AR226_3307

CHROMOSOMAL ABERRATION TEST RESULTS USING MAMMALIAN CULTURE CELLS

1. General information

Nomenclature of novel chemical substance (according to IUPAC nomenclature)	[redacted]		
Other name	[redacted]		
Structural formula and empirical formula (outline of preparation method when unknown)	[redacted]		
Purity of novel chemical substance used in the test	[redacted]	Lot No. of novel chemical substance used in test	[redacted]
Name and concentration of impurities	Uncertain 0.4%		
CAS No.	[redacted]	Vapor pressure	–
Molecular weight	370.37	Distribution coefficeint	–
Melting point	–	Appearance at room temperature	Brown viscous solid
Boiling point	–		
Stability	Unknown		

Solubility in solvents, etc.	Solvent	Solubility	Stability in solvents
	Water	<50 mg/mL (measured on site)	*
	DMSO	<50 mg/mL (measured on site)	–
	Acetone	–	–
	Other (ethanol)	Soluble (solubility unknown)	–

[Note]
Hydrolysis property: unknown
* The 50-mg/mL solution was confirmed to be free of discoloration, heat emission, etc., up to 2 h after preparation (confirmed on site at Hita Works)

2. Cell type and culture conditions

Cell name	CHL/IU	Acquisition		Human Science Shinko Foundation, Research Resources Bank
Species	Chinese hamster	Acquisition date		April 17, 2002
Culture solution	Eagle MEM	Producer		Nippon Seiyaku K.K.
Serum type and amount added	Newborn calf serum (NBCS), 10%	Producer (Lot No.)		Miko Junyaku K.K. (27020859)
Cell cycle	About 15 h	Freeze conditions		-196°C
No. of generations	<20 after acquisition	Incubation conditions	Container	Plastic dish of 90 mm in diameter
			Temperature	37 ± 0.5°C
No. of chromosomes (mode)	25		CO_2 concentration	5%
Note:				

3. S9 mix

(1) S9 acquisition, etc.

Self-made or acquisition	1. Self-made ② Acquisition (Oriental Kobo Kogyo K.K.)
Production date	May 9, 2003
Lot No. for acquisition	03050902
Storage temperature	-80°C

(2) S9 preparation

Animal used		Derivative material	
Species-origin	Rat-SD	Name	PB and 5,6-PF
Sex	male	Dosage	Abdominal cavity
Age	7 weeks	Dose period and dosage (g/kg-body weight)	PB 30 mg/kg one time PB 60 mg/kg three times 5,6-PF 80 mg/kg one time
Body weight	233.5 ± 8.4 g		

(3) S9 mix composition

Component	Amount in 1 mL of S9	Component	Amount in 1 mL of S9
S9	0.3 mL	NADP	4 μmol
$MgCl_2$	5 μmol	Na phosphate buffer	–
KCl	33 μmol	HEPES (pH 7.2)	4 μmol
Glucose-6-phosphoric acid	5 μmol	Others (–)	–

(4) S9 mix treatment conditions

① Plate method 2. Flotation cell method 3. Other ( )	
S9 (final concentration)	5%
S9 protein (final concentration)	1.17* mg/mL
Treatment time	6 h
Recovery time	18 h
Time	* S9 Assay Data (Oriental Kobo Kogyo K.K.)

4. Preparation of test substance solution

Solvent used	Name	Producer	Lot No.	Grade	Purity (%)
	Distilled water	Otsuka Seiyaku K.K.	K2J77	Injectable water	–
Reasons for solvent selection	The test substance was not dissolved to [sic] 50 mg/mL in distilled water or 500 mg/mL in DMSO and acetone, while 50 mg/mL in distilled water showed a good suspension state; furthermore, there was no heat emission or discoloration at room temperature up to 2 h after preparation, thus distilled water was chosen as the solvent.				
Test substance solution	Dissolution (Suspension) Other ( )				
Method for suspension, etc., when the test substance is not soluble	Stirring alone gave a good suspension, thus the Labomixer was used for making the suspension				
Storage time and temperature of the solution after preparation	Within 2 h, room temperature				
Purity conversion	Yes (No)				

5. Short-term treatment test

(1) Conditions for cell growth inhibition test

		Without metabolism activation	With metabolism activation
Test duration		6/25/03 to 6/26/03	6/25/03 to 6/26/03
Incubator	Shape	Round plastic dish	Round plastic dish
	Size	Diameter: 60 mm	Diameter: 60 mm
	Culture solution [a)]	3 mL/dish	3 mL/dish
	No. of incubators per dose	2	2
Cells	No. of inoculated cells	1.5×10^4/mL	1.5×10^4/mL
	Incubation duration	2 days	2 days
Treatment conditions	Amount of test substance solution added [b)]	0.3 mL/dish	0.3 mL/dish
	Amount of S9 mix added	–	0.5 mL/dish
	S9 final concentration	–	5%
	S9 protein final concentration	–	1.17 mg/mL
	Treatment time	6 h	6 h
	Recovery time	18 h	18 h
Cell growth inhibition measurement	Electric cell counter (Microcell Counter CDA-500) was used for counting the number of cells		

