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Final Report

Study Title	2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Induction of micronuclei in cultured human peripheral blood lymphocytes
Author	G Clare BSc PhD
Sponsor	
Study Monitor	
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY ENGLAND
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STUDY DIRECTOR AUTHENTICATION AND GLP COMPLIANCE STATEMENT

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Induction of micronuclei in cultured human peripheral blood lymphocytes

I, the undersigned, hereby declare that the work was performed by me or under my supervision and that the findings provide a true and accurate record of the results obtained.

The study was performed in accordance with the agreed protocol and with Covance Laboratories Limited Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The study was conducted in compliance with the United Kingdom Statutory Instrument No. 3106, The Good Laboratory Practice Regulations 1999, as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004 and the OECD Principles on Good Laboratory Practice (revised 1997, issued January 1998) ENV/MC/CHEM (98) 17.

Gill Clare — 20 July 2005

G Clare BSc PhD — Date
Study Director

QUALITY ASSURANCE STATEMENT

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Induction of micronuclei in cultured human peripheral blood lymphocytes

This study has been reviewed by the Quality Assurance Unit of Covance Laboratories Ltd. and the report accurately reflects the raw data. The following inspections were conducted and findings reported to the study director (SD) and associated management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a "process" inspection program. This can be in addition to phases scheduled on an individual study basis. Selected process inspections conducted and considered applicable to this study are included below.

In addition to the inspection programmes details below, a facility inspection programme is also operated. Details of this programme, which covers all areas of the facility annually (at a minimum), are set out in standard operating procedures.

Inspection Dates			Date Reported to SD and SD Management
From	To	Phase	
01 Dec 2004	01 Dec 2004	Protocol Review	01 Dec 2004
15 Feb 2005	16 Feb 2005	Draft Report and Data Review	17 Feb 2005
20 Jul 2005	20 Jul 2005	Final Report Review	20 Jul 2005

Process

Inspection Dates			Date Reported to SD and SD Management
From	To	Phase	
24 Nov 2004	24 Nov 2004	Dose Preparation	24 Nov 2004
26 Nov 2004	26 Nov 2004	Slide Preparation	26 Nov 2004
09 Dec 2004	09 Dec 2004	Media Preparation	09 Dec 2004
22 Dec 2004	22 Dec 2004	Dose Preparation	22 Dec 2004

20/07/05

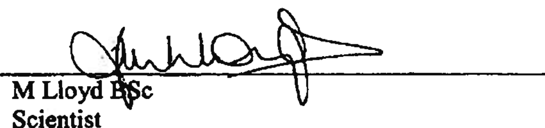
D Allison BSc (Hons) MSc
QA Team Manager

Date

REVIEWING SCIENTIST'S STATEMENT

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Induction of micronuclei in cultured human peripheral blood lymphocytes

I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.



M Lloyd BSc
Scientist

19 July 2005
Date

RESPONSIBLE PERSONNEL

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Induction of micronuclei in cultured human peripheral blood lymphocytes

The following personnel were responsible for key elements of the study:

Study Director	G Clare BSc PhD
Study Supervisor	S Coates BSc
Slide Analysts	L Fiddler BSc
	L Hudson BSc

ARCHIVE STATEMENT

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Induction of micronuclei in cultured human peripheral blood lymphocytes

All primary data, or authenticated copies thereof, specimens and the final report will be retained in the Covance Laboratories Limited archives for **ten** years after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

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SUMMARY

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two female donors in a single experiment. Treatments covering a broad range of doses, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9). The test article was dissolved in water for injection (purified water) and the highest dose level used, 1822 µg/mL, was equivalent to 10 mM free acid.

In the main Experiment, treatment of cells commenced approximately 24 hours following mitogen stimulation by PHA. In the absence of S-9 this was for 20 hours followed by a 28-hour recovery period prior to harvest (20+28). Treatment in the presence of S-9 was for 3 hours followed by a 45-hour recovery period prior to harvest (3+45). The S-9 used was prepared from a rat liver post-mitochondrial fraction (S-9) from Aroclor 1254 induced animals. The test article dose levels for micronucleus analysis were selected by evaluating the effect of 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) on the replication index (RI). Micronuclei were analysed at three dose levels (see below). The highest concentrations chosen for analysis, 8.00 µg/mL in the absence of S-9 and 150 µg/mL in the presence of S-9, induced approximately 68% and 63% reduction in RI, respectively.

Main Experiment (24 hour PHA) – Treatment summary

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081)	Positive control(s)
-	20+28	0[a]	3.00, 5.00, 8.00	NQO, 5.00 µg/mL VIN, 0.08 µg/mL
+	3+45	0[a]	25.00,100.0, 150.0	CPA, 6.25 µg/mL

[a] Vehicle control was purified water only

A summary of the micronucleus data is presented in the following table:

Main Experiment (24 hour PHA) – Result summary

Treatment	Concentration (µg/mL)	Cytotoxicity (%)	Mean MNBN cell frequency (%)	Historical Control Range (%)	Statistical significance
20+28 hour -S-9	Vehicle[a]	-	0.55	0.1-1.7	-
	3.00	11	0.75		NS
	5.00	31	0.80		NS
	8.00	68	1.40		$P \leq 0.01$
	*Vinblastine, 0.08	ND	9.75		$P \leq 0.001$
	*NQO, 5.00	ND	11.70		$P \leq 0.001$
3+45 hour +S-9	Vehicle[a]	-	0.85	0.1-1.7	-
	25.00	26	1.00		NS
	100.0	41	1.90		$P \leq 0.01$
	150.0	63	2.75		$P \leq 0.001$
	*CPA, 6.25	ND	11.80		$P \leq 0.001$

[a] Vehicle control was purified water only
* Positive control
NS = not significant
ND = not determined

Appropriate negative (solvent) control cultures were included in the test system under each treatment condition. The proportion of binucleate cells with micronuclei in these cultures fell within historical solvent control ranges. 4-Nitroquinoline 1-oxide (NQO) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of liver S-9. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of liver S-9. Cells receiving these were sampled at 48 hours after the start of treatment; all compounds induced statistically significant increases in the proportion of cells with micronuclei. There was no second main experiment, because of the positive results in the first main experiment.

Treatment of cultures with 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) in the absence of S-9 resulted in frequencies of micronucleated binucleate (MNBN) cells that were generally similar to those of the concurrent vehicle controls and within the historical vehicle control range. Exposure to 8 µg/mL, the highest concentration tested, was associated with a small increase in the frequency of MNBN cells ($P \leq 0.01$), and the value in one culture only exceeded the historical vehicle control range. This positive effect was associated with high cytotoxicity, namely 68%, was marginal, and did not fully meet the criteria for a positive response. The biological relevance of this effect is unclear.

Treatment of cultures with 100 and 150 µg/mL 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) in the presence of S-9 was associated with 41 and 63% cytotoxicity respectively and resulted in frequencies of MNBN cells that were higher

than those of the concurrent vehicle controls. Both cultures at each of the two concentrations exceeded the historical vehicle control range. There was a concentration-related increase and the data fulfilled the criteria for a positive response.

It is concluded that 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081), when tested at concentrations causing at least 41% cytotoxicity, induced micronuclei in cultured human peripheral blood lymphocytes in the presence of a rat liver metabolic activation system (S-9). Exposure to a concentration of 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) causing 68% cytotoxicity was associated with a marginal increase in the frequency of micronuclei in the absence of S-9, the biological relevance of which is unclear, under the experimental conditions described.

INTRODUCTION

Chromosome defects are recognised as the basis of a number of human genetic diseases (1).

The purpose of the *in vitro* chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. No one assay has been extensively evaluated on the same compounds in several laboratories but there is a large database on the use of chromosomal assays for screening purposes (2). The use of human peripheral blood lymphocytes is recommended because the cells are only used in short-term culture and maintain a stable karyotype (3). Experiments with these cells can also be performed in conjunction with a rat liver metabolising system since, for short incubation periods, no toxicity is induced by the liver homogenate itself. Increases in numerical micronuclei can be detected but the assay is not specifically designed to evaluate potential to induce aneuploidy or polyploidy.

An alternative to measuring structural aberrations in mitotic cells is to measure micronuclei. These are produced from whole chromosomes or acentric fragments which are unable to attach to the spindle at mitosis and appear during the next interphase as small darkly staining bodies adjacent to the main daughter nucleus. These are more easily counted than structural aberrations at mitosis and analysis can be performed rapidly on large numbers of cells. The technique as described suffers a disadvantage, however, because the production of a micronucleus is dependent on nuclear division thus the frequency of micronuclei will vary with the proportion of cells which divide. This problem is solved if cytochalasin B is added to the cultures (4). Cytochalasin B inhibits cytokinesis (cell division) but not karyokinesis (nuclear division) resulting in the formation of binucleate cells. If micronuclei are only counted in binucleate cells then a true measurement of their induction can be obtained.

