STUDY TITLE

HR-001: *In Vitro* Cytogenetics Test

DATA REQUIREMENT

Required under U.S. EPA FIFRA Guidelines, Subdivision F

AUTHOR

[redacted]

STUDY COMPLETED ON

May 29, 1995

PERFORMING LABORATORY

The Institute of Environmental Toxicology
2-772 Suzuki-cho, Kodaira, Tokyo 187, Japan

LABORATORY PROJECT ID

IET 94-0143

SPONSOR

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7-12, 2-chome, Ginza, Chuo-ku,
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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

HR-001: *In Vitro* Cytogenetics Test
(IET 94-0143)

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GLP STATEMENT

HR-001: *In Vitro* Cytogenetics Test
(IET 94-0143)

This study was conducted in conformity to Good Laboratory Practice standards (GLPs) of MAFF in Japan (59 NohSan No. 3850, 1984), EPA in U.S.A. (FIFRA: 40 CFR 160, 1989), and OECD (OECD Principles of GLP, 1981).

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FLAGGING STATEMENT

HR-001: *In Vitro* Cytogenetics Test
(IET 94-0143)

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HR-001: *In Vitro* Cytogenetics Test
(IET 94-0143)

OBJECTIVE

The purpose of this study was to evaluate the clastogenic potential of HR-001 in cultured mammalian cells.

SPONSOR

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HR-001: *In Vitro* Cytogenetics Test
(IET 94-0143)

STUDY DIRECTOR AND SUPERVISORY PERSONNEL

We, the undersigned, hereby declare that the study was performed under our supervision in conformity to the GLPs of MAFF in Japan (59 NohSan No. 3850, 1984), EPA in U.S.A. (FIFRA: 40 CFR 160, 1989), and OECD (OECD Principles of GULP, 1981) and the Guidelines of MAFF in Japan (59 NohSan No. 4200, 1985), EPA in U.S.A. (Pesticide Assessment Guidelines, Subdivision F, 1991) and OECD (OECD Guideline No. 473, 1983).

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Date

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May 29, 1995
Date

HR-001: *In Vitro* Cytogenetics Test
(IET 94-0143)

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QUALITY ASSURANCE AUTHORIZATION

HR-001: *In vitro* Cytogenetics Test (IET 94-0143)

Report

	Inspection date	Report date to the study director	Report date to the administrator
Protocol	1/12/1995	1/12/1995	1/12/1995
	1/24/1995	1/24/1995	1/24/1995
Study procedure	4/11/1995	4/11/1995	4/11/1995
	4/13/1995	4/13/1995	4/13/1995
Raw data	5/19-22/1995	5/22/1995	5/22/1995
Report	5/19-22/1995	5/22/1995	5/22/1995
	5/29/1995	5/29/1995	5/29/1995

By the above inspections, it was assured that the reported methods and procedures were found to describe those used and the results to reflect the raw data generated during the conduct of this study accurately.

Quality Assurance Manager:

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Chief, Quality Assurance Unit

May 29, 1995
Date

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1. SUMMARY

The *in vitro* cytogenetics test using cultured Chinese hamster lung (CHL) cells was performed to evaluate the clastogenic potential of HR-001. In the direct method, the cells were continuously treated with HR-001 at 4 doses of 125, 250, 500 and 1000 µg/ml for 24 hours, and at 4 doses of 62.5, 125, 250 and 500 µg/ml for 48 hours. In the metabolic activation method, the cells were treated with HR-001 at 4 doses of 250, 500, 1000 and 2000 µg/ml for 6 hours in the presence of S9 mix and fixed 18 hours after the end of the treatment. The highest dose of HR-001 for each treatment was chosen on the basis of the results of the preliminary cell growth inhibition test. The dose of 1000 µg/ml in the 24-hr treatment and the dose of 2000 µg/ml in the metabolic activation system gave high cytotoxicity to the cells so that chromosome preparations were not obtained from these cultures.

The metaphase analysis showed that there were no significant increases in the frequencies of the metaphases with structural chromosome aberrations or polyploid cells at any analyzable doses of HR-001 in any treatments when compared with the concurrent solvent controls.

On the other hand, chromosome aberrations were observed at a significantly high frequency in the positive controls treated with

mitomycin C (in the direct method) and benzo(a)pyrene (in the metabolic activation system).

It was concluded from the above results that, under the test conditions, HR-001 did not induce either structural or numerical chromosome aberrations in the presence or absence of the metabolic activation system.

2. OBJECTIVE

The objective of this study was to evaluate the clastogenic potential of HR-001 in cultured Chinese hamster lung cells.

3. TEST SUBSTANCE

Code name:	HR-001
Lot No.:	940908-1
Purity:	95.68%
Appearance at normal temperature:	White crystal
Melting point:	200℃
Solubility:	Water, 12 g/l (25℃)
Storage condition:	Dark cold room (5℃)

4. MATERIALS AND METHODS

1) Cell line

The CHL cells[1)] established from the lung of Chinese hamster were used. The model number of chromosomes was 25 and doubling time was about 15 hours. The cells of which the passage number was 11 were obtained from Dr. [redacted], National Institute of

Hygienic Science, Tokyo, Japan, on January 12, 1984, and stored in liquid nitrogen to prevent any changes of their characteristics and properties. The cells were thawed immediately before the initiation of this study and confirmed not to be contaminated with *Mycoplasma* before use.

