ORIGINAL

TSCA NON-CONFIDENTIAL BUSINESS INFORMATION

DOCUMENT DESCRIPTION	DOCUMENT CONTROL NUMBER	DATE RECEIVED
8EHQ-10-18198	89110000191	3/23/11

COMMENTS: COMMUN S (DECLASS)

DOES NOT CONTAIN CBI

334163

RECEIVED
OPPT CBIC

11 MAR 23 AM 6:37

From: Scott Sherlock/DC/USEPA/US
To: Jeffrey Santacroce/DC/USEPA/US@EPA
Cc: Aakruti Shah/DC/USEPA/US@EPA, Pamela Myrick/DC/USEPA/US@EPA, Ryan Wallace/DC/USEPA/US@EPA, Matthew Leopard <leopard.matthew@epa.gov>
Date: 03/22/2011 02:23 PM
Subject: Fw: CBI declassification request re: PMN P94-1487 and 8(E) 8EHQ-10-18198

Just spoke to Dr. Olson who confirmed that the intent of his email of 3/11/11 (below) was to consent to (1) the placing of the substance on the public portion of the inventory and also to (2) dropping the CBI claim for chemical name in the above referenced TSCA section 8(e) filing.

See language in Dr. Olson's letter highlighted in blue.

How can we effectuate this?

Scott M. Sherlock, Attorney Advisor
Environmental Assistance Division
Office of Pollution Prevention and Toxics
202.564-8257 (telephone)
202.564-8251 (facsimile)
sherlock.scott@epa.gov (e-mail)

8EHQ-10-18198

----- Forwarded by Scott Sherlock/DC/USEPA/US on 03/22/2011 02:18 PM -----

From: Aakruti Shah/DC/USEPA/US
To: CFRSRV@aol.com
Cc: Scott Sherlock/DC/USEPA/US@EPA
Date: 03/15/2011 03:23 PM
Subject: Re: CBI declassification request re: PMN P94-1487 and 8(E) 8EHQ-10-18198

Dear Dr. Olson:

Thank you for your prompt reply. Confirming the various emails and conversations, EPA intends to place the chemical substance subject to the premanufacture notice and notice of substantial risk on the public portion of the TSCA Inventory on Monday, March 21st.

As we discussed, the CBI status of the substance expired by its own terms as expressed in the substantiation which was included in the notice of commencement filed in 1995.

If you have any questions or comments about this please advise no later than Friday, March 18th.

Thank you.

Aakruti Shah

Aakruti Shah
Environmental Protection Specialist
Environmental Assistance Division
Office of Pollution Prevention and Toxics/OCSPP

8911000019 1

Contains No CBI

Ph: 202.564.8183
Fax: 202.564.8197
shah.aakruti@epa.gov

From: CFRSRV@aol.com
To: Aakruti Shah/DC/USEPA/US@EPA
Cc: Scott Sherlock/DC/USEPA/US@EPA
Date: 03/11/2011 03:18 PM
Subject: CBI declassification request re: PMN P94-1487 and 8(E) 8EHQ-10-18198

Dear Ms. Shah:

We have received communication from the PMN notifier and the submitter of TSCA 8(e) Reports for the substance which is the subject of PMN P94-1487.

The notifier has accepted the request for declassification of the substance identification.

Therefore, we do agree that the U.S. EPA may disclose the full chemical name and the CAS Registry Number for the substance which is the subject of PMN P94-1487 and any notifications made with respect to TSCA Section 8(e) relating to this substance.

William A. Olson, Ph.D.
Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Road
Woodbridge, VA 22192
Telephone: 703 590 7337
Faxline: 703 580 8637

331421 Sanitized Submission

Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road * Woodbridge, VA 22192-5755
Telephone 703 590 7337 * Fax 703 580 8637
cfrsrv@aol.com

8EHQ-10-18198

November 30, 2010

U.S. Environmental Protection Agency – East
TSCA Section 8(e)
Room 6428
1201 Constitution Avenue, NW
Washington, DC 20460-0001

SUBJECT: Notice in Accordance with TSCA Section 8(e)
4-mercaptomethyl-3,6-dithia-1,8-octanedithiol
In Vitro Micronucleus Test in Cultured Human Lymphocytes

Inc., has received the final report, November 24, 2010, regarding in vitro micronucleus test in cultured human lymphocytes for the substance 4-mercaptomethyl-3,6-dithia-1,8-octanedithiol, which was the subject of PMN P94-1487. The full chemical name of this substance is considered CBI, generically identified as *thia alkanethiol.*

The results of this test when the substance as administered for 20 hours in the absence of exogenous metabolic activation (S9 mix) showed clear evidence of causing increase in the induction of micronuclei.

Please note that the complete **chemical identity** of this substance is considered **Confidential Business Information**. Also enclosed is a sanitized copy of the report.

Sincerely,

William A. Olson, Ph.D.
Consultant

, Inc.

WAO:gbt
MTCA33-8E

CASRN-131538-00-6

Enclosure
CBI Report, 11-24-10, 39 pages
Sanitized Report, 11-24-10, 39 pages

8811000008 2/S

Cc:

Company Sanitized

BY JCOTT M. SHENLOCK, EAD, OPPT, OCSPP, USEPA
3/22/11 PER E-MAIL.

Working for a better future

GST

In Vitro Micronucleus Test In Cultured Human Lymphocytes

HLS study number:	
Version ID:	Final report
Issue date:	24 November 2010

Sponsor and Test Facility Details

Sponsor

Test facility

Test facility (statistics)

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Compliance with Good Laboratory Practice standards

GST: *In Vitro* Micronucleus Test in Cultured Human Lymphocytes

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards, with the exception stated below, and I consider the data generated to be valid.

