

Ref #14

**SafePharm
Laboratories**

#12

N-Acetyl-L-hydroxyproline:

**CHROMOSOME ABERRATION TEST IN
CHL CELLS *IN VITRO***

SPL PROJECT NUMBER: 732/092

AUTHORS: Ms N P Wright
Mrs S Nolan

STUDY SPONSOR:

Kyowa Hakko Kogyo Co., Ltd.
6-1, Ohtemachi, 1-chome
Chiyoda-ku
TOKYO 100-8185
JAPAN

TEST FACILITY:

Safepharm Laboratories Limited
P.O. Box No. 45
DERBY
DE1 2BT
U.K.

Telephone: (01332) 792896

Facsimile: (01332) 799018

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 Safeparm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, 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:

24 January 2001	Standard Test Method Compliance Audit
27 September 2001	Test Material Preparation
11 September 2001	Test System Preparation
06,13 September 2001	Exposure
21 September 2001	Assessment of Response
21 September 2001	Cell Harvest/Staining/Slide Preparation
§ 06 November 2001	Draft Report Audit
§ Date of QA Signature	Final Report Audit
§	Evaluation specific to this study



DATE: 26 NOV 2001

For Safeparm Quality Assurance Unit*

***Authorised QA Signatures:**

Head of Department:

JR Pateman CBiol MIBiol DipRQA FRQA

Deputy Head of Department:

JM Crowther MIScT MRQA

Senior Audit Staff:

JV Johnson BSc MRQA; G Wren ONC MRQA; R Hurst 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)). 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 87/18/EEC (as amended by Directive 1999/11/EC) and 88/320/EEC (as amended by Directive 1999/12/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, 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.

..... *P. Wright* DATE: **26 NOV 2001**

Ms N P Wright BSc (Hons)

Study Director

CONTENTS

QUALITY ASSURANCE REPORT	2
GLP COMPLIANCE STATEMENT	3
CONTENTS	4
SUMMARY	5
1. INTRODUCTION	6
2. TEST MATERIAL	6
2.1 Description, Identification and Storage Conditions	6
3. METHODS	7
3.1 Cell Line	7
3.2 Cell Culture	7
3.3 Preparation of Test and Control Materials	7
3.4 Cell Growth Inhibition Test	8
3.5 Microsomal Enzyme Fraction	8
3.6 Culture Conditions	8
3.7 Cell Harvest	9
3.8 Preparation of Metaphase Spreads, Staining and Coding	9
3.9 Mitotic Index	9
3.10 Scoring of Chromosome Damage	9
3.11 Statistical Analysis	10
4. ARCHIVES	10
5. RESULTS AND DISCUSSION	11
5.1 Cell Growth Inhibition Test	11
5.2 Short Term Treatment Test - Experiment 1	11
5.3 Continuous Treatment Test - Experiment 2	12
6. CONCLUSION	13
7. REFERENCES	14
Appendix 1 Report of Results of Chromosomal Aberration Test in Cultured Mammalian Cells	15
Appendix 2 Results of Chromosome Aberration Test	26
Appendix 3 Dose Response Curves	31
Appendix 4 Chromosome Structural Aberrations: Classification, Evaluation Criteria and Historical Control Data	36
Appendix 5 Statement of GLP Compliance in Accordance with Directive 88/320/EEC	39

N-Acetyl-L-hydroxyproline:
CHROMOSOME ABERRATION TEST IN
CHL CELLS *IN VITRO*

SUMMARY

Introduction. This study was conducted according to a method which was designed to assess the potential chromosomal mutagenicity of a test material on the metaphase chromosomes of the Chinese Hamster Lung (CHL) cell line according to the requirements of the Japanese New Chemical Substance Law (METI).

Methods. Duplicate cultures of Chinese Hamster Lung (CHL) cells were treated with the test material at several dose levels, together with vehicle and positive controls. Four treatment regimens were used: Experiment 1 used a 6(18)-hours exposure, both with and without the addition of an induced rat liver homogenate metabolising system; Experiment 2 used both 24 and 48 hours continuous exposures.

The dose levels used were selected on the basis of the results of a preliminary toxicity test and were in the range of 216.25 to 1730 µg/ml for all exposure groups.

Results. The vehicle (solvent) controls gave frequencies of cells with aberrations within the range expected for the CHL cell line. All the positive control treatments gave highly 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 did not induce any significant increases in the frequency of cells with aberrations in any of the exposure groups. The test material was shown to be non-toxic to CHL cells *in vitro* in all four exposure groups.

Conclusion. The test material was shown to be non-clastogenic to CHL cells *in vitro*.

N-Acetyl-L-hydroxyproline:
CHROMOSOME ABERRATION TEST IN
CHL CELLS *IN VITRO*

1. INTRODUCTION

This study was conducted according to a method which was designed to assess the potential chromosomal mutagenicity of a test material, on the metaphase chromosomes of the Chinese Hamster Lung (CHL) cell line according to the requirements of the Japanese New Chemical Substance Law (METI).

Numerical and structural chromosome aberrations are implicated in the pathology of neoplasia (Radman *et al*, 1982; Cairns, 1981) and also occur in a high proportion of spontaneous abortions and abnormal live births (Chandley, 1981). Furthermore, most carcinogens are capable of inducing such changes in chromosome fidelity (Ishidate and Odashima, 1977; Ishidate and Sofuni, 1985). Metaphase analysis *in vitro* involves the evaluation of chromosomes of exposed cells for structural damage. Many of these changes are accompanied by more subtle changes (translocations, inversions, small deletions) which are not cell lethal, and therefore represent a hazard. The ability to induce chromosome aberrations also correlates with the induction of gene mutations (Hollstein *et al*, 1979).

The experimental phases of the study were performed between 18 May 2001 and 11 October 2001.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	N-Acetyl-L-hydroxyproline
Description	:	white solid
Purity	:	100%
Batch number	:	000703
Date received	:	23 April 2001
Storage conditions	:	room temperature, in the dark

Data relating to the identity, purity and stability of the test material are the responsibility of the Sponsor.

3. METHODS

3.1 Cell Line

The Chinese Hamster Lung (CHL) cell line, isolated by Koyama *et al* (1970) and cloned by Ishidate and Sofuni (1985), was used. The CHL cell line has an average generation time of approximately 17 hours when growing under normal experimental conditions.

3.2 Cell Culture

Cells were grown in Eagle's Minimal Essential Medium (MEM) with HEPES buffer and Earle's Salts and supplemented "in-house" with 10% foetal bovine serum and antibiotics, at 37°C with 5% CO₂ in air.

3.3 Preparation of Test and Control Materials

The test material was accurately weighed and formulated in MEM and appropriate dilutions made. Analysis for concentration, homogeneity and stability of the test material preparations was not a requirement of the test guidelines and therefore was not performed.

The molecular weight of the test material was 173.17, therefore a dose level of 1730 µg/ml gave a 10 mM concentration, which was the maximum dose level tested.

The initial purity of the test material was 100%, therefore an allowance for test material purity was not required when the dosing solutions were prepared. There was no observable change in pH when the test material was dosed into culture media and the osmolality was not increased by more than 50 mOsm at the 10 mM maximum dose level.

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

- Mitomycin C (MMC, Sigma Batch Nos. 30K2508 and 90K2511) 0.1, 0.05 and 0.025 µg/ml for cultures treated for 6(18), 24 or 48 hours respectively in the absence of metabolising enzymes.
- Cyclophosphamide (CP, Sigma Batch No. 108H0568) 5.0 µg/ml for cultures treated for 6(18) hours with metabolic activation.

