

STUDY TO EVALUATE THE CHROMOSOME
DAMAGING POTENTIAL OF UVINUL T 150
BY ITS EFFECTS IN THE PRESENCE
OF ULTRA VIOLET LIGHT ON
CULTURED CHINESE HAMSTER OVARY (CHO) CELLS
USING AN *IN VITRO* CYTOGENETICS ASSAY

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STUDY DIRECTOR'S STATEMENT

I, the undersigned, declare that this study was carried out according to the principles of Good Laboratory Practice set out by the Department of Health (London), United Kingdom Compliance Programme, 1989.

I further declare that this report constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of this study.

STUDY DIRECTOR:



DATE: 27-7-94

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QUALITY ASSURANCE STATEMENT

I hereby confirm that this report is a full and accurate representation of the study data. The procedures involved in this type of study are regularly inspected. Dates of inspections of critical phases/procedures relevant to this study and dates of the findings observed during the inspections and reported to the Study Director are listed. All findings are reported to Management within 3 weeks of inspection. The date of the report audit is also stated.

Hazleton Europe will be responsible for archiving (at Otley Road, Harrogate, North Yorkshire) raw data, reports and relevant specimens connected with the study, unless otherwise requested by the Sponsor, for a maximum of 10 years. Following this period, the situation regarding continued archive facilities will be discussed with the Sponsor.

QUALITY ASSURANCE OFFICER: *Clare Winter*.....

DATE: *27 July 1994*

Date(s) of inspections:

16 February 1994

Date(s) of findings
reported to Study Director:

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1 SUMMARY

Uvinul T 150 was tested in an *in vitro* cytogenetics assay for its effects on the chromosomes of CHO cells in combination with irradiation by filtered or unfiltered UV light. The effects of the test chemical on mitotic index in the absence of light were first investigated in a preliminary range-finding experiment using a broad range of doses, separated by narrow intervals. The highest dose level used, 80 µg/ml was close to (just above) the solubility limit of Uvinul T 150 in culture medium. Treatment was continuous until harvest (20 hours). Negligible mitotic inhibition was seen at the highest dose tested and a maximum concentration of 80 µg/ml was selected for the main study.

In the main study, cultures were treated with Uvinul T 150 for 2 hours during which they were irradiated with 200 mJ/cm² UVA, 33 mJ/cm² UVB (unfiltered) or 700 mJ/cm² UVA (glass-filtered) light. Cultures were harvested 18 hours later.

Appropriate negative control cultures were included in the test system. The proportion of cells with structural aberrations in unirradiated and irradiated solvent control cultures, unirradiated 8-methoxypsoralen (8-MOP) treated cultures and unirradiated test chemical treated cultures in the main study had frequencies of cells with chromosome aberrations which fell within the historical negative control range. In contrast, positive control treatments (4-nitroquinoline 1-oxide [NQO], 8-MOP + light) induced large increases in the incidence of aberrant cells indicating that the test system was functioning effectively.

Treatment of cultures with concentrations of Uvinul T 150 up to 80 µg/ml in the presence of ultra violet light did not result in significant increases in the incidence of cells with chromosome aberrations when compared with concurrent negative (solvent) controls. Frequencies of cells with aberrations fell within the normal range in all treated cultures.

It is concluded that Uvinul T 150 was not photoactivated to a clastogenic form detectable in this assay.

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2 INTRODUCTION

The European Scientific Committee for Cosmetology (SCC) has recommended the *in vitro* testing of sunscreens for photomutagenic potential (1). These requirements have been explained and summarized by Loprieno (2). In response to these suggestions, published by the SCC, Molecular Toxicology (Microtest) have developed protocols for the testing of sunscreens for photomutagenicity (3,4), this report describes the chromosome aberration assay.

Chromosome defects are recognised as being the basis of a number of human genetic diseases (5). While there are a number of assays which detect genotoxic chemicals, there are relatively few that detect chemicals which either interfere with the process of mitosis or cause chromosome aberrations.

No one assay has been extensively evaluated on the same compounds in several laboratories (6) but there is a large database on the use of chromosomal assays for screening purposes. The use of CHO cells (a permanent line originally derived from a biopsy of Chinese hamster ovary) is recommended because the cells have a low number of chromosomes making scoring relatively easy (7), the modal chromosome number for the clone of CHO cells used at Molecular Toxicology (Microtest) being 21.

As some test chemicals may become potent clastogens when photosensitised (8,9), the clastogenic potential of Uvinul T 150 was assessed by its effects on the chromosomes of CHO cells treated in the absence and presence of UV light, both unfiltered and filtered through 3 mm of glass.

3 MATERIALS

3.1 Test chemical

Uvinul T 150, batch number 08-0083 (ZHT number 93/246), was an off-white powder. It was received on 3 February 1994 and stored desiccated and refrigerated in the dark. Purity was stated as 97%. Determinations of stability and characteristics of the test chemical were the responsibility of the Sponsor.

Preliminary solubility data indicated that Uvinul T 150 was soluble in dimethyl sulphoxide (DMSO) at a concentration of approximately 450 mg/ml following sonication, vortexing and warming to 37°C. Dilutions from this solution in DMSO were further diluted 100-fold into culture medium. Limited precipitation was observed at a final concentration of approximately 60 µg/ml and a maximum concentration of 80 µg/ml was selected for the range-finder.

