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SILICONES ENVIRONMENTAL, HEALTH AND SAFETY COUNCIL NORTH AMERICA

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8EHQ-12-18604

April 5, 2012

TSCA Confidential Business Information Center (7407M)
EPA East – Room 6428 Attn: Section 8(e)
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

89120000324

Re: 8EHQ-12-18604: Supplement TSCA Section 8(e) Notification of Substantial Risk: Triethoxy(3-thiocyanatopropyl)silane (CAS No. 34708-08-2)

Dear TSCA Section 8(e) Coordinator:

In accordance with the provisions of Section 8(e) of the Toxic Substances and Control Act (TSCA), as interpreted in the TSCA Section 8(e) Policy Statement and Guidance, Fed. Reg. 33129 (June 3, 2003) and other Agency guidance, the Silicones Environmental, Health and Safety Council (SEHSC)[1] submits, on behalf of its member companies, a final study report as a supplemental submission to our March 9, 2012, TSCA 8(e) notification.

Chemical Substances

Triethoxy(3-thiocyanatopropyl)silane (CAS No. 34708-08-2)

Completed Study

1. Final report: *In Vitro* Mammalian Gene Cell Mutation Assay Thymidine Kinase Locus (TK +/-) in Mouse Lymphoma L5178Y cells with Triethoxy(3-thiocyanatopropyl)silane
2. Draft report: *In Vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 Cells with Triethoxy(3-thiocyanatopropyl)silane

Summary

Results from an in vitro mammalian gene cell mutation assay conducted with Triethoxy(3-thiocyanatopropyl)silane (CAS No. 34708-08-2; the test substance) indicate the test substance is mutagenic, but not clastogenic. Results from an in vitro mammalian chromosome aberration test in Chinese Hamster V79 Cells indicate the test substance induced structural chromosomal aberrations and is considered to be clastogenic.

[1] SEHSC is a not-for-profit trade association whose mission is to promote the safe use of silicones through product stewardship and environmental, health, and safety research. The Council is comprised of North American silicone chemical producers and importers.

CONTAINS NO CBI

If you have any questions concerning this submission, please contact me at (703) 788-6570, kthomas@sehsc.com, or at the address provided herein.

Sincerely,

Karluss V. Thomas

Karluss Thomas
Executive Director

BIOSERVICE
SCIENTIFIC LABORATORIES GmbH

In vitro Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/TK$^{+/-}$) in Mouse Lymphoma L5178Y Cells with Triethoxy(3-thiocyanatopropyl)silane

Report

Version: Final

Date: 12 December 2011

BSL BIOSERVICE Study No.: 114186

Sponsor:

ReachCentrum SPRL

For account of the members of the Reconsile Consortium

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1160 Brussels

Belgium

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GLP zertifiziert

Akkreditiert durch Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten ZLG-P-986.96.01

1. Copy of the GLP Certificate

BAYERISCHES LANDESAMT
FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT,
LANDESINSTITUT FÜR ARBEITSSCHUTZ UND PRODUKTSICHERHEIT
Pfarrstraße 3 · 80538 München · Telefon (089) 21 84-0

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

BSL Bioservice Scientific Laboratories GmbH
Behringstrasse 6 - 8
82152 Planegg

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen auf toxikologische Eigenschaften
3 Prüfungen auf mutagene Eigenschaften
9 Sonstige Prüfungen:
a) Mikrobiologische Sicherheitsprüfungen
b) Wirksamkeitsprüfungen an Zellkulturen

Datum der Inspektion/Date of Inspection
(Tag.Monat.Jahr/day.month year)
16./17.09.2008

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/ diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP

München, 06.04.2009

Ritter
Leitender Gewerbedirektor

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4. Preface

4.1. Abbreviations

B[*a*]P	Benzo[*a*]pyrene
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
DMSO	Dimethylsulfoxide
CE	Cloning Efficiency
EC	European Community
EMS	Ethylmethanesulfonate
EPA	Environmental Protection Agency
GEF	Global Evaluation Factor
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
HS	Horse Serum
IWGT	International Workshop on Genotoxicity Testing
MLA	Mouse Lymphoma Assay
MMS	Methylmethanesulfonate
Nr.	Nummer *(number)*
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate Buffered Saline
PE	Plating Efficiency
QA	Quality Assurance
QAU	Quality Assurance Unit
RCE	Relative Cloning Efficiency
RPMI	Roswell Park Memorial Institute medium
RSG	Relative Suspension Growth
RTG	Relative Total Growth
S9	Microsomal fraction of rat liver homogenate
SG	Suspension Growth
SOP	Standard Operating Procedures
TFT	Trifluorothymidine
TK	Thymidine Kinase

4.2. General

Sponsor:	ReachCentrum SPRL For account of the members of the Reconsile Consortium Avenue E. van Nieuwenhuyse 6 1160 Brussels Belgium
Study Monitor:	Dr. Nils Krueger Evonik Industries AG Department IM-PT-PS Rodenbacher Chaussee 4 63457 Hanau Germany
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	114186
Test Item:	Triethoxy(3-thiocyanatopropyl)silane
Title:	*In vitro* Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/$TK^{+/-}$) in Mouse Lymphoma L5178Y Cells with Triethoxy(3-thiocyanatopropyl)silane

4.3. Project Staff

Study Director:	Dr. Kristina Trenz
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Head of Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann

4.4. Schedule

Arrival of the Test Item:	29 July 2011
Date of Draft Study Plan:	30 August 2011
Date of Final Study Plan:	21 September 2011
Start of Experiment:	26 September 2011
End of Experiment:	15 November 2011
Date of Draft Report:	28 November 2011
Date of 2nd Draft Report	09 December 2011
Date of Final Report:	12 December 2011

5. Project Staff Signatures

Study Director

Dr. Kristina Trenz

.....

Date: 12 Dec 2011

Management

.....

Print Name: Dr. Angela Lutterbach

Date: 12 Dec 2011

6. Quality Assurance

6.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility were periodically inspected by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

6.2. Guidelines

This study followed the procedures indicated by internal BSL BIOSERVICE SOPs and the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 476, "*In vitro* Mammalian Cell Gene Mutation Tests" adopted July 21, 1997.

Commission Regulation (EC) No 440/2008, L142, Annex Part B, B 17, "*In vitro* Mammalian Cell Gene Mutation Test", dated May 30, 2008.

EPA Health Effects Test Guidelines, OPPTS 870.5300 "*In vitro* Mammalian Cell Gene Mutation Test" EPA 712-C-98-221, August 1998.

IWGT recommendations (11, 12, 13, 14, 15).

6.3. Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP regulations:

A copy of the final report, the original study plan and documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study.

If test item is left, a sample will be stored according to the period fixed by the GLP regulations. Material and samples that are unstable may be disposed of before that time and without sponsor's prior consent. Raw data relating to the study will be discarded only with the prior consent of the sponsor. Unless otherwise agreed upon, the remaining test item will be discarded three months after the release of the report.

7. Statement of Compliance

BSL BIOSERVICE Study No.:	114186
Test Item:	Triethoxy(3-thiocyanatopropyl)silane
Title:	*In vitro* Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/$TK^{+/-}$) in Mouse Lymphoma L5178Y Cells with Triethoxy(3-thiocyanatopropyl)silane
Study Director:	Dr. Kristina Trenz

This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998.

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dr. Kristina Trenz

Kristina Trenz

Date: 19 Dec 2011

8. Statement of the Quality Assurance Unit

BSL BIOSERVICE

Study No.:	114186
Test Item:	Triethoxy(3-thiocyanatopropyl)silane
Title:	*In vitro* Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/$TK^{+/-}$) in Mouse Lymphoma L5178Y Cells with Triethoxy(3-thiocyanatopropyl)silane
Study Director:	Dr. Kristina Trenz

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phases of QAU Inspections	*Dates of QAU Inspections*	*Dates of Reports to the Study Director and Management*
Audit Final Study Plan:	21 September 2011	21 September 2011
Audit Experimental Phase (process-based):	1 July 2011	1 July 2011
Audit Final Report:	15 December 2011	15 December 2011

This report reflects the raw data.

Member of the
Quality Assurance Unit:

.....

Print Name: Gwendolyn Pretzsch

Date: 16 Dec 2011

9. Summary

9.1. Summary Results

The test item Triethoxy(3-thiocyanatopropyl)silane was assessed for its potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The selection of the concentrations used in the main experiment was based on data from the pre-experiment. In the main experiment 1.3 mM (**with** metabolic activation) and 1 mM (**without** metabolic activation) were selected as the highest concentrations. The experiment **with** and **without** metabolic activation was performed as a 4 h short-term exposure assay.

The test item was investigated at the following concentrations:

with metabolic activation:

0.05, 0.1, 0.2, 0.5, 0.7, 0.9, 1.1 and 1.3 mM

and **without** metabolic activation:

0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mM

Precipitation of the test item was noted in the pre-experiment at a concentration of 2.5 mM.

Growth inhibition was observed in the main experiment **with** and **without** metabolic activation.

