

**Sublimed Undensified Molybdenum Trioxide:
In-vitro Micronucleus Assay in Human
Lymphocytes**

Study No. CTL/SV1230 conducted in 2004

by

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Prepared for:



INTERNATIONAL MOLYBDENUM ASSOCIATION

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**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK**

CTL/SV1230/REGULATORY/REPORT

**SUBLIMED UNCONDENSED MOLYBDENUM TRIOXIDE:
IN VITRO MICRONUCLEUS ASSAY IN HUMAN
LYMPHOCYTES**

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IN VITRO MICRONUCLEUS ASSAY IN HUMAN
LYMPHOCYTES**

STUDY DETAILS

Sponsor: International Molybdenum Association
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Sponsor Reference: CO3162/3
CTL Test Substance Reference Number: Y12618/001
CTL Study Number: SV1230
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AUTHOR

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DATE OF ISSUE

6 May 2005

STATEMENT OF DATA CONFIDENTIALITY CLAIM

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STATEMENT OF GLP COMPLIANCE AND AUTHENTICATION

I, the undersigned, declare that the objectives laid down in the protocol were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.

The study (SV1230) was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Regulations 1999, Statutory Instrument No. 3106, as amended 2004, Statutory Instrument No.994) except for the deviation listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

The following GLP deviation is considered not to affect the integrity of the study or the validity of the conclusions drawn:

- (i) the stability, homogeneity and achieved concentration of the test substance in the vehicle used were not determined by analysis.

V Fox
Study Director


.....

6 May 2005
Date

This page may be
Required by some
Regulatory Authorities

QUALITY ASSURANCE STATEMENT

In accordance with CTL policy and QA procedures for Good Laboratory Practice, this report has been audited and the conduct of this study has been inspected as follows:

Date	Audit/Inspection	Date of QA Report
05 Mar 2004	Slide preparation	09 Mar 2004
24 May 2004	Draft report	28 May 2004
04 May 2005	Final report review	05 May 2005

In addition, inspections associated with this type of study were made as follows:

15 Jan 2004	Protocol	15 Jan 2004
21 Jan 2004	Slide coding	22 Jan 2004
22 Jan 2004	Slide scoring	22 Jan 2004
30 Jan 2004	Transfer	30 Jan 2004
12 Feb 2004	Dilutions	13 Feb 2004
04 Mar 2004	Dose administration	04 Mar 2004
18 Mar 2004	Dose preparation	18 Mar 2004
18 Mar 2004	Addition of S9	18 Mar 2004

Facilities and process based procedures associated with this type of study were inspected in accordance with QA Standard Operating Procedures.

So far as can be reasonably established, the methods described and the results given in the final report accurately reflect the raw data produced during the study, SV1230.

V L Wright

..... 6 May 2005

(CTL Quality Assurance Unit)

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

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1. SUMMARY

1.1 Study design

Sublimed Undensified Molybdenum Trioxide (CAS No. 1313-27-5) was evaluated for its clastogenic or aneugenic potential in an *in vitro* micronucleus assay in three separate experiments using human lymphocytes treated in the presence and absence of a rat liver-derived metabolic activation system (S9-mix). In Experiments 1 and 2, cultures were treated for a period of 3 hours both in the presence and absence of S9-mix. In an additional experiment (Experiment 3), cultures were treated for 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix.

Cultures treated with Sublimed Undensified Molybdenum Trioxide at the following concentrations were selected for micronucleus analysis along with the appropriate solvent and positive control cultures.

Experiment 1		Experiment 2		Experiment 3		
+S9-mix	-S9-mix	+S9-mix	-S9-mix	+S9-mix	-S9-mix	-S9-mix
3 hour treatment	20 hour treatment					
1439µg/ml	1439µg/ml	1000µg/ml	1000µg/ml	1439µg/ml	1439µg/ml	1000µg/ml
1000µg/ml	1000µg/ml	500µg/ml	500µg/ml	1000µg/ml	1000µg/ml	500µg/ml
250µg/ml	250µg/ml	100µg/ml	100µg/ml	250µg/ml	250µg/ml	100µg/ml

1.2 Results

The highest concentration selected for micronucleus analysis was 10mM (1439µg/ml), which is the limit concentration for the assay, or was limited by toxicity.