Test chemical stock solutions were prepared by dissolving (with warming to 37°C as appropriate) Uvinul T 150 in anhydrous analytical grade DMSO to give 8 mg/ml. The stock solutions were membrane filter-sterilized (Sartorius Minisart SRP, pore size 0.2 µm) and dilutions made using sterile DMSO. The test chemical solutions were then used (in the main study) within 1½ hours of initial dissolution as follows:

Experiment	Concentration of treatment solutions (mg/ml)	Final concentration (µg/ml)
Range-finder	0.5498	5.498
	0.6872	6.872
	0.8590	8.590
	1.074	10.74
	1.342	13.42
	1.678	16.78
	2.097	20.97
	2.621	26.21
	3.277	32.77
	4.096	40.96
	5.120	51.20 P
	6.400	64.00 P
8.000	80.00 P	
Main study	3.277	32.77
	4.096	40.96
	5.120	51.20 P
	6.400	64.00 P
	8.000	80.00 P

P = limited precipitation observed

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3.2 Controls

Negative controls were treated with the solvent as described in 4.1. Additional negative controls were treated with 8-MOP in the absence of UV irradiation. The positive control chemicals were dissolved in sterile anhydrous analytical grade dimethyl sulphoxide immediately prior to use, as follows:

Chemical	Source	Concentration of treatment solution (mg/ml)	Final concentration ($\mu\text{g/ml}$)*	UV light
4-nitroquinoline 1-oxide (NQO)	Fluka Chemical Co, Glossop, UK	0.00625	0.0625	-
		0.0125	0.125	
		0.0250	0.250	
8-methoxypsoralen (8-MOP)	Sigma Chemical Co, Poole, Dorset, UK.	0.3125	3.125	+
		0.6250	6.250	
		1.250	12.50	

* Cells treated with 0.25 μg NQO/ml and 3.125 μg 8-MOP/ml gave satisfactory preparations which were analysed.

3.3 UV irradiation

Irradiation of cultures was performed using the apparatus described in Figure 1. Flasks (25 cm²) were exposed either directly or through a sheet of 3 mm glass. (The absorbance spectrum of the glass is shown in Appendix 2, that of tissue culture flask plastic is shown in Appendix 3). The light source was calibrated using an Osram Centra UV meter before and after each experiment. Measurements were taken through culture medium and flasks and the results used to calculate the doses delivered as described in Appendix 1.

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The doses used for irradiation were as follows:

Experiment	Duration of treatment		mJ/cm ²		
	min	sec	UVA	UVB	UVA + glass
Range-finder	-	-	-	-	-
Main study	9	31	200	33	-
	36	50	-	-	700

3.4 Cell cultures

CHO cells, supplied by Dr S Galloway, West Point, PA, USA, are maintained at Hazleton Microtest in tissue culture flasks containing McCoy's 5A medium including 10% (v/v) foetal calf serum (FCS), and 100 µg/ml gentamycin. They are subcultured regularly at low density, and before overgrowth occurs, to maintain low aberration frequencies, the range for which is shown in Appendix 4. Stocks of cells preserved in liquid nitrogen were reconstituted for each experiment so as to maintain karyotypic stability. The cells were screened for mycoplasma contamination.

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4 METHODS

4.1 Treatment

Cell sheets were removed from stock cultures using EDTA solution and subcultured at low density in 25 cm² tissue culture flasks. After one day of incubation in an atmosphere of 5% (v/v) CO₂ in air and at 37°C, cultures were of acceptably low confluence and considered suitable for treatment.

For the range-finder, duplicate cultures were treated with the solvent and single cultures with test chemical as detailed in 3.1. All cultures then received 0.25 ml KCl buffer. Cells were incubated for a further 20 hours before harvesting.

For the main study, 9 or 6 cultures (solvent or chemical treatments as appropriate: triplicate or duplicate cultures without light, with filtered light and with unfiltered light) were treated with the solvent or with the test chemical as detailed in 3.1 (0.05 ml per culture). Additional duplicate cultures were treated with the positive control chemical NQO and 6 (see above) flasks with 8-MOP as detailed in 3.2. Cultures then received 0.25 ml KCl buffer. Flasks were treated and irradiated in groups as detailed in Appendix 5. All flasks were exposed to light, at least 15 minutes but no more than 2 hours after addition of chemical treatment to ensure medium change occurred on time and to permit equilibration of the chemicals into the cells. Flasks were kept in the incubator before and after irradiation.

After 2 hours of treatment at 37°C, culture media were removed and cells washed and re-fed with fresh McCoy's 5A medium containing FCS and gentamycin. They were then incubated for approximately 18 hours before harvesting.

4.2 Harvesting

One and a half hours prior to harvest, colchicine was added at a final concentration of approximately 1 µg/ml to arrest dividing cells in metaphase. Cells were removed from flasks using EDTA. The suspension from each flask was transferred to a plastic centrifuge tube and the cells

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pelleted by centrifuging at 200 x 'g' for 5 minutes. The supernatant was carefully removed and the cells were resuspended in 4 ml of 0.075 M KCl at 37°C for 5 minutes to allow swelling to occur.

Cells were fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed several times by centrifugation (initially at 200 x 'g' for 5 minutes and then at 1250 x 'g' for 2-3 minutes) and resuspension.

4.3 Preparation of metaphase spreads

The cells were kept refrigerated in fixative before slides were prepared but slides were not made on the day of harvest to ensure cells were adequately fixed. Cells were pelleted and resuspended in a minimal amount of fresh fixative so as to give a milky suspension. A few drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance spreading, and 3 or 4 drops of suspension were dropped on to clean microscope slides which had been dipped in water.

After the slides had been dried, the cells were stained for 5 minutes in 4% (v/v) Giemsa in pH 6.8 buffer. The slides were then rinsed, dried and mounted with coverslips.

4.4 Rationale for dose selection

Slides from the range-finder cultures were examined uncoded. A selection of random fields were observed, using a x40 objective, from enough treatments to determine whether chemically induced mitotic inhibition had occurred. This was defined as a clear decrease in the percentage of cells in mitosis (MI, based on 1000 cells counted, where possible), preferably dose-related. The results from the range-finder were used to select doses for the main study.

Slides from the main study were examined as described above. The top dose for analysis was to be one at which a 50-80% reduction in MI occurred. If this could not be identified, the solubility limit or highest non-inhibitory concentration was to be used. Mitotic index data and the results of dose selection are presented in 5.1.

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4.5 Scoring of aberrations

Twenty-five cells from each of the selected NQO and 8-MOP positive control treatments (see 3.2) were analysed to ensure that the system was operating satisfactorily. Slides from the selected treatments and from negative controls were coded by a person not connected with the scoring of the slides. Sticky labels were used to cover any identification marks on the slides so that the cytogeneticists could only see the study reference number and the code.