In the main experiment **with** metabolic activation the relative total growth (RTG) was 9.4% for the highest concentration (1.3 mM) evaluated. The highest concentration evaluated **without** metabolic activation was 1 mM with a RTG of 13.1%.

In the main experiment a biologically relevant increase of mutants was found after treatment with the test item **with** metabolic activation, **without** metabolic activation no biologically relevant increase of mutants was found after treatment with the test item. The Global Evaluation Factor (GEF; defined as the mean of the negative/vehicle mutant frequency plus one standard deviation; data gathered from ten laboratories [13, 14, 15]) was exceeded by the induced mutant frequency at a concentration of 1.1 mM and higher in the main experiment **with** metabolic activation.

A dose-response relationship was observed.

However, in the main experiment colony sizing showed no clastogenic effects induced by the test item under the experimental conditions (**with** and **without** metabolic activation).

EMS, MMS and B[*a*]P were used as positive controls and showed distinct and biologically relevant effects in mutation frequency. Additionally, MMS and B[*a*]P significantly increased the number of small colonies, thus proving the efficiency of the test system to indicate potential clastogenic effects.

9.2. Conclusion

In conclusion, in the described mutagenicity test under the experimental conditions reported, the test item Triethoxy(3-thiocyanatopropyl)silane is considered to be mutagenic in the *in vitro* mammalian cell gene mutation assay (thymidine kinase locus) in mouse lymphoma L5178Y cells.

9.3. Summary Tables

Table 1: Summary: Main Experiment, *with* metabolic activation

	Test Group	Conc. [mM]	RCE[a] [%]	RTG[b] [%]	MF[c] [mutants/ 10^6 cells]	IMF[d] [mutants/ 10^6 cells]	GEF[e] exceeded	Statistical Significance[f]	Precipitate
Main Exp	C1	0	100.0	93.4	88.0	/	/	/	-
	C2		100.0	106.2		/	/	/	-
	S1	0	100.0	100.0	84.6	/	/	/	-
	S2					/	/	/	-
	3	0.05	103.5	97.3	72.7	-11.9	-	-	-
	4	0.1	90.2	91.3	107.2	22.6	-	-	-
	5	0.2	100.7	65.2	127.5	42.9	-	+	-
	6	0.5	93.7	34.8	161.0	76.4	-	+	-
	7	0.7	101.4	35.8	130.0	45.5	-	+	-
	8	0.9	95.8	30.1	186.3	101.8	-	+	-
	9	1.1	95.8	22.5	226.7	142.1	+	+	-
	10	1.3	86.0	9.4	236.2	151.6	+	+	-
	B[a]P	2.5	82.5	44.2	817.8	733.3	+	+	-

C. Negative Controls
S: Solvent Controls
a: Relative Cloning Efficiency, RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
b: Relative Total Growth, RTG = (RSG x RCE)/100
c: Mutant Frequency,
MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non selective medium)]}x800
d: Induced Mutant Frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls
e: Global Evaluation Factor, GEF (126); +: GEF exceeded, -: GEF not exceeded
f: statistical significant difference in mutant frequency compared to negative/solvent controls (Mann Whitney test , $p<0.05$). +: significant; -not significant
B[*a*]P: Benzo[a]pyrene [µg/mL]

Table 2: Summary: Main Experiment, *without* metabolic activation

	Test Group	Conc. [mM]	RCE[a] [%]	RTG[b] [%]	MF[c] [mutants/ 10^6 cells]	IMF[d] [mutants/ 10^6 cells]	GEF[e] exceeded	Statistical Significance[f]	Precipitate
Main Exp	C1	0	103.2	122.9	69.4	/	/	/	-
	C2		100.4	121.0		/	/	/	-
	S1	0	100.0	100.0	66.4	/	/	/	-
	S2					/	/	/	-
	3	0.005	101.1	110.1	67.1	0.8	-	-	-
	4	0.01	99.6	104.1	69.1	2.7	-	-	-
	5	0.02	109.5	119.3	50.8	-15.5	-	-	-
	6	0.05	103.2	108.4	85.9	19.5	-	-	-
	7	0.1	112.3	61.3	74.7	8.3	-	-	-
	8	0.2	92.6	35.8	93.0	26.7	-	-	-
	9	0.5	96.8	26.1	113.1	46.7	-	+	-
	10	1	96.8	13.1	143.3	76.9	-	+	-
	EMS	300	94.0	99.6	622.7	556.3	+	+	-
	MMS	10	92.6	88.1	456.8	390.5	+	+	-

C: Negative Controls
S Solvent Controls
a: Relative Cloning Efficiency, RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
b: Relative Total Growth, RTG = (RSG x RCE)/100
c: Mutant Frequency,
MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non selective medium)]}x800
d: Induced Mutant Frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls
e: Global Evaluation Factor, GEF (126); +: GEF exceeded, -: GEF not exceeded
f: statistical significant difference in mutant frequency compared to negative/solvent controls (Mann Whitney test , $p<0.05$). +: significant; -not significant
EMS: Ethylmethanesulfonate [µg/mL]
MMS: Methylmethanesulfonate [µg/mL]

10. Introduction

10.1. Aim of the Study

Mammalian cell culture systems are used to detect mutations induced by chemical substances. This *in vitro* experiment is carried out to assess the potential of the test item to induce gene mutations by means of a Thymidine Kinase assay using the mouse lymphoma cell line L5178Y. The Thymidine Kinase (TK) system detects base pair mutations, frameshift mutations, small deletions as well as large, non-lethal deletions and rearrangements of the relevant chromosomes [1, 8, 9, 10].

TK catalyses the conversion of TFT (trifluorothymidine) to its cytostatic and cytotoxic trifluoro-thymidine-monophosphate derivative. Cells deficient in the heterozygous TK-locus due to the forward mutation $TK^{+/-} \rightarrow TK^{-/-}$ are resistant to the cytotoxic effects of pyrimidine analogues such as TFT. The deficiency of the "salvage" enzyme TK means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained solely from de novo synthesis. However, in the presence of TK, TFT is incorporated into the nucleotides, resulting in inhibition of cellular metabolism and cytotoxicity. Thus, mutant cells are able to proliferate in the presence of TFT, whereas normal cells which contain TK, are not. Cells as suspension cultures are exposed to the test item for a defined period of time (4 h for a short-term exposure or 24 h for a long-term exposure). Cytotoxicity is determined by measuring the colony-forming ability and the growth rate of cultures. The treated cultures are maintained 2 days to allow near optimal phenotypic expression of induced mutations.

Mutant frequency is determined by seeding defined numbers of cells in medium containing the selective agent (TFT) to detect mutant cells and in medium without selective agent to determine the cloning efficiency. After a suitable incubation time all colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in non-selective medium to derive the mutant frequency [5].

The size of the colonies is usually characterised as follows [3, 7]:
Small colonies approximately $\leq$ ¼ of well diameter.
Large colonies approximately > ¼ of well diameter.
Size is the key factor and morphology should be secondary.

There is no requirement for the verification of positive results. Equivocal results should be clarified by further testing preferably using modified experimental conditions. Negative results need to be confirmed on a case-by case basis. In those cases where confirmation of negative results is not considered necessary, justification has to be provided by the sponsor. Study parameters which might be changed are concentrations, treatment time or metabolic activation conditions.

To establish a concentration response of the test item, at least eight concentrations are tested. These concentration levels should yield a concentration-related toxic effect. The highest concentration should induce a reduced level of survival of approximately 10-20% relative survival. The lowest concentration should be in the range of the negative control with respect to cell viability and proliferation.

For soluble, non-toxic test compounds, the recommended maximum test concentration is 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest. Negative and/or solvent controls are tested in duplicate.

Reference mutagens are tested in parallel to the test item in order to demonstrate the sensitivity of the test system.

10.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals Section 4, No 476 – *In Vitro* Mammalian Cell Gene Mutation Test – recommends using cell line L5178Y (Mouse Lymphoma Cells).

11. Materials and Methods

11.1. Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor. All data related to the test item are the responsibility of the sponsor and have not been verified by the test facility.

Name:	Triethoxy(3-thiocyanatopropyl)silane
Batch No.:	809901
CAS No.:	34708-08-2
Physical State at RT:	liquid
Colour:	light brown
Expiry Date:	June 2012
Molecular Weight:	263.43
Purity:	98.3%
Storage conditions:	at room temperature
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

11.2. Preparation of the Test Item

A solubility test was performed with different solvents and vehicles up to the maximum recommended concentration of 10 mM. Based on the solubility test DMSO was used as solvent (final concentration 1% DMSO v/v). Different test item stock solutions were prepared and added to the samples. When adding the stock solutions to the samples precipitation was noted. The solvent was compatible with the survival of the cells of the S9 activity.

11.3. Controls

Negative and/or solvent as well as positive controls were included in each experiment.

Negative/Solvent Control

Negative controls (treatment medium) and solvent controls (1% DMSO v/v) were treated in the same way as the treatment groups.