> The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).
>
> OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17.
>
> EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

..... 24 November 2010

Study Director Date

Quality Assurance Statement

GST: *In Vitro* Micronucleus Test in Cultured Human Lymphocytes

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date(s) of Inspection	Date of Reporting to Study Director and Management
Protocol	30 Jul 2008-31 Jul 2008	31 Jul 2008
Formulation & Treatment (including Cytochalasin B)	20 Aug 2008-21 Aug 2008	21 Aug 2008
Harvesting & Slide Preparation	22 Aug 2008-26 Aug 2008	26 Aug 2008
Report audit	15 Jun 2010 – 17 Jun 2010	17 Jun 2010
Protocol amendment no. 1	04 Feb 2009	04 Feb 2009
Protocol amendment no. 2	18 Dec 2009	18 Dec 2009

In addition, process based inspections were conducted of other routine and repetitive procedures employed on this type of study at or about the time this study was in progress. Similarly an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were reported to Company Management.

..... 24 November 2010

Group Manager Date

Contributing Scientists

GST: *In Vitro* Micronucleus Test in Cultured Human Lymphocytes

Study Management

Study Director

Statistics

Senior Statistician

Summary

This study was designed to assess the potential of GST to cause an increase in the induction of micronuclei in cultured human lymphocytes *in vitro*.

Human lymphocytes in whole blood culture, stimulated to divide by addition of phytohaemagglutinin (PHA) 48 hours prior to treatment, were exposed to GST for 3 hours in both the absence and presence of exogenous metabolic activation (S9 mix) and for 20 hours in the absence of S9 mix. The maximum final concentration to which the cells were exposed was 2600 μg/mL, dosed at 1% v/v (10 mM). Vehicle (dimethyl sulphoxide) and positive control cultures were included in all test conditions.

Cytokinesis was blocked following mitosis using Cytochalasin B, the cells harvested and slides prepared, so that both mononucleate and binucleate cells could be examined for micronucleus induction. In order to assess the toxicity of GST to cultured human lymphocytes, the cytokinesis-block proliferative index (CBPI) was calculated for cultures treated with the test substance, the vehicle and positive controls. At least three test concentrations were assessed for determination of induction of micronuclei. The highest GST concentration selected was that causing 55 ± 5% cytotoxicity compared with the concurrent vehicle control. In both the absence and presence of S9 mix following 3-hour treatment (48-hour PHA stimulation), reductions in CBPI equivalent to 58.02% and 53.94% cytotoxicity, were obtained with GST at 81 μg/mL, in the absence and presence of S9 mix respectively. Concentrations of GST selected for micronucleus analysis were 10, 20, 41 and 81 μg/mL. In the absence of S9 mix following 20 hour treatment (48-hour PHA stimulation), a reduction in CBPI equivalent to 59.80% cytotoxicity, was obtained with GST at 30 μg/mL. Concentrations of GST selected for micronucleus analysis were 1, 10, 20, 25 and 30 μg/mL.

In both the absence and presence of S9 mix, following 3-hour treatment (48-hour PHA stimulation), GST did not cause any statistically significant increases in the number of mononucleate or binucleate cells containing micronuclei when compared with the vehicle controls. In the absence of S9 mix, following 20 hour treatment (48 hour PHA stimulation), GST induced statistically significant increases in the number of mononucleate or binucleate cells containing micronuclei when compared to the vehicle controls. For mononucleate cells the trend test was significant at GST concentrations of 20 μg/mL ($p<0.01$), 25 μg/mL ($p<0.001$) and 30 μg/mL ($p<0.001$), and for binucleate cells the trend test was significant at a GST concentration of 30 μg/mL ($p<0.01$).

Although the binucleate cell was the principal treatment unit in this assay, it was apparent that the ratio of the number of mononucleate cells to the number of binucleate cells increased with increasing concentrations of GST, indicating that GST had the effect of retarding the action of Cytochalasin B, resulting in the proliferation of mononucleate cells and the consequent induction of micronuclei in mononucleate cells as well as in binucleate cells.

The positive control compounds (mitomycin C, colchicine and cyclophosphamide) caused significant increases in the number of mononucleate (colchicine) and binucleate cells (mitomycin C, colchicine and cyclophosphamide) containing micronuclei, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

It is concluded that GST administered for 3 hours at concentrations of up to 10 mM, in both the absence and presence of S9 mix, did not show evidence of causing an increase in the

induction of micronuclei in cultured human lymphocytes, in this *in vitro* test system under the experimental conditions described. It is, however, concluded that GST administered for 20 hours, in the absence of S9 mix, showed clear evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes, in this *in vitro* test system under the experimental conditions described.

1. Introduction

1.1 Objective

The purpose of this study was to assess the potential of GST to induce an increase in the induction of micronuclei in cultured human lymphocytes *in vitro*.

1.2 Regulatory Compliance

The treatment schedule is in accordance with the International Working Group of Genotoxicity Testing Procedure (IWGTP): Guidance on the *in vitro* micronucleus test using human lymphocytes.

The performance of this study was in compliance with the following guideline:

OECD Guideline for the testing of chemicals. Draft proposal for a new guideline 487: *In vitro* micronucleus test.

1.3 Test System

In mitotic cells in which chromosomal breakage has been caused by the test substance or its metabolites, acentric fragments of the chromosomes do not separate at the anaphase stage of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and hence will form single or multiple micronuclei in the cytoplasm of these cells.

Substances which interfere with the mitotic spindle cause non-disjunction or lagging chromosomes at anaphase which may not be incorporated into the daughter nuclei. These lagging chromosomes are not excluded from the cell with the main nucleus and hence form single or multiple micronuclei in the cytoplasm of these cells.

Human lymphocytes are cultured *in vitro* and are stimulated to divide by adding phytohaemagglutinin (PHA) to the culture, resulting in a high mitotic yield (Nowell 1960).