One hundred metaphases from each culture were analysed for chromosome aberrations. The modal number of this CHO line is 21; cells with 19-23 chromosomes were considered acceptable for scoring although any cell with more than 23 chromosomes, that is polyploid, endoreduplicated and hyperdiploid cells, was noted and recorded separately. Aberrations were classified according to the scheme described by ISCN (10) and detailed in Appendix 6. Observations were recorded on raw data sheets (summarised in Tables 1-3) kept within the study file and the microscope stage coordinates of any cell with one or more aberrations were recorded.

4.6 Analysis of results

4.6.1 Treatment of data

After completion of scoring and decoding of slides, the numbers of aberrations of each type in each culture were totalled. These data were then summarised into 3 categories as follows:

- 1 cells with structural aberrations including gaps
- 2 cells with structural aberrations excluding gaps
- 3 polyploid, endoreduplicated and hyperdiploid cells.

The category totals for negative control cultures were used to determine whether the assay is acceptable or not (see 4.6.2). The proportions of aberrant cells in each replicate were used to establish acceptable homogeneity between replicates by means of a binomial dispersion test (11).

The proportion of cells in category 2 for each treatment condition was compared with the proportion in negative controls by using Fisher's exact test (11). Probability values of $p \leq 0.05$ are

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accepted as significant. The proportions of cells in categories 1, 2 and 3 were examined in relation to historical control ranges.

4.6.2 Acceptance criteria

The assay is usually considered valid if the following criteria are met:

- 1) the binomial dispersion test provides evidence of acceptable homogeneity between replicate cultures, particularly where no positive responses are seen
- 2) the proportion of cells with structural aberrations (without gaps) in negative control cultures falls within the normal range, and the percentage of polyploid/endoreduplicated/hyperdiploid cells is <10%
- 3) at least 160 cells out of an intended 200 are analyzable for each treatment condition
- 4) the positive control treatments induce statistically significant increases in the proportion of cells with structural aberrations.

4.6.3 Evaluation criteria

A test chemical is considered to be photosensitised to a mutagen if:

- 1) statistically significant increases in the proportion of cells with structural aberrations occur at one or more treatment combinations
- 2) the incidence of aberrations at such data points exceeds the normal range
- 3) significant chromosome aberrations are induced in the presence of UV but not in its absence
- 4) cells with chromosomal aberrations occur with significantly higher frequencies, or at lower doses, in the presence of UV.

Increased incidences of gaps and/or numerical aberrations, or increased numbers of structural aberrations not exceeding the normal range, or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal" or "probably of no biological importance". Any positive responses are more likely to lead to confident conclusions if they are dose-related and occur at non-toxic doses.

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5 **RESULTS**

5.1 **Selection of doses**

The mitotic index determination for the treatments in the range-finder and main study experiments are shown below:

Experiment	Treatment ($\mu\text{g/ml}$)	Mitotic index (%)					
		No light		200 mJ/cm ² UVA 33 mJ/cm ² UVB		700 mJ/cm ² UVA ⁺	
	Replicate	A	B	A	B	A	B
Range-finder	5.498	2.8	3.3	-	-	-	-
	6.872	NS	NT	-	-	-	-
	8.59	NS	NT	-	-	-	-
	10.74	NS	NT	-	-	-	-
	13.42	NS	NT	-	-	-	-
	16.78	NS	NT	-	-	-	-
	20.97	NS	NT	-	-	-	-
	26.21	NS	NT	-	-	-	-
	32.77	NS	NT	-	-	-	-
	40.96	NS	NT	-	-	-	-
	51.2	NS	NT	-	-	-	-
	64	NS	NT	-	-	-	-
80	2.9	NT	-	-	-	-	
Main study	Solvent	8.2	7.8	5.8	6.3	8.6	8.4
	32.77	NS	NS	NS	NS	NM	NM
	40.96	NS	NS	NS	NS	NM	NM
	51.2	3.0	6.5	6.7	5.4	7.4	9.7
	64	6.9	6.3	8.5	7.2	4.7	7.3
	80	6.0	7.1	8.4	7.2	9.4	10.6

NS = not scored NT = not treated NM = not made

No clear evidence of mitotic inhibition was apparent at the highest dose level in the range-finder and a reduced dose range was selected for the main study. Marked mitotic inhibition was not clearly apparent under any treatment conditions in this study.

5.2 **Chromosome aberration assay**

5.2.1 **Slide codes**

These are detailed in Appendix 7.

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5.2.2 Raw data

The raw data for the observations on the test agent plus positive and negative controls are retained at Hazleton Europe and are summarised in Table 1 for treatments in the absence of light and in Tables 2 and 3 for treatments in the presence of unfiltered and glass-filtered light respectively. Appendices 8 and 9 show a detailed breakdown of the aberrations observed.

5.2.3 Validity of study

Acceptance criteria for the assay are listed in 4.6.2. The data in Tables 1 to 3 and Appendices 9 and 10 confirm that:

- 1) the binomial dispersion test provided evidence of acceptable homogeneity between replicate cultures
- 2) the proportion of cells with structural aberrations (without gaps) in negative control cultures fell within the normal range and the percentage of polyploid/endoreduplicated/hyperdiploid cells was <10%
- 3) at least 160 cells out of an intended 200 were analysable for each treatment condition
- 4) the positive control treatments NQO, and 8-MOP with light, induced large and statistically significant increases in numbers of aberrations.

5.2.4 Analysis of data

Structural aberrations

Cultures treated with Uvinul T 150 in the absence of light had frequencies of cells with aberrations which were similar to and not significantly different from those seen in concurrent solvent controls (Table 1).

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In the presence of 200 mJ/cm² UVA, 33 mJ/cm² UVB, there was no significant increase in the proportion of cells with structural chromosome aberrations (Table 2).

Cultures exposed to 700 mJ/cm² UVA showed no significant increase in the proportion of cells with structural aberrations (Table 3).

No cultures receiving Uvinul T 150 had frequencies of aberrant cells outside the normal range (Appendix 4).