Positive Controls

Without metabolic activation

Name:	EMS; Ethylmethanesulfonate
Supplier:	Sigma
Catalogue No.:	M 0880
Batch No.:	BCBC4573V
Dissolved in:	Medium
Final concentration:	300 µg/mL

Name:	MMS; Methylmethanesulfonate
Supplier:	Sigma
Catalogue No.:	M 4016
Batch No.:	87596LJ
Dissolved in:	0.9% NaCl
Final concentration:	10 µg/mL

With metabolic activation

Name:	B[*a*]P, Benzo[*a*]pyrene
Supplier:	Sigma
Catalogue No.:	12780
Batch No.:	097K1293
Dissolved in:	DMSO, Dimethylsulfoxide; final concentration in RPMI medium 1%
Final concentration:	2.5 µg/mL

The dilutions of the stock solutions of the positive controls were prepared on the day of the experiment and used immediately.

The stability of the positive control substances in solution is proven by the mutagenic response in the expected range.

11.4. Test System

11.4.1. Cells

Mouse Lymphoma L5178Y cells have been used successfully in *in vitro* experiments for many years. These cells are characterised by their high proliferation rate (10-12 h doubling time of the BSL BIOSERVICE stock cultures) and their cloning efficiency, usually more than 50%. The cells obtain a near diploid karyotype (40 ± 2 chromosomes). They are heterozygous at the Thymidine Kinase (TK) locus in order to detect mutation events at the TK- locus.

To prevent high backgrounds arising from spontaneous mutation, cells lacking TK can be eliminated by culturing cells in RPMI 1640 supplemented with:

9.0	µg/mL	hypoxanthine
15.0	µg/mL	thymidine
22.5	µg/mL	glycine
0.1	µg/mL	methotrexate

The cells are resuspended in medium without methotrexate but thymidine, hypoxanthine and glycine for 1-3 days.

Large stock cultures of the cleansed L5178Y cell line are stored over liquid nitrogen (vapour phase) in the cell bank of BSL BIOSERVICE. This allows the repeated use of the same cell batch in experiments. Each cell batch is routinely checked for mycoplasma infection.

Thawed stock cultures are maintained in plastic culture flasks in RPMI 1640 complete medium and subcultured three times per week.

11.4.2. Mammalian Microsomal Fraction S9 Homogenate

An advantage of using *in vitro* cell cultures is the accurate control of concentration and exposure time of cells to the test item under study. However, due to the limited capacity of cells growing *in vitro* for metabolic activation of potential mutagens, an exogenous metabolic activation system is necessary [2]. Many substances only develop mutagenic potential when they are metabolized by the mammalian organism. Metabolic activation of substances can be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix). Since the L5178Y cells used in this assay do not have the cytochrome-based P450 metabolic oxidation system, an exogenous metabolic activation system, the S9 microsomal fraction was added. The cells were exposed to the test substance both, in the presence and the absence of metabolic activation.

The S9 liver microsomal fraction was prepared at BSL BIOSERVICE GmbH. Male Wistar rats were induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route.

The following quality control determinations were performed:

a) Biological activity in:
- the *Salmonella typhimurium* assay using 2-aminoanthracene
- the mouse lymphoma assay using benzo[*a*]pyrene
- the chromosome aberration assay using cyclophosphamide.

b) Sterility Test

A stock of the supernatant containing the microsomes was frozen in aliquots of 2 and 4.5 mL and stored at ≤ -75°C.

The protein concentration in the S9 preparation (Lot: 210711) was 38.5 mg/mL.

11.4.3. S9 Mix

An appropriate quantity of the S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the concentrations below:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	Glucose-6-phosphate
5	mM	NADP

in 100 mM sodium-phosphate-buffer pH 7.4. During the experiment the S9 mix was stored on ice.

11.5. Experimental Design

11.5.1. Pre-Experiment for Toxicity

The toxicity of the test item was determined in a pre-experiment up to a maximum concentration of 10 mM. For the main experiment four concentrations [0.2, 0.5, 1 and

2 mM] were reported **without** metabolic activation, **with** metabolic activation three concentrations [0.2, 0.5 and 2.5 mM] were reported. Dose groups that were not evaluable due to very high toxicity of the test item are not reported. The experimental conditions in this pre-experiment were the same as described below in the paragraph experimental performance.

11.5.2. Exposure Concentrations

The selection of the concentrations used in the main experiment was based on data from the pre-experiment. In the main experiment 1.3 mM (**with** metabolic activation) and 1 mM (**without** metabolic activation) were selected as the highest concentrations. The main experiment **with** and **without** metabolic activation was performed as 4 h short-term exposure assay.

The test item was investigated at the following concentrations:

with metabolic activation:

0.05, 0.1, 0.2, 0.5, 0.7, 0.9, 1.1 and 1.3 mM

and **without** metabolic activation:

0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mM

According to OECD Guidelines at least 8 concentrations of the test item were set up in the experiments **with** and **without** metabolic activation.

11.5.3. Experimental Performance

For a short-term exposure experiment 1 x 10^7 cells were suspended in 11 mL RPMI medium with 5% horse serum (25 cm^2 flasks) and exposed to designated concentrations of the test item either in the presence or absence of metabolic activation in the mutation experiment. After 4 h the test item was removed by centrifugation (200 x g, 10 min) and the cells were washed twice with PBS. Subsequently the cells were suspended in 30 mL complete culture medium and incubated for an expression and growth period of 2 days in total at 37 °C in 5% CO_2/95% humidified air. The cell density was determined each day and adjusted to 3 x 10^5 cells/mL in a total culture volume of 20 mL, if necessary.

Due to the positive result in the short-term exposure experiment it was not necessary to perform a long-time exposure experiment.

After the expression period the cloning efficiency (CE) of the cells was determined by seeding a statistical number of 1.6 cells/well in two 96-well plates. The cells were incubated for at least 6 days at 37 °C in a humidified atmosphere with 5% CO_2. Analysis of the results was based on the number of cultures with cell growth (positive wells) and those without cell growth (negative wells) compared to the total number of cultures seeded. Additionally, cultures were seeded in selective medium. Cells from each experimental group were seeded in four 96-well plates at a density of approximately 2000 cells/well in 200 µL selective medium (see below) with TFT. The plates were scored after an incubation period of ~14 days at 37 °C in 5% CO_2/95% humidified air.

The mutant frequency is calculated by dividing the number of TFT resistant colonies by the number of cells plated for selection, corrected for the plating efficiency of cells from the same culture grown in the absence of TFT. For the microwell method used here the Poisson distribution is used to calculate the plating efficiencies for cells cloned without and with TFT selection. Based on the null hypothesis of the Poisson distribution, the

probable number of clones/well (P) is equal to –ln(negative wells/total wells) and the plating efficiency (PE) equals P/(number of cells plated per well). Mutant frequency then is calculated as MF = (PE(cultures in selective medium)/PE(cultures in non-selective medium)). The mutant frequency is usually expressed as "mutants per 10^6 viable cells"(3).

$$\text{Mutant frequency} = \frac{-\ln\,[NW/TW_{\text{(selective medium)}}]}{-\ln\,[NW/TW_{\text{(non-selective medium)}}]} \times 800$$

NW: number of negative wells
TW: number of total wells

Suspension growth (SG) of the cell cultures reflects the number of times the cell number increases from the starting cell density. When carrying out a short-term treatment (4 h) a 2 day growth period is considered, when carrying out a long-term treatment the treatment period of 24 h and the 2 day growth period is considered. The relative total growth (RTG) is the product of the relative suspension growth (RSG; calculated by comparing the SG of the dose groups with the SG of the control) and the relative cloning efficiency (RCE) for each culture: RTG = RSG x RCE /100.

The mutant frequencies obtained from the experiments are compared with the Global Evaluation Factor (GEF). To arrive at a GEF, the workgroup (IWGT MLA Workgroup; [11-15]) analyzed distributions of negative/vehicle mutant frequencies of the MLA that they gathered from ten laboratories. The GEF is defined as the mean of the negative/vehicle mutant frequency plus one standard deviation. Applying this definition to the collected data, the GEF arrived to be 126 for the microwell method (13, 14, 15).

The non-parametric Mann-Whitney test is applied to the mutation data to prove the dose groups for any significant difference in mutant frequency compared to the negative /solvent controls. Mutant frequencies of the solvent/negative controls are used as reference.

Cell Culture Media

For preparation of the different media horse serum (HS) was heat-inactivated for 30 min. at 56 °C prior usage.

Complete Culture Medium

RPMI 1640 medium supplemented with

10	%	horse serum (HS)
100 U/100	µg/mL	penicillin/streptomycin
1	mM	sodium pyruvate
2	mM	L-glutamine
25	mM	HEPES
2.5	µg/mL	amphotericin B

Treatment Medium

RPMI 1640 medium supplemented with

5	%	HS: in case of short term exposure
7.5	%	HS: in case of long term exposure
100 U/100	µg/mL	penicillin/streptomycin

1	mM	sodium pyruvate
2	mM	L-glutamine
25	mM	HEPES
2.5	µg/mL	amphotericin B

Selective Medium

RPMI 1640 medium supplemented with

20	%	horse serum (HS)
100 U/100	µg/mL	penicillin/streptomycin
1	mM	sodium pyruvate
2	mM	L-glutamine
25	mM	HEPES
2.5	µg/mL	amphotericin B
5	µg/mL	TFT

11.6. Data Recording

The data generated are recorded in the raw data. The results are presented in tabular form, including experimental groups with the test item, negative and/or solvent as well as positive controls. Individual colony counts for the treated and control groups are presented for both mutation induction and survival. Small colonies (produced predominantly by chromosomal rearrangements) and large colonies (produced by point mutations) are determined for the relevant dose groups.

