



PPG Industries, Inc.  
USA

P.O. Box 9

4325 Rosanna Drive

Allison Park, Pennsylvania

15101

Company Sanitized

June 4, 2007

Certified Mail No. 70053110000089435886  
Return Receipt Requested

Document Processing Center (7407M)  
EPA East – Room 6428  
Attn: Section 8(e) and FYI  
U.S. Environmental Protection Agency  
1200 Pennsylvania Avenue, NW  
Washington, DC 20460-0001



8 E H Q - 0 7 - 1 6 8 5 7

RECEIVED  
OPPT/CSIC  
07 JUN 11 AM 6:11

To whom it may concern:

PPG Industries Inc., (PPG), is submitting this information pursuant to Section 8(e) of TSCA.

PPG is submitting a report on a toxicology study conducted concerning [ ], a PPG Aerospace product which is an adhesion promoter for an aerospace sealant. This substance was described in a premanufacture notification (PMN) which also was assigned EPA Accession No. 105534 upon listing on the TSCA Inventory, using the specific chemical name: [ ].<sup>1</sup> Enclosed you will find the full report, including summary, entitled “[ ]: Chromosome Aberration Test in Human Lymphocytes In-Vitro”. This substance was tested as 100% solids material, and considered to be clastogenic to human lymphocytes in-vitro.

In addition, we are submitting two additional study reports on this same substance as “For Your Information” (FYI) submissions. The first report is an In-Vivo study entitled “[ ]: Micronucleus Test in the Mouse”. This substance was tested as 100% solids material, and considered to be non-genotoxic. The second report is entitled “[ ]: Reverse Mutation Assay (Ames Test) Using Salmonella Typhimurium and Escherichia Coli”. This substance was tested as 100% solids material, and considered to be mutagenic to one bacterial strain, TA100, under the test conditions.<sup>2</sup>

Consistent with claims made in the Company’s PMN, and reasserted at the time the substance was added to the Inventory, PPG is claiming the specific chemical identity of the substance, its composition, and its trade name/code number identifier, as Confidential Business Information (CBI). A sanitized copy of this correspondence has been included.

As previously indicated, the [ ] composition is being claimed as CBI. The rationale for this claim is as follows: 1) the complete disclosure of the [ ] substance identity has never

<sup>1</sup> The generic chemical name for this substance is “resin for adhesion promotion”.

<sup>2</sup> The positive Ames Test study has not been submitted previously to the Agency because EPA’s guidance concerning TSCA Section 8(e) has made clear that a positive in vitro genotoxicity study does not trigger a notification requirement as such studies alone lack sufficient corroborative information to reasonably support the conclusion of substantial risk. PPG is submitting this information at this time to provide the Agency with a comprehensive set of data concerning this substance.



8 8 0 7 0 0 0 0 2 7 5 1 5

305160

Document Processing Center (7407M)  
EPA East – Room 6428  
Attn: Section 8(e) and FYI  
Page Two  
June 4, 2007

been made available to our competitors and 2) revealing this information would lead to a significant competitive disadvantage to PPG. This CBI strategy will allow our company to protect sensitive information while giving the Agency and the public information about the chemical nature of the substance that is the subject of this submission. Additional CBI justification is presented in Attachment 1.

PPG provides our customers with labeling and MSDS, which specify procedures for proper handling and disposal of products containing the substance including the use of personal protective equipment.

Please telephone me at [                      ] if you have any questions.

Sincerely,

*attachments*

**SafePharm  
Laboratories**

**CHROMOSOME ABERRATION TEST IN  
HUMAN LYMPHOCYTES *IN VITRO***

**SPL PROJECT NUMBER: 2246/0017**

**AUTHOR:** R Durward

**STUDY SPONSOR:**

PPG Industries, Inc.  
440 College Park Drive  
Monroeville  
PA 15146  
UNITED STATES OF AMERICA

**TEST FACILITY:**

Safepharm Laboratories Limited  
Shardlow Business Park  
Shardlow  
Derbyshire  
DE72 2GD

Telephone: +44 (0) 1332 792896

Facsimile: +44 (0) 1332 799018

**TEST SITE:**

Microptic Cytogenetic Services  
2 Langland Close  
Mumbles  
Swansea  
SA3 4LY

## QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safepharma Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, unless otherwise indicated, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

21 March 2002	Standard Test Method Compliance Audit
13 September 2006	Test Material Preparation
11 September 2006	Test System Preparation
13 September 2006	Exposure
05 September 2006	Assessment of Response
05 September 2006	Cell Harvest/Staining/Slide Preparation
Φ 24 May 2005, 25 May 2005, 30 May 2006	Cytogenetic facilities
§ 28 November 2006	Draft Report Audit
§ Date of QA Signature	Final Report Audit
§ Evaluation specific to this study	
Φ Inspection by Test Site QA(Reported to Management on 01 June 2005 and 31 May 2006)	

  
 .....  
 For Safepharma Quality Assurance Unit\*

DATE:

17 APR 2007

---

**\*Authorised QA Signatures:**

Head of Department:

JR Pateman CBiol MIBiol DipRQA AIQA FRQA

Deputy Head of Department:

JM Crowther MIScT MRQA

Senior Audit Staff:

JV Johnson BSc MRQA; G Wren ONC MRQA

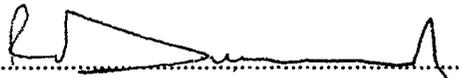
---

**GLP COMPLIANCE STATEMENT**

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

 DATE: 23 MAR 2007

R Durward HNC  
Study Director

**CONTENTS**

<b>QUALITY ASSURANCE REPORT</b>	<b>2</b>
<b>GLP COMPLIANCE STATEMENT</b>	<b>3</b>
<b>CONTENTS</b>	<b>4</b>
<b>SUMMARY</b>	<b>5</b>
<b>1. INTRODUCTION</b>	<b>6</b>
<b>2. TEST MATERIAL</b>	<b>6</b>
2.1 Description, Identification and Storage Conditions	6
<b>3. METHODS</b>	<b>7</b>
3.1 Cells	7
3.2 Cell Culture	7
3.3 Preparation of Test and Control Materials	7
3.4 Microsomal Enzyme Fractions	8
3.5 Culture Conditions	8
3.6 Preliminary Toxicity Test	9
3.7 Experiment 1	9
3.8 Experiment 2	10
3.9 Cell Harvest	10
3.10 Preparation of Metaphase Spreads	10
3.11 Staining	10
3.12 Qualitative Slide Assessment	10
3.13 Coding	11
3.14 Mitotic Index	11
3.15 Scoring of Chromosome Damage	11
3.16 Statistical Analysis	11
<b>4. ARCHIVES</b>	<b>11</b>
<b>5. RESULTS</b>	<b>12</b>
5.1 Preliminary Toxicity Test	12
5.2 Chromosome Aberration Test – Experiment 1	12
5.3 Chromosome Aberration Test - Experiment 2	14
<b>6. CONCLUSION</b>	<b>16</b>
<b>7. REFERENCES</b>	<b>17</b>
Appendix 1 Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells	18
Appendix 2 Results of Chromosome Aberration Test	29
Appendix 3 Dose Response Curves	34
Appendix 4 Chromosome Structural Aberrations: Classification, Evaluation Criteria and Historical Control Data	38
Appendix 5 Statement of GLP Compliance in Accordance with Directive 2004/9/EC	41

**CHROMOSOME ABERRATION TEST IN  
HUMAN LYMPHOCYTES *IN VITRO***

**SUMMARY**

**Introduction.** This report describes the results of an *in vitro* study for the detection of structural chromosomal aberrations in cultured mammalian cells. It supplements microbial systems insofar as it identifies potential mutagens that produce chromosomal aberrations rather than gene mutations (Scott et al, 1990). This study was conducted according to the requirements of the Japanese New Chemical Substance Law (METI), OECD Guidelines for Testing of Chemicals (1997) No. 473 "Genetic Toxicology: Chromosome Aberration Test" and Method B10 of Commission Directive 2000/32/EC.

**Methods.** Duplicate cultures of human lymphocytes, treated with the test material, were evaluated for chromosome aberrations at up to four dose levels, together with vehicle and positive controls. Four treatment conditions were used for the study, i.e. in Experiment 1, 4 hours in the presence of an induced rat liver homogenate metabolising system (S9) (at a 2% final concentration) with cell harvest after a 20-hour expression period and a 4-hour exposure in the absence of metabolic activation (S9), with an 20-hour expression period. In Experiment 2, in the absence and presence of metabolic activation (S9) the exposure time was increased to 6 hours with an 18-hour expression period, with the S9 final concentration increased to 5%.

**Results.** All vehicle (solvent) control groups had frequencies of cells with aberrations within the range expected for normal human lymphocytes.

All the positive control materials induced statistically significant increases in the frequency of cells with aberrations indicating the satisfactory performance of the test and of the activity of the metabolising system.

The test material induced statistically significant increases in the frequency of cells with aberrations, in two separate experiments, using a dose range that included dose levels which induced approximately 50% mitotic inhibition.

**Conclusion.** The test material was considered to be clastogenic to human lymphocytes *in vitro*.

## CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

### 1. INTRODUCTION

This report describes the results of an *in vitro* study for the detection of structural chromosomal aberrations in cultured mammalian cells. It supplements microbial systems insofar as it identifies potential mutagens that produce chromosomal aberrations rather than gene mutations (Scott *et al*, 1990).