2) Cell culture

Cells were cultured in 100-mm Falcon tissue culture dishes (Becton Dickinson Labware) at 37°C in a humidified atmosphere of 5% CO_2 in air. The growth medium was Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% newborn calf serum (Lot No. 30520007, ICN Biomedicals Japan Co., Ltd.). Sub-cultivation of the cells was done using 0.25% trypsin solution (ICN Biomedicals Japan Co., Ltd.).

3) Preparations of the test substance and positive control compounds solutions

The test substance HR-001 was dissolved in Hanks' balanced salt solution (HBSS) (Nissui Pharmaceutical Co., Ltd., Japan) or directly dissolved in culture medium when needed to be prepared at 2000 µg/ml or more. Mitomycin C (MMC, 2 mg/vial, Kyowa Hakko Kogyo Co., Japan) used as a positive control compound in the direct method was dissolved in physiological saline (Otsuka Pharmaceutical Co.,

Ltd., Japan). Benzo(a)pyrene (B(a)P, 98%, Sigma Chemical Co., U.S.A.) used as a positive control compound in the metabolic activation method was dissolved in dimethyl sulfoxide (DMSO, >99%, Tokyo Kasei Kogyo Co., Japan). A volume of the test substance solutions added to the culture medium was 10% (v/v). All the above solutions were prepared immediately before the experiments. The residual solutions were discarded in each experiment.

4) Preparations of S9 mix

A metabolic activation system (S9 mix) was prepared by mixing the 9000×*g* supernatant fraction of the rat liver homogenate (S9 fraction) and co-factor (freeze-dried co-factor mixture, Lot No. 718; [redacted], Japan) immediately before use. S9 fraction with following data was purchased from Kikkoman Corporation (Chiba, Japan), stored at -80℃, and used within 6 months after purchase.

(1) Animal: Sprague-Dawley rat (Slc:SD)
male, 7 weeks old

(2) Body weight: 192 - 229 g

(3) Inducer: phenobarbital (PB: Wako Pure Chemical Industries Ltd., Japan)
5,6-benzoflavone (BF: Aldrich Chemical Co., Inc., U.S.A.)

(4) Route of administration: intraperitoneal injection

(5) Dosage: Day 1: PB 30 mg/kg

Day 2: PB 60 mg/kg

Day 3: PB 60 mg/kg and BF 80 mg/kg

Day 4: PB 60 mg/kg

(6) Protein content: 24.40 mg/ml

(7) P-450 content: 1.04 nmol/mg protein

(8) Date of preparation: October 6, 1994

(9) Lot No.: RAA-316

(10) Sterility test: passed

(11) Enzyme activity measured by mutagenicity: good

(12) B(a)P enzyme activity: 11.20 (relative value, non-induced control = 1.00)

The enzyme activity of this S9 fraction was re-evaluated by microbial mutagenicity in our laboratory and its sterility was checked again before use. The components of S9 mix were as follows: 30% (v/v) S9 fraction, 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADH, 4 mM NADPH, and 100 mM sodium phosphate buffer (pH 7.4).

5) Preliminary growth inhibition test

A preliminary growth inhibition test was performed to determine doses of the test substance in the cytogenetics test. CHL cells were seeded at a density of 1.0×10^5 cells in 60-mm Falcon

tissue culture dishes with 5 ml of medium, and incubated for about 48 hours. The cultures were treated with HR-001 at the highest dose of 1 mg/ml and 8 more doses separated by a factor 2. A solvent control treated with HBSS was included in this test.

In a direct method, the cells were treated with the test substance for 24 or 48 hours. After the treatment, the medium was removed and the cells were fixed with ethanol for 5 minutes. Fixed cells were stained with 3% Giemsa solution (Merck Ltd,. U.S.A., diluted in pH 6.8 phosphate buffer) for about 20 minutes at room temperature, rinsed with tap water, and then air-dried. Staining intensities (= cell densities) were optically measured by Monocellater (Olympus Optical Corporation, Tokyo, Japan) and a relative cell growth value of each culture was measured by comparing with the staining density in the concurrent solvent control.

In a metabolic activation method, the medium was replaced with 3 ml of the medium containing S9 mix (fresh medium:S9 mix=5:1) and then the test substance solutions were added to the cultures. Six hours later, the treatment medium was removed and the cells were rinsed twice with HBSS, and incubated in fresh medium for a further 18 hours. After fixation and staining in the same way as the direct method, the relative cell growth values were obtained.

The second growth inhibition test with the metabolic activation system was carried out with higher doses of the test

substance, where 5 mg/ml[2] was set as the highest dose, because no reduction of cell growth was seen at any doses in the first growth inhibition test with the metabolic activation system.

Duplicate cultures were used for each experimental point and their relative values were averaged.

6) Cytogenetics tests[3), 4)]

(1) Direct method

CHL cells were seeded at a density of 2×10^5 cells in 100-mm Falcon tissue culture dishes with 10 ml of medium, and the test substance solutions were added to the cultures 48 hours after seeding. On the basis of the results of the preliminary growth inhibition test, 4 doses of 125, 250, 500 and 1000 µg/ml for the 24-hr treatment and 4 doses of 62.5, 125, 250 and 500 µg/ml for the 48-hr treatment were selected. An untreated negative control, a solvent control treated with HBSS, and a positive control treated with MMC at a final concentration of 0.1 µg/ml were included in the experiment. Chromosome preparations were made at 24 and 48 hours after addition of the test substance.

Duplicate cultures were used for each dose.

