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April 12, 2006

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Environmental Protection Agency
1200 Pennsylvania Ave., NW
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Attention: Section 8(e) Coordinator

8EHQ-06-16439

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Re: Section 8(e) Reporting

Dear Sir or Madam:

On behalf of XXXXXXXXXXXXX, we submit the following TSCA Section 8(e) report and enclosed studies.

COMPANY

XXXXXXXXXXX
XXXXXXXXXXXXXXXX
XXXXXXXXXXX

88060000211

Contact: XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

RPW

IDENTITY OF TESTED CHEMICAL

The chemical identity of the substance is:
CAS Registry Number: 4450-68-4
p-Nitrobenzyl tosylate (PNBT)

XXXXXXXXXXXXXXXXXXXXXXXXXXXX

USE OF THE TESTED CHEMICAL/LOW VOLUME/LOW PROBABILITY OF EXPOSURE

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XXXXXXXXX.

I. TITLE OF ATTACHED REPORT

FINAL REPORT: *In vitro* chromosome aberration test of XXXX using mammalian cultured cell. Study No. B05483.

CATEGORY OF REPORT

- Type of study: *in vitro* study of mutagenic potential of subject chemical to induce chromosome aberrations.
- Tested cell line: Chinese hamster lung cell (CHL/IU).

SUMMARY OF ADVERSE EFFECT(S) OBSERVED

Chinese hamster lung cells (CHL/IU) were cultured in fresh medium and plated. All plates were divided into three categories: (1) 24-hour continuous treatment with the test material; (2) no test material; and (3) in the presence of metabolic activation system of short time treatment. For short time treatments, cells were treated with no test material, 7.5 ug/ml test material and 15.0 ug/ml test material. For continuous treatment, cells were treated with no test material, 3.5 ug/ml test material and 7.0 ug/ml test material. The positive control test material consisted of mitomycin C and benzo[a]pyrene. After culture completion, cells were treated with 0.25% trypsin-EDTA, centrifuged, treated with a hypotonic solution, and then fixed. Cell suspensions were dropped onto slides, dried overnight at room temperature and stained with 5% Giemsa solution.

In each slide, 100 metaphases (200 metaphases per dose) were examined under microscope for chromosome aberrations (structural aberrations and numerical aberrations). Averages of chromosomal aberrations in the test groups were observed at 3-11.5%, dose-dependently, regardless of metabolic activation system.

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II. TITLE OF ATTACHED REPORT

FINAL REPORT: *In vitro* micronucleus test of XXXX in mice. Study No. B05512

CATEGORY OF REPORT

Type of report: *In vivo* assay of mutagenic potential of XXXX

Tested animal: Crljbgi:CD1 (ICR) mouse

SUMMARY OF ADVERSE EFFECT(S) OBSERVED

500, 1000 and 2000 mg/kg dose levels of the test material were administered to male ICR mice (6 mice in each dosage group) orally by gavage. Six male ICR mice were treated with water only administered by gavage (negative control) and six male ICR mice were treated with 2 mg/kg mitomycin C (positive control) administered intraperitoneally. All animals were observed once a day for appearance, moribund and mortality. Individual body weights were recorded just before administration and the day before bone marrow collection. Bone marrow was collected 24 hours after administration. Two specimens of bone marrow cells were collected per animal, air-dried on slides and stained with 5% Giemsa stain. The slides were observed under a microscope.

A specimen was judged "positive" when micronucleated polychromatic erythrocytes (MNPCE) were increased significantly in a dose-dependent manner or there was a reproducible increase in the number of MNPCE at least one test level. The appearance rate of MNPCE in polychromatic erthrocytes (PCE) was significantly increased at the 2000 mg/kg dosage level.

III. TITLE OF ATTACHED REPORT

FINAL REPORT: Bacterial reverse mutation test of XXXX. Study No. B05482.

CATEGORY OF REPORT

Type of report: *In vitro* assay of mutagenic potential of XXXX

Tested cell lines: *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2uvrA(pKM101))

SUMMARY OF ADVERSE EFFECT(S) OBSERVED

The assay was conducted on the above identified bacterial strains at 5 dose levels: 0 ug/plate, 312.5 ug/plate, 625 ug/plate, 1250 ug/plate, 2500 ug/plate and

5000 ug/plate. Positive controls consisted of 2-nitrofluorene, sodium azide, 2-aminoanthracene, 9-aminoacridine and 4-nitroquinoline 1-oxide as appropriate for each bacterial strain.

After incubation, an automatic colony counter was used for counting revertant colonies. The number of revertant colonies:

a. in the TA98 strain increased more than two-fold at all dose levels compared to the negative control regardless of application of the metabolic activation system;

b. in the WP2uvrA (pKM101) strain increased more than two-fold compared to the negative control group, with metabolic activation;

c. in the TA100 strain increased more than two-fold compared to the negative control group at all dose levels except 2500 and 5000 ug/plate without metabolic activation, and 312.5 ug/plate with activation;

d. in the TA1535 strain, increased more than two-fold compared to the negative control at dose levels of 2500 and 5000 ug/plate with and without metabolic activation; and

e. in the TA1537 strain, increased more than two-fold compared to the negative control group at 5000 ug/plate without metabolic activation and at 1250, 2500, and 5000 ug/plate with activation.

Very truly yours,

Ann G. Grimaldi

Enclosures
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Biotoxtech

FINAL REPORT

***In vitro* chromosome aberration test of [redacted] using mammalian cultured cell**

Study No.: B05483

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Biotoxtech Co., Ltd.
58-1Block Ochang Scientific Industrial Complex, Ochang-myeon,
Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

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CERTIFICATE OF TRANSLATION

This report is translated from Korean into English as an original contents, therefore, is not assigned on the relevant pages. This report is partially modified the Korean report for an expression of exact meaning but it did not influence on the original copy.

Translated by _____ Date Dec. 21. 2005

Hye-Jin Lee
Biotoxtech Co., Ltd.

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PREPARATION OF FINAL REPORT

Study Title : *In vitro* chromosome aberration test of [redacted] using mammalian cultured cell
Study No. : B05483

This study was performed in accordance with the agreed protocol and the study objectives were achieved in compliance with the Standard Operating Procedures (SOPs) of Biotoxtech Co., Ltd, the following Good Laboratory Practice and Guideline.

- Good Laboratory Practice
  - TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998)

- Guideline
  - The Section 10 of Chapter 4 in Annex 5, in compliance with TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998) '*In vitro* chromosome aberration test of using mammalian cultured cell'

The study described in this report was performed under the supervision of the Study Director and this report was written based on the raw data obtained from the study.

Mei-Shu Zheng	Nov. 21, 2005
Biotoxtech Co., Ltd. Study Director	Date
Byeong-Kyu Lee	Nov. 22, 2005
Biotoxtech Co., Ltd. Testing Facility Management	Date

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OUTLINE OF STUDY

Study Title : *In vitro* chromosome aberration test of [redacted] using mammalian cultured cell
Study No. : B05483

1. Purpose

This study was performed to examine a mutagenic potential of [redacted] to induce chromosome aberrations in CHL/IU cell.

2. GLP

TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998)

3. Guideline

The Section 10 of Chapter 4 in Annex 5, in compliance with TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998) '*In vitro* chromosome aberration test of using mammalian cultured cell'

4. Sponsor

Name [redacted]
Address [redacted]

5. Test management

Name Biotoxtech Co., Ltd.
Address 58-1Block Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

6. Test facility

Name Biotoxtech Co., Ltd.
Address 58-1Block Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

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7. Record and archives of data

(1)Duration : 5 years after the completion of test

(2) Record and data :

Study related protocol, raw data, final report (draft and original) and documents of GLP and all correspondences.

(3) Storage place and address :

Place : Archives of Biotoxtech Co., Ltd.

Address : 58-1Block Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

8. Schedule

Initiation of study	Oct. 17, 2005
Initiation of experiments	Oct. 27, 2005
Test substance treatment	Oct. 27, 2005
Slide preparation & observation	Oct. 28, 2005 ~ Nov. 10, 2005
Completion of experiments	Nov. 10, 2005
Presentation of final report	Nov. 21, 2005
Completion of study	Nov. 21, 2005

9. Responsible personnel

Preparation of protocol, management of study, and preparation of final report	Mei-Shu Zheng
Identity confirmation and formulation of test substance	Eun-Ae Kim, Jin-Ah kim
Treatment of test substance	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee
Experimentation, medium & chemical formulation	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee
Slide preparation & observation	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee
Arrangement of raw data & evaluation	Mi-Young Lee, Hye-Jin Lee

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QUALITY ASSURANCE RECORD AND AUTHENTICATION STATEMENT

Study Title : *In vitro* chromosome aberration test of [redacted] using mammalian cultured cell
Study No. : B05483

This study was subject to audit by the independent Quality Assurance Unit of BIOTOXTECH as indicated below. The findings of each audit were reported to the Study Director and Management as prescribed by Standard Operating Procedures.

The final report audit was designed to confirm that as far as can be reasonably established the methods described and results incorporated in the final report accurately reflect the raw data produced during the study.

Audit phases and dates reported to the responsible personnel were as indicated below and these were based upon the audit records.

Audit type	Audit phase	Audit date	To Study Director	To Management
Study based audit	Protocol	Oct. 17, 2005	Oct. 17, 2005	Oct. 17, 2005
	Protocol Amendment	Oct. 26, 2005	Oct. 26, 2005	Oct. 26, 2005
	Pre-culture	Oct. 26, 2005	Oct. 29, 2005	Oct. 29, 2005
	Storage and preparation of test substance	Oct. 27, 2005	Oct. 29, 2005	Oct. 29, 2005
	Treatment	Oct. 27, 2005	Oct. 29, 2005	Oct. 29, 2005
	Slide preparation	Oct. 28, 2005	Oct. 29, 2005	Oct. 29, 2005
	Observation	Nov.10, 2005	Nov.10, 2005	Nov.10, 2005
	Raw data	Nov.18, 2005	Nov.19, 2005	Nov.19, 2005
	Final report (Draft)	Nov.18, 2005	Nov.19, 2005	Nov.19, 2005
	Final report	Nov.21, 2005	Nov.21, 2005	Nov.21, 2005

Biotoxtech Co., Ltd.
Director, Quality Assurance Young-Hyun Lee Nov. 21, 2005
Date

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I. SUMMARY

This study was designed to examine a mutagenic potential of [redacted] in the chromosome aberration test system using Chinese hamster lung cell (CHL/IU).

According to the MTT assay, treatment level for the short time treatments was determined as 15.0 μg/mL for the highest concentration, regardless of application of metabolic activation system; and for the continuous treatments was determined as 7.0 μg/mL. The highest concentration was diluted by 2, common ratio, to prepare 3 treatment levels including the highest concentration accompanying negative and positive control in the main test.

As a result, the structural and numerical chromosome aberrations were significantly increased dose-dependently in the short time treatment and the continuous treatment without and with metabolic activation system as compared with that of the negative control.

In the positive control, the structural and numerical chromosome aberration was significantly increased.

In conclusion, [redacted] did show the chromosome aberrations regardless of application of metabolic activation system in the chromosome aberration assay system using Chinese hamster lung cell(CHL/IU) under the conditions of this study.

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II. PURPOSE

This study was designed to examine mutagenic potential of [redacted] in the chromosome aberration assay system using Chinese hamster lung cell (CHL/IU).

III. MATERIAL AND METHOD

1. Test substance

(1)	Name	[redacted]
(2)	Lot No.	072805-2
(3)	Appearance	Off white solid
(4)	Degree of Purity	>99 %
(5)	Molecular formula	[redacted]
(6)	Solubility in water	Hydrophobic
(7)	Degree of storage	Room temperature
(8)	Conditions of storage	Store in dry place (Avoid light).
(9)	Handling & notice	Do not breathe and ingest of test substance. Avoid contact with eyes and skin
(10)	Supplier	
	Name	[redacted]
	Address	[redacted]
(11)	Residue treatment	After the study, any remaining test substance will be discarded.
(12)	Certificate of analysis	Provided by the sponsor (Appendix 1).