In The main experiment, cells were exposed to the test article for 3 hours in the presence of liver S-9 (from rats induced with Aroclor 1254) and for 20 hours in the absence of S-9. The test chemical was added 24 hours following culture initiation and cells were harvested at 72 hours.

The responses in Experiment 1 were discussed with the Sponsor and no further testing was carried out.

The micronucleus test also allows one to distinguish between aneugenic (5) and clastogenic events by using fluorescence *in situ* hybridization (FISH) (6, 7). If an increase in the frequency of micronucleated cells was observed in this study then provision was made to further investigate this effect by determining the relative proportions of micronuclei formed by clastogenic and aneugenic events using a pan-centromeric probe (specific for all human centromeres). This identifies micronuclei which comprise whole chromosomes (i.e. carry a centromere) and the data can be used to evaluate any aneugenic potential.

The objective of this study was to evaluate the clastogenic and aneugenic potential of 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) by examining its ability to induce of micronuclei in the lymphocytes of human donors, cultured *in vitro* and treated in the absence and presence of a rat liver metabolising system (S-9).

Although no definitive regulatory guidelines exist for this assay, the test methodology was in accordance with the recommendations of the International Workshop on Genotoxicity Testing (8), draft OECD guideline 487 (9) and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro* (10, 11, 12, 13).

With the exception of the minor deviations detailed in Appendix 7, none of which in any way prejudice the validity of the study, this study was performed according to the protocol.

The study was initiated on 24 November 2004. Experimental work started on 29 November 2004 and was completed on 4 January 2005. The study completion date is considered to be the date on which the Study Director signs the final report.

MATERIALS

Test article

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081), Lehmann Blau), batch number 57 (R96000196) was a pale grey powder. It was received on 21 October 2004 and stored at room temperature in the dark, under desiccant. Purity was specified as 99.6%. The expiry date was specified as July 2005. The Manufacturer's certificate of analysis is given in Appendix 6. The test article information and certificate of analysis provided by the Sponsor are considered an adequate description of the characterisation, purity and stability of the test article. Determinations of stability and characteristics of the test article were the responsibility of the Sponsor.

Preliminary solubility data indicated that 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) was soluble in water for injection (purified water), with the aid of warming at 37°C and vortex mixing, performed under yellow light, at a concentration of at least 32.1 mg/mL. A 10-fold dilution of this purified water solution into culture medium at a final concentration of approximately 3210 µg/mL did not result in visible precipitation. A top concentration of 1822 µg/mL (equivalent to 10 mM, free acid, molecular weight 182.2) was chosen as the maximum for the cytotoxicity range-finding experiment. Doses for the main experiment were selected based on the results of this cytotoxicity range-finding experiment.

Test article stock solutions were prepared by dissolving 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) in purified water, with the aid of warming at 37°C and vortex mixing, under yellow light, to give the top concentrations as specified in the tables overleaf. All test article formulations were adjusted for free acid content, using a correction factor of 1.5383. The stock solutions were membrane filter-sterilised (Pall Acrodisc 32, pore size, 0.2 µm) and subsequent dilutions made using sterile purified water. The test article solutions were protected from light and used within 4¼ hours of initial formulation as follows overleaf:

Cytotoxicity Range-Finder Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)	Hours treatment + hours recovery			
		24 hour PHA prior to treatment		48 hour PHA prior to treatment	
		20+28 –S-9	3+45 +S-9	20+28 –S-9	3+45 +S-9
0.00896	0.8896	T	T	T	T
0.01779	1.779	T	T	T	T
0.03559	3.559	T	T	T	T
0.07117	7.117	T	T	T	T
0.1423	14.23	T	TH	T	TH
0.2847	28.47	T	TH	T	TH
0.5694	56.94	T	TH	T	TH
1.139	113.9	T	TH	T	TH
2.277	227.8	T	T	T	T
4.555	455.5	T	T	T	T
9.110	911.0	T	T	T	T
18.22	1822	T	T	T	T

T Indicates concentration tested
H Indicates precipitation observed at harvest

Main Experiment Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)	24 hour PHA prior to treatment	
		Hours treatment + hours recovery	
		20+28 –S-9	3+45 +S-9
0.005	0.500	T	
0.010	1.000	T	
0.020	2.000	T	
0.030	3.000	T	
0.040	4.000	T	
0.050	5.000	T	
0.060	6.000	T	
0.070	7.000	T	
0.080	8.000	T	
0.090	9.000	T	
0.100	10.00	T	
0.110	11.00	T	
0.250	25.00		TH
0.500	50.00		TH
1.000	100.0		T
1.500	150.0		T
1.750	175.0		T
2.000	200.0		T
2.250	225.0		T
2.500	250.0		T
2.750	275.0		T
3.000	300.0		T
3.500	350.0		T
4.000	400.0		T

T Indicates concentration tested
H Indicates precipitation observed at harvest

Shifts of pH of more than one unit or increases in osmolality of more than 50 mOsm/kg may be associated with increased frequencies of chromosome damage

(14). Post-treatment media, at final concentrations of 455.5, 911.0 and 1822 µg/mL in the absence and presence of S-9 were measured for changes in pH and osmolality. These measurements indicated that the test article had no marked effect on osmolality (greater than a shift of 50 mOsm/kg) or pH (shift of greater than 1 pH unit) as compared to concurrent vehicle controls, although there was a small reduction in pH associated with exposure to the test article.

There was no second main experiment, because of the positive results in the first main experiment.

Controls

Sterile purified water was added to cultures designated as negative controls as described in the methods section of this report. The positive control chemicals NQO and CPA were dissolved in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), frozen down and thawed out prior to use, and Vinblastine was dissolved in purified water shortly prior to use as follows (see Appendix 7):

Chemical	Supplier	Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)	S-9
4-Nitroquinoline 1-oxide	Sigma-Aldrich Chemical Co,	0.250	2.50	-
(NQO)	Poole, UK	0.500	5.00	-
Cyclophosphamide	Sigma-Aldrich Chemical Co,	0.625	6.25	+
(CPA)	Poole, UK	1.25	12.5	+
Vinblastine	Sigma-Aldrich Chemical Co,	0.006	0.06	-
(VIN)	Poole, UK	0.008	0.08	-

Cells treated with 5.00 µg NQO/mL, 0.08 µg VIN/mL and 6.25 µg CPA/mL gave satisfactory responses in terms of quality and quantity of preparations and frequency of micronucleated binucleate cells. These were selected for analysis.

Metabolic activation system

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. The batches of MolTox™ S-9 were stored frozen in aliquots at -80°C and thawed just prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities

(alkoxyresorufin-O-dealkylase activities). The quality control statement, relating to the batch of S-9 preparation used, is included in Appendix 5 of this report.

Preparation of S-9 mix

Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 mM KCl and rat liver S-9 were mixed in the ratio 1:1:1:2. An aliquot of the resulting S-9 mix was added to each cell culture designated for treatment in the presence of S-9 to achieve the required final concentration in a total of 10 mL. The final concentration of liver homogenate in the test system was 2%. Cultures treated in the absence of S-9 received an equal volume of 150 mM KCl.

Blood cultures

Blood from two healthy, non-smoking female volunteers (under 35 years of age) was used in this study:

Experiment	Donor Sex	Donor Identity
Range-Finder	Female	JM, KC
1	Female	EH, RM

No donor was suspected of any virus infection nor had been exposed to high levels of radiation or hazardous chemicals. For each experiment, an appropriate volume of whole blood was drawn from the peripheral circulation within two days prior to culture initiation. Blood was stored refrigerated and pooled prior to use. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood into 8.1 mL Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 μg/mL gentamycin. Phytohaemagglutinin (PHA, reagent grade) was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated at 37°C and rocked continuously.

For treatments conducted as part of the cytotoxicity Range-Finder, blood cultures were incubated in the presence of PHA for approximately 24 or 48 hours prior to treatment.

For treatments conducted as part of The main experiment blood cultures were incubated in the presence of PHA for approximately 24 hours prior to treatment.

METHODS

Treatment

For the cytotoxicity range-finder experiment, S-9 mix or KCl (0.5 mL) was added appropriately as detailed previously. Duplicate cultures (A, B) were treated with the solvent, and single cultures treated with the test article at appropriate concentrations (0.1 mL per culture). Positive control treatments were not included.

Duration of treatment (hours)	S-9	Hours after culture initiation			
		Addition of test chemical	Removal of test chemical	Addition of Cytochalasin B	Harvest time
20	-	24	44	44*	72
3	+	24	27	44*	72
20	-	48	68	68*	96
3	+	48	51	68*	96

* Approximate times

For the main experiment, S-9 mix or KCl (0.5 mL) was added appropriately as detailed previously. Quadruplicate cultures (A, B, C and D) were treated with the solvent, and duplicate cultures treated with the test article at appropriate concentrations (0.1 mL per culture). Additional duplicate cultures for treatments in the absence of S-9 and in its presence, were treated with 0.1 mL of the positive control chemicals.

