date	S9 protein content (mg/mL)
RAA201701A	Kikkoman Biochemifa Company	January 27, 2017	24.14
RAA201702A		February 24, 2017	24.05

Animal

Species and strain	Rat, SD
Sex	Male
Age in weeks	7 weeks old
Body weight	199 - 247 g (Lot number RAA201701A)
	191 - 253 g (Lot number RAA201702A)

Inducing substances

Name	Phenobarbital (PB) and 5,6-benzoflavone (5,6-BF)	
Method of administration	Intraperitoneal administration	
Administration period and administered dose (mg/kg body weight)		
	PB	30 mg/kg, one time
		60 mg/kg, three times
	5,6-BF	80 mg/kg, one time

5) Composition of S9 mix

Components	Amount in 1 mL of S9 mix	Components	Amount in 1 mL of S9 mix
S9	0.3 mL	NADP	4 μmol
$MgCl_2$	5 μmol	Na-Phosphoric acid buffering solution	–
KCl	33 μmol	HEPES (pH7.2)	4 μmol
Glucose-6-phosphate	5 μmol	Other (–)	–

6) Usage of S9 mix

S9 mix was prepared just before use.

12.4 Cell Pre-culture

Culture vessel	60-mm diameter plastic dish (Asahi Glass)
Cell density at seeding	5×10^3 cells/mL
Volume of cell suspension	5 mL
Pre-culture period	3 days

12.5 Preparation of Test Substance Solution and Positive Control Substance Solutions

a) Preparation of test substance solution

1) Preparation method

The test substance was weighted and dissolved in distilled water to prepare the test substance solution. The test substance solutions of each required concentrations were prepared with the same vehicle. The prepared concentrations were 100 times as high as in the medium. The concentrations were corrected by the purity of the test substance because the purity was below 95%.

2) Timing of preparation

The test substance solutions were prepared just before use, stored at room temperature and used within 2 hours after preparation.

b) Preparation of positive control substance solutions

1) Preparation method and storage condition

MMC and CPA were dissolved in distilled water (Lot number K6B72, for injection, Otsuka Pharmaceutical Factory) at 0.01 and 1.00 mg/mL, respectively. The positive control substance solutions were stored in a frozen place in the cell experimental room No. 2 (permissible range: −80°C or below).

2) Timing of preparation

The positive control substance solutions were thawed just before use, stored at room temperature and used within 2 hours after thawing.

13. TEST PROCEDURE

13.1 Cell Growth Inhibition Test

a) Procedures

One dish was used for each dose. For calculation of Relative Population Doubling (RPD) and Relative Increase in Cell Count (RICC), the number of the cells in one dish was measured at the start of the treatment and the end of the culture using the same procedure as that at the end of the culture. Specimens were prepared at doses which were referred for the dose setting of the chromosomal aberration test.

1) Treatment

(1) For the short-term treatment without S9 mix (−S9 mix), the medium was removed from a pre-culture, and the cells were treated in well-mixed medium made of 3 mL of the fresh medium with 30 μL of the test substance solution or the vehicle.

(2) For the short-term treatment with S9 mix (+S9 mix), the medium was removed from a pre-culture, and the cells were treated in well-mixed medium made of 2.8 mL of fresh medium, 30 μL of the test substance solution or the vehicle, and 0.2 mL of S9 mix.

(3) For "−S9 mix" and "+S9 mix", after treatment for 6 hours, 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.

(4) For the 24 hours continuous treatment, the medium was removed from a pre-culture, and the cells were treated for 24 hours with well-mixed medium made of 5 mL of fresh medium and 50 μL of the test substance solution or the vehicle.

2) Fifty microliters of a 10 μg/mL demecolcine solution was added to each dish at 2 hours before the end of the culture.

3) 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 dishes were observed macroscopically.

4) At the end of the culture, cells were suspended 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), the number of the cells was measured using a Microcell counter (CDA-500, Sysmex) and the cell growth rate compared with a negative control (Relative Cell Count, RCC), RPD and RICC were calculated. Population doubling (PD), RPD and RICC were calculated by the following formula.

PD = [log {(Number of cells at the end of the culture) / (Number of cells at the start of the treatment)}] / log 2

RPD = (PD in test substance group) / (PD in negative control group) × 100

RICC = [Increase in number of cells in test substance group {(the end of the culture) - (the start of the treatment)}] / [Increase in number of cells in negative control group{(the end of the culture) - (the start of the treatment)}] × 100

5) Specimen preparation

(1) Leftover cells were collected by a centrifugation at 1000 rpm for 5 minutes and were treated hypotonically with 3 mL of 0.075 mol/L KCl at 37°C for 15 minutes. After 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 solutions. Then, the cell suspension was prepared with appropriate amount of a fixative solution, the suspension was dropped onto a glass slide. One specimen per dose was prepared.

(2) A specimen was dried and stained for about 15 minutes with 2 vol% Giemsa solution made with 1/15 mol/L phosphate buffer solution (pH 6.8).

b) Dose levels

Maximum dose: 2000 μg/mL, as the maximum dose in the case of no cytotoxicity on the test method.

Treatment method		Setting dose of test substance
Short-term treatment	Without S9 mix	31.3, 62.5, 125, 250, 500, 1000 and 2000 μg/mL (geometric progression: 2)
	With S9 mix	
24 hours continuous treatment		

c) Observation

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

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.

(1) Chromatid break

(2) Chromatid exchange

(3) Chromosome break

(4) Chromosome exchange (dicentric, ring and translocation)

(5) Fragmentation

2) Numerical aberration

The numbers of polyploid cells with triploid or more (38 or more chromosomes) and endoreduplicated cells were scored.