In this study, blood taken from healthy male non-smoking donors was pooled and diluted with tissue culture medium. The cultures were incubated in the presence of PHA before being treated with the test substance. Following treatment (3 hour exposure) or during treatment (20 hour exposure) cytokinesis was blocked at metaphase using the inhibitor, Cytochalasin B. Mononucleate and binucleate cells were then examined for the presence of micronuclei.

Some substances do not exert a mutagenic effect until they have been metabolised by enzyme systems that are not found in cultured cells. Therefore the cultures and test substance were incubated in both the absence and presence of a supplemented liver fraction (S9 mix).

The study comprised two tests, a 3-hour treatment time in both the absence and the presence of S9 mix and a 20-hour treatment time in the absence of S9 mix only.

Any toxic effects of the test substance on the cells may lead either to a reduction in cell replication or to cell death. Cytokinesis-block proliferative index (CBPI) values significantly less than the concurrent vehicle control value are indicative of toxicity.

The following criteria for selection of analysable cells were used: cells are included in the analysis provided that the cytoplasm has remained essentially intact and any micronuclei present are separate in the cytoplasm or only just touching the main nucleus (not connected to the nucleus by a nucleoplasmic bridge). The micronuclei should lie in the same focal place as the cell, with no micronucleus debris in the surrounding area. The main nuclei of the binucleate cells scored for micronuclei should be of approximately equal size. The diameter of the micronucleus should be <1/3 that of the main nucleus with a generally rounded shape with a clearly defined outline. The colour of the micronuclei should be the same or lighter than the main nucleus.

The protocol was approved by and the Study Director on 15 July 2008 and by the Sponsor on 1 August 2008.

Experimental start date: 5 August 2008.
Experimental completion date: 17 March 2010.

2. Experimental Procedure

2.1 Test substance

Identity:	GST
Chemical name:	
Molecular weight:	
Appearance:	
Storage conditions:	
Batch number:	
Expiry:	
Purity:	
Date received:	
Certificate of Analysis:	

2.2 Culture of lymphocytes

Blood was taken from 2 healthy non-smoking male donors into Lithium Heparin tubes and diluted with tissue culture medium (RPMI 1640 containing 10% foetal calf serum, heparin and antibiotics). Prior to the culture establishment, the blood was pooled using equal volumes from each donor. Lymphocytes, which do not normally undergo cell division, were stimulated to do so by the addition of the naturally occurring mitogen, phytohaemagglutinin (PHA), (Evans and O'Riordan 1975, Nowell 1960). The cultures were prepared as 5 mL aliquots (0.4 mL blood: 4.5 mL medium: 0.1 mL PHA solution) in sterile universal containers and incubated at 37°C. The cultures were shaken occasionally to re-suspend the cells.

2.3 Positive controls

In the absence of S9 mix

Identity:	Mitomycin C
Solvent:	Sterile purified (reverse osmosis) water
CAS No.:	50-07-7
Exposure concentrations:	0.05 and 0.075 µg/mL

Identity:	Colchicine
Solvent:	Sterile purified (reverse osmosis) water
CAS No.:	64-86-8
Exposure concentrations:	0 02, 0.03 and 0.04 µg/mL

In the presence of S9 mix

Identity:	Cyclophosphamide
Solvent:	Sterile purified (reverse osmosis) water
CAS No.:	6055-19-2
Exposure concentration:	5 and 10 µg/mL

2.4 S9 metabolising system

2.4.1 Preparation of S9 fraction

S9 fraction, prepared from male Sprague-Dawley derived rats, dosed with phenobarbital and 5,6-benzoflavone to stimulate mixed-function oxidases in the liver, was purchased from a commercial source and stored at ca -80°C. The quality control statement relating to each batch of S9 preparation used is included in the raw data and a copy is included in this report as Annex 2.

Lot No.: 2273 (Date of preparation: 1 May 2008)

2.4.2 Preparation of S9 mix

S9 mix contains: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). The cofactors were filter-sterilised with a 0.2 µm non pyrogenic sterile filter prior to use.

2.5 Selection of solvent and formulation of test substance

Dimethyl sulphoxide (DMSO; ACS spectrophotometric grade) was used as the vehicle for this study.

The highest concentration of GST tested in this study was 260 mg/mL in the chosen vehicle, which provided a final concentration of 2600 µg/mL when dosed at 1% v/v. This is the standard limit concentration recommended in the regulatory guidelines that this assay follows. The highest concentration in each test was diluted with DMSO to produce a series of lower concentrations.

On addition to culture media, it was noted that precipitate that did not clear after shaking was present at final GST concentrations of 2600 and 1300 µg/mL. No precipitate was present at any of the GST concentrations assessed for micronucleus induction.

All concentrations cited in this report are expressed in terms of pure GST, and were corrected for the stated purity of 93.9%.

2.6 *In vitro* micronucleus test

2.6.1 3-hour treatment in the absence and presence of S9 mix

Lymphocyte cultures were incubated for approximately 48 hours following stimulation with PHA, before addition of the test substance. The test substance was prepared in the vehicle and dilutions made for both sets of cultures. Two sets of duplicate cultures were used for

each treatment level, solvent and positive control cultures. S9 homogenate was present in appropriate cultures at a final concentration of 2% v/v. All cultures were identified using unique number/colour codes.

Before treatment in the absence and presence of S9 mix all cultures were re-suspended in fresh media. For cultures in the presence of S9 mix, 1 mL of medium was removed from the final volume. This was replaced with 1 mL of S9 mix immediately prior to treatment.

Test substance preparations were added to cultures at 1% v/v. Cultures were incubated at 37°C for 3 hours.

The cells were centrifuged and the medium was replaced with fresh medium. The cultures were incubated for a further 17 hours in the absence of the test substance. Cytochalasin B, at a final concentration of 6 µg/mL, was then added to all cultures. The cultures were incubated for a further 28 hours until the scheduled harvest time.