The main Experiment comprised a 20-hour treatment - S-9 and a 3 hour treatment +S-9. The test chemical was added 24 hours following culture initiation and cells were harvested at 72 hours. The final approximately 28 hours of incubation was in the presence of Cytochalasin B, at a final concentration of 6 μg/mL.

This scheme is illustrated overleaf:

Treatment	S-9	Number of cultures 3+45*	20+28*
Cytotoxicity Range-Finder	-		2
Negative control	+	2	
Test article	-		1
(all doses/)	+	1	
Main Experiment	-		4
Negative control	+	4	
Test article	-		2
(doses as appropriate)	+	2	
Positive controls	-		2#
(all doses)	+	2	

* Hours treatment + hours recovery
# Two cultures for each positive control.

Summary of treatment conditions

	Duration of treatment (hours)	S-9	Hours after culture initiation: Addition of test chemical	Removal of test chemical	Addition of Cytochalasin B	Harvest time
Main Experiment	20	-	24	44	44*	72
	3	+	24	27	44*	72

* Approximate times

For removal of the test chemical, cells were pelleted (approximately 300 x 'g', 10 minutes), washed twice with sterile saline, and resuspended in fresh medium containing foetal calf serum and gentamycin. At the appropriate times, Cytochalasin B, formulated in dimethyl sulphoxide, was added in medium to give a final concentration of 6 μg/mL (see Appendix 7).

Harvesting and slide preparation

At the defined sampling time, cultures were centrifuged at approximately 300 x 'g' for 10 minutes; the supernatant carefully removed and cells resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37°C for 4 minutes (see Appendix 7) to allow cell swelling to occur. Cells were then 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 by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x 'g', 2-3 minutes) until the cell pellets were clean.

Lymphocytes were kept in fixative in the refrigerator 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 (if required) to give a milky suspension. Several drops of suspension were transferred to clean microscope slides.

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

Selection of doses for the main study experiment

Slides (from the cytotoxicity range-finding experiment) were initially examined, uncoded, for proportions of mono-, bi- and multinucleate cells, to a minimum of 200 cells per concentration. From these data the replication index (RI) was determined using the formula described below.

A suitable range of concentrations was selected for the main experiment based on these toxicity data.

Selection of doses for micronucleus analysis

Slides were examined, uncoded, for proportions of mononucleate, binucleate and multinucleate cells and the replication index (RI) calculated based on the analysis of 500 cells per replicate (1000 per dose). A selection of random fields were observed from enough treatments to determine whether chemically-induced cell cycle delay or cytotoxicity has occurred. The RI was determined using the following formula:

Replication Index (RI) indicates the relative number of nuclei in treated cultures compared to control cultures. Individual replicate calculations are performed using the formulae below:

$$RI = \frac{\text{number binucleate cells} + 2 \times \text{number multinucleate cells}}{\text{Total number of cells}}$$

Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{(\text{number binucleate cells} + 2 \times \text{number multinucleate cells}) / \text{total number of cells in treated cultures}}{(\text{number binucleate cells} + 2 \times \text{number multinucleate cells}) / \text{total number of cells in control cultures}} \times 100$$

The top dose for analysis was to be one at which at least 60% (approximately) reduction in RI occurred or the highest dose tested. Analysis of slides from highly cytotoxic concentrations is avoided, if possible.

The rationale for the limit of approximately 60% cytotoxicity is based on limited data (discussed by the IWGTP (9)) which show that in some cases (i.e. some aneugens), a very steep toxicity curve is observed and very closely spaced doses in the range of 50-60% toxicity need to be evaluated. Data from certain validation experiments have demonstrated that the lowest observed effective dose (LOEDs) for the aneugens diethylstilbestrol and vincristine showed a relative cell count (RCC) of 42% and 43% respectively; this corresponding to a toxicity of approximately 60%. These compounds might not have been found to be micronucleus inducers if they had not been tested up to the 60% toxicity level.

For poorly soluble compounds, slides from precipitating cultures were checked to confirm that the presence of precipitate did not preclude analysis.

Slides from the highest selected dose and at least two lower doses, such that a range of cytotoxicity from maximum to little or none is covered, were taken for microscope analysis.

Slides from solvent control cultures C and D were only analysed for micronuclei if necessary to help resolve an equivocal result or increased frequencies of micronuclei were observed in solvent controls.

Slide analysis

Acceptance criteria

Binucleate cells were only included in the analysis if all of the following criteria were met:

1) the cytoplasm remained essentially intact, and

2) the daughter nuclei were of approximately equal size.

A micronucleus was only recorded if it met the following criteria:

1) the micronucleus has the same staining characteristics and a similar morphology to the main nuclei, and

2) any micronucleus present was separate in the cytoplasm or only just touching a main nucleus, and

3) micronuclei were smooth edged and smaller than approximately one third the diameter of the main nuclei.

Procedure

Slides from the CPA, NQO and Vinblastine- treated positive control cultures were initially examined for micronuclei to ensure that the system had responded satisfactorily. Slides from the selected treatments and from solvent and positive controls were then coded, using randomly generated letters, by a person(s) not connected with the scoring of the slides. Labels were used to cover any treatment details on the slides, so that the cytogeneticists could only see the study number and the code.

One thousand binucleate cells from each culture (2000 per dose level) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei per cell on each slide was noted. Observations were recorded on raw data sheets.

Micronucleus analysis was not conducted on slides generated from the range-finder treatments.

Slide analysis was performed by competent analysts trained in the applicable Covance Laboratories Harrogate (CLEH) standard operating procedures. Although physically located remote from the CLEH facility, all analysts participating in this study were subject to CLEH management and GLP control systems, and all slides and raw data were returned to CLEH for archiving on completion of their analysis.

Analysis of results

Treatment of data

After completion of scoring and decoding of slides, the numbers of binucleate cells with micronuclei in each culture were obtained.

The proportions of micronucleated cells in each replicate were used to establish acceptable homogeneity between replicates by means of a binomial dispersion test (15).

The proportion of cells with micronuclei for each treatment condition were compared with the proportion in solvent controls by using Fisher's exact test (15). Probability values of $p \leq 0.05$ were accepted as significant.

Additionally, the number of micronuclei per binucleate cell were obtained and recorded. This data was also used as a potential additional tool for the interpretation of the study data.

Acceptance criteria

The assay was to be considered valid if the following criteria were met:

1) the binomial dispersion test demonstrated acceptable heterogeneity between replicate cultures, particularly where no positive responses are seen, and

2) the frequency of cells with micronuclei in solvent controls fell within the laboratories historical negative control (normal) range, and

3) the positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei.

4) a minimum of 50% binucleate cells was achieved in negative control cultures at the time of harvest.

Evaluation criteria

A test chemical is considered as clearly positive in this assay if:

1) a statistically significant increase in the proportion of cells with micronuclei occurs at one or more concentrations, and

2) the incidence of micronucleated cells at such data points exceeds the normal range.

RESULTS

Selection of doses for micronucleus analysis

The results of the RI determinations taken from the cytotoxicity range-finder were as follows:

24-hour PHA

Treatment (µg/mL)	RI (Replication Index)					
	20+28 hours, -S-9			3+45 hours, +S-9		
	A	B	Cytotoxicity (%) *	A	B	Cytotoxicity (%) *
Solvent	1.02	1.02	-	1.04	1.14	-
0.8896	1.02	NT	0	NS	NT	-
1.779	0.86	NT	16	NS	NT	-
3.559	0.89	NT	13	0.98	NT	10
7.117	0.16	NT	85	0.99	NT	9
14.23	0.09	NT	91	0.79	NT	28
28.47	0.00	NT	100	0.72	NT	34
56.94	T	NT	-	0.81	NT	26
113.9	T	NT	-	0.68	NT	38
227.8	T	NT	-	0.35	NT	68
455.5	T	NT	-	0.07	NT	93
911.0	T	NT	-	0.01	NT	100
1822	T	NT	-	T	NT	-

T = Toxic NS = Slides not scored NT = not tested
Shaded concentrations selected for analysis

*Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \times 100$$

48-hour PHA

Treatment (µg/mL)	RI (Replication Index)					
	20+28 hours, -S-9			3+45 hours, +S-9		
	A	B	Cytotoxicity (%) *	A	B	Cytotoxicity (%) *
Solvent	1.34	1.42	-	1.54	1.42	-
0.8896	1.26	NT	9	NS	NT	-
1.779	1.15	NT	17	NS	NT	-
3.559	1.14	NT	17	1.30	NT	12
7.117	0.41	NT	71	1.35	NT	9
14.23	0.02	NT	99	0.78	NT	47
28.47	0.03	NT	98	1.16	NT	22
56.94	0.02	NT	98	1.17	NT	21
113.9	T	NT	-	0.93	NT	37
227.8	T	NT	-	0.93	NT	37
455.5	T	NT	-	1.05	NT	29
911.0	T	NT	-	0.87	NT	41
1822	T	NT	-	0.28	NT	81

T = Toxic NS = Slides not scored NT = not tested

*Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \times 100$$

There were dark pellets and media at all concentrations of test article at medium change. At harvest, there were dark pellets and media in the cultures exposed to the four highest concentrations (227.8-1822 µg/mL) in the absence of S-9.