13.2 Chromosomal Aberration Test

a) Procedures

The chromosomal aberration test was carried out using the same procedure as that described in 13.1 a), with the following positive controls. Specimen preparation was carried out for the negative and positive controls and all doses of the test substance which RPD was 30% or more. Two dishes were used for each dose and four specimens per dose (two specimens per dish) were prepared. Two dishes were used for calculation of RPD and RICC.

The following volume of positive control substance solution per dish was added.

Treatment method		Substance	Concentration of frozen solution	Volume	Dose
Short-term treatment	Without S9 mix	MMC	0.01 mg/mL	15 μL	0.05 μg/mL
	With S9 mix	CPA	1.00 mg/mL	12 μL	4.00 μg/mL
24 hours continuous treatment		MMC	0.01 mg/mL	25 μL	0.05 μg/mL

b) Dose levels

The dose levels of the test substance were set on the basis of the results of the cell growth inhibition test. The setting doses and the reason for selection are shown below. When

RPD or RICC was less than 50%, it was judged that cytotoxicity was defined.

The cytotoxicity was not observed in "−S9 mix" and "+S9 mix". Therefore, the following doses were set.

In 24 hours continuous treatment, the cytotoxicity was observed. Therefore, the following doses were set to obtain the dose which RPD or RICC was 40% or more and 50% or less.

Treatment method		Setting doses of test substance
Short-term treatment	Without S9 mix	250, 500, 1000 and 2000 μg/mL (geometric progression: 2)
	With S9 mix	
24 hours continuous treatment		62.5, 125, 250, 354, 500, 707, 1000, 1410 and 2000 μg/mL (geometric progression: $\sqrt{2}$ or 2)

c) Observation

1) Dose for observation

All specimens of the negative and the positive controls were observed.

The cytotoxicity was observed in "−S9 mix". Therefore, the dose which RPD or RICC was 40% or more and 50% or less was selected as the maximum dose and the following doses were selected for observation.

The cytotoxicity was not observed in "+S9 mix". Therefore, the maximum concentration was selected to be 2000 μg/mL and the following doses were selected for observation.

In 24 hours continuous treatment, the cytotoxicity was observed. Therefore, the lowest dose which RPD or RICC was 40% or more and 50% or less was selected as the maximum dose and the following doses were selected for observation.

Treatment method		Observation doses of test substance
Short-term treatment	Without S9 mix	500, 1000 and 2000 μg/mL
	With S9 mix	500, 1000 and 2000 μg/mL
24 hours continuous treatment		250, 354 and 500 μg/mL

Slide numbers were allocated randomly to all observed specimens. All specimens were observed in a blinded manner.

2) Structural aberration

Three hundred metaphase cells per dose (75 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.

3) Numerical aberration

The numbers of polyploid cells with triploid or more (38 or more chromosomes) and endoreduplicated cells among 300 metaphase cells per dose (75 cells per specimen) were recorded.

13.3 Statistical Method

Statistical method was performed in order to compare the frequencies of cells with structural aberrations and numerically aberrant cells. Significance level of these tests was both sides of 1% and 5%.

Fisher's exact test was performed in order to compare in the negative control group with positive control group.

Fisher's exact test was performed in order to compare in the negative control group with test substance group when the frequencies of cells with chromosomal aberrations in each test substance group were outside the distribution of the historical data of the negative control group. As a result of Fisher's exact test, increased significantly compared to the negative control group, it was evaluated by using Cochran-Armitage trend test that the dose-dependency was exhibited.

14. JUDGEMENT CRITERIA OF TEST RESULTS

The acceptability criteria was considered to be negative, which were either in a) or in b):

a) all results are inside the distribution of the historical data of the negative control group,
b) outside the distribution of the historical data of the negative control group, but none of the doses of the test substance exhibits a statistically significant increase compared with the concurrent negative control.

Providing that all acceptability criteria in case of c) to e) or f) were fulfilled, a test substance was considered to be positive:

c) outside the distribution of the historical data of the negative control group,
d) the doses of the test substance exhibits a statistically significant increase compared with the concurrent negative control,
e) the increase of the frequencies of cells with chromosomal aberrations is dose-related,
f) both chromosomal aberration test and confirmation test are fulfilled in case of c) and d).

15. ACCEPTABILITY CRITERIA OF THE TEST

a) The frequencies of cells with chromosomal aberrations in the concurrent negative control are "under control" in quality control method used for control chart in the historical data.
b) The frequencies of cells with structural aberrations in the concurrent positive control are "under control" in quality control method used for control chart in the historical data. Concurrent positive controls produce a statistically significant increase compared with

the concurrent negative control.

c) Cell proliferation criteria in the negative control should be fulfilled.

d) If the findings are not judged to be positive by the result of the short-term treatments, the continuous treatment is carried out.

16. FACTORS AFFECTED RELIABILITY OF TEST

There were no factors that might have affected the reliability of the test.

17. RESULTS

17.1 Cell Growth Inhibition Test

The test results are shown in Table 1 and Fig. 1.

The precipitation of the test substance , the color change of the medium and the corrosion of the culture dish were not observed in all treatment methods.

17.2 Chromosomal Aberration Test

a) Short-term treatments

The test results are shown in Table 2 and Figs. 2 and 3.

1) −S9 mix

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

The precipitation of the test substance , the color change of medium and the corrosion of the culture dish were not observed.

(2) Frequency of cells with structural aberrations

Negative control:	The frequency was within the range of the historical data.
Positive control:	The frequency was within the range of the historical data. A statistically significant increase was observed at both sides of 1% level.
Test substance:	The frequencies were within the range of the historical data of the negative control. Therefore, it was judged to be negative.