2.6.2 20-hour treatment in the absence of S9 mix

Human lymphocyte cultures were set up as previously described. A 20-hour continuous treatment (1.5 to 2 normal cell cycles) at 37°C was used in the absence of S9 mix. Test substance preparations were added to cultures at 1% v/v in the presence of Cytochalasin B (6 µg/mL).

2.6.3 Harvesting and fixation

The cells were harvested by centrifugation at 500 g for 5 minutes. The supernatant was removed and the cell pellet re-suspended and treated with a 4 mL hypotonic solution (0.075M KCl) at 37°C, cultures were then incubated for 3 minutes at 37°C to cause swelling. Cultures were agitated, 4 mL of ice-cold fixative (3:1 v/v methanol: acetic acid) was added slowly onto the culture surface and the cultures were slowly inverted to mix.

The cultures were centrifuged at 500 g for five minutes. The supernatant was removed, and the cell pellet re-suspended. A further 4 mL of fresh fixative was then added and the cells stored at 4°C until slide preparation.

2.6 4 Slide preparation

The cultures were centrifuged at 500 g for 5 minutes and the supernatant removed. A homogeneous cell suspension was prepared. Microscope slides (pre-cleaned in methanol for ten minutes and left to air dry) were prepared for each culture by aliquoting the re-suspended cells onto the slides, and allowing the slides to air-dry. Four slides were prepared per culture. The remaining cell cultures were stored at approximately 4°C until slide analysis was complete.

2.6.5 Slide staining

1 Rinsed in purified water

2 Stained in acridine orange solution (0.0125 mg/mL using purified water) for 4 minutes

3 Washed in purified water for 5 minutes

4 Rinsed in cold tap water for 2 minutes

5 Stored at room temperature protected from light until required

6 Immediately prior to scoring, slides are wet mounted with glass coverslips using purified water

2.6.6 Microscopic examination

The prepared slides were examined by fluorescence microscopy. The incidence of mononucleate, binucleate and polynucleate cells per 500 cells was assessed per culture. The presence of an unusual number of, for example, cells undergoing mitosis, polyploid cells, necrotic cells and debris was also noted.

From these results, concentrations were selected for micronucleus analysis. The highest concentration was intended to be that which caused a depression in the cytokinesis-block proliferative index (CBPI) equivalent to 55 ± 5% cytotoxicity (approximately) when compared with the concurrent vehicle control.

Prior to micronucleus analysis, all slides were randomly coded. Interphase cells were examined by fluorescence microscopy and the incidence of micronucleated cells per 1000 mononucleate and 1000 binucleate cells per culture were scored where possible.

The analysis for micronucleated cells was based on the following criteria (Fenech & Morley, 1985, Fenech, 1993, Fenech *et al*, 2003 and Albertini *et al*, 1997):

- Only cells where the cytoplasm has remained essentially intact are analysed.
- Any micronuclei present should be separate in the cytoplasm or just touching the main nucleus (not connected via a cytoplasmic bridge).
- The main nuclei of the binucleate cells scored for micronuclei should be of approximately equal size.
- Micronuclei should lie in the same focal plane as the cell.
- Micronuclei should be < 1/3 of the nucleus area.
- Micronuclei should possess a generally rounded shape with a clearly defined outline.
- The colour of the micronuclei should be the same or lighter than the main nucleus.
- There should be no micronucleus like debris in the surrounding area.

2.7 Stability, homogeneity and formulation analysis

The stability of GST and the stability and homogeneity of GST in the vehicle were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

2.8 Assessment of results

2.8.1 Acceptance criteria

The following criteria were applied for assessment of assay acceptability:

Positive controls must show clear unequivocal positive responses.

The negative control (solvent, vehicle control or untreated cultures) must show reproducible low and consistent micronucleus frequencies.

Tests that did not fulfil the required criteria were rejected and therefore are not reported.

2.8.2 Analysis of data

Cytotoxicity

$$\text{Cytotoxicity} = 100\text{-}100\{(CBPI_T - 1)/(CBPI_C - 1)\}$$

Where $$CBPI = \frac{N^o \text{ mononucleate cells} + 2 \times N^o \text{ binucleate cells} + 3 \times N^o \text{ multinucleate cells}}{\text{Total number of cells}}$$

T = test chemical treatment culture
C = solvent control culture

Thus, a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytotoxicity.

Genotoxicity

The analysis assumed that the replicate is the experimental unit. GST was compared to control using an exact one-tailed Linear-by-Linear association test (Cytel 1995). Positive controls were compared to control using exact one-tailed Permutation tests (Cytel 1995).

Statistical significance was declared at the 1% level for all tests.

The data were received in an Excel spreadsheet and analysed using StatXact 3 (Cytel 1995).

2.8.3 Criteria for assessing genotoxic potential

Although statistical methods were used as an aid in evaluating the micronucleus test results, biological relevance of the results were considered first. Statistical significance should not be the only determining factor for a positive response. A test substance is considered to be positive if the following conditions are met:

Statistically significant increases in the frequency of micronucleated cells are observed at one or more test concentration compared to the solvent control.

The increases are reproducible between replicate cultures.

The increases are not associated with large changes in pH, osmolality of the treatment medium or extreme toxicity.

There is evidence of a dose-response relationship.

A negative response is claimed if no statistically significant increases in the number of micronucleated cells above concurrent solvent control frequencies are observed at any dose level, and there is no evidence of a dose-response relationship.

2.9 Maintenance of records

Records and documentation relating to this study (including electronic records) will be maintained in the archives of Huntingdon Life Sciences for a period of one year from the date on which the Study Director signs the final report. This will include but may not be limited to the Study Protocol, raw data, a copy of the final report and other samples and specimens generated during the course of this study. If requested, Huntingdon Life Sciences will continue to retain the materials at additional cost.