The results of the cytotoxicity range-finding experiment were used to select suitable top concentrations for The main experiment.

The results of the RI determinations taken from The main experiment were as follows:

Treatment (µg/mL)	RI (Replication Index)					
	20+28 hours, -S-9			3+45 hours, +S-9		
	A/C	B/D	Cytotoxicity (%) *	A/C	B/D	Cytotoxicity (%) *
Solvent	0.91/0.94	0.93/1.02	-	0.97/0.90	0.87/1.02	-
0.500	NS	NS	-	NT	NT	-
1.000	1.02	0.98	0	NT	NT	-
2.000	0.90	1.00	0	NT	NT	-
3.000	0.84	0.86	11	NT	NT	-
4.000	0.70	0.80	21	NT	NT	-
5.000	0.65	0.66	31	NT	NT	-
6.000	0.63	0.46	43	NT	NT	-
7.000	0.44	0.82	34	NT	NT	-
8.000	0.28	0.34	68	NT	NT	-
9.000	0.23	0.24	76	NT	NT	-
10.00	0.19	0.19	80	NT	NT	-
11.00	0.33	0.24	70	NT	NT	-
25.00	NT	NT	-	0.57	0.82	26
50.00	NT	NT	-	0.60	0.59	37
100.0	NT	NT	-	0.52	0.59	41
150.0	NT	NT	-	0.35	0.35	63
175.0	NT	NT	-	0.39	0.53	51
200.0	NT	NT	-	0.36	0.42	59
225.0	NT	NT	-	0.27	0.33	68
250.0	NT	NT	-	0.25	0.36	67
275.0	NT	NT	-	0.23	0.57	57
300.0	NT	NT	-	0.20	0.20	79
350.0	NT	NT	-	0.17	0.31	75
400.0	NT	NT	-	0.17	0.17	82

NS = Slides not scored NT = Not treated
Shaded concentrations selected for analysis

*Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} x100$$

A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for vehicle controls and two [A, B] for test article and positive controls)

In both the absence and presence of S-9, dark cell pellets were noted in all cultures exposed to 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) at the end of treatment. At harvest, in the presence of S-9 only, dark cell pellets were noted in all cultures exposed to 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081), while dark supernatant was observed at concentrations of 25 and 50 µg/mL.

The following doses were selected for analysis:

	Reduction in RI†
20+28 hours, -S-9: 3.00, 5.00, 8.00 µg/mL	68%
3+45 hours, +S-9: 25.00, 100.0, 150.0 µg/mL	63%

† At highest analysed dose

Micronucleus analysis

Raw data

The raw data for the observations on the test article plus positive and negative controls are retained by Covance Laboratories Limited. A summary of the number of cells containing micronuclei is given in Appendix 1 for the treatment regimes.

Validity of study

The data in Appendix 1 and Appendix 3 indicate that:

1) the binomial dispersion test demonstrated acceptable heterogeneity between replicate cultures, the slight heterogeneity in The main experiment in the absence of S-9 being ascribed to the small increases between duplicate cultures treated with 8 µg/mL test article or differences between duplicates of the positive controls, and

2) the frequency of cells with micronuclei in negative controls fell within the normal range, and

3) the positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei.

4) a minimum of 50% binucleate cells was achieved in negative control cultures at the time of harvest.

Analysis of data

Treatment of cultures with 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) in the absence of S-9 resulted in frequencies of micronucleated binucleate (MNBN) cells that were generally similar to those of the concurrent vehicle controls and within the historical vehicle control range. Exposure to 8 µg/mL, the highest concentration tested, was associated with a small increase in the frequency of MNBN cells ($P \leq 0.01$), and the value in one culture only exceeded the historical vehicle control range. This positive effect was associated

with high cytotoxicity, namely 68%, was marginal, and did not fully meet the criteria for a positive response. The biological relevance of this effect is unclear.

Treatment of cultures with 100 and 150 µg/mL 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) in the presence of S-9, associated with 41% and 63% cytotoxicity respectively, resulted in frequencies of MNBN cells that were higher than those of the concurrent vehicle controls. Both cultures at each of the two concentrations exceeded the historical vehicle control range. There was a concentration-related increase and the data fulfilled the criteria for a positive response.

No second main experiment was considered necessary, owing to the positive results seen in the first main experiment.

CONCLUSION

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081), when tested at concentrations causing at least 41% cytotoxicity, induced micronuclei in cultured human peripheral blood lymphocytes in the presence of a rat liver metabolic activation system (S-9). Exposure to a concentration of 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) causing 68% cytotoxicity was associated with a marginal in the frequency of micronuclei in the absence of S-9, the biological relevance of which is unclear, under the experimental conditions described.

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APPENDICES

Appendix 1

2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Binucleate cells with micronuclei

Table 1
Data for 20+28 hour treatments -S-9, Main Experiment - 24 hour PHA Female Donors

Treatment (µg/mL)	Replicate	Total BN Cells Scored	Total MN BN Cells Scored	No. of micronuclei observed per MN BN cell 1	2	>2	Frequency of MN BN Cells/ Cells Scored (%)	Significance	Cytotoxicity %
Solvent	A	1000	4	4	0	0	0.40		
	B	1000	7	5	2	0	0.70		
	Total	2000	11		-		0.55	-	
3.00	A	1000	10	10	0	0	1.00		
	B	1000	5	5	0	0	0.50		
	Total	2000	15		-		0.75	NS	11
5.00	A	1000	7	6	1	0	0.70		
	B	1000	9	9	0	0	0.90		
	Total	2000	16		-		0.80	NS	31
8.00	A	1000	7	7	0	0	0.70		
	B	1000	21	20	1	0	2.10		
	Total	2000	28		-		1.40	p ≤ 0.01	68
VIN, 0.08	A	1000	84	69	14	1	8.40		
	B	1000	111	81	30	0	11.10		
	Total	2000	195		-		9.75	p ≤ 0.001	
NQO, 5.00	A	1000	102	72	25	5	10.20		
	B	1000	132	114	16	2	13.20		
	Total	2000	234		-		11.70	p ≤ 0.001	

BN = Binucleate
MN = Micronucleated
§ Statistical significance (Appendix 3)
NS = not significant
Numbers highlighted exceed historical negative control range (Appendix 4)

*Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \times 100$$

Table 2
Data for 3+45 hour treatments +S-9, Main Experiment- 24 hour PHA
Female Donors

Treatment (µg/mL)	Replicate	Total BN Cells Scored	Total MN BN Cells Scored	No. of micronuclei observed per MN BN cell 1	2	>2	Frequency of MN BN Cells/ Cells Scored (%)	Significance	Cytotoxicity %
Solvent	A	1000	8	7	1	0	0.80		
	B	1000	9	8	1	0	0.90		
	Total	2000	17		-		0.85	-	-
25.00	A	1000	5	5	0	0	0.50		
	B	1000	15	15	0	0	1.50		
	Total	2000	20		-		1.00	NS	26
100.0	A	1000	19	19	0	0	1.90		
	B	1000	19	19	0	0	1.90		
	Total	2000	38		-		1.90	$p \leq 0.01$	41
150.0	A	1000	21	21	0	0	2.10		
	B	1000	34	34	0	0	3.40		
	Total	2000	55		-		2.75	$p \leq 0.001$	63
CPA, 6.25	A	1000	116	91	19	6	11.60		
	B	1000	120	94	20	6	12.00		
	Total	2000	236		-		11.80	$p \leq 0.001$	

BN = Binucleate
MN = Micronucleated
§ Statistical significance (Appendix 3)
NS = not significant
Numbers highlighted exceed historical negative control range (Appendix 4)

*Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \times 100$$

Appendix 2
2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Determination of RI

Table 3
Data for 20+28 hour treatments -S-9, Range-Finder- 24 hour PHA Female Donors

Dose (µg/mL)	Replicate	Mono	Bi	Multi	Total Number of Cells	RI	Cytotoxicity (%)
Solvent	A	35	127	38	200	1.02	-
	B	37	122	41	200	1.02	
0.8896	A	37	122	42	201	1.02	0
1.779	A	52	128	23	203	0.86	16
3.559	A	43	140	20	203	0.89	13
7.117	A	170	29	1	200	0.16	85
14.23	A	182	18	0	200	0.09	91
28.47	A	200	0	0	200	0.00	100
56.94	A	NS					-
113.9	A	NS					-
227.8	A	NS					-
455.5	A	NS					-
911.0	A	NS					-
1822	A	NS					-