Notes

a) Liquid quantity at treatment time. Recovery period [sic; level] after treatment: 5 mL/dish

b) Composition of medium used in the treatment of the test substance:

Without metabolism activation:

Medium 2.7 mL (= NBCS 0.3 mL + 2-fold concentration MEM (x 2 MEM) 1.35 mL + distilled water 1.05 mL) + test substance solution 0.3 mL

With metabolism activation:

Medium 2.2 mL (= NBCS 0.25 mL + x2 MEM 1.125 mL + distilled water 0.825 mL) + S9 mix 0.5 mL + test substance solution 0.3 mL

(2) Cell growth and aberration cell appearance frequency

Without metabolism activation (6-18)					With metabolism activation (6-18)				
Dose (μg/mL)	Cell growth[a] (%)	Incidence of split cells[b]	Aberration cell appearance frequency[c]		Dose (μg/mL)	Cell growth[a] (%)	Incidence of split cells[b]	Aberration cell appearance frequency[c]	
			Structural aberration	Numerical aberration				Structural aberration	Numerical aberration
0	100	abundant	0	2	0	100	abundant	2	0
19.5	93.3	abundant	n.o.	n.o.	19.5	101.9	abundant	n.o.	n.o.
39.1	84.7	abundant	n.o.	n.o.	39.1	90.5	abundant	n.o.	n.o.
78.1	84.2	abundant	n.o.	n.o.	78.1	92.3	abundant	n.o.	n.o.
156	70.9	abundant	n.o.	n.o.	156	86.4	abundant	n.o.	n.o.
313	66.9	abundant	n.o.	n.o.	313	87.4	abundant	n.o.	n.o.
625	59.1	abundant	0	0	625	79.1	abundant	0	0
1250	53.0	abundant	6	0	1250	75.3	abundant	4	2
2500†	64.2	abundant	2	2	2500†	72.6	abundant	4	0
5000†	59.7	abundant	6	0	5000†	66.0	abundant	4	0

a) Average value of 2 sheets
b) abundant: sufficient split cells　　rare: very few split cells
a few: few split cells　　none: no split cells at all
c) 50 cells were observed
n.o.: not observed under microscope
†: Test substance precipitation occurred at the beginning and end of the treatment.
Note) Upper limit of dosage was set at 5000 μg/mL

(3) Chromosome aberration test conditions

		Without metabolism activation	With metabolism activation
Test duration		7/2/03 to 7/3/03	7/2/03 to 7/3/03
Incubator	Shape	Round plastic dish	Round plastic dish
	Size	Diameter: 60 mm	Diameter: 60 mm
	Culture solution [a)]	3 mL/dish	3 mL/dish
	No. of incubators per dose	2	2
Cells	No. of inoculated cells	1.5×10^4/mL	1.5×10^4/mL
	Incubation duration	2 days	2 days
Treatment conditions	Amount of test substance solution added [b)]	0.3 mL/dish	0.3 mL/dish
	Amount of S9 mix added	–	0.5 mL/dish
	S9 final concentration	–	5%
	S9 protein final concentration	–	1.17 mg/mL
	Treatment time	6 h	6 h
	Recovery time	18 h	18 h

Notes
a) Liquid quantity at treatment time. Recovery period [sic] after treatment: 5 mL/dish
b) Composition of medium used in the treatment of the test substance:
Without metabolism activation:
Medium 2.7 mL (= NBCS 0.3 mL + x2 MEM 1.35 mL + distilled water 1.05 mL) + test substance solution 0.3 mL
With metabolism activation:
Medium 2.2 mL (= NBCS 0.25 mL + x2 MEM 1.125 mL + distilled water 0.825 mL) + S9 mix 0.5 mL + test substance solution 0.3 mL
Cell growth was calculated from the number of cells counted by electric cell counter (Microcell Counter CDA-500)

(4) Chromosome aberration test results (Table 1)

6. Continuous treatment test

(1) Cell growth inhibition test conditions

Test duration		6/25/03 to 6/26/03
Incubator	Shape	Round plastic dish
	Size	Diameter: 60 mm
	Culture solution	5 mL/dish
	No. of incubators per dose	2
Cells	No. of inoculated cells	1.5×10^4/mL
	Incubation duration	2 days
Treatment conditions	Amount of test substance solution added [a)]	0.5 mL/dish
	Treatment time	24 h
	Recovery time	0 h
Cell growth inhibition measurement	Number of cells counted by electric cell counter (Microcell Counter CDA-500)	

Note

a) Composition of medium used in treatment of test substance
Medium 4.5 mL (= NBCS 0.5 mL + x2 MEM 2.25 mL + distilled water 1.75 mL) + test substance solution 0.5 mL

