Appendix 18

A CHROMOSOMAL ABERRATION TEST OF PROTEASE P IN CULTURED CHINESE HAMSTER CELLS

February 14, 1994

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INTRODUCTION

PROTEASE P is an enzyme developed for food industry use by Amano Pharmaceutical Co., Ltd. The purpose of this study was to examine the clastogenicity potential of PROTEASE P in cultured Chinese Hamster Cells.

This study was performed in accordance with the Guidelines for Toxicity Studies Required to Manufacture (Import) Drugs (Notification No. 24 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, effective September 11, 1989), OECD Guidelines for Testing of Chemicals (1987), and the Methods for the Determination of Physico-Chemical Properties, Toxicity and Ecotoxicity (Annex V to directive 79/831/EEC., 1989). Date of initiation of Dose Finding Test was on December 7, 1993, and date of completion of Chromosomal Aberration Observation on January 24, 1994.

The study was conducted between December 6, 1993 and February 14, 1994.

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- FINAL REPORT -

Study Title: A CHROMOSOMAL ABERRATION TEST OF PROTEASE P IN CULTURED CHINESE HAMSTER CELLS

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PREPARATION OF THE FINAL REPORT

Study Title: A CHROMOSOMAL ABERRATION TEST OF PROTEASE P IN CULTURED CHINESE HAMSTER CELLS

Study Number: SBL 34-00

Responsibility for the Final Report:

[redacted]

Study Director

February 14, 1994
Date

STATEMENT

Study Title: A CHROMOSOMAL ABERRATION TEST OF PROTEASE P IN CULTURED CHINESE HAMSTER CELLS

Study Number: SBL 34-00

We, the undersigned, hereby state that the study was performed in accordance with the Good Laboratory Practice Regulations (Notification No. 313 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, effective March 31, 1982 and Notification No. 776, partially amended as of October 1, 1983), the Amendment to Regulations of Drug GLP and Inspection (Notification No. 870 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, effective October 5, 1988), the Guidelines for Toxicity Studies Required to Manufacture (Import) Drugs (Notification No. 24 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, effective September 11, 1989), OECD Guidelines for Testing of Chemicals (1983), and the Methods for the Determination of Physico-Chemical Properties, Toxicity and Ecotoxicity (Annex V to directive 79/831/EEC., 1984).

We further warrant that there were no unexpected conditions that may have had an adverse effect on the study, and no significant deviations from the approved protocol.

[redacted] _____ February 14, 1994

Study Director Date

[redacted] _____ February 14, 1994

Management Director Date

QAU Statement

Study Title: A CHROMOSOMAL ABERRATION TEST OF PROTEASE P IN CULTURED CHINESE HAMSTER CELLS

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I further warrant that there were no unexpected conditions that may have had an adverse effect on the study, and no significant deviations from the approved protocol.

Items of inspections	Dates of inspection	Dates of reports to study director	Dates of reports to management
Protocol	December 6, 1993	December 6, 1993	December 6, 1993
Schedule	December 20, 1993	January 12, 1994	January 17, 1994
Storage of test article	January 10, 1994	January 12, 1994	January 17, 1994
Preparation of test article	January 10, 1994	January 12, 1994	January 17, 1994
Administration of test article	January 10, 1994	January 12, 1994	January 17, 1994
Chromosome preparation	January 11, 1994	January 12, 1994	January 17, 1994
Analysis of chromosomal aberration	January 12, 1994	January 12, 1994	January 17, 1994
Records and Data	February 1 and 3, 1994	February 3, 1994	February 7, 1994
Final report (Draft)	February 1 and 3, 1994	February 3, 1994	February 7, 1994
Final report	February 14, 1994	February 14, 1994	February 14, 1994

QAU :

Signature [redacted] Date February 14, 1994

STUDY PERSONNEL

Study Director: [redacted]

Preparation of the Final Report: [redacted]

Conduct of the Chromosomal Aberration Test: [redacted]

Data Management: [redacted]

Storage of Records and Specimens

The records and specimens are stored for 7 years in the Archives of Shin Nippon Biomedical Laboratories, Ltd.

CONTENTS

SUMMARY

In order to evaluate the clastogenicity potential of PROTEASE P, CHL/IU cells, which were derived from the lungs of newborn female Chinese Hamsters, were exposed *in vitro* to PROTEASE P, using the direct method test (22-hour and 46-hour treatments) and the metabolic activation method test (with and without S 9 Mix). The dose levels of PROTEASE P used in the Chromosomal Aberration Test were 19.5, 39.1, 78.1 and 156.3 µg/ml (Direct method test: 22-hour treatment); 4.9, 9.8, 19.5 and 39.1 µg/ml (Direct method test: 46-hour treatment); 78.1, 156.3, 312.5 and 625 µg/ml [Metabolic activation method test: with S 9 Mix (recovery time 16 hours) and without S 9 Mix (recovery time 16 hours)]; and 78.1, 156.3, 312.5 and 625 µg/ml [Metabolic activation method test: with S 9 Mix (recovery time 40 hours) and without S 9 Mix (recovery time 40 hours)].

In the direct method test, no significant difference was noted in the average frequencies of structural aberrations or polyploidy between the test article treated groups and the control group.

In the metabolic activation method test, no significant difference was noted in the average frequencies of structural aberrations or polyploidy between the results of the metabolic activation method test with S 9 Mix and the results of the metabolic activation method test without S 9 Mix. Further, in the metabolic activation method test, no significant difference was noted in the average frequencies of structural aberrations or polyploidy between the test article treated groups and the control group.

Under the study conditions, the results clearly indicate that PROTEASE P is not clastogenic to the CHL/IU cell line.

INTRODUCTION

PROTEASE P is a enzyme in food industry use under development by Amano Pharmaceutical Co., Ltd. The purpose of this study was to examine the clastogenicity potential of PROTEASE P in cultured Chinese Hamster Cells.

This study was performed in accordance with the Guidelines for Toxicity Studies Required to Manufacture (Import) Drugs (Notification No. 24 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, effective September 11, 1989), OECD Guidelines for Testing of Chemicals (1983), and the Methods for the Determination of Physico-Chemical Properties, Toxicity and Ecotoxicity (Annex V to directive 79/831/EEC., 1984). Date of initiation of Dose Finding Test was on December 7, 1993, and date of completion of Chromosomal Aberration Observation on January 24, 1994.

The study was conducted between December 6, 1993 and February 14, 1994.

MATERIALS AND METHODS

I. TEST ARTICLE

The test article, PROTEASE P (Lot No.: PZR08524), was supplied by Amano Pharmaceutical Co., Ltd. on October 14, 1993. Protease activity of PROTEASE P was 999000 u/g under the condition of pH 8.0B, and loss on drying was 6.80%. PROTEASE P was stored at room temperature (20 ± 4°C) in Shin Nippon Biomedical Laboratories, Ltd. Test Article Storage Room.