3.4 Cell Growth Inhibition Test

A preliminary toxicity test was performed on cell cultures using 24 and 48-hour continuous exposure times without metabolic activation and a 6-hour exposure period (both with and without metabolic activation) followed by an 18-hour recovery period in treatment-free media. The dose range used was 6.76 to 1730 $\mu\text{g/ml}$. Precipitate observations were noted at the beginning and end of the treatment period. Growth inhibition was estimated by counting the number of cells at the end of the culture period on an electronic cell counter (Coulter) and expressing the cell count as a percentage of the concurrent vehicle control value. Slides were also prepared to check for the presence, number and quality of cells in metaphase. Selected dose levels were scored for mitotic index.

3.5 Microsomal Enzyme Fraction

Lot No. PB/BNF S9/17/03/01 was prepared in-house at Safepharm Laboratories 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 β -naphthoflavone (100 mg/kg), before S9 preparation. The S9 was stored at -196°C in a liquid nitrogen freezer.

3.6 Culture Conditions

Cultures were established 16 to 72 hours prior to treatment; a nominal concentration of 0.25×10^6 cells were seeded per flask for the 6-hour cultures, 0.15×10^6 cells were seeded per flask for the 24-hour cultures and 0.1×10^6 cells were seeded per flask for the 48-hour cultures. The cells were exposed to at least four doses of the test material, vehicle and positive controls, both with and without metabolic activation. All treatments were performed in duplicate (A + B). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

3.6.1 Experiment 1 - Short Term Exposure Both With and Without Activation

- i) 6 hours exposure to the test material without S9 mix followed by 18 hours culture in treatment-free media prior to cell harvest.
- ii) 6 hours exposure to the test material with S9 mix followed by 18 hours culture in treatment-free media prior to cell harvest.

3.6.2 Experiment 2 - Continuous Exposure Without Activation

- i) 24 hours continuous exposure to the test material prior to cell harvest.
- ii) 48 hours continuous exposure to the test material prior to cell harvest.

3.7 Cell Harvest

Mitosis was arrested by addition of demecolcine (Colcemid 0.1 $\mu\text{g/ml}$) two hours before the required harvest time. After incubation with demecolcine, the cells were trypsinised to detach them from the tissue culture flask and suspended in culture medium. A sample of the cell suspension from each harvest time was counted to estimate growth inhibition at each concentration. The cells were centrifuged, the culture medium drawn off and discarded, and the cells resuspended in 0.075M hypotonic KCl. After ten to fifteen minutes (including five minutes centrifugation), most of the hypotonic solution was drawn off and discarded. The cells were resuspended and then fixed by dropping the cell suspension into fresh methanol/glacial acetic acid (3:1 v/v). The fixative was changed several times and the cells stored at 4°C for sufficient time to ensure complete fixation.

3.8 Preparation of Metaphase Spreads, Staining and Coding

The cells were resuspended in fresh fixative before centrifugation and 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. When the slides were dry they were stained in Giemsa for 5 minutes, rinsed, dried and coverslipped using mounting medium. After checking that the slide preparations were of good quality, they were coded using a computerised random number generator.

3.9 Mitotic Index

A total of 1000 cells 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.

3.10 Scoring of Chromosome Damage

Where possible the first 100 consecutive well-spread metaphases from each culture were counted, and if the cell had 23 to 27 chromosomes, any gaps, breaks or rearrangements were noted according to the simplified system of Savage (1976) recommended in the 1983 UKEMS guidelines for mutagenicity testing (Appendix 4).

Aberrations recorded by the slide scorer were checked by a senior cytogeneticist. Cells with 38 or more chromosomes were classified as polyploid cells and the % incidence of polyploid cells reported. Endoreduplicated cells are recorded and are included in the polyploid cell total number. If there was a dose-related increase in endoreduplicated cells then they are reported separately. The percentage of cells showing structural chromosome aberrations (breaks and exchanges) was calculated and reported. The number of gap-type aberrations was recorded.

3.11 Statistical Analysis

The frequency of cells with aberrations excluding gaps and the frequency of polyploid cells was compared, where necessary, with the concurrent vehicle control value using Fisher's Exact test.

4. ARCHIVES

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

5. RESULTS AND DISCUSSION

5.1 Cell Growth Inhibition Test

The results of the cell counts and the mitotic index of the cell growth inhibition test are presented in sections 5.2 and 6.2 of Appendix 1. In all cases the test material showed no evidence of cell toxicity. No precipitate of the test material was observed at any dose level. Microscopic assessment of the slides prepared from the treatment cultures showed that metaphases were present at all dose levels in all the treatment groups.

The maximum dose level was selected according to the test method guideline and was 1730 µg/ml, which was equivalent to a 10 mM concentration.

5.2 Short Term Treatment Test - Experiment 1

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

Group	Final Concentration of Test Material (µg/ml)
6(18) hour without S9	0*, 216.25, 432.5*, 865*, 1730*, MMC 0.1*
6(18)-hour with S9	0*, 216.25, 432.5*, 865*, 1730*, CP 5.0*

The results of the cell counts and the mitotic indices (MI) from the cultures after their respective exposures are presented as cell growth indices in Form 1, Appendix 2. The test material demonstrated no evidence of toxicity, as was observed in the Cell Growth Inhibition Test.

No precipitate of the test material was observed at the end of the treatment period.

Therefore, the maximum dose level selected for metaphase analysis was the 10 mM concentration of 1730 µg/ml for both the absence and presence of metabolic activation.

* Dose levels selected for metaphase analysis
MMC = mitomycin C
CP = cyclophosphamide

The chromosome aberration data are given in Form 1, Appendix 2. Both of the vehicle control groups 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 satisfactory and the test method itself was operating as expected.

The test material did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of metabolic activation.

The test material did not induce any statistically significant increases in the number of polyploid cells at any dose level in either exposure group.

5.3 Continuous Treatment Test - Experiment 2

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

Group	Final Concentration of Test Material ($\mu\text{g/ml}$)
24-hour	0*, 216.25, 432.5*, 865*, 1730*, MMC 0.05*
48-hour	0*, 216.25, 432.5*, 865*, 1730*, MMC 0.025*

The results of the cell counts from the cultures after their respective exposures are presented as cell growth indices in Form 2, Appendix 2. The test material demonstrated no evidence of toxicity, as was observed in the Cell Growth Inhibition Test.

No precipitate of the test material was observed at the end of the treatment period.

Therefore the maximum dose level selected for metaphase analysis was the 10 mM concentration of 1730 $\mu\text{g/ml}$, for both exposure groups.

The chromosome aberration data are given in Form 2, Appendix 2. Both of the vehicle control groups 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 test method was therefore considered to be operating as expected.

* Dose levels selected for metaphase analysis
MMC = mitomycin C

The test material did not induce any statistically significant increases in the frequency of cells with aberrations in either the 24 or the 48-hour continuous exposure groups.

The test material did not induce any statistically significant increases in the numbers of polyploid cells at any dose level in either exposure group.

6. CONCLUSION

The test material did not induce any statistically significant, dose-related increases in the frequency of cells with chromosome aberrations either in the presence or absence of a liver enzyme metabolising system or after various exposure times. The test material was therefore considered to be non-clastogenic to CHL cells *in vitro*.