Table 4
Data for 3+45 hour treatments +S-9, Range-Finder- 24 hour PHA Female Donors

Dose (µg/mL)	Replicate	Mono	Bi	Multi	Total Number of Cells	RI	Cytotoxicity (%)
Solvent	A	31	132	39	202	1.04	-
	B	22	128	51	201	1.14	
0.8896	A	NS					-
1.779	A	NS					-
3.559	A	44	118	40	202	0.98	10
7.117	A	42	118	40	200	0.99	9
14.23	A	61	124	18	203	0.79	28
28.47	A	76	104	20	200	0.72	34
56.94	A	55	128	17	200	0.81	26
113.9	A	81	108	15	204	0.68	38
227.8	A	140	53	9	202	0.35	68
455.5	A	186	15	0	201	0.07	93
911.0	A	199	1	0	200	0.01	100
1822	A	NS					-

NS = not scored
Mono = mononucleate
Bi = binucleate
Multi = multinucleate
RI = Replication index

Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \times 100$$

Table 5
Data for 20+28 hour treatments -S-9, Range-Finder- 48 hour PHA Female Donors

Dose (µg/mL)	Replicate	Mono	Bi	Multi	Total Number of Cells	RI	Cytotoxicity (%)
Solvent	A	7	119	74	200	1.34	-
	B	8	101	94	203	1.42	
0.8896	A	22	105	73	200	1.26	9
1.779	A	26	118	56	200	1.15	17
3.559	A	24	125	52	201	1.14	17
7.117	A	130	62	10	202	0.41	71
14.23	A	196	4	0	200	0.02	99
28.47	A	195	5	0	200	0.03	98
56.94	A	199	1	2	202	0.02	98
113.9	A	NS					-
227.8	A	NS					-
455.5	A	NS					-
911.0	A	NS					-
1822	A	NS					-

Table 6
Data for 3+45 hour treatments +S-9, Range-Finder- 48 hour PHA Female Donors

Dose (µg/mL)	Replicate	Mono	Bi	Multi	Total Number of Cells	RI	Cytotoxicity (%)
Solvent	A	5	83	112	200	1.54	-
	B	7	102	91	200	1.42	
0.8896	A	NS					-
1.779	A	NS					-
3.559	A	19	103	80	202	1.30	12
7.117	A	12	108	82	202	1.35	9
14.23	A	64	118	19	201	0.78	47
28.47	A	19	131	50	200	1.16	22
56.94	A	21	124	56	201	1.17	21
113.9	A	47	121	32	200	0.93	37
227.8	A	45	129	30	204	0.93	37
455.5	A	32	126	42	200	1.05	29
911.0	A	56	115	29	200	0.87	41
1822	A	147	51	2	200	0.28	81

NS = not scored
Mono = mononucleate
Bi = binucleate
Multi = multinucleate
RI = Replication index

Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \times 100$$

Table 7
Data for 20+28 hour treatments -S-9, Main Experiment- 24 hour PHA Female Donors

Dose (µg/mL)	Replicate	Mono	Bi	Multi	Total Number of Cells	RI	Cytotoxicity (%)
Solvent	A	127	290	84	501	0.91	-
	B	126	286	91	503	0.93	
	C	121	287	93	501	0.94	
	D	96	300	107	503	1.02	
0.50	A	NS					-
	B						
1.00	A	84	320	96	500	1.02	0
	B	100	312	88	500	0.98	
2.00	A	124	301	76	501	0.90	0
	B	105	296	106	507	1.00	
3.00	A	131	329	50	510	0.84	11
	B	124	331	54	509	0.86	
4.00	A	197	267	45	509	0.70	21
	B	141	320	40	501	0.80	
5.00	A	204	266	31	501	0.65	31
	B	206	272	31	509	0.66	
6.00	A	222	254	32	508	0.63	43
	B	289	196	18	503	0.46	
7.00	A	304	186	18	508	0.44	34
	B	163	263	75	501	0.82	
8.00	A	370	123	8	501	0.28	68
	B	341	149	10	500	0.34	
9.00	A	399	89	12	500	0.23	76
	B	398	96	12	506	0.24	
10.00	A	417	73	12	502	0.19	80
	B	414	76	10	500	0.19	
11.00	A	359	118	24	501	0.33	70
	B	396	99	11	506	0.24	

NS = not scored
Mono = mononucleate
Bi = binucleate
Multi = multinucleate
RI = Replication index

Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \times 100$$

A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for vehicle controls and two [A, B] for test article and positive controls)

Table 8
Data for 3+45 hour treatments +S-9, Main Experiment- 24 hour PHA
Female Donors

Dose (µg/mL)	Replicate	Mono	Bi	Multi	Total Number of Cells	RI	Cytotoxicity (%)
Solvent	A	113	301	96	510	0.97	-
	B	142	279	79	500	0.87	
	C	160	244	107	511	0.90	
	D	92	312	103	507	1.02	
25.00	A	233	257	14	504	0.57	26
	B	146	297	58	501	0.82	
50.00	A	225	260	21	506	0.60	37
	B	229	251	23	503	0.59	
100.00	A	272	204	29	505	0.52	41
	B	235	242	28	505	0.59	
150.00	A	339	153	11	503	0.35	63
	B	338	153	11	502	0.35	
175.00	A	310	187	4	501	0.39	51
	B	259	234	17	510	0.53	
200.00	A	335	153	13	501	0.36	59
	B	302	187	11	500	0.42	
225.00	A	374	128	5	507	0.27	68
	B	343	156	5	504	0.33	
250.00	A	384	108	8	500	0.25	67
	B	330	172	6	508	0.36	
275.00	A	391	106	6	503	0.23	57
	B	234	250	17	501	0.57	
300.00	A	406	90	4	500	0.20	79
	B	408	90	5	503	0.20	
350.00	A	428	79	3	510	0.17	75
	B	357	139	8	504	0.31	
400.00	A	424	81	2	507	0.17	82
	B	416	81	3	500	0.17	

Mono = mononucleate
Bi = binucleate
Multi = multinucleate
RI = Replication index

Cytotoxicity is 100 –RI where RI is calculated using the formulae below:

$$RI = \frac{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in treated cultures}}{\text{(number binucleate cells + 2 x number multinucleate cells) / total number of cells in control cultures}} \; x100$$

A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for vehicle controls and two [A, B] for test article and positive controls)

Appendix 3
2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Statistical analysis of test article data

Table 9
Data for 20+28 hour treatments -S-9, Main Experiment- 24 hour PHA Female Donors

Binomial Dispersion Test ?² = 9.85	DF = 4
Significance: p = 0.05	

Treatment (µg/mL)	Total BN Cells	BN Cells with micronuclei	Proportion	Fisher's exact test	Significance
Solvent	2000	11	0.006	-	-
3.00	2000	15	0.008	0.220	NS
5.00	2000	16	0.008	0.172	NS
8.00	2000	28	0.014	0.003	$p \leq 0.01$
VIN, 0.08	2000	195	0.098	0.000	$p \leq 0.001$
NQO, 5.00	2000	234	0.117	0.000	$p \leq 0.001$

NS = not significant DF = degrees of freedom BN = binucleate

Table 10
Data for 3+45 hour treatments +S-9, Main Experiment- 24 hour PHA Female Donors

Binomial Dispersion Test ?² = 8.27	DF = 4
Significance: p = 0.05	

Treatment (µg/mL)	Total BN Cells	BN Cells with micronuclei	Proportion	Fisher's exact test	Significance
Solvent	2000	17	0.009	-	-
25.00	2000	20	0.010	0.313	NS
100.0	2000	38	0.019	0.002	$p \leq 0.01$
150.0	2000	55	0.028	0.000	$p \leq 0.001$
CPA, 6.25	2000	236	0.118	0.000	$p \leq 0.001$

NS = not significant DF = degrees of freedom BN = binucleate

Appendix 4
Historical vehicle control ranges for human lymphocyte micronucleus (HLM) assay

24 hour PHA

		Micronucleated binucleates observed in 1000 binucleates scored		
		Male donors	Female donors	Male / female donors
-S9	Number of cultures	52	82	134
	Median	3.00	10.00	7.00
	Mean	4.00	9.61	7.43
	SD	2.66	3.92	4.43
	Observed range	**0 - 14**	**2 - 19**	**0 - 19**
	95% reference range	**0 - 10**	**3 - 17**	**1 - 16**
+S9	Number of cultures	70	74	144
	Median	3.00	8.00	4.50
	Mean	3.17	8.57	5.94
	SD	1.75	4.14	4.19
	Observed range	**0 - 8**	**2 - 17**	**0 - 17**
	95% reference range	**0 - 7**	**3 - 17**	**0 - 15**

Reference ranges are calculated from percentiles of the observed distributions.

Calculated in February 2005 by CLEH Statistics, for studies started between December 2003 and October 2004

Note: Female data used for highlighting MNBN frequencies exceeding 'normal' values in this assay.