II. PREPARATION OF TEST ARTICLE

The test article amount necessary for the high dose level was first weighed out, and the high dose level solutions were prepared by mixing the test article with appropriate amounts of saline solution (Lot No. 3E88N, Otsuka Pharmaceutical Factory, Inc.). The other dose levels were prepared by sequential-multiple dilution (common ratio of 2) of the high dose level solutions with appropriate amounts of saline solution. The preparation of high dose level solutions and the sequential-multiple dilutions were performed immediately prior to use. The test article solutions were stored in an ice water bath immediately prior to use, and were used within 30 minutes after the preparation. Confirmation of the stability and the concentration of the test article was performed by Amano Pharmaceutical Co., Ltd.(Appendix 1).

III. CONTROL SUBSTANCES

A saline solution was used as the negative control. In the direct method test, Mitomycin C (MMC, Lot No. TWK1373, Wako Pure Chemical Industries, Ltd.) was used as the positive control. In the metabolic activation method test, Benzo(a)pyrene (B(a)P, Lot No. ECF6942, purity of 98%, Wako Pure Chemical Industries, Ltd.) was used as the positive control.

IV. CULTURED CELL LINE

CHL/IU (Passage number: 14), which was derived from the lungs of newborn female Chinese Hamsters, was bought from the Division of Laboratory Products, Dai Nippon Pharmaceutical Co., Ltd. on September 1, 1992. The cell line was cultured (Passage

number: 15) and then stored frozen in a freezer (MDF-290AT, Sanyo) set at –80°C in the Mutagenicity Testing Facility.

V. CULTURE MEDIUM

The culture medium was prepared by mixing 9.5 g Eagles' MEM powder (Cont. No. 60K1214, Gibco Laboratories), 2.2 g $NaHCO_3$ and 1000 ml distilled water. After adjusting to pH 7.2 with 0.1 N HCl, the MEM was sterilized using a 0.2 µm filter (Lot No. 114924: Iwaki). The 10% CS-MEM was then prepared by mixing 900 ml of sterilized MEM and 100 ml of calf serum (CS, Cont. No. 34P7216, Gibco Laboratories), which was inactivated at 56°C for 30 minutes.

VI. PREPARATION OF S 9 MIX

In the metabolic activation method, S 9 Mix as the metabolic activation system, which consisted mainly of a preparation (S 9) from rat liver homogenate, was prepared in an ice water bath immediately prior to use. Composition was as shown in the table below. S 9 (Lot No.: RAA-300, Production date: October 15, 1993, Kikkoman) was prepared from the livers of male rats (SD strain, 7 weeks old, body weight 190-218 g) that had been injected intraperitoneally with phenobarbital and 5,6-benzoflavone to induce drug metabolizing enzymes.

Composition of S 9 Mix per 10 ml:

Component	Amount
S 9	3 ml
20 mM HEPES (pH 7.2)	2 ml
50 mM $MgCl_2$	1 ml
330 mM KCl	1 ml
50 mM G-6-P (Lot No. 115104, Oriental Yeast Co., Ltd.)	1 ml
40 mM NADP (Lot No. 040009, Oriental Yeast Co., Ltd.)	1 ml
Distilled water	1 ml

VII. CULTURE CONDITION

Cultures were incubated in a humidified incubator set at 37°C, and in an atmosphere containing 5% CO_2.

VIII. EXPERIMENTAL DESIGN

In accordance with Ishidate's testing methods[1),2)], the MHW Guideline and the EEC Guideline, the Dose Finding Test and the Chromosomal Aberration Test were performed using the direct and the metabolic activation methods by means of the plate method. The incubation time was estimated to be 22 hours, because the sampling time was 1.5 cell cycles from beginning of treatment in accordance with EC guideline. 0.11 ml of the test article solution, the negative control solution or the positive control solution (the direct method: MMC) was added per 1.0 ml of culture medium. 0.005 ml of the positive control solution (the metabolic activation method: B(a)P) was added per 1.0 ml culture medium.

IX. DOSE FINDING TEST

A. Preliminary Test I

The dose levels for the Chromosomal Aberration Test were selected based on the results of the Dose Finding Test using mitogenesis inhibition as the indicator. The Preliminary Test I used 2 plastic petri dishes (diameter of 6 cm) per dose level. The CHL cells were seeded (inoculated) at approximately 2 x 10^4 cells per petri dish in 5 ml of culture medium. After 72 hours of incubation, the Preliminary Test I was initiated as follows:

1. Direct Method Test

   0.55 ml of the test article solution at the dose levels of 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 μg/ml or 0.55 ml of the negative control was added to petri dishes that were then incubated for either 22 or 46 hours. Two hours before the termination of the 22-hour or 46-hour incubation treatment, 0.1 μg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

2. Metabolic Activation Method Test

   With S 9 Mix: Exactly 2.5 ml of the culture medium was removed from the petri dishes. S 9 Mix (0.575 ml) was added to the cells and the culture medium

remaining in the petri dishes. 0.343 ml of the test article solution at the dose levels of 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/ml or 0.343 ml of the negative control was added to the petri dishes, and incubation continued for 6 hours. After these 6 hours of incubation, the reaction mixture (culture medium and test article solution or negative control) was poured out of the petri dishes. The cells were washed with saline solution, 5 ml of fresh culture medium was added to the cells, and incubation continued for an additional 16 or 40 hours. Two hours before the termination of this 16-hour or 40-hour incubation treatment, 0.1 µg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

Without S 9 Mix: Exactly 2.5 ml of the culture medium was removed from the petri dishes. Fresh culture medium (0.575 ml) was added to the cells and the culture medium remaining in the petri dishes. 0.343 ml of the test article solution at the dose levels of 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/ml or 0.343 ml of the negative control was added the petri dishes, and incubation continued for 6 hours. After these 6 hours of incubation, the culture mixture (culture medium and test article solution or negative control) was poured out of the petri dishes. The cells were washed with saline solution, 5 ml of fresh culture medium was added to the cells, and incubation continued for an additional 16 or 40 hours. Two hours before the termination of this 16-hour or 40-hour incubation treatment, 0.1 µg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

B. Preliminary Test II

It was not possible to determine the mitotic index at the dose levels used for the direct method test (46-hour treatment) in Preliminary Test I, because of the cytotoxicity caused by the test article. Therefore, the direct method test (46-hour treatment) was reperformed in Preliminary Test II using the doses of 0, 2.4, 4.9, 9.8, 19.5, 39.1, 78.1 and 156.3 µg/ml. Preliminary Test II used 2 plastic petri dishes (diameter of 6 cm) per dose level. The CHL cells were seeded (inoculated) at approximately 2 x 10^4 cells per petri dish in 5 ml of culture medium. After 72 hours of incubation, the Preliminary Test II was initiated as follows:

0.55 ml of the test article solution at the dose levels of 0, 2.4, 4.9, 9.8, 19.5, 39.1, 78.1 and 156.3 µg/ml or 0.55 ml of the negative control was added to the petri dishes that were then incubated for 46 hours. Two hours before the termination of the 46-hour incubation treatment, 0.1 µg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

C. Chromosome Preparation

After the final incubation treatment, the cells were harvested using trypsinization (0.25% trypsin solution) and put into test tubes. The cells were centrifuged at 1000 r.p.m. for 5 minutes and the supernatant removed, leaving only the cells in the test tube. Approximately 5 ml of hypotonic potassium chloride (0.075 M) was added to swell the cells. The cells were incubated for 20 minutes in a 37°C water bath. The hypotonic solution was then replaced with a cooled fixative solution (3:1 mixture of methanol and glacial acetic acid). The cells were centrifuged at 1000 r.p.m. for 5 minutes. After the supernatant was removed, 4 ml of the fixation solution was added. This fixation, centrifugation, supernatant removal process was repeated 4 times. Thereafter, one drop of fixed cells from the slightly turbid solution of prepared suspended cells was placed onto a clean glass slide (2 slides per dose) positioned on a table and allowed to dry over night. The cells were then stained with 3.0% Giemsa staining solution (pH 6.8) for 15 minutes, rinsed in water and allowed to dry.

D. Conclusion of the Dose Finding Test

The number of metaphase cells in one thousand cells was counted per slide. After the ratio [the mitotic index (%)] of the number of metaphase cells in the test article treated groups to the number of metaphase cells in the negative control groups was calculated, the concentrations producing 50% mitogenesis inhibition were estimated. The results of the Dose Finding Test are shown in Tables 1 and Fig. 1. The concentrations inducing 50% growth inhibition were estimated to be 100 µg/ml (Direct method test: 22-hour treatment); 17 µg/ml (Direct method test: 46-hour treatment); 520 µg/ml [Metabolic activation method test: with S 9 Mix (recovery time 16 hours)]; 980 µg/ml [Metabolic activation method test: with S 9 Mix (recovery time 40 hours)]; 610 µg/ml [Metabolic activation method test: without S 9 Mix (recovery time 16 hours)]; and 1100 µg/ml [Metabolic activation method test: with S 9 Mix (recovery time 40 hours)].

Based on the results of the Dose Finding Test, the dose levels selected for the Chromosomal Aberration Test were 19.5, 39.1, 78.1 and 156.3 μg/ml (Direct method test: 22-hour treatment); 4.9, 9.8, 19.5 and 39.1 μg/ml (Direct method test: 46-hour treatment); 78.1, 156.3, 312.5 and 625 μg/ml [Metabolic activation method test: with S 9 Mix (recovery time 16 hours) and without S 9 Mix (recovery time 16 hours)]; and 312.5, 625, 1250 and 2500 μg/ml [Metabolic activation method test: with S 9 Mix (recovery time 40 hours) and without S 9 Mix (recovery time 40 hours)].

X. CHROMOSOMAL ABERRATION TEST-1[3),4),5),6)]

A. Test Procedure

This test used 2 plastic petri dishes per dose, each with a diameter of 6 cm. The CHL cells were seeded (inoculated) at approximately 2 x 10^4 cells per petri dish in 5 ml of culture medium, and the Chromosomal Aberration Test-1 was performed as follows:

1. Direct Method

   After an initial 72 hours of incubation, 0.55 ml of the test article solution (at the established dose levels), the negative control or the positive control solution was added to the petri dishes which were then incubated for 22 hours. Two hours before the termination of the 22-hour incubation treatment, 0.1 μg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

2. Metabolic Activation Method Test with S 9 Mix

   After an initial 72 hours of incubation, 2.5 ml of culture medium was removed from the petri dishes. S 9 Mix (0.575 ml), at a final concentration of 5%, was added to the cells and the culture medium remaining in the petri dishes (2.5 ml). 0.343 ml of the test article solution (at the established dose levels) or 0.343 ml of the negative control was added to the petri dishes, and incubation continued for 6 hours. The positive control culture consisted of 2.5 ml of culture medium, 0.5 ml of S 9 Mix and 0.015 ml of the positive control solution. After these 6 hours of incubation, the reaction mixture (S 9 Mix, culture medium, and test article solution, negative control, or positive control) was poured out of the petri dishes. The cells were washed with physiological saline, 5 ml of fresh culture medium was added to the cells, and incubation continued for an additional 16 hours. Two hours before the termination of this 16-hour incubation treatment,

0.1 μg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

3. Metabolic Activation Method Test without S 9 Mix

After an initial 72 hours of incubation, 2.5 ml of culture medium was removed from the petri dishes. Fresh culture medium (0.575 ml) was added to the cells and the culture medium remaining in the petri dishes (2.5 ml). 0.343 ml of the test article solution (at the established dose levels) or 0.343 ml of the negative control was added to the petri dishes, and incubation continued for 6 hours. The positive control culture consisted of 2.5 ml of culture medium, 0.5 ml of fresh culture medium and 0.015 ml of the positive control solution. After these 6 hours of incubation, the culture mixture (culture medium and test article solution, negative control, or positive control) was poured out of the petri dishes. The cells were washed with physiological saline, 5 ml of fresh culture medium was added to the cells, and incubation continued for an additional 16 hours. Two hours before the termination of this 16-hour incubation treatment, 0.1 μg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

B. Chromosome Preparation

After the final incubation treatment, the cells were harvested using trypsinization (0.25% trypsin solution) and put into test tubes. The cells were centrifuged at 1000 r.p.m. for 5 minutes and the supernatant removed, leaving only the cells in the test tube. Approximately 5 ml of hypotonic potassium chloride (0.075 M) was added to swell the cells. The cells were incubated for 20 minutes in a 37°C water bath. The hypotonic solution was then replaced with a cooled fixative solution (3:1 mixture of methanol and glacial acetic acid). The cells were centrifuged at 1000 r.p.m. for 5 minutes. After the supernatant was removed, 4 ml of the fixation solution was added. This fixation, centrifugation, supernatant removal process was repeated 4 times. Thereafter, one drop of fixed cells from the slightly turbid solution of prepared suspended cells was placed onto a clean glass slide (2 slides per dose) positioned on a table and allowed to dry over night. The cells were then stained with 3.0% Giemsa staining solution (pH 6.8) for 15 minutes, rinsed in water and allowed to dry. Each slide was labeled with the study number and a code which identifies the test or control article, the dose level and the culture method.