(2) Cell growth and aberration cell appearance frequency

Without metabolism activation (24-0)					
Dose (μg/mL)	Cell growth [a] (%)	Relative split index [b] (%)	Incidence of split cells [c]	Aberration cell appearance frequency [d] (%)	
				Structural aberration	Numerical aberration
0	100	100	abundant	0	0
19.5	95.2	93.9	abundant	n.o.	n.o.
39.1	98.8	65.3	abundant	0	0
78.1	91.5	20.4	a few	0	0
156	83.2	n.o.	rare	n.o.	n.o.
313	61.7	n.o.	none	n.o.	n.o.
625	33.5	n.o.	none	n.o.	n.o.
1250	24.0	n.o.	none	n.o.	n.o.
2500†	16.5	n.o.	none	n.o.	n.o.
5000†	11.7	n.o.	none	n.o.	n.o.

a) Average value of 2 sheets
b) Calculated by split cells in 500 cells
c) abundant: sufficient split cells　　rare: very few split cells
a few: few split cells　　none: no split cells at all
d) 50 cells were observed
n.o.: not observed under microscope
†: Test substance precipitation occurred at the beginning and end of the treatment.
Note) Upper limit of dosage was set at 5000 μg/mL

(3) Chromosome aberration test conditions

Test duration		7/2/03 to 7/3/03
Incubator	Shape	Round plastic dish
	Size	Diameter: 60 mm
	Culture solution	5 mL/dish
	No. of incubators per dose	2
Cells	No. of inoculated cells	1.5×10^4/mL
	Incubation duration	2 days
Treatment conditions	Amount of test substance solution added [a)]	0.5 mL/dish
	Treatment time	24 h
	Recovery time	0 h

Note
a) Composition of medium used in treatment of test substance
Medium 4.5 mL (= NBCS 0.5 mL + x2 MEM 2.25 mL + distilled water 1.75 mL) + test substance solution 0.5 mL
Number of cells counted by electric cell counter (Microcell Counter CDA-500) for cell growth

(4) Chromosome aberration test results (Table 2)

(5) Split index in chromosome aberration test (Table 3)

7. Result evaluation and notes

(1) Result evaluation

Evaluation (surrounded by a circle)	Positive	Negative (circled)
Reasons for evaluation The evaluation is given because of no chromosomal aberration increase in both the short-term treatment and continuous treatment		

(2) Notes

1. Reasons for setting dosage of test substance

Cell growth inhibition test results

Treatment method		IC_{50} value	Maximum dosage used for observation of needed split image and [cell growth]
Short-term treatment	- S9 mix	>5,000 μg/mL	5,000 μg/mL [59.7%]
	+ S9 mix	>5,000 μg/mL	5,000 μg/mL [66.0%]
Continuous treatment	24-0 h	About 440 μg/mL	78.1 μg/mL [91.5%]

In short-term treatment, the maximum dosage for observing mid-term split images needed for analysis of chromosomal aberrations had an upper limit of 5000 μg/mL in the presence or absence of the S9 mix. Both in the presence and absence of the S9 mix, with a maximum dosage at 5000 μg/mL, and with the appearance of light cell toxicity, 1580 and 500 μg/mL diluted at a nominal ratio [sic] of $\sqrt{10}$ were set.

In the continuous treatment, the maximum dosage for the observation of the mid-term split image needed for analysis of chromosomal aberrations was 78.1 μg/mL. At 78.1 μg/mL, cell growth was 91.5%, and the relative split index was inhibited to 20.4% of the negative control, thus a distinct cell toxicity was judged to occur. Thus, the concentration for inhibiting 50% of split cells in the cell growth inhibition test was about 52 μg/mL; with a maximum dosage of 78.1 μg/mL, 39.1 and 19.5 μg/mL diluted by a nominal ratio of 2 were set.

2. Toxicity evaluation in continuous treatment

At 78.1 μg/mL set as the maximum dosage used in the continuous treatment, the cell growth in the chromosome aberration test is 86.9%; cell growth inhibition exceeding 50% is not observed, while a relative split index compared with the negative control is decreased to 38.3%; thus, it is judged that a distinct cell toxicity needed for evaluating chromosomal aberrations occurred.

3. Chromosome aberration evaluation standard

A positive judgment is given when the appearance frequency of cells having structural aberrations and numerical* aberrations increased more than 10% with a dependence on the dosage or when an increase exceeding 5% is regenerated in the chromosome aberration test and confirmation test; all others are judged as negative.

4. Figures

Figure 1: Cell growth inhibition test results of short-term treatment using [redacted]

Figure 2: Chromosome aberration test results of short-term treatment using [redacted]

Figure 3: Cell growth inhibition test results of continuous treatment using [redacted]

Figure 4: Chromosome aberration test results of continuous treatment using [redacted]

* [Not certain if "numerical" in this case indicates the "number" of aberrations.]