Numerical aberrations

Frequencies of cells with numerical aberrations fell within the normal range under all treatment conditions (Appendix 9).

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6 CONCLUSION

It is concluded that Uvinul T 150 was not photoactivated to a clastogenic form detectable in this assay.

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Metaphase Analysis in vitro

TABLE 1

Cells with structural aberrations: no light treatment

Test chemical: Uvinul T 150

Treatment (µg/ml)	Replicate	Cells scored	Cells with aberrations including gaps	Cells with Signifi- aberrations cance § excluding gaps	Mitotic index (mean)
Solvent	A	100	1	0	8.2
	B	100	0	0	7.8
	Totals	200	1	0	(8.0)
51.2	A	100	2	2	3.0
	B	100	1	0	6.5
	Totals	200	3	2 NS	(4.8)
64	A	100	0	0	6.9
	B	100	4	1	6.3
	Totals	200	4	1 NS	(6.6)
80	A	100	1	0	6.0
	B	100	2	0	7.1
	Totals	200	3	0 NS	(6.6)
8-MOP, 3.125	A	100	2	0	
	B	100	1	0	
	Totals	200	3	0 NS	
NQO, 0.25	A	25	11	10	
	B	25	17	15	
	Totals	50	28	25 p ≤0.001	

§ Statistical significance (Appendix 10)

NS = not significant

Numbers highlighted exceeded historical negative control range (Appendix 4)

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Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

Metaphase Analysis in vitro

TABLE 2

Cells with structural aberrations: 200 mJ/cm² UVA, 33 mJ/cm² UVB

Test chemical: Uvinul T 150

Treatment (µg/ml)	Replicate	Cells scored	Cells with aberrations including gaps	Cells with aberrations excluding gaps	Significance §	Mitotic index (mean)
Solvent	A	100	3	1		5.8
	B	100	2	0		6.3
	Totals	200	5	1		(6.1)
51.2	A	100	3	0		6.7
	B	100	2	1		5.4
	Totals	200	5	1	NS	(6.1)
64	A	100	2	0		8.5
	B	100	3	0		7.2
	Totals	200	5	0	NS	(7.9)
80	A	100	1	0		8.4
	B	100	5	0		7.2
	Totals	200	6	0	NS	(7.8)
8-MOP, 3.125	A	25	14	12		
	B	25	21	20		
	Totals	50	35	32	p ≤ 0.001	

§ Statistical significance (Appendix 10)

NS = not significant

Numbers highlighted exceeded historical negative control range (Appendix 4)

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

Metaphase Analysis in vitro

TABLE 3

Cells with structural aberrations: 700 mJ/cm² UVA

Test chemical: Uvinul T 150

Treatment (µg/ml)	Replicate	Cells scored	Cells with aberrations including gaps	Cells with aberrations excluding gaps	Signifi- cance §	Mitotic index (mean)
Solvent	A	100	5	3		8.6
	B	100	3	2		8.4
	Totals	200	8	5		(8.5)
51.2	A	100	6	1		7.4
	B	100	1	1		9.7
	Totals	200	7	2	NS	(8.6)
64	A	100	4	2		4.7
	B	100	2	1		7.3
	Totals	200	6	3	NS	(6.0)
80	A	100	1	1		9.4
	B	100	4	0		10.6
	Totals	200	5	1	NS	(10.0)
8-MOP, 3.125	A	25	19	19		
	B	25	24	24		
	Totals	50	43	43	p ≤0.001	

§ Statistical significance (Appendix 10)

NS = not significant

Numbers highlighted exceeded historical negative control range (Appendix 4)

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 1

Light calibration and dose calculations

Dosimetry for UVA and UVB was measured before and after exposure of flasks, in the centre and towards opposite sides of the 20 cm diameter field (see Figure 1). Duplicate readings were taken before exposure, at the 3 positions and a single measurement taken at position B following treatments. These measurements were recorded as a check on the performance of the lamp.

The cells received light through the top of the flask and 5 ml of culture medium. The amount of light was measured by placing a bottomless, 25 cm² flask containing 4.95 ml McCoy's 5A medium and 0.05 ml DMSO on top of each sensor at position A, B and C. The data were used to calculate exposure times to obtain the desired doses. The data presented for treatments with 200 mJ/cm² UVA and 33 mJ/cm² UVB are from Trial 1. Those for 700 mJ/cm² UVA are from Trial 2.

Trial 1

Treatment		EXPOSURE TIME		
		A	B	C
UVA (0.1 J/cm ²)	1	4 min 58 sec	4 min 34 sec	5 min 00 sec
	2	5 min 04 sec	4 min 41 sec	5 min 01 sec
	mean	5 min 01 sec	4 min 37.5 sec	5 min 00.5 sec
mJ/cm ² /min		20	22	20
UVA (0.1 J/cm ² + glass)	1	-	5 min 29 sec	-
	2	-	5 min 30 sec	-
	mean	-	5 min 29.5 sec	-
mJ/cm ² /min		-	18	-
UVB (0.01 J/cm ²)	1	3 min 02 sec	2 min 46 sec	3 min 03 sec
	2	3 min 01 sec	2 min 45 sec	3 min 00 sec
	mean	3 min 01.5 sec	2 min 45.5 sec	3 min 01.5 sec
mJ/cm ² /min		3	4	3