11.7. Acceptability of the Assay

A mutation assay is considered acceptable if it meets the criteria mentioned in current international guidelines and the current recommendations of the IWGT (11, 12, 13, 14, 15):

- At least three out of four 96-well plates from the TFT resistance-testing portion of the experiment are scorable.
- The cloning efficiency of the negative and/or solvent controls is in the range 65%-120%.
- The spontaneous mutant frequency in the negative and/or solvent controls is in the range 50-170 per 10^6 cells.
- The cell number of the negative/solvent controls should undergo 8-32 fold increase during a 2 day growth period (short-term treatment) or 32-180 fold increase during a 3 day growth period (long-term treatment).
- The clastogenic positive controls (MMS and B[*a*]P) have to produce an induced mutant frequency (total mutant frequency minus concurrent negative control mutant frequency) of at least 300 per 10^6 cells with at least 40% of the colonies being small colonies or with an induced small colony mutant frequency of at least 150 per 10^6 cells. The RTG must be greater than 10%.

11.8. Evaluation of Results

The test item is considered mutagenic if following criteria are met (13, 14, 15):

- The induced mutant frequency meets or exceeds the Global Evaluation factor (GEF) of 126 per 10^6 cells
- A dose-dependent increase in mutant frequency is detected.

Besides, combined with a positive effect in the mutant frequency, an increased occurrence of small colonies (≥40% of total colonies) is an indication for potential clastogenic effects and/or chromosomal aberrations.

According to the OECD guideline, the biological relevance is considered first for the interpretation of results. Statistical methods might be used as an aid in evaluation the test result.

A test item is considered to be negative if the induced mutant frequency is below the GEF and the trend test is negative.

12. Deviations from the Study Plan

There were no deviations from the study plan.

13. Results

Table 3: Pre-Experiment for Toxicity, *with* metabolic activation

Test Group	Concentration [mM]	Number of Cells 4 h after Treatment	Number of Cells 24 h after Treatment	Number of Cells 48 h after Treatment	SG[a]	RSG[b] [%]
C1	0	329000	864000	1350000	11.7	103.4
C2		337000	879000	1400000	12.3	109.1
S1	0	305000	834000	1370000	11.4	100.0
S2		315000	831000	1340000	11.1	
1	0.2	295000	732000	1370000	10.0	88.9
2	0.5	288000	558000	1340000	7.5	66.3
3 (P)	2.5	135000	107000	80500	0.2	2.1

C: Negative control
P: Precipitation
a: Suspension Growth, SG = [((value 24h x 30) / $1x10^7$) x ((value 48 h x 20) / (value 24 h*x20))];
* : for value 24 h > $3x10^5$ then value 24 h = $3x10^5$
b: Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]

Table 4: Pre-Experiment for Toxicity, *without* metabolic activation

Test Group	Concentration [mM]	Number of Cells 4 h after Treatment	Number of Cells 24 h after Treatment	Number of Cells 48 h after Treatment	SG[a]	RSG[b] [%]
C1	0	346000	827000	1360000	11.2	104.2
C2		366000	872000	1340000	11.7	108.3
S1	0	364000	758000	1390000	10.5	100.0
S2		370000	767000	1440000	11.0	
1	0.2	339000	573000	1090000	6.2	57.9
2	0.5	282000	377000	1040000	3.9	36.3
3	1	234000	227000	600000	1.8	16.7
4	2	107000	77500	596000	1.8	16.6

C. Negative control
S: Solvent control
a Suspension Growth, SG = [((value 24h x 30) / $1x10^7$) x ((value 48 h x 20) / (value 24 h*x20))];
* : for value 24 h > $3x10^5$ then value 24 h = $3x10^5$
b: Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]

Table 5: Main Experiment - Toxicity Data, *with* metabolic activation

Test Group	Concentration [mM]	Number of Cells 4 h after Treatment	Number of Cells 24 h after Treatment	Number of Cells 48 h after Treatment	SG[a]	RSG[b] [%]	RCE[c] [%]	RTG[d] [%]
C1	0	269000	707000	1450000	10.3	93.4	100.0	93.4
C2		298000	762000	1530000	11.7	106.2	100.0	106.2
S1	0	272000	675000	1520000	10.3	100.0	100.0	100.0
S2		312000	764000	1530000	11.7			
3	0.05	302000	697000	1480000	10.3	94.0	103.5	97.3
4	0.1	314000	726000	1530000	11.1	101.2	90.2	91.3
5	0.2	236000	497000	1430000	7.1	64.8	100.7	65.2
6	0.5	218000	329000	1240000	4.1	37.2	93.7	34.8
7	0.7	241000	340000	1140000	3.9	35.3	101.4	35.8
8	0.9	236000	284000	1150000	3.5	31.4	95.8	30.1
9	1.1	185000	235000	860000	2.6	23.5	95.8	22.5
10	1.3	186000	183000	400000	1.2	10.9	86.0	9.4
B[a]P	2.5	234000	452000	1300000	5.9	53.5	82.5	44.2

C: Negative control
S: Solvent control
a: Suspension Growth, SG = [((value 24hx30)/1x10^7) x ((value 48 h x 20)/(value 24 h*x20))];
* : for value 24h > 3x10^5 then value 24h = 3x10^5
b: Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]
c: Relative Cloning Efficiency, RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
d: Relative Total Growth, RTG = (RSG x RCE)/100
B[a]P: Benzo[a]pyrene [µg/mL]

Table 6: Main Experiment - Mutagenicity Data, *with* metabolic activation

Test Group	Concentration [mM]	Cloning Efficiency (CE)			Mutagenicity Data						
		Plate 1[e]	Plate 2[e]	RCE[f] [%]	Number of cultures / 96 wells					MF[g] [mutants / 10^7 cells]	IMF[h] [mutants / 10^7 cells]
					Plate 1[e]	Plate 2[e]	Plate 3[e]	Plate 4[e]	Mean		
C1	0	69	74	100.0	13	12	14	11	12.5	81.8	/
C2		71	72	100.0	14	13	13	17	14.3	94.2	/
S1	0	70	76	100.0	13	15	11	12	12.8	79.9	/
S2		74	66		12	13	16	11	13.0	89.3	/
3	0.05	74	74	103.5	12	9	12	15	12.0	72.7	-11.9
4	0.1	62	67	90.2	12	14	9	18	13.3	107.2	22.6
5	0.2	72	72	100.7	16	22	19	19	19.0	127.5	42.9
6	0.5	70	64	93.7	18	19	20	25	20.5	161.0	76.4
7	0.7	70	75	101.4	25	22	18	13	19.5	130.0	45.5
8	0.9	71	66	95.8	24	24	25	24	24.3	186.3	101.8
9	1.1	74	63	95.8	26	23	34	31	28.5	226.7	142.1
10	1.3	60	63	86.0	22	27	27	24	25.0	236.2	151.6
B[a]P	2.5	61	57	82.5	64	55	55	64	59.5	817.8	733.3

C: Negative control
S: Solvent control
e: Number of cultures with cell growth.
f: Relative Cloning Efficiency, RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
g: Mutant frequency,
MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non selective medium)]}x800
h: Induced mutant frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls
B[a]P: Benzo[a]pyrene [µg/mL]

Table 7: Main Experiment - Colony Sizing, *with* metabolic activation

Test Group	Concentration [mM]	Wells with at least 1 colony	Large colonies	Small colonies	% small colonies
C1	0	50	43	7	14.0
C2		57	50	7	12.3
S1	0	51	47	4	7.8
S2		52	47	5	9.6
8	0.9	97	74	23	23.7
9	1.1	114	76	38	33.3
10	1.3	100	65	35	35.0
B[a]P	2.5	238	120	118	49.6

C: Negative control
S: Solvent control
B[a]P: Benzo[a]pyrene [µg/mL]

Table 8: Main Experiment - Toxicity Data, *without* metabolic activation

Test Group	Concentration [mM]	Number of Cells 4 h after Treatment	Number of Cells 24 h after Treatment	Number of Cells 48 h after Treatment	SG[a]	RSG[b] [%]	RCE[c] [%]	RTG[d] [%]
C1	0	323000	895000	1420000	12.7	119.2	103.2	122.9
C2		341000	869000	1480000	12.9	120.6	100.4	121.0
S1	0	315000	779000	1380000	10.8	100.0	100.0	100.0
S2		331000	820000	1290000	10.6			
3	0.005	329000	807000	1440000	11.6	109.0	101.1	110.1
4	0.01	335000	819000	1360000	11.1	104.4	99.6	104.1
5	0.02	327000	836000	1390000	11.6	109.0	109.5	119.3
6	0.05	343000	830000	1350000	11.2	105.1	103.2	108.4
7	0.1	299000	451000	1290000	5.8	54.6	112.3	61.3
8	0.2	299000	396000	1040000	4.1	38.6	92.6	35.8
9	0.5	216000	282000	959000	2.9	27.0	96.8	26.1
10	1	153000	150000	482000	1.4	13.6	96.8	13.1
EMS	300	323000	763000	1480000	11.3	105.9	94.0	99.6
MMS	10	327000	714000	1420000	10.1	95.1	92.6	88.1