Reductions in mitotic activity were observed in cultures from all experiments, thus demonstrating that Sublimed Undensified Molybdenum Trioxide is biologically active in this test system.

Precipitation of the test substance in the culture medium was visible on the slides of cultures treated with Sublimed Undensified Molybdenum Trioxide at concentrations of 1000µg/ml and above.

The culture medium was at an acceptable pH (within the normal control culture range) for the duration of treatment with the test substance. Changes in pH have been reported to result in the production of chromosomal aberrations (Scott *et al*, 1991). As the test substance was acidic, the pH of the stock test substance was adjusted to between 7.0 and 7.3 using sodium hydroxide and checked at the end of the treatment periods.

Statistically significant increases in the percentage of aberrant cells, compared to the solvent control, were recorded at 1439 μ g/ml in Experiment 1 both in the presence and absence of S9-mix.

No statistically significant increases in micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 2 in either the presence or absence of S9-mix.

As the highest concentration used in Experiment 2 was not as high as that used in Experiment 1, a third experiment was conducted to clarify the results obtained in Experiment 1. In Experiment 3, cultures were treated for 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix. No statistically significant increases in the incidence of micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 3 showing that the increases in micronuclei observed in Experiment 1 were not reproducible.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in micronucleated binucleate cells induced by the positive control agents, mitomycin C and cyclophosphamide.

1.3 Conclusion

It is concluded that, under the conditions of this assay, Sublimed Undensified Molybdenum Trioxide is not reproducibly clastogenic or aneugenic to cultured human lymphocytes treated *in vitro* in either the presence or absence of S9-mix.

2. INTRODUCTION

2.1 Purpose

The purpose of this study was to investigate whether the test substance induced genetic damage (clastogenic as a result of chromosome breakage or aneugenic as a result of chromosome lagging) *in vitro* in the human peripheral blood lymphocyte micronucleus assay.

2.2 Regulatory guidelines

There are no specific Regulatory Guidelines relating to studies of this type. The study design is based on the recommendations of Kirsh-Volders *et al* (2003).

2.3 Justification for test system selection

The *in vitro* human lymphocyte micronucleus assay is designed to detect chemically induced changes in cells treated in culture. The human peripheral lymphocyte used is a sensitive target cell for the induction of *in vitro* genetic damage when stimulated to provide large numbers of rapidly dividing cells in culture. When the cells are in exponential growth they are exposed to various concentrations of the test substance. The cells are harvested, slides are prepared and analysed for the presence or absence of micronucleated binucleate cells. Since micronuclei can also be seen in solvent control cultures, the assay is based on the observation of an increased incidence of micronucleated cells over and above that seen in the solvent control cultures.

Molybdenum salts have been reported to induce micronuclei *in vitro* in human lymphocytes (Titenko-Holland *et al*; 1998) and syrian hamster embryo cells (Gibson *et al*; 1997). In neither of these studies was any adjustment made for possible changes in pH. Changes in pH have been reported to result in the production of chromosomal aberrations (Scott *et al*, 1991). As the test substance was acidic, the pH of the stock test substance was adjusted to between 7.0 and 7.3 using sodium hydroxide and checked at the end of the treatment periods.

2.4 Study dates

The study was initiated on 3 February 2004. The experimental phase started on 3 February 2004 and was completed on 8 April 2004.

2.5 Data storage

An original report, the study protocol and all raw data, samples and specimens pertaining to this study are retained in the CTL Archives, Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK for a minimum of 5 years from the date of issue of the final report. At the end of this period the Sponsor will be contacted regarding the further retention, return to the Sponsor or destruction of the archived materials.

3. TEST AND CONTROL SUBSTANCES

3.1 Test substance

Name:	Sublimed Undensified Molybdenum Trioxide (CAS No. 1313-27-5)
Source:	IMOA
Colour:	Pale green
Physical state:	Powder
Batch reference:	Lot 1MS
CTL test substance reference number:	Y12618/001
Purity:	99.9% w/w
Storage conditions:	Cool, dark well ventilated area
Expiry date:	In excess of 1 year

From information supplied by the Sponsor, the test substance was used within the expiry date.