HE study number: 729/186
Sponsor's project number: 31M0246/939008

HM study number: BLG 9/CHU

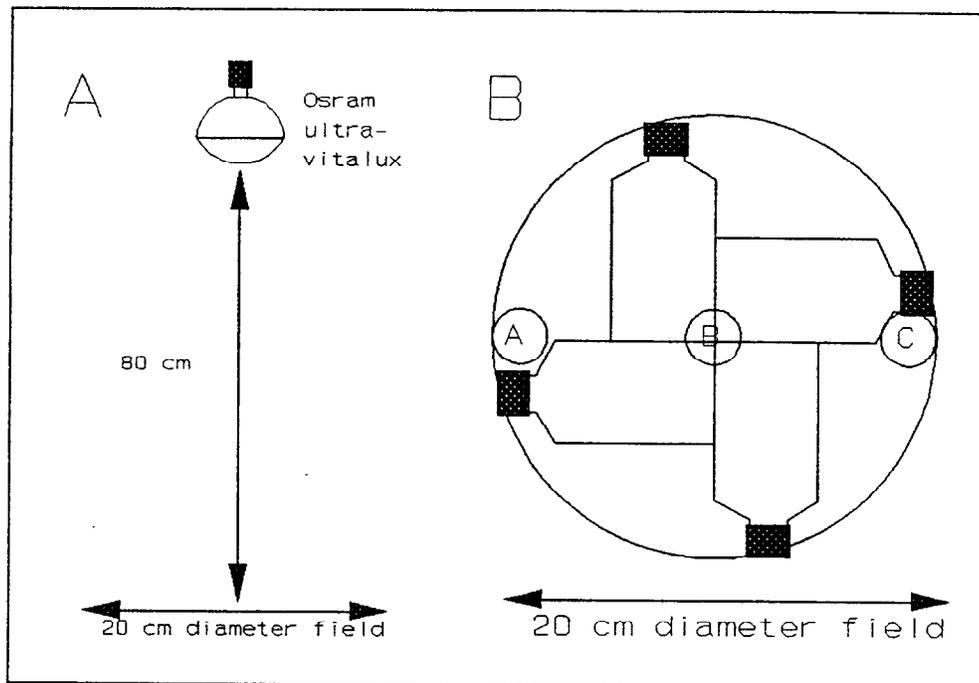
APPENDIX 1 (continued)

Trial 2

Treatment		EXPOSURE TIME					
		A		B		C	
UVA (0.1 J/cm ²)	1	4 min	50 sec	4 min	31 sec	4 min	58 sec
	2	5 min	00 sec	4 min	39 sec	5 min	00 sec
	mean	4 min	55 sec	4 min	35 sec	4 min	59 sec
mJ/cm ² /min		20		22		20	
UVA (0.1 J/cm ² + glass)	1	-		5 min	18 sec	-	
	2	-		5 min	16 sec	-	
	mean	-		5 min	17 sec	-	
mJ/cm ² /min		-		19		-	
UVB (0.01 J/cm ²)	1	2 min	56 sec	2 min	37 sec	2 min	53 sec
	2	3 min	02 sec	2 min	42 sec	2 min	52 sec
	mean	2 min	59 sec	2 min	39.5 sec	2 min	52.5 sec
mJ/cm ² /min		3		4		3	

FIGURE 1

Diagram showing the irradiation apparatus



A Details of the lamp and setup

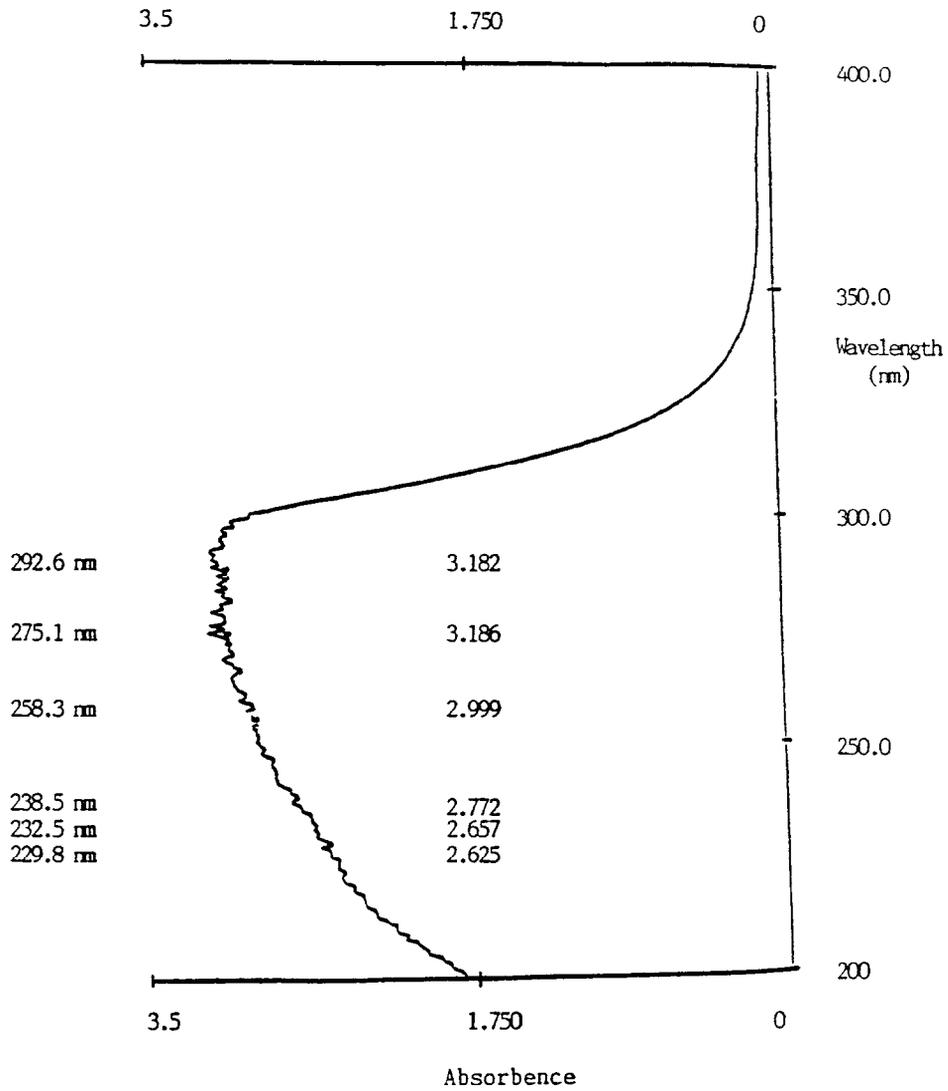
B Irradiation field and arrangement of flasks