Samples and specimens that no longer afford evaluation will be discarded in accordance with Standard Operating Procedures.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

3. Results

3.1 3-hour treatment in the absence and presence of S9 mix

Summaries of the results and statistical analysis of the *in vitro* micronucleus test, 3-hour treatment (48-hour PHA stimulation), are presented in Table 1 and Table 2.

3.1.1 Cytotoxicity

In both the absence and presence of S9 mix, following 3-hour treatment (48-hour PHA stimulation), there were very few or no viable cells with GST at concentrations of 325 µg/mL and above. Reductions in CBPI, compared with vehicle control values, equivalent to 58.02% and 53.94% cytotoxicity, were obtained with GST at 81 µg/mL, in the absence and presence of S9 mix respectively. Concentrations of GST selected for micronucleus analysis were 10, 20, 41 and 81 µg/mL.

Mean CPBI values obtained in the solvent control cultures in both the absence and presence of S9 mix were within the historical control range.

On the basis of the results obtained following CBPI analysis, the concentrations of mitomycin C, colchicine and cyclophosphamide analysed for micronucleus induction were 0.075, 0.03 and 5 µg/mL respectively.

The results of the cytotoxicity evaluation are presented in Table 4 and Table 6.

3.1.2 Micronucleus Analysis

In both the absence and presence of S9 mix, following 3-hour treatment (48-hour PHA stimulation), GST did not cause any statistically significant increases in the number of

mononucleate or binucleate cells containing micronuclei when compared with the vehicle controls.

In both the absence and presence of S9 mix, mean micronucleus induction in the vehicle control and GST treated cultures was within or close to the historical control range.

The positive control compounds (mitomycin C, colchicine and cyclophosphamide) caused significant increases in the number of mononucleate (colchicine) and binucleate cells (mitomycin C, colchicine and cyclophosphamide) containing micronuclei, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

The effects of GST on the induction of micronuclei in cultured human lymphocytes are presented in Table 5 and Table 7.

3.2 20-hour treatment in the absence of S9 mix

A summary of the results and statistical analysis of the *in vitro* micronucleus test, 20-hour treatment (48-hour PHA stimulation), is presented in Table 3.

3.2.1 Cytotoxicity

In the absence of S9 mix following 20-hour treatment (48-hour PHA stimulation), there were substantial reductions in CBPI, compared with vehicle control values, with GST at concentrations of 35 µg/mL and above. A reduction in CBPI, compared with vehicle control values, equivalent to 59.80% cytotoxicity, was obtained with GST at 30 µg/mL. Concentrations of GST selected for micronucleus analysis were 1, 10, 20, 25 and 30 µg/mL.

Mean CPBI values obtained in the solvent control cultures were slightly below, but very close to, the historical control range.

On the basis of the results obtained following CBPI analysis, the concentrations of mitomycin C and colchicine analysed for micronucleus induction were 0.075 and 0.02 µg/mL respectively.

The results of the cytotoxicity evaluation are presented in Table 8.

3.2.2 Micronucleus Analysis

In the absence of S9 mix, following 20-hour treatment (48-hour PHA stimulation), GST induced statistically significant increases in the number of both mononucleate and binucleate cells containing micronuclei when compared to the vehicle controls. For mononucleate cells the trend test was significant at GST concentrations of 20 µg/mL ($p<0.01$), 25 µg/mL ($p<0.001$) and 30 µg/mL ($p<0.001$), and for binucleate cells the trend test was significant at a GST concentration of 30 µg/mL ($p<0.01$).

Although the binucleate cell was the principal treatment unit in this assay, it was apparent that the ratio of the number of mononucleate cells to the number of binucleate cells increased with increasing concentrations of GST, indicating that GST had the effect of retarding the action of Cytochalasin B, resulting in the proliferation of mononucleate cells and the consequent induction of micronuclei in mononucleate cells as well as in binucleate cells.

Mean micronucleus induction in the vehicle control was within the historical control range.

The positive control compounds (mitomycin C, and colchicine) caused significant increases in the number of mononucleate (colchicine) and binucleate cells (mitomycin C and colchicine) containing micronuclei, demonstrating the sensitivity of the test system.

The effects of GST on the induction of micronuclei in cultured human lymphocytes are presented in Table 9.

Appendix 1 summarises the current historical control data.

4. Conclusion

It is concluded that GST administered for 3 hours at concentrations of up to 10 mM, in both the absence and presence of S9 mix, did not show evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes, in this *in vitro* test system under the experimental conditions described. It is, however, concluded that GST administered for 20 hours, in the absence of S9 mix, showed clear evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes, in this *in vitro* test system under the experimental conditions described.

5. References

ALBERTINI, S., MILLER, B., CHETELAT, A-A. and LOCHER, F. (1997) Detailed data on *in vitro* MNT and in vitro CA: industrial experience. *Mutation Research,* **392**, 187-208.

EVANS, H.J. and O'RIORDAN, M.L. (1975) Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. *Mutation Research,* **31**, 135.

CYTEL (1995) *StatXact 3 for Windows: Statistical Software for Exact Nonparametric Inference.* Cytel Software Corporation, NC, USA.

FENECH, M. (1993) The cytokinesis-block micronucleus technique: A detailed description of the method and its application to genotoxicity studies in human populations. *Mutation Research,* **285**, 35-44.