(2) Metabolic activation method

CHL cells were seeded at a density of 2×10^5 cells in 100-mm

Falcon tissue culture dishes with 10 ml of medium. After about 48 hours the medium was replaced with 5 ml of the medium containing S9 mix (fresh medium:S9 mix=5:1) and then the test substance solutions were added. On the basis of the results of the preliminary growth inhibition tests, 4 doses of 250, 500, 1000 and 2000 µg/ml were selected. The experiment system included an untreated negative control, a solvent negative control, and a positive control treated with B(a)P at a final concentration of 40 µg/ml. The medium was removed after 6 hours of the treatment. The cells were rinsed twice with HBSS and re-incubated in fresh medium. Chromosome preparations were made at 18 hours after the medium change.

A concurrent control experiment against S9 mix treatment was also conducted, where all conditions were the same as the above metabolic activation method except for not adding S9 mix.

Duplicate cultures were used for each dose.

(3) Chromosome preparations and staining

Chromosome preparations were made by a conventional air-drying method. Colchicine (Wako Pure Chemical Industries, Ltd., Japan) was added to the cultures at a final concentration of 0.5 µg/ml 2 hours before harvest to arrest metaphase. The cells were detached by 0.25% trypsin and swollen with a hypotonic solution

(0.075 M KCl) for about 15 minutes at room temperature. The cells were fixed with Carnoy's solution (methanol:acetic acid=3:1), dropped on two glass slides per culture, and air dried. The chromosome preparations were labeled with code numbers and stained with 2% Giemsa solution for 15 minutes at room temperature.

(4) Analysis of chromosome aberrations

Total number of 200 well-spread metaphase cells for each dose (100 metaphase cells/culture) were examined under 1000 × magnification by light microscopy. Diploid metaphase cells which possessed the typical karyotype of CHL cells (number of chromosome=25±2) and polyploid metaphase cells were analyzed for a structural chromosome aberration. Structural chromosome aberrations were classified into different types. Each number of aberrations was recorded. The number of chromosomes of polyploid cells was also recorded. Mitotic index was assessed by examination of 1000 cells from each culture. The following data per culture were summarized in a tabular form:

1) Number and frequency of polyploid cells.
2) Number and frequency of each structural chromosome aberration.
3) Number and frequency of metaphase cells with structural chromosome aberrations (both including and excluding gaps).

(5) Classification of chromosome aberrations

Chromosome aberrations were classified according to the following criteria:

A) Structural chromosome aberrations

(a) Gap (g): An achromatic (unstained) region in one chromatid or both sister chromatids at the identical loci without dislocation of the segment. The size of achromatic region is shorter than the width of chromatid.

(b) Chromatid break (ctb): An achromatic (unstained) region in one chromatid. The size of achromatic region is greater than the width of chromatid, or lateral dislocation of the fragment is observed. Chromatid fragments are included.

(c) Chromatid exchange (cte): Rearrangement as a consequence of breakage and misreunion between two or more chromatids of different chromosome (interchromosomal exchange) or within a chromosome (intrachromosomal exchange).

(d) Chromosome break (isochromatid break, csb): Same as chromatid break but at the same loci in both sister chromatids. Acentric chromosome fragments are included.

(e) Chromosome exchange (cse): Chromosomes with multiple centromeres, and chromosome ring.

(f) Fragmentation (frg): A metaphase containing a large number of gaps and breaks. This aberrant metaphase was scored as one

aberrant cell.

(g) Others (oth): Other structural chromosome aberrations such as multiple aberration.

B) Numerical chromosome aberrations

Only polyploid cell having 3 or more copies of haploid number of chromosomes was scored as a numerical chromosome aberration cell. Since the CHL cells have 25 chromosomes in the modal number, the cell with 37 or more chromosomes was recorded as a polyploid cell.

(6) Statistical analysis

The number of aberrant metaphases and polyploid cells at each dose were statistically compared with those of corresponding solvent controls using a chi-square test.

7) Criteria of a valid test

Conducted cytogenetics tests were considered to be valid if the negative and positive control values met the following criteria:

(a) The frequencies of the aberrant metaphases in the solvent control groups are within our historical negative control range (mean ±3×S.D.).

(b) The frequency of the aberrant metaphases in the positive control groups are 10% or more.

8) Evaluation of results

Evaluations for structural and numerical chromosome aberrations were done separately. A test substance was judged negative if there was no significant increase in the frequencies of aberrant metaphases or polyploid cells at any doses. A test substance was judged positive if reproducible and significant increases in the frequencies of aberrant metaphases or polyploid cells were observed with a dose-related response. Both biological and statistical significance were considered together in a final evaluation.

5. RESULTS

1) Preliminary growth inhibition test

The results of the preliminary growth inhibition test are summarized in Table 1. Figures 1 to 4 represent the relationship between cell growth and dose levels.

In the 24-hr and 48-hr treatments of the direct method, the doses of HR-001 which showed a reduction of the relative cell growth by 50% or more were 1000 and 500 µg/ml, respectively. Therefore, the doses of 1000 and 500 µg/ml were chosen as the highest doses in the 24-hr and 48-hr treatments of the cytogenetics test, respectively.

In the metabolic activation method, no cell growth inhibition effect was observed at the dose of 1000 µg/ml or less, but at 2000 µg/ml or more, their cell growth was inhibited over 50%. Therefore, the dose of 2000 µg/ml was determined to be used as the highest dose in the cytogenetics test with the metabolic activation system.

It was noticed that in the both methods the color of the culture medium was turned to yellow at 500 µg/ml or more, indicating a decline of pH.