The study was conducted according to the requirements of the Japanese New Chemical Substance Law (METI), OECD Guidelines for Testing of Chemicals (1997) No. 473 "Genetic Toxicology: Chromosome Aberration Test" and Method B10 of Commission Directive 2000/32/EC.

The experimental phases of the study were performed between 08 May 2006 and 12 October 2006.

### 2. TEST MATERIAL

#### 2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	
Description	:	Brown extremely viscous liquid
Purity	:	100%
Lot number	:	261147
Date received	:	21 April 2006
Storage conditions	:	Room temperature, in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

### 3. METHODS

#### 3.1 Cells

For each experiment, sufficient whole blood was drawn from the peripheral circulation of a volunteer who had been previously screened for suitability. The volunteer had not been exposed to high levels of radiation or hazardous chemicals and had not knowingly recently suffered from a viral infection. The cell-cycle time for the lymphocytes from the donors used in this study was determined using BrdU (bromodeoxyuridine) incorporation to assess the number of first, second and third division metaphase cells and so calculate the average generation time (AGT). The average AGT for the regular donors used in this laboratory has been determined to be approximately 17 hours under typical experimental exposure conditions.

#### 3.2 Cell Culture

Cells were grown in Eagle's minimal essential medium with HEPES buffer (MEM), supplemented "in-house" with L-glutamine, penicillin/streptomycin, amphotericin B and 15% foetal calf serum, at 37°C with 5% CO<sub>2</sub> in air. The lymphocytes of fresh heparinised whole blood were stimulated to divide by the addition of phytohaemagglutinin (PHA) at 90 µg/ml final concentration.

#### 3.3 Preparation of Test and Control Materials

The test material was accurately weighed, dissolved/dispersed in dimethyl sulphoxide (DMSO) and serial dilutions prepared. The molecular weight of the test material was given as greater than 500, therefore the maximum dose level was 5000 µg/ml, the maximum recommended dose level. There was no significant change in pH when the test material was dosed into media and the osmolality did not increase by more than 50 mOsm. Chemical analysis of the test material formulations was not performed because it is not a requirement of the test method.

Vehicle and positive controls were used in parallel with the test material. The positive control materials were as follows:

In the absence of S9, mitomycin C (MMC) (Sigma, Batch No. 103K0498) at 0.4 µg/ml for 4(20)-hour and 6(18)-hour cultures. It was dissolved in Minimal Essential Medium.

In the presence of S9, Cyclophosphamide (CP) (Acros Batch No. A0164185 and Sigma, Batch No. 084K1328) at 5 µg/ml in Experiment 1 and 2. It was dissolved in dimethyl sulphoxide.

### 3.4 Microsomal Enzyme Fractions

PB/BNF S9 was prepared in-house at Safepharma Laboratories on 01 April 2006 and 08 July 2006 from the livers of male Sprague-Dawley rats weighing ~250g. These had received three daily oral doses of a mixture of phenobarbitone (80 mg/kg) and  $\beta$ -naphthoflavone (100 mg/kg), prior to S9 preparation on the fourth day. The S9 was stored at -196°C in a liquid nitrogen freezer.

### 3.5 Culture Conditions

Duplicate lymphocyte cultures (A and B) were established for each dose level by mixing the following components, giving, when dispensed into sterile plastic flasks for each culture:

9.05 ml MEM, 15% (FCS)  
0.1 ml Li-heparin  
0.1 ml phytohaemagglutinin  
0.75 ml heparinised whole blood

#### 3.5.1 With Metabolic Activation (S9) Treatment

After approximately 48 hours incubation at 37°C, 5% CO<sub>2</sub> in humidified air, the cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed, reserved, and replaced with the required volume of MEM (including serum) and 0.1 ml of the appropriate solution of vehicle control, test material or positive control, was added to the cultures. 1 ml of 20% S9-mix (ie 2% final concentration of S9 in standard co-factors) was added to the cultures of the Preliminary Toxicity Test and of Experiment 1.

After 4 hours at 37°C, the cultures were centrifuged, the treatment medium removed by suction and replaced with an 8 ml wash of MEM culture medium. After a further centrifugation the wash medium was removed by suction and replaced with the original culture medium. The cells were then re-incubated for a further 20 hours at 37°C in 5% CO<sub>2</sub> in humidified air.

In Experiment 2 the cultures were processed as above, with the S9-mix increased to 50% (giving a final concentration of 5%) and exposure increased to 6 hours with an 18 hour recovery period.

### 3.5.2 Without Metabolic Activation (S9) Treatment

In Experiment 1, after approximately 48 hours incubation at 37°C with 5% CO<sub>2</sub> in humidified air, the cultures were decanted into tubes and centrifuged. Approximately 9 ml of the culture medium was removed and reserved. The cells were then re-suspended in the required volume of fresh MEM (including serum) and dosed with 0.1 ml of the appropriate vehicle control, test material solution or positive control solution. The total volume for each culture was a nominal 10 ml.

After 4 hours at 37°C, the cultures were centrifuged; the treatment medium was removed by suction and replaced with an 8 ml wash of MEM culture medium. After a further centrifugation the wash medium was removed by suction and replaced with the reserved original culture medium. The cells were then returned to the incubator for a further 20 hours.

In Experiment 2, in the absence of metabolic activation cultures were processed as in Experiment 1 and the exposure was increased to 6 hours with an 18 hour recovery period after washing

### 3.6 Preliminary Toxicity Test

A preliminary toxicity test was performed on cell cultures using a 4-hour exposure time with and without metabolic activation followed by an 20-hour recovery period, and a continuous exposure of 24 hours without metabolic activation. The dose range of test material used was 19.5 to 5000 µg/ml. Parallel flasks, containing culture medium without whole blood, were established for all three exposure conditions so that test material precipitate observations could be made. Precipitate observations were recorded at the beginning and end of the exposure periods.

Using a qualitative microscopic evaluation of the microscope slide preparations from each treatment culture, appropriate dose levels were selected for mitotic index evaluation. Mitotic index data was used to estimate test material toxicity.

### 3.7 Experiment 1

- i) 4-hour exposure to the test material without S9-mix followed by 20-hour culture in treatment-free media prior to cell harvest.
- ii) 4-hour exposure to the test material with S9-mix (2% final S9 concentration) followed by 20-hour culture in treatment-free media prior to cell harvest.

### 3.8 Experiment 2

- i) 6-hour exposure to the test material without S9-mix followed by 18-hour culture in treatment-free media prior to cell harvest.
- ii) 6-hour exposure to the test material with S9-mix (5% final S9 concentration) followed by 18-hour culture in treatment-free media prior to cell harvest.

### 3.9 Cell Harvest

Mitosis was arrested by addition of demecolcine (Colcemid 0.1 µg/ml) two hours before the required harvest time. After incubation with demecolcine, the cells were centrifuged, the culture medium was drawn off and discarded, and the cells re-suspended in 0.075M hypotonic KCl. After approximately fourteen minutes (including centrifugation), most of the hypotonic solution was drawn off and discarded. The cells were re-suspended and then fixed by dropping the KCl cell suspension into fresh methanol/glacial acetic acid (3:1 v/v). The fixative was changed at least three times and the cells stored at 4°C for at least four hours to ensure complete fixation.

### 3.10 Preparation of Metaphase Spreads

The lymphocytes were re-suspended in several ml of fresh fixative before centrifugation and re-suspension in a small amount of fixative. Several drops of this suspension were dropped onto clean, wet microscope slides and left to air dry. Each slide was permanently labelled with the appropriate identification data.

### 3.11 Staining

When the slides were dry they were stained in 5% Gurr's Giemsa for 5 minutes, rinsed, dried and coverslipped using mounting medium.

### 3.12 Qualitative Slide Assessment

The slides were checked microscopically to determine the quality of the metaphases and also the toxicity and extent of precipitation, if any, of the test material. These observations were used to select the dose levels for mitotic index evaluation.

### 3.13 Coding

The slides were coded using a computerised random number generator.

### 3.14 Mitotic Index

A total of 2000 lymphocyte cell nuclei were counted and the number of cells in metaphase recorded and expressed as the mitotic index and as a percentage of the vehicle control value.

### 3.15 Scoring of Chromosome Damage

The metaphase analysis for the study was sub-contracted to Microptic Cytogenetic services, Swansea, UK (Principal Investigator: Dr N Danford).

Where possible the first 100 consecutive well-spread metaphases from each culture were counted. Where there were approximately 50% of cells with aberrations, slide evaluation was terminated at 50 cells. If the cell had 44-48 chromosomes, any gaps, breaks or rearrangements were noted according to the International System for Chromosome Nomenclature (1985) as described by Scott *et al* and compatible and equitable to the simplified system of Savage (1976) recommended in the 1983 UKEMS guidelines for mutagenicity testing (Appendix 4). Cells with chromosome aberrations were reviewed as necessary by a senior cytogeneticist prior to decoding the slides.

### 3.16 Statistical Analysis

The frequency of cells with aberrations excluding gaps, and the frequency of polyploid cells were compared, where necessary, with the concurrent vehicle control value using Fisher's Exact test or Chi-squared analysis.