FENECH, M., BONASSI, S., TURNER, J., LANDO, C., CEPPI, M., CHANG, W.P., HOLLAND, N., KIRSCH-VOLDERS, M., ZEIGER, E., BIGATTI, M.P., BOLOGNESI, C., CAO, J., DE LUCA, G., DI GIOGIO, M., FERGUSON, L.R., FUCIC, A., LIMA, O.G., HADJIDEKOVA, V.V., HRELIA, P., JAWORSKA, A., JOKSIC, G., KRISHNAJA, A.P., LEE, T-K., MARTELLI, A., MCKAY, M.J., MIGLIORE, L., MIRKOVA, E., MÜLLER, W-U., ODAGIRI, Y., ORSIERE, T., SCARFI, M.R., SILVA, M.J., SOJUNI, T., SURRALLES, J., TRENTA, G., VOROBTSOVA, I., VRAL, A. and ZIJANO, A. (2003) Intra- and inter-laboratory variation in the scoring of micronuclei and nucleoplasmic bridges in binucleated human lymphocytes. Results of an international slide-scoring exercise by the HUMN project. *Mutation Research,* **534**, 45-64.

FENECH, M. and MORLEY, A.A. (1985) Measurement of micronuclei in human lymphocytes.
Mutation Research **147**, 29-36.

International Working Group of Genotoxicity Testing Procedure (IWGTP). Guidance on the in vitro micronucleus test using human lymphocytes.

NOWELL, P.C. (1960) Phytohaemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Research* **20**, 462.

Table 1 Summary of results and statistical analysis: 3-hour treatment in the absence of S9 mix

Concentration (µg/mL)	CBPI	Mean CBPI	Cytotoxicity (%)	Mononucleate micronuclei				Binucleate micronuclei			
				per 1000 cells	Mean (%)	Pairwise *p*-value	Trend test *p*-value	per 1000 cells	Mean (%)	Pairwise *p*-value	Trend test *p*-value
Control (DMSO)	2.10	2.17	0.00	0	0.05			2	0.25		
	2.17			2				5			
	2.23			0				0			
	2.18			0				3			
GST (10)	2.19	2.18	-1.11	2	0.10	0.600		4	0.40	0.267	
	2.17			0				4			
GST (20)	2.15	2.12	3.81	1	0.15	0.200	0.200	1	0.15	0.800	0.702
	2.09			2				2			
GST (41)	1.88	1.92	21.48	2	0.20	0.200	0.046	3	0.25	0.667	0.676
	1.95			2				2			
GST (81)	1.53	1.49	58.02	1	0.25	0.133	0.019	5	0.50	0.200	0.168
	1.45			4				5			
Mitomycin C (0.075)	2.22	2 22	-4.57	2	0.20	0.200		17	2.65	0.067	
	2.22			2				36			
Colchicine (0.03)	2 16	2.20	-2.83	17	1.80	0.067		1	0.30	0.467	
	2.24			19				5			

CBPI: Cytokinesis-Block Proliferative Index
Pairwise *p*-values are for comparisons to control us: :g Linear-by-Linear trend test for GST and Exact Permutation tests otherwise

Results presented above were obtained in a repeat test. An earlier test was rejected as invalid because of failure of PHA induction, and is not reported.

Table 2 Summary of results and statistical analysis: 3-hour treatment in the presence of S9 mix

Concentration (µg/mL)	CBPI	Mean CBPI	Cytotoxicity (%)	Mononucleate micronuclei				Binucleate micronuclei			
				per 1000 cells	Mean (%)	Pairwise *p*-value	Trend test *p*-value	per 1000 cells	Mean (%)	Pairwise *p*-value	Trend test *p*-value
Control (DMSO)	2.21 2.24 2.37 2.31	2.28	0.00	10 0 1 0	0.28			4 1 4 3	0.30		
GST (10)	2.31 2.31	2.31	-2.31	0 2	0.10	0.600		7 3	0.50	0.267	
GST (20)	2.17 2.12	2.15	10.62	2 2	0.20	0.400	0.536	3 3	0.30	0.667	0.429
GST (41)	1.98 1.97	1.97	24.23	1 4	0.25	0.466	0.507	5 7	0.60	0.067	0.094
GST (81)	1.59 1.59	1.59	53.94	5 2	0.35	0.400	0.393	7 5	0.60	0.067	0.028
Cyclophosphamide (5)	2.34 2.37	2.35	-5.39	2 1	0.15	0.466		37 31	3.40	0.067	

CBPI: Cytokinesis-Block Proliferative Index

Pairwise *p*-values are for comparisons to control using Linear-by-Linear trend test for GST and Exact Permutation tests otherwise

Results presented above were obtained in a repeat test. An earlier test was rejected as invalid because of failure of PHA induction, and is not reported.

Table 3 **Summary of results and statistical analysis: 20-hour treatment in the absence of S9 mix**

Concentration (µg/mL)	CBPI	Mean CBPI	Cytotoxicity (%)	Mononucleate micronuclei				Binucleate micronuclei			
				per 1000 cells	Mean (%)	Pairwise *p*-value	Trend test *p*-value	per 1000 cells	Mean (%)	Pairwise *p*-value	Trend test *p*-value
Control	1.83	1.84	0.00	2[a]	0.30			5	0.65		
(DMSO)	1.89			1[a]				6			
	1 82			2[a]				5			
	1.82			3[a]				10			
GST	1.80	1.84	-0.05	5[a]	0.65	0.800		10	1.50	>0.999	
(1)	1.88			1[a]				20			
GST	1.80	1 80	4.38	7[a]	1.20	>0.999	0.014	12	1.55	>0.999	
(10)	1.80			11[a]				19			
GST	1.47	1.46	45.45	24	1.50**	>0.999	0.004	14	1.70	>0.999	
(20)	1.44			6				20			
GST	1.39	1.40	52.21	20	1.95***	>0.999	<0.001	18[a]	1.85	0.933	0.051
(25)	1.41			19				7[a]			
GST	1.33	1.34	59.80	34	2.95***	0 067	<0.001	25[a]	4.60**	0.067	0.007
(30)	1.35			25				18[a]			
Mitomycin C	1.72	1.72	14.25	7	0.70	0.067		32	3.90	0.067	
(0.075)	1.72			5[a]				46			
Colchicine	1.41	1.39	53.27	45	4.05	0.067		12[a]	3.75	0.067	
(0.02)	1.37			36				21[a]			

CBPI Cytokinesis-Block Proliferative Index a <1000 cells scored ** $p<0.01$, *** $p<0.001$ for Linear-by-Linear trend test
Pairwise *p*-values are for comparisons to control using Linear-by-Linear trend test for GST and Exact Permutation tests otherwise

Results presented above were obtained in a repeat test. Earlier tests were rejected as invalid because of excessive heterogeneity in the vehicle controls or unsatisfactory cytostasis, and are not reported.