2) Cytogenetics test (direct method)

The results of the cytogenetics test by the direct method are

shown in Table 2 (24-hr treatment) and Table 3 (48-hr treatment). The frequency of the aberrant metaphases (excluding gaps) was 0.5% at any doses of 125, 250 and 500 μg/ml in the 24-hr treatment, showing no significant increases when compared with the concurrent solvent control. The dose of 1000 μg/ml gave high cytotoxicity to the cells so that chromosome preparations were not obtained from the cultures treated at this dose.

In the 48-hr treatment, the frequencies of the aberrant metaphases were ranging from 0.0% to 1.0%, showing no significant increases when compared with the concurrent solvent control.

There was no increase in the frequencies of polyploid metaphases at any doses of HR-001 in both 24-hr and 48-hr treatments.

It was noticed that in both methods the color of the culture medium was turned to yellow at 500 and 1000 μg/ml, indicating a decline of pH.

On the other hand, MMC used as a positive control caused a great increase in a frequency of aberrant metaphases. As shown in Appendix 1, the frequencies of aberrant metaphases in the negative controls were consistent with our historical control data. These control results supported the validity of this study.

3) Cytogenetics test (metabolic activation method)

The results of the cytogenetics test by the metabolic

activation method are shown in Table 4 (with S9 mix) and Table 5 (without S9 mix). In the presence of a metabolic activation system, the frequencies of the aberrant metaphases (excluding gaps) were in a range of 0.5% - 1.0%, showing no significant increases when compared with the concurrent solvent control.

In the concurrent control experiment (without S9 mix), the frequencies of the aberrant metaphases were also in a range of 0.5% - 1.0%, showing no significant increases when compared with the solvent control.

The frequencies of polyploid cells did not significantly increase in either presence or absence of S9 mix.

It was noticed that in both treatments (with and without S9 mix) pH of the culture medium of the cultures treated at 500, 1000, and 2000 µg/ml went down. The dose of 2000 µg/ml gave high cytotoxicity to the cells so that chromosome preparations were not obtained from the cultures treated at this dose.

On the other hand, B(a)P used as a positive control caused a remarkable increase in the frequency of aberrant metaphases in the presence of S9 mix. As shown in Appendix 1, the frequencies of aberrant metaphase in the negative controls were consistent with our historical control data. These control results supported the validity of this study.

6. CONCLUSION

In the direct and the metabolic activation system, there was not a significant increase in the frequencies of abnormal metaphases with structural chromosome aberrations or polyploid metaphases.

Based on the results obtained, it was concluded that, under the conditions of this study, the test substance HR-001 did not induce chromosome aberrations in Chinese hamster CHL cells with or without the metabolic activation system.

7. REFERENCES

1) Koyama H., et al. (1970) A new cell line derived from newborn Chinese hamster lung tissue. Gann, 61: 161 - 167.

2) Ishidate M. Jr., and Odashima S. (1977) Chromosome tests with 134 compounds on Chinese hamster cell *in vitro*: a screening for chemical carcinogens. Mutation Res., 48: 337 - 354.

3) Testing guidelines for toxicology studies for medical drugs (1990), The Ministry of Health and Welfare, Japan, Yakuji-Nippo (1990) (in Japanese)

4) Ishidate M. Jr., Sofuni T., Yoshikawa K. (1981) The results of

mutagenicity of food additives (No.2). Mutagens and Toxicology, 4:80-89 (in Japanese).

Table 1 Preliminary growth inhibition test

Test substance : HR-001

Concentration (μg/ml)	Relative cell growth (%)			
	Direct method 24 h	Direct method 48 h	Metabolic activation method 6-18 h[a] (1st Exp.)	Metabolic activation method 6-18 h[a] (2nd Exp.)
Solvent control (Hanks)	100	100	100	100
3.9	100	100	100	—
7.8	97	101	99	—
15.6	100	101	108	—
31.3	96	110	104	—
62.5	100	106	97	—
125	102	99	103	—
250	92	82	106	—
500[b]	74	46	112	—
1000[b]	22	9	106	100
2000[b]	—	—	—	22
3000[b]	—	—	—	38
4000[b]	—	—	—	27
5000[b]	—	—	—	25

a) CHL cells were treated with the test substance for 6 hours in the presence of S9 Mix and then cultured in fresh medium for further 18 hours.

b) The color of the culture medium was turned to yellow after addition of the test substance solutions, indicating a decline of pH.

Table 2 Cytogenetics test (direct method, 24-hr treatment) (IET 91-0143)

Test substance : IIR-001

Treatment	Time (h)	S9 Mix	Concentration (μg/ml)	Number of metaphases	Mitotic index (%)	Polyploid		Number of chromosome aberrations						Number of aberrant metaphases		
							Judge	Gap g	Chromatid type ctb	Chromatid type cte	Chromosome type csb	Chromosome type cse	Fragmentation	Others	+g	−g	Judge
Untreated control	24	−	0	100 100 Total(Mean)	5.8 6.3 (6.1)	0 0 0(0)	−	0 2 2(1.0)	0 0 0(0)	0 0 0(0)	0 1 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 3 3(1.5)	0 1 1(0.5)	−
Solvent control (Hanks)	24	−	10%	100 100 Total(Mean)	6.2 5.7 (6.0)	0 1 1(0.5)		0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	
IIR-001	24	−	125	100 100 Total(Mean)	6.2 7.2 (6.7)	1 0 1(0.5)	−	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	1 0 1(0.5)	1 0 1(0.5)	−
			250	100 100 Total(Mean)	6.4 5.4 (5.9)	0 1 1(0.5)	−	0 2 2(1.0)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 2 3(1.5)	1 0 1(0.5)	−
			500[a]	100 100 Total(Mean)	4.5 6.7 (5.6)	0 0 0(0)	−	2 1 3(1.5)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	3 1 4(2.0)	1 0 1(0.5)	−
			1000[a, b]														
Positive control (MMC)	24	−	0.1	100 100 Total(Mean)	3.0 3.5 (3.3)	1 0 1(0.5)	−	4 4 8(4.0)	26 17 43(21.5)	40 39 79(39.5)	2 3 5(2.5)	1 1 2(1.0)	0 0 0(0)	1 0 1(0.5)	51 50 101(50.5)	50 48 98(49.0)	+[c]