## 4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

## 5. RESULTS

### 5.1 Preliminary Toxicity Test

The mitotic index data are presented in Appendix 1 (5) and (6). It can be seen that the test material showed evidence of toxicity in all exposure groups. A precipitate of the test material was observed in the parallel blood-free cultures at the end of the exposure period, at and above 312.5 µg/ml, in both of the 4(20)-hour pulse exposure groups and at and above 625 µg/ml in the 24-hour continuous exposure group. Microscopic assessment of the slides prepared from the treatment cultures showed that metaphase cells were present up to 1250 µg/ml in the 4(20)-hour treatment in the presence and absence of metabolic activation (S9). The maximum dose with metaphases present in the 24-hour continuous exposure was 156.25 µg/ml.

Dose selection for both experiments was based on toxicity .

### 5.2 Chromosome Aberration Test – Experiment 1

The dose levels of the controls and the test material are given in the table below:

Group	Final concentration of (µg/ml)
4(20)-hour without S9	0*, 156.25*, 312.5*, 625*, 937.5, 1250, 1875, MMC 0.4*
4(20)-hour with S9 (2%)	0*, 156.25*, 312.5*, 625*, 937.5, 1250, 1875, CP 5*

The qualitative assessment of the slides determined that the toxicity was similar to that observed in the Preliminary Toxicity Test and that there were metaphases suitable for scoring present up to 625 µg/ml in the absence of metabolic activation (S9). In the presence of metabolic activation (S9) the maximum test material dose level with metaphases suitable for scoring was 937.5 µg/ml.

---

\* Dose levels selected for metaphase analysis  
MMC = Mitomycin C  
CP = Cyclophosphamide

CONCENTRATION ( $\mu\text{g/ml}$ )	4(20)h WITHOUT S9	CONCENTRATION ( $\mu\text{g/ml}$ )	4(20)h WITH 2% S9
0	Metaphases present	0	Metaphases present
156.25	Metaphases present	156.25	Metaphases present
312.5	Metaphases present	312.5	Metaphases present
625	Metaphases present	625	Metaphases present
937.5	NSM	937.5	Metaphases present
1250	NSM	1250	NSM
1875	NSM	1875	NSM
MMC 0.4	Metaphases present	CP 5	Metaphases present

The results of the mitotic indices (MI) from the cultures after their respective treatments are presented in Form 1, Appendix 2. These data show an approximate 50% mitotic inhibition was achieved at 625  $\mu\text{g/ml}$  in the absence of S9. In the presence of S9 28% and 81% mitotic inhibition were achieved at 625 and 937.5  $\mu\text{g/ml}$  respectively. The test material toxicity observed in the presence of S9 at 937.5  $\mu\text{g/ml}$  was considered to be excessive and, therefore, the dose level was not selected for metaphase analysis.

Precipitate observations from the Preliminary Toxicity Test were considered to be representative for the whole study and, therefore test material precipitate would have been present at and above 625  $\mu\text{g/ml}$  in both exposure groups.

The maximum dose level selected for metaphase analysis was based on toxicity for both exposure groups.

---

NSM = No metaphases suitable for scoring  
 MMC = Mitomycin C  
 CP = Cyclophosphamide

The chromosome aberration data are given in Appendix 2, Form 1. All of the vehicle control cultures had frequencies of cells with chromosome aberrations within the expected range. The positive control materials induced statistically significant increases in the frequency of cells with aberrations. The metabolic activation system was therefore shown to be functional and the test method itself was operating as expected.

The test material induced statistically significant increases in the frequency of cells with aberrations in both the absence and presence of metabolic activation (S9) at 625 µg/ml. The increases were part of a modest dose-related response in both treatment groups, though statistical significance was not noted at any other dose levels.

The polyploid cell frequency data are given in Appendix 2, Form 1. The test material induced a statistically significant increase in the numbers of polyploid cells in the absence of metabolic activation at 312.5 and 625 µg/ml, though no clear dose-relationship was observed.

### 5.3 Chromosome Aberration Test - Experiment 2

The dose levels of the controls and the test material are given in the table below:

Group	Final concentration of (µg/ml)
6(18)-hour without S9	0*, 78.1, 156.25, 312.5*, 468.75*, 625*, 937.5, MMC 0.4*
6(18)-hour with S9 (5%)	0*, 78.1, 156.25, 312.5*, 468.75*, 625*, 937.5*, CP 5*

The qualitative assessment of the slides determined that there were metaphases suitable for scoring present at the maximum test material dose level of 937.5 µg/ml in the absence and presence of S9.

---

\* Dose levels selected for metaphase analysis  
MMC = Mitomycin C  
CP = Cyclophosphamide

CONCENTRATION ( $\mu\text{g/ml}$ )	6(18)h WITHOUT S9	CONCENTRATION ( $\mu\text{g/ml}$ )	6(18)h WITH 5% S9
0	Metaphases present	0	Metaphases present
78.1	Metaphases present	78.1	Metaphases present
156.25	Metaphases present	156.25	Metaphases present
312.5	Metaphases present	312.5	Metaphases present
468.75	Metaphases present	468.75	Metaphases present
625	Metaphases present	625	Metaphases present
937.5	Metaphases present	937.5	Metaphases present
MMC 0.4	Metaphases present	CP 5	Metaphases present

The results of the mitotic indices (MI) from the cultures after their respective treatments are presented in Form 2, Appendix 2. These data show an approximate 50% growth inhibition was achieved at 625  $\mu\text{g/ml}$  in the absence of S9, the toxicity seen at 937.5  $\mu\text{g/ml}$ , 80% mitotic inhibition, precluded the dose level from selection for metaphase analysis. In the presence of S9 the maximum level of mitotic inhibition achieved was 31% at 937.5  $\mu\text{g/ml}$ , the maximum dose level tested. The slight differences in toxicity between the two experiments are most likely due to the slight modifications in the exposure times and change in final S9 concentration.

Precipitate observations from the Preliminary Toxicity Test were considered to be representative for the whole study and, therefore test material precipitate would have been present at and above 625  $\mu\text{g/ml}$  in both exposure groups.

The maximum dose level selected for metaphase analysis in the absence of S9 (625  $\mu\text{g/ml}$ ) was the same as Experiment 1, and was based on toxicity. In the presence of S9 the maximum dose level selected for metaphase analysis was 937.5  $\mu\text{g/ml}$ , the maximum dose level investigated.

---

MMC = Mitomycin C  
CP = Cyclophosphamide

The chromosome aberration data are given in Appendix 2, Form 2. All of the vehicle control cultures had frequencies of cells with chromosome aberrations within the expected range. The positive control materials induced statistically significant increases in the frequency of cells with aberrations. The metabolic activation system was therefore shown to be functional and the test method itself was operating as expected.

The test material induced modest, but statistically significant increases in the frequency of cells with chromosome aberrations at 468.75 and 625  $\mu\text{g/ml}$ , without a dose-relationship, in the absence of metabolic activation. In the presence of S9 the test material induced a statistically significant increase in the frequency of cells with chromosome aberrations at 937.5  $\mu\text{g/ml}$ . This was taken to confirm the reproducibility of the responses seen in Experiment 1.

The polyploid cell frequency data are given in Appendix 2, Form 2. The test material did not induce any statistically significant increase in the numbers of polyploid cells at any dose level in either of the exposure groups. The fact that the Experiment 1 response in the absence of S9 was not reproduced may be due to the reduced recovery period not allowing sufficient time for the response to surface.

## 6. CONCLUSION

The test material induced statistically significant reproducible increases in the frequency of cells with chromosome aberrations in both the absence and presence of a liver enzyme metabolising system in two separate experiments. The test material was therefore considered to be clastogenic to human lymphocytes *in vitro*.

---

7. REFERENCES

1. ISCN (1985) An International System for Human Cytogenetic Nomenclature, ed Harnden D G *et al*, Karger S, Switzerland.
2. Savage J R K (1976). Annotation: Classification and relationships of induced chromosomal structural changes. *J. Med. Genet.*, **13**, 103 - 122.
3. Scott D, Dean B J, Danford N D and Kirkland D J (1990). Metaphase chromosome aberration assays *in vitro*. In: Basic mutagenicity tests: UKEMS recommended procedures (Kirkland D J Ed), *Cambridge University Press Report*. Part 1 revised, 62 - 84.
4. Richardson C, Williams D A, Amphlett G, Phillips B, Allen J A, Chanter D O (1989). Analysis of data from *in vitro* cytogenetics assays: Statistical evaluation of mutagenicity test data: UKEMS sub-committee on guidelines for mutagenicity testing (Kirkland D J Ed) *Cambridge University Press*.