7. REFERENCES

- 1) Cairns J (1981) The origin of human cancers, *Nature* **289**, 353 - 357.
- 2) Chandley A C (1981) The origin of chromosomal aberrations in man and their potential for survival and reproduction in the adult human population. *Ann. Genet.*, **24**, 5-11.
- 3) Hollstein M, McCann J, Angelosanto F A, and Nichols W W (1979) Short-term tests for carcinogens and mutagens. *Mutation Res.*, **65**, 133-226.
- 4) Ishidate M and Odashima S (1977) Chromosome tests with 134 compounds on Chinese hamster cells *in vitro* - a screening for chemical carcinogens. *Mutation Res.*, **48**, 337-354.
- 5) Ishidate M and Sofuni T (1985) The *in vitro* chromosomal aberration test using Chinese hamster lung (CHL) fibroblast cells in culture, P.427-432. J. Ashby, F.J. de Serres et al. (Eds.) *Progress in Mutation Research*, Vol. 5. Elsevier, Amsterdam.
- 6) Koyama H *et al* (1970) A new cell line derived from new-born Chinese hamster lung tissue. *Gann*, **61**, 161-167.
- 7) Radman M, Jeggo P, and Wagner R (1982) Chromosomal rearrangement and carcinogenesis. *Mutation Res.*, **98**, 249-264.
- 8) Savage J R K (1976) Annotation: Classification and relationships of induced chromosomal structural changes. *J. Med. Genet.*, **13**, 103-122.

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *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)	N-Acetyl-L-hydroxyproline					
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		000703		
Name of impurities and concentration	-					
CAS No.	-	Vapour pressure		-		
Molecular weight	173.17	Partition coefficient		-		
Melting point (°C)	-	Appearance at ordinary temperature		White crystalline powder		
Boiling point (°C)	-					
Stability	-					
Degree of solubility in solvent	Solvent	Degree of solubility	Stability in solvent	Solvent	Degree of solubility	Stability in solvent
	Water	Soluble at 17.3 mg/ml	-	DMSO	Soluble at 173 mg/ml	-
	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

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

2. KIND OF A CELL LINE - CULTURE CONDITION

Name of Cell Line	CHL	Obtained from	National Institute of Health Science - Cell Bank	
Species	Chinese Hamster (Lung)	Date obtained	11 March 1988	
Medium	Eagles Minimal Essential Media	Manufacturer	GIBCO BRL	
Serum and %	Foetal Bovine (10%)	Manufacturer (lot no.)	PAA (A01920-518)	
Doubling time	17 hr	Freezing condition	-196°C 10% DMSO	
Passage number	Experiment 1: 16 Experiment 2: 17	Culture Condition	Container	25 cm ² TC flask
			Temperature	37°C
			CO ₂	5% (Humidified)
Number of chromosomes (Mode)	25			
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	①. Made in-house	2. Purchase (Supplier)
Prepared on	17 March 2001	
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/ β -naphthoflavone
Sex	Male	Administration method	Oral
Age (in weeks)	7 weeks	Administration period and amount (mg/kg bodyweight)	3 days
Weight	~ 250g		80/100 mg per kg per day

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

(3) Composition of S9-Mix

Constituents	Amount in 1 ml S9-Mix	Constituents	Amount in 1 ml S9-Mix
S9	0.5 ml	NADPH	- μmol
MgCl ₂	8.0 μmol	NADP	5.0 μmol
KCl	33.0 μmol	NADH	- μmol
Glucose-6-phosphate	5.0 μmol	Na-phosphate buffer	100 μmol
		Others ()	- μmol

(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)	5%		
Amount of S9 Protein (final concentration)	1 mg/ml		
Culture time	6 hr		
Culture time after treatment of the test substance	18 hr		
Remarks			

4. PREPARATION OF THE TEST MATERIAL IN SOLUTION

(encircle the applicable response regarding purity conversion)

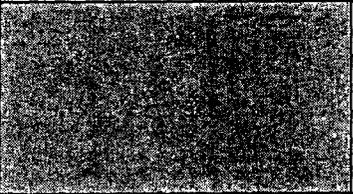
	Name	Supplier	Lot Nos.	Grade	Purity (%)
Solvent used	MEM	Gibco BRL	3054943 3058937	Not applicable	Not applicable
Reason for selection of solvent	Soluble at 17.3 mg/ml				
Appearance of the test material preparation	<u>solution</u>	suspension	others ()		
Suspension and other methods when test substance difficult to dissolve	Vortex				
Storage time and temperature of solution from preparation until use	15 minutes at room temperature: Experiment 1 15 minutes at room temperature: Experiment 2				
Conversion of purity	Yes		<u>No</u>		

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

5. SHORT TERM TREATMENT TEST

(1) Test Condition of Cell Growth Inhibition Test

		Without metabolic activation	With metabolic activation
Period of experiment		From: 21/05/01 To: 24/05/01	From: 21/05/01 To: 24/05/01
Plate	Form	Flask	Flask
	Size	25 cm ²	25 cm ²
	Final volume of medium	5 ml/plate	5 ml/plate
	Number of plates for each concentration	1	1
Cell	Number of cells seeded (final concentration)	0.5 x 10 ⁵ cells/ml	0.5 x 10 ⁵ cells/ml
	Days after initiation of culture	2 days	2 days
Condition of treatment	Added volume of the prepared solution	0.5 ml/plate	0.5 ml/plate
	Added volume of S9 mix		0.5 ml/plate
	Final concentration of S9		5%
	Final concentration of S9 protein		1.0 mg/ml
	Period of treatment	6 hours	6 hours
	Period of recovery	18 hours	18 hours
Determination method of cell growth inhibition	Cell count with Coulter electronic cell counter and Giemsa staining [Fixing solution: methanol:glacial acetic acid (3:1 v/v), staining solution: Gurr's Giemsa R66]		
Remarks:			

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

(2) Results of Cell Growth Inhibition Test

6(18) hours without metabolic activation			6(18) hours with metabolic activation		
Dose Level ($\mu\text{g/ml}$)	Cell Growth Index (%)	Mitotic Index (%)	Dose Level ($\mu\text{g/ml}$)	Cell Growth Index (%)	Mitotic Index (%)
0	100	100	0	100	100
6.76	87	-	6.76	89	-
13.52	89	-	13.52	95	-
27.03	85	-	27.03	95	-
54.06	84	-	54.06	98	-
108.12	90	-	108.12	99	-
216.25	81	-	216.25	92	-
432.5	74	77	432.5	87	126
865	88	88	865	92	99
1730	82	87	1730	96	100

[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 maximum dose level was 1730 $\mu\text{g/ml}$ which was the 10 mM maximum recommended dose level.