C: Negative control
S: Solvent control
a: Suspension Growth, SG = [((value 24hx30)/1x10^7) x ((value 48 h x 20)/(value 24 h*x20))];
* : for value 24h > 3x10^5 then value 24h = 3x10^5
b: Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]
c. Relative Cloning Efficiency, RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
d: Relative Total Growth, RTG = (RSG x RCE)/100
EMS: Ethylmethanesulfonate [µg/mL]
MMS: Methylmethanesulfonate [µg/mL]

Table 9: Main Experiment - Mutagenicity Data, *without* metabolic activation

Test Group	Concentration [mM]	Cloning Efficiency (CE) Plate 1[e]	Plate 2[e]	RCE[f] [%]	Mutagenicity Data: Number of cultures / 96 wells Plate 1[e]	Plate 2[e]	Plate 3[e]	Plate 4[e]	Mean	MF[g] [mutants / 10^6 cells]	IMF[h] [mutants / 10^6 cells]
C1	0	72	75	103.2	11	7	13	15	11.5	70.7	/
C2		71	72	100.4	15	9	9	9	10.5	68.1	/
S1	0	72	72	100.0	8	9	10	6	8.3	51.9	/
S2		68	73		13	8	13	14	12.0	80.8	/
3	0.005	69	75	101.1	13	6	12	11	10.5	67.1	0.8
4	0.01	72	70	99.6	12	10	7	13	10.5	69.1	2.7
5	0.02	81	75	109.5	6	8	19	5	9.5	50.8	-15.5
6	0.05	75	72	103.2	16	11	19	9	13.8	85.9	19.5
7	0.1	79	81	112.3	17	16	11	15	14.8	74.7	8.3
8	0.2	70	62	92.6	12	19	12	5	12.0	93.0	26.7
9	0.5	69	69	96.8	16	16	14	17	15.8	113.1	46.7
10	1	67	71	96.8	17	21	20	20	19.5	143.3	76.9
EMS	300	69	65	94.0	58	64	55	55	58.0	622.7	556.3
MMS	10	67	65	92.6	45	47	51	43	46.5	456.8	390.5

C: Negative control
S: Solvent control
e: Number of cultures with cell growth.
f: Relative Cloning Efficiency, RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
g: Mutant frequency,
MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non selective medium)]} x800
h: Induced mutant frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls
EMS: Ethylmethanesulfonate [µg/mL]
MMS: Methylmethanesulfonate [µg/mL]

Table 10: Main Experiment - Colony Sizing, *without* metabolic activation

Test Group	Concentration [mM]	Wells with at least 1 colony	Large colonies	Small colonies	% small colonies
C1	0	46	39	7	15.2
C2		42	35	7	16.7
S1	0	33	23	10	30.3
S2		48	40	8	16.7
8	0.2	48	39	9	18.8
9	0.5	63	52	11	17.5
10	1	78	62	16	20.5
MMS	10	186	90	96	51.6

C: Negative control
S: Solvent control
MMS: Methylmethanesulfonate [µg/mL]

Table 11: Biometry - Main Experiment, *with* metabolic activation

Statistical significance at the 5% level ($p < 0.05$) was evaluated by means of the non-parametric Mann-Whitney test.

Test Group	Concentration [mM]	mean Mutant Frequency	mean Induced Mutant Frequency	p-value	statistical Significance
C1	0	81.8	/	/	/
C2		94.2	/	/	/
S1	0	79.9	/	/	/
S2		89.3	/	/	/
3	0.05	72.7	-11.9	0.214	-
4	0.1	107.2	22.6	0.214	-
5	0.2	127.5	42.9	0.008	+
6	0.5	161.0	76.4	0.004	+
7	0.7	130.0	45.5	0.028	+
8	0.9	186.3	101.8	0.004	+
9	1.1	226.7	142.1	0.004	+
10	1.3	236.2	151.6	0.004	+
B[a]P	2.5	817.8	733.3	0.004	+

C: Negative control
S. Solvent control
B[*a*]P: Benzo[a]pyrene
+: significant; -:not significant

Table 12: Biometry - Main Experiment, *without* metabolic activation

Statistical significance at the 5% level ($p < 0.05$) was evaluated by means of the non-parametric Mann-Whitney test.

Test Group	Concentration [mM]	mean Mutant Frequency	mean Induced Mutant Frequency	p-value	statistical Significance
C1	0	70.7	/	/	/
C2		68.1	/	/	/
S1	0	51.9	/	/	/
S2		80.8	/	/	/
3	0.005	67.1	0.8	0.933	-
4	0.01	69.1	2.7	1.000	-
5	0.02	50.8	-15.5	0.283	-
6	0.05	85.9	19.5	0.214	-
7	0.1	74.7	8.3	0.808	-
8	0.2	93.0	26.7	0.368	-
9	0.5	113.1	46.7	0.004	+
10	1	143.3	76.9	0.004	+
EMS	300	622.7	556.3	0.004	+
MMS	10	456.8	390.5	0.004	+

C: Negative control
S: Solvent control
EMS: Ethylmethanesulfonate
MMS: Methylmethanesulfonate
+: significant; -:not significant

14. Discussion

The test item Triethoxy(3-thiocyanatopropyl)silane was assessed for a possible potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The assay was conducted **with** and **without** metabolic activation. The experiment **with** metabolic activation were performed by including liver microsomes and NADP for efficient detection of a wide variety of carcinogens requiring metabolic activation.

The selection of the concentrations used in the main experiment was based on data from the pre-experiment according to the OECD guideline 476.

The selection of the concentrations used in the main experiment was based on data from the pre-experiment. In the main experiment 1.3 mM (**with** metabolic activation) and 1 mM (**without** metabolic activation) were selected as the highest concentrations. The main experiment **with** and **without** metabolic activation was performed as 4 h short-term exposure assay.

The test item was investigated at the following concentrations:

with metabolic activation:

0.05, 0.1, 0.2, 0.5, 0.7, 0.9, 1.1 and 1.3 mM

and **without** metabolic activation:

0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mM

The pH-value detected with the test item was within the physiological range.

Precipitation:

Precipitation of the test item was noted in the pre-experiment at a concentration of 2.5 mM.

Toxicity:

Growth inhibition was observed in the main experiment **with** and **without** metabolic activation.

In the main experiment **with** metabolic activation the relative total growth (RTG) was 9.4% for the highest concentration (1.3 mM) evaluated. The highest concentration evaluated **without** metabolic activation was 1 mM with a RTG of 13.1%.

Mutagenicity:

The mutant frequencies obtained from all experiments were compared with the Global Evaluation Factor (GEF) and a statistical analysis (Table 11, 12) was performed. The GEF is defined as the mean of the negative/vehicle mutant frequency plus one standard deviation; data are gathered from ten laboratories [13, 14, 15]. For the microwell method the GEF was defined to be 126. Criterion for mutagenicity is the extension of the GEF by the induced mutant frequency as well as a dose-dependent increase in mutant frequency. The positive controls EMS (300 µg/mL), MMS (10 µg/mL) and B[*a*]P (2.5 µg/mL) showed distinct increases in mutation frequency, thus proving the ability of the test system to detect potential mutagenic effects.

In the main experiment **with** metabolic activation all validity criteria were met. The negative and solvent controls showed mutant frequencies within the acceptance range of

50-170 mutants/10^6 cells, according to the IWGT criteria of 81.8 and 94.2 mutants/10^6 cells for negative controls and 79.9 and 89.3 mutants/10^6 cells for solvent controls, respectively. The positive control B[*a*]P induced a distinct increase in mutant frequency with 817.8 mutants/10^6 cells (Table 6).

The mutant frequencies induced by the test item did show a biologically relevant increase. The GEF of 126 was exceeded in two of the dose groups showing induced mutant values between –11.9 and 151.6 mutants/10^6 cells (Table 6). A statistical analysis (Table 11) confirmed that six of the mutant values were significantly different compared to the mutant values of the solvent controls. There was evidence for a dose-response relationship. Therefore this effect was considered as biologically relevant.

In the main experiment **without** metabolic activation all validity criteria were met. The negative and solvent controls showed mutant frequencies within the acceptance range of 50-170 mutants/10^6 cells, according to the IWGT criteria of 70.7 and 68.1 mutants/10^6 cells and 51.9 and 80.8 mutants/10^6 cells, respectively. The positive controls EMS and MMS induced a distinct increase in mutant frequency with 622.7 and 456.8 mutants/10^6 cells (Table 9).