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## 1. GENERAL ITEMS (to be completed by the sponsor)

Name of the new chemical substance (IUPAC nomenclature)						
Other name						
Structural formula or rational formula (or outline of manufacturing method, in case both are unknown)						
Purity of the new chemical substance tested	100	Lot No. of the new chemical substance tested	261147			
Name of impurities and concentration						
CAS No.	Not applicable	Vapour pressure				
Molecular weight	762	Partition coefficient	64.7% log <sub>10</sub> Pow <0 20.3% log <sub>10</sub> Pow 0 to 1.32 15.0% log <sub>10</sub> Pow >6.50			
Melting point (°C)		Appearance at ordinary temperature	Brown extremely viscous liquid			
Boiling point (°C)	176.65					
Stability	Stable in light, heat, air and water					
Degree of solubility in solvent	Solvent	Degree of solubility	Stability in solvent	Solvent	Degree of solubility	Stability in solvent
	Water (MEM)	Insoluble at 50 mg/ml		DMSO	Soluble at 500 mg/ml	Stable*
	Acetone			Others ()		

[REMARKS] Because physicochemical properties are for reference, fill in spaces where possible

1. "STABILITY" - Fill in the stability for heat, light etc.
  2. "VAPOUR PRESSURE" - Fill in the vapour pressure of the test substance at 25°C.
  3. "PARTITION COEFFICIENT" - Fill in the value, the temperature used and the name of solvent used for measurement.
  4. "DEGREE OF SOLUBILITY" - Fill in the solubility for each solvent and the stability in each solvent.
- \* It was confirmed that there was no change such as colour, generation of heat etc up to 2 hours after preparation.

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## 2. KIND OF A CELL LINE - CULTURE CONDITION

Name of Cell Line	Lymphocytes	Obtained from	Blood donor	
Species	Human	Date obtained	Ex 1: 03 July 2006 Ex 2: 14 August 2006	
Medium	Eagles Minimal Essential Media	Manufacturer	GIBCO BRL	
Serum and %	Foetal Bovine (15%)	Manufacturer (lot no.)	Gibco (3107260K)	
Doubling time	17 hr	Freezing condition	Not applicable	
Passage number	1	Culture Condition	Container	25 cm <sup>2</sup> TC flask
			Temperature	37°C
			CO <sub>2</sub>	5% (Humidified)
Number of chromosomes (Mode)	46			
Remarks	Cell doubling time determined under normal experimental conditions			

## 3. S-9 MIX

## (1) Source of S9

(encircle the applicable number and fill in the relevant entries)

Made in-house or purchase	<input checked="" type="radio"/> 1. Made in-house	<input type="radio"/> 2. Purchase (Supplier)
Prepared on	01 April 2006 Ex 1 08 July 2006 Ex 2	
Lot No. (in case of purchase)		
Storage temperature	-196°C	

## (2) Preparation of S9

(if purchased material, fill in spaces to extent possible)

Animal used		Inducing substance	
Species strain	Rat, Sprague-Dawley	Name	Phenobarbitone/ $\beta$ -naphthoflavone
Sex	Male	Administration method	Oral doses daily
Age (in weeks)	6-8 weeks	Administration period and amount (mg/kg bodyweight)	3 days
Weight	~ 250g		Phenobarbitone/ $\beta$ -naphthoflavone (80/100 mg/kg)

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## (3) Composition of S9-Mix

Constituents	Amount in 1 ml S9-Mix	Constituents	Amount of 1 ml S9-Mix
S9	0.2 ml Ex 1 0.5 ml Ex2	NADPH	- $\mu$ mol
MgCl <sub>2</sub>	8.0 $\mu$ mol	NADP	5.0 $\mu$ mol
KCl	33.0 $\mu$ mol	NADH	- $\mu$ mol
Glucose-6-phosphate	5.0 $\mu$ mol	Na-phosphate buffer	30.0 $\mu$ mol
		Others ( )	- $\mu$ mol

## (4) Treatment Condition of S9 Mix

(encircle the applicable number and fill in the relevant entries)

	1. Plate method	② Suspension method	3. Others ( )
Amount of S9 (final concentration)	2% Ex1 5% Ex2		
Amount of S9 Protein (final concentration)	0.4 mg/ml Ex1 1.0 mg/ml Ex2		
Culture time	4 hr Ex1 6 hr Ex2		
Culture time after treatment of the test substance	20 hr Ex1 18 hr Ex2		
Remarks			

## 4. PREPARATION OF THE TEST MATERIAL IN SOLUTION

(encircle the applicable response regarding purity conversion)

	Name	Supplier	Lot No.	Grade	Purity (%)
Solvent used	DMSO	Fluka	1196880 23505027	pfs	99.9
Reason for selection of solvent	Test material was soluble in it at the required concentration				
Appearance of the test material preparation	① solution      suspension      others ( )				
Suspension and other methods when test substance difficult to dissolve	Sonicate at 40°C for 5 to 10 minutes				
Storage time and temperature of solution from preparation until use	10 minutes at room temperature Ex 1 and Ex2				
Conversion of purity	Yes				① No

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## 5. SHORT TERM TREATMENT TEST

## (1) Preliminary Toxicity Test

		Without metabolic activation	With metabolic activation
Period of experiment		From: 16 May 2006 To: 19 May 2006	From: 16 May 2006 To: 19 May 2006
Plate	Form	Flask	Flask
	Size	25 cm <sup>2</sup>	25 cm <sup>2</sup>
	Volume of medium	10 ml/plate	10 ml/plate
	Number of plates for each concentration	1	1
Cell	Number of cells seeded (final concentration)	0.75 ml of blood	0.75 ml of blood
	Days after initiation of culture	2 days	2 days
Condition of treatment	Added volume of the prepared solution	0.1 ml/plate	0.1 ml/plate
	Added volume of S9 mix		1 ml/plate
	Final concentration of S9		2%
	Final concentration of S9 protein		0.4 mg/ml
	Period of treatment	4 hours	4 hours
	Period of recovery	20 hours	20 hours
Determination method of cell growth inhibition	The number of metaphase cells per 2000 interphase cells was counted per culture.		
Remarks:	A qualitative evaluation was made from slides prepared from each culture. Giemsa staining [Fixing solution: methanol/glacial acetic acid (3:1 v/v), staining solution: Gurr's Giemsa R66]		

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## (2) Results of Preliminary Toxicity Test

4(20)-hour without metabolic activation		4(20)-hour with metabolic activation	
Dose Level ( $\mu\text{g/ml}$ )	Cell Growth Index (%)	Dose Level ( $\mu\text{g/ml}$ )	Cell Growth Index (%)
0	100	0	100
19.5	-	19.5	-
39	-	39	-
78.1	-	78.1	-
156.25	-	156.25	-
312.5	- P	312.5	- P
625	33 P	625	65 P
1250	21 P	1250	39 P
2500	NM P H	2500	NM P H
5000	NM P H	5000	NM P H

## [Remarks]

- Record the period of treatment and the period of recovery in parentheses
- Fill in the value in order beginning with low concentrations of the test substance, designating the value of the solvent-treated group as 100%

The observations of precipitate were taken at the end of the exposure period.

The maximum dose level was 5000  $\mu\text{g/ml}$ , which was the maximum recommended dose.

---

P = precipitate  
 NM = no scorable metaphases  
 H = haemolysis  
 - = not determined

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## (3) Test Condition of Chromosome Aberration Test Experiment 1

		Without metabolic activation	With metabolic activation
Period of experiment		From: 04 July 2006 To: 07 July 2006	From: 04 July 2006 To: 07 July 2006
Plate	Form	Flask	Flask
	Size	25 cm <sup>2</sup>	25 cm <sup>2</sup>
	Final volume of medium	10 ml/plate	10 ml/plate
	Number of plates for each concentration	2	2
Cell	Number of cells seeded (final concentration)	0.75 ml of blood	0.75 ml of blood
	Days after initiation of culture	2 days	2 days
Condition of treatment	Added volume of the prepared solution	0.1 ml/plate	0.1 ml/plate
	Added volume of S9 mix		1 ml/plate
	Final concentration of S9		2%
	Final concentration of S9 protein		0.4 mg/ml
	Period of treatment	4 hours	4 hours
	Period of recovery	20 hours	20 hours
Remarks	The test material dose ranges were as follows: 4(20)h without S9-mix: 0, 156.25, 312.5, 625, 937.5, 1250, 1875 µg/ml 4(20)h with S9 mix: 0, 156.25, 312.5, 625, 937.5, 1250, 1875 µg/ml		

## (4) Results of Chromosome Aberration Test

(Test results should be reported on the attached Form 1)

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## (5) Test Condition of Chromosome Aberration Test Experiment 2

		Without metabolic activation	With metabolic activation
Period of experiment		From: 14 August 2006 To: 17 August 2006	From: 14 August 2006 To: 17 August 2006
Plate	Form	Flask	Flask
	Size	25 cm <sup>2</sup>	25 cm <sup>2</sup>
	Final volume of medium	10 ml/plate	10 ml/plate
	Number of plates for each concentration	2	2
Cell	Number of cells seeded (final concentration)	0.75 ml of blood	0.75 ml of blood
	Days after initiation of culture	2 days	2 days
Condition of treatment	Added volume of the prepared solution	0.1 ml/plate	0.1 ml/plate
	Added volume of S9 mix		1 ml/plate
	Final concentration of S9		5%
	Final concentration of S9 protein		1 mg/ml
	Period of treatment	6 hours	6 hours
	Period of recovery	18 hours	18 hours
Remarks	The test material dose ranges were as follows: 6(18)h without S9-mix: 0, 78.1, 156.25, 312.5, 468.75, 625, 937.5 µg/ml 6(18)h with S9 mix: 0, 78.1, 156.25, 312.5, 468.75, 625, 937.5 µg/ml		

## (6) Results of Chromosome Aberration Test

(Test results should be reported on the attached Form 2)

. CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## 6. CONTINUOUS TREATMENT TEST

## (1) Preliminary Toxicity Test

Period of experiment		From: 16 May 2006 To: 19 May 2006
Plate	Form	Flask
	Size	25 cm <sup>2</sup>
	Volume of medium	10 ml/plate
	Number of plates for each concentration	1
Cell	Number of cells seeded (final concentration)	0.75 ml of blood
	Days after initiation of culture	2 days
Condition of treatment	Added volume of the prepared solution	0.1 ml/plate
	Period of treatment	24 hours
	Period of recovery	- hours
Determination method of cell growth inhibition	The number of metaphase cells per 2000 interphase cells was counted per culture.	
Remarks:	A qualitative evaluation was made from slides prepared from each culture. Giemsa staining [Fixing solution: methanol/glacial acetic acid (3:1 v/v), staining solution: Gurr's Giemsa R66]	

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## (2) Results of preliminary Toxicity Test

24 Hour Treatment	
Dose Level ( $\mu\text{g/ml}$ )	Cell Growth Index (%)
0	100
19.5	-
39	-
78.1	69
156.25	32
312.5	NM
625	NM P
1250	NM P
2500	NM P
5000	NM P

## [Remarks]

- Record the period of treatment and the period of recovery in parentheses
- Continuous treatment tests should be conducted without metabolic activation
- Fill in the value in order beginning with low concentrations of the test substance, designating the value of the solvent-treated group as 100%

The observations of precipitate were taken at the end of the exposure period.