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

(3) Test Condition of Chromosome Aberration Test

		Without metabolic activation	With metabolic activation
Period of experiment		From: 20/06/01 To: 23/06/01	From: 20/06/01 To: 23/06/01
Plate	Form	Flask	Flask
	Size	25 cm ²	25 cm ²
	Final volume of medium	5 ml/plate	5 ml/plate
	Number of plates for each concentration	2	2
Cell	Number of cells seeded (final concentration)	0.5 x 10 ⁵ cells/ml	0.5 x 10 ⁵ cells/ml
	Days after initiation of culture	2 days	2 days
Condition of treatment	Added volume of the prepared solution	0.5 ml/plate	0.5 ml/plate
	Added volume of S9 mix		0.5 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: 216.25 to 1730 µg/ml 6(18)h with S9 mix: 216.25 to 1730 µg/ml		

(4) Results of Chromosome Aberration Test

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

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

6. CONTINUOUS TREATMENT TEST

(1) Test Condition of Cell Growth Inhibition Test

Period of experiment		From: 21/05/01 To: 24/05/01	From: 21/05/01 To: 25/05/01
Plate	Form	Flask	Flask
	Size	25 cm ²	25 cm ²
	Final volume of medium	5 ml/plate	5 ml/plate
	Number of plates for each concentration	1	1
Cell	Number of cells seeded (final concentration)	0.4 x 10 ⁵ cells/ml	0.3 x 10 ⁵ cells/ml
	Days after initiation of culture	2 days	2 days
Condition of treatment	Added volume of the prepared solution	0.5 ml/plate	0.5 ml/plate
	Period of treatment	24 hours	48 hours
	Period of recovery	- hours	- hours
Determination method of cell growth inhibition	Cell count with Coulter electronic cell counter and Giemsa staining [Fixing solution: methanol:glacial acetic acid (3:1 v/v), staining solution: Gurr's Giemsa R66]		
Remarks:			

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

(2) Results of Cell Growth Inhibition Test

24 Hour Treatment			48 Hour Treatment		
Dose Level ($\mu\text{g/ml}$)	Cell Growth Index (%)	Mitotic Index (%)	Dose Level ($\mu\text{g/ml}$)	Cell Growth Index (%)	Mitotic Index (%)
0	100	100	0	100	100
6.76	100	-	6.76	113	-
13.52	109	-	13.52	118	-
27.03	104	-	27.03	130	-
54.06	104	-	54.06	140	-
108.12	96	-	108.12	149	-
216.25	108	-	216.25	133	66
432.5	109	153	432.5	135	34
865	107	129	865	140	100
1730	111	163	1730	132	93

[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 maximum dose level was 1730 $\mu\text{g/ml}$ which was the 10 mM maximum recommended dose level.

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

(3) Test Condition of Chromosome Aberration Test

Period of experiment		From: 27/08/01 To: 30/08/01	From: 27/08/01 To: 31/08/01
Plate	Form	Flask	Flask
	Size	25 cm ²	25 cm ²
	Final volume of medium	5 ml/plate	5 ml/plate
	Number of plates for each concentration	2	2
Cell	Number of cells seeded (final concentration)	0.3 x 10 ⁵ cells/ml	0.2 x 10 ⁵ cells/ml
	Days after initiation of culture	2 days	2 days
Condition of treatment	Added volume of the prepared solution	0.5 ml/plate	0.5 ml/plate
	Period of treatment	24 hours	48 hours
	Period of recovery	- hours	- hours
Remarks	The test material dose ranges were as follows: 24-hour without S9-mix: 216.25 to 1730 µg/ml 48-hour without S9-mix: 216.25 to 1730 µg/ml		

(4) Results of Chromosome Aberration Test

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

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

7. JUDGEMENT OF RESULTS AND REFERENTIAL MATTERS

(1) Judgement of the Results

Judgement (Encircle one)		Positive		Negative	
Reason for judgement		The test material did not induce any significant increases in the frequency of cells with aberrations in any of the treatment cases.			
D ₂₀	Structural Aberration	Short term treatment	-S9 mix	- hr treatment	NA
			+S9 mix	- hr treatment	NA
		Continuous treatment	[REDACTED]	- hr treatment	NA
			[REDACTED]	- hr treatment	NA
	Numerical Aberration	Short term treatment	-S9 mix	- hr treatment	NA
			+S9 mix	- hr treatment	NA
Continuous treatment		[REDACTED]	- hr treatment	NA	
		[REDACTED]	- hr treatment	NA	

[Remarks]

D₂₀ value is the presumed dose level of the test substance which is required to induce aberrations in 20% of metaphases. D₂₀ value of the test system judged positive should be noted based upon a type of aberration.

(2) Referential Matters

MEM was selected as the solvent as the test material was readily soluble in it at a 10 mM concentration. The molecular weight of the test material was 173.17 therefore a maximum dose of 1730 µg/ml gave a 10 mM concentration, which was the maximum recommended dose level. No precipitate of the test material was observed at any dose level in any treatment group. The test material was shown to be non-toxic to CHL cells *in vitro* in all treatment cases. The study was conducted according to the requirements of Japanese New Chemical Substance Law (METI).

[Remarks]

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

NA = Not applicable

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

8. OTHERS

Testing Institution	Name	Safeparm Laboratories Ltd.	
	Address	P.O. Box 45, Derby, United Kingdom	Tel No: 0044 1332 792896 Fax No: 0044 1332 799018
Study Director	Title:	Senior Genetic Toxicologist	Signed: 
	Name:	Ms N P Wright BSc (Hons)	
	Years of experience:	16	
Study number	732/092		
Test dates	26 April 2001		26 NOV 2001
	Protocol 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

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *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 "TOX" 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

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 2 Results of Chromosome Aberration Test (continued)
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		Number and Percentages of Cells Showing Numerical Aberrations (%)			
				Observed	ctb	cte	csb	cse	Others	Total		Cell Count %	Mitotic Index (%)	Observed	Polyploids	Others	Total
6	-	Negative Control (Media) 0	A	100	0	0	0	0	0	0	1	100	6.20	107	7	0	7
			B	100	0	0	0	1	0	1	0	100	5.90	105	5	0	5
			Total %	200 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)	1 (0.5)	100 (100)	6.05	212	12	0	12 (5.7)
	-	432.5	A	100	0	0	1	0	0	1	0	101	4.70	100	0	0	0
			B	100	0	0	0	1	0	1	0	116	4.80	109	9	0	9
			Total %	200 (100)	0 (0.0)	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	2 (1.0)	0 (0.0)	109 (79)	4.75	209	9	0	9 (4.3)
	-	865	A	100	1	0	0	2	0	3	0	91	6.10	103	3	0	3
			B	100	1	0	0	0	0	1	0	112	5.70	100	0	0	0
			Total %	200 (100)	2 (1.0)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	4 (2.0)	0 (0.0)	102 (98)	5.90	203	3	0	3 (1.5)
	-	1730	A	100	0	0	2	0	0	2	2	90	6.20	105	5	0	5
			B	100	0	0	0	0	0	0	1	114	4.80	100	0	0	0
			Total %	200 (100)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	0 (0.0)	2 (1.0)	3 (1.5)	102 (91)	5.50	205	5	0	5 (2.4)
	-	Positive Control (MMC) 0.1	A	50	10	20	2	0	0	24	6	50	3.40	51	1	0	1
			B	100	16	22	2	1	0	35	12	50	3.20	103	3	0	3
			Total %	150 (100)	26 (17.3)	42 (28.0)	4 (2.7)	1 (0.7)	0 (0.0)	59*** (39.3)	18 (12.0)	50 (55)	3.30	154	4	0	4 (2.6)

MMC = Mitomycin C
 *** = p < 0.001

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 2 Results of Chromosome Aberration Test (continued)
Form 1 Short Term Treatment Test - Experiment 1 With Metabolic Activation (S9)