The mutant frequencies induced by the test item did not show a biologically relevant increase. The GEF of 126 was not exceeded in any of the dose groups showing induced mutant values between –15.5 and 76.9 mutants/10^6 cells (Table 9). A statistical analysis (Table 12) revealed that two of the mutant values were significantly different compared to the mutant values of the solvent controls, however these values did not exceed the GEF of 126. There was evidence for a dose-response relationship, however this effect was considered as no biologically relevant.

Clastogenicity

Colony sizing was performed for the highest concentrations of the test item and for the negative and positive controls. An extension of the GEF by the induced mutant frequency in combination with an increased occurrence of small colonies (defined by slow growth and/or morphological alteration of the cell clone) is an indication for potential clastogenic effects and/or chromosomal aberrations. The positive controls MMS (10 µg/mL) and B[*a*]P (2.5 µg/mL) induced a significant increase in mutant frequency and a biologically significant increase of small colonies (≥40%), thus proving the ability of the test system to indicate potential clastogenic effects.

In the main experiment **with** metabolic activation the percentage of small colonies in the negative controls was found to be 14.0% and 12.3% and in solvent controls was found to be 7.8% and 9.6%, respectively. The percentage of small colonies of the positive control B[*a*]P was found to be 49.6%. In the highest dose groups 23.7% (0.9 mM), 33.3% (1.1 mM) and 35.0% (1.3 mM) of small colonies were found (Table 7). As none of the values exceeded 40%, all dose groups were considered as not clastogenic.

Without metabolic activation the percentage of small colonies in the negative controls was found to be 15.2% and 16.7% and in the solvent controls was found to be 30.3% and 16.7%, respectively. The percentage of small colonies of the positive control MMS was found to be 51.6%. In the highest dose groups 18.8% (0.2 mM), 17.5% (0.5 mM) and 20.5% (1 mM) of small colonies were found (Table 10). As none of the values exceeded 40%, all dose groups were considered as not clastogenic.

14.1. Conclusion

In conclusion, in the described mutagenicity test under the experimental conditions reported, the test item Triethoxy(3-thiocyanatopropyl)silane is considered to be mutagenic in the *in vitro* mammalian cell gene mutation assay (thymidine kinase locus) in mouse lymphoma L5178Y cells.

15. Distribution of the Report

1 original (paper):	Sponsor
1 copy (paper):	BSL BIOSERVICE

16. References

16.1. Guidelines

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Bedienung und Wartung der Zellzählgeräte ALM871 und AL 2000 (SOP 4-6-5)

Herstellung und Charakterisierung von Rattenleberhomogenat (S9) (SOP 15-1-1)

Verwendung von S9 Mix bei *in vitro* Systemen (SOP 15-1-2)

Maus-Lymphoma-Test (SOP 15-2-4)

17. Appendix – Certificate of Analysis

Inspection Certificate

according to EN 10204-3.1

EVONIK INDUSTRIES

Certificate-No. page 1 of 1

Product:	**VP Si 264**
Product No.:	**25.6025.0000.xx**
Specification No:	**79/1**
Valid from:	01.11.2005
Order No.:	
Delivery No.:	
Shipment Date:	
Packaging:	
Quantity:	
Control-/Lot No.:	**CH 809901**
Customer Order No.:	
Customer Mat. No.:	
Licence Plate:	
Shelf Life:	12 months
Retesting Date:	June 2011
Expiry Date:	June 2012

Test Methods / Properties	Units	Target Value (Limits)	Actual Values
Sulfur content following ASTM D 6741-01	%	12,5 (11,5-13,5)	12,3
Residue on ignition following ASTM D 6740-01	%	23 (21,5-24,5)	23,1

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eMail: elke.taesler@evonik.com

09.12.2011

This certificate has been established automatically and contains no signature.
The data given above reflect the results of our internal quality tests. We do not hereby make any express or implied warranty, whether of quality or of fitness for any specific purpose. All warranty claims are subject to the terms of contract and to our "General Conditions of Sale and Delivery". All values refer to the material when leaving the plant.

In vitro Mammalian Chromosome Aberration Test

in Chinese Hamster V79 cells

with

Triethoxy(3-thiocyanatopropyl)silane

Report

Version: 2nd Draft

Date: 30 January 2012

BSL BIOSERVICE Study No.: 114184

Sponsor:

ReachCentrum SPRL

For account of the members of the Reconsile Consortium

Avenue E. van Nieuwenhuyse 6

1160 Brussels

1. Copy of the GLP Certificate

A copy of the GLP certificate will be included in the final report.

2. Contents

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4. Preface

4.1. Abbreviations

ATCC	American Type Culture Collection
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
bw	Body weight
CA	Chromosome aberration
conc.	concentration
CPA	Cyclophosphamide
DNA	Desoxyribonucleic acid
e.g.	exempli gratia (for example)
EC	European Commission
EMS	Ethylmethanesulfonate
EPA	Environmental Protection Agency
FBS	Fetal Bovine Serum
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
i.e.	id est (that is)
KCl	Potassium Chloride
MEM	Minimum Essential Medium
NADP	Nicotinamide Adenine Di-Phosphate
Nr.	Nummer *(number)*
OECD	Organisation of Economic Cooperation and Development
PBS	Phosphate buffered saline
QA	Quality Assurance
QAU	Quality Assurance Unit
S9	Microsomal fraction of rat liver homogenate
SOP	Standard Operating Procedures

4.2. General

Sponsor:	ReachCentrum SPRL For account of the members of the Reconsile Consortium Avenue E. van Nieuwenhuyse 6 1160 Brussels Belgium
Study Monitor:	Dr. Nils Krueger Evonik Industries AG Department IM-PT-PS Rodenbacher Chaussee 4 63457 Hanau Germany
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	114184
Test Item:	Triethoxy(3-thiocyanatopropyl)silane
Title:	*In vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 cells with Triethoxy(3-thiocyanatopropyl)silane

4.3. Project Staff

Study Director:	Dipl.-Biol. Margit Oppong-Nketiah
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Head of Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann

4.4. Schedule

Arrival of the Test Item:	29 July 2011
Date of Draft Study Plan:	20 September 2011
Date of Final Study Plan:	14 October 2011
Start of Experiment:	19 October 2011
End of Experiment:	29 December 2011
Date of Draft Report:	02 January 2012
Date of 2nd Draft Report:	30 January 2012

5. Project Staff Signatures

Study Director: Dipl.-Biol. Margit Oppong-Nketiah

.....

Date: .....

Management

.....
Print Name:

Date: .....

6. Quality Assurance

6.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. 1 Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility were periodically inspected by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

6.2. Guidelines

This study followed the procedures indicated by internal BSL BIOSERVICE SOPs and the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473, „*In vitro* Mammalian Chromosome Aberration Test", adopted 21st July, 1997

Commission Regulation (EC) No. 440/2008 B.10: "Mutagenicity – *In vitro* Mammalian Chromosome Aberration Test", dated May 30, 2008.

EPA Health Effects Test Guidelines, OPPTS 870.5375, „*In vitro* Mammalian Chromosome Aberration Test" EPA 712-C-98-223, August 1998.

6.3. Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP regulations:

A copy of the final report, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study.

If test item is left, a sample will be stored according to the period fixed by the GLP regulations. Material and samples that are unstable may be disposed of before that time and without sponsor's prior consent. Raw data relating to the study will be discarded only with the prior consent of the sponsor. Unless otherwise agreed upon, the remaining test item will be discarded three months after the release of the report.

7. Statement of Compliance

BSL BIOSERVICE Study No.:	114184
Test Item:	Triethoxy(3-thiocyanatopropyl)silane
Study Director:	Dipl.-Biol. Margit Oppong-Nketiah
Title:	*In vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 cells with Triethoxy(3-thiocyanatopropyl)silane

This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998.

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dipl.-Biol. Margit Oppong-Nketiah

.....

Date: .....

8. Statement of the Quality Assurance Unit

BSL BIOSERVICE

Study No.:	114184
Test Item:	Triethoxy(3-thiocyanatopropyl)silane
Study Director:	Dipl.-Biol. Margit Oppong-Nketiah
Title:	*In vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 cells with Triethoxy(3-thiocyanatopropyl)silane

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phases of QAU Inspections	*Dates of QAU Inspections*	*Dates of Reports to the Study Director and Management*
Audit Final Study Plan:	14 October 2011	14 October 2011
Audit Experimental Phase (process-based):	03 March 2011	03 March 2011
Audit Final Report:		

This report reflects the raw data.

Member of the
Quality Assurance Unit:

.....
Print Name:

Date: .....

9. Summary

9.1. Summary Results

To investigate the potential of Triethoxy(3-thiocyanatopropyl)silane to induce structural chromosome aberrations in Chinese hamster V79 cells, an *in vitro* chromosome aberration assay was carried out.

The chromosomes were prepared 20 h after start of treatment with the test item. The treatment interval was 4 h **with** and **without** metabolic activation in the main experiment. Duplicate cultures were treated at each concentration. 100 metaphases per culture were scored for structural chromosomal aberrations.