2. Control

(1) Negative control

0.5% Methylcellulose 1500 (MC1500, Lot No.: ASQ6053, Wako, Japan)

(2) Positive control

MMC : Mitomycin C (Lot No. : 103K0496, Sigma, USA)

B[a]P : Benzo[a]pyrene (Lot No. : 111K3454, Sigma, USA)

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3. Preparation of test substance and analysis

(1) Vehicle and reason for selection

0.5% MC1500 was used as a vehicle, since the test substance was suspended through the pre-preparation of the test substance, based on the information of test substance.

(2) Preparation of test substance

The provided test substance was weighed using a semi-micro electronic balance (BJ210S, Sartorius, Germany) and suspended with 0.5% MC1500. Then it was further diluted in accordance with fixed common ratio for lower dose. The test substance was prepared just before the treatment.

(3) Formulation stability, homogeneity and concentration analysis

① Stability	Generation of heat, coloring and foaming were not observed.
② Homogeneity	The test substance was homogeneously dissolved.
③ Concentration analysis	Not done.

4. Preparation of control

(1) Negative control

0.5% MC 1500 was used as a negative control, which was prepared that 0.5 g of MC1500 was dissolved in 100 mL of distilled water and disinfected through the autoclave (121 ℃, 20 min).

(2) Positive control

MMC was used for the continuous treatment and for the short time treatment in the absence of metabolic activation system. MMC was dissolved in the water for injection (Lot No.: AAW5AF, Choongwae Pharma Corp., Korea). They were kept in -20 ℃ and thawed out immediately when treated(MMC concentration was 0.05 μg/mL).

B[a]P was used for the short time treatment in the presence of a metabolic activation system. B[a]P was dissolved in the DMSO (Lot No.: K32997731 413, Merck, USA). They were kept in -20 ℃ and thawed out immediately when treated (B[a]P concentration was 20 μg/mL).

5. Cell line and culture condition

Chinese hamster lung (CHL/IU) cells were used, for they have higher detection sensitivity. They were received on Nov. 15, 2002 from the American Type Culture collection (ATCC) (Lot No.: 2184656, USA) and subcultured. Then cell vials were kept in the liquid nitrogen (XC34/18, MVE. USA) tank.

Modal chromosome number of Chinese hamster lung (CHL/IU) cells is 25, and cell-cycle is

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15-17 hours. Cells were cultured with EMEM in the CO_2 incubator, (Mco-20AIC, SANYO, Japan), which was set up as 95% humidity, 37 ℃, 5% CO_2.
0.25 % Trypsin-EDTA solution was added evenly on to the cultured cells to separate and a medium was added to make cell suspension. Then it was centrifuged to sub-culture in a culture flask with fresh medium. The cultured cells were checked regularly whether they are contaminated by mycoplasma.

6. Culture medium

Culture medium was used modified Eagle's Mininum Essential Medium (EMEM, Lot No.: 01107936. Cambrex Bio Science Walker sville Inc. Walkersville, MD, USA) with 10 % fetal bovine serum (FBS, Lot No.: 1215188, Gibco, USA) and penicillin-streptomycin (Lot No.: 1244471, Gibco, USA) added a ratio of 100:1.

7. Metabolic activation system

(1) Source and storage of S9

S9 (Lot No.: 1883) originated from hormone-induced liver homogenized solution of Sprague-Dawley rat using Aroclor 1254, in which was purchased from Molecular Toxicology, Inc., was used. it was kept in -80 ℃(DFU-657CL, Operon, Korea) till using.

(2) Composition and preparation of S9 mix

The composition of S9 mix in 1 mL was followed.

Component	Formation	Amount
Rat liver S9 (Aroclor-1254 induced)	50%	0.5 mL
$MgCl_2$	8.0 μmol	0.02 mL
KCl	33.0 μmol	
Glucose-6-phosphate	5.0 μmol	0.005 mL
NADP	4.0 μmol	0.04 mL
Sodium phosphate buffer, pH 7.4	50.0 μmol	0.25 mL
Water for injection	-	0.185 mL
Total volume		1.0 mL

It was prepared as follows; 1.0 mol/L Glucose-6-phosphate (Lot No.: 052K7043, Sigma, USA) and 0.1 mol/L NADP (Lot No.: 054K7036, Sigma, USA) were dissolved in water for injection (Lot No.: AAW5AF, Choongwae Pharma Corp., Korea) and mix solution of 0.4 mol/L $MgCl_2$ and 1.65 mol/L KCl, then added 0.2 mol/L Na-phosphate buffer (pH 7.4). Finally they were filtered (0.22 μm. Millipore, USA) under the sterilized condition and thawed S9 was added. It was used in an ice-cooling condition.

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8. Dose

The growth inhibition test (MTT assay) was conducted in 12 dose levels(0, 4.9, 9.8, 19.6, 39.1, 78.2, 156.3, 312.5, 625, 1250, 2500 and 5000 μg/mL). According to the growth inhibition test, the inhibition concentration 50 % (IC_{50}) for the short time treatments was determined as 15.0 μg/mL for the highest concentration, regardless of application of metabolic activation system; and for the continuous treatments was determined as 7.0 μg/mL. According to a result of growth inhibition test, the main test would be conducted in 3 dose levels containing inhibition concentration 50 % (IC_{50}) as the highest dose, and 2 additional dose levels, which are divided by common ratio 2; and negative and positive control would be included in the main test.

9. Method

MTT assay[1)] and chromosome aberration test[2, 3)] were conducted as follows;

(1) MTT assay[1)]

Cultured cells in 75 cm^2 cell culture flask (Nunc, USA) were cultured in a fresh medium to make final density for 5×10^4 cell/mL using the hemocytometer in 96 well plate (200 μL/well; Nunc, USA). Cells were incubated in the 5% CO_2 incubator containing 95% relative humidity and 37 ℃. All plates were divided into three as follows; (a) the continuous treatment, (b) in the absence and (c) the presence of metabolic activation system of short time treatment. Well number and others were recorded in surfaces and sides of the plates. For this experiments, 4 wells per dose were made.

In the short time treatment, both negative control and the test substance were added in the wells regardless of application of the metabolic activation system. In addition, S9 mix and 0.1 mol/L phosphate buffer (pH 7.4) were treated to make 5 % of the final concentration without and with metabolic activation system. In 6 hours cultivation, each well was washed by a culture medium and then mediums were replaced to culture for 18 hours more.

In the continuous treatment system, both negative control and the test substance were added in the wells as the short time treatment and cultured for 24 hours.

Both the short time treatment and continuous treatment systems were incubated in a 5 % CO_2 incubator containing 95 % of relative humidity and 37 ℃.

In 24 hours (culture completion time), 50 μL of MTT (5 mg/mL PBS) were treated and cultured for 4 hours. Then the wells were washed with PBS and dried. DMSO (150 μL/well) was added and dissolved in it. The spectrophoto-metrical absorbance of the sample was measured, using the ELISA reader (VERSA mat™, Molecular Devices, USA) at 540 nm. The results were expressed as a graph for an absorbance and calculated IC_{50}.

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(2) *In vitro* Chromosome aberration test[2, 3)]

The cultured cells in a 75 cm^2 cell culture flask (Nunc, USA) were cultured in fresh mediums to make the final density for 5×10^4 cell/mL using the hemocytometer (5 mL/dish; Nunc, USA) in 60 mm culture dish. All dishes were divided into three as follows; (a) the 24 hour culture system of continuous treatment, (b) in the absence and (c) presence of metabolic activation system of short time treatment. In the surfaces and sides of the dishes, well number and others were recorded. In the experiments, 2 slides per dish were prepared and used as the negative control and positive control in each group.

In the short time treatment, the negative control, the test substance (all dose levels), In addition, S9 mix and 0.1 mol/L phosphate buffer (pH 7.4) were treated to make 5 % of the final concentration without and with metabolic activation system. and 0.05 μg/mL of MMC were treated in the absence of metabolic activation system and it was cultured for 6 hours. In addition, of the negative control, the test substance (all dose levels), 20 μg/mL of B[a]P and 5%(final concentration) of S9 mix were treated in the presence of metabolic activation system. Then culture mediums were removed from dishes and washed. Fresh mediums were newly placed and cultured for 18 hours.

In the continuous treatment, the negative control, the test substance, and 0.05 μg/mL of MMC were treated and cultured for 24 hours.

The short time treatment and continuous treatment system were incubated in a 5 % CO_2 incubator containing 95 % of relative humidity and 37 ℃.

About 2 hours before culture completion, 0.25 μg/mL of colcemid (Gibco, USA) was treated. After the culture completion, cells were treated by 0.25 % trypsin-EDTA and were sedimented by the centrifuger at 1000 rpm, 4 ℃ for 5 minutes. The cells were treated with a hypotonic solution (0.075 mol/L, KCl) for 30 minutes at 37 ℃, and then were fixed using 1 mL of freshly prepared fixative (methanol: glacial acetic acid, 3:1). After the centrifugation (at 2000 rpm, 4 ℃ for 5 minutes), the supernant was removed and 5 mL of cooling fixative was added. This process was repeated twice. Then 1 ~ 2 drops of cell suspensions were dropped on the slides.

The slides were dried at a room temperature overnight and stained with 5 % Giemsa solution (manufactured with 0.1 mol/L Sörenson Phosphate buffer (pH 6.8)) for 30 minutes. The numbers of slides were randomized.

10. Observation

In each slide, 100 metaphases (200 metaphases/dose) were examined using the biological microscope of differential interference type (BX-51, Olympus, Japan) of 1000-fold magnification.

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There are two types of chromosome aberrations namely, structural aberration and numerical aberration and these were counted. Structural aberration was classified as follows; chromatid break (ctb), chromatid exchange (cte), chromosome break (csb), chromosome exchange (cse) and etc(o). These two types aberration such as chromatid and chromosome gap(g) were recorded separately. In the meta phase, if there are several gap or cuttings, it was recorded as a fragment (frg). For a numerical aberration, any cell with 1 or more aberration was counted as 1 aberrant cell.

For numerical aberration, any cell with 1 or more polyploidy (pol) aberration was counted as 1 aberrant cell.

11. Results judgment

If the number of chromosome aberration cells were increased clearly as compared with that of negative control or increased significantly dose-dependently or increased significantly at any dose level several times, then we confirmed that it was positive.

Evaluation of results did not include gaps while the gaps were recorded in raw data of structural aberration.

The final decision of chromosome aberration in relation to the test substance was carried out in accordance with Toshio Sofuni and *etc*[4]. If an appearance rate was below 5 %; between 5 and 10 %; and over 10 %, it was judged as a negative, equivocal, and positive, respectively.

12. Statistical analysis

Any specific statistical analysis for the mean value was not performed in this test. The mean value and standard deviation was calculated in relation to the measured value in the main test.

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IV. RESULTS

The MTT assay was conducted with 12 dose levels (0, 4.9, 9.8, 19.6, 39.1, 78.2, 156.3, 312.5, 625, 1250, 2500 and 5000 μg/mL) to examine a cytotoxicity of the test substance without and with metabolic activation system (Table 1).

According to the MTT assay, treatment level for the short time treatments was determined as 15.0 μg/mL for the highest concentration regardless of application of metabolic activation system; and for the continuous treatments was determined as 7.0 μg/mL. The highest concentration was diluted by 2, common ratio, to prepare 3 treatment levels including the highest concentration accompanying negative and positive control in the main test.

According to the chromosome aberration test regardless of metabolic activation system, averages of chromosomal aberration cells were 3.0 ~ 11.5 %, therefore, they were increased as compared with the negative control group (Fig. 1, 2, Table 2).

Averages of chromosomal aberration cells in the positive control group were calculated as 14.0, 18.0 and 19.0 % for short time treatment without and with metabolic activation system; and the continuous treatment, respectively. The positive control induced undeniable frequency of the chromosomal aberration (Table 2).

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V. DISCUSSION AND CONCLUSION

This study was designed to examine a mutagenic potential of [redacted] in the chromosome aberration test system using Chinese hamster lung cell (CHL/IU).