Treatment Period (hours)	S9 mix	Concentration µg/ml		Number and Percentages of Cells Showing Structural Chromosome Aberrations (%)							g	Cell Growth Index		Number and Percentages of Cells Showing Numerical Aberrations (%)			
				Observed	ctb	cte	csb	cse	Others	Total		Cell Count %	Mitotic Index (%)	Observed	Polyploids	Others	Total
6	+	Negative Control (Media) 0	A	100	0	0	0	1	0	1	0	100	4.90	108	8	0	8
			B	100	0	1	0	0	0	1	1	100	6.70	105	5	0	5
			Total	200	0	1	0	1	0	2	1	5.80	213	13	0	13	
			%	(100)	(0.0)	(0.5)	(0.0)	(0.5)	(0.0)	(1.0)	(0.5)	100	(100)				(6.1)
	+	432.5	A	100	1	0	0	0	0	1	0	112	6.40	100	0	0	0
			B	100	0	0	0	1	0	1	0	106	4.00	101	1	0	1
			Total	200	1	0	0	1	0	2	0	5.20	201	1	0	1	
			%	(100)	(0.5)	(0.0)	(0.0)	(0.5)	(0.0)	(1.0)	(0.0)	109	(90)				(0.5)
	+	865	A	100	0	0	1	0	0	1	1	107	4.9	103	3	0	3
			B	100	0	0	0	1	0	1	2	101	7.8	100	0	0	0
			Total	200	0	0	1	1	0	2	3	6.4	203	3	0	3	
			%	(100)	(0.0)	(0.0)	(0.5)	(0.5)	(0.0)	(1.0)	(1.5)	104	(109)				(1.5)
	+	1730	A	100	1	0	0	1	0	2	2	110	4.90	105	5	0	5
			B	100	0	0	0	1	0	1	0	104	3.50	106	6	0	6
			Total	200	1	0	0	2	0	3	2	4.20	211	11	0	11	
			%	(100)	(0.5)	(0.0)	(0.0)	(1.0)	(0.0)	(1.5)	(1.0)	107	(72)				(5.2)
	+	Positive Control (CP) 5	A	50	13	17	7	0	0	30	7	51	1.50	51	1	0	1
			B	50	11	15	2	5	0	24	9	52	1.30	53	3	0	3
Total			100	24	32	9	5	0	54***	16	1.40	104	4	0	4		
%			(100)	(24.0)	(32.0)	(9.0)	(5.0)	(0.0)	(54.0)	(16.0)	52	(24)				(3.8)	

CP = Cyclophosphamide
*** = p < 0.001

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 2 Results of Chromosome Aberration Test (continued)

Form 2 Continuous Treatment Test - Experiment 2

Treatment Period (hours)	S9 mix	Concentration µg/ml		Number and Percentages of Cells Showing Structural Chromosome Aberrations (%)							g	Cell Growth Index		Number and Percentages of Cells Showing Numerical Aberrations (%)			
				Observed	ctb	cte	csb	cse	Others	Total		Cell Count %	Mitotic Index (%)	Observed	Polyploids	Others	Total
24	-	Negative Control (Media) 0	A	100	2	0	2	1	0	4	0	100	3.10	102	2	0	2
			B	100	0	0	0	0	0	0	0	100	5.60	100	0	0	0
			Total	200	2	0	2	1	0	4	0	0	4.35	202	2	0	2
			%	(100)	(1.0)	(0.0)	(1.0)	(0.5)	(0.0)	(2.0)	(0.0)	100	(100)				
	-	432.5	A	100	1	0	3	0	0	4	0	112	3.90	101	1	0	1
			B	100	0	0	0	1	0	1	1	116	3.20	100	0	0	0
			Total	200	1	0	3	1	0	5	1	1	3.55	201	1	0	1
			%	(100)	(0.5)	(0.0)	(1.5)	(0.5)	(0.0)	(2.5)	(0.5)	114	(82)				
	-	865	A	100	0	0	0	1	0	1	0	95	3.90	100	0	0	0
			B	100	1	0	1	2	0	4	1	105	3.40	101	1	0	1
			Total	200	1	0	1	3	0	5	1	1	3.65	201	1	0	1
			%	(100)	(0.5)	(0.0)	(0.5)	(1.5)	(0.0)	(2.5)	(0.5)	100	(84)				
	-	1730	A	100	0	0	1	0	0	1	0	103	3.60	100	0	0	0
			B	100	1	1	0	2	0	3	0	105	4.60	100	0	0	0
			Total	200	1	1	1	2	0	4	0	0	4.10	200	0	0	0
			%	(100)	(0.5)	(0.5)	(0.5)	(1.0)	(0.0)	(2.0)	(0.0)	104	(94)				
-	Positive Control (MMC) 0.05	A	50	8	2	3	1	0	14	12	73	3.40	50	0	0	0	
		B	50	4	9	8	2	0	21	9	85	3.70	50	0	0	0	
		Total	100	12	11	11	3	0	35***	21	3.55	100	0	0	0		
		%	(100)	(12.0)	(11.0)	(11.0)	(3.0)	(0.0)	(35.0)	(21.0)	79	(82)					(0.0)

MMC = Mitomycin C

*** = p < 0.001

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 2 Results of Chromosome Aberration Test (continued)

Form 2 Continuous Treatment Test - Experiment 2

Treatment Period (hours)	S9 mix	Concentration µg/ml		Number and Percentages of Cells Showing Structural Chromosome Aberrations (%)							g	Cell Growth Index		Number and Percentages of Cells Showing Numerical Aberrations (%)			
				Observed	ctb	ctc	csb	cse	Others	Total		Cell Count %	Mitotic Index (%)	Observed	Polyploids	Others	Total
48	-	Negative Control (Media) 0	A	100	0	0	1	0	0	1	0	100	2.90	100	0	0	0
			B	100	0	0	1	0	0	1	1	100	1.20	101	1	0	1
			Total %	200	0	0	2	0	0	2	1	100	2.05	201	1	0	1
				(100)	(0.0)	(0.0)	(1.0)	(0.0)	(0.0)	(1.0)	(0.5)	(100)					(0.5)
	-	432.5	A	100	1	0	2	2	0	5	1	93	2.30	102	2	0	2
			B	100	0	0	0	0	0	0	0	86	1.50	100	0	0	0
			Total %	200	1	0	2	2	0	5	1	90	1.90	202	2	0	2
				(100)	(0.5)	(0.0)	(1.0)	(1.0)	(0.0)	(2.5)	(0.5)	(93)					(1.0)
	-	865	A	100	2	0	3	1	0	5	2	95	1.50	102	2	0	2
			B	100	0	1	0	1	0	2	1	92	1.80	100	0	0	0
			Total %	200	2	1	3	2	0	7	3	94	1.65	202	2	0	2
				(100)	(1.0)	(0.5)	(1.5)	(1.0)	(0.0)	(3.5)	(1.5)	(80)					(1.0)
	-	1730	A	100	1	0	1	1	0	3	1	88	2.40	102	2	0	2
			B	100	0	1	0	0	0	1	2	92	3.20	100	0	0	0
			Total %	200	1	1	1	1	0	4	3	90	2.80	202	2	0	2
				(100)	(0.5)	(0.5)	(0.5)	(0.5)	(0.0)	(2.0)	(1.5)	(137)					(1.0)
	-	Positive Control (MMC) 0.025	A	100	11	3	3	0	0	15	8	77	4.40	100	0	0	0
			B	100	3	6	0	1	0	10	6	83	3.40	100	0	0	0
Total %			200	14	9	3	1	0	25***	14	80	3.90	200	0	0	0	
			(100)	(7.0)	(4.5)	(1.5)	(0.5)	(0.0)	(12.5)	(7.0)	(190)					(0.0)	