8. Others

Test facilities	Name	Hita Works; Kagaku Busshitsu Heikakenkjukiku
	Address	3-822 Ishii, Hita, Oita Tel 0973 (24) 7211 Fax 0973 (23) 9800
Principal tester	Department and Name	Test Department 3 Yoshikazu Anjimi
	Experience	17 years
Test duration	6/23/03 to 11/13/03	
Test No.	K06-1022	

Table 1: Chromosome aberration test results (short-term treatment)

Treatment time (h)	S9 mix	Dosage (μg/mL)	Number of cells with chromosome structural aberrations (appearance frequency %)							Gap appearance frequency (appearance frequency %)	Cell growth (%)	Number of cells of [with] chromosome numerical aberrations (appearance frequency %)			
			Number of cells observed	Chromosome fragment cleavage	Chromosome fragment exchange	Chromosome cleavage	Chromosome exchange	Other	Total number of aberration cells			Number of cells observed	Multiple	Other	Total number of aberration cells
6 - 18	–	negative control (D.W.) 0	100	0	0	1	0	0	1	0	100	100	1	0	1
			100	2	0	0	0	0	2	1		100	2	0	2
			200	2 (1.0)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	3 (1.5)	1 (0.5)		200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	–	500	100	1	0	0	0	0	1	0	79.9	100	0	0	0
			100	1	1	0	0	0	2	0	76.5	100	1	0	1
			200	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	0 (0.0)	(78.2)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	–	1,580	100	0	0	1	0	0	1	1	72.6	100	0	0	0
			100	3	0	0	0	0	3	0	75.0	100	3	0	3
			200	3 (1.5)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	4 (2.0)	1 (0.5)	(73.8)	200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	–	5,000 †	100	1	0	0	0	0	1	1	77.8	100	2	0	2
			100	1	0	0	0	0	1	1	68.9	100	0	0	0
			200	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	2 (1.0)	(73.4)	200	2 (1.0)	0 (0.0)	2 (1.0)
6 - 18	–	positive control (MMC) 0.1	100	31	40	1	0	0	57	3		100	1	0	1
			100	19	37	3	0	0	52	1		100	0	0	0
			200	50 (25.0)	77 (38.5)	4 (2.0)	0 (0.0)	0 (0.0)	109 (54.5)	4 (2.0)		200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	+	negative control (D.W.) 0	100	1	1	0	0	0	2	1	100	100	0	0	0
			100	0	0	0	0	0	0	0		100	1	0	1
			200	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	1 (0.5)		200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	+	500	100	0	0	0	0	0	0	1	90.4	100	0	0	0
			100	0	0	0	0	0	0	0	92.8	100	1	0	1
			200	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	(91.6)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	+	1,580	100	0	0	1	0	0	1	0	91.8	100	1	0	1
			100	0	0	0	0	0	0	0	90.4	100	2	0	2
			200	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	(91.1)	200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	+	5,000 †	100	1	0	0	0	0	1	0	71.7	100	0	0	0
			100	0	1	0	0	0	1	0	79.2	100	0	0	0
			200	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	(75.5)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	positive control (CPA) 6	100	32	42	3	0	0	54	1		100	1	0	1
			100	39	64	0	0	0	75	1		100	0	0	0
			200	71 (35.5)	106 (53.0)	3 (1.5)	0 (0.0)	0 (0.0)	129 (64.5)	2 (1.0)		200	1 (0.5)	0 (0.0)	1 (0.5)

The treatment duration is treatment time – recovery time.
Data for aberration cell numbers in plates of each group are given in the first and second rows, with their sum in the third row.
The numerical value of cell growth in plates of each test substance group are given in the first and second rows, with their sum in the third row.
D.W.: distilled water
MMC: mitomycin C
CPA: dichlorophosphamide hydrate
†: Test substance precipitation occurred at the beginning and end of the treatment.

Table 2: Chromosome aberration test results (continuous treatment)

Test substance name: [redacted]

Treatment time (h)	Dosage (μg/mL)	Number of cells with chromosome structural aberrations (appearance frequency %)							Gap appearance frequency (appearance frequency %)	Cell growth (%)	Number of cells of [with] chromosome numerical aberrations (appearance frequency %)			
		Number of cells observed	Chromosome fragment cleavage	Chromosome fragment exchange	Chromosome cleavage	Chromosome exchange	Other	Total number of aberration cells			Number of cells observed	Multiple	Other	Total number of aberration cells
	negative control	100	0	1	0	0	0	1	1		100	1	0	1
24 - 0	(D.W.)	100	1	0	0	0	0	1	0	100	100	1	0	1
	0	200	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	1 (0.5)		200	2 (1.0)	0 (0.0)	2 (1.0)
		100	2	0	0	0	0	2	0	91.9	100	0	0	0
24 - 0	19.5	100	0	0	0	0	0	0	0	90.5	100	1	0	1
		200	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	(91.2)	200	1 (0.5)	0 (0.0)	1 (0.5)
		100	2	0	0	0	0	2	0	83.8	100	1	0	1
24 - 0	39.1	100	0	0	0	0	0	0	0	78.8	100	0	0	0
		200	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	(81.3)	200	1 (0.5)	0 (0.0)	1 (0.5)
		100	0	0	0	0	0	0	0	76.4	100	2	0	2
24 - 0	78.1	100	0	0	0	0	0	0	0	97.4	100	1	0	1
		200	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	(86.9)	200	3 (1.5)	0 (0.0)	3 (1.5)
	positive control	100	28	41	2	0	0	56	2		100	1	0	1
24 - 0	(MMC)	100	16	53	3	0	0	60	0		100	0	0	0
	0.05	200	44 (22.0)	94 (47.0)	5 (2.5)	0 (0.0)	0 (0.0)	116 (58.0)	2 (1.0)		200	1 (0.5)	0 (0.0)	1 (0.5)