Abbreviations : ctb, chromatid break ; cte, chromatid exchange ; csb, chromosome break ; cse, chromosome exchange ; +g, including gaps ; −g, excluding gaps ; MMC, mitomycin C.

[a] : The color of the culture medium was turned to yellow after addition of the test substance solutions, indicating a decline of pH.

[b] : No chromosome preparations because of cytotoxicity.

[c] : Significantly different from the solvent control at $p \leq 0.001$.

Table 3 Cytogenetics test (direct method, 48-hr treatment)

Test substance : IR-001

Treatment	Time (h)	S9 Mix	Concentration (μg/ml)	Number of metaphases	Mitotic index (%)	Polyploid	Polyploid Judge	Gap g	Chromatid type ctb	Chromatid type cte	Chromosome type csb	Chromosome type cse	Fragmentation	Others	Number of aberrant metaphases +g	Number of aberrant metaphases −g	Judge
Untreated control	48	−	0	100 100 Total (Mean)	2.2 2.8 (2.5)	0 1 1(0.5)	−	1 0 1(0.5)	1 1 2(1.0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	2 1 3(1.5)	1 1 2(1.0)	−
Solvent control (Hanks)	48	−	10%	100 100 Total (Mean)	3.1 3.6 (3.4)	0 1 1(0.5)		0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	
IR-001	48	−	62.5	100 100 Total (Mean)	2.7 3.3 (3.0)	0 0 0(0)	−	2 1 3(1.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	2 1 3(1.5)	0 0 0(0)	−
			125	100 100 Total (Mean)	3.4 2.4 (2.9)	0 0 0(0)	−	1 0 1(0.5)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	2 0 2(1.0)	1 0 1(0.5)	−
			250	100 100 Total (Mean)	3.4 2.7 (3.1)	0 2 2(1.0)	−	2 1 3(1.5)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 1 1(0.5)	0 0 0(0)	0 0 0(0)	3 2 5(2.5)	1 1 2(1.0)	−
			500[a)]	100 100 Total (Mean)	3.5 2.5 (3.0)	0 0 0(0)	−	1 2 3(1.5)	1 1 2(1.0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	2 3 5(2.5)	1 1 2(1.0)	−
Positive control (MMC)	48	−	0.1	100 100 Total (Mean)	2.8 2.3 (2.6)	2 0 2(1.0)	−	6 5 11(5.5)	44 42 86(43.0)	53 46 99(49.5)	2 3 5(2.5)	5 1 6(3.0)	0 3 3(1.5)	2 1 3(1.5)	74 70 144(72.0)	72 69 141(70.5)	+[b)]

Abbreviations : ctb, chromatid break ; cte, chromatid exchange ; csb, chromosome break ; cse, chromosome exchange ; +g, including gaps ; −g, excluding gaps ; MMC, mitomycin C.

a) : The color of the culture medium was turned to yellow after addition of the test substance solutions, indicating a decline of pH

b) : Significantly different from the solvent control at $p \leq 0.001$.

Table 4 Cytogenetics test (metabolic activation method, 6-hr treatment, +S9 Mix)

(IET 91-0143)

Test substance : HR-001

Treatment	Time (h)	S9 Mix	Concentration (μg/ml)	Number of metaphases	Mitotic index (%)	Polyploid	Judge	Number of chromosome aberrations						Number of aberrant metaphases		
								Gap g	Chromatid type ctb	Chromatid type cte	Chromosome type csb	Chromosome type cse	Fragmentation	Others	+g	−g	Judge
Untreated control	6 a)	+	0	100 100 Total(Mean)	3.9 5.9 (4.9)	0 0 0(0)	−	1 2 3(1.5)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	2 2 4(2.0)	1 0 1(0.5)	−
Solvent control (Hanks)	6 a)	+	10%	100 100 Total(Mean)	5.1 7.4 (6.3)	0 0 0(0)		1 2 3(1.5)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 2 3(1.5)	1 0 1(0.5)	
HR-001	6 a)	+	250	100 100 Total(Mean)	6.6 6.8 (6.7)	0 0 0(0)	−	0 0 0(0)	1 1 2(1.0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 1 2(1.0)	1 1 2(1.0)	−
			500 b)	100 100 Total(Mean)	5.7 5.4 (5.6)	0 0 0(0)	−	1 1 2(1.0)	0 2 2(1.0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 3 4(2.0)	0 2 2(1.0)	−
			1000 b)	100 100 Total(Mean)	7.2 7.2 (7.2)	1 0 1(0.5)	−	1 1 2(1.0)	0 1 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 2 3(1.5)	0 1 1(0.5)	−
			2000 b, c)														
Positive control [B(a)P]	6 a)	+	40	100 100 Total(Mean)	3.4 4.2 (3.8)	0 0 0(0)	−	4 2 6(3.0)	20 22 42(21.0)	30 30 60(30.0)	2 2 4(2.0)	0 1 1(0.5)	0 0 0(0)	0 0 0(0)	39 40 79(39.5)	38 39 77(38.5)	+d)