Table 4 Cytotoxicity evaluation: 3-hour treatment in the absence of S9 mix

Concentration (µg/mL)	Mononucleate cells	Binucleate cells	Polynucleate cells	Total number of cells	CBPI	Mean CBPI	Cytotoxicity (%)
Control	32	419	83	534	2.10	2.17	0.00
(DMSO)	24	411	116	551	2.17		
	13	377	130	520	2.23		
	17	404	113	534	2.18		
GST	32	367	134	533	2.19	2.18	-1.11
(10)	9	511	116	636	2.17		
GST	33	368	110	511	2.15	2.12	3.81
(20)	52	35	100	511	2.09		
GST	105	360	45	510	1.88	1.92	21.48
(41)	89	451	59	599	1.95		
GST	252	255	8	515	1.53	1.49	58.02
(81)	291	233	3	527	1.45		
GST	432	72	0	504	1.14	1.14	88.31
(163)	435	65	0	500	1.13		
Mitomycin C	31	353	147	531	2.22	2.22	-4.57
(0 075)	47	348	173	568	2.22		
Colchicine	30	371	113	514	2.16	2.20	-2.83
(0 03)	28	363	159	550	2.24		

CBPI: Cytokinesis-Block Proliferative Index

Table 5 Micronucleus analysis: 3-hour treatment in the absence of S9 mix

Concentration (µg/mL)	Mononucleate micronuclei		Binucleate micronuclei	
	per 1000 cells	Mean (%)	per 1000 cells	Mean (%)
Control (DMSO)	0 2 0 0	0.05	2 5 0 3	0.25
GST (10)	2 0	0.10	4 4	0.40
GST (20)	1 2	0.15	1 2	0.15
GST (41)	2 2	0.20	3 2	0.25
GST (81)	1 4	0.25	5 5	0.50
Mitomycin C (0.075)	2 2	0.20	17 36	2.65
Colchicine (0.03)	17 19	1.80	1 5	0.30

Table 6 Cytotoxicity evaluation: 3-hour treatment in the presence of S9 mix

Concentration (µg/mL)	Mononucleate cells	Binucleate cells	Polynucleate cells	Total number of cells	CBPI	Mean CBPI	Cytotoxicity (%)
Control (DMSO)	30	359	144	533	2.21	2.28	0.00
	33	377	172	582	2.24		
	10	297	200	507	2.37		
	13	329	170	512	2.31		
GST (10)	23	34[illegible]	199	563	2.31	2.31	-2.31
	21	308	181	510	2.31		
GST (20)	27	366	116	509	2.17	2.15	10.62
	44	369	106	519	2.12		
GST (41)	80	502	67	649	1.98	1.97	24.23
	81	432	61	574	1.97		
GST (81)	229	287	13	529	1.59	1.59	53.94
	217	296	5	518	1.59		
GST (163)	410	145	0	555	1.26	1.23	82.19
	424	101	1	526	1.20		
Cyclophosphamide (5)	27	296	204	527	2.34	2.35	-5.39
	70	215	278	563	2.37		

CBPI. Cytokinesis-Block Proliferative Index

Table 7 Micronucleus analysis: 3-hour treatment in the presence of S9 mix

Concentration (µg/mL)	Mononucleate micronuclei		Binucleate micronuclei	
	per 1000 cells	Mean (%)	per 1000 cells	Mean (%)
Control (DMSO)	10 0 1 0	0.28	4 1 4 3	0.30
GST (10)	0 2	0.10	7 3	0.50
GST (20)	2 2	0.20	3 3	0.30
GST (41)	1 4	0.25	5 7	0.60
GST (81)	5 2	0.35	7 5	0.60
Cyclophosphamide (5)	2 1	0.15	37 31	3.40

Table 8 Cytotoxicity evaluation: 20-hour treatment in the absence of S9 mix

Concentration (µg/mL)	Mononucleate cells	Binucleate cells	Polynucleate cells	Total number of cells	CBPI	Mean CBPI	Cytotoxicity (%)
Control (DMSO)	134	373	39	546	1.83	1.84	0.00
	120	394	56	570	1.89		
	137	317	46	500	1.82		
	141	309	50	500	1.82		
GST (1)	157	298	54	509	1.80	1.84	-0.05
	119	323	58	500	1.88		
GST (5)	147	299	54	500	1.81	1.83	0.83
	133	314	56	503	1.85		
GST (10)	150	300	50	500	1.80	1.80	4.38
	155	294	55	504	1.80		
GST (20)	290	189	23	502	1.47	1.46	45.45
	301	197	16	514	1.45		
GST (25)	326	159	21	502	1.39	1.40	52.21
	314	168	18	500	1.41		
GST (30)	353	133	15	501	1.33	1.34	59.80
	342	142	16	500	1.35		
GST (35)	400	96	6	502	1.22	1.23	72.52
	391	106	9	506	1.25		
GST (40)	413	74	13	500	1.20	1.22	73.97
	401	80	19	500	1.24		
GST (50)	426	65	9	500	1.17	1.15	82.15
	441	59	4	504	1.13		
Mitomycin C (0.075)	180	329	30	530	1.72	1.72	14.25
	167	341	19	527	1.72		
Colchicine (0.02)	304	196	6	506	1.41	1.39	53.27
	321	182	3	506	1.37		