Appendix 5
Quality control statement for S-9

MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9) QUALITY CONTROL & PRODUCTION CERTIFICATE

LOT NO.: 1737
PART NO.: 11-101
VOLUME: 5ml
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
TISSUE: Liver
PREPARATION DATE: July 29, 2004
EXPIRATION DATE: July 29, 2006
BUFFER: 0.154 M KCl
INDUCING AGENT(s): Aroclor 1254 (Monsanto KL615), 500 mg/kg i.p.
REFERENCE: Maron, D & Ames, B. *Mutat Res* 113:173, 1983
STORAGE: At or below -70°C

BIOCHEMISTRY:

- PROTEIN

38.0 mg/ml

Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	55.0
PROD	2B1	17.6
BROD	2B1	35.4
MROD	1A2	44.9

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 81.2, 23.4, 48.7, & 21.3 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
EtBr/ TA98	CPA/ TA1535
155.6	1136

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

µl S9 per plate/number *his*+ revertants per plate

Promutagen	0	1	5	10	20	50
BP (5 µg)	136	296	289	709	919	1409
2-AA (2.5 µg)	154	999	1032	2547	2605	2388

MOLECULAR TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28607
(828) 264-9099

B.Ward 10/29/04

Appendix 6
Manufacturer's certificate of analysis

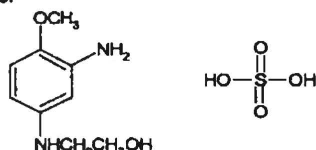
COSMITAL SA

Marly, 19.02.2001 LSY/BR/DOU/bs
modified 03.08.2004

CERTIFICATE OF ANALYSIS

aw material no.: 23081 **Code:** A000157

Structure:



Molecular formula: $C_9H_{14}N_2O_2.H_2O_4S$ **Molecular weight:** 280.30
Molecular weight of free acid: 182.22

LEHMANN BLAU

Trade name: HC BLAU AC (ROBINSON). HC BLUE AC (CLARIANT)

Chemical name: 2-Amino-4-(2-hydroxyethyl)amino-anisole-sulfate

Name (INCI): 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE

CAS-No: 83763-48-8 **EINECS/ELINCS-No:** 280-734-8

Testing material

Sample name: **Sample no:** R96000196

Batch: 57 **Study no.:** A9803/086 and A2004/237

Date of entry: 08.01.96 **Expiry date:** July, 2005

Results

Aspect: pale grey powder

Identity: The ^{1}H-NMR spectra confirmed the chemical identity of the test substance.

Purity: 99.6 area% (by HPLC)

Content: 99.1 weight% ($C_9H_{14}N_2O_2xH_2SO_4xH_2O$) by
93.5 weight% ($C_9H_{14}N_2O_2xH_2SO_4$) NMR

By-products: 0.03 weight% 4-methoxy-1,3-phenylenediaminesulfate (2,4-diaminoanisole)
4-methoxyaniline was not detectable
4-methoxy-3-nitroaniline was not detectable
2-methoxy-5-nitroaniline was not detectable
10.6 weight% of water
28 ppm of sodium

Solubility: 10g/l in water pH 2.8 (>5 weight% pH 8)
1 weight% in acetone/water 1:1 (pH 2.1)
9-10 weight% in DMSO
0.2 weight% in ethanol

Stability: The substance on storage in dryness and darkness to be stable → July 2005.

Stability in solution:
The stability over a total period of seven days was tested by HPLC. The test stock solutions (approx. 5 weight%) were stored at room temperature and in the absence of light.
Water solution: the results (t = 0h: 100.0%; 6h: 93.8%; 2d: 95.1%; 7d: 79.7%) confirm a low degradation (G2000/003)
DMSO solution: the results (t = 0h: 100.0%; 6h: 98.8%; 2d: 92.0%; 7d: 80.5%) confirm a low degradation (G2000/003)

P. Dougoud

Appendix 7
Minor deviations from protocol

Protocol section	Subject	Deviation
Materials	Positive control	The protocol states that three concentrations of Vinblastine would be used, but in The main experiment, the lowest of the three concentrations was omitted. The response to Vinblastine was satisfactory, and so this does not prejudice the integrity of the study in any way.
Methods	Treatment	The protocol states that Cytochalasin B, made up in dimethyl sulphoxide, will be added (0.1 mL/culture) to give a final concentration of 6 μg/mL. Owing to a minor change in laboratory procedure, which in no way compromises the validity of the study, Cytochalasin B was added by media replacement.
Methods	Harvesting	Cells were incubated in KCl for 4 minutes rather 5 minutes, as stated in the protocol. This was a change in procedure introduced to improve the quality of the cells for analysis. The deviation has no adverse impact on the validity of the study.

Definitive Protocol

Study Title	2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081): Induction of micronuclei in cultured human peripheral blood lymphocytes
Study Director	G Clare
Sponsor	
Study Monitor	
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY ENGLAND
Covance Study Number	213/48
Version	Definitive
Page Number	1 of 19

COVANCE.
THE DEVELOPMENT SERVICES COMPANY

OBJECTIVES

To evaluate the clastogenic and aneugenic potential of 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081) by its effects on the frequency of micronuclei in cultured human peripheral blood lymphocytes treated in the absence and presence of a rat liver metabolising system.

TEST SYSTEM

Chromosome defects are recognised as the basis of a number of human genetic diseases (1).

The purpose of the *in vitro* chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells and there is a large database on the use of chromosomal assays for screening purposes (2). The use of human peripheral blood lymphocytes is recommended because the cells are only used in short-term culture and maintain a stable karyotype (3). Experiments with these cells can also be performed in conjunction with a rat liver metabolising system since, for short incubation periods, no toxicity is induced by the liver homogenate itself. Increases in numerical chromosome aberrations can be detected but the assay is not specifically designed to evaluate potential to induce aneuploidy or polyploidy.

An alternative to measuring structural aberrations in mitotic cells is to measure micronuclei. These are produced from whole chromosomes or acentric fragments which are unable to attach to the spindle at mitosis and appear during the next interphase as small darkly staining bodies adjacent to the main daughter nucleus. These are more easily counted than structural aberrations at mitosis and analysis can be performed rapidly on large numbers of cells. The technique as described suffers a disadvantage, however, because the production of a micronucleus is dependent on nuclear division thus the frequency of micronuclei will vary with the proportion of cells which divide. This problem is solved if cytochalasin B is added to the cultures (4). Cytochalasin B inhibits cytokinesis (cell division) but not karyokinesis (nuclear division) resulting in the formation of binucleate cells. If micronuclei are only counted in binucleate cells then a true measurement of their induction can be obtained.

In the first instance cells are exposed for 20 hours in the absence of S-9 (from rats induced with Aroclor) and for 3 hours in the presence of S-9. The test chemical will

be added at 24 hours following culture initiation and cells will be harvested at 72 hours.

If the first experiment is negative or equivocal, provision is made for a second experiment in which cells are treated at 48 hours following culture initiation and harvested at 96 hours. Again, treatment in the absence of S-9 will be for 20 hours and in the presence of S-9 for 3 hours.

The micronucleus test allows one to detect and distinguish between aneugenic (5) and clastogenic events by using fluorescence *in situ* hybridization (FISH) (6,7). If an increase in the frequency of micronucleated cells is observed in this study then provision may be made to further investigate this effect by determining the relative proportions of micronuclei formed by clastogenic and aneugenic events using a pan-centromeric probe (specific for all human centromeres). This identifies micronuclei which comprise whole chromosomes (ie carry a centromere) and the data can be used to evaluate any aneugenic potential. If this additional analysis is required, it will be addressed in a protocol amendment.

In this study, the clastogenic and aneugenic potential of the test chemical will be assessed by its effects on the frequency of micronuclei in lymphocytes of human donors, cultured *in vitro* and treated in the presence and absence of a rat liver metabolising system (S-9).

Although no definitive regulatory guidelines exist for this assay, the test methodology is in accordance with the recommendations of the International Workshop on Genotoxicity Testing (8), draft OECD guideline 487 (9) and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro* (10,11,12,13).

MATERIALS

Test article

The test article will be 2-Amino-4-Hydroxyethylamino-Anisole Sulfate (WR 23081).

The sponsor will supply sufficient test article to allow completion of the study. The sponsor has supplied the following information about the test article:

- Molecular weight: 280.30
- Molecular weight of free acid: 182.22
- Appearance: pale grey powder
- Batch number: 57
- Expiry date: July 2005
- Content: 93.5% weight (by NMR)
- Purity: 99.6% area by HPLC
- Solubility in a variety of common solvents, such as water, DMSO; stated to be soluble in water (approx. 5% w/w)
- Stability under various conditions of heat, light and pH: stated to be stable to expiry date when stored in the dark under dry conditions.
- Storage conditions: room temperature (10-30°C) dry under dark conditions
- any special handling precautions

The concentrations will be calculated and expressed in terms of the free acid, using a conversion factor of 1.5383.