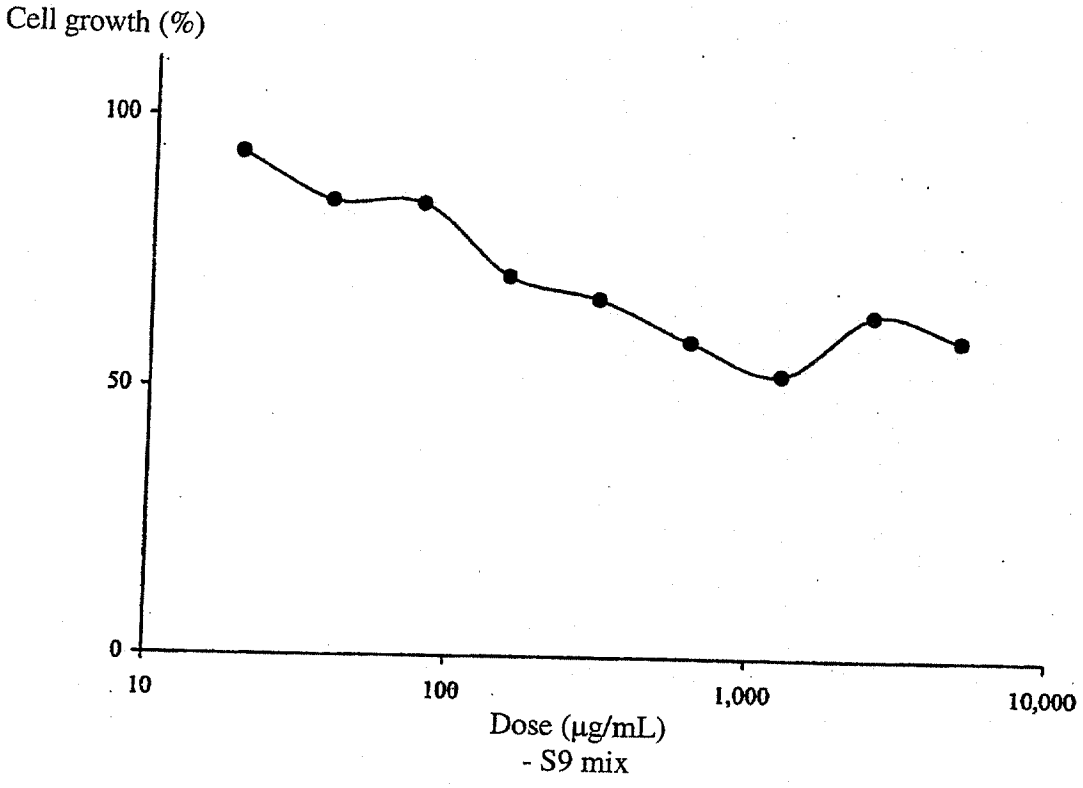
The treatment duration is treatment time – recovery time.
Data for aberration cell numbers in plates of each group are given in the first and second rows, with their sum in the third row.
The numerical value of cell growth in plates of each test substance group are given in the first and second rows, with their sum in the third row.
D.W.: distilled water
MMC: mitomycin C

Table 3: Split index in chromosome aberration test (continuous treatment)

Treatment time (h)	S9 mix	Dosage (µg/mL)	Relative split index [a] (%)
24-0	–	Negative control (distilled water) 0	100
		19.5	101.8
		39.1	80.0
		78.1	38.3

Relative split index is calculated based on 100% as the split index of the negative control.

a) Calculated with the observation of 1000 cells per dose.