(3) Frequency of numerically aberrant cells

Negative control:	The frequency was within the range of the historical data.
Test substance:	The frequencies were within the range of the historical data of the negative control. Therefore, it was judged to be negative.

2) +S9 mix

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

The precipitation of the test substance , the color change of medium and the corrosion of the culture dish were not observed.

(2) Frequency of cells with structural aberrations

Negative control:	The frequency was within the range of the historical data.
Positive control:	The frequency was within the range of the historical data. A statistically significant increase was observed at both sides of 1% level.
Test substance:	The frequencies were within the range of the historical data of the negative control. Therefore, it was judged to be negative.

(3) Frequency of numerically aberrant cells

Negative control:	The frequency was within the range of the historical data.
Test substance:	The frequencies were within the range of the historical data of the negative control. Therefore, it was judged to be negative.

b) 24 hours continuous treatment

The test results are shown in Table 3 and Figs. 2 and 4.

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

The precipitation of the test substance , the color change of medium and the corrosion of the culture dish were not observed.

2) Frequency of cells with structural aberrations

Negative control:	The frequency was within the range of the historical data.
Positive control:	The frequency was within the range of the historical data. A statistically significant increase was observed at both sides of 1% level.
Test substance:	The frequencies were within the range of the historical data of the negative control. Therefore, it was judged to be negative.

3) Frequency of numerically aberrant cells

Negative control:	The frequency was within the range of the historical data.
Test substance:	The frequencies were within the range of the historical data of the negative control. Therefore, it was judged to be negative.

18. HISTORICAL DATA

Historical data of negative control and positive control in the testing facility was attached in Appendix 1.

19. DISCUSSION

In each treatment method, the frequencies of cells with chromosomal aberrations in the negative and the positive control groups were within the range of the historical data. The frequency of cells with structural aberrations in the positive control was showed a statistically significant increased. Cell proliferation criteria in the negative control met the criteria in the testing facility. In addition, because the findings were judged to be negative by the result of the short-term treatments, the continuous treatment was carried out, indicating that the present study was appropriately performed.

As a result of the chromosomal aberration test, the frequencies of cells with structural aberrations and numerically aberrant cells at all observation doses of the test substance in all treatment methods were within the range of the historical data of the negative control. Therefore, structural aberration and numerical aberration were judged to be negative.

20. CONCLUSION

It was concluded that APFHx(C-1500N) did not induce chromosomal aberrations under the present test conditions.

21. REFERENCES

1.Sofuni, T. (ed.) Data book of chromosomal aberration test *in vitro*. Revised edition, 1998 (in Japanese). Life-science Information Center. (1999).

2.Japan Environmental Mutagenicity Society/Mammalian Mutagenicity Study Group (ed.) Atlas of chromosomal aberration induced with chemical substance (in Japanese). Asakura Publishing Co., Tokyo. (1988).

Table 1 Results of cell growth inhibition test

Substance	Dose (μg/mL)	Treatment-recovery time (hour)	S9 mix	Cell growth rate (%)	RPD (%)	RICC (%)	Precipitation of test substance in medium[a]			Index of metaphase cells	Frequency of cells with aberrations (%)[b]	
							Treatment start	Treatment end	Culture end		Structural aberration	Numerical aberration
Distilled water	0	6-18	-	100	100	100	-	-	-	n.s.	n.s.	n.s.
APFHx (C-1500N)	31.3	6-18	-	93.3	90.7	87.2	-	-	-	n.s.	n.s.	n.s.
	62.5	6-18	-	98.1	97.4	96.4	-	-	-	n.s.	n.s.	n.s.
	125	6-18	-	100.8	101.1	101.6	-	-	-	n.s.	n.s.	n.s.
	250	6-18	-	94.9	93.1	90.4	-	-	-	n.s.	n.s.	n.s.
	500	6-18	-	94.3	92.2	89.2	-	-	-	n.s.	n.s.	n.s.
	1000	6-18	-	82.5	74.3	66.8	-	-	-	abundant	n.o.	n.o.
	2000	6-18	-	73.9	59.5	50.4	-	-	-	abundant	2.0	0.0
Distilled water	0	6-18	+	100	100	100	-	-	-	n.s.	n.s.	n.s.
APFHx (C-1500N)	31.3	6-18	+	105.0	106.2	109.2	-	-	-	n.s.	n.s.	n.s.
	62.5	6-18	+	102.2	102.7	104.0	-	-	-	n.s.	n.s.	n.s.
	125	6-18	+	99.8	99.7	99.6	-	-	-	n.s.	n.s.	n.s.
	250	6-18	+	96.8	95.9	94.1	-	-	-	n.s.	n.s.	n.s.
	500	6-18	+	97.2	96.4	94.9	-	-	-	n.s.	n.s.	n.s.
	1000	6-18	+	90.6	87.5	82.8	-	-	-	n.s.	n.s.	n.s.
	2000	6-18	+	74.7	63.3	53.8	-	-	-	abundant	0.0	2.0
Distilled water	0	24-0	-	100	100	100	-	-		n.s.	n.s.	n.s.
APFHx (C-1500N)	31.3	24-0	-	98.0	97.7	96.5	-	-		n.s.	n.s.	n.s.
	62.5	24-0	-	94.1	93.1	89.9	-	-		n.s.	n.s.	n.s.
	125	24-0	-	83.6	79.6	71.9	-	-		abundant	n.o.	n.o.
	250	24-0	-	78.2	72.1	62.8	-	-		abundant	n.o.	n.o.
	500	24-0	-	64.6	50.3	39.4	-	-		abundant	4.0	0.0
	1000	24-0	-	64.8	50.6	39.7	-	-		abundant	4.0	0.0
	2000	24-0	-	49.1	19.0	12.9	-	-		no meta	n.o.	n.o.