According to information received from the sponsor, the stability of the test article in water (approx. 5% w/w) solution was demonstrated over a 7-day period (under darkened conditions at room temperature).

All test articles are handled as though they were potential carcinogens and appropriate safety procedures are employed

Determinations of the stability and characteristics of the test article, where defined in GLP regulations, are the responsibility of the sponsor. Archiving a sample of the test article will be the responsibility of the Sponsor.

Stability and solubility information provided by the Sponsor, indicate that water is a suitable vehicle. As such, preliminary solubility assessments will be performed using water in the first instance. If other solvents or volumes of organic solvents exceeding

1% (v/v) need to be used, the effects on viability and spontaneous aberration frequencies may need to be checked first.

Choice of solvent will be confirmed in the study file.

Most chemicals that are clastogenic *in vivo* have been shown to (or would be expected to) produce positive responses *in vitro* at concentrations <10 mM (14). For freely soluble test articles, therefore, testing will not proceed above 10 mM, in accordance with current regulatory guidelines for *in vitro* cytogenetic assays.

If the final concentration in the culture medium is equivalent to 10 mM, then the osmolality of the medium will be measured, as increases in osmolality of more than 50 mOsm/kg can be responsible for chromosomal aberrations and thus micronuclei (14). The effect of the test article on the pH of the culture medium will be assessed as fluctuations in pH of more than one unit can give rise to chromosome aberrations (15).

Testing compounds in the precipitating range can be problematical with respect to defining the exposure periods for assays where cells grow in suspension because the test article is difficult to remove by centrifugation and resuspension. If a precipitate is present, the compound will be carried through to later stages of the assay, making control of exposure impossible. For pulse treatments (where the test article is removed and fresh culture medium replaced for recovery) dilutions will be arranged that allow maximum exposure up to the solubility limit only. This is defined as a concentration where precipitate is visible to the naked eye at the end of the treatment period.

Initially, a preliminary toxicity range-finder experiment will be performed using a series of doses, ranging down from the upper limit (spacing intervals may vary). Should the test article be freely soluble, the following range of doses may be tested.

Concentration of treatment solution (mg/mL)	Final concentration (μg/mL)
0.0089	0.8897
0.0178	1.779
0.0356	3.559
0.0712	7.118
0.1424	14.24
0.2847	28.47
0.5694	56.94
1.139	113.9
2.278	227.8
4.556	455.6
9.111	911.1
18.22	1822

Alternative dose ranges will be detailed in the raw data. When possible, stock test solutions will be filter-sterilized.

Concentrations for the main experiment will be based on the toxicity data from the cytotoxicity range-finder experiment.

Controls

Negative controls will comprise treatments with the chosen solvent diluted to the same extent as the test article solutions. Untreated controls will not be included in this study unless a solvent is chosen that is not commonly used in this laboratory.

The positive control chemicals will be prepared (for the main study experiments only) in DMSO (NQO or CPA) or purified water (Vinblastine) to be used as shown in the following table:

Chemical	Supplier*	Concentration of treatment solution (mg/mL)	Final concentration** (µg/mL)	S-9
4-Nitroquinoline 1-oxide (NQO)	Sigma-Aldrich Chemical Co, Poole, UK	0.250 0.500	2.50 5.00	- -
Cyclophosphamide (CPA)	Sigma-Aldrich Chemical Co, Poole, UK	0.625 1.25	6.25 12.5	+ +
Vinblastine (VIN)	Sigma-Aldrich Chemical Co, Poole, UK	0.004 0.006 0.008	0.04 0.06 0.08	- - -

* Source of supply as indicated, or a preparation of equivalent standard
** The treatments giving satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage will be analysed

Positive controls will not be included in the range-finder experiment.

Metabolic activation system

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation is obtained from Molecular Toxicology Incorporated, USA, where it is prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox™ S-9 are stored frozen in aliquots at -80°C prior to use. Each batch is checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin O-dealkylase activities). The quality control statement(s), relating to the batch(es) of S-9 preparation used, will be included in the report.

Treatment will be carried out both in the absence and presence of S-9, prepared in the following way. Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 mM KCl and rat liver S-9 will be mixed in the ratio 1:1:1:2. An aliquot of the resulting S-9 mix will be added to each cell culture to achieve the required final concentration of the test article in a total of 10 mL. The final concentration of the liver homogenate in the test system will be 2%. Cultures treated in the absence of S-9 will receive an equivalent volume of 150 mM KCl.

Blood cultures

Two healthy, non-smoking male/female volunteers will be used for this study. Donors less than 35 years of age (where possible) will be used. No volunteer suspected of any virus infection or exposed to high levels of radiation or hazardous chemicals is used. The measured cell cycle time of the donors used at Covance falls within the range 13 +/- 1.5 hours. An appropriate volume of whole blood will be drawn from the peripheral circulation into heparinised tubes, and if not used immediately, may be stored at 1-10°C for up to two days prior to establishing cultures. Prior to culture, the blood will be pooled using equal volumes from each donor. Wherever possible, the same donors will be used throughout the study.

Whole blood cultures will be established in sterile disposable centrifuge tubes by placing 0.4 mL of pooled heparinised blood into a sufficient volume of HEPES-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg/mL gentamycin, so that the final volume following addition of S-9 mix/KCl and the test article in its chosen solvent is 10 mL. Phytohaemagglutinin (PHA, reagent grade) is included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures will be incubated at 37°C ± 1°C and rocked continuously.

METHODS

Treatment

The test system will be suitably labelled (using a colour-coded procedure) to clearly identify the study number, experiment number, treatment time, test article concentration (if applicable), positive and negative controls.

For the cytotoxicity range-finder experiment, duplicate cultures (A, B) will be treated with the solvent and single cultures treated with the test article at appropriate concentrations. S-9 mix or KCl (0.5 mL) will be added appropriately. The conditions will be those for Experiments 1 and 2, as described in the table in this section.

For the main study treatments, quadruplicate cultures (A, B, C and D) will be treated with the solvent, and duplicate cultures treated with the test article at appropriate concentrations. Additional duplicate cultures are treated with the positive control chemicals. S-9 mix or KCl (0.5 mL) will be added appropriately.

Experiment 1 will comprise a 20-hour treatment - S-9 and a 3 hour treatment +S-9. The test chemical will be added at 24 hours following culture initiation and cells will be harvested at 72 hours. The final 27-28 hours (approximately) of incubation will be in the presence of Cytochalasin B (at a final concentration of 6 μg/mL).

If the result of treatment in Experiment 1 is negative or equivocal, cells will be treated in Experiment 2, at 48 hours following culture initiation and harvested at 96 hours. Again, treatment in the absence of S-9 will be for 20 hours and in the presence of S-9 for 3 hours and the final 28 hours (approximately) of incubation will be in the presence of Cytochalasin B.

Clear positive effects observed in Experiment 1 need not be confirmed in a further experiment.

Summary of treatment conditions

	Duration of treatment (hours)	S-9	Hours after culture initiation			
			Addition of test chemical	Removal of test chemical	Addition of Cytochalasin B	Harvest time
Experiment 1	20	-	24	44	44-45*	72
	3	+	24	27	44-45*	72
Experiment 2	20	-	48	68	68-69*	96
	3	+	48	51	68-69*	96

*Approximate times

For removal of the test chemical, cells will be pelleted (approximately 300 x 'g', 10 minutes), washed twice with sterile saline, and resuspended in fresh medium containing foetal calf serum and gentamycin. At the appropriate times, Cytochalasin B, made up in dimethyl sulphoxide, will be added (0.1 mL/culture) to give a final concentration of 6 µg/mL.

Harvesting

At the defined sampling time, cultures will be centrifuged at approximately 300 x 'g' for 10 minutes, the supernatant carefully removed and cells resuspended in 4 mL (hypotonic) 0.075 M KCl at 37°C for 5 minutes to allow cell swelling to occur. Cells will be fixed by dropping the KCl suspension into fresh, cold methanol/glacial acetic acid (3:1, v/v). The fixative will be changed by centrifugation (at approximately 300 x 'g' for 10 minutes) and resuspension. This procedure will be repeated several times (centrifuging at approximately 1250 x 'g', two to three minutes) until the cell pellets are clean.

Slide preparation

Lymphocytes will be kept in fixative in the refrigerator before slides are made, but slides will not be made on the day of harvest in order to ensure that cells are adequately fixed. Cells will be pelleted and resuspended in a minimal amount of fresh fixative (if required) so as to give a milky suspension. Several drops of suspension will be gently spread onto multiple clean, dry microscope slides. After the slides have dried the cells will be stained for 5 minutes in filtered 4% (v/v) Giemsa in pH 6.8 buffer. The slides will be rinsed, dried and mounted with coverslips.