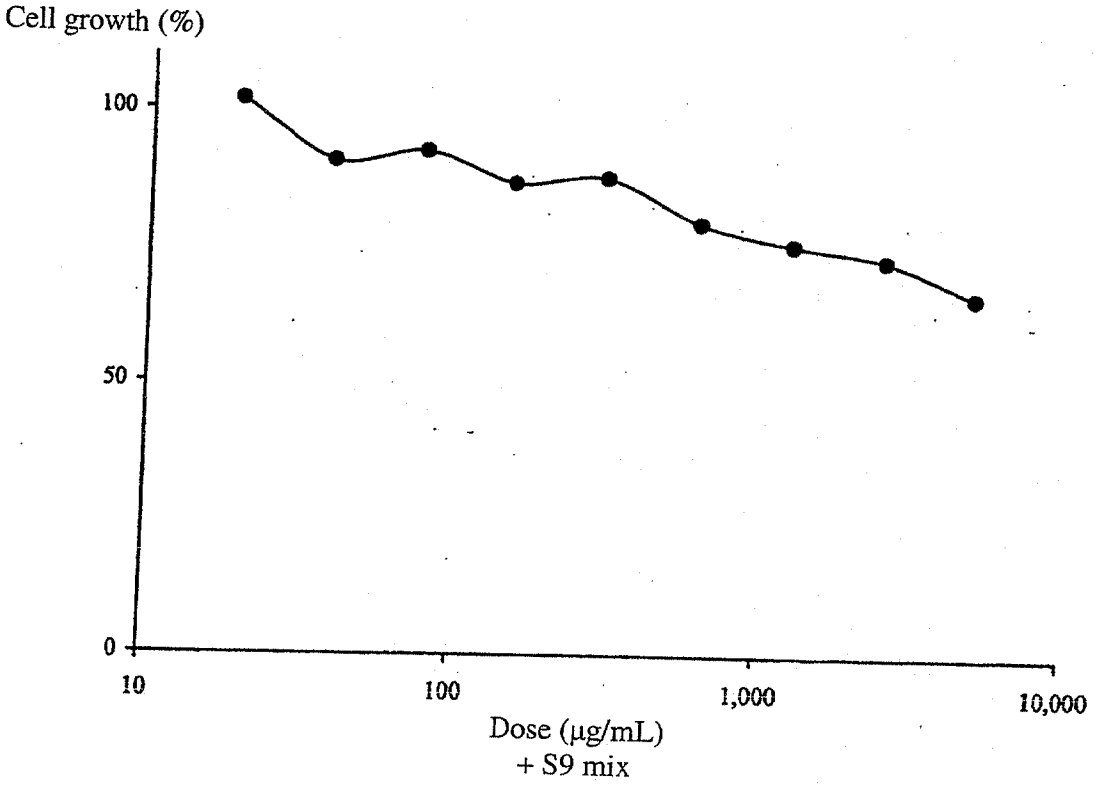
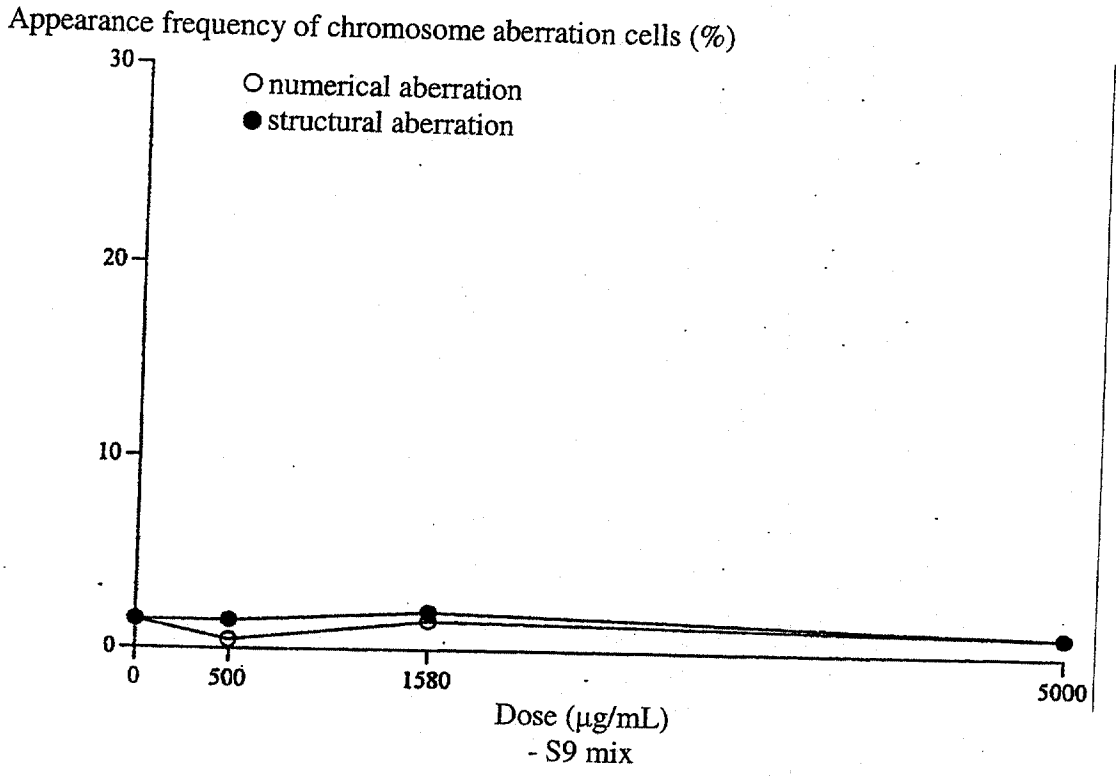


Figure 1: Cell growth inhibition test results of short-term treatment using [redacted]