According to the MTT assay, treatment level for the short time treatments was determined as 15.0 μg/mL for the highest concentration regardless of application of metabolic activation system; and continuous treatments was determined as 7.0 μg/mL. The highest concentration was diluted by 2, common ratio, to prepare 3 treatment levels including the highest concentration accompanying negative and positive control in the main test.

As a result, the structural and numerical chromosome aberrations were significantly increased dose-dependently in the short time treatment and the continuous treatment without and with metabolic activation system as compared with that of the negative control.

In the positive control, the structural and numerical chromosome aberration was significantly increased.

The number of structural and numerical chromosome aberration cells of the negative and positive control was within the range of the standard value of the historical data in our laboratory. These results indicate that the test has been properly carried out.

In conclusion, [redacted] did show the chromosome aberrations regardless of application of metabolic activation system in the chromosome aberration assay system using Chinese hamster lung cell (CHL/IU) under the conditions of this study.

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VI. REFERENCES

(1) Mosmann, T.(1983): Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. Journal of Immunological Methods, 65, 55-63.

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

(3) Sofuni, T., Matsuoka, A., Sawada, M., Ishidate, M. Jr., Zeiger, E. and Shelby, M.D.(1990): A comparison of chromosome aberration induction by 25 compounds tested by two Chinese hamster cell (CHL and CHO) systems in culture. Mutation Research, 241, 175-213.

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FIGURES

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Figure1. Dose – response curve for the incidence of chromosome aberrations in CHL/IU cells treated with [redacted] [without and with S9 mix for 6 hrs]

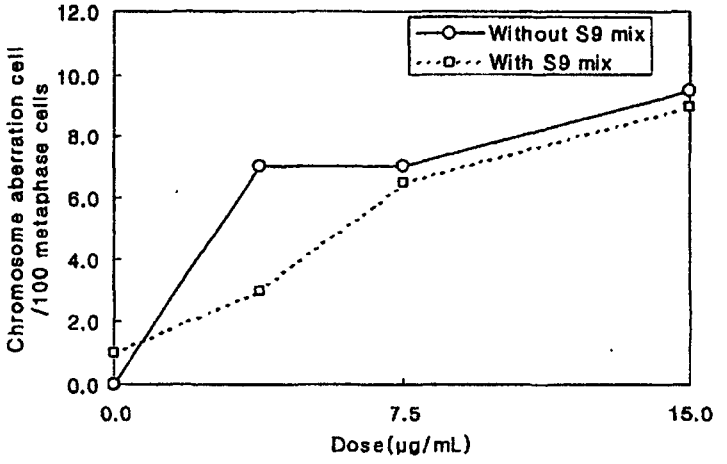
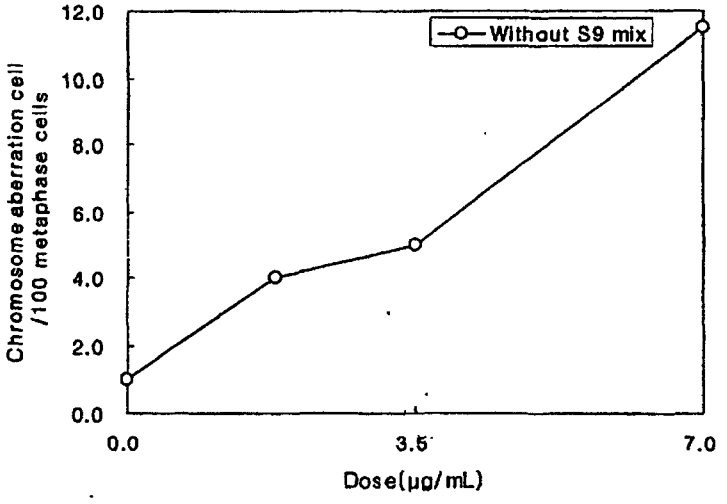


Figure 2. Dose – response curve for the incidence of chromosome aberrations in CHL/IU cells treated with [redacted] [without S9 mix for 24 hrs]



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TABLES

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Table 1. Results of growth inhibition test in CHL/IU cells treated with [redacted]

S9 mix	Test substance	Dose(μg/mL)	Growth of CHL/IU cells(OD) (Mean±S.D.)
S9 mix (−) 6+18 hrs	0.5% MC 1500	0	0.950 ± 0.020
	[redacted]	4.9	0.741 ± 0.013
		9.8	0.556 ± 0.013
		19.6	0.436 ± 0.100
		39.1	0.327 ± 0.019
		78.2	0.318 ± 0.027
		156.3	0.103 ± 0.029
		312.5	0.112 ± 0.009
		625	0.088 ± 0.008
		1250	0.069 ± 0.002
		2500	0.064 ± 0.003
		5000	0.062 ± 0.002
S9 mix (+) 6+18 hrs	0.5% MC 1500	0	0.962 ± 0.024
	[redacted]	4.9	0.771 ± 0.013
		9.8	0.525 ± 0.023
		19.6	0.363 ± 0.021
		39.1	0.158 ± 0.025
		78.2	0.071 ± 0.005
		156.3	0.064 ± 0.002
		312.5	0.067 ± 0.002
		625	0.069 ± 0.005
		1250	0.069 ± 0.003
		2500	0.050 ± 0.004
		5000	0.051 ± 0.006
S9 mix (−) 24+0 hrs	0.5% MC 1500	0	0.959 ± 0.038
	[redacted]	4.9	0.507 ± 0.027
		9.8	0.364 ± 0.044
		19.6	0.267 ± 0.024
		39.1	0.129 ± 0.010
		78.2	0.106 ± 0.010
		156.3	0.085 ± 0.002
		312.5	0.065 ± 0.004
		625	0.060 ± 0.001
		1250	0.058 ± 0.001
		2500	0.059 ± 0.002
		5000	0.054 ± 0.002

0.5% MC 1500 : 0.5% Methyl cellulose 1500

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Table 2. Results of *in vitro* chromosome aberration test in CHL/IU cells treated with [redacted]

S9 mix/ Time	Test substance	Dose (μg/mL)	No. of cell scored	No. of aberration cell							Aberration Cell(%)	Chromosome aberration cell /100 metaphase cells(%) (Mean ± S.D.)
				ctb	csb	cte	cse	frg	o	pol		
S9 mix(-) /6+18hrs	0.5% MC1500	0	100	0	0	0	0	0	0	0	0	0.0 ± 0.0
			100	0	0	0	0	0	0	0	0	
	[redacted]	3.8	100	1	0	4	1	0	0	0	6	7.0 ± 1.4
			100	2	2	3	1	0	0	0	8	
		7.5	100	3	0	2	0	0	0	0	5	7.0 ± 2.8
			100	1	4	3	1	0	0	0	9	
		15.0	100	1	1	6	0	0	0	0	8	9.5 ± 2.1
			100	2	2	4	3	0	0	0	11	
	MMC	0.05	100	7	2	5	0	0	0	0	14	14.0 ± 0.0
			100	7	2	4	1	0	0	0	14	
S9 mix(+) /6+18hrs	0.5% MC1500	0	100	0	0	0	0	0	0	0	0	1.0 ± 1.4
			100	2	0	0	0	0	0	0	2	
	[redacted]	3.8	100	0	0	2	0	0	0	0	2	3.0 ± 1.4
			100	1	2	1	0	0	0	0	4	
		7.5	100	0	4	2	1	0	0	0	7	6.5 ± 0.7
			100	0	1	4	1	0	0	0	6	
		15.0	100	2	4	4	1	0	0	0	11	9.0 ± 2.8
			100	2	0	4	1	0	0	0	7	
	B[a]P	20	100	5	4	4	2	0	0	0	15	18.0 ± 4.2
			100	6	4	5	5	1	0	0	21	
S9 mix(-) /24+0hrs	0.5% MC1500	0	100	1	0	0	0	0	0	0	1	1.0 ± 0.0
			100	0	0	1	0	0	0	0	1	
	[redacted]	1.8	100	1	0	1	2	0	0	0	4	4.0 ± 0.0
			100	0	1	3	0	0	0	0	4	
		3.5	100	2	0	1	1	0	0	0	4	5.0 ± 1.4
			100	2	1	1	2	0	0	0	6	
		7.0	100	6	2	3	1	0	0	0	12	11.5 ± 0.7
			100	2	2	4	2	1	0	0	11	
	MMC	0.05	100	6	5	4	3	2	0	0	20	19.0 ± 1.4
			100	6	4	5	2	1	0	0	18	

MMC : Mitomycin C, B[a]P : Benzo[a]pyrene, ctb : chromatid break, csb : chromosome break, cte : chromatid exchange, cse : chromosome exchange, frg : fragment, o : other, pol : polyploidy
0.5% MC 1500 : 0.5% Methyl cellulose 1500

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APPENDIX

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Appendix 1. Certificate of analysis

CERTIFICATE OF ANALYSIS

1. TEST MATERIAL IDENTIFICATION

(1) TEST MATERIAL (LOT No.) : [redacted]
(2) QUANTITY : 20 g
(3) PRODUCER : [redacted]
5-6-10 Tsukiji, Chuo-Ku, Tokyo 104-8410 Japan

(4) CHEMICAL NAME (Generic name). :
[redacted]

(5) PURITY : >99%
(6) APPEARANCE : Off white solid
(7) MOLECULAR FORMULA : [redacted]

2. SOLUBILITY

(1) INSOLUBLE : Water
(2) SOLUBLE : No information

3. STORAGE

Room temperature

We hereby certify that the data stated here above are true and correct.

Oct. 2005
[redacted]

[redacted]

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Company Sanitized

Biotoxtech

FINAL REPORT

***In vivo* micronucleus test of [redacted] in mice**

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Study No.: B05512

Biotoxtech Co., Ltd.
58-1 Block, Ochang Scientific Industrial Complex, Ochang-myeon,
Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

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294800

CERTIFICATE OF TRANSLATION

This report is translated from Korean into English as an original contents, therefore, is not assigned on the relevant pages. This report is partially modified the Korean report for an expression of exact meaning but it did not influence on the original copy.

Translated by _____ Date Jan. 23, 2006
Mei-Shu Zheng
Biotoxtech Co., Ltd.

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PREPARATION OF FINAL REPORT

Study Title : *In vivo* micronucleus test of [redacted] in mice

Study No. : B05512

This study was performed in accordance with the agreed protocol and the study objectives were achieved in compliance with the Standard Operating Procedures (SOPs) of Biotoxtech Co., Ltd., the following Good Laboratory Practice and Guideline.

- Good Laboratory Practice
  - TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998)

- Guideline
  - The Section 10 of Chapter 4 in Annex 5, in compliance with TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998) '*In vivo* micronucleus test in mice'

The study described in this report was performed under the supervision of the Study Director and this report was written based on the raw data obtained from the study.

Mei-Shu Zheng	Dec. 28, 2005
Biotoxtech Co., Ltd. Study Director	Date
Byeong-Kyu Lee	Dec. 28, 2005
Biotoxtech Co., Ltd. Testing Facility Management	Date

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OUTLINE OF STUDY

Study Title : *In vivo* micronucleus test of [redacted] in mice

Study No. : B05512

1. Purpose

The objective of this study was to determine a mutagenic potential of [redacted] in the micronucleus test system using bone marrow cells in mice.

2. GLP

The study was performed in accordance with the following Good Laboratory Practice(GLP) regulation.

- TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998)

3. Guideline

The study was performed in accordance with the following test guidelines.

- The Section 10 of Chapter 4 in Annex 5, in compliance with TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998) '*In vivo* micronucleus test in mice'

4. Sponsor

Name [redacted]

Address [redacted]

5. Test management

Name Biotoxtech Co., Ltd.

Address 58-1Block, Ochang Scientific Industrial Complex. Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do. 363-883 Korea

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6. Test facility

Name	Biotoxtech Co., Ltd.
Address	58-1Block, Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

7. Record and archives of data

(1) Duration : 5 years after the completion of test

(2) Record and data :

Protocol, raw data, specimen, final report (draft and original), documents related to the study (documents of GLP and all correspondences).

(3) Storage place and address :

Place : Archives of Biotoxtech Co., Ltd.