RPD: relative population doubling, RICC: relative increase in cell count

n.s.: no specimens, n.o.: not observed, abundant: metaphases existed abundantly, 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 to be 2000 μg/mL, as the maximum dose in the case of no cytotoxicity on the test method, the dose levels based on a geometric progression of 2 were selected.

Table 2 Results of chromosomal aberration test (short-term treatments)

Name of test substance: APFHx(C-1500N)

K06-1564

Treatment time (h)	S9 mix	Dose (μg/mL)	Number of cells with structural chromosomal aberrations (frequency%)							Number of gaps (frequency%)	Cell growth rate (%)	RPD (%)	RICC (%)	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 (D.W.)	150	2	0	0	0	0	2	0	100	100	100	150	0	0	0
			150	0	0	0	0	0	0	1				150	1	0	1
		0	300	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)	1 (0.3)				300	1 (0.3)	0 (0.0)	1 (0.3)
6 - 18	-	250	0								100.1	100.2	100.2	0			
			0								94.9	92.6	89.9	0			
			0								(97.5)	(96.4)	(95.1)	0			
6 - 18	-	500	150	0	1	0	0	0	1	0	94.3	91.6	88.6	150	0	0	0
			150	0	0	0	0	0	0	0	91.0	86.4	82.0	150	0	0	0
			300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)	0 (0.0)	(92.7)	(89.0)	(85.3)	300	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	-	1000	150	2	0	0	0	0	2	0	90.2	85.1	80.3	150	1	0	1
			150	0	0	0	0	0	0	0	94.5	91.9	89.0	150	0	0	0
			300	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)	0 (0.0)	(92.4)	(88.5)	(84.7)	300	1 (0.3)	0 (0.0)	1 (0.3)
6 - 18	-	2000	150	1	0	0	0	0	1	1	77.8	63.7	55.5	150	0	0	0
			150	1	1	0	0	0	2	0	69.3	47.0	38.5	150	0	0	0
			300	2 (0.7)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.0)	1 (0.3)	(73.6)	(55.4)	(47.0)	300	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	-	Positive control (MMC)	150	19	23	0	0	0	36	1	94.1	91.3	88.2	150	0	0	0
			150	15	27	0	0	0	39	0	88.5	82.4	77.0	150	1	0	1
		0.05	300	34 (11.3)	50 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	75 (25.0)**	1 (0.3)	(91.3)	(86.9)	(82.6)	300	1 (0.3)	0 (0.0)	1 (0.3)
6 - 18	+	Negative control (D.W.)	150	0	1	0	0	0	1	0	100	100	100	150	0	0	0
			150	2	0	0	0	0	2	0				150	1	0	1
		0	300	2 (0.7)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.0)	0 (0.0)				300	1 (0.3)	0 (0.0)	1 (0.3)
6 - 18	+	250	0								102.3	103.3	104.5	0			
			0								102.5	103.6	105.0	0			
			0								(102.4)	(103.5)	(104.8)	0			
6 - 18	+	500	150	1	0	0	0	0	1	0	95.9	93.9	91.7	150	0	0	0
			150	1	0	0	0	0	1	0	97.3	96.1	94.6	150	3	0	3
			300	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)	0 (0.0)	(96.6)	(95.0)	(93.2)	300	3 (1.0)	0 (0.0)	3 (1.0)
6 - 18	+	1000	150	2	1	0	0	0	3	0	94.4	91.7	88.8	150	1	0	1
			150	1	0	1	0	0	2	0	89.3	83.6	78.5	150	0	0	0
			300	3 (1.0)	1 (0.3)	1 (0.3)	0 (0.0)	0 (0.0)	5 (1.7)	0 (0.0)	(91.9)	(87.7)	(83.7)	300	1 (0.3)	0 (0.0)	1 (0.3)
6 - 18	+	2000	150	1	0	0	0	0	1	0	81.4	70.3	62.8	150	0	0	0
			150	1	1	0	0	0	2	0	77.7	63.6	55.4	150	0	0	0
			300	2 (0.7)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.0)	0 (0.0)	(79.6)	(67.0)	(59.1)	300	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	Positive control (CPA)	150	15	26	0	0	0	37	0	84.5	75.7	69.0	150	0	0	0
			150	20	23	0	0	0	40	0	73.6	55.7	47.1	150	1	0	1
		4	300	35 (11.7)	49 (16.3)	0 (0.0)	0 (0.0)	0 (0.0)	77 (25.7)**	0 (0.0)	(79.1)	(65.7)	(58.1)	300	1 (0.3)	0 (0.0)	1 (0.3)

Treatment times comprised treatment-time and recovery-time.

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

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

RPD: relative population doubling, RICC: relative increase in cell count, D.W.: distilled water, MMC: mitomycin C, CPA: cyclophosphamide monohydrate

In all treatment methods, the specimens at 250 μg/mL was not observed.