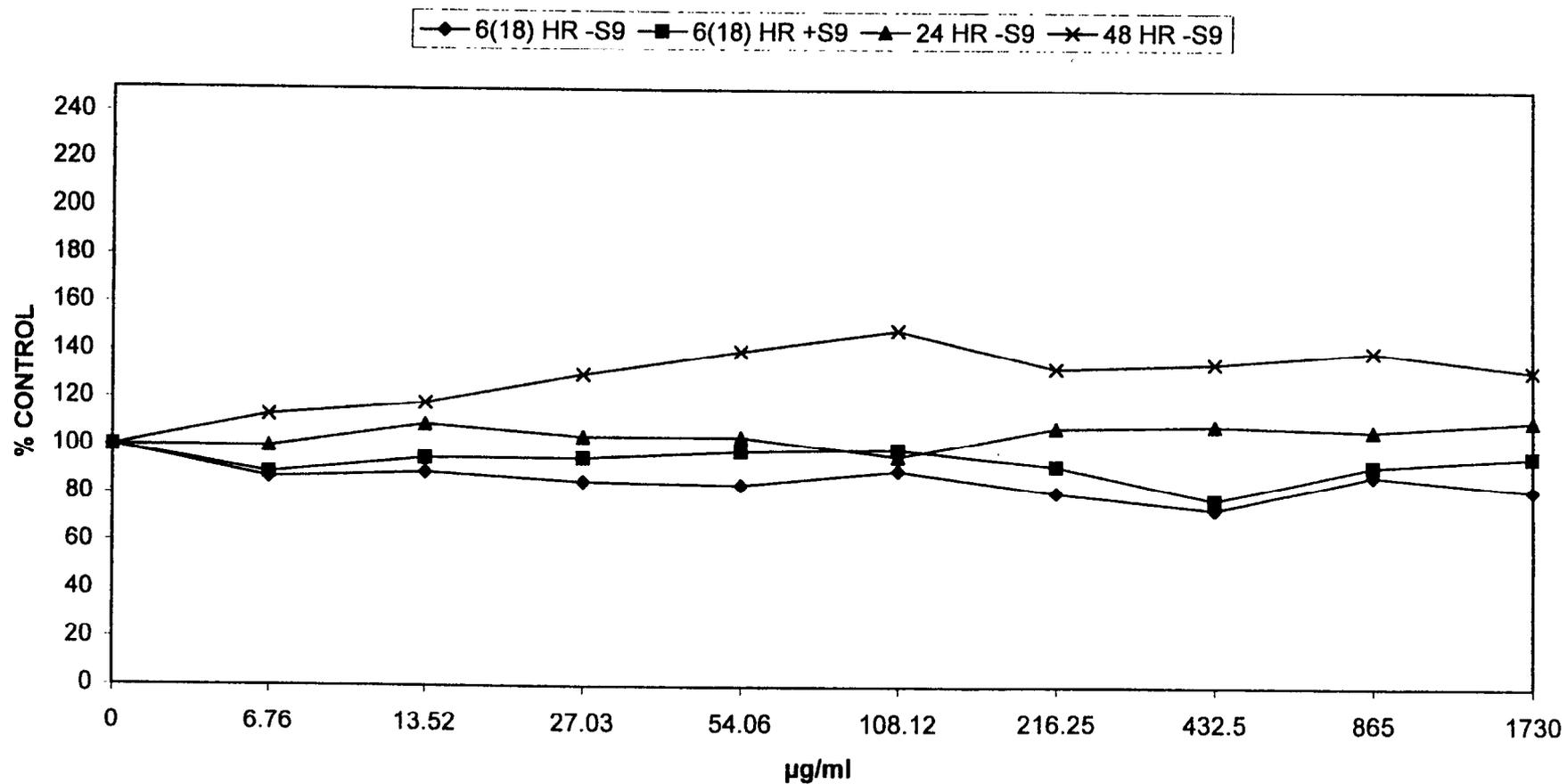
MMC = Mitomycin C

*** = p < 0.001

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 3 Dose Response Curves

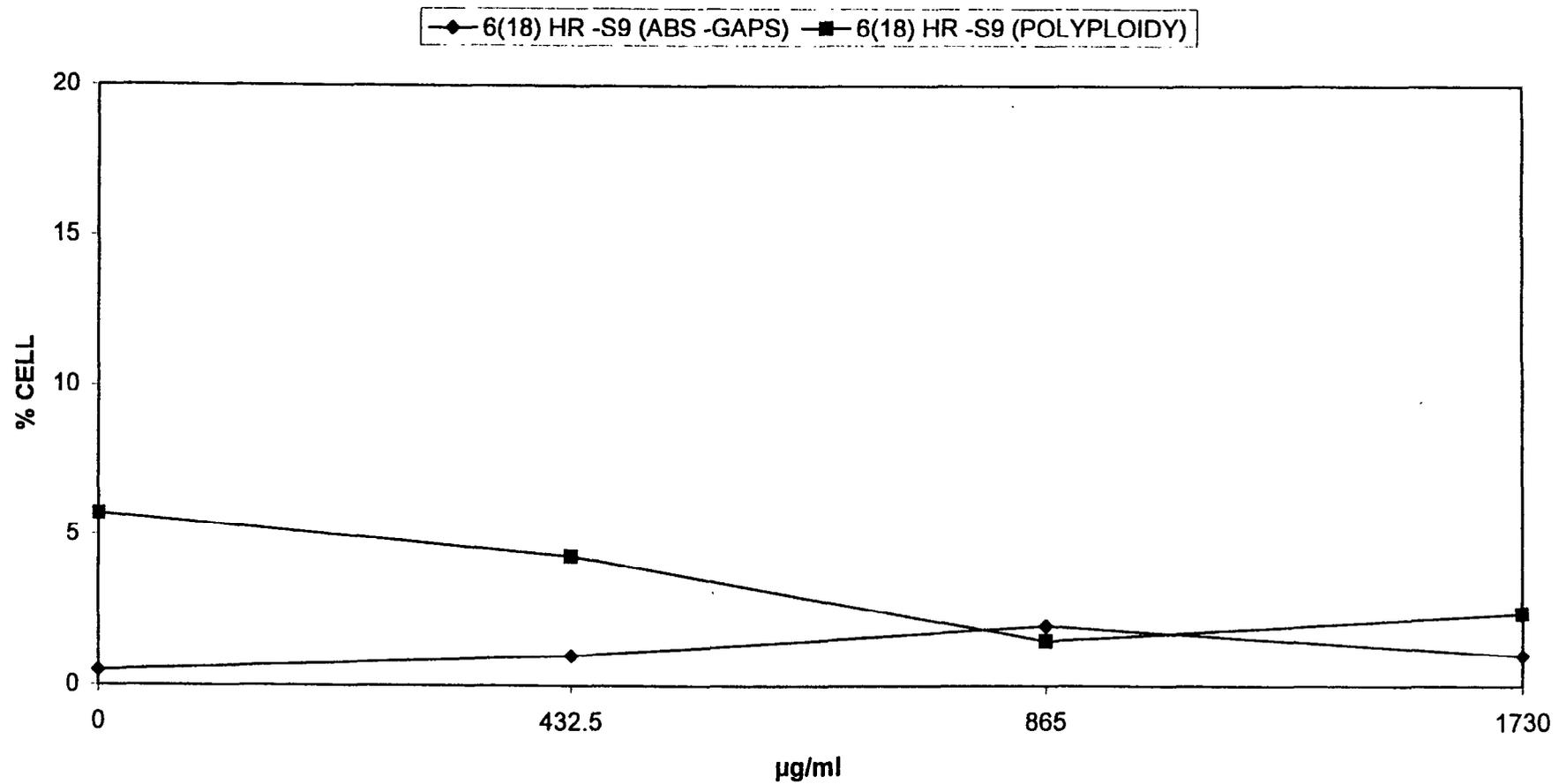
Figure 1 Cell Growth Inhibition Test



N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 3 Dose Response Curves (continued)