The characterisation of the test substance is the responsibility of the Sponsor.

In all cases where the concentration of test substance is quoted, this concentration refers to the concentration of test substance not corrected for purity.

3.2 Control substances

The control substance and solvent for the test sample was supplemented RPMI-1640 culture medium (CTL test substance reference number Y08056/001).

The positive control substances, mitomycin C (CTL test substance reference number Y02201/004) and cyclophosphamide (CTL test substance reference number Y01259/042) were obtained from Sigma Chemical Company.

4. EXPERIMENTAL PROCEDURES

4.1 Dosing preparations

An individual stock suspension of the test substance was prepared for each experiment in supplemented RPMI-1640 culture medium and the pH was adjusted to 7.0 in Experiment 1 to neutralise the culture medium and 7.3 in Experiments 2 and 3 (which is closer to the normal pH of culture medium) using sodium hydroxide. Serial dilutions were carried out as required in each case.

Both positive control substances were prepared as solutions in sterile double deionised water (CTL test substance reference number Y04517/035).

All test and positive control substance dosing preparations were prepared as close to the time of culture treatment as possible and were dosed at 8ml/10ml culture following removal of 8ml of the culture medium.

4.2 Analysis of dosing preparations

In view of the short term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration were carried out on the preparations of the test or positive control substances either prior to or after addition to the human peripheral blood lymphocyte cultures.

4.3 Experimental design

Duplicate human peripheral blood cultures were exposed to the solvent, test substance or positive control substances at appropriate concentrations in the following experiments:

- a) An *in vitro* micronucleus experiment (Experiment 1) was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a

period of 3 hours, both in the presence and absence of S9-mix. Solvent, untreated and positive control cultures were included.

- b) A second independent *in vitro* micronucleus experiment (Experiment 2) was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a period of 3 hours, both in the presence and absence of S9-mix. Solvent, untreated and positive control cultures were included.
- c) A third independent *in vitro* micronucleus experiment (Experiment 3) was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a period of 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix. Solvent, untreated and positive control cultures were included.

Treatment of the cultures started approximately 24 hours after culture initiation. A single sampling time, 68 hours after culture initiation, was used.

In all three experiments a range of concentrations of Sublimed Undensified Molybdenum Trioxide was used in order to define suitable concentrations for micronucleus analysis.

4.4 Culture establishment

Human blood samples were obtained by venepuncture in lithium heparin tubes on the day prior to culture initiation or the day of culture initiation from healthy, non-smoking donors. Equal volumes of blood from 2 x 2 female donors were pooled together for Experiments 1 and 2 and blood from 2 male donors was pooled together for Experiment 3. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes and therefore an assumed low incidence of micronuclei caused by chromosomal breakage.

At 0 hours, cultures (10ml) were established by the addition of 0.5ml of whole blood to RPMI-1640 (Dutch modification) tissue culture medium supplemented with approximately 10% foetal bovine serum (FBS), 1.0IU/ml heparin, L-glutamine (2mM), 100IU/ml penicillin and 100µg/ml streptomycin. The lymphocytes were stimulated to enter cell division by addition of phytohaemagglutinin (PHA; at 5% v/v) and the cultures were maintained at approximately 37°C for 24 hours with gentle daily mixing where possible.

4.5 Metabolic activation system

The metabolic activation system (S9-mix) used in this study was prepared as required (on each day of culture treatment) as a 1:1 mixture of S9 fraction and cofactor solution.

S9 was purchased from MolTox Inc., and was prepared from male Sprague Dawley rats, dosed once daily (by oral gavage) for 3 days with a combined phenobarbital (80mg/kg bodyweight) and β -naphthoflavone (100mg/kg bodyweight) corn oil preparation. The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared according to the method given in Callander *et al* (1995).