Selection of doses for the main study experiments

Slides from the cytotoxicity range-finding experiment will be examined, uncoded, for proportions of mono-, bi- and multinucleate cells, to a minimum of 200 cells per concentration. From these data the replication index (RI) will be determined using the formula described below.

A suitable range of concentrations will be selected for the main experiment based on these toxicity data.

Selection of doses for micronucleus analysis

Slides from the main study experiments will be examined, uncoded, for proportions of mono-, bi- and multinucleate cells, to a minimum of 500 cells per culture. From these data the replication index (RI) will be determined using the following formula:
Replication Index (RI) indicates the relative number of nuclei compared with controls:

$$RI = \frac{\text{number binucleate cells} + 2\ (\text{number multinucleate cells})/\text{total number of cells in treated cultures}}{\text{number binucleate cells} + 2\ (\text{number multinucleate cells})/\text{total number of cells in controls}} \times 100$$

This indicates a value relative to the control. Expressed as a percentage cytotoxicity, the value is: 100 –RI = %Cytotoxicity

A selection of random fields will be observed from enough treatments to determine whether chemically induced cell cycle delay or cytotoxicity has occurred.

The highest dose for micronucleus analysis should be one at which at least 60% (approximately) reduction in RI has occurred or should be the highest dose tested. Analysis of slides from highly cytotoxic concentrations will be avoided.

The rationale for the limit of approximately 60% cytotoxicity is based on limited data (discussed by the IWGTP (8)) which show that in some cases (i.e. some aneugens), a very steep toxicity curve is observed and very closely spaced doses in the range of 50-60% toxicity need to be evaluated. Data from certain validation experiments have demonstrated that the lowest observed effective dose (LOEDs) for the aneugens diethylstilbestrol and vincristine showed a relative cell count (RCC) of 42% and 43% respectively; this corresponding to a toxicity of approximately 60%. These compounds might not have been found to be micronucleus inducers if they had not been tested up to the 60% toxicity level.

For poorly soluble compounds, slides from precipitating cultures will be checked to confirm that the presence of precipitate does not preclude analysis.

Slides from the highest selected dose and two lower doses, such that a range of cytotoxicity from maximum to little or none is covered, will be taken for microscope analysis.

Slide analysis

Acceptance criteria

Binucleate cells will only be included in the analysis if all of the following criteria are met:

1) the cytoplasm has remained essentially intact, and

2) the daughter nuclei are of approximately equal size.

A micronucleus will only be recorded if it meets the following criteria:

1) the micronucleus should have the same staining characteristics and a similar morphology to the main nuclei, and

2) any micronucleus present is separate in the cytoplasm or only just touching a main nucleus, and

3) micronuclei should be smooth edged and smaller than approximately one third the diameter of the main nuclei.

Procedure

Slides from the CPA, NQO and Vinblastine-treated positive control cultures will initially be examined for micronuclei to ensure that the system has responded satisfactorily. Slides from the selected treatments and from solvent and positive controls will then be coded, using randomly generated letters, by a person(s) not connected with the scoring of the slides. Labels will be used to cover any treatment details on the slides, so that the cytogeneticists can only see the study number and the code.

One thousand binucleate cells from each culture (2000 per dose level) will be analysed for micronuclei. The number of cells containing micronuclei and the

number micronuclei per cell on each slide will be noted. Observations will be recorded on raw data sheets.

Slide analysis will be performed by competent analysts trained in the applicable Covance Laboratories Harrogate (CLEH) standard operating procedures. Analysts may be physically located remote from the CLEH facility, but regardless of location, all analysts will be subject to CLEH management and GLP control systems (including QA inspection). All slides and raw data generated by remote analysts will be returned to CLEH for archiving. Details of the analysts involved in scoring will be recorded in the study data, and the use of any remote analysts will be specified in the final report.

Analysis of results

Treatment of data

After completion of scoring and decoding of slides, the numbers of binucleate cells with micronuclei in each culture will be obtained and tabulated in the report.

The proportions of micronucleated cells in each replicate will be used to establish acceptable homogeneity between replicates by means of a binomial dispersion test (16).

The proportion of cells with micronuclei for each treatment condition will be compared with the proportion in negative controls by using Fisher's exact test (16). Probability values of p #0.05 will be accepted as significant. Additionally the number of micronuclei per binucleate cell will also be presented,

Acceptance criteria

The assay will be considered valid if the following criteria are met:

1) the binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, particularly where no positive responses are seen, and

2) the frequency of cells with micronuclei in negative controls falls within the normal range, and

3) the positive control chemicals induce statistically significant increases in the proportion of cells with micronuclei, and

4) a minimum of 50% of cells have undergone cell division (binucleate or multinucleate) in negative control cultures at the time of harvest.

Acceptance under any other criteria would be discussed in the report.

Evaluation criteria

A test chemical will be considered as clearly positive in this assay if:

1) a statistically significant increase in the proportion of cells with micronuclei occurs at one or more concentrations, and

2) the incidence of micronucleated cells at such data points exceeds the normal range.

GLP COMPLIANCE

The study will be performed in compliance with:

United Kingdom Statutory Instrument 1999 No. 3106, The Good Laboratory Practice Regulations 1999, as amended by the Good Laboratory Practice (Codification Amendments Etc) Regulations 2004.

OECD Principles on Good Laboratory Practice (revised 1997, issued Jan 1998) ENV/MC/CHEM (98) 17.

All procedures will be performed in accordance with Covance Laboratories Limited standard operating procedures (SOPs). The study will be subject to Quality Assurance evaluation by the Covance Laboratories Limited Quality Assurance Unit (QAU) in accordance with SOPs. Where appropriate, any change to this protocol will be made by an amendment issued in agreement with the Sponsor.

Following completion of the study, a draft report will be issued. Client comments should be supplied for inclusion into a final document within six months of receipt of the draft document.

In accordance with the requirements of the UK Medicines and Healthcare Products Regulatory Agency, if no client comments are received within six months of issue, the report will be finalised. Any further changes after this time will be addressed as an amended final report, which may result in additional costs.

A list of study records to be maintained by Covance Laboratories Limited is detailed in Appendix 1.

PROPOSED TIME SCHEDULE

It is estimated that the study will start in the week beginning 29 November 2004 such that the experimental phase will be completed during the week beginning 24 January 2005. An audited draft report will be issued to the sponsor during the week beginning 28 February 2005 (an electronic copy will also be included). Following comments from the sponsor and from the Covance Laboratories Limited QA Unit a final report will be agreed. One signed copy (bound, single sided) and an electronic copy of the final report will be issued.

Should any unforeseen circumstance alter the proposed timescale, the sponsor will be informed immediately. Covance Laboratories Limited cannot guarantee to meet the proposed timescale unless this protocol is approved (by signature and return or by telefax) by 25 November 2004.

Following receipt of the signed protocol approval and the test article, a detailed schedule confirming timings of the experimental phase and issue date of the draft report will be sent to the Sponsor.

The study schedule may not be possible should the test article either, demonstrate an overtly steep toxicity profile, or demonstrate insufficient toxicity (according to the requirements of the protocol). If additional work is required to identify suitable concentrations for micronucleus analysis, the experimental and reporting schedule may be affected. In such cases, the Sponsor Monitor will be contacted and a revised schedule issued by protocol amendment.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, specimens and the final report will be retained in the Covance Laboratories Limited archives for **ten** years after issue of the final report. At this time the sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

On studies in which the test article is tested for longer than four weeks duration, it is the responsibility of the Sponsor to ensure that a sample from each test article batch is retained for analytical purposes.

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Appendix 1
Study records to be maintained

Definitive protocol
Amendment(s)*
File note(s)*
Study schedule
Report schedule
Study correspondence
Client correspondence
Test article description
Test article formulation assessment*
Dispensary requests
Test article utilisation
Details of formulation preparation
Formulation storage and dispatch*
Analysis of formulations*
Metrology#
Records for reagents and stock solutions#
Blood donor records*
Culture records
Slide preparation*
Cytogenetic data*
Statistical analysis

some records held centrally
* where appropriate

Appendix 2
Responsible personnel

STUDY MANAGEMENT[1]	NAME
Study Director	G Clare +44 (0)1423 848892 gillian.clare@covance.com REF
Study Monitor	S Balakrishnan 1-818-710-5681 sbalakrishnan@wellacorp.com

QUALITY ASSURANCE[2]	
Director, Quality Systems	C Clare + 44 (0)1423 848399 christopher.clare@covance.com

1 = Any change documented by protocol amendment
2 = Any change documented in study records

DISTRIBUTION: Personnel above

PROTOCOL APPROVAL

Please sign both approval pages; return one to the Study Director and retain one for your records.

03 December 2004
Date

G Clare
Study Director
Covance Laboratories Ltd

24th November 2004
Date

J Clements
CLE Management
Covance Laboratories Ltd

24 November 2004
Date