XI. CHROMOSOMAL ABERRATION TEST-2 [3),4),5),6)]

As the results of Chromosomal Aberration Test-1 were negative, the Chromosomal Aberration Test-2 was performed as follows:

A. Test Procedure

This test used 2 plastic petri dishes per dose, each with a diameter of 6 cm. The CHL cells were seeded (inoculated) at approximately 2 x 10^4 cells per petri dish in 5 ml of culture medium, and the Chromosomal Aberration Test-2 was performed as follows:

1. Direct Method Test

   After an initial 72 hours of incubation, 0.55 ml of the test article solution (at the established dose levels), the negative control or the positive control solution was added to the petri dishes which were then incubated for 46 hours. Two hours before the termination of the 46-hour incubation treatment, 0.1 μg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

2. Metabolic Activation Method Test with S 9 Mix

   After an initial 72 hours of incubation, 2.5 ml of culture medium was removed from the petri dishes. S 9 Mix (0.575 ml), at a final concentration of 5%, was added to the cells and the culture medium remaining in the petri dishes (2.5 ml). 0.343 ml of the test article solution (at the established dose levels) or 0.343 ml of the negative control was added to the petri dishes, and incubation continued for 6 hours. The positive control culture consisted of 2.5 ml of culture medium, 0.5 ml of S 9 Mix and 0.015 ml of the positive control solution. After these 6 hours of incubation, the reaction mixture (S 9 Mix, culture medium, and test article solution, negative control, or positive control) was poured out of the petri dishes. The cells were washed with physiological saline, 5 ml of fresh culture medium was added to the cells, and incubation continued for an additional 40 hours. Two hours before the termination of this 40-hour incubation treatment, 0.1 μg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

3. Metabolic Activation Method Test without S 9 Mix

   After an initial 72 hours of incubation, 2.5 ml of culture medium was removed from the petri dishes. Fresh culture medium (0.575 ml) was added to the cells and the culture medium remaining in the petri dishes (2.5 ml). 0.343 ml of the test article solution (at the established dose levels) or 0.343 ml of the negative control

was added to the petri dishes, and incubation continued for 6 hours. The positive control culture consisted of 2.5 ml of culture medium, 0.5 ml of fresh culture medium and 0.015 ml of the positive control solution. After these 6 hours of incubation, the culture mixture (culture medium and test article solution, negative control, or positive control) was poured out of the petri dishes. The cells were washed with physiological saline, 5 ml of fresh culture medium was added to the cells, and incubation continued for an additional 40 hours. Two hours before the termination of this 40-hour incubation treatment, 0.1 μg/ml (final concentration) of Colcemid (Control No. 21K3011, Gibco) was added and incubation continued for the remaining 2 hours.

B. Chromosome Preparation

After the final incubation treatment, the cells were harvested using trypsinization (0.25% trypsin solution) and put into test tubes. The cells were centrifuged at 1000 r.p.m. for 5 minutes and the supernatant removed, leaving only the cells in the test tube. Approximately 5 ml of hypotonic potassium chloride (0.075 M) was added to swell the cells. The cells were incubated for 20 minutes in a 37°C water bath. The hypotonic solution was then replaced with a cooled fixative solution (3:1 mixture of methanol and glacial acetic acid). The cells were centrifuged at 1000 r.p.m. for 5 minutes. After the supernatant was removed, 4 ml of the fixation solution was added. This fixation, centrifugation, supernatant removal process was repeated 4 times. Thereafter, one drop of fixed cells from the slightly turbid solution of prepared suspended cells was placed onto a clean glass slide (2 slides per dose) positioned on a table and allowed to dry over night. The cells were then stained with 3.0% Giemsa staining solution (pH 6.8) for 15 minutes, rinsed in water and allowed to dry. Each slide was labeled with the study number and a code which identifies the test or control article, the dose level and the culture method.

XII. ANALYSIS OF CHROMOSOMAL ABERRATIONS

One hundred well-spread metaphases were then analyzed microscopically at over x600 and x1000 magnification under blind study conditions. Chromosomal aberrations were recorded as structural aberrations and polyploid cells. In accordance with the Atlas of Chromosomal Aberration[7], structural aberrations were classified as gap (Chromatid and Chromosome gaps: g), Chromatid break (ctb), Chromosome break (csb), Chromatid exchange (cte), Chromosome exchange (cse) and Others (o). The cell having any of

these aberrations was counted as an aberrant cell. The calculation of the number of aberrant cells was made including TAG (Total number of cells with aberrations including gap) and TA (Total number of cells with aberrations except gap). A chromosomal gap was characterized by a section of the chromosome that was separated from the chromosome, but remains on the axis. A chromosomal break was characterized by a section of the chromosome that had broken off and was not on the axis.

XIII. JUDGMENT

If the frequency of aberrant cells found in the test article treated groups was significantly greater than that of the negative control group, and if dose-response was confirmed, the results of the Chromosomal Aberration Test would be interpreted as positive.

XIV. STATISTICAL ANALYSIS

The X^2-Test was used to compare the percentage of cells with aberrations in the test article treated groups or the positive control groups with that in the negative control group. The difference would be considered significant if $p < 0.05$.

RESULTS

I. CHROMOSOMAL ABERRATION TEST-1

The results of the Chromosomal Aberration Test-1 are shown in Table 2-1 (Direct method) and Table 3-1 (Metabolic activation method). The dose-response curves of the average frequencies of structural aberrations and polyploidy are shown in Figures 2~3.

In the direct method test (22-hour treatment), the average frequencies of structural aberrations in the test article treated groups (0-1.5%) were not significantly different from those of the negative control groups (1%). Further, in the metabolic activation method test, no significant difference was noted in the average frequencies of structural aberrations between the metabolic activation method test with S 9 Mix (recovery time 16 hours; 1-2%) and the metabolic activation method test without S 9 Mix (recovery time 16 hours; 0.5-2%).

The average frequencies of polyploidy (0-1%) that were found using the direct method and the metabolic activation method tests, at all dose levels of the test article, were the same as those of the negative control group.

In the positive control group, using MMC (0.05 μg/ml) in the direct method test and B(a)P (20 μg/ml) in the metabolic activation method test with S 9 Mix, the average frequency of structural aberrations was significantly greater than that in the negative control group. The frequency of structural aberrations in the negative control group was within acceptable limits.

II. CHROMOSOMAL ABERRATION TEST-2

The results of the Chromosomal Aberration Test-2 are shown in Table 2-2 (Direct method) and Table 3-2 (Metabolic activation method). The dose-response curves of the average frequencies of structural aberrations and polyploidy are shown in Figures 2~3.