The cofactor solution was prepared as a single stock solution of Na₂HPO₄, KCl, glucose-6-phosphate, NADP (Na salt) and MgCl₂ (150 : 49.5 : 7.5 : 6 : 12mM) in sterile double deionised water and adjusted to a final pH of 7.4.

4.6 Culture treatment

Approximately 24 hours after culture establishment, the cultures were centrifuged and 8ml of the culture medium was removed and replaced with 8ml aliquots of supplemented culture medium containing the appropriate concentrations of the test substance. The culture medium was removed from the solvent control and positive controls and replaced with fresh supplemented medium. The positive controls were then treated with the appropriate concentration of cyclophosphamide or mitomycin C. In addition, 200 μ l of a 1:1 mix of S9 and co-factor solution was added to each culture to be treated in the presence of S9-mix.

Cultures from all experiments in the presence and absence of S9-mix were treated for a period of 3 hours at 37°C, after which the culture medium was removed following centrifugation and replaced with fresh supplemented RPMI-1640 culture medium. In addition, cultures from Experiment 3 in the absence of S9-mix were treated for a period of 20 hours, after which the culture was removed following centrifugation and replaced with fresh supplemented RPMI-1640 culture medium. The cultures were re-incubated at 37°C for the remainder of the 68 hour growth period.

The pH of the stock suspension of the test substance was adjusted to pH 7.0 in Experiment 1 and pH 7.3 in Experiments 2 and 3. In Experiment 1, the culture medium was neutralised to pH 7.0 and in subsequent experiments, it was decided to adjust the pH to a value representing

the normal pH of culture medium. The pH of the culture medium was checked in all experiments at the end of the treatment periods, using single treated cultures (minus blood, PHA and S9-mix) treated with the test substance. The osmolality of the culture medium was determined immediately following treatment in Experiment 2. The solubility of the test substance in the treated blood cultures and in media-only cultures was assessed by eye immediately after treatment and at the end of the treatment periods.

4.7 Culture harvesting

Approximately 24 hours prior to harvesting, the cultures were treated with cytochalasin B at a final concentration of 5.4µg/ml. Sixty-eight hours after culture establishment, the cultures were centrifuged, the supernatant was removed and the cells were treated with 5ml of pre-warmed (37°C) 0.075M KCl for approximately 5 minutes at room temperature. The blood culture/KCl was mixed whilst adding pre-cooled (4°C) freshly prepared methanol/glacial acetic acid fixative (3:1 v/v) dropwise and made up to a volume of approximately 10ml with methanol/acetic acid. The blood culture/KCl/fixative mixture was incubated for 10 minutes at room temperature. The fixative was removed following centrifugation and replaced with freshly prepared fixative. After at least two subsequent changes of fixative, slides were made by dropping the cell suspension on to clean, moist, labelled microscope slides. The slides were air dried, stained in filtered Giemsa stain (10% Gurr's R66 in buffered [pH 6.8] double deionised water) for 7 minutes, rinsed in water, air-dried and mounted with coverslips in DPX.

4.8 Slide analysis

Slides were examined to determine that they were of suitable quality and, where appropriate, the cytokinesis-block proliferation index (CBPI) was determined by examining 500 cells per culture. The CBPI is defined as:

$$\begin{array}{l} 1 \times \text{number of mononucleate cells} \\ + 2 \times \text{number of binucleate cells} \\ + 3 \times \text{number of tri- and tetranucleate cells} \\ \hline \text{total number of cells scored} \end{array}$$

For each experiment, both in the presence and absence of S9-mix, duplicate cultures treated with Sublimed Undensified Molybdenum Trioxide at three concentrations were selected for micronucleus scoring along with the appropriate solvent and positive control cultures.

The slides were coded and five hundred binucleate cells were analysed from each selected culture for the incidence of micronuclei.

5. DATA EVALUATION

The Fisher Exact Probability Test (one-sided) was used to evaluate statistically the percentage of binucleate cells with micronuclei. Data from each treatment group, in the presence and absence of S9-mix, was compared with the respective solvent control group value. The data from each experiment have been interpreted as follows:

- a) No statistically significant increase in micronucleated binucleate cells (at any concentration) above concurrent solvent control values - **NEGATIVE**.
- b) A statistically significant increase in micronucleated binucleate cells, at least at one concentration - **POSITIVE**.