**: significant increase compared to negative control at $p<0.01$ [Fisher's exact test]

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Table 3 Results of chromosomal aberration test (contiunous treatment)

Name of test substance: APFHx(C-1500N)

K06-1564

Treatment time (h)	Dose (μg/mL)	Number of cells with structural chromosomal aberrations (frequency%)							Number of gaps (frequency%)	Cell growth rate (%)	RPD (%)	RICC (%)	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 (D.W.)	150	1	0	0	0	0	1	0	100	100	100	150	0	0	0
		150	1	0	0	0	0	1	1				150	0	0	0
	0	300	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)	1 (0.3)				300	0 (0.0)	0 (0.0)	0 (0.0)
24 - 0	62.5	0								91.2	88.8	84.3	0			
		0								98.1	97.7	96.6	0			
		0								(94.7)	(93.3)	(90.5)	0			
24 - 0	125	0								90.1	87.4	82.4	0			
		0								92.5	90.5	86.6	0			
		0								(91.3)	(89.0)	(84.5)	0			
24 - 0	250	150	2	0	0	0	0	2	0	81.6	75.3	67.2	150	0	0	0
		150	3	2	0	0	0	5	0	84.5	79.5	72.4	150	0	0	0
		300	5 (1.7)	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	7 (2.3)	0 (0.0)	(83.1)	(77.4)	(69.8)	300	0 (0.0)	0 (0.0)	0 (0.0)
24 - 0	354	150	1	1	0	0	0	2	1	72.9	61.6	51.7	150	0	0	0
		150	2	0	0	0	0	2	1	74.9	64.9	55.3	150	0	0	0
		300	3 (1.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.3)	2 (0.7)	(73.9)	(63.3)	(53.5)	300	0 (0.0)	0 (0.0)	0 (0.0)
24 - 0	500	150	3	0	0	0	0	3	0	64.6	46.8	36.8	150	0	0	0
		150	1	0	0	0	0	1	0	66.8	50.9	40.7	150	0	0	0
		300	4 (1.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.3)	0 (0.0)	(65.7)	(48.9)	(38.8)	300	0 (0.0)	0 (0.0)	0 (0.0)
24 - 0	707	0								62.6	43.0	33.3	0			
		0								62.8	43.4	33.6	0			
		0								(62.7)	(43.2)	(33.5)	0			
24 - 0	1000	0								56.7	30.8	22.6	0			
		0	n.s.							55.2	27.7	20.0	0	n.s.		
		0								(56.0)	(29.3)	(21.3)	0			
24 - 0	1410	0								51.2	18.6	12.9	0			
		0	n.s.							48.1	11.0	7.4	0	n.s.		
		0								(49.7)	(14.8)	(10.2)	0			
24 - 0	2000	0								41.4	-7.2	-4.5	0			
		0	n.s.							40.5	-9.9	-6.1	0	n.s.		
		0								(41.0)	(-8.6)	(-5.3)	0			
24 - 0	Positive control (MMC)	150	38	59	1	0	0	82	1	78.0	69.8	60.7	150	0	0	0
		150	41	77	0	0	0	94	1	77.1	68.4	59.1	150	0	0	0
	0.05	300	79 (26.3)	136 (45.3)	1 (0.3)	0 (0.0)	0 (0.0)	176 (58.7)**	2 (0.7)	(77.6)	(69.1)	(59.9)	300	0 (0.0)	0 (0.0)	0 (0.0)

Treatment times comprised treatment-time and recovery-time.

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

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

RPD: relative population doubling, RICC: relative increase in cell count, D.W.: distilled water, MMC: mitomycin C, n.s.: no specimens

The specimens at 62.5, 125 and 707 μg/mL were not observed.

**: significant increase compared to negative control at $p<0.01$ [Fisher's exact test]

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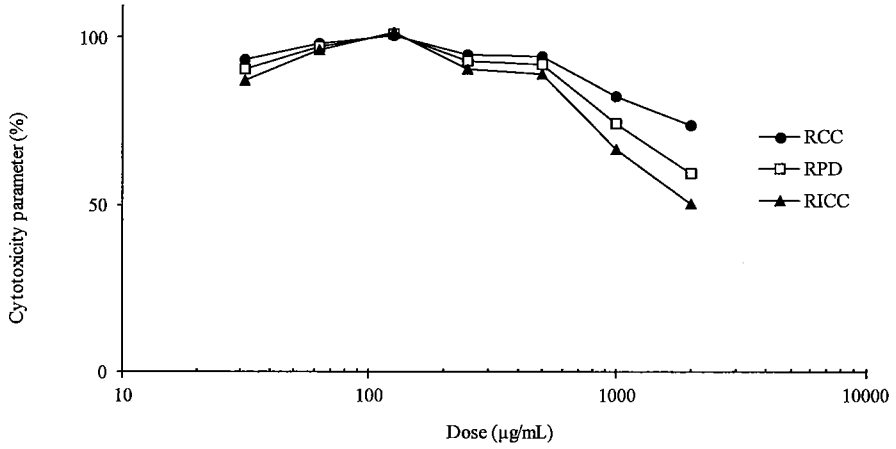
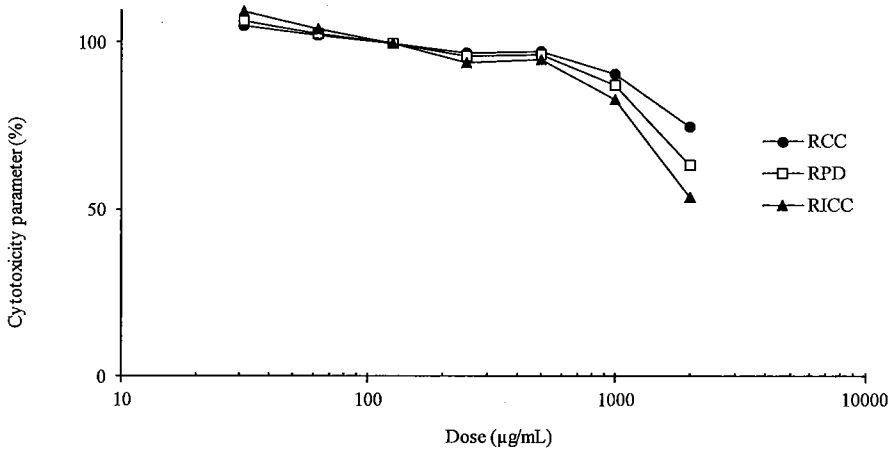