In the direct method test (46-hour treatment), the average frequencies of structural aberrations in the test article treated groups (1-2%) were not significantly different from those of the negative control groups (1%). Further, in the metabolic activation method test, no significant difference was noted in the average frequencies of structural

aberrations between the metabolic activation method test with S 9 Mix (recovery time 40 hours; 0.5-1.5%) and the metabolic activation method test without S 9 Mix (recovery time 40 hours; 1-2%).

The average frequencies of polyploidy (0-1%) that were found using the direct method and the metabolic activation method tests, at all dose levels of the test article, were the same as those of the negative control group.

In the positive control group, using MMC (0.05 μg/ml) in the direct method test and B(a)P (20 μg/ml) in the metabolic activation method test with S 9 Mix, the average frequency of structural aberrations was significantly greater than that in the negative control group. The frequency of structural aberrations in the negative control group was within acceptable limits.

DISCUSSION

In order to evaluate the clastogenicity potential of PROTEASE P, cultured Chinese Hamster Cells (CHL/IU cell line) were exposed *in vitro* to PROTEASE P, using the direct method test (22-hour and 46-hour treatments) and the metabolic activation method test (with and without S 9 Mix). The dose levels of PROTEASE P used the Chromosomal Aberration Test were 19.5, 39.1, 78.1 and 156.3 µg/ml (Direct method test: 22-hour treatment); 4.9, 9.8, 19.5 and 39.1 µg/ml (Direct method test: 46-hour treatment); 78.1, 156.3, 312.5 and 625 µg/ml [Metabolic activation method test: with S 9 Mix (recovery time 16 hours) and without S 9 Mix (recovery time 16 hours)]; and 312.5, 625, 1250 and 2500 µg/ml [Metabolic activation method test: with S 9 Mix (recovery time 40 hours) and without S 9 Mix (recovery time 40 hours)].

In the direct method test, PROTEASE P was judged to have no clastogenicity potential on the CHL/IU cell line, because the frequencies of structural aberrations and polyploidy found in the test article treated groups were not significantly different from those of the negative control group.

In the metabolic activation method test, PROTEASE P was judged to have no clastogenicity potential, because the frequencies of structural aberrations and polyploidy found in the test article treated groups were not significantly different from those of the negative control group, and because there was no significant difference in the average frequencies of structural aberrations and polyploidy between the metabolic activation test with S 9 Mix and the metabolic activation test without S 9 Mix.

The results of the negative and positive control groups were within the acceptable limits of SNBL's background data. Therefore, the proper performance of this study was confirmed.

Under the study conditions, the results clearly indicate that PROTEASE P is not clastogenic to the CHL/IU cell line.

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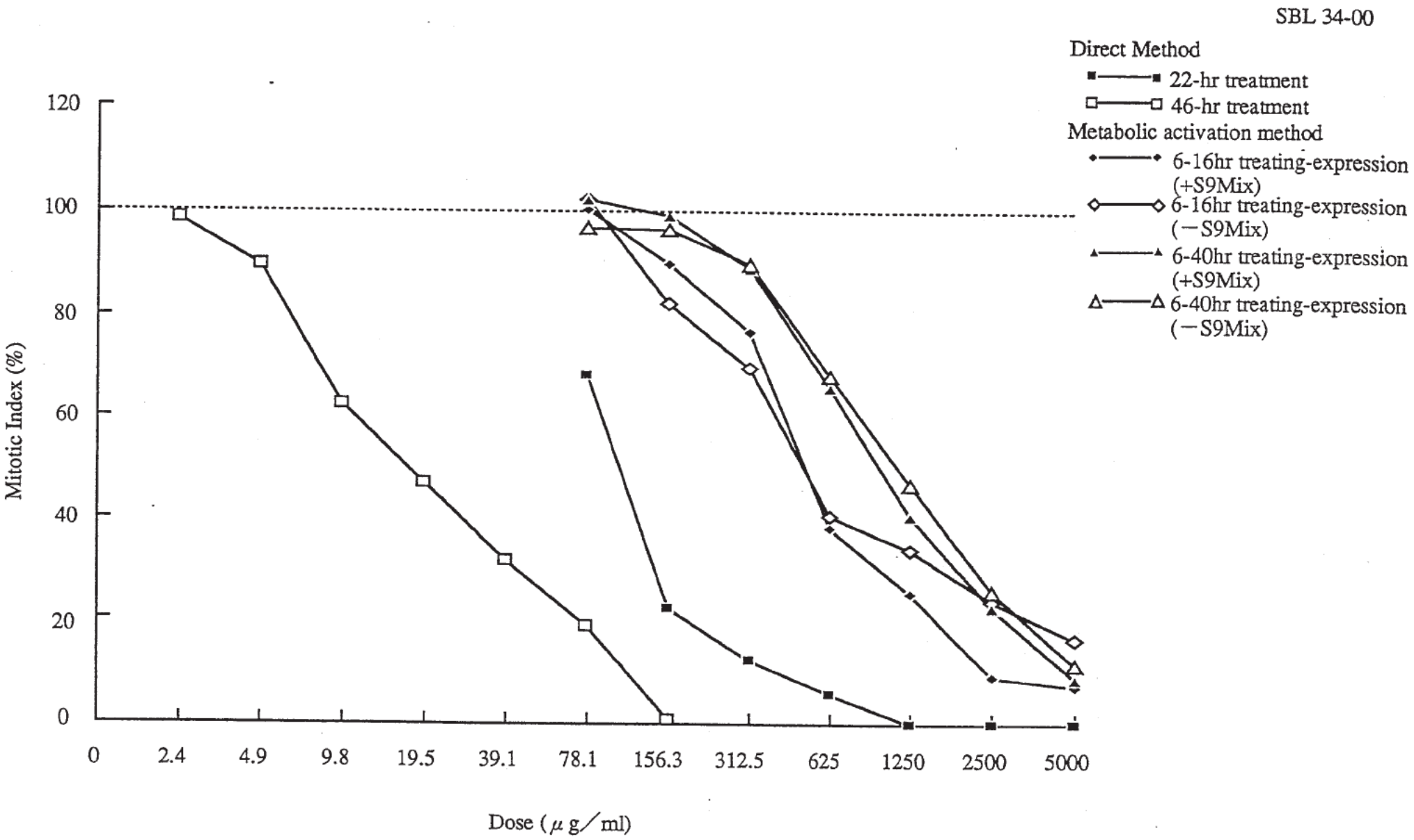