CBPI. Cytokinesis-Block Proliferative Index

Table 9 Micronucleus analysis: 20-hour treatment in the absence of S9 mix

Concentration (µg/mL)	Mononucleate micronuclei		Binucleate micronuclei	
	per 1000 cells	Mean (%)	per 1000 cells	Mean (%)
Control (DMSO)	2[a] 1[a] 2[a] 3[a]	0.30	5 6 5 10	0.65
GST (1)	5[a] 1[a]	0.65	10 20	1.50
GST (10)	7[a] 11[a]	1.20	12 19	1.55
GST (20)	24 6	1.50	14 20	1.70
GST (25)	20 19	1.9	18[a] 7[a]	1.85
GST (30)	34 25	2.95	25[a] 18[a]	4.60
Mitomycin C (0.075)	7 5[a]	0.70	32 46	3.90
Colchicine (0.02)	45 36	4.05	12[a] 21[a]	3.75

a <1000 cells scored

Appendix 1 Historical control data in the absence of S9 mix

Historical control data – April 2006 – September 2008

Solvent Control Values – 27 cultures

Mononucleate cells			Binucleate cells			Cytokinesis-block proliferative index			Mononucleate Micronuclei (%)			Binucleate Micronuclei (%)		
Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
13	136	60.04	322	568	423.96	1.86	2.23	2.06	0.00	0.60	0.16	0.00	0.90	0.37

Positive Control Values - Mitomycin C (0.075 µg/mL) – 20 cultures

Mononucleate cells			Binucleate cells			Cytokinesis-block proliferative index			Mononucleate Micronuclei (%)			Binucleate Micronuclei (%)		
Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
28	136	79.45	263	472	359.05	1.89	2.24	2.09	0.00	2.00	0.23	1.20	8.70	4.61

Positive Control Values - Colchicine (0.02 - 0.03 µg/mL) – 13 cultures

Mononucleate cells			Binucleate cells			Cytokinesis-block proliferative index			Mononucleate Micronuclei (%)			Binucleate Micronuclei (%)		
Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
28	469	279.15	66	589	233.38	1.19	2.24	1.62	1.10	2.70	1.87	0.00	2.00	0.42

Appendix 2 Historical control data in the presence of S9 mix

Historical control data – April 2006 – September 2008

Solvent Control Values – 23 cultures

Mononucleate cells			Binucleate cells			Cytokinesis-block proliferative index			Mononucleate Micronuclei (%)			Binucleate Micronuclei (%)		
Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
10	214	56.57	227	650	382.52	1.66	2.50	2.15	0.00	1.00	0.11	0.10	0.40	0.27

Positive Control Values – Cyclophosphamide (10 µg/mL) – 15 cultures

Mononucleate cells			Binucleate cells			Cytokinesis-block proliferative index			Mononucleate Micronuclei (%)			Binucleate Micronuclei (%)		
Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
46	231	104.40	250	430	362.47	1.66	2.17	2.00	0.00	0.60	0 09	3.10	5.90	4.46

Appendix 3 Certificate of Analysis

[redacted]

CERTIFICATE OF ANALYSIS

NAME OF SAMPLE : GST
LOT NO. OF SAMPLE : Lot number K0821081
DATE OF ANALYSIS : 2008/02/25

COMPOSITION

Identity	Conc.
#1	93.9%
#2	0.7%
#3	2.7%
#4 GST dimer	1.6%
#5 GST trimer	0.2%
#6 unknown	0.9%
TOTAL	100%

NAME : [illegible]

SIGNATURE [illegible] DATE 2008 / 4 / 11

1

Annex 1 GLP Compliance Statements

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

TEST TYPE

Analytical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Testing
Toxicology

DATE OF INSPECTION

28th January 2008

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

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

Head,

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Testing
Toxicology

DATE OF INSPECTION

17-19 February 2009

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

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

Head,

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Physico-chemical Testing
Residue Studies
Toxicology

DATE OF INSPECTION

26 January 2010

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

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

Head,

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity (terrestrial only)
Flow Cytometry
Histology/pathology (slide reading only)
Microbiology
Toxicology

DATE OF INSPECTION

17 September 2007

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

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

Head,

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity (terrestrial only)
Flow Cytometry
Histology/pathology (slide reading only)
Microbiology
Toxicology

DATE OF INSPECTION

23rd – 25th September 2008

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

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

Head.

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

TEST TYPE

Analytical Chemistry
Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Toxicology

DATE OF INSPECTION

2nd November 2009

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

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

Head,

Annex 2 S9 Quality Control Certificate

TRINOVA Biochem GmbH

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

LOT NO.: 2273 **SPECIES:** Rat **PREPARATION DATE:** May 1, 2008
PART NO.: 11-105 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** May 1, 2010
VOLUME: 1 & 2 ml **SEX:** Male **BUFFER:** 0.154 M KCl
TISSUE: Liver **INDUCING AGENT(s):** Phenobarbital - 5,6-Benzoflavone
REFERENCE: Matsushima, et. al., In In Vitro Metabolic Activation in Mutagenesis Testing (F.J. de Serres, Ed.), Elsevier, 1976 p. 85
STORAGE: At or below 70°C

BIOCHEMISTRY:

- PROTEIN

37.6 mg/ml — Assayed according to the method of Lowry et al., JBC 193 265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-O-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	1A1 1A2	55.5
PROD	2B1	19.7
BROD	2B1	19.9
MROD	1A2	11.2

Assays for ethoxyresorufin-O-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-O-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 31.0, 14.2, 57.4, 10.4 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 F, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. *his*- Revertants EtBr/ CPA/	
TA98	TA1535
342.8	1528

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lescar et al., *Mutat. 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)	181	297	387	650	855	1065
2-AA (2.5 µg)	184	365	1075	1727	2026	1283

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