Short-term treatment without S9 mix



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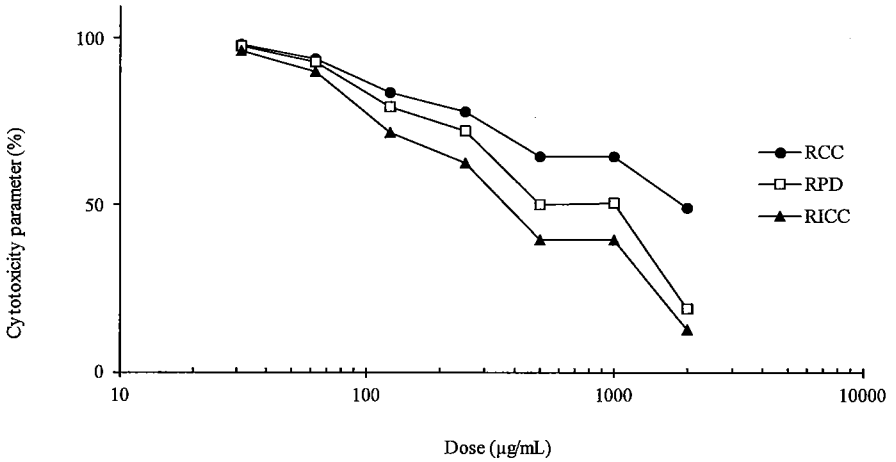
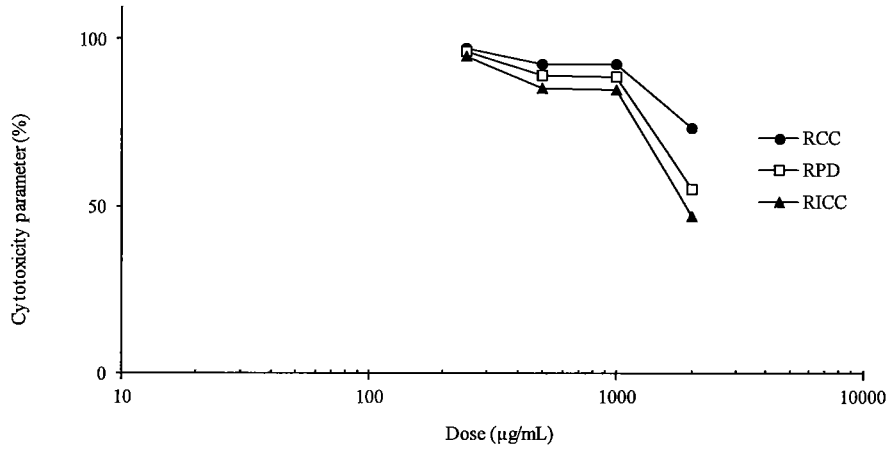


24 hours continuous treatment

Fig. 1 Results of cell growth inhibition test



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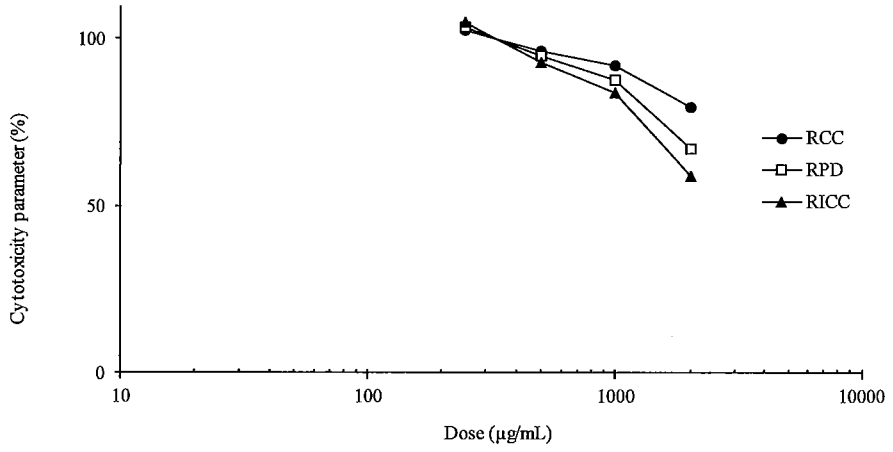
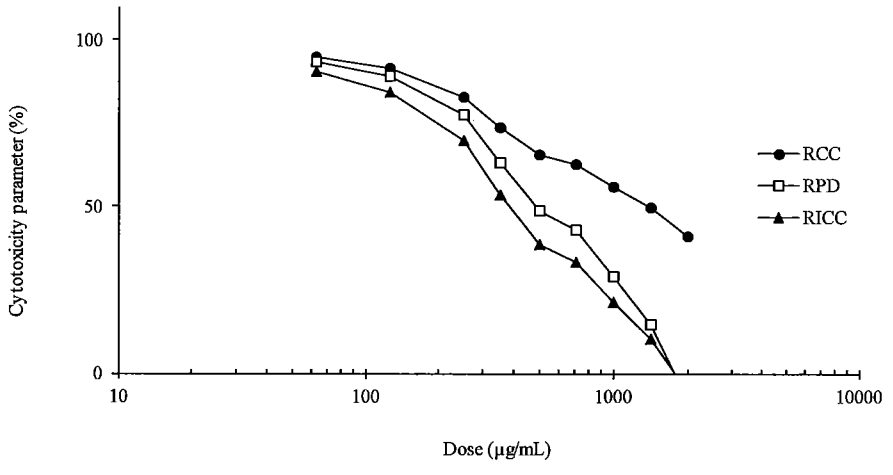
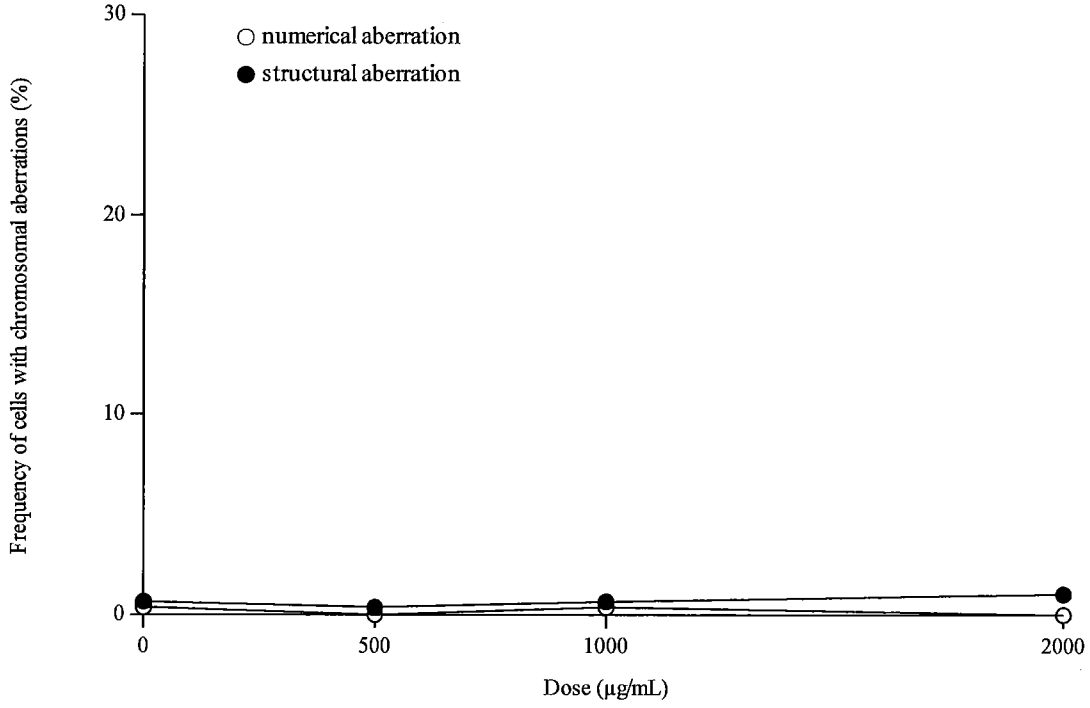