Fig. 1 Results of the Dose Finding Test

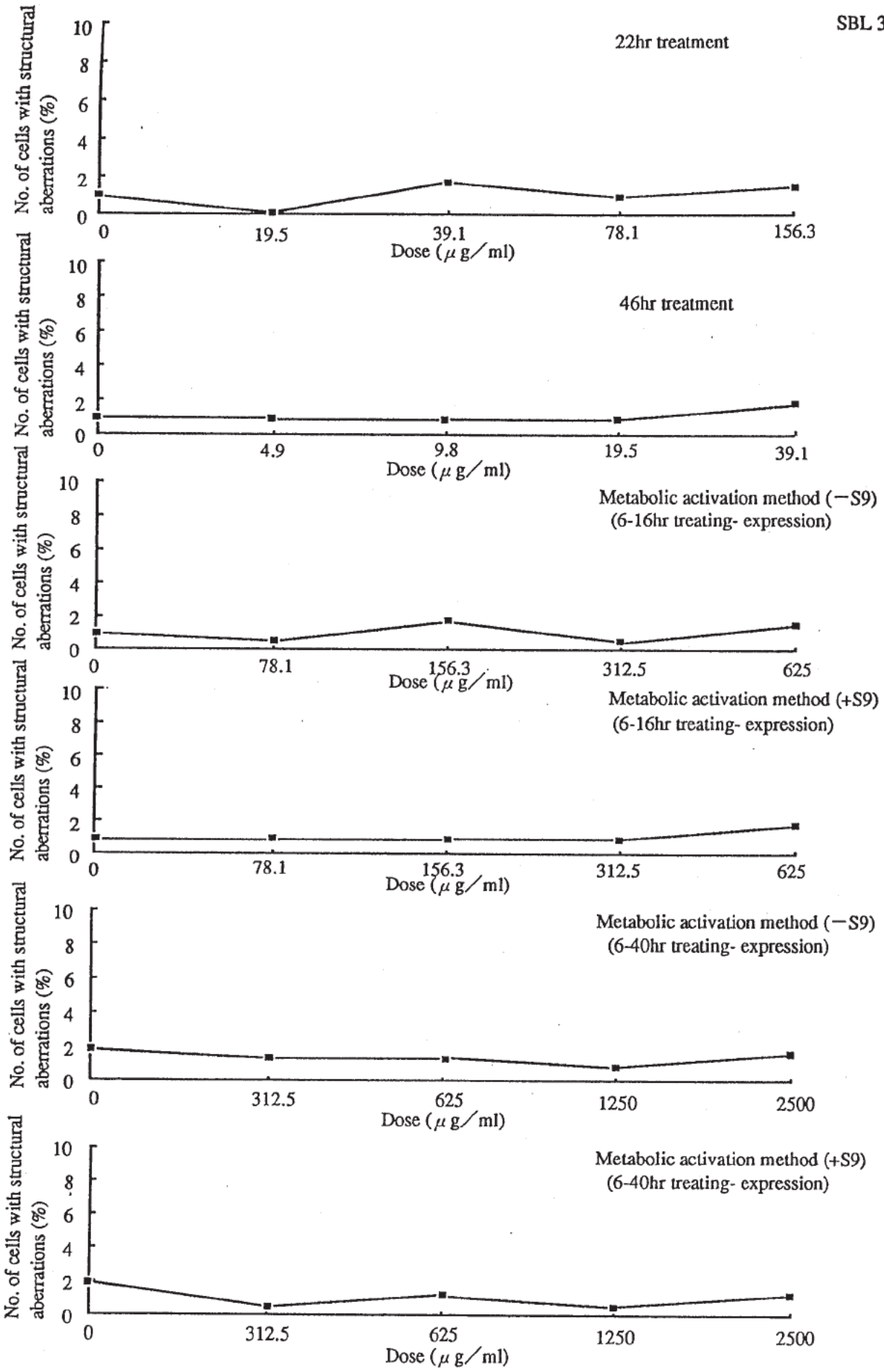

Fig.2 Dose－frequency of appearance curves (Structural aberrations)