Abbreviations : ctb, chromatid break ; cte, chromatid exchange ; csb, chromosome break ; cse, chromosome exchange ; +g, including gaps ; −g, excluding gaps ; B(a)P, benzo(a)pyrene.

a) : CHL cells were treated with the test substance for 6 hours in the presence of S9 Mix and then cultured in fresh medium for further 18 hours.

b) : The color of the culture medium was turned to yellow after addition of the test substance solutions, indicating a decline of pH.

c) : No chromosome preparations because of cytotoxicity.

d) : Significantly different from the solvent control at $p \leq 0.001$.

Table 5 Cytogenetics test (concurrent control, 6-hr treatment, −S9 Mix) (IET 91-0143)

Test substance : HR-001

Treatment	Time (h)	S9 Mix	Concentration (μg/ml)	Number of metaphases	Mitotic index (%)	Polyploid	Judge	Number of chromosome aberrations						Number of aberrant metaphases		
								Gap	Chromatid type		Chromosome type		Fragmentation	Others			
								g	ctb	cte	csb	cse			+g	−g	Judge
Untreated control	6 a)	−	0	100 100 Total(Mean)	5.0 5.6 (5.3)	0 0 0(0)	−	1 0 1(0.5)	0 2 2(1.0)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	2 2 4(2.0)	1 2 3(1.5)	−
Solvent control (flanks)	6 a)	−	10%	100 100 Total(Mean)	5.9 5.5 (5.7)	2 0 2(1.0)		0 2 2(1.0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 2 2(1.0)	0 0 0(0)	
HR-001	6 a)	−	250	100 100 Total(Mean)	4.7 5.5 (5.1)	0 0 0(0)	−	2 1 3(1.5)	2 0 2(1.0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	3 1 4(2.0)	2 0 2(1.0)	−
			500 b)	100 100 Total(Mean)	4.8 5.0 (4.9)	0 0 0(0)	−	0 1 1(0.5)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 1 2(1.0)	1 0 1(0.5)	−
			1000 b)	100 100 Total(Mean)	4.9 6.5 (5.7)	1 0 1(0.5)	−	1 0 1(0.5)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	2 0 2(1.0)	1 0 1(0.5)	−
			2000 b, c)														
Positive control [B(a)P]	6 a)	−	40	100 100 Total(Mean)	5.0 4.3 (4.7)	0 1 1(0.5)	−	0 0 0(0)	1 0 1(0.5)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	0 0 0(0)	1 0 1(0.5)	1 0 1(0.5)	−

Abbreviations : ctb, chromatid break ; cte, chromatid exchange ; csb, chromosome break ; cse, chromosome exchange ; +g, including gaps ; −g, excluding gaps ; B(a)P, benzo(a)pyrene.

a) : CHL cells were treated with the test substance for 6 hours in the absence of S9 Mix and then cultured in fresh medium for further 18 hours.

b) : The color of culture medium was turned to yellow after addition of the test substance solutions, indicating a decline of pH.

c) : No chromosome preparations because of cytotoxicity.

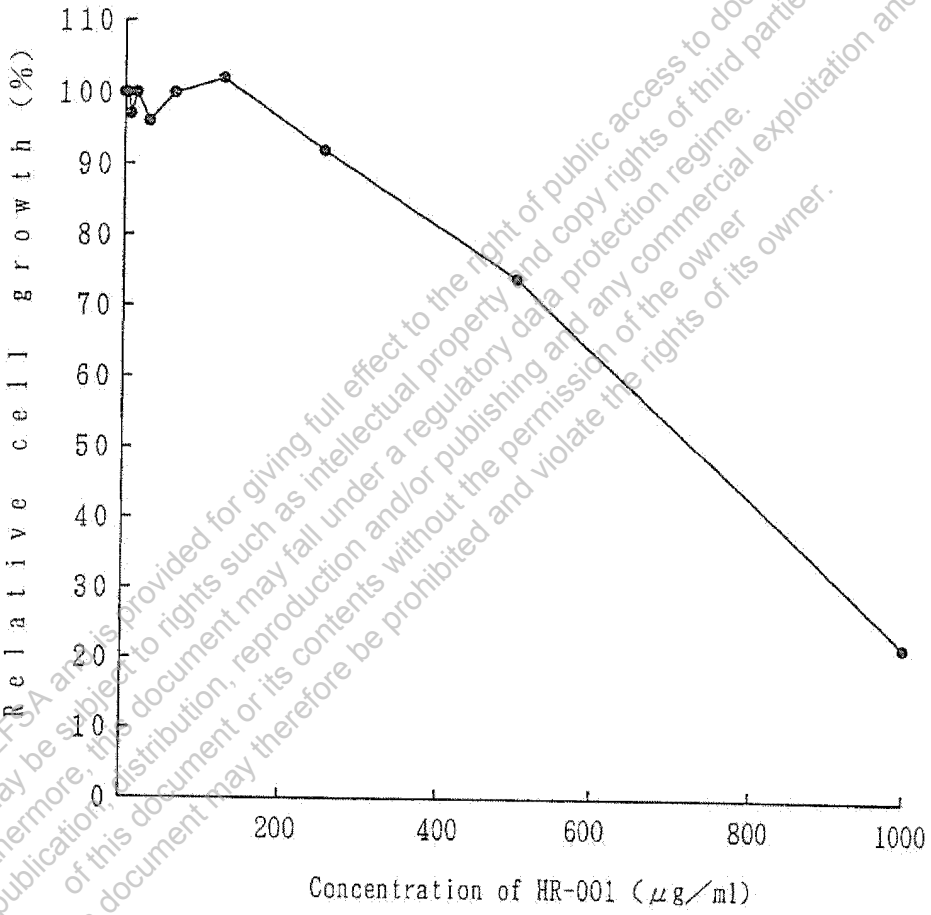

Fig. 1 Relative cell growth values (direct method, 24-hr treatment)