Short-term treatment with S9 mix

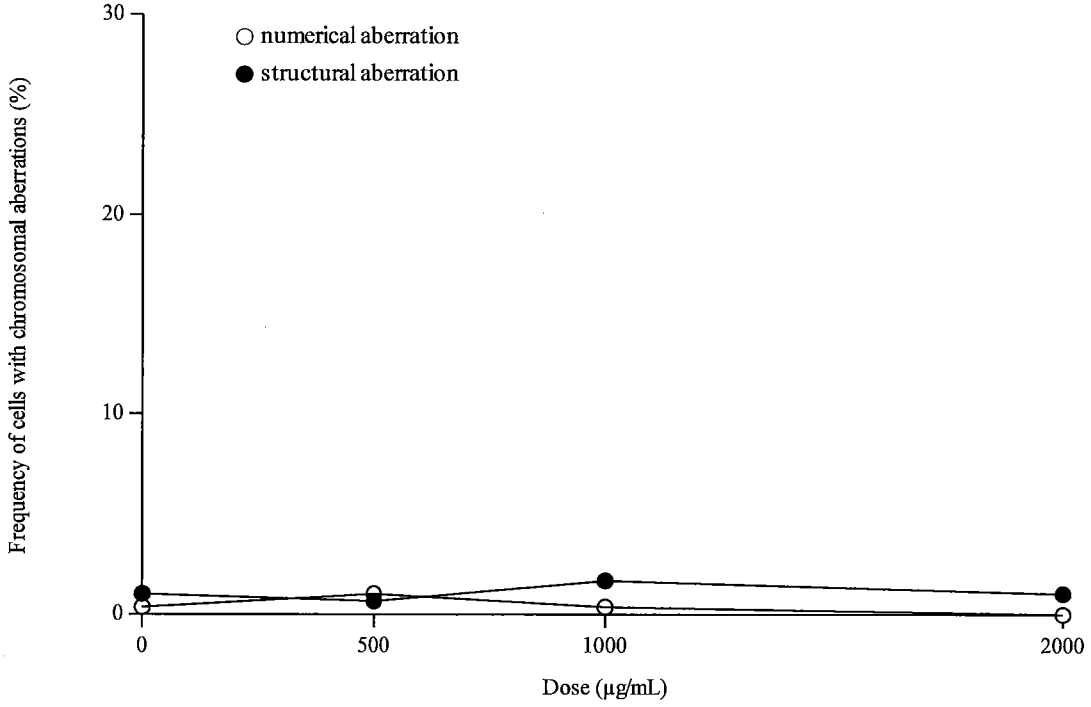


24 hours continuous treatment

Fig. 2 Cytotoxicity parameter in chromosomal aberration test



Without S9 mix



With S9 mix

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

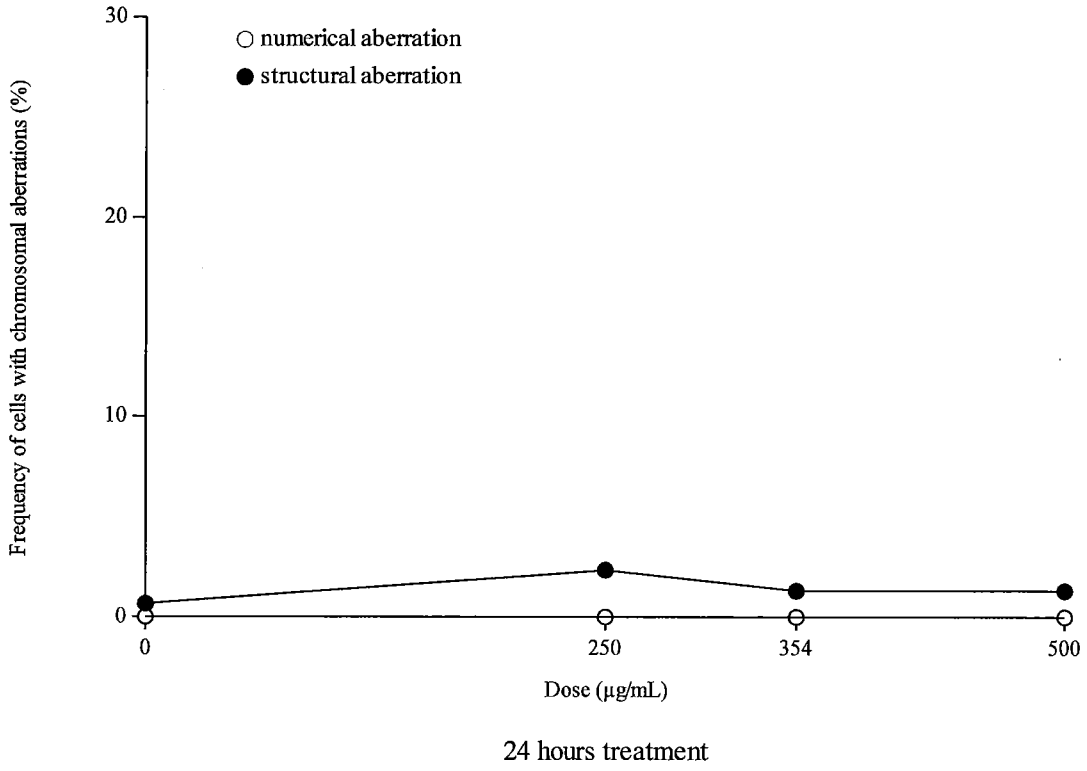


Fig. 4 Results of chromosomal aberration test in continuous treatment

Appendix 1 Historical data in the testing facility

Negative control

Treatment method		Frequency of cells with chromosomal aberrations (%)			
		Structural aberration		Numerical aberration	
		Mean	SD	Mean	SD
Short-term treatment	Without S9 mix	1.9	0.54	0.4	0.31
	With S9 mix	1.5	0.65	0.5	0.38
24 hours continuous treatment		1.5	0.52	0.4	0.33

Treatment method		Range of frequency of cells with chromosomal aberrations (%, Values in parentheses indicate 95% control limit)			
		Structural aberration		Numerical aberration	
		Lower control limit	Upper control limit	Lower control limit	Upper control limit
Short-term treatment	Without S9 mix	< 0 (< 0)	4.3 (4.2)	< 0 (< 0)	1.6 (1.5)
	With S9 mix	< 0 (< 0)	3.7 (3.6)	< 0 (< 0)	1.8 (1.7)
24 hours continuous treatment		< 0 (< 0)	3.6 (3.5)	< 0 (< 0)	1.6 (1.5)

The minimum range below 0 was shown "< 0".

Positive control

Treatment method		Substance	Dose (μg/mL)	Frequency of cells with chromosomal aberrations (%)	
				Structural aberration	
				Mean	SD
Short-term treatment	Without S9 mix	MMC	0.05	28.8	3.50
	With S9 mix	CPA	4.00	32.2	3.41
24 hours continuous treatment		MMC	0.05	64.0	3.05

Treatment method		Range of frequency of cells with chromosomal aberrations (%, Values in parentheses indicate 95% control limit)	
		Structural aberration	
		Lower control limit	Upper control limit
Short-term treatment	Without S9 mix	19.5 (19.9)	38.1 (37.6)
	With S9 mix	22.4 (22.9)	42.1 (41.6)