The following concentrations were evaluated for the microscopic analysis of chromosomal aberrations:

without metabolic activation: 0.40, 0.60 and 0.80 mM

with metabolic activation: 0.15, 0.30 and 0.50 mM

In the main experiment precipitation of the test item was noted after incubation with the test item **without** metabolic activation at a concentration of 0.80 mM, **with** metabolic activation no precipitation was observed.

Toxic effects of the test item were observed in the main experiment **without** metabolic activation at concentrations of 0.60 mM and higher, **with** metabolic activation no toxic effects were noted at the concentrations evaluated.

A clear increase of aberrant cells was found in the main experiment **with** and **without** metabolic activation at all concentrations evaluated. In addition, a dose response relationship was observed.

In the main experiment **with** and **without** metabolic activation no biologically relevant increase in the frequencies of polyploid cells was observed after treatment with the test item as compared to the controls.

EMS (900 µg/mL) and CPA (0.83 µg/mL) were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations.

9.2. Conclusion

In conclusion, it can be stated that during the described *in vitro* chromosomal aberration test and under the experimental conditions reported, the test item Triethoxy(3-thiocyanatopropyl)silane induced structural chromosomal aberrations in the V79 Chinese hamster cell line.

Therefore, the test item Triethoxy(3-thiocyanatopropyl)silane is considered to be clastogenic in this chromosome aberration test.

10. Introduction

10.1. Aim of the Study

The *in vitro* chromosomal aberration (CA) test is a mutagenicity test system for the detection of chromosomal aberration in cultured mammalian cells (1)(2)(3).

Chromosome aberration assays aim to detect the induction of chromosome breakage (clastogenesis). Although mutagenic substances produce structural chromosome aberrations by a variety of mechanisms, the endpoint is a discontinuity in the chromosomal DNA which is left unrejoined, or rejoined inaccurately, thus producing a mutated chromosome. Many of these changes are lethal to the cells during the first few cell cycles after their induction but are used as indicators of the presence of non-lethal changes such as reciprocal translocations, inversions and small deletions. These more subtle changes may have important consequences in both germ and somatic cells. Chromosomal mutations and related events are the cause of many human genetic diseases and there is substantial evidence that these changes, including oncogens and tumor suppressor genes, are involved in carcinogenesis in humans and experimental systems. CA are generally evaluated in first post treatment mitosis. The majority of chemical mutagens induce aberrations of the chromatid type, but chromosome type aberrations also occur.

For treatment, an asynchronous population of V79 cells in exponential growth should be used. A fixation time of around 20 h after treatment is appropriate since the guidelines recommend fixation times of about 1.5-fold of the normal cell cycle and the normal cell cycle of the cells is 12 - 14 h. However, because there may be substances which induce very extensive mitotic delay at clastogenic concentrations or may be clastogenic only when cells have passed through more than one cell cycle since the beginning of treatment in the second experiment, an additional later sampling time (28 h) should be included.

At least three concentrations of the test item should be used at the 20 h fixation time. The highest concentration should be in the toxic range and should show a significant reduction in mitotic index or in the degree of cell confluency (50% or greater). The lowest dose should be in the range of the negative control.

If an additional sampling time is carried out due to the highly toxic effects of the test item, the same concentration which induced a suitable degree of mitotic inhibition at the earlier fixation time should be chosen.

Although the purpose of the assay is to detect structural chromosome aberrations, it is important to report polyploidy and/or endoreduplication when this is seen.

To validate the test, reference mutagens are tested in parallel to the test item.

10.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals Section 4, No 473 – *In Vitro* Mammalian Chromosome Aberration Test – recommends using a variety of cell lines or primary cell cultures (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes).

11. Materials and Methods

11.1. Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor. All data related to the test item are the responsibility of the sponsor and have not been verified by the test facility.

Name:	Triethoxy(3-thiocyanatopropyl)silane
Batch No.:	809901
CAS No.:	34708-08-2
Physical State at RT:	liquid
Colour:	light brown
Expiry Date:	June 2012
Molecular Weight:	263.43
Purity:	98.3%
Storage conditions:	at room temperature
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

11.2. Preparation of the Test Item

The test item was dissolved and diluted in DMSO prior to treatment. The solvent was compatible with the survival of the cells and the S9 activity.

11.3. Controls

Positive and negative controls were included in each experiment.

Negative Controls

A negative control, consisting of cell culture medium (solvent or vehicle) alone and treated in the same way as the treatment groups was included.

Positive Controls

Without metabolic activation

Name	EMS; Ethylmethanesulfonate
Supplier	Sigma
Catalogue No.	M 0880
Lot No.	BCBF0736V
Dissolved in	nutrient medium
Final concentration	900 µg/mL

The stability of the positive control substance in solution is proven by the mutagenic response in the expected range. The solution was prepared on the day of experiment.

With metabolic activation

Name	CPA; Cyclophosphamide
Supplier	Sigma
Catalogue No.	C0768
Lot No.	079K1569
Dissolved in	nutrient medium
Final concentration	0.83 µg/mL

The stability of CPA at room temperature is good. At 25 °C only 3.5% of its potency is lost after 24 h (9). The solution is aliquoted and stored at ≤ -15 °C.

11.4. Test System

11.4.1. The Cells

V79 cells *in vitro* were widely used to examine the ability of chemicals to induce cytogenetic changes and thus identify potential carcinogens or mutagens. These cells were chosen because of their relatively small number of chromosomes (diploid number, 2n = 22), their high proliferation rate (doubling time of the BSL BIOSERVICE V79 done in stock cultures: 12 – 14 h) and the high plating efficiency of untreated cells (normally more than 50%). These facts were necessary for the appropriate performance of the study.

The V79 (ATCC, CCL-93) cells are stored over liquid nitrogen (vapour phase) in the cell bank of BSL BIOSERVICE, as large stock cultures allowing the repeated use of the same cell culture batch in experiments. Routine checking for mycoplasma infections was carried out before freezing.

For the experiment, thawed cultures were set up in 75 cm^2 cell culture plastic flasks at 37 °C in a 5% carbon dioxide atmosphere (95% air). 5 x 10^5 cells per flask were seeded in 15 mL of MEM (minimum essential medium) supplemented with 10% FBS (foetal calf serum) and subcultures were made every 3 - 4 days.

11.4.2. Mammalian Microsomal Fraction S9 Homogenate

An advantage of using *in vitro* cell cultures is the accurate control of the concentration and exposure time of cells to the test item under study. However, due to the limited capacity of cells growing *in vitro* for metabolic activation of potential mutagens an exogenous metabolic activation system is necessary (2). Many substances only develop mutagenic potential when they are metabolized by the mammalian organism. Metabolic activation of substances can be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix).

The S9 liver microsomal fraction was prepared at BSL BIOSERVICE GmbH. Male Wistar rats were induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route.

The following quality control determinations were performed:

a) Biological activity in:
   - the *Salmonella typhimurium* assay using 2-aminoanthracene
   - the mouse lymphoma assay using benzo[*a*]pyrene
   - the chromosome aberration assay using cyclophosphamide.

b) Sterility Test.

A stock of the supernatant containing the microsomes is frozen in ampoules of 2 and 4.5 mL and stored at ≤-75° C.

The protein concentrations in the S9 preparation (Lot Numbers: 050511 and 210711) were 31 and 38.5 mg/mL, respectively.

11.4.3. S9 Mix

An appropriate quantity of the S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures.

Cofactors were added to the S9 mix to reach the concentrations below:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	Glucose-6-phosphate
5	mM	NADP

in 100 mM sodium-phosphate-buffer pH 7.4. During the experiment the S9 mix was stored on ice.

11.5. Experimental Design

11.5.1. Solubility of the Test Item

The test item Triethoxy(3-thiocyanatopropyl)silane could be dissolved and diluted in DMSO at a concentration of 1 M. To reach a final concentration of 1% solvent v/v in the test item stock solution the solution was diluted in cell culture medium (MEM). After transfer precipitation of the test item was indicated at a concentration of 10 mM. Based on the results of the solubility test, 10 mM was chosen as the highest dose group of the test item for the pre-experiment.

11.5.2. Pre-Experiment for Toxicity

A pre-experiment was conducted under identical conditions as described for the main experiment. The following concentrations were tested **with** and **without** S9 mix:

0.020, 0.039, 0.078, 0.16, 0.31, 0.63, 1.25, 2.50, 5.0 and 10.0 mM

11.5.3. Exposure Concentrations

Duplicate cultures were treated at each concentration. The selection of the concentrations used in the main experiment was based on data from the pre-experiment.

The following concentrations were used in the main experiment:

without metabolic activation:

0.015, 0.03, 0.06, 0.08, 0.10, 0.20, 0.40, 0.60 and 0.80 mM

with metabolic activation:

0.02, 0.05, 0.075, 0.15, 0.30, 0.50, 0.75, 1.0, 1.25 and 1.5 mM

In the experiment the cells were treated with the test item for 4 h **with** and **without** metabolic activation. The metaphases were prepared 20 h after the treatment. The dose group selection for microscopic analyses of chromosomal aberrations was based on the mitotic index in accordance with the guidelines.