6. RESULTS

6.1 Determination of CBPI and selection of concentrations

Three independent *in vitro* micronucleus experiments were carried out using ranges of Sublimed Undensified Molybdenum Trioxide concentrations as detailed in Tables 1 - 2 which also detail the individual culture and mean CBPI and the number of binucleate cells observed for the solvent controls and the Sublimed Undensified Molybdenum Trioxide treated cultures.

The highest concentration selected for micronucleus analysis was 10mM (1439µg/ml), which is the limit concentration for the assay, or was limited by toxicity. Where the limit concentration was not selected, this was because the cultures at 1439µg/ml were considered

not to be suitable for micronucleus analysis due to lack of binucleate cells as a result of toxicity (Tables 1 - 2).

Reductions in mean binucleate index, compared to the solvent control values, were observed in cultures from both Experiment 1 (46% +S9-mix; 18% 3 hour -S9-mix), Experiment 2 (53% +S9-mix; 32%; 3 hour -S9-mix) and Experiment 3 (68% +S9-mix; 56% 3 hour -S9-mix, 73% 20 hour -S9-mix) treated with the highest concentrations of Sublimed Undensified Molybdenum Trioxide selected for micronucleus analysis.

Precipitation of the test substance in the culture medium was visible on the slides of cultures treated with Sublimed Undensified Molybdenum Trioxide at concentrations of 1000µg/ml and above.

The culture medium was at an acceptable pH (within the normal control culture range) for the duration of treatment with the test substance. Changes in pH have been reported to result in the production of chromosomal aberrations (Scott *et al*, 1991). As the test substance was acidic, the pH of the stock test substance was adjusted to between 7.0 and 7.3 using sodium hydroxide and checked at the end of the treatment periods (Table 5).

Treatment of the culture medium with Sublimed Undensified Molybdenum Trioxide up to 1439µg/ml (10mM) had no significant effect on osmolality (Table 6).

6.2 Results of micronucleus analyses

From the results detailed in Section 6.1, cultures treated with following concentrations of Sublimed Undensified Molybdenum Trioxide were selected for micronucleus analysis.

Experiment 1		Experiment 2		Experiment 3		
+S9-mix	-S9-mix	+S9-mix	-S9-mix	+S9-mix	-S9-mix	-S9-mix
3 hour treatment	20 hour treatment					
1439µg/ml	1439µg/ml	1000µg/ml	1000µg/ml	1439µg/ml	1439µg/ml	1000µg/ml
1000µg/ml	1000µg/ml	500µg/ml	500µg/ml	1000µg/ml	1000µg/ml	500µg/ml
250µg/ml	250µg/ml	100µg/ml	100µg/ml	250µg/ml	250µg/ml	100µg/ml

The results of the micronucleus analysis are shown in Tables 3 – 4 expressed as the mean percentage of micronucleated binucleate cells at each concentration. Individual data from each culture are given in Tables 7 - 8.

Statistically significant increases in the percentage of aberrant cells, compared to the solvent control, were recorded at 1439µg/ml in Experiment 1 both in the presence and absence of S9-mix.

No statistically significant increases in micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 2 in either the presence or absence of S9-mix.

Although the highest concentration used in Experiment 2 (1000µg/ml) was not as high as that used in Experiment 1 (1439µg/ml), the degree of biological activity (toxicity) as measured by reduction in the percentage of binucleate cells and CBPI, was comparable or greater.

However, a third experiment was conducted to clarify the results. In Experiment 3, cultures were treated for 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix. No statistically significant increases in the incidence of micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 3.

The positive control materials, mitomycin C and cyclophosphamide induced statistically and biologically significant increases in the percentage of aberrant cells, compared to the solvent control cultures.

7. DISCUSSION

The test substance, Sublimed Undensified Molybdenum Trioxide, was assessed in an *in vitro* micronucleus assay in cultured human peripheral blood lymphocytes to determine if it induces genetic damage.