The maximum dose level was 5000  $\mu\text{g/ml}$ , which was the maximum recommended dose.

With a positive clastogenic response being observed in the short term tests the 24-hour continuous exposure test was not performed.

---

P = precipitate  
NM = no scorable metaphases  
- = not determined

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 1 (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## 7. JUDGEMENT OF RESULTS AND REFERENTIAL MATTERS

## (1) Judgement of the Results

Judgement (Encircle one)		Positive		Negative	
Reason for judgement		The test material induced statistically significant and reproducible increases in the frequency of cells with aberrations in all of the treatment cases.			
D <sub>20</sub>	Structural Aberration	Short term 4 Hr treatment	-S9 mix	4- hr treatment	1.42 mg/ml
			+S9 mix	4- hr treatment	1.55 mg/ml
		Short term 6Hr treatment	-S9 mix	6- hr treatment	2.39 mg/ml
			+S9 mix	6- hr treatment	3.20 mg/ml
	Numerical Aberration	Short term 4Hr treatment	-S9 mix	4- hr treatment	2.98 mg/ml
			+S9 mix	4- hr treatment	NA
		Short term 6Hr treatment	-S9 mix	6- hr treatment	NA
			+S9 mix	6- hr treatment	NA

[Remarks]

D<sub>20</sub> value is the presumed dose level of the test substance that is required to induce aberrations in 20% of metaphases. D<sub>20</sub> value of the test system judged positive should be noted based upon a type of aberration.

## (2) Referential Matters

DMSO was selected as the solvent because the test material was soluble in it at the required concentrations. A correction for the purity of the test material was not required when the test material formulations were prepared. Precipitate of the test material was observed at and above 312.5 µg/ml. The chromosome aberration data were analysed statistically using Fishers Exact Test or Chi-squared analysis. The test material was shown to be toxic to human lymphocyte cells *in vitro* in all treatment cases. The maximum dose level in the cell growth inhibition test was 5000 µg/ml, the maximum recommended dose. The study was conducted according to the requirements of Japanese New Chemical Substance Law (METI) and OECD 473 guidelines.

[Remarks]

"Referential matters" - Fill in the view etc of the Study Director on the test results.

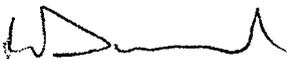
---

NA = Not applicable

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix I (continued) Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells

## 8. OTHERS

Testing Institution	Name	Safepharm Laboratories Ltd.	
	Address	Shardlow Business Park, , Shardlow, Derbyshire, DE72 2GD	Tel No: 0044 1332 792896 Fax No: 0044 1332 799018
Study Director	Title:	Senior Genetic Toxicologist	Signed: 
	Name:	R Durward	
	Years of experience:	31	
Study number	2246/0017		
Test dates	04 May 2006		<b>23 MAR 2007</b>
	Protocol authorised by Study Director		Final report authorised by Study Director

[Remarks]

1. Any information in this format should be accurately transferred from a final report
2. The same study number as that of the final report should be used in the format

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 2 Results of Chromosome Aberration Test

## [Remarks]

- Record the period of treatment and the period of recovery in the column of TREATMENT PERIOD.
- Fill in the dose level of the test substance in order beginning with low concentrations.
- Record the name of the solvent (negative) control or the positive control in parentheses. If an abbreviation is used, record the full substance in the key.
- Record the data for each plate in the first line or the second line, and record the total in the third line.
- When precipitate of the test substance is found, the applicable dose level is marked with a P.
- When it is not possible to observe chromosomes due to cell toxicity, record "TOXIC" in the column of "OBSERVED" of the applicable dose level.
- When the column of "OTHERS" is used, record the contents in the margin.

## [KEY]

OBSERVED	:	The number of the observed cells
ctb	:	Chromatid breaks
cte	:	Chromatid exchanges
csb	:	Chromosome breaks
cse	:	Chromosome exchanges
g	:	Gaps
TOTAL (%)	:	the total and percentage of cells with structural aberrations excluding gaps or numerical aberrations
DMSO	:	Dimethyl sulphoxide

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 2 (continued) Results of Chromosome Aberration Test

Form 1 Short Term Treatment Test - Experiment 1 Without Metabolic Activation (S9)

Treatment Period (hours)	S9 mix	Concentration µg/ml	Number and Percentages of Cells Showing Structural Chromosome Aberrations (%)										g	Cell Growth Index Mitotic Index (%)	Number and Percentages of Cells Showing Numerical Aberrations (%)					
			Observed	ctb	cte	csb	csc	Others	Total	Observed	Polyploids	Others			Total					
	-	Negative Control (DMSO) 0	A	100	2	0	0	0	0	0	0	2	1	6.40	100	0	0	0		
			B	100	1	0	0	0	0	0	0	1	2	3.15	101	1	0	0	1	
			Total %	200	3	0	0	0	0	0	0	3	3	4.78 (1.5)	201	1	0	0	1 (0.5)	
	-	-	156.25	A	100	1	0	0	0	0	0	1	1	0	7.10	102	2	0	0	2
				B	100	0	0	1	0	0	0	0	1	1	4.40	100	0	0	0	0
				Total %	200	1	0	1	0	0	0	0	2	2	5.75 (1.0)	202	2	0	0	2 (1.0)
4(20)Hr	-	312.5	A	100	6	0	0	1	0	0	7	0	5.10	104	4	0	0	4		
			B	100	0	0	1	0	0	1	0	2	3.60	106	6	0	0	6		
			Total %	200	6	0	1	1	0	0	8	2	4.35 (1.0)	210	10	0	0	10 (4.8)		
	-	-	625	A	100	8	2	1	0	0	0	10	4	2.80	105	5	0	0	5	
				B	89 <sup>a</sup>	7	1	0	0	0	0	8	2	2.50	92	3	0	0	3	
				Total %	189	15	3	1	0	0	0	18***	6	2.65 (3.2)	197	8	0	0	8* (4.1)	
-	-	Positive Control (MMC) 0.4	A	50 <sup>a</sup>	14	11	4	0	0	0	24	3	2.65	50	0	0	0	0		
			B	50 <sup>a</sup>	16	12	0	0	0	0	24	1	3.40	50	0	0	0	0		
			Total %	100	30	23	4	0	0	0	48***	4	3.03 (63)	100	0	0	0	0 (0.0)		

MMC = Mitomycin C

a = Slide evaluation terminated at 50 cells because approximately 50% cells with aberrations had been observed

b = Insufficient metaphases suitable for scoring available to score 100 metaphases

\* = p < 0.05

\*\* = p < 0.01

\*\*\* = p < 0.001

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES IN VITRO

Appendix 2 (continued) Results of Chromosome Aberration Test

Form 1 Short Term Treatment Test - Experiment 1 With Metabolic Activation (2% S9)

Treatment Period (hours)	S9 mix	Concentration µg/ml	Number and Percentages of Cells Showing Structural Chromosome Aberrations (%)						g	Number and Percentages of Cells Showing Numerical Aberrations (%)							
			Observed	ctb	cte	csb	cse	Others		Total	Mitotic Index (%)	Observed	Polyploids	Others	Total		
	+	Negative Control (DMSO) 0	A	0	0	0	1	0	0	0	1	6.25	100	0	0	0	0
			B	0	1	0	0	0	0	0	1	4.30	101	1	0	0	1
			Total %	0	1	0	0	0	0	0	2	5.28	201	1	0	0	1
	+	156.25	A	0	0	0	0	0	0	0	0	(100)	101	1	0	0	1
			B	0	0	0	0	0	0	0	0	5.75	100	0	0	0	0
			Total %	0	0	0	0	0	0	0	0	3.10	201	1	0	0	1
4(20)Hr	+	312.5	A	1	0	0	0	0	0	0	1	4.43	101	1	0	0	1
			B	1	0	0	0	0	0	0	0	(84)	101	1	0	0	1
			Total %	1	0	0	0	0	0	0	0	4.80	202	2	0	0	2
	+	625	A	2	0	0	0	0	0	0	2	4.40	103	3	0	0	3
			B	10	2	2	1	1	0	0	11	3.60	101	1	0	0	1
			Total %	15	4	4	1	1	0	0	18***	3.78	204	4	0	0	4
	+	937.5	A	7	2	2	1	1	0	0	12	(72)	103	3	0	0	3
			B	15	4	4	1	1	0	0	18***	1.25	101	1	0	0	1
			Total %	22	6	6	2	2	0	0	30	0.80	204	4	0	0	4
	+	Positive Control (CP) 5	A	16	5	5	1	0	0	0	21	1.03	50	0	0	0	0
			B	24	11	11	3	1	0	0	25	(19)	50	0	0	0	0
			Total %	40	16	16	4	1	0	0	46***	1.50	100	0	0	0	0