Address : 58-1Block Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

8. Schedule

Initiation of study	Nov. 18, 2005
Animal receipt (Preliminary test and Induction of micronucleus)	Nov. 18, 2005
Preliminary test	Nov. 24, 2005~ Nov. 27, 2005
Animal receipt (main test)	Nov. 30, 2005
Completion of quarantine and acclimatization	Dec. 7, 2005
Group assignment	Dec. 7, 2005
Initiation of experiments	Dec. 7, 2005
Administration of test substance	Dec. 7, 2005
Specimen preparation and observation	Dec. 8, 2005 ~ Dec. 21, 2005
Completion of experiments	Dec. 21, 2005
Presentation of final report	Dec. 28, 2005
Completion of study	Dec. 28. 2005

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9. Personnel

Preparation of protocol, management of study, and preparation of final report	Mei-Shu Zheng
Evaluation of health condition for animal	Joong-Hyun Kim
Management, formulation and identity confirmation of test substance	Eun-Ae Kim, Jin-Ah Kim
Administration of test substance and observation	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee
Specimen preparation and observation	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee
Arrangement of raw data and statistics	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee

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QUALITY ASSURANCE RECORD AND AUTHENTICATION STATEMENT

Study Title : *In vivo* micronucleus test of [redacted] in mice
Study No. : B05512

This study was subject to audit by the independent Quality Assurance Unit of BIOTOXTECH as indicated below. The findings of each audit were reported to the Study Director and Management as prescribed by Standard Operating Procedures.

The final report audit was designed to confirm that as far as can be reasonably established the methods described and results incorporated in the final report accurately reflect the raw data produced during the study.

Audit phases and dates reported to the responsible personnel were as indicated below and these were based upon the audit records.

Audit type	Audit phase	Audit date	To Study Director	To Management
Study based audit	Protocol	Nov. 18, 2005	Nov. 18, 2005	Nov. 18, 2005
	Animal receipt	Nov. 30, 2005	Dec. 3, 2005	Dec. 3, 2005
	Quarantine	Dec. 7, 2005	Dec. 8, 2005	Dec. 8, 2005
	Group assignment	Dec. 7, 2005	Dec. 8, 2005	Dec. 8, 2005
	Animal identification	Dec. 7, 2005	Dec. 8, 2005	Dec. 8, 2005
	Storage and preparation of test substance	Dec. 7, 2005	Dec. 8, 2005	Dec. 8, 2005
	Administration	Dec. 7, 2005	Dec. 8, 2005	Dec. 8, 2005
	Clinical signs	Dec. 8, 2005	Dec. 9, 2005	Dec. 9, 2005
	Specimens preparation	Dec. 8, 2005	Dec. 9, 2005	Dec. 9, 2005
	Observation	Dec. 21, 2005	Dec. 21, 2005	Dec. 21, 2005
	Raw data	Dec. 27, 2005	Dec. 28, 2005	Dec. 28, 2005
	Final report (Draft)	Dec. 27, 2005	Dec. 28, 2005	Dec. 28, 2005
	Final report	Dec. 28, 2005	Dec. 28, 2005	Dec. 28, 2005

Biotoxtech Co., Ltd.
Director. Quality Assurance Young-Hyun Lee Dec. 28, 2005
Date

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1. SUMMARY

This study was designed to examine a mutagenic potential of [redacted] in the micronucleus test system using bone marrow cells in mice.

In the main test, 8 week-old 6 male mice of per group were used, and three dose levels such as 500, 1000 and 2000 mg/kg for the low dose, middle dose and high dose, respectively of the test substance were administrated once by gavage in accordance with preliminary test. The negative control (0.5% MC 1500) is administrated once by gavage and the positive control (MMC, 2mg/kg) is administrated once intraperitoneally.

In 24 hours after the administration, all mice were sacrificed and bone marrow cells in thigh were collected. The specimens of bone marrow cells were stained with 5% Giemsa solution and observed by a microscope.

An appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) was significantly increased in the dosage of 2000 mg/kg as compared with that of the control group, but an appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) for other treatment groups was significantly different as compared with that of the negative control. The proportions of [PCE/(PCE+NCE)] were not significantly different between administration groups and control group.

In the positive control group, an appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) was significantly increased as compared with that of the negative control group.

In conclusion, the test substance, [redacted] did influence on the micronucleus formation in the bone marrow cells in mice under the condition of this study.

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II. PURPOSE

The objective of this study was to examine a mutagenic potential of [redacted] in the micronucleus test system using bone marrow cells in mice.

III. MATERIALS AND METHODS

1. Test substance

(1)	Name	[redacted]
(2)	Lot No.	072805-2
(3)	Appearance	Off white solid
(4)	Degree of Purity	>99 %
(5)	Molecular formula	[redacted]
(6)	Solubility in water	Hydrophobic
(7)	Degree of storage	Room temperature
(8)	Conditions of storage	Store in dry place (Avoid light).
(9)	Handling & notice	Do not breathe and ingest of test substance. Avoid contact with eyes and skin
(10)	Supplier	
	Name	[redacted]
	Address	[redacted]
(11)	Residue treatment	After the study, any remaining test substance was will be discarded.
(12)	Certificate of analysis	Provided by the sponsor (Appendix 1).

2. Control

(1) Negative control

0.5% Methyl cellulose 1500 (MC 1500, Lot No.: ASQ6053, Wako, Japan)

(2) Positive control

Mitomycin C (MMC, Lot No.: 103K0496, Sigma, USA)

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3. Preparation and analysis of test substance

(1) Vehicle and reason for selection

0.5% MC1500 was used as a vehicle, since the test substance was suspended through the pre-preparation of the test substance, based on the information of test substance.

(2) Preparation of test substance

The test substance was weighed using a semi-micro electronic balance (BP410S, BP310S, Sartorius, Germany) and suspended with 0.5% MC1500. Then it is further diluted by the fixed common ratio for lower doses. The test substance was prepared just before the administration.

(3) Formulation stability, homogeneity and concentration analysis

① Stability	Generation of heat, coloring and foaming were not observed.
② Homogeneity	The test substance was homogeneously dissolved.
③ Concentration analysis	Not done.

4. Preparation of control

(1) Negative control

0.5% MC 1500 was used as a negative control, which was prepared that 0.5 g of MC 1500 was dissolved in 100 mL of distilled water.

(2) Positive control

MMC was dissolved into water for injection (Lot No.: AAW5AL, Choongwae Pharma Corp. Korea)

5. Test system

(1) Species, family	Mouse, Crljbgi:CD1(ICR)
(2) Supplier and Producer	
Name	ORIENTBIO INC.
Address	143-1 Sangdaewon-dong, Jungwon-gu, Sungnam-si, Gyunggi-do, Korea

(3) Justification of test system

The ICR mouse was selected because it was widely used in toxicity studies and plenty of historical background data were accumulated.

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(4) Sex and Number of animals

1) Preliminary test and Induction of micronucleus : 27 mice, male

2) Main test : 33 mice, male

(5) Age and body weight range (receipt)

7 week-old, 28.12 ~ 32.94g

(6) Animal receipt and Labeling

When animals were received, they were weighed using the animal balance (BP3100S, Sartorius, Germany). Their tails were labeled using an oil based pen in accordance with the identification method (SOP/SGE/110 and140).

(7) Quarantine and acclimation

Animals were quarantined and acclimatized for 8 days and general signs were observed once a day. At the time of completion of the acclimation period, the body weights were measured using the animal balance (BP3100S, Sartorius, Germany) to select healthy animals suitable for the test.

(8) Group assignment

After the quarantine and acclimatization, 15, 9 and 30 animals for preliminary test, induction of micronucleus and main test respectively were selected. They were normal in body weight and general signs.

(9) Disposition of remaining animals

Remaining animals will be euthanized or disposed (SOP/SGE/220).

(10) Animal identification

An identification card was attached on a cage. To identify among the animals, their tails will be labeled using an indelible pen in accordance with the method of identification (SOP/SGE/140).

6. Environmental conditions

(1) Room No. A333

(2) Type and size of a cage

Acclimatization period : Polycarbonate cage (260W × 420D × 180H (mm), MJ. Ltd)

Testing period : Polycarbonate cage (200W × 260D × 130H (mm). MJ. Ltd)

(3) Number of animals in a cage

8~9 (Acclimatization period) / 5 (main test)

(4) Temperature 21.0 ~ 22.1 ℃

(5) Humidity 38.4 ~ 49.2 %

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(6) Frequency and method of ventilation

10~15 times/hr, All fresh air method

(7) Lighting — 12 hours per day (light on: 7 a.m., light off: 7 p.m.).

(8) Intensity of illumination — 150 ~ 300 Lux

(9) Cleansing. sterilization and frequency of breeding materials and animal room

1) Cage, diet tub and water bottles

A Cage, diet tub and water bottle were replaced 1 time/week. Ultra-Clean Neighbour-PC (alkali surface active agent, NE-SARANG Co., Ltd.) and Ultra-Clean Neighbour-RM300 (surface active agent, NE-SARANG Co., Ltd.) were used for washing; and high pressure steam was used for sterilization.

2) Sterilization of the animal room

After daily work, Pacom A (the principal element: 200 fold Octyldecyl dimethyl ammonium chloride, SAMU MEDIAN CO., Ltd), Mikro-Quat (the principle element: 400 fold Alkyldimethylbenzyl ammonium chloride, Ecolab), and Bromo-sept (the principal element: 300 fold Di-decyl di-methyl ammonium bromide, DAE SUNG MICROBIOLOGICAL LABS. CO., Ltd) solution were used to disinfect the test room. Each antiseptic solution was replaced every 10 days.

7. *Diet*

(1) Type

TEKLAD CERTIFIED GLOBAL 18% PROTEIN RODENT DIET 2918C

(Lot No.: 2918C 091305MA)

(2) Producer

Name	Harlan TEKLAD
Address	Madison, Wisconsin 53744-4220, USA

(3) Method of supply and amount of diets

Experimental diet was provided *ad libitum* throughout the acclimation period and during the study.

(4) Analysis of compound and contaminant in diet

The diet was analyzed periodically by manufacturer for nutrient components and environmental contaminants. Upon receipt of the certificate from a manufacturer, the contaminants in the diet were checked 2 times/year in Korea Testing and Research Institute for Chemical Industry (539-3, Pyeongchon-dong. Daedeok-gu. Daejeon, Korea). These results of all analytical items were confirmed within the range of the contaminant limit set in our facility (SOP/ANC/230).

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8. Water

(1) Type

Purified water using a filter and sterilizer was provided.

(2) Method of water supply

Purified water in a polycarbonate bottle (500 mL) was freely supplied.

(3) Analysis and validation of compound and contaminant in water

Chungcheongbuk-do Institute of Health and Environment Research (140-50 Songjeong-Dong, Cheongju-si Chungcheongbuk- Do Korea) analyzed the supplied water in our test facility in accordance with a Regulation of Quality Criteria for Potable Water and Test」 (Ministry of Environment Ordinance No. 122, 2002) on 2 times/year. The results were confirmed within the range of the contaminant limit set by the Korea Ministry of Environment (SOP/ANC/250).

9. Administration

(1) Route

Oral administration and Intraperitoneal administration

(2) Reason for selection

According to the appearance of the test substance, the administration route was chosen.

(3) Method of administration

The test substance and negative control were administrated once by gavage and the positive control was administrated intraperitoneally.

10. Test group

	Group	Dose (mg/kg)	Route	Dosing volume (mL/kg)	Administration times	Animal No. (Identification No.)
G1	Negative Control	0	P.O.	10	1	6 (1M01-1M06)
G2	Low	500	P.O.	10	1	6 (2M07-2M12)
G3	Middle	1000	P.O.	10	1	6 (3M13-3M18)
G4	High	2000	P.O.	10	1	6 (4M19-4M24)
G5	Positive control	2	I.P.	10	1	6 (5M25-5M30)

The preliminary test was conducted in 5 dose levels (125, 250, 500, 1000, and 2000

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mg/kg). There were no dead animal and no clinical signs in the treatment groups. The main test was conducted in 3 treatment groups containing 2000 mg/kg as a high dose and 2 additional lower dose levels such as 1000, 500 mg/kg, negative control and positive control. The dosing volume was 10 mL/kg.