Sublimed Undensified Molybdenum Trioxide was tested over a range of concentrations, both in the presence and absence of S9-mix, in three independent cytogenetic tests.

Increases in micronucleated binucleate cells observed in Experiment 1 were not reproducible in Experiment 2 or in Experiment 3 in which the same concentration as that used in

Experiment 1 was evaluated for cultures treated for 3 hours in the presence and absence of S9-mix and a 20 hour treatment period in the absence of S9-mix was also used.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in the frequencies of aberrant cells induced by the positive control agents, mitomycin C and cyclophosphamide.

The data obtained in this study therefore show that the test sample of Sublimed Undensified Molybdenum Trioxide did not induce reproducible genetic damage in human peripheral blood lymphocytes following *in vitro* treatment in either the presence or absence of S9-mix.

8. CONCLUSION

It is concluded that, under the conditions of this assay, Sublimed Undensified Molybdenum Trioxide (CAS No. 1313-27-5) is not reproducibly clastogenic or aneugenic to cultured human lymphocytes treated *in vitro* in either the presence or absence of S9-mix.

9. REFERENCES

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TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 – 3 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.664	1.587	260	238
	1.510		215	
Sublimed Undensified Molybdenum Trioxide (µg/ml)	1.422	1.414	199b	194
	1439 1.406		189b	
1000	1.506	1.496	221b	221
	1.486		221b	
500	a	1.678	a	268
	a		a	
250	1.682	1.678	265	268
	1.674		271	
175	a	1.678	a	268
	a		a	
100	a	1.678	a	268
	a		a	
50	a	1.678	a	268
	a		a	
10	a	1.678	a	268
	a		a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Precipitate of test substance visible on the slides

TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 2 – 3 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.654	1.694	301	316
	1.734		331	
Sublimed Undensified Molybdenum Trioxide (µg/ml)	1.160	1.160 Δ	80c	80
	b		c	
1439	1.474	1.433	231c	214
	1.392		196c	
1000	1.682	1.665	311d	306
	1.648		300d	
500	a			
	a			
250	a		a	
	a		a	
175	1.776	1.756	342	340
	1.736		338	
100	a		a	
	a		a	
50	a		a	
	a		a	
10	a		a	
	a		a	

- a - CBPI and binucleate cell count not required for selection of concentrations for analysis
- b - Too few binucleate cells and excessive reduction in binucleate cells observed in Culture A
- c - Precipitate of test substance visible on the slides
- d - Light precipitate visible on the slides
- Δ - Mean determined from a single culture

TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 – 3 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.514	1.519	245	245
	1.524		244	
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
	1439	1.206 1.230	1.218	103b 113b
1000	1.296	1.329	148b	164
	1.362		179b	
500	a	1.547	a	247
	a		a	
250	1.510	1.547	229	247
	1.584		264	
175	a	1.547	a	247
	a		a	
100	a	1.547	a	247
	a		a	
50	a	1.547	a	247
	a		a	
10	a	1.547	a	247
	a		a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
 b - Precipitate of test substance visible on the slides

TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3-- 20 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.460	1.531	230	244
	1.602		257	
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	b		bc	
	b		bc	
1000	1.114	1.134	57c	67
	1.154		77c	
500	1.248	1.277	124	138
	1.306		151	
250	a		a	
	a		a	
175	a		a	
	a		a	
100	1.536	1.570	226	244
	1.604		262	
50	a		a	
	a		a	
10	a		a	
	a		a	

- a - CBPI and binucleate cell count not required for selection of concentrations for analysis
- b - Too few binucleate cells for micronucleus analysis
- c - Precipitate of test substance visible on the slides

TABLE 2 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 – 3 hour treatment (+S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.534	1.489	229	215
	1.444		200	
Sublimed Undensified Molybdenum Trioxide (µg/ml)	1.222	1.232	111b	116
	1.242		121b	
1439	1.460	1.469	208b	217
	1.478		226b	
1000	a	1.517	a	233
	a		a	
500	1.526	1.517	237	233
	1.508		228	
250	a	1.517	a	233
	a		a	
175	a	1.517	a	233
	a		a	
100	a	1.517	a	233
	a		a	
50	a	1.517	a	233
	a		a	
10	a	1.517	a	233
	a		a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Precipitate of test substance visible on the slides