CP = Cyclophosphamide  
a = Slide evaluation terminated at 50 cells because approximately 50% cells with aberrations had been observed  
\*\*\* = p < 0.001

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 2 (continued) Results of Chromosome Aberration Test

Form 2 Short Term Treatment Test - Experiment 2 Without Metabolic Activation (S9)

Treatment Period (hours)	S9 mix	Concentration µg/ml	Number and Percentages of Cells Showing Structural Chromosome Aberrations (%)										g	Cell Growth Index Mitotic Index (%)	Number and Percentages of Cells Showing Numerical Aberrations (%)					
			Observed	ctb	cte	csb	cse	Others	Total	Observed	Polyploids	Others			Total					
	-	Negative Control (DMSO) 0	A	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0		
			B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
			Total	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
			(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.5)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)		
	-	312.5	A	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	
			B	100	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
			Total	200	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
			(100)	(2.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(2.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	
6(18)hr	-	468.75	A	100	6	1	2	0	0	0	0	9	0	0	0	0	0	0	0	
			B	100	1	1	0	0	0	0	0	0	2	0	0	0	0	0	0	0
			Total	200	7	2	2	0	0	0	0	0	11**	0	0	0	0	0	0	0
			(100)	(3.5)	(1.0)	(1.0)	(0.0)	(0.0)	(0.0)	(0.0)	(5.5)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(1.0)	
	-	625	A	100	4	0	0	0	0	0	4	0	0	0	0	0	0	0	0	
			B	100	5	0	1	0	0	0	0	6	0	0	0	0	0	0	0	0
			Total	200	9	0	1	0	0	0	0	10**	0	0	0	0	0	0	0	0
			(100)	(4.5)	(0.0)	(0.5)	(0.0)	(0.0)	(0.0)	(5.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.5)	
	-	937.5	A	TOXIC																
			B	TOXIC																
			Total																	
	-	Positive Control (MMC) 0.4	A	50 <sup>a</sup>	12	17	0	0	0	0	0	26	0	0	0	0	0	0	0	
			B	50 <sup>a</sup>	16	21	2	0	0	0	0	30	1	0	0	0	0	0	0	0
			Total	100	28	38	2	0	0	0	0	56***	1	0	0	0	0	0	0	0
			(100)	(28.0)	(38.0)	(2.0)	(0.0)	(0.0)	(0.0)	(56.0)	(1.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	

MMC = Mitomycin C

a = Slide evaluation terminated at 50 cells because approximately 50% cells with aberrations had been observed

\*\* = p < 0.01

\*\*\* = p < 0.001

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 2 (continued) Results of Chromosome Aberration Test

Form 2 Short Term Treatment Test - Experiment 2 With Metabolic Activation (5% S9)

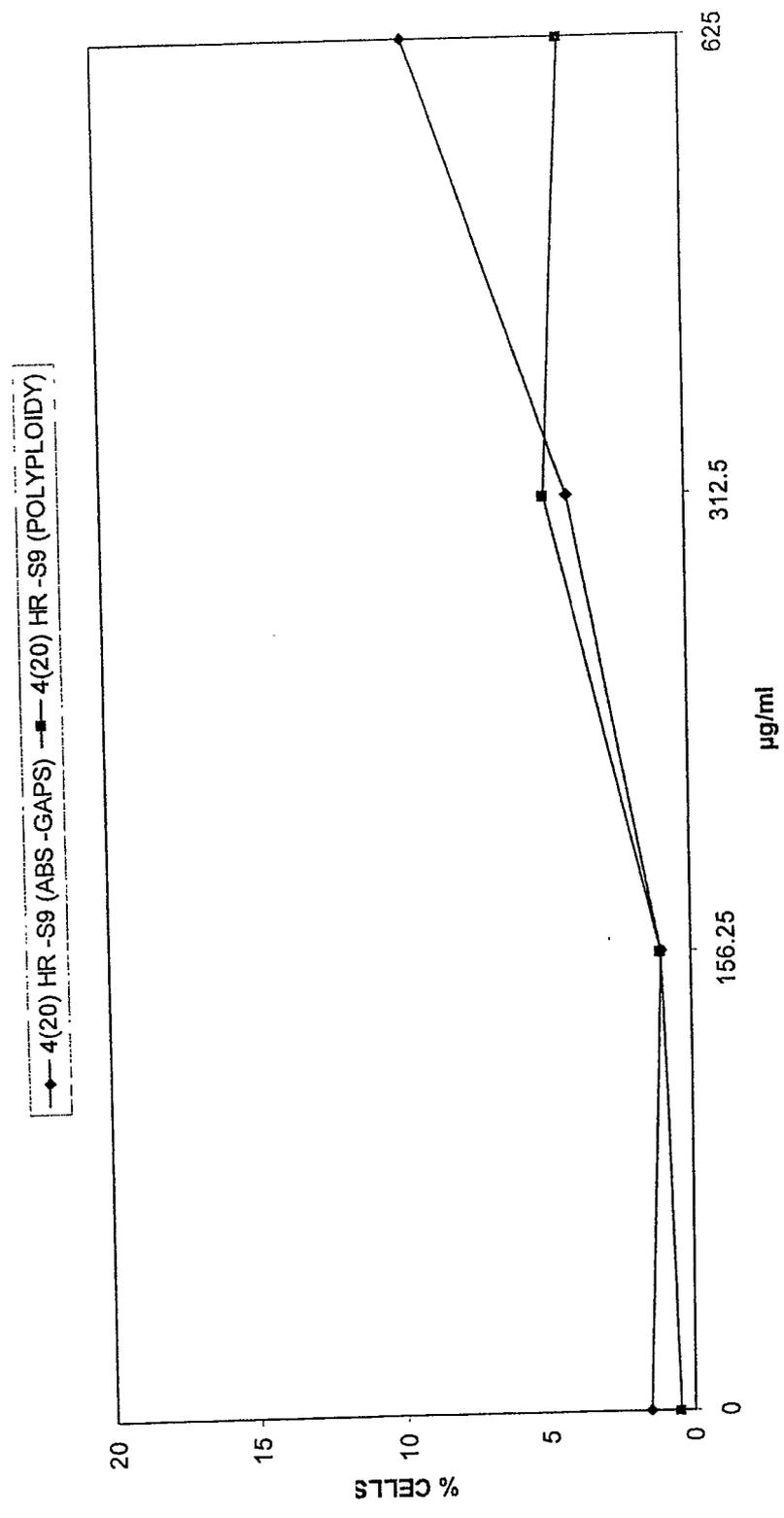
Treatment Period (hours)	S9 mix	Concentration µg/ml	Number and Percentages of Cells Showing Structural Chromosome Aberrations (%)										B	Cell Growth Index				Number and Percentages of Cells Showing Numerical Aberrations (%)									
			Observed	ctb	ctc	csb	csc	Others	Total	Mitotic Index (%)	Observed	Polyploids		Others	Total	Cell Growth Index		Number and Percentages of Cells Showing Numerical Aberrations (%)									
																Observed	Others	Polyploids	Others	Total							
6(18) Hr	+	Negative Control (DMMSO) 0	A	100	1	0	1	0	0	0	2	0	0	0	0	6.05	100	0	0	0	0	0	0	0			
			B	100	2	0	1	0	0	3	0	0	0	0	0	6.45	102	2	0	0	0	0	0	2	0		
			Total %	200	3	0	2	0	0	5	0	0	(0.0)	(2.5)	0	6.25	202	2	0	0	0	0	0	0	2	(1.0)	
	A	100	1	0	0	0	0	1	0	0	1	0	0	0	5.40	100	0	0	0	0	0	0	0	0	0		
	B	100	1	0	2	0	0	3	0	0	0	0	0	5.85	100	0	0	0	0	0	0	0	0	0	0		
	Total %	200	2	0	2	0	0	4	0	0	(0.0)	(2.0)	0	5.63	200	0	0	0	0	0	0	0	0	0	0	(0.0)	
	A	100	0	0	1	0	0	1	0	0	0	0	0	5.45	100	0	0	0	0	0	0	0	0	0	0	0	
	B	100	0	0	0	0	0	0	0	0	0	0	0	6.05	100	0	0	0	0	0	0	0	0	0	0	0	
	Total %	200	0	0	0	1	0	2	0	0	0	0	0	5.75	200	0	0	0	0	0	0	0	0	0	0	0	(0.0)
	A	100	2	0	0	1	0	3	0	0	0	0	0	5.00	101	1	0	0	0	0	0	0	0	0	0	0	
	B	100	2	0	0	1	0	3	0	0	0	0	0	5.20	100	0	0	0	0	0	0	0	0	0	0	0	
	Total %	200	4	0	0	2	0	6	0	0	0	0	0	5.10	201	1	0	0	0	0	0	0	0	0	0	0	(0.5)
A	100	8	0	0	0	0	8	0	0	0	0	0	4.85	100	0	0	0	0	0	0	0	0	0	0	0	0	
B	100	8	1	0	0	0	9	0	0	0	0	0	3.80	100	0	0	0	0	0	0	0	0	0	0	0	0	
Total %	200	16	1	0	0	0	17**	0	0	0	0	0	4.33	200	0	0	0	0	0	0	0	0	0	0	0	0	
A	100	21	2	0	0	0	23	0	0	0	0	0	3.05	100	0	0	0	0	0	0	0	0	0	0	0	0	
B	100	21	3	0	0	0	24	0	0	0	0	0	2.65	100	0	0	0	0	0	0	0	0	0	0	0	0	
Total %	200	42	5	0	0	0	46***	0	0	0	0	0	2.85	200	0	0	0	0	0	0	0	0	0	0	0	0	
			(21.0)	(2.5)	(0.0)	(0.0)	(23.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(4.6)													(0.0)	