11. Observation and examination

(1) Clinical signs

All animals were observed once a day for appearance, moribund and mortality.

(2) Body weight

Individual body weights were recorded just before the administration and before the day for the bone marrows cells collection.

12. Induction of micronucleus

2000 mg/kg, high dose level, which was selected in the preliminary test, was administered into animals to establish the collection time of bone marrow cells. Then a micronucleus formation was observed in 24, 48 and 72 hours after the administration.

13. Collection of bone marrow cells and preparation of micronucleus slides

Bone marrow cells collection was carried out at the time decided in the induction of micronucleus, 24 hours. To collect the bone marrow cells, mice were euthanized by dislocation of cervical vertebrae, after that the 200 μL of fetal bovine serum (Hyclone, USA) was run through an extracted femur using a 1 mL disposable syringe with a needle and collected. Finally, collected samples were centrifuged at 1000 rpm, 4 ℃, for 5 min. The supernatant was discarded and the cell pellet is mixed well and then smeared on the slide glass. Two specimens per animal are produced, which were air-dried and stained with a 5 % Giemsa solution for 30. They were observed under 1000 fold magnification of the microscope(BX51, OLYMPUS, Japan).

14. Result analysis

(1) Observation of specimens

To calculate the proportion of PCE in the total erythrocytes [PCE/(PCE+NCE)], sum of polychromatic erythrocyte (PCE) and normochromatic erythrocyte (NCE) should be 500; and to calculate the proportion of MNPCE in the micronucleated polychromatic erythrocyte [MNPCE/(2000PCE)], polychromatic erythrocyte (PCE) were counted 2000.

(2) Judgment criteria

It was judged as a positive when MNPCE was increased significantly in dose-dependent manner or there was reproducible increase in number of MNPCE at

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least one test level.

15. Statistics analysis

In accordance with the table of Kastenbaum&Bowman[2)], statistical significance was tested for the induction frequency of micronucleus and the t-test was conducted for an appearance rate for polychromatic erythrocyte (PCE) in 5 % significant level in p-value statistically. We conducted the ANOVA test for a body weight change within 5 % significant level in p-value statistically.

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IV. RESULTS

1. Clinical signs and mortality

There were no treatment related mortality and clinical signs for all groups. No dead animals were found in all groups.

2. Measurement of body weight (Table1)

There was no significant body weight change for all test groups during the test period.

3. *In vivo* micronucleus test (Table 2, 3)

The preliminary test was performed to establish a collection time of bone marrow cells, in which MNPCE were increased at 24 hours after the administration of the test substance; therefore a collection time of bone marrow cells was decided in 24 hour.

The frequency of micronucleated polychromatic erythrocytes (MNPCE) induction per 12000 polychromatic erythrocytes (PCE) was 6, 10, 14 and 33 for negative control group. 500, 1000 and 2000 mg/kg of treatment groups, respectively. Therefore, an appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) was significantly increased in the dosage of 2000 mg/kg as compared with that of the control group, but appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) for other was not significantly different as compared with that of the control group. In the positive control group, the frequency of micronucleated polychromatic erythrocytes (MNPCE) induction per 12000 polychromatic erythrocytes (PCE) was 991; and an appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) was significantly increased in contrast with that of the negative control.

The PCE/(PCE+NCE) ratio, criterion of cytotoxicity, for the following dose levels 0(negative control), 500, 1000, 2000 mg/kg and positive control (MMC) were 0.483, 0.479, 0.462, 0.454 and 0.473 respectively. There was no significant different between the treatment and control groups.

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V. DISCUSSIONS AND CONCLUSIONS

This study was designed to determine the mutagenic potential of [redacted] in the micronucleus test using bone marrow cells in ICR mice.

The serial doses of the test substance, [redacted], such as 500, 1000 and 2000 mg/kg were administrated by gavage. 10 mL/kg of water for injection was also administrated by gavage to negative control group and 2 mg/kg of Mitomycin C was administrated intraperitoneally to positive control group. In 24 hours after the treatment, all mice were sacrificed then bone marrow cells in thigh were collected. The specimens were stained with 5% Giemsa solution. The frequency of micronucleus induction was observed by a microscope.

An appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) was significantly increased in the dosage of 2000 mg/kg as compared with that of the control group, but appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) for other was not significantly different as compared with that of the control group.

However, in positive control group, an appearance rate of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) was increased significantly in contrast with that of the negative control group.

The PCE/(PCE+NCE) ratio for all treatment groups was not different significantly in contrast with that of the negative control.

In conclusion, the test substance, [redacted], did influence the micronucleus formation in the bone marrow cells in mice.

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VI. REFERENCES

1) Makoto Hayashi (1999) : The micronucleus test, Scientist Inc. Monograph series No. 2

2) Kastenbaum M.A. and Bowman K.O. (1970) : Tables for determining the statistical significance of mutation frequencies, Mutation Research, 9, 527- 549

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TABLES

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Table 1. Body weight *in vivo* micronucleus test with [redacted] in male ICR mice

Groups	Test substance	Dose (mg/kg)	Route	Sampling time (hrs)	Animal I.D.	Body weight (g) at the time of	
						Administration	Sacrifice
G1	0.5% MC1500	0	P.O.	24	1M01	31.91	32.14
					1M02	33.44	32.24
					1M03	33.59	33.46
					1M04	34.07	34.05
					1M05	34.75	34.59
					1M06	35.84	35.19
					Mean±S.D.	33.93 ± 1.32	33.61 ± 1.24
G2	[redacted]	500	P.O.	24	2M07	31.97	32.64
					2M08	33.41	33.33
					2M09	33.69	33.32
					2M10	34.02	34.33
					2M11	34.89	34.40
					2M12	35.84	35.64
					Mean±S.D.	33.97 ± 1.32	33.94 ± 1.07
G3	[redacted]	1000	P.O.	24	3M13	32.23	33.07
					3M14	33.33	31.88
					3M15	33.70	34.01
					3M16	34.00	34.75
					3M17	34.94	35.34
					3M18	35.82	36.09
					Mean±S.D.	34.00 ± 1.25	34.19 ± 1.54
G4	[redacted]	2000	P.O.	24	4M19	32.65	32.99
					4M20	33.27	34.86
					4M21	33.72	34.10
					4M22	33.95	34.11
					4M23	34.97	34.95
					4M24	35.61	36.22
					Mean±S.D.	34.03 ± 1.09	34.54 ± 1.08
G5	MMC	2	I.P.	24	5M25	32.91	33.11
					5M26	33.01	33.02
					5M27	33.73	33.97
					5M28	33.86	34.88
					5M29	35.29	34.79
					5M30	35.48	34.71
					Mean±S.D.	34.05 ± 1.10	34.08 ± 0.85

MMC : Mitomycin C
0.5% MC 1500 : 0.5% Methyl celluose 1500

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Table 2. Result of preliminary micronucleus test with [redacted] in male ICR mice

Groups	Test substance	Dose (mg/kg)	Route	Sampling time (hrs)	Animal ID	MNPCE/ PCE
BG1	[redacted]	2000	P.O.	24	BM01	6 / 2000
					BM02	4 / 2000
					BM03	4 / 2000
					Total	14 / 6000
					Mean±S.D.	4.7 ± 1.2
BG2	[redacted]	2000	P.O.	48	BM04	3 / 2000
					BM05	2 / 2000
					BM06	1 / 2000
					Total	6 / 6000
					Mean±S.D.	2.0 ± 1.0
BG3	[redacted]	2000	P.O.	72	BM07	0 / 2000
					BM08	1 / 2000
					BM09	2 / 2000
					Total	3 / 6000
					Mean±S.D.	1.0 ± 1.0

MNPCE : Micronucleated polychromatic erythrocyte
PCE : Polychromatic erythrocyte

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Table 3. Result of *in vivo* micronucleus test with [redacted] in male ICR mice

Groups	Test substance	Dose (mg/kg)	Sampling time(hrs)	Animal I.D.	Counted PCE : NCE	PCE/ (PCE+NCE)	MNPCE/ PCE
G1	0.5% MC1500	0	24	1M01	236 : 264	0.472	0 / 2000
				1M02	224 : 276	0.448	1 / 2000
				1M03	237 : 263	0.474	2 / 2000
				1M04	246 : 254	0.492	1 / 2000
				1M05	229 271	0.458	1 / 2000
				1M06	278 : 222	0.556	1 / 2000
				Total	- : -	-	6 / 12000
				Mean±S.D.	- : -	0.483 ± 0.039	1.0 ± 0.6
G2	[redacted]	500	24	2M07	228 : 272	0.456	1 / 2000
				2M08	265 : 235	0.530	3 / 2000
				2M09	237 : 263	0.474	2 / 2000
				2M10	242 : 258	0.484	0 / 2000
				2M11	230 : 270	0.460	2 / 2000
				2M12	234 : 266	0.468	2 / 2000
				Total	- : -	-	10 / 12000
				Mean±S.D.	- : -	0.479 ± 0.027	1.7 ± 1.0
G3	[redacted]	1000	24	3M13	242 : 258	0.484	2 / 2000
				3M14	239 : 261	0.478	1 / 2000
				3M15	244 : 256	0.488	3 / 2000
				3M16	233 : 267	0.466	4 / 2000
				3M17	219 : 281	0.438	3 / 2000
				3M18	208 : 292	0.416	1 / 2000
				Total	- : -	-	14 / 12000
				Mean±S.D.	- : -	0.462 ± 0.029	2.3 ± 1.2
G4	[redacted]	2000	24	4M19	230 : 270	0.460	6 / 2000
				4M20	205 : 295	0.410	7 / 2000
				4M21	209 : 291	0.418	4 / 2000
				4M22	226 : 274	0.452	8 / 2000
				4M23	242 : 258	0.484	4 / 2000
				4M24	249 : 251	0.498	4 / 2000
				Total	- : -	-	33* / 12000
				Mean±S.D.	- : -	0.454 ± 0.035	5.5 ± 1.8
G5	MMC	2	24	5M25	252 : 248	0.504	146 / 2000
				5M26	205 : 295	0.410	200 / 2000
				5M27	234 : 266	0.468	153 / 2000
				5M28	219 : 281	0.438	161 / 2000
				5M29	235 : 265	0.470	196 / 2000
				5M30	274 : 226	0.548	135 / 2000
				Total	- : -	-	991 / 12000
				Mean±S.D.	- : -	0.473 ± 0.049	165.2 ± 26.9

MNPCE : Micronucleated polychromatic erythrocyte
PCE : Polychromatic erythrocyte
NCE : Normochromatic erythrocyte
MMC : Mitomycin C
0.5% MC 1500 : 0.5% Methyl celluose 1500
* :Significantly different with vehicle (P<0.05, Kastenbaum & Bowman test)

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APPENDIX

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Appendix 1. Certificate of analysis

CERTIFICATE OF ANALYSIS

1. TEST MATERIAL IDENTIFICATION

(1) TEST MATERIAL (LOT No.) : [redacted]
(2) QUANTITY : 20 g
(3) PRODUCER : [redacted]

(4) CHEMICAL NAME (Generic name). :
PNBT

(5) PURITY : >99%
(6) APPEARANCE : Off white solid
(7) MOLECULAR FORMULA : [redacted]

2. SOLUBILITY

(1) INSOLUBLE : Water
(2) SOLUBLE : No information

3. STORAGE

Room temperature

We hereby certify that the data stated here above are true and correct.

Oct. 2005

[redacted]

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Biotoxtech

FINAL REPORT

Bacterial reverse mutation test of [redacted]

Study No.: B05482

Biotoxtech Co., Ltd.
58-1Block Ochang Scientific Industrial Complex, Ochang-myeon,
Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

294800

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CERTIFICATE OF TRANSLATION

This report is translated from Korean into English as an original contents, therefore, is not assigned on the relevant pages. This report is partially modified the Korean report for an expression of exact meaning but it did not influence on the original copy.