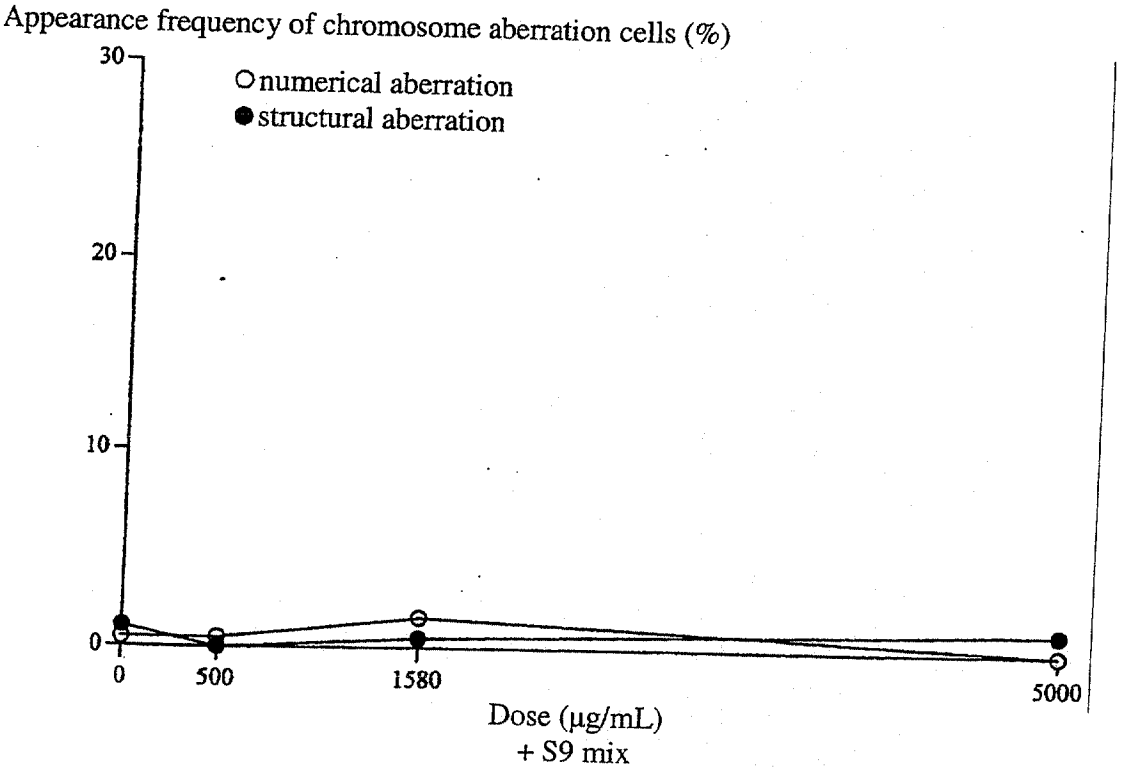


Figure 2: Chromosome aberration test results of short-term treatment using [redacted]

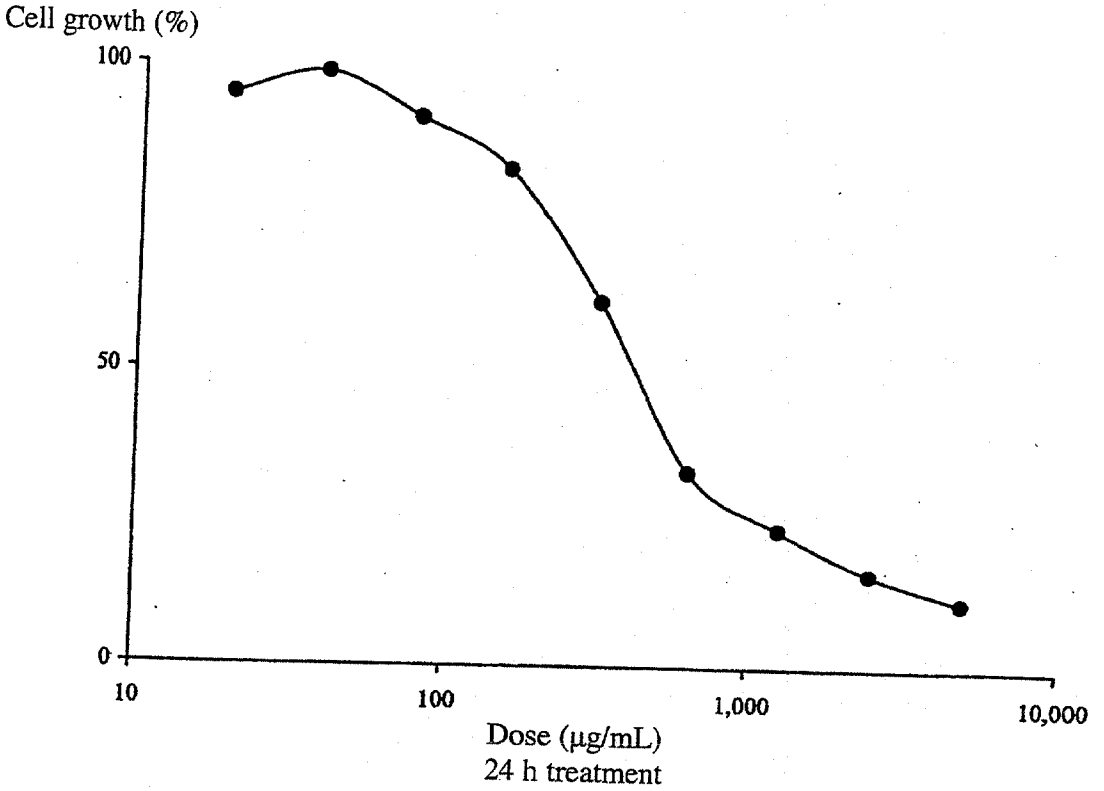


Figure 3: Cell growth inhibition test results of continuous treatment using [redacted]