TABLE 2 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 2 – 3 hour treatment (+S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.784	1.787	338	333
	1.790		327	
Sublimed Undensified Molybdenum Trioxide (µg/ml)	b	1.314	bc	157
	1439		b	
1000	1.320	1.534	160c	244
	1.308		154c	
500	1.524	1.729	242d	309
	1.544		246d	
250	a	1.714	a	301
	a		a	
175	a	1.744	a	316
	a		a	
100	a	a	a	a
	a		a	
50	a	a	a	a
	a		a	
10	a	a	a	a
	a		a	

- a - CBPI and binucleate cell count not required for selection of concentrations for analysis
- b - Too few binucleate cells for micronucleus analysis
- c - Precipitate of test substance visible on the slides
- d - Light precipitate visible on the slides

TABLE 2 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 – 3 hour treatment (+S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.696	1.693	250	263
	1.690		275	
Sublimed Undensified Molybdenum Trioxide (µg/ml)	1.158	1.170	79b	85
	1439		1.182	
1000	1.368	1.377	174b	181
	1.386		187b	
500	a	1.511	a	227
	a		a	
250	1.494	1.511	223	227
	1.528		230	
175	a	1.511	a	227
	a		a	
100	a	1.511	a	227
	a		a	
50	a	1.511	a	227
	a		a	
10	a	1.511	a	227
	a		a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
 b - Precipitate of test substance visible on the slides

TABLE 3 - MEAN MICRONUCLEATED BINUCLEATE CELLS AND REDUCTION IN BINUCLEATE CELLS IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

Treatment	Mean % Micronucleated binucleate Cells	Mean % Reduction in binucleate cells
Experiment 1 – 3 hour treatment		
Solvent Control	1.9	-
Mitomycin C 0.5µg/ml	20.0**	-
Sublimed Undensified Molybdenum Trioxide 1439µg/ml	4.2**	18
1000µg/ml	1.5	7
250µg/ml	1.7	No reduction
Experiment 2 – 3 hour treatment		
Solvent Control	0.4	-
Mitomycin C 0.5µg/ml	22.0**	-
Sublimed Undensified Molybdenum Trioxide 1000µg/ml	0.4	32
500µg/ml	1.0	3
100µg/ml	0.8	No reduction
Experiment 3 – 3 hour treatment		
Solvent Control	0.7	-
Mitomycin C 0.5µg/ml	23.8**	-
Sublimed Undensified Molybdenum Trioxide 1439µg/ml	1.0	56
1000µg/ml	0.4	33
250µg/ml	0.6	No reduction
Experiment 3 – 20 hour treatment		
Solvent Control	1.0	-
Mitomycin C 0.25µg/ml	28.0**	-
Sublimed Undensified Molybdenum Trioxide 1000µg/ml	0.8	73
500µg/ml	0.6	43
100µg/ml	0.6	No reduction

** Statistically significant increase in the percentage of aberrant cells at $p < 0.01$ using Fisher's Exact Test (one-sided).

Δ Positive control mitotic index and % aberrant cells are determined from a single culture.

TABLE 4 - MEAN MICRONUCLEATED BINUCLEATE CELLS AND REDUCTION IN BINUCLEATE CELLS IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

Treatment	Mean % Micronucleated binucleate Cells	Mean % Reduction in binucleate cells
Experiment 1 – 3 hour treatment		
Solvent Control	0.7	-
Cyclophosphamide 25µg/ml	6.0**	-
Sublimed Undensified 1439µg/ml	3.7**	46
Molybdenum Trioxide 1000µg/ml	1.4	No reduction
250µg/ml	1.5	No reduction
Experiment 2 - 3 hour treatment		
Solvent Control	0.9	-
Cyclophosphamide 25µg/ml	8.0**	-
Sublimed Undensified 1000µg/ml	0.5	53
Molybdenum Trioxide 500µg/ml	0.4	27
100µg/ml	0.5	7
Experiment 3 – 3 hour treatment		
Solvent Control	0.7	-
Cyclophosphamide 50µg/ml	12.2**	-
Sublimed Undensified 1439µg/ml	0.6	68
Molybdenum Trioxide 1000µg/ml	0.4	31
250µg/ml	0.6	14

** Statistically significant increase in the percentage of aberrant cells at $p < 0.01$ using Fisher's Exact Test (one-sided).