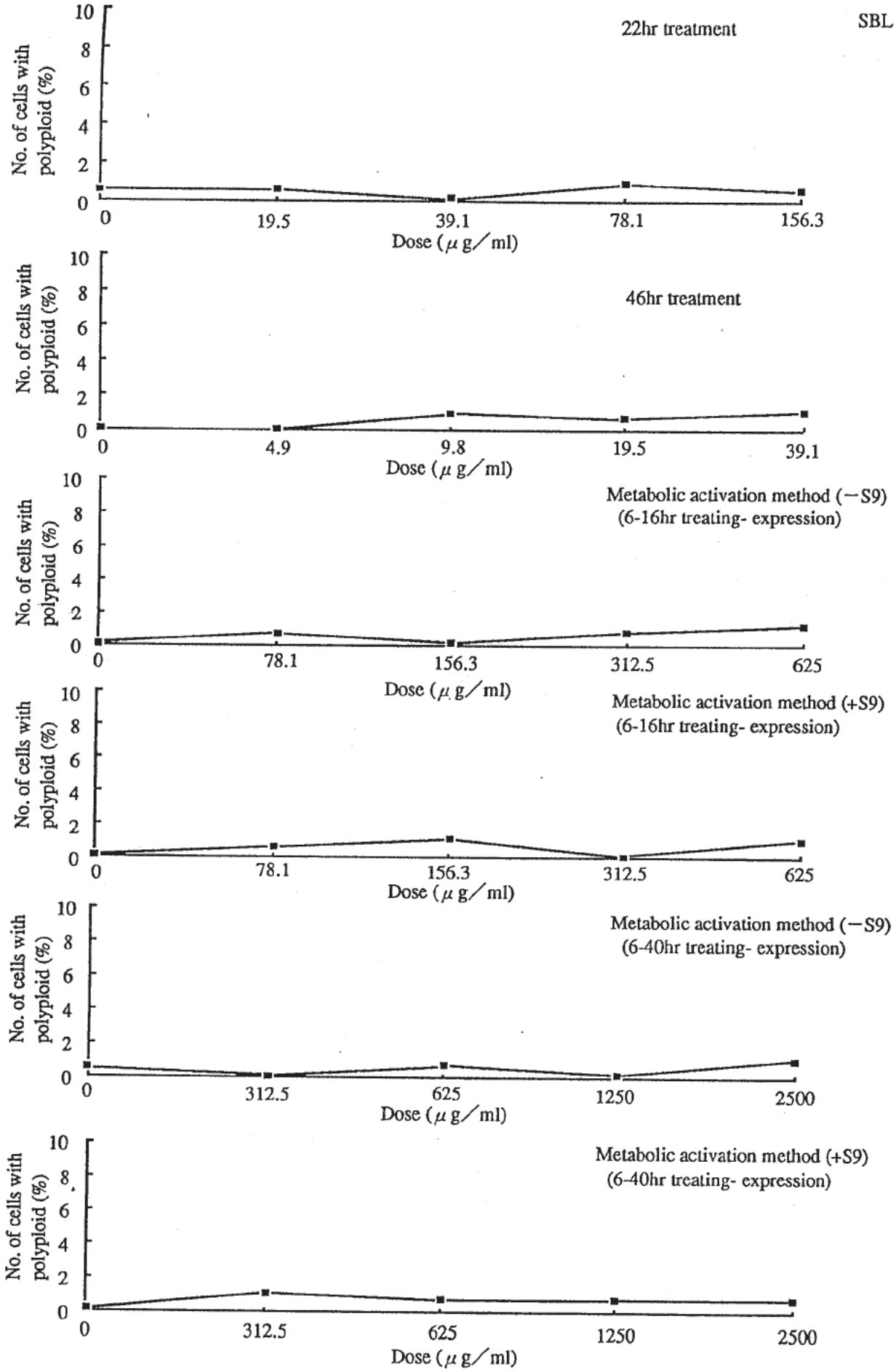

Fig. 3 Dose−frequency of appearance curves (Polyploid cells)

Table 1. Results of cell inhibitory effects of PROTEASE P

Test article : PROTEASE P

Direct methods	22hr treatment	Dose (μg/ml)	0	78.1	156.3	312.5	625	1250	2500	5000	50% mitogenesis inhivition (μg/ml)
		Mitotic index (%) *	100	68.1	22.5	12.3	5.8	0	0	0	100
	46hr treatment	Dose (μg/ml)	0	2.4	4.9	9.8	19.5	39.1	78.1	156.3	—
		Mitotic index (%) *	100	98.3	89.5	62.3	46.9	31.8	18.8	0.8	17
Metabolic activation method (+S9Mix)	6 - 16hr treating-expression	Dose (μg/ml)	0	78.1	156.3	312.5	625	1250	2500	5000	—
		Mitotic index (%) *	100	100.5	90.0	76.8	38.2	25.5	9.1	7.3	520
Metabolic activation method (−S9Mix)	6 - 16hr treating-expression	Dose (μg/ml)	0	78.1	156.3	312.5	625	1250	2500	5000	—
		Mitotic index (%) *	100	102.5	82.0	69.6	41.0	34.2	24.2	16.8	610
Metabolic activation method (+S9Mix)	6 - 40hr treating-expression	Dose (μg/ml)	0	78.1	156.3	312.5	625	1250	2500	5000	—
		Mitotic index (%) *	100	102.4	99.2	89.0	65.9	40.8	22.7	9.0	980
Metabolic activation method (−S9Mix)	6 - 40hr treating-expression	Dose (μg/ml)	0	78.1	156.3	312.5	625	1250	2500	5000	—
		Mitotic index (%) *	100	97.0	96.5	90.4	68.3	47.0	26.1	11.7	1100

* : Average of 2 culture flasks

Table 2-1. Results of chromosomal aberration test-1 on CHL/IU cells treated with Protease P (Direct methods)

SBL 34-00

Treatment time (hr)	Compound	Dose (μg/ml)	No. of cells observed	No. of poly-ploid cells	Judgement	No. of structural aberrations						No. of cells with structural aberrations		Judgement
						gap	Chromatid type		Chromosome type					
						g	ctb	cte	csb	cse	o	TA	TAG	
22	Saline	0	100	0	−	0	0	0	0	0	0	0	0	−
			100	1		1	1	0	0	0	0	1	2	
			200	1 (0.5)		1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
	Protease P	19.5	100	0	−	0	0	0	0	0	0	0	0	−
			100	1		0	0	0	0	0	0	0	0	
			200	1 (0.5)		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
		39.1	100	0	−	0	1	0	0	0	0	1	1	−
			100	0		2	0	0	0	0	0	0	2	
			200	0 (0)		2 (1)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	3 (1.5)	
		78.1	100	2	−	0	1	0	0	0	0	1	1	−
			100	0		0	0	1	0	0	0	1	1	
			200	0 (1)		0 (0)	1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	2 (1)	2 (1)	
		156.3	100	0	−	1	0	0	0	0	0	0	1	−
			100	1		2	0	0	0	0	0	0	2	
			200	1 (0.5)		3 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.5)	
	MMC	0.05	100	0	−	8	14	22	0	0	1	34	42	+
			100	0		12	15	23	0	0	0	32	40	
			200	0 (0)		20 (10)	29 (14.5)	45 (22.5)	0 (0)	0 (0)	1 (0.5)	66* (33)	82* (41)	

Aberrations : gap; Chromatid gap and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange, o; others
TAG; Total No. of cells with aberrations including gap, TA; Total No. of cells with aberrations except gap, Saline: negative control, MMC; Mitomycin C: positive control
* : Significantly different from negative control ($p<0.05$).

Table 2-2. Results of chromosomal aberration test-2 on CHL/IU cells treated with Protease P (Direct methods)

SBL 34-00

Treatment time (hr)	Compound	Dose (μg/ml)	No. of cells observed	No. of poly-ploid cells	Judgement	No. of structural aberrations						No. of cells with structural aberrations		Judgement
						gap	Chromatid type		Chromosome type					
						g	ctb	cte	csb	cse	o	TA	TAG	
46	Saline	0	100	0	–	0	0	0	0	0	0	0	0	–
			100	0		1	0	1	0	0	0	1	2	
			200	0 (0)		1 (0.5)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
	Protease P	4.9	100	0	–	1	1	0	0	0	0	1	2	–
			100	0		0	0	0	0	0	0	0	0	
			200	0 (0)		1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
		9.8	100	2	–	0	2	0	0	0	0	2	2	–
			100	0		0	0	0	0	0	0	0	0	
			200	2 (1)		0 (0)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1)	2 (1)	
		19.5	100	1	–	0	0	0	0	0	0	0	0	–
			100	0		1	1	0	0	0	0	1	2	
			200	0 (0.5)		1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
		39.1	100	2	–	0	1	0	0	0	0	1	1	–
			100	0		2	0	1	0	0	0	1	3	
			200	2 (1)		2 (1)	1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	2 (1)	4 (2)	
	MMC	0.05	100	0	–	12	19	27	0	1	0	33	40	+
			100	0		16	23	33	0	0	1	38	47	
			200	0 (0)		28 (14)	42 (21)	60 (30)	0 (0)	1 (0.5)	1 (0.5)	71* (35.5)	87* (43.5)	

Aberrations : gap; Chromatid gap and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange, o; others
TAG; Total No. of cells with aberrations including gap, TA; Total No. of cells with aberrations except gap, Saline: negative control, MMC; Mitomycin C: positive control
* : Significantly different from negative control ($p<0.05$).