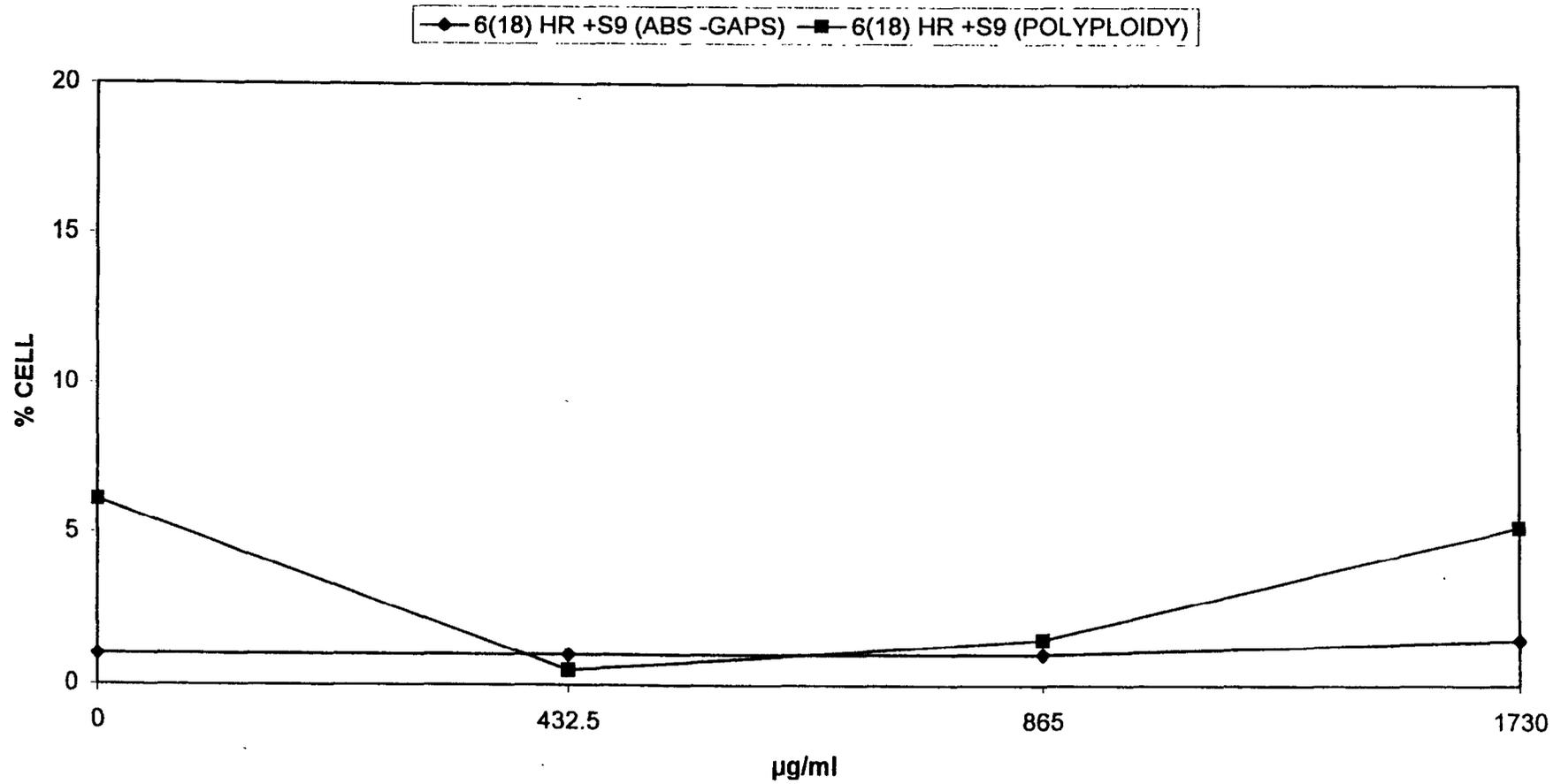
Figure 2 Chromosome Aberration Test - Experiment 1 Without S9



N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 3 Dose Response Curves (continued)

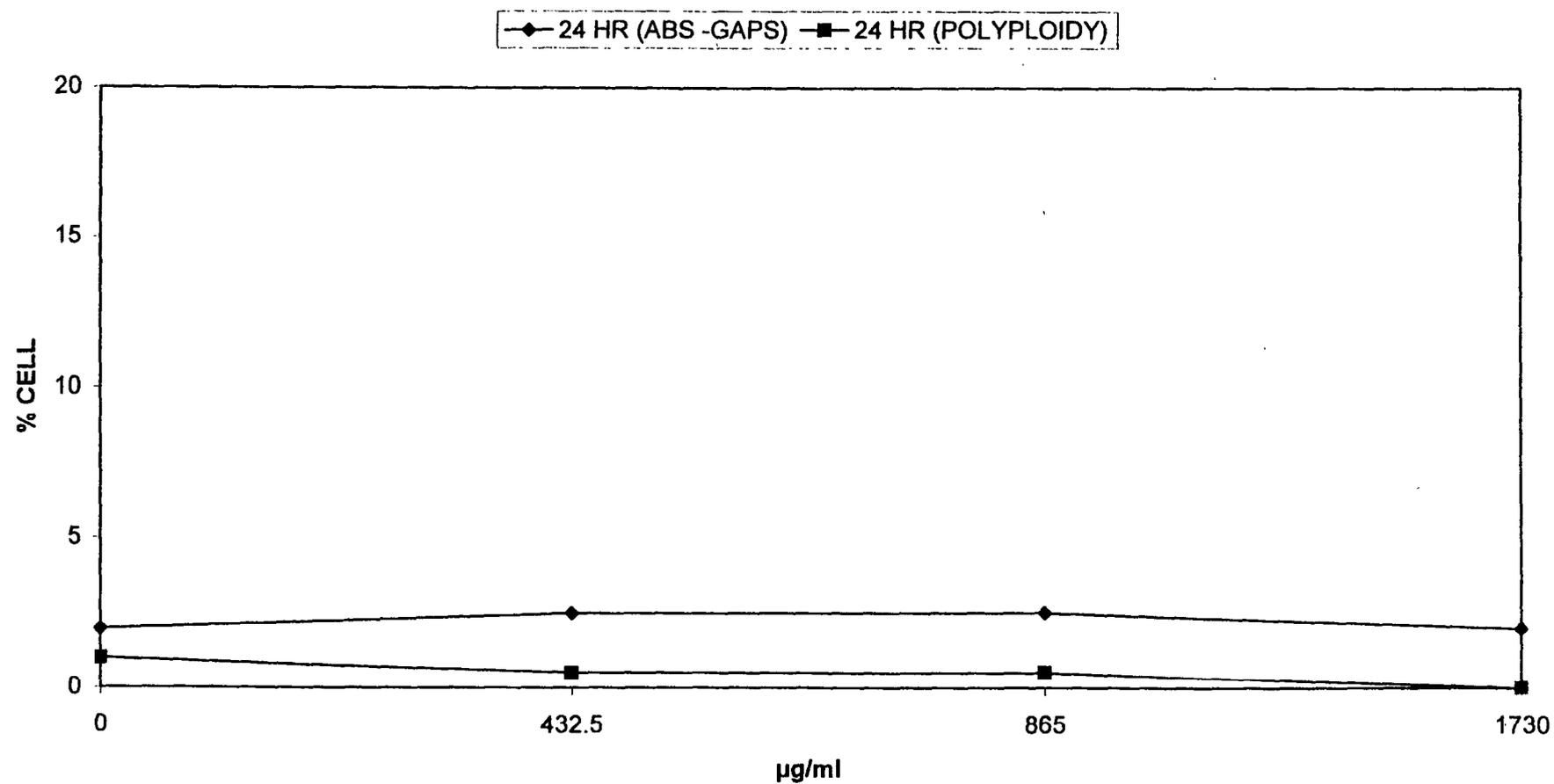
Figure 3 Chromosome Aberration Test - Experiment 1 With S9