24 hours continuous treatment		50.1 (50.8)	77.8 (77.1)

The minimum range below 0 was shown "< 0".

The latest 20 test data completed by February, 2017 were used.

Upper and lower control limits were calculated from number of cells with chromosomal aberrations using c chart.

The value was converted to the frequency from the number of cells with chromosomal aberrations.

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Hita

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: Chromosomal aberration test of APFHx(C-1500N) using cultured mammalian cells

Study Number: K06-1564

I assure that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study. The inspections of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance Unit as follows.

Item of inspection	Date of inspection	Date of report
Study plan	March 6, 2017	March 6, 2017
Preparation of test substance solution and treatment of cell	March 13, 2017	March 13, 2017
Study plan amendment No. 1	April 4, 2017	April 4, 2017
Observation of specimens	May 29, 2017	May 29, 2017
Raw data and draft final report	June 29, 2017	June 29, 2017
Final report	July 3, 2017	July 3, 2017

The inspection result of following item was reported to the Study Director and the Test Facility Management based on the report of facility-based inspection and/or process-based inspection relevant to this study type and timeframe.

Item of inspection	Date of inspection	Date of report
Preparation and management of positive control substance solutions	December 7, 2016	July 3, 2017
Preparation of medium and reagents	March 9 and 10, 2017	July 3, 2017
Cell subculture	March 3, 2017	July 3, 2017
Cell pre-culture	March 3, 2017	July 3, 2017
Cell collection and preparation of specimens	March 3 and 6, 2017	July 3, 2017

Date: July 3, 2017

Quality Assurance Manager: _