Table 3-1. Results of chromosomal aberration test-1 on CHL/IU cells treated with Protease P (Metabolic activation methods)

SBL 34-00

Treating-expression time(hr)	S9Mix	Compound	Dose (μg/ml)	No. of cells observed	No. of poly-ploid cells	Judgement	No. of structural aberrations						No. of cells with structural aberrations		Judgement
							gap	Chromatid type		Chromosome type					
							g	ctb	cte	csb	cse	o	TA	TAG	
6 - 16	−	Saline	0	100	0	−	0	1	0	0	0	0	1	1	−
				100	0		0	0	1	0	0	0	1	1	
				200	0 (0)		0 (0)	1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	2 (1)	2 (1)	
		Protease P	78.1	100	0	−	1	0	0	0	0	0	0	1	−
				100	1		0	0	0	0	0	0	0	0	
				200	1 (0.5)		1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	
			156.3	100	0	−	0	0	1	0	0	0	1	1	−
				100	0		3	0	0	0	0	0	0	3	
				200	0 (0)		3 (1.5)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	1 (0.5)	4 (2)	
			312.5	100	0	−	0	0	0	0	0	0	0	0	−
				100	1		0	1	0	0	0	0	1	1	
				200	1 (0.5)		0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	1 (0.5)	
			625	100	0	−	1	1	0	0	0	0	1	2	−
				100	2		0	2	0	0	0	0	2	2	
				200	2 (1)		1 (0.5)	3 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.5)	4 (2)	
		B(a)P	20	100	0	−	2	1	0	0	0	0	1	3	−
				100	0		0	1	1	0	0	0	2	2	
				200	0 (0)		2 (1)	2 (1)	1 (0.5)	0 (0)	0 (0)	0 (0)	3 (1.5)	5 (2.5)	
6 - 16	+	Saline	0	100	0	−	1	0	1	0	0	0	1	2	−
				100	0		0	0	0	0	0	0	0	0	
				200	0 (0)		1 (0.5)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
		Protease P	78.1	100	1	−	0	0	0	0	0	0	0	0	−
				100	0		0	2	0	0	0	0	2	2	
				200	1 (0.5)		0 (0)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1)	2 (1)	
			156.3	100	1	−	0	0	0	0	0	0	0	0	−
				100	1		1	1	0	0	0	0	1	2	
				200	2 (1)		1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
			312.5	100	0	−	0	1	0	0	0	0	1	1	−
				100	0		1	0	0	0	0	0	0	1	
				200	0 (0)		1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
			625	100	2	−	0	2	0	0	0	0	2	2	−
				100	0		2	0	0	0	0	0	0	2	
				200	2 (1)		2 (1)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1)	4 (2)	
		B(a)P	20	100	0	−	3	17	32	0	1	2	43	46	+
				100	0		5	20	35	0	0	1	39	42	
				200	0 (0)		8 (4)	37 (18.5)	67 (33.5)	0 (0)	1 (0.5)	3 (1.5)	82* (41)	88* (44)	

Aberrations : gap; Chromatid gap and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange,o; others
TAG; Total No. of cells with aberrations including gap, TA; Total No. of cells with aberrations except gap, Saline:negative control, B(a)P; Benzo(a)Pyrene:positive control
* : Significantly different from negative control ($p<0.05$).

Table 3-2. Results of chromosomal aberration test-2 on CHL／IU cells treated with Protease P (Metabolic activation methods)

SBL 34-00

Treating-expression time(hr)	S9Mix	Compound	Dose (μg/ml)	No. of cells observed	No. of poly-ploid cells	Judgement	No. of structural aberrations						No. of cells with structural aberrations		Judgement
							gap	Chromatid type		Chromosome type					
							g	ctb	cte	csb	cse	o	TA	TAG	
6 - 40	−	Saline	0	100	0	−	1	1	0	0	0	0	1	2	−
				100	1		0	2	0	0	0	0	2	2	
				200	1 (0.5)		1 (0.5)	3 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.5)	4 (2)	
		Protease P	312.5	100	0	−	0	0	0	0	0	0	0	0	−
				100	0		2	0	1	0	0	0	1	3	
				200	0 (0)		2 (1)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	1 (0.5)	3 (1.5)	
			625	100	0	−	0	0	0	0	0	0	0	0	−
				100	1		2	1	0	0	0	0	1	3	
				200	1 (0.5)		2 (1)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	3 (1.5)	
			1250	100	0	−	0	0	0	0	0	0	0	0	−
				100	0		1	1	0	0	0	0	1	2	
				200	0 (0)		1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	2 (1)	
			2500	100	0	−	2	0	1	0	0	0	1	3	−
				100	2		1	0	0	0	0	0	0	1	
				200	2 (1)		3 (1.5)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	1 (0.5)	4 (2)	
		B(a)P	20	100	0	−	0	2	0	0	0	0	2	2	−
				100	0		1	1	1	0	0	0	2	3	
				200	0 (0)		1 (0.5)	3 (1.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	4 (2)	5 (2.5)	
6 - 40	+	Saline	0	100	0	−	0	1	1	0	0	0	2	2	−
				100	0		2	0	0	0	0	0	0	2	
				200	0 (0)		2 (1)	1 (0.5)	1 (0.5)	0 (0)	0 (0)	0 (0)	2 (1)	4 (2)	
		Protease P	312.5	100	2	−	0	0	0	0	0	0	0	0	−
				100	0		0	1	0	0	0	0	1	1	
				200	2 (1)		0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	1 (0.5)	
			625	100	1	−	0	0	1	0	0	0	1	1	−
				100	0		2	0	0	0	0	0	0	2	
				200	1 (0.5)		2 (1)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	1 (0.5)	3 (1.5)	
			1250	100	0	−	0	0	0	0	0	0	0	0	−
				100	1		0	1	0	0	0	0	1	1	
				200	1 (0.5)		0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	1 (0.5)	
			2500	100	0	−	3	0	0	0	0	0	0	3	−
				100	1		0	0	0	0	0	0	0	0	
				200	1 (0.5)		3 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.5)	
		B(a)P	20	100	0	−	3	6	12	0	0	0	15	18	+
				100	0		4	10	12	0	0	0	18	22	
				200	0 (0)		7 (3.5)	16 (8)	24 (12)	0 (0)	0 (0)	0 (0)	33* (16.5)	40* (20)	

Aberrations : gap; Chromatid gap and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange,o; others
TAG; Total No. of cells with aberrations including gap, TA; Total No. of cells with aberrations except gap, Saline:negative control, B(a)P; Benzo(a)pyrene:positive control
* : Significantly different from negative control ($p<0.05$).