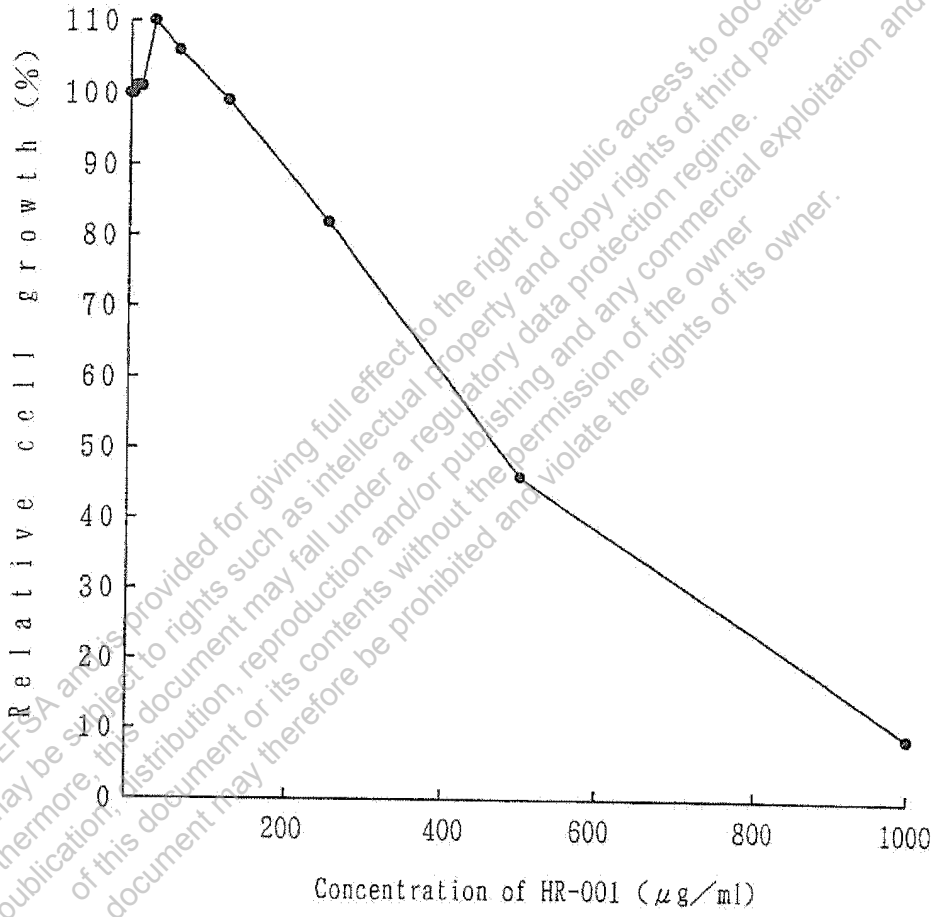

Fig. 2 Relative cell growth values (direct method, 48-hr treatment)