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 2

Absorbance characteristics of the 3 mm thick sheet of glass



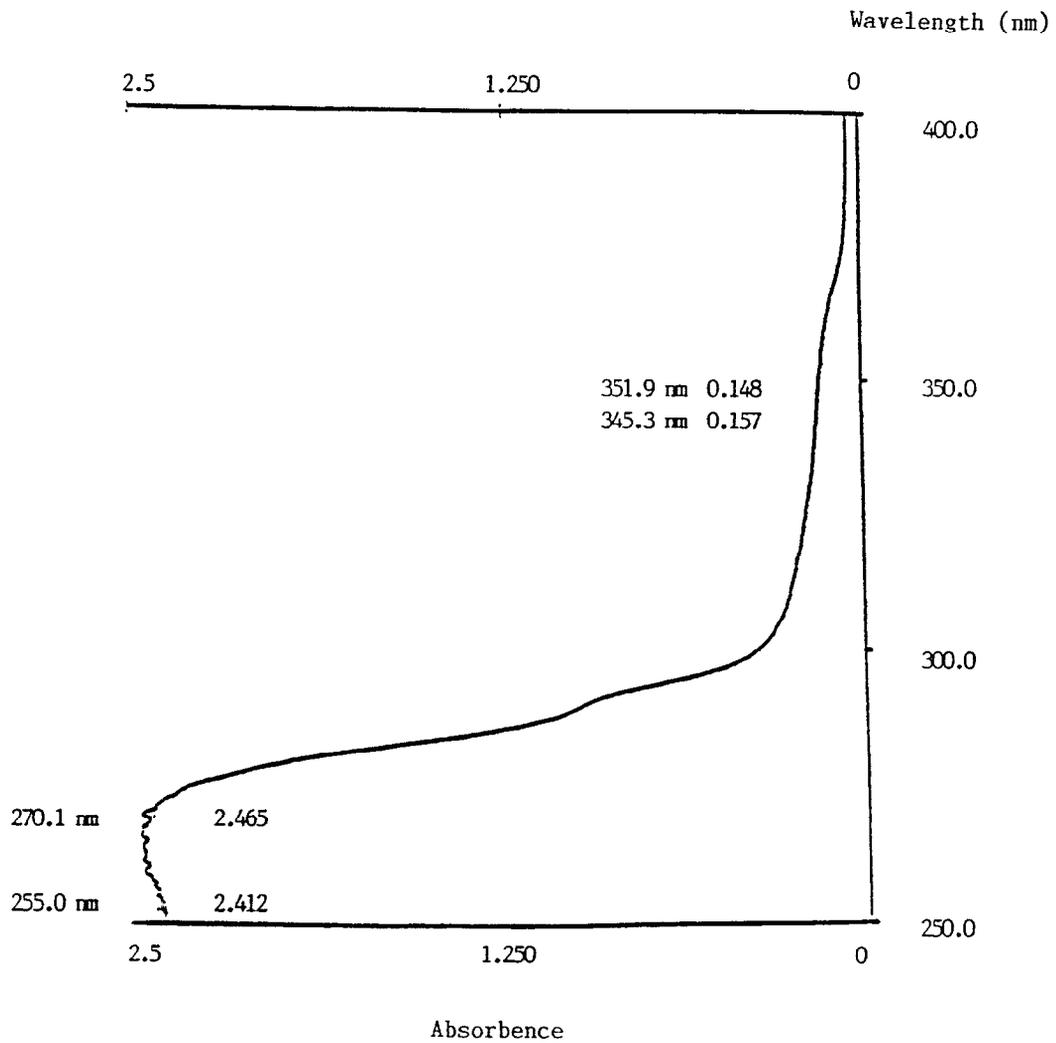
See reference number 3.

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 3

Absorbance characteristics of a sample 25 cm² tissue culture flask



See reference number 3.

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 4

Historical solvent control data for CHO cultures*

	Mean	Defined normal range
Cells with structural aberrations including gaps per 100 cells	3.3	0 - 9
Cells with structural aberrations excluding gaps per 100 cells	1.2	0 - 4
Cells with numerical aberrations per 100 cells	2.1	0 - 6

* Calculated on the basis of 31 of the most recent negative control cultures on 25 June 1993.

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 5

Treatment and irradiation schedule

Flasks were treated in groups such that both chemical and light treatments could be achieved within 2 hours. Freshly prepared test chemical, negative and (where appropriate) positive control solutions were used and treatment times separated by approximately 2 hours. Following the addition of chemical, flasks were incubated at 37°C in the dark for at least 15 minutes before irradiation. Flasks were replaced in the incubator immediately after light treatment. Within each light treatment, flasks were irradiated in batches consisting of 2, 3 or 4 flasks in the order shown such that replicates of a particular treatment were not exposed together. Flasks receiving no light were treated with Group 1 in Trial 1. The data presented for treatments with 200 mJ/cm² UVA and 33 mJ/cm² UVB are from Trial 1. The data for 700 mJ/cm² UVA are from Trial 2.

Main study irradiation groups: Trial 1

Dose (µg/ml)	Chemical	Time treated (approximate)					
		10.00			10.00		
		Group number					
		1			1		
		Minutes of treatment (+/- glass)					
		0 min			9 min 31 sec (-)		
Replicate		A	B	C	A	B	C
DMSO		1	1	1	1	3	5
32.77	Uvinul T 150	1	1		1	3	
40.96		1	1		1	3	
51.2		1	1		1	4	
64		1	1		2	4	
80		1	1		2	4	
3.125	8-MOP	1	1		2	4	
6.25		1	1		2	5	
12.5		1	1		3	5	
0.0625	NQO	1	1				
0.125		1	1				
0.25		1	1				

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 5 (continued)

Main study irradiation groups: Trial 2

Dose ($\mu\text{g/ml}$)	Chemical	Time treated (approximate)						
		11.00		12.40		14.00		
		Group number						
		1		2		3		
		Minutes of treatment (+/- glass)						
		36 min 50 sec (+)						
	Replicate	A	B	A	B	A	B	C
0	DMSO	1			3			5
32.77	Uvinul T 150	1			3			
40.96		1			3			
51.2		1			4			
64		2			4			
80		2			4			
3.125	8-MOP	2			4			
6.25		2						5
12.5					3			5