Translated by _____ Date Dec. 21. 2005

Hye-Jin Lee
Biotoxtech Co., Ltd.

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PREPARATION OF FINAL REPORT

Study Title : Bacterial reverse mutation study of [redacted]
Study No. : B05482

This study also was performed in accordance with the agreed protocol and the study objectives were achieved in compliance with Standard Operating Procedures (SOPs) of Biotoxtech Co., Ltd. and the following Good Laboratory Practice and test guidelines.

- Good Laboratory Practice
  - TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998)

- Guideline
  - The Section 10 of Chapter 4 in Annex 5, in compliance with TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998) 'Bacterial Reverse Mutation Test'

The study described in this report was performed under the supervision of the Study Director and this report was written based on the raw data obtained from the study.

Mei-Shu Zheng	Nov. 21, 2005
Biotoxtech Co., Ltd. Study Director	Date
Byeong-Kyu Lee	Nov. 22, 2005
Biotoxtech Co., Ltd. Testing Facility Management	Date

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OUTLINE OF STUDY

Study Title : Bacterial reverse mutation study of [redacted] -

Study No. : B05482

1. Purpose

The objective of this study was to determine a mutagenic potential of [redacted] by histidine-requiring bacterial strain, *Salmonella typhimurium* and tryptophan-requiring bacterial strain, *Escherichia coli*.

2. GLP

TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998)

3. Guideline

The Section 10 of Chapter 4 in Annex 5, in compliance with TCCA(Toxic Chemical Control Act)-Good Laboratory Practice described in Annex 1 of TCCA-Good Laboratory Practice Standards and Test Guidelines (NIER Public Notice No. 1998-41, Korean National Institute of Environmental Research, December 23, 1998) 'Bacterial Reverse Mutation Test'

4. Sponsor

Name [redacted]

Address [redacted]

5. Test management

Name Biotoxtech Co., Ltd.

Address 58-1Block Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

6. Test facility

Name Biotoxtech Co., Ltd.

Address 58-1Block Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

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7. Record and archives of data

(1) Duration : 5 years after the completion of test

(2) Record and data :

Study related protocol, raw data, final report (draft and original) and documents of GLP and all correspondences.

(3) Storage place and address :

Place : Archives of Biotoxtech Co., Ltd.

Address : 58-1Block Ochang Scientific Industrial Complex, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, 363-883 Korea

8. Schedule

Initiation of study	Oct. 17, 2005
Initiation of experiments	Oct. 27, 2005
Test substance treatment	Oct. 27, 2005, Nov. 2, 2005
Colony counting	Oct. 29, 2005, Nov. 4, 2005
Completion of experiments	Nov. 4, 2005
Presentation of final report	Dec.10, 2005
Completion of study	Dec.10, 2005

9. Responsible personnel

Preparation of protocol, management of study, and preparation of final report

Mei-Shu Zheng

Strains maintenance	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee

Identity confirmation and formulation of test substance

Eun-Ae Kim, Jin-Ah kim

Treatment of test substance	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee
Colony counting	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee
Arrangement of raw data & evaluation	Mi-Young Lee, Hye-Jin Lee, Ji-Eun Lee

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QUALITY ASSURANCE RECORD AND AUTHENTICATION STATEMENT

Study Title : Bacterial reverse mutation study of [redacted]

Study No. : B05482

This study was subject to audit by the independent Quality Assurance Unit of BIOTOXTECH as indicated below. The findings of each audit were reported to the Study Director and Management as prescribed by Standard Operating Procedures.

The final report audit was designed to confirm that as far as can be reasonably established the methods described and results incorporated in the final report accurately reflect the raw data produced during the study.

Audit phases and dates reported to the responsible personnel were as indicated below and these were based upon the audit records.

Audit type	Audit phase	Audit date	To Study Director	To Management
Study based audit	Protocol	Oct. 17, 2005	Oct. 17, 2005	Oct. 17, 2005
	Preliminary dose range finding test			
	Treatment	Oct. 27, 2005	Oct. 29, 2005	Oct. 29, 2005
	Colony counting	Oct. 29, 2005	Oct. 29, 2005	Oct. 29, 2005
	Main test			
	Pre-incubation	Nov. 1, 2005	Nov. 2, 2005	Nov. 2, 2005
	Storage and preparation of test substance	Nov. 2, 2005	Nov. 2, 2005	Nov. 2, 2005
	Treatment	Nov. 2, 2005	Nov. 2, 2005	Nov. 2, 2005
	Colony counting	Nov. 4, 2005	Nov. 4, 2005	Nov. 4, 2005
	Record & Raw data	Nov.21, 2005	Nov.21, 2005	Nov.21, 2005
	Final report (Draft)	Nov.21, 2005	Nov.21, 2005	Nov.21, 2005
	Final report	Nov.21, 2005	Nov.21, 2005	Nov.21, 2005

Biotoxtech Co., Ltd.
Director, Quality Assurance Young-Hyun Lee Nov. 21, 2005
Date

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Figure 3. Dose-response curve of revertant colonies by [redacted] in differential bacterial strains [with S9 mix][TA98, TA100, WP2uvrA(pKM101)] ... 14

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I. SUMMARY

This study was designed to examine the mutagenic potential of [redacted] in the bacterial reverse mutation study without and with metabolic activation system (S9 mix) using *Salmonella typhimurium* strains (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2uvrA (pKM101)).

According to the preliminary dose range-finding test, the number of revertant colonies in the test strains was not reduced at all dose levels without and with metabolic activation system. In addition, a deposition was observed at 1250, 2500 and 5000 μg/plate without metabolic activation system and was also observed at 2500 and 5000 μg/plate with metabolic activation system but those were not influenced on the number of colonies. As a result, the main test was carried out with 5 dose levels (312.5, 625, 1250, 2500 and 5000 μg/plate) for all test strains accompanying with negative and positive controls.

According to the main test, the numbers of colonies with reference to the bacterial reverse mutation in the test strains was increased dose-dependently. The number of revertant colonies in TA98 strain at all does levels was increased more than two-fold as compared with that of the negative control group regardless of application of metabolic activation system. The number of revertant colonies in WP2uvrA (pKM101) strain at all does levels was increased more than two-fold as compared with that of the negative control group with metabolic activation system. In TA100 strain, a number of revertant colonies all dose levels except 2500 and 5000 μg/plate without metabolic activation system, and 312.5 μg/plate with metabolic activation were increased more than two-fold as compared with that of the negative control group. The number of revertant colonies at 2500 and 5000 μg/plate without and with metabolic activation system in TA1535 strain; and at 5000 μg/plate without metabolic activation system and at 1250, 2500 and 5000 μg/plate with metabolic activation system in TA1537 strain was increased more than two-fold as compared with that of the negative control group. In addition, the number of revertant colonies in the test strains was not reduced at all dose levels without and with metabolic activation system. A deposition in relation to the test substance was observed at 1250, 2500 and 5000 μg/plate without metabolic activation system, and at 2500 and 5000 μg/plate with metabolic activation system but no influence on the counting of colonies.

In conclusion, the test substance, [redacted], showed the mutagenic potential under the conditions of this study.

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II. PURPOSE

The objective of this study was to determine a mutagenic potential of [redacted] by histidine-requiring bacterial strain, *Salmonella typhimurium* and tryptophan-requiring bacterial strain, *Escherichia coli*.

III. TEST MATERIALS AND METHODS

1. Test substance

(1)	Name	[redacted]
(2)	Lot No.	072805-2
(3)	Appearance	Off white solid
(4)	Degree of Purity	>99 %
(5)	Molecular formula	[redacted]
(6)	Solubility in water	Hydrophobic
(7)	Degree of storage	Room temperature
(8)	Conditions of storage	Store in dry place (Avoid light).
(9)	Handling & notice	Do not breathe and ingest of test substance. Avoid contact with eyes and skin
(10)	Supplier	
	Name	[redacted]
	Address	[redacted], [redacted]
(11)	Residue treatment	After the study, any remaining test substance will be discarded.
(12)	Certificate of analysis	Provided by the sponsor (Appendix 1).

2. Control

(1) Negative control

Dimethyl sulfoxide (DMSO, Lot No.: K32997731 413, Merck, USA)

(2) Positive control

2-NF : 2-Nitrofluorene (Lot No.: DA09213BA, Aldrich, USA)
SA : Sodium azide (Lot No.: 121K0047, Sigma, USA)
2-AA : 2-Aminoanthracene (Lot No.: 20505HA, Aldrich, USA)

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9-AA : 9-Aminoacridine (Lot No.: 106F06681, Sigma, USA)

4-NQO : 4-nitroquinoline 1-oxide (Lot No.: 101K2191, Sigma, USA)

3. Preparation of test substance and analysis

(1) Vehicle and reason for selection

DMSO was used as a vehicle, since the test substance was dissolved through the pre-preparation of the test substance based on the information of test substance.

(2) Preparation of test substance

In the study, the test substance was weighed using the semi-micro electronic balance (BP310S, Sartorius, Germany) and dissolved with DMSO to prepare the highest concentration. Then it was further diluted with DMSO for lower concentrations.

(3) Formulation stability, homogeneity and concentration analysis

① Stability	Generation of heat, coloring and foaming were not observed.
② Homogeneity	The test substance was homogeneously dissolved.
③ Concentration analysis	Not done.

4. Preparation of control

(1) Negative control

DMSO was used as a negative control.

(2) Positive control

SA was dissolved in a water for injection (Lot No.: AAW5AF, Choongwae Pharma Corp., Korea); and 2-NF, 2-AA, 9-AA and 4-NQO were dissolved in a Dimethyl Sulfoxide (DMSO, Lot No.: K32960231 504, Merck, USA). They were kept at -80℃ (DFU-657CL, Operon, Korea) and thawed out immediately when treated.

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5. Test strain

(1) Strain, reason for selection and storage

We used below strains, which had following characteristics;

	Amino acid requiring [a]	rfa mutation [b]	Ampicillin resistance test [c]	UV sensitivity [d]
Salmonella typhimurium				
TA98	his−	rfa	+pKM101	uvrB
TA100	his−	rfa	+pKM101	uvrB
TA1535	his−	rfa	-	uvrB
TA1537	his−	rfa	-	uvrB
Escherichia coli				
WP2uvrA(pKM101)	trp−	wild	+pKM101	uvrA

a : Indicate a gene of a histidine-requiring bacterial strain (his−) and a gene of a tryptophan-requiring bacterial strain (trp−).

b : The rfa had a mutation characteristic, in which indicated a crystal violet excision sensitivity.

c : +pKM101 had a chemical resistance plasmid, in which indicated ampicillin resistance.

d : uvrA, and uvrB indicated both excision of DNA repair gene and UV deletion sensitivity.

The test strains are the most useful for a bacterial mutagenicity study since they have a high sensitivity for a mutagen. Both *Salmonella typhimurium* and *Escherichia coli* were purchased from Molecular Toxicology, Inc. (address: 157 Industrial Park Drive, P.O. Box 1189 Boone, NC28607, USA / Aug. 25, 2004. After that, the rate of each 8 mL of cultivated strain for 8 hours to 0.7 mL of DMSO were quickly frozen and kept at −80℃(DFU-657CL, Operon, Korea). In addition, a suitable route of preservation for each strain in reference to their genetic characteristics was used.

(2) Pre-incubation

Each strain was inoculated with a sterilized condition into 15 mL of liquid culture medium. They were cultured in a shaking state overnight using shaking water bath for pre-incubation. After the pre-incubation, the absorbance of a suspension for each strain was measured to examine the number of strains using the UV spectrophotometer (660 nm, V-550, Jasco, Japan). The number of strains above 1.0 x 10^9 cells/mL was used in the test.

6. Medium

(1) Nutrient broth medium

A nutrient broth No.2 (Lot No.: 298714, Oxoid, UK) was dissolved in a distilled

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water to prepare 2.5% of the final concentration and sterilized by an autoclave.

(2) Minimum glucose agar plate medium

In an erlenmeyer flask, 15 g of bacto Agar (Lot No.: 4149282, BD, USA) and 800 mL of distilled water were mixed and sterilized using an autoclave. Then 100 mL of sterilized VB salt (10X) and 100 mL of 20% glucose were added, and dispensed into a petri dish.