CP = Cyclophosphamide  
 \*\* = p < 0.01  
 \*\*\* = p < 0.001

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 3 Dose Response Curves

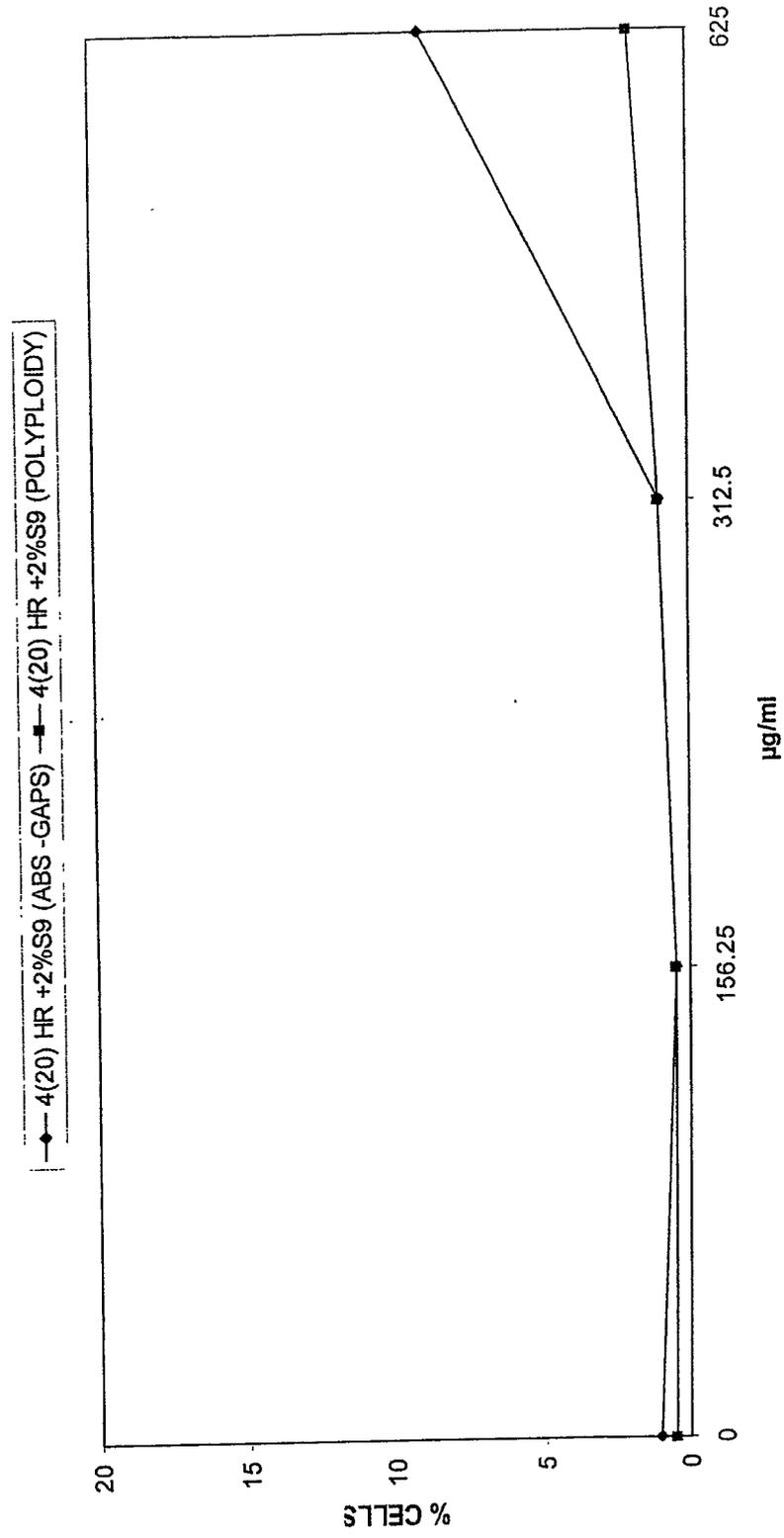
Figure 1 Chromosome Aberration Test - Experiment 1 Without S9



CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 3 (continued) Dose Response Curves

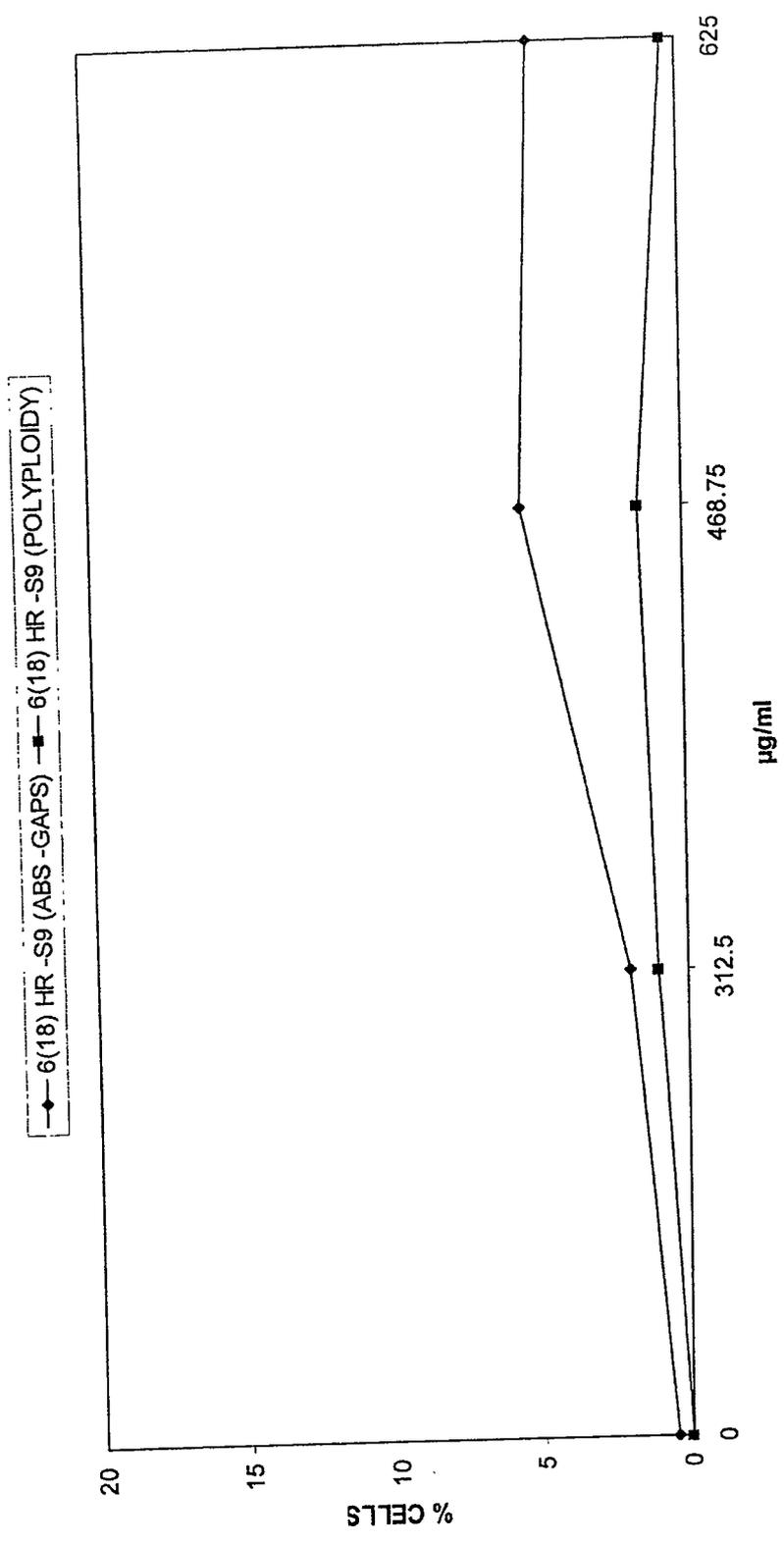
Figure 2 Chromosome Aberration Test - Experiment 1 With S9



CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 3 (continued) Dose Response Curves

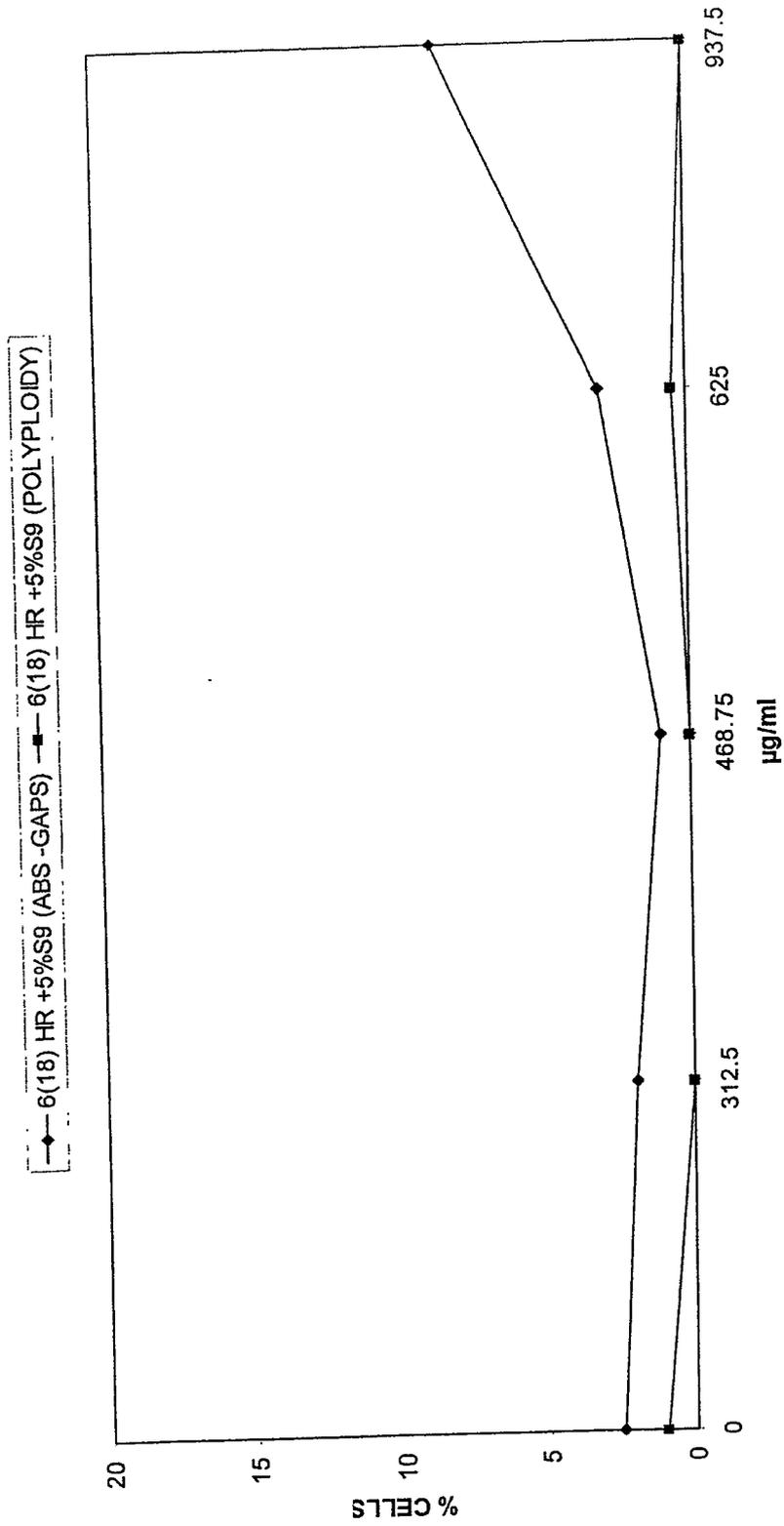
Figure 3 Chromosome Aberration Test - Experiment 2 Without S9



CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 3 (continued) Dose Response Curves

Figure 4 Chromosome Aberration Test - Experiment 2 With S9



CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

## Appendix 4 Chromosome Structural Aberrations: Classification, Evaluation Criteria and Historical Control Data

## 1. CLASSIFICATION

## 1.1 Gaps (g)

Gaps are small areas of the chromosome which are unstained. The chromatids remain aligned as normal and the gap does not extend along the chromatid for a distance greater than the width of a chromatid. If the gap occurs on one chromatid only it is a chromatid gap (g). If a gap appears in both chromatids at the same position it is a chromosome gap (G).

## 1.2 Chromatid Breaks (ctb)

Chromatid breaks (ct) vary in appearance. The chromatid may remain aligned but show a gap which is too large to classify as a gap. Alternatively, the chromatid may be broken so that the broken fragment is displaced. In some cases, the fragment is not seen at all. A chromatid fragment (f) should be scored if the chromosome of origin cannot be identified. Very small fragments are scored as minutes (m)

## 1.3 Chromosome Breaks (csb)

Chromosome breaks (CS) are breaks in both chromatids of the chromosome. A fragment with two chromatids is formed and this may be displaced by varying degrees. Breaks are distinguished from gaps by the size of the unstained region. A chromosome break is scored if the fragment is associated with a chromosome from which it was probably derived. However, fragments are often seen in isolation and are then scored as chromosome fragments (F). Very small fragments are scored as minutes (M).

## 1.4 Exchanges (cte and cse)

Exchanges are formed by faulty rejoining of broken chromosomes and may be of the chromosome or chromatid type. Chromatid exchanges (c/c,r) have numerous different forms but are generally not further classified. Where multiple exchanges have occurred each exchange point is counted as one chromatid exchange. Chromosome exchanges generally appear as either a dicentric (D) or a ring (R) form, either of which can be associated with a fragment, which if possible should be scored as part of the exchange.

. CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 4 (continued)    Chromosome Structural Aberrations: Classification,  
Evaluation Criteria and Historical Control Data

### 1.5 Multiple Aberrations

If many aberrations are present in one metaphase, the exact details may not be scorable. This is particularly the case when chromosome pulverisation occurs. If the number of aberrations is 10 or more then the cell is classified as X.

### 1.6 Chromosome Number

If the chromosome (centromere) number is 44-48 then it is classified as a diploid cell and scored for aberrations. If less than 44 chromosomes are counted then the cell is ignored under the assumption that some chromosomes may have been lost for technical reasons. If greater than 44 chromosomes are scored and the number is a multiple of the haploid count then the cell is classified as a polyploid cell. If the chromosomes are arranged in closely apposed pairs, ie. 4 chromatids instead of 2, the cell is scored as endoreduplicated (E).

## 2. HISTORICAL CONTROL DATA AND EVALUATION CRITERIA

### 2.1 Historical Aberration Ranges for Vehicle Control Cultures

Many experiments with human lymphocytes have established a range of aberration frequencies acceptable for control cultures. The current in-house historical aberration ranges are presented below:

	Pulse exposure -S9		Pulse exposure +S9 (1% S9)		24-Hour -S9		Pulse exposure +S9 (2% S9)	
	% cells with aberrations (-gaps)	% cells with polyploids						
Minimum	0	0	0	0	0	0	0	0
Maximum	3	1.5	3.5	2	3	1.5	3.5	1.5
Mean	0.7	0.1	0.8	0.1	0.6	0.1	0.6	0.1
Standard Deviation	0.7	0.3	0.8	0.3	0.6	0.3	0.6	0.2
Number	100	100	100	100	97	97	96	96

· CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 4 (continued)      **Chromosome Structural Aberrations: Classification,  
Evaluation Criteria and Historical Control Data**

**2.2      Historical Aberration Range for Positive Control Cultures**

	Pulse exposure -S9 MMC 0.4 µg/ml		Pulse exposure +S9 (1% S9) CP 5 - 10 µg/ml		24-Hour -S9 MMC 0.2 - 0.25 µg/ml		Pulse exposure +S9 (2% S9) CP 5 - 10 µg/ml	
	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids
Minimum	11	0	10	0	12	0	13	0
Maximum	53	1.5	42	0.5	68	1	52	2
Mean	34	0.1	23	0.1	38	0	31	0.1
Standard Deviation	10.1	0.3	9.5	0.2	13.8	0.2	10.4	0.4
Number	46	46	22	22	48	48	26	26

**2.3      Evaluation Criteria**

A positive response was recorded for a particular treatment if the % cells with aberrations, excluding gaps, markedly exceeded that seen in the concurrent control, either with or without a clear dose-relationship. For modest increases in aberration frequency a dose response relationship is generally required and appropriate statistical tests may be applied in order to record a positive response.

## Appendix 5 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT  
OF THE UNITED KINGDOM****GOOD LABORATORY PRACTICE****STATEMENT OF COMPLIANCE  
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC**

LABORATORY	TEST TYPE
SafePharm Laboratories Ltd. Shardlow Business Park London Road Shardlow Derby DE72 2GD	Analytical Chemistry Environmental Fate Environmental Toxicity Mutagenicity Phys/Chem Testing Toxicology

**DATE OF INSPECTION****30<sup>th</sup> August 2005**

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Handwritten signature of Bryan J. Wright, dated 21/11/05.

Mr. Bryan J. Wright  
Head, UK GLP Monitoring Authority