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 6

Abbreviations and classification of observations

abs = aberrations
tot = total
rep = replicate
num = numerical

Gaps (g)

csg = chromosome gap
ctg = chromatid gap

Chromosome deletions (Chr del)

del = chromosome deletion
d min = double minute
f = isolocus fragment

Chromosome exchanges (Chr exch)

t = interchange between chromosomes (eg reciprocal translocation)
inv = chromosome intrachange (eg pericentric inversion)
dic = dicentric
dic+f = dicentric with accompanying fragment
acr = acentric ring
r = centric ring
r+f = centric ring with accompanying fragment

Chromatid deletions (Ctd del)

del = chromatid deletion
su = isochromatid deletion with sister union of broken ends
nud = isochromatid deletion with non-union of broken ends distally
nup = isochromatid deletion with non-union of broken ends proximally
min = single minute

Chromatid exchanges (Ctd exch)

qr = interchange between chromatids of different chromosomes (eg quadriradial)
cx = obligate complex interchange
e = chromatid intrachange
tr/tr+f = isochromatid/chromatid interchange (eg triradial)

Other structural aberrations

pvz = pulverised
mabs = multiple aberrations (greater than 7 aberrations per cell or too many aberrations to permit accurate analysis)

Numerical aberrations (num abs)

E = endoreduplicated
H = hyperdiploid (24-32 chromosomes)
P = polyploid (greater than 32 chromosomes)

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 7

Slide codes

No light treatment

Solvent
51.2 µg/ml
64 µg/ml
80 µg/ml
8-MOP, 3.125 µg/ml

Replicate A

Replicate B

E U
O M
T B
L J
F C

200 mJ/cm² UVA, 33 mJ/cm² UVB

Solvent
51.2 µg/ml
64 µg/ml
80 µg/ml

Replicate A

Replicate B

I D
W A
P R
X S

700 mJ/cm² UVA

Solvent
51.2 µg/ml
64 µg/ml
80 µg/ml

Replicate A

Replicate B

B E
A H
F C
J D

Slides from 700 mJ/cm² UVA treatments were performed in a separate Trial (2) and this was noted on the slide labels.

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 8

Summary of structural aberrations observed: no light treatment

Test chemical: Uvinul T 150

Treatment (µg/ml)	Rep	Cells *	g	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	A	100	1	0	0	0	0	0	1	0
	B	100	0	0	0	0	0	0	0	0
	A+B	200	1	0	0	0	0	0	1	0
51.2	A	100	1	0	1	0	1	0	3	2
	B	100	1	0	0	0	0	0	1	0
	A+B	200	2	0	1	0	1	0	4	2
64	A	100	0	0	0	0	0	0	0	0
	B	100	3	0	0	1	0	0	4	1
	A+B	200	3	0	0	1	0	0	4	1
80	A	100	1	0	0	0	0	0	1	0
	B	100	2	0	0	0	0	0	2	0
	A+B	200	3	0	0	0	0	0	3	0
8-MOP, 3.125	A	100	2	0	0	0	0	0	2	0
	B	100	1	0	0	0	0	0	1	0
	A+B	200	3	0	0	0	0	0	3	0
NQO, 0.25	A	25	5	1	0	4	10	2	22	17
	B	25	5	0	0	7	0	11	23	18
	A+B	50	10	1	0	11	10	13	45	35

* = Total cells examined for structural aberrations

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 8 (continued)

Summary of structural aberrations observed: 200 mJ/cm² UVA, 33 mJ/cm² UVB

Test chemical: Uvinul T 150

Treatment (µg/ml)	Rep	Cells *	g	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	A	100	2	0	0	1	0	0	3	1
	B	100	3	0	0	0	0	0	3	0
	A+B	200	5	0	0	1	0	0	6	1
51.2	A	100	3	0	0	0	0	0	3	0
	B	100	2	1	0	0	0	0	3	1
	A+B	200	5	1	0	0	0	0	6	1
64	A	100	2	0	0	0	0	0	2	0
	B	100	3	0	0	0	0	0	3	0
	A+B	200	5	0	0	0	0	0	5	0
80	A	100	1	0	0	0	0	0	1	0
	B	100	5	0	0	0	0	0	5	0
	A+B	200	6	0	0	0	0	0	6	0
8-MOP, 3.125	A	25	5	2	0	8	12	5	32	27
	B	25	8	1	0	3	3	15	30	22
	A+B	50	13	3	0	11	15	20	62	49

* = Total cells examined for structural aberrations

HE study number: 729/186
 Sponsor's project number: 31M0246/939008

HM study number: BLG 9/CHU

APPENDIX 8 (continued)

Summary of structural aberrations observed: 700 mJ/cm² UVA

Test chemical: Uvinul T 150

Treatment (µg/ml)	Rep	Cells *	g	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	A	100	2	2	0	2	0	0	6	4
	B	100	1	0	2	0	0	0	3	2
	A+B	200	3	2	2	2	0	0	9	6
51.2	A	100	5	1	0	0	0	0	6	1
	B	100	0	1	1	0	0	0	2	2
	A+B	200	5	2	1	0	0	0	8	3
64	A	100	2	4	0	1	0	0	7	5
	B	100	3	1	0	1	0	0	5	2
	A+B	200	5	5	0	2	0	0	12	7
80	A	100	0	0	0	2	0	0	2	2
	B	100	6	0	0	0	0	0	6	0
	A+B	200	6	0	0	2	0	0	8	2
8-MOP, 3.125	A	25	0	1	0	8	3	17	29	29
	B	25	0	2	0	2	8	21	33	33
	A+B	50	0	3	0	10	11	38	62	62