(3) Identification

Marking a treatment group and S9 treatment using oil based pen.

(4) Top agar

Top agar solution, 0.6 % bacto agar (Lot No.: 4149282, BD, USA) including 0.5 % NaCl, was prepared. In case of *Salmonella typhimurium*, the ratio of 10 to 1 for the top agar solution and 0.5 mmol/L-Histidine (Lot No.: 126H1093, Sigma, USA)-0.5 mmol/L D-biotin (Lot No.: 1256A99, Amresco, USA) solution, respectively, were mixed. In case of *Escherichia coli*, the ratio of 10 to 1 for the top agar solution and 0.5 mmol/L L-Tryptophan (Lot No.: J14575, Research Organics Inc, USA) solution were mixed, respectively.

7. Metabolic activation system

(1) Source and storage of S9

S9 (Lot No.: 1883) originated from hormone-induced liver homogenized solution of Sprague-Dawley rat using Aroclor 1254, in which was purchased from Molecular Toxicology, Inc., was used. it was kept in -80 ℃(DFU-657CL, Operon, Korea) till using.

(2) Composition and preparation of S9 mix

Component	Formation	Amount
Homogenous liver fraction of rat (S9)	10%	0.1 mL
$MgCl_2$	8.0 μmol	0.02 mL
KCl	33.0 μmol	
Glucose-6-phosphate	5.0 μmol	0.005 mL
NADP	4.0 μmol	0.04 mL
Na-phosphate buffer, pH 7.4	100.0 μmol	0.5 mL
Water for injection	-	0.335 mL
Total volume		1.0 mL

It was prepared as follows; 1.0 mol/L Glucose-6-phosphate (Lot No.: 052K7043,

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Sigma, USA) and 0.1 mol/L NADP(Lot No.: 054K7036, Sigma. USA) were dissolved in water for injection(Lot No.: AAW5AF, Choongwae Pharma Corp., Korea) and mix solution of 0.4 mol/L $MgCl_2$ and 1.65 mol/L KCl, then added 0.2 mol/L Na-phosphate buffer(pH 7.4). Finally they were filtered (0.22 μm, Millipore, USA) under the sterilized condition and thawed S9 was added. It was used in an ice-cooling condition.

8. Dose

A preliminary dose range-finding test would be conducted in 5 dose levels containing 5000 μg/mL as a highest dose, which are divided by common ratio 2 and negative control. According to a result, the number of revertant colonies was not reduced without and with metabolic activation system at all dose levels in the test strain. The main test would be conducted in 5 dose levels containing 5000 μg/mL as a highest dose, 2 additional dose levels which were divided by common ratio 2; and negative and positive control would be included in the main test.

9. Method

The preliminary dose range-finding test for a starting dose level and main test were carried out as a pre-incubation method[1, 2]. Both studies were carried out either without or with metabolic activation system.

10. Study management [1, 2]

(1) Method of treatment

100 μL of test solution (negative control, test substance, positive control), either 500 μL of 0.1 mol/L of Na-phosphate buffer (pH 7.4, without metabolic activation system) or 500 μL of S9 mix (with metabolic activation system), and 100 μL of suspension for cultured strains (1×10^9 cells/mL) were mixed in a dry heat sterilized glass tube (13 mm×100 mm). And this mixture was incubated in the shaking water bath at 37 ℃ for 20 minutes. After that, they were mixed and stirred with 2.0 mL of warmed top agar (45 ℃), and they were poured onto the minimal glucose agar plate and consolidated. The preliminary dose range-finding test for an anti-microbial action and dose finding study, 2 plates per dose were used and in the main test including the negative and positive controls, 2 plates per dose were used.

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(2) Positive control for each strain and the amount of used

S9 mix	Strain	Material	Dose (μg/plate)
	TA98	2-Nitrofluorene(2-NF)	5.0
	TA100	Sodium azide (SA)	1.5
−	TA1535	Sodium azide (SA)	1.5
	TA1537	9-Aminoacridine (9-AA)	80.0
	WP2uvrA(pKM101)	4-nitroquinoline 1-oxide (4-NQO)	5.0
	TA98	2-Aminoanthracene (2-AA)	1.0
	TA100	2-Aminoanthracene (2-AA)	1.0
+	TA1535	2-Aminoanthracene (2-AA)	2.0
	TA1537	2-Aminoanthracene (2-AA)	2.0
	WP2uvrA(pKM101)	2-Aminoanthracene (2-AA)	2.0

(3) Aseptic test

An aseptic test was carried out in relation to the test substance (the highest dose) and S9 mix.

(4) A method of culture and time assigned

After solidification of the top agar, the minimal glucose agar plate was turned over and cultured in the incubator at 37℃ for 48 hours. After that, it was refrigerated before counting the colony.

11. Observation and measurement

(1) Colony counting

After the incubation, an automatic colony counter (ProtoCOL, SYNBIOSIS, UK) was used for counting revertant colonies. If either the test substance is detected in a plate or cytotoxicity is observed, the automatic counting is not accurate; therefore, the counting is performed with the unaided eyes.

The number of revertant colony was expressed as a mean number for 2 plates/dose for the preliminary dose range-finding test and 2 plates/dose for the main test.

(2) Observation of background lawn

Background lawn was examined by the unaided eye.

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12. Results judgment

It was judged as a positive, if the number of colonies increased significantly at least in a tester strain in dose-dependently without and with S9 mix.

The judgment criteria of the cytotoxicity were that a background lawn of the test substance was done certainly or clearing comparing negative control group.

13. Statistical analysis

Any specific statistical analysis for the mean value was not performed in this test. The mean value and standard deviation was calculated in relation to the measured value of revertant colonies in the main test.

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IV. RESULTS

The number of revertant colonies and deposition of [redacted] was verified in the test strains. The preliminary dose range-finding test was conducted in 5 dose levels containing 5000 μg/plate as a highest dose, which are divided by common ratio 2 for all test strains accompanying with negative and positive controls.

According to the preliminary dose range-finding test, the number of revertant colony was not reduced without and with metabolic activation system at all dose levels in the test strains. A deposition in relation to the test substance was observed at 1250, 2500 and 5000 μg/plate without metabolic activation system, and at 2500 and 5000 μg/plate with metabolic activation system but no influence on the counting of colonies(Table 1).

As a result, the main test was conducted in 5 dose levels containing 5000 μg/plate as a highest dose, which are divided by common ratio 2 for all test strains accompanying with negative and positive controls.

According to the main test, the numbers of colonies with reference to the bacterial reverse mutation in the test strains was increased dose-dependently. The number of revertant colonies in TA98 strain at all does levels was increased more than two-fold as compared with that of the negative control group regardless of application of metabolic activation system. The number of revertant colonies in WP2uvrA (pKM101) strain at all does levels was increased more than two-fold as compared with that of the negative control group with metabolic activation system. In TA100 strain, a number of revertant colonies all dose levels except 2500 and 5000 μg/plate without metabolic activation system, and 312.5 μg/plate with metabolic activation were increased more than two-fold as compared with that of the negative control group. The number of revertant colonies at 2500 and 5000 μg/plate without and with metabolic activation system in TA1535 strain; and at 5000 μg/plate without metabolic activation system and at 1250, 2500 and 5000 μg/plate with metabolic activation system in TA1537 strain was increased more than two-fold as compared with that of the negative control group.

In addition, the number of revertant colonies in the test strains was not reduced at all dose levels without and with metabolic activation system. A deposition in relation to the test substance was observed at 1250, 2500 and 5000 μg/plate without metabolic activation system, and at 2500 and 5000 μg/plate with metabolic activation system but no influence on the counting of colonies (Fig. 1, 2, Table 2, 3).

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V. DISCUSSION AND CONCLUSION

The principle of this bacterial reverse mutation study is to detect chemicals that induce mutations in the tester strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria were detected by their ability to grow in the absence of the amino acid required by the parent tester strain.

This study was designed to examine the mutagenic potential of [redacted] in the bacterial reverse mutation study without and with metabolic activation system (S9 mix) using *Salmonella typhimurtum* strains (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2uvrA(pKM101)).

According to the preliminary dose range-finding test, the number of revertant colony was not reduced without and with metabolic activation system at all dose levels in the test strains. A deposition in relation to the test substance was observed at 1250, 2500 and 5000 μg/plate without metabolic activation system, and at 2500 and 5000 μg/plate with metabolic activation system but no influence on the counting of colonies. As a result, the main test was conducted in 5 dose levels containing 5000 μg/plate as a highest dose, which are divided by common ratio 2 for all test strains accompanying with negative and positive controls.

According to the main test, the numbers of colonies with reference to the bacterial reverse mutation in the test strains was increased dose-dependently. The number of revertant colonies in TA98 strain at all does levels was increased more than two-fold as compared with that of the negative control group regardless of application of metabolic activation system. The number of revertant colonies in WP2uvrA (pKM101) strain at all does levels was increased more than two-fold as compared with that of the negative control group with metabolic activation system. In TA100 strain, a number of revertant colonies of all dose levels except 2500 and 5000 μg/plate without metabolic activation system, and 312.5 μg/plate with metabolic activation were increased more than two-fold as compared with that of the negative control group. The number of revertant colonies at 2500 and 5000 μg/plate without and with metabolic activation system in TA1535 strain; and at 5000 μg/plate without metabolic activation system and at 1250, 2500 and 5000 μg/plate with metabolic activation system in TA1537 strain was increased more than two-fold as compared with that of the negative control group. In addition, the number of revertant colonies in the test strains was not reduced at all dose levels without and with metabolic activation system. A deposition in relation to the test substance was observed at 1250, 2500 and 5000 μg/plate without metabolic activation system and at 2500 and 5000 μg/plate with metabolic activation system but no influence on the counting of colonies.

The number of revertant colonies of the negative control and positive control was within the range of the standard value for the historical data in our laboratory. These results indicate that the test has been properly carried out.

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Also, the number of revertant colony was observed at least 4 does levels, the test was judged that it was conducted adequately. In addition, a contamination did not show in the aseptic test in the preliminary test and main test.

In conclusion, the test substance, [redacted], did show the mutagenic potential under the conditions of this study.

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VI. REFERENCES

1) Maron, D. M. and Ames, B.N. (1983): Revised methods for the Salmonella mutagenicity test, Mutation Research, 113, 173-215.

2) Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T. and Okada, M. (1977) : Mutagenicies of N-nitrosamines of Salmonella, Mutation Research, 48, 121-130.

3) Claxton L. D., Allen J., Auletta A., Mortelmams K., Nestmann E. and Zeiger E.(1987): Guide for the *Salmonella typhimurium*/mammalian microsome tests for bacterial mutagenicity. Mutation Research, Oct;189(2):83-91.