N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 3 Dose Response Curves (continued)

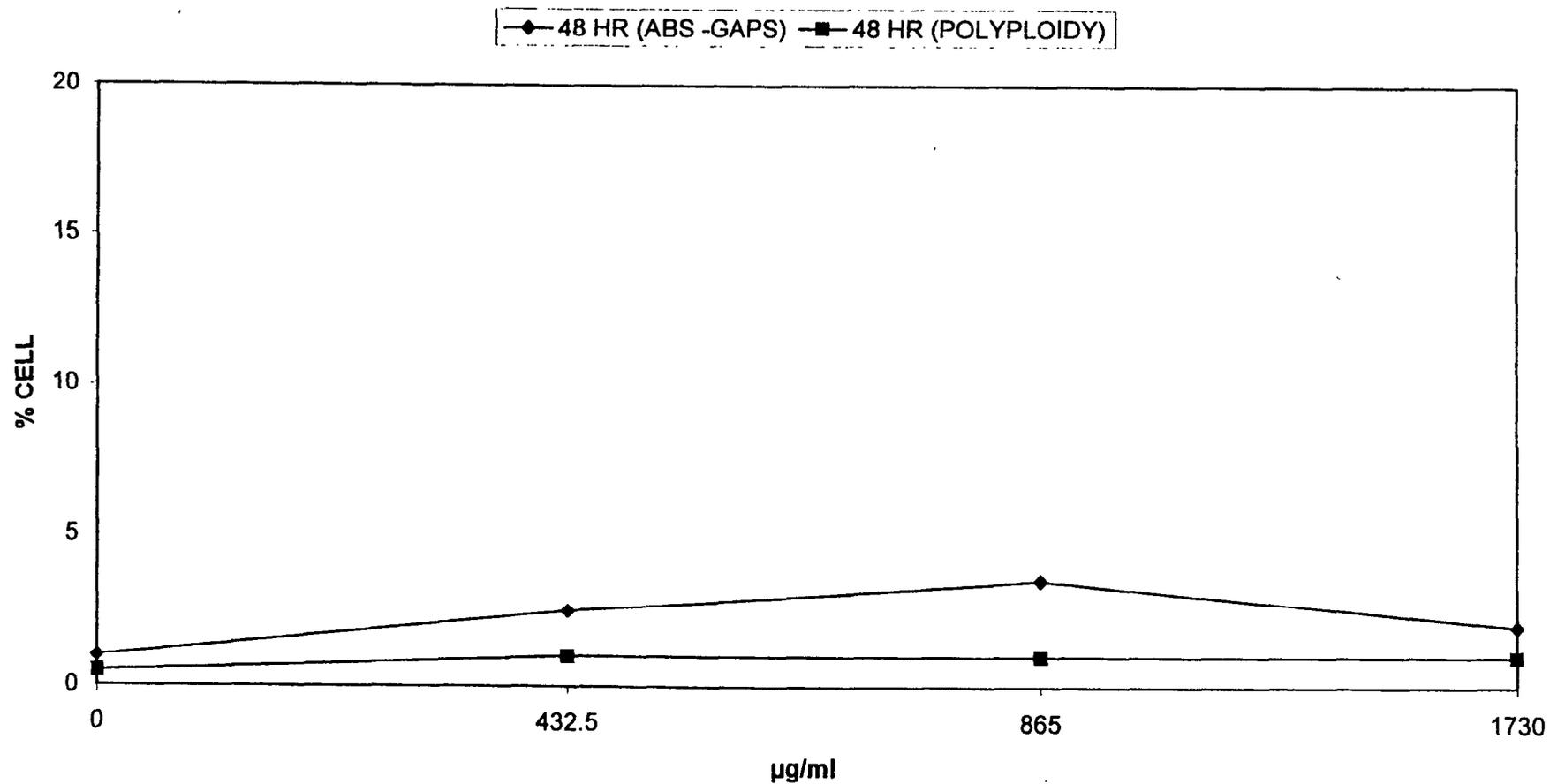
Figure 4 Chromosome Aberration Test - Experiment 2 (24-Hour)



N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

Appendix 3 Dose Response Curves (continued)

Figure 5 Chromosome Aberration Test - Experiment 2 (48 Hour)



N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *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.

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO*

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

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 between 23 and 27 inclusive then it is classified as a diploid cell and scored for aberrations. If less than 23 chromosomes are counted then the cell is ignored under the assumption that some chromosomes may have been lost for technical reasons. If 38 chromosomes or more are scored 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 the CHL cell line have established a range of aberration frequencies acceptable for control cultures. The current in-house historical aberration ranges are presented below:

	24 Hour	48 Hour	6 Hour -S9	6 Hour +S9
	% Cells with Aberrations (-gaps)			
Minimum	0	0	0	0
Maximum	3.0	4.5	4.0	4.5
Mean	0.58	0.63	0.66	1.10
Standard Deviation	0.79	0.84	0.79	0.98
Number of experiments	85	86	86	86

N-Acetyl-L-hydroxyproline: CHROMOSOME ABERRATION TEST IN CHL CELLS *IN VITRO***Appendix 4 Chromosome Structural Aberrations: Classification, Evaluation Criteria and Historical Control Data (continued)****2.2 Evaluation Criteria**

In all circumstances where increases in the frequency of cells with aberrations are seen, statistical comparisons will be made with the vehicle. 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 88/320/EEC

**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM****GOOD LABORATORY PRACTICE****STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC****LABORATORY**

**SafePharm Laboratories Ltd
Shardlow Business Park
London Road
Shardlow
Derbyshire
DE72 2GD**

TEST TYPE

**Analytical Chemistry
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Tests
Toxicology**

DATE OF INSPECTION

28 February 2000

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

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

Roger G. Alexander
26/4/00

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

SAFEPHARM LABORATORIES LIMITED

N-Acetyl-L-hydroxyproline:

**CHROMOSOME ABERRATION TEST IN
CHL CELLS *IN VITRO***

SPL PROJECT NUMBER: 732/092

I verify that this is an exact copy of the original report which is located in the Archives of SafePharm Laboratories Limited, Derby, UK.

Ms N P Wright BSc (Hons)
Study Director



29 NOV 2001

DATE