Δ Positive control mitotic index and % aberrant cells are determined from a single culture.

TABLE 5 - pH DATA

Treatment	Experiment 1	Experiment 2		Experiment 3		
	After 3 hrs	After treatment	After 3 hrs	After treatment	After 3 hrs	After 20 hrs
Solvent Control	7.09	7.13	7.16	7.11	7.12	7.18
Sublimed Undensified Molybdenum Trioxide						
1439µg/ml	6.95	7.04	6.95	7.19	7.13	7.17
1000µg/ml	7.11	7.09	7.00	7.22	7.18	7.23
500µg/ml	7.22	7.17	7.14	7.20	7.17	7.23
250µg/ml	7.28	7.21	7.18	7.20	7.18	7.24
175µg/ml	7.32	7.23	7.22	7.20	7.21	7.26
100µg/ml	7.36	7.25	7.24	7.21	7.20	7.26
50µg/ml	7.35	7.25	7.23	7.20	7.19	7.25
10µg/ml	7.30	7.23	7.21	7.16	7.16	7.23

Stock formulation in Experiment 1 adjusted to pH 7.0

Stock formulation in Experiments 2 and 3 adjusted to pH 7.3 as this as this is closer to the normal pH of culture medium

Culture medium pH not adjusted either prior to treatment of the control cultures or prior to use in the serial dilutions of the stock test substance formation

TABLE 6 - OSMOLALITY DATA

Treatment	Osmolality (mmol/kg) Recorded in Experiment 2
Solvent Control	302
Sublimed Undensified Molybdenum Trioxide	
1439µg/ml	304
1000µg/ml	302
500µg/ml	301
250µg/ml	299
175µg/ml	305
100µg/ml	302
50µg/ml	305
10µg/ml	303

TABLE 7 - INDIVIDUAL CULTURE DATA IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	9
		B	500	10
Mitomycin C	0.5µg/ml	A	500	100
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	23
		B	500	19
	1000µg/ml	A	500	10
		B	500	5
	250µg/ml	A	500	9
		B	500	8

EXPERIMENT 2 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	2
		B	500	2
Mitomycin C	0.5µg/ml	A	500	110
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	A	500	3
		B	500	1
	500µg/ml	A	500	9
		B	500	1
	100µg/ml	A	500	4
		B	500	4

TABLE 7 - INDIVIDUAL CULTURE DATA IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	3
		B	500	4
Mitomycin C	0.5µg/ml	A	500	119
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	5
		B	500	5
	1000µg/ml	A	500	2
		B	500	2
	250µg/ml	A	500	2
		B	500	4

EXPERIMENT 3- 20 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	3
		B	500	7
Mitomycin C	0.25µg/ml	B	500	140
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	A	500	3
		B	500	5
	500µg/ml	A	500	4
		B	500	2
	100µg/ml	A	500	5
		B	500	1

TABLE 8 - INDIVIDUAL CULTURE DATA IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	4
		B	500	3
Cyclophosphamide	25µg/ml	A	500	30
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	23
		B	500	14
	1000µg/ml	A	500	7
		B	500	7
	250µg/ml	A	500	10
		B	500	5

EXPERIMENT 2 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	5
		B	500	4
Cyclophosphamide	25µg/ml	B	500	40
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	A	500	2
		B	500	3
	500µg/ml	A	500	1
		B	500	3
	100µg/ml	A	500	2
		B	500	3

TABLE 8 - INDIVIDUAL CULTURE DATA IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	3
		B	500	4
Cyclophosphamide	50µg/ml	A	500	61
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	3
		B	500	3
	1000µg/ml	A	500	2
		B	500	2
	250µg/ml	A	500	2
		B	500	4