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FIGURES

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Figure 1. Dose-response curve of revertant colonies by [redacted] in differential bacterial strains [without S9 mix][TA98, TA100, WP2uvrA(pKM101)]

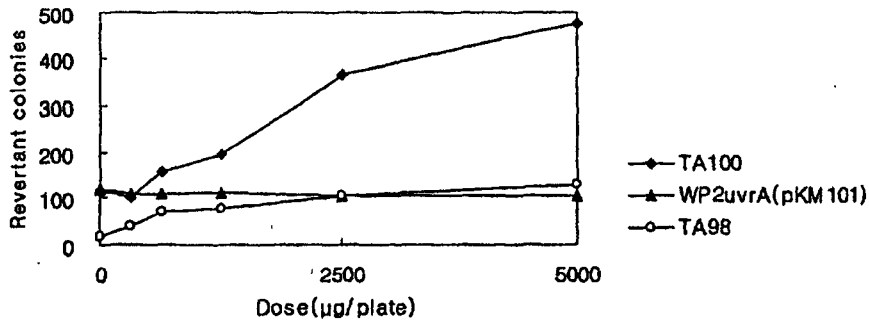

Figure 2. Dose-response curve of revertant colonies by [redacted] in differential bacterial strains [without S9 mix][TA1535, TA1537]

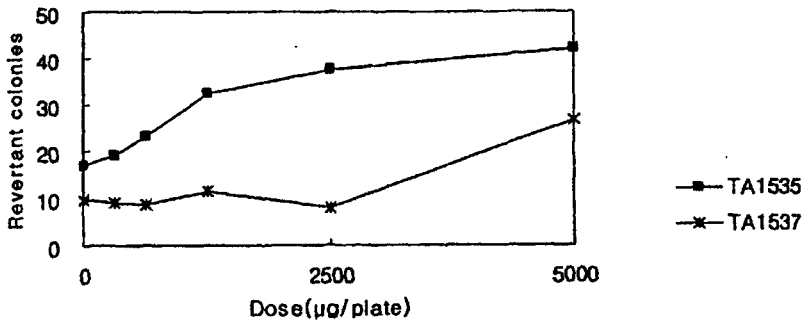

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Figure 3. Dose-response curve of revertant colonies by [redacted] in differential bacterial strains [with S9 mix][TA98, TA100, WP2uvrA(pKM101)]

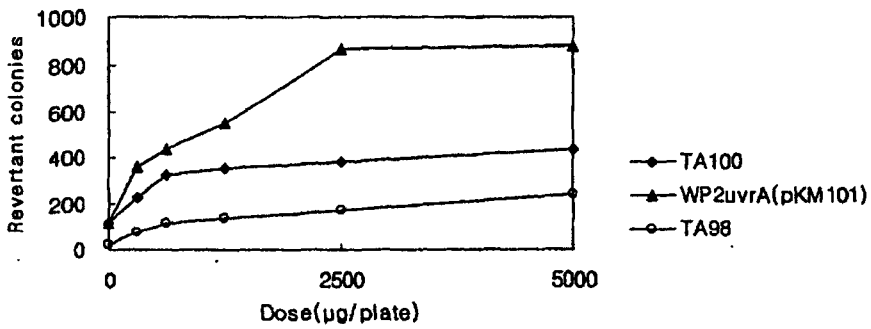

Figure 4. Dose-response curve of revertant colonies by [redacted] in differential bacterial strains [with S9 mix][TA1535, TA1537]

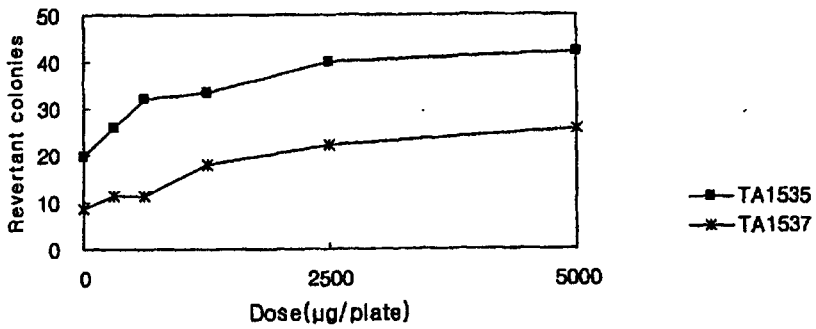

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TABLE

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Table 1. Summary data of preliminary dose range-finding test of [redacted] [without and with S9 mix]

Dose (μg/plate)	Base-pair substitution type			Frame-shift type	
	TA100	TA1535	WP2uvrA (pKM101)	TA98	TA1537
Number of revertant colonies per plate [N=2] (without S9 mix)					
0	116 , 117 [117 ± 1]	17 , 15 [16 ± 1]	120 , 119 [120 ± 1]	19 , 17 [18 ± 1]	9 , 7 [8 ± 1]
312.5	111 , 108 [110 ± 2]	20 , 19 [20 ± 1]	109 , 115 [112 ± 4]	44 , 38 [41 ± 4]	8 , 9 [9 ± 1]
625	169 , 146 [157 ± 17]	24 , 21 [23 ± 2]	108 , 106 [107 ± 1]	68 , 77 [73 ± 6]	9 , 8 [9 ± 1]
1250	261 , 294 [278 ± 23]†	31 , 38 [35 ± 5]†	105 , 117 [111 ± 8]†	88 , 78 [83 ± 7]†	10 , 10 [10 ± 0]†
2500	386 , 387 [386 ± 1]†	32 , 33 [33 ± 1]†	108 , 97 [103 ± 8]†	105 , 101 [103 ± 3]†	8 , 9 [9 ± 1]†
5000	454 , 576 [515 ± 86]†	42 , 47 [45 ± 4]†	111 , 101 [106 ± 7]†	120 , 143 [132 ± 16]†	28 , 21 [25 ± 5]†
Positive control	497 , 467 [482 ± 21]	435 , 436 [436 ± 1]	566 , 596 [581 ± 21]	442 , 408 [425 ± 24]	486 , 486 [486 ± 0]
Number of revertant colonies per plate [N=2] (with S9 mix)					
0	119 , 118 [119 ± 1]	20 , 18 [19 ± 1]	119 , 121 [120 ± 1]	18 , 21 [20 ± 2]	7 , 9 [8 ± 1]
312.5	272 , 254 [263 ± 13]	23 , 24 [24 ± 1]	372 , 387 [380 ± 11]	73 , 77 [75 ± 3]	11 , 10 [11 ± 1]
625	313 , 382 [348 ± 49]	32 , 32 [32 ± 0]	450 , 457 [454 ± 5]	122 , 105 [114 ± 12]	12 , 11 [12 ± 1]
1250	354 , 330 [342 ± 17]	31 , 35 [33 ± 3]	634 , 598 [616 ± 25]	157 , 148 [153 ± 12]	18 , 18 [18 ± 0]
2500	388 , 382 [385 ± 4]†	39 , 40 [40 ± 1]†	840 , 801 [821 ± 28]†	178 , 165 [172 ± 9]†	20 , 23 [22 ± 2]†
5000	447 , 430 [439 ± 12]†	40 , 42 [41 ± 1]†	831 , 885 [858 ± 38]†	244 , 215 [230 ± 21]†	26 , 25 [26 ± 1]†
Positive control	453 , 454 [454 ± 1]	144 , 113 [129 ± 22]	468 , 511 [490 ± 30]	519 , 520 [520 ± 1]	142 , 151 [147 ± 6]

Strain (without S9)	Positive control	Dose (μg/plate)
TA100	: Sodium azide (SA)	1.5
TA1535	: Sodium azide (SA)	1.5
WP2uvrA(pKM101)	: 4-nitroquinoline 1-oxide(4-NQO)	5.0
TA98	: 2-Nitrofluorene (2-NF)	5.0
TA1537	: 9-Aminoanthracene (9-AA)	80.0

Strain (with S9)	Positive control	Dose (μg/plate)
TA100	: 2-Aminoanthracene(2-AA)	1.0
TA1535	: 2-Aminoanthracene(2-AA)	2.0
WP2uvrA(pKM101)	: 2-Aminoanthracene(2-AA)	2.0
TA98	: 2-Aminoanthracene(2-AA)	1.0
TA1537	: 2-Aminoanthracene(2-AA)	2.0

N : Number of bacterial reverse examined

[ ] : Mean±S.D.

† : Deposition

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Table 2. Results of the bacterial reverse mutation test of [redacted] [without S9 mix]

Dose (μg/plate)	Number of revertant colonies per plate [N=2] (without S9 mix)				
	Base-pair substitution type			Frame-shift type	
	TA100	TA1535	WP2uvrA (pKM101)	TA98	TA1537
0	117 , 114 [116 ± 2]	17 , 17 [17 ± 0]	120 , 117 [119 ± 2]	18 , 17 [18 ± 1]	9 , 10 [10 ± 1]
312.5	105 , 100 [103 ± 4]	20 , 18 [19 ± 1]	112 , 108 [110 ± 3]	38 , 39 [39 ± 1]	10 , 8 [9 ± 1]
625	142 , 176 [159 ± 24]	23 , 23 [23 ± 0]	106 , 111 [109 ± 4]	70 , 72 [71 ± 1]	7 , 10 [9 ± 2]
1250	189 , 200 [195 ± 8]†	35 , 30 [33 ± 4]†	114 , 107 [111 ± 5]†	73 , 83 [78 ± 7]†	13 , 10 [12 ± 2]†
2500	381 , 345 [363 ± 25]†	37 , 38 [38 ± 1]†	102 , 105 [104 ± 2]†	108 , 102 [105 ± 4]†	9 , 7 [8 ± 1]†
5000	441 , 508 [475 ± 47]†	42 , 42 [42 ± 0]†	112 , 99 [106 ± 9]†	141 , 117 [129 ± 17]†	26 , 27 [27 ± 1]†
Positive control	436 , 499 [468 ± 45]	478 , 464 [471 ± 10]	589 , 564 [577 ± 18]	413 , 420 [417 ± 5]	444 , 483 [464 ± 28]

Strain	Positive control	Dose (μg/plate)
TA100	: Sodium azide (SA)	1.5
TA1535	: Sodium azide (SA)	1.5
WP2uvrA(pKM101)	: 4-nitroquinoline 1-oxide(4-NQO)	5.0
TA98	: 2-Nitrofluorene (2-NF)	5.0
TA1537	: 9-Aminoanthracene (9-AA)	80.0

N : Number of bacterial reverse examined

[] : Mean±S.D.

† : Deposition

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Table 3. Results of the bacterial reverse mutation test of [redacted] [with S9 mix]

Dose (μg/plate)	Number of revertant colonies per plate [N=2] (with S9 mix)				
	Base-pair substitution type			Frame-shift type	
	TA100	TA1535	WP2uvrA (pKM101)	TA98	TA1537
0	119 , 118 [119 ± 1]	20 , 19 [20 ± 1]	118 , 123 [121 ± 4]	21 , 20 [21 ± 1]	8 , 9 [9 ± 1]
312.5	229 , 227 [228 ± 1]	28 , 24 [26 ± 3]	337 , 385 [361 ± 34]	86 , 64 [75 ± 16]	11 , 12 [12 ± 1]
625	315 , 343 [329 ± 20]	34 , 30 [32 ± 3]	458 , 422 [440 ± 25]	114 , 126 [120 ± 8]	13 , 10 [12 ± 2]
1250	322 , 387 [355 ± 46]	33 , 34 [34 ± 1]	515 , 589 [552 ± 52]	133 , 145 [139 ± 8]	19 , 17 [18 ± 1]
2500	392 , 377 [385 ± 11]†	38 , 42 [40 ± 3]†	836 , 899 [868 ± 45]†	167 , 175 [171 ± 6]†	22 , 22 [22 ± 0]†
5000	414 , 458 [436 ± 31]†	40 , 44 [42 ± 3]†	877 , 880 [879 ± 2]†	215 , 266 [241 ± 36]†	24 , 27 [26 ± 2]†
Positive control	459 , 486 [473 ± 19]	153 , 123 [138 ± 21]	464 , 444 [454 ± 14]	540 , 513 [527 ± 19]	122 , 142 [132 ± 14]

Strain	Positive control	Dose (μg/plate)
TA100	: 2-Aminoanthracene(2-AA)	1.0
TA1535	: 2-Aminoanthracene(2-AA)	2.0
WP2uvrA(pKM101)	: 2-Aminoanthracene(2-AA)	2.0
TA98	: 2-Aminoanthracene(2-AA)	1.0
TA1537	: 2-Aminoanthracene(2-AA)	2.0

N : Number of bacterial reverse examined

[] : Mean±S.D.

† : Deposition

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APPENDIX

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Appendix 1. Certificate of analysis

CERTIFICATE OF ANALYSIS

1. TEST MATERIAL IDENTIFICATION

(1) TEST MATERIAL (LOT No.) : [redacted]
(2) QUANTITY : 20 g
(3) PRODUCER : [redacted]

(4) CHEMICAL NAME (Generic name). :
PNBT

(5) PURITY : >99%
(6) APPEARANCE : Off white solid
(7) MOLECULAR FORMULA : [redacted]S

2. SOLUBILITY

(1) INSOLUBLE : Water
(2) SOLUBLE : No information

3. STORAGE

Room temperature

We hereby certify that the data stated here above are true and correct.

Oct. 2005

[redacted]

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