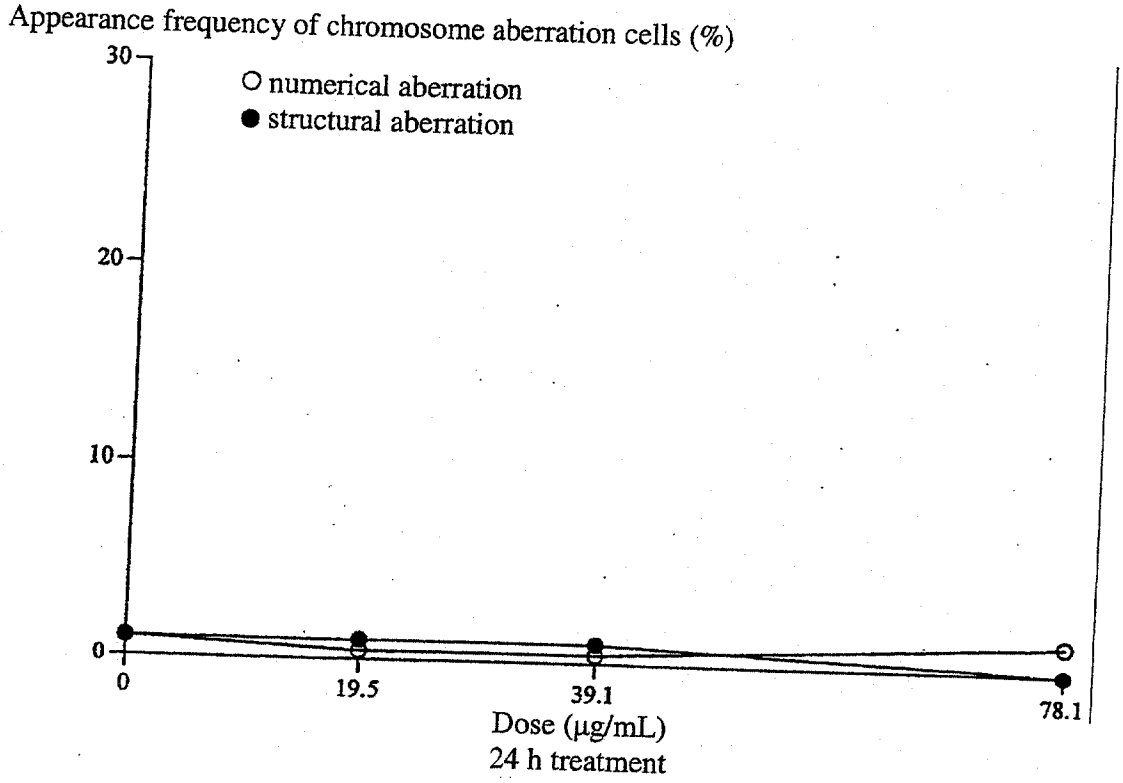


Figure 4: Chromosome aberration test results of continuous treatment using [redacted]

Responsibility Chart

Title: Chromosomal aberration test of [redacted] using mammalian cultured cells

Name	Responsibility
Principal tester Yoshikazu Ajimi	Test planning, management of overall test, specimen observation, results analysis, evaluation, report preparation
Tester Junko Kawaguchi	Preparation and treatment of test substance, specimen observation
Shinya Wakabayashi	Specimen observation

RELIABILITY WARRANTY

Kagakubusshitsu Heikakenkyukiko
Hita Works

Test Requester: Dupont K.K.
Test Title: Chromosomal aberration test of [redacted] using mammalian cultured cells
Test Code: K06-1022

This test has been audited or inspected by the QA Division of Hita Works, Kagakubusshitsu Heikakenkyukiko. Supervision or inspection dates and dates reported to the principal tester and management are given below.

Auditing or inspection items	Auditing or inspection date	Auditing or inspection results report date
Test plan documented	6/25/2003	6/26/2003
Test substance preparation and test start	7/2/2003	7/2/2003
Amendment of test plan document	7/2/2003	7/3/2003
Recording and report draft	10/21/2003	10/21/2003
Amendment of test plan document (No. 2)	10/24/2003	10/27/2003
Final report draft review	10/29/2003	10/29/2003
Amendment of test plan document (No. 3)	10/30/2003	10/30/2003
Final report draft review	10/30/2003	10/30/2003
Final report	11/13/2003	11/13/2003

This report accurately describes the testing method and procedure and the reported results accurately reflect the raw data of the tests.

November 13, 2003
Keiji Shiraishi
Reliability Warranty Officer

Language Services Unit
Phoenix Translations
January 14, 2004