* = Total cells examined for structural aberrations

HE study number: 729/186
 Sponsor's project number: 31M0246/939008

HM study number: BLG 9/CHU

APPENDIX 9

Summary of numerical aberrations observed: no light treatment

Test chemical: Uvinul T 150

Treatment (µg/ml)	Rep	Cells **	H	E	P	Tot abs	% with num abs
Solvent	A	101	0	0	1	1	1.0
	B	101	0	0	1	1	1.0
	A+B	202	0	0	2	2	1.0
51.2	A	102	0	0	2	2	2.0
	B	100	0	0	0	0	0
	A+B	202	0	0	2	2	1.0
64	A	100	0	0	0	0	0
	B	100	0	0	0	0	0
	A+B	200	0	0	0	0	0
80	A	100	0	0	0	0	0
	B	100	0	0	0	0	0
	A+B	200	0	0	0	0	0
8-MOP, 3.125	A	102	0	0	2	2	2.0
	B	101	0	0	1	1	1.0
	A+B	203	0	0	3	3	1.5
NQO, 0.25	A	25	0	0	0	0	0
	B	26	0	0	1	1	3.8
	A+B	51	0	0	1	1	2.0

** = Total cells examined for numerical aberrations

HE study number: 729/186
Sponsor's project number: 31M0246/939008

HM study number: BLG 9/CHU

APPENDIX 9 (continued)

Summary of numerical aberrations observed: 200 mJ/cm² UVA, 33 mJ/cm² UVB

Test chemical: Uvinul T 150

Treatment (µg/ml)	Rep	Cells **	H	E	P	Tot abs	% with num abs
Solvent	A	100	0	0	0	0	0
	B	101	0	0	1	1	1.0
	A+B	201	0	0	1	1	0.5
51.2	A	100	0	0	0	0	0
	B	100	0	0	0	0	0
	A+B	200	0	0	0	0	0
64	A	101	0	0	1	1	1.0
	B	101	0	0	1	1	1.0
	A+B	202	0	0	2	2	1.0
80	A	100	0	0	0	0	0
	B	100	0	0	0	0	0
	A+B	200	0	0	0	0	0
8-MOP, 3.125	A	25	0	0	0	0	0
	B	25	0	0	0	0	0
	A+B	50	0	0	0	0	0

** = Total cells examined for numerical aberrations

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 9 (continued)

Summary of numerical aberrations observed: 700 mJ/cm² UVA

Test chemical: Uvinul T 150

Treatment (µg/ml)	Rep	Cells **	H	E	P	Tot abs	% with num abs
Solvent	A	101	0	0	1	1	1.0
	B	101	0	0	1	1	1.0
	A+B	202	0	0	2	2	1.0
51.2	A	102	0	0	2	2	2.0
	B	101	0	0	1	1	1.0
	A+B	203	0	0	3	3	1.5
64	A	101	0	0	1	1	1.0
	B	100	0	0	0	0	0
	A+B	201	0	0	1	1	0.5
80	A	104	0	0	4	4	3.8
	B	102	0	0	2	2	2.0
	A+B	206	0	0	6	6	2.9
8-MOP, 3.125	A	25	0	0	0	0	0
	B	25	0	0	0	0	0
	A+B	50	0	0	0	0	0

** = Total cells examined for numerical aberrations

HE study number: 729/186
 Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 10

Statistical analysis of test chemical data

Cells with structural aberrations excluding gaps

No light treatment

Test chemical: Uvinul T 150

Binomial Dispersion Test $\chi^2 = 3.03$	DF = 4
Significance	NS

Treatment ($\mu\text{g/ml}$)	Cells	Aberrant cells	Proportion	Fisher's exact test	Significance
Solvent	200	0	---		
51.2	200	2	0.0100	0.125	NS
64	200	1	0.0050	0.250	NS
80	200	0	---	0.500	NS
8-MOP, 3.125	200	0	---	0.500	NS
NQO, 0.25	50	25	0.5000	0.000	p \leq 0.001

200 mJ/cm² UVA, 33 mJ/cm² UVB

Test chemical: Uvinul T 150

Binomial Dispersion Test $\chi^2 = 2.01$	DF = 4
Significance	NS

Treatment ($\mu\text{g/ml}$)	Cells	Aberrant cells	Proportion	Fisher's exact test	Significance
Solvent	200	1	0.0050		
51.2	200	1	0.0050	0.500	NS
64	200	0	---	0.750	NS
80	200	0	---	0.750	NS
8-MOP, 3.125	50	32	0.6400	0.000	p \leq 0.001

NS = not significant
 DF = degrees of freedom

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 10 (continued)

Statistical analysis of test chemical data

Cells with structural aberrations excluding gaps

700 mJ/cm² UVA

Test chemical: Uvinul T 150

Binomial Dispersion Test $\chi^2 = 1.55$	DF = 4
Significance	NS

Treatment ($\mu\text{g/ml}$)	Cells	Aberrant cells	Proportion	Fisher's exact test	Significance
Solvent	200	5	0.0250		
51.2	200	2	0.0100	0.857	NS
64	200	3	0.0150	0.748	NS
80	200	1	0.0050	0.939	NS
8-MOP, 3.125	50	43	0.8600	0.000	p \leq 0.001

NS = not significant
DF = degrees of freedom

HE study number: 729/186
Sponsor's project number: 31MO246/939008

HM study number: BLG 9/CHU

APPENDIX 11

Minor deviations from protocol

Protocol section	Subject	Deviation
4.1 and 4.2	Treatment	Flasks were established in 4.7 ml medium to allow for the addition of 0.25 ml KCl buffer and 0.05 ml of the test chemical to give a total of 5 ml. Buffer was added in error, but will have had no bearing on the outcome of the assay. The test chemical was not washed off after 2 hours in the range-finder. Insofar as essentially no toxicity was apparent at the solubility limit of the test chemical then it was assumed that a similar (or lower) response would be seen after 2 hours of treatment. This was confirmed in the main study.
	Controls	Solvent controls were treated in duplicate in the range-finder and in triplicate in the main study to ensure that at least 1 or 2 cultures respectively were available for analysis.
	Experimental repeats	Main study treatments were repeated: 1) as a result of unacceptably high frequencies of aberrant cells in a solvent control culture and 2) because exposures in the presence of glass filtered UV were omitted from the experiment.