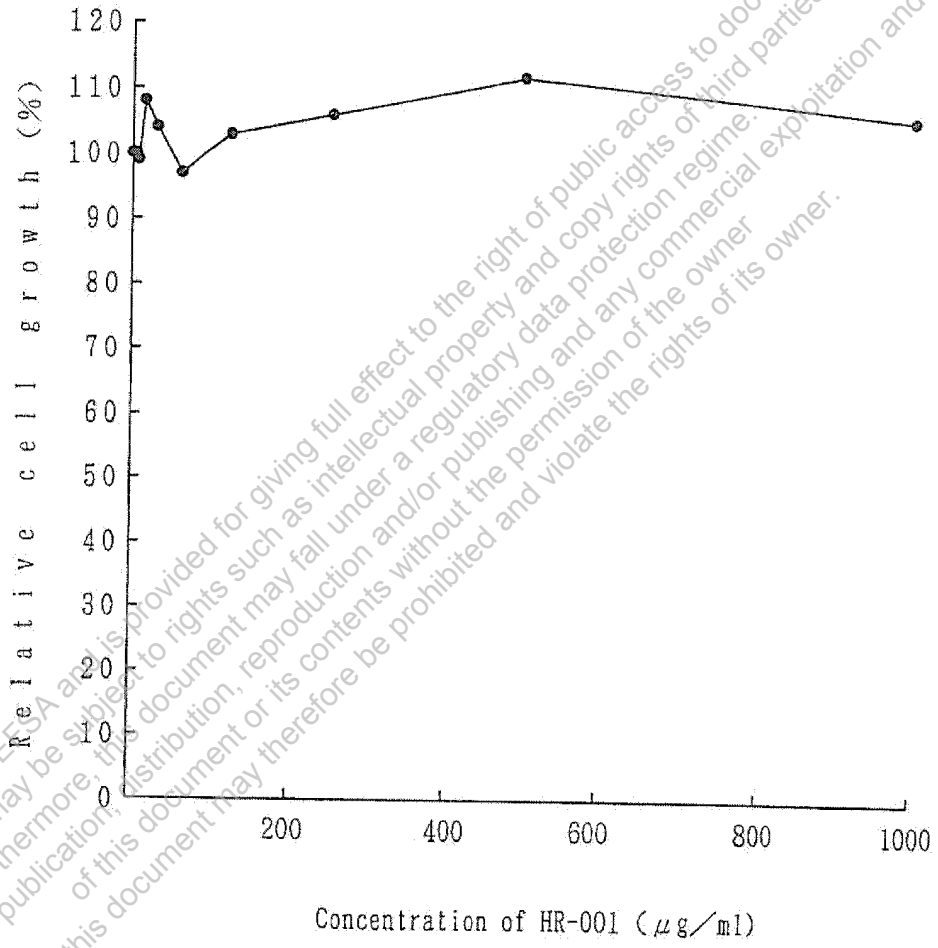

Fig. 3 Relative cell growth values (metabolic activation method, 1st Exp.)

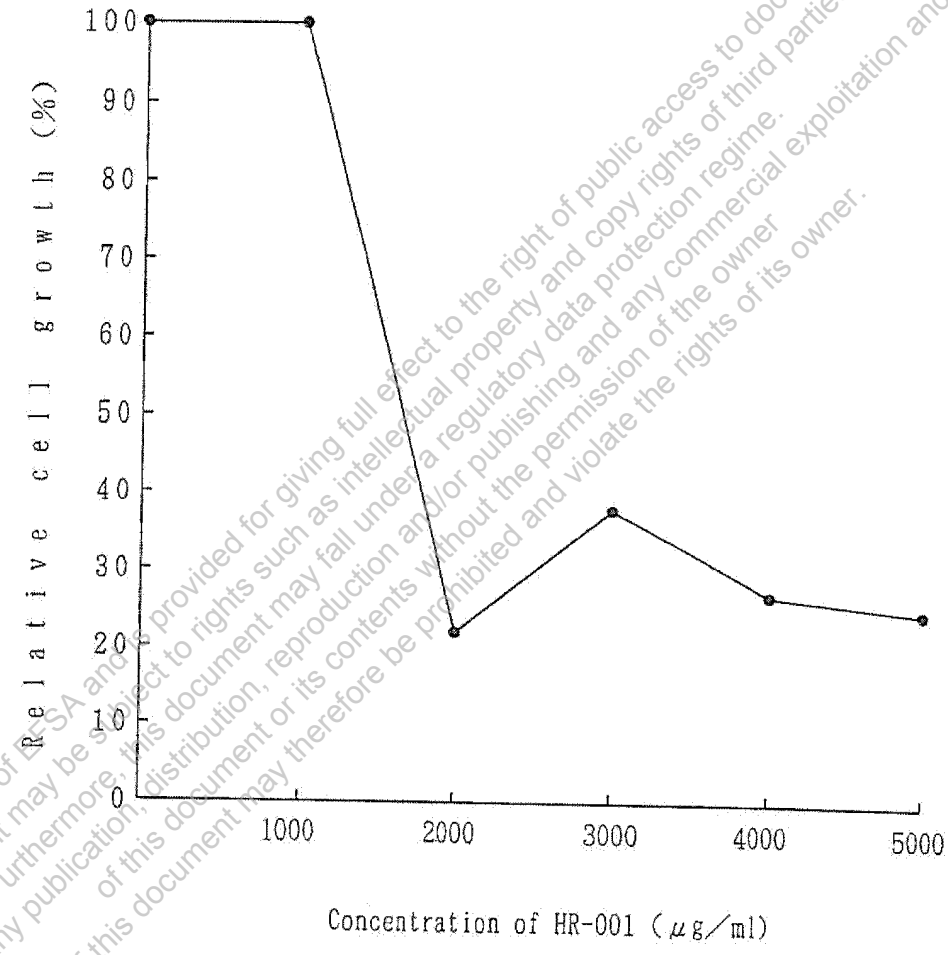

Fig. 4 Relative cell growth values (metabolic activation method, 2nd Exp.)

Appendix 1 Laboratory historical control values[a] — in vitro cytogenetics test —

(I E T 94-0143)

Treatment		Cells with structural chromosome aberrations (%) (Mean ± S.D.)		Polyploid cells (%) (Mean ± S.D.)
		+g	−g	
Direct method 24 h	Untreated control	1.12 ± 0.93	0.50 ± 0.59	0.37 ± 0.56
	Solvent control (DMSO 0.5%)	1.32 ± 0.75	0.61 ± 0.55	0.43 ± 0.52
Direct method 48 h	Untreated control	1.18 ± 1.22	0.50 ± 0.66	0.48 ± 0.51
	Solvent control (DMSO 0.5%)	1.18 ± 0.86	0.65 ± 0.61	0.47 ± 0.49
Metabolic activation method(+S9) 6-18 h	Untreated control	1.38 ± 1.22	0.52 ± 0.69	0.48 ± 0.70
	Solvent control (DMSO 0.5%)	1.41 ± 0.98	0.72 ± 0.67	0.43 ± 0.38

Abbreviations: +g, including gaps; −g, excluding gaps; DMSO, dimethylsulfoxide.
[a] The values are based on 39 cytogenetics tests independently conducted from December, 1988 to December, 1994.