The following concentrations were selected in the main experiment for the microscopic analyses:

without metabolic activation: 0.40, 0.60 and 0.80 mM

with metabolic activation: 0.15, 0.30 and 0.50 mM

11.6. Experimental Performance

11.6.1. Seeding of the Cultures

Three or four day-old stock cultures (in exponential growth), more than 50% confluent, were rinsed with Ca-Mg-free PBS solution prior to the trypsin treatment. Cells subsequently were trypsinised with a solution of 0.2% trypsin in Ca-Mg-free PBS at 37 °C for 5 minutes. By adding complete culture medium the detachment was stopped and a single cell suspension was prepared.

The cells were seeded into Quadriperm dishes which contain microscopic slides (at least 2 chambers per dish and test group). Into each chamber 1×10^4 - 5×10^4 cells were seeded with regard to preparation time. The medium was minimum essential medium supplemented with 10% FBS.

11.6.2. Treatment

<u>Exposure time 4 hours (with and without S9 mix; first experiment – with S9 mix; second experiment):</u>

Two days after seeding of the cells, the culture medium was replaced with serum-free medium containing the test item and 50 µL/mL S9 mix (with metabolic activation). Additional negative and positive controls were performed with and without exogenic metabolic activation.

4 h after treatment the cultures were washed twice with PBS and cultured in complete medium for the remaining culture time.

All cultures were incubated at 37 °C in a humidified atmosphere with 5.0% CO_2 (95.0% air).

11.6.3. Preparation of the Cultures

Colcemid® was added to the cultures (0.2 µg/mL culture medium) 17.5 hours after the start of each treatment (4 h and 20 h treatment). 2.5 h later, the cells were treated on the slides in the chambers with hypotonic solution (0.4% KCl) for 20 min at 37 °C. After incubation in the hypotonic solution the cells were fixed with 3 + 1

methanol + glacial acetic acid (v/v). All the steps were carried out on precision hot plates at 37 °C. After fixation the cells were stained with Giemsa.

11.6.4. Analysis of Metaphase Cells

All slides, including those of positive and negative controls, were independently coded before microscopic analysis. Evaluation of the cultures was performed according to the standard protocol of the "Arbeitsgruppe der Industrie, Cytogenetik" (9) using microscopes with 100x oil immersion objectives. If observed, structural chromosomal aberrations, including breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded. Gaps were recorded as well but not included in the calculation of the aberration rates. The definition of a gap is as follows: an achromatic region (occurring in one or both chromatids) independent of its width. The remaining visible chromosome regions should not be dislocated either longitudinally or laterally. At least 200 well spread metaphases per concentration and validity controls were scored for cytogenetic damage.

The cells scored contained 22 ± 1 centromeres. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined by counting the number of mitotic cells in 1000 cells. Additionally the number of polyploid cells was scored. Polyploid means a near tetraploid karyotype in the case of this aneuploid cell line.

11.6.5. Analysis of Relative Cell Density

As an additional parameter for cytotoxic effects of the test item, the relative cell density was calculated as the mean of twenty cell counts per test group (cells within the visual field at a 400-fold magnification, Table 2).

11.7. Data Recording

The data generated are recorded in the raw data file. The results are presented in tables, including experimental groups with the test item, negative and positive controls. The experimental unit is the cell and therefore, the percentage of cells with structural aberrations is evaluated. Different types of chromosome aberrations are listed with their numbers of frequencies for the experimental and control groups. Gaps are recorded separately and reported but generally not included in the aberration frequency. Concurrent measurements of cytotoxicity are also recorded

11.8. Acceptability of the Assay

The chromosomal aberration assay is considered acceptable if it meets the following criteria:

- the number of aberrations found in the negative and/or solvent controls falls within the range of historical laboratory control data: 0.0% - 4.0% (with and without metabolic activation),
- the positive control substance should produce biologically relevant increases in the number of cells with structural chromosome aberrations.

11.9. Evaluation of Results

There are several criteria for determining a positive result:

- a clear and dose-related increase in the number of cells with aberrations,
- a biologically relevant response for at least one of the dose groups, which is higher than the laboratory negative control range (0.0% - 4.0% aberrant cells (with and without metabolic activation)).

According to the OECD guidelines, the biological relevance of the results is the criterion for the interpretation of results and a statistical evaluation of the results is not regarded as necessary (3)(10). However, for the interpretation of the data, both biological and thoroughly evaluated statistical significance should be considered together.

A test item is considered to be negative if there is no biologically relevant increase in the percentages of aberrant cells above concurrent control levels, at any dose group. Although most experiments will give clearly positive or negative results, in some cases the data set will preclude making a definitive judgement about the activity of the test substance.

12. Deviations from the Study Plan

There were no deviations from the study plan.

13. Results

13.1. Abbreviations

The following abbreviations are used in the tables with structural chromosomal aberrations:

g = gap and **ig** = iso-gap; gaps are achromatic lesions of chromatid or chromosome type where no dislocation of chromosomal material is visible (independent of the size of the achromatic region).

b = break and **ib** = iso-break

f = fragment; **if** = iso-fragment

d = deletion; **id** = iso-deletion

ma = multiple aberration is defined as a metaphase containing more than 4 events [excluding gaps]; only exchanges are recorded additionally in these cells

ex = chromatid type exchange

cx = chromosome type exchange

cd = chromosomal disintegration (pulverisation)

13.2. Pre-Experiment for Toxicity

According to the guidelines used the highest recommended dose is 10 mM. The test item was dissolved in DMSO and further diluted in cell culture medium. Precipitation of the test item was observed at a concentration of 10 mM. The highest dose group evaluated in the pre-experiment was 10 mM. The relative mitotic index was used as parameter for toxicity. The cell density was determined in addition. The concentrations used in the main experiment were based on the results obtained in the pre-experiment (Table 1).

Table 1: Test for Cytotoxicity

Dose Group	[illegible]	Mitotic Index relative [%]	[illegible]	[illegible]	[illegible]
without metabolic activation					
C	0	84	111	88	102
S	0	76	100	86	100
1	0.020	70	92	89	103
2	0.039	62	82	86	100
3	0.078	78	103	90	104
4	0.16	56	74	57	66
5	0.31	76	100	77	89
6	0.63	60	79	57	65
7	1.25	40	53	43	50
8	2.50	36	47	39	45
9	5.0	0	0	2	3
10	10.0	0	0	2	2
with metabolic activation					
C	0	89	109	88	101
S	0	82	100	87	100
1	0.020	96	117	87	100
2	0.039	83	101	85	97
3	0.078	64	78	81	93
4	0.16	70	85	66	76
5	0.31	45	55	45	52
6	0.63	36	44	35	40
7	1.25	0	0	27	31
8	2.50	6	7	27	31
9	5.0	0	0	20	23
10	10.0	0	0	5	5

The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the solvent control.
C: Negative Control
S: Solvent Control (DMSO)

13.3. Main Experiment

Table 2: Main Experiment - Number of Polyploid Cells, Mitotic Index and Cell Density: 4 h treatment, 20 h fixation period.

Dose Group	[illegible]	Polyploid Cells			Mitotic Index				Cell Density			
		Culture 1	Culture 2	Mean	Culture 1	Culture 2	Mean	relative [%]	Culture 1	Culture 2	Mean	relative [%]
without metabolic activation												
C	0	1	0	0.5	68	70	69	87	86	88	87	100
S	0	0	1	0.5	82	76	79	100	86	87	87	100
1	0.02	n.d.	n.d.	n.d.	80	80	80	101	n.d.	n.d.	n.d.	n.d.
2	0.03	n.d.	n.d.	n.d.	78	72	75	95	n.d.	n.d.	n.d.	n.d.
3	0.06	n.d.	n.d.	n.d.	72	82	77	97	n.d.	n.d.	n.d.	n.d.
4	0.08	n.d.	n.d.	n.d.	70	64	67	85	n.d.	n.d.	n.d.	n.d.
5	0.10	n.d.	n.d.	n.d.	74	70	72	91	n.d.	n.d.	n.d.	n.d.
6	0.20	n.d.	n.d.	n.d.	70	56	63	80	n.d.	n.d.	n.d.	n.d.
7	0.40	1	1	1	62	54	58	73	74	80	77	89
8	0.60	0	1	0.5	34	42	38	48	62	71	66	77
9	0.80	0	0	0	24	26	25	32	46	38	42	48
EMS	900 µg/mL	0	1	0.5	92	74	83	105	72	74	73	84
with metabolic activation												
C	0	1	0	0.5	84	84	84	101	88	86	87	101
S	0	0	1	0.5	84	82	83	100	86	87	87	100
1	0.02	n.d.	n.d.	n.d.	76	86	81	98	n.d.	n.d.	n.d.	n.d.
2	0.05	n.d.	n.d.	n.d.	88	72	80	96	n.d.	n.d.	n.d.	n.d.
3	0.08	n.d.	n.d.	n.d.	74	74	74	89	n.d.	n.d.	n.d.	n.d.
4	0.15	0	0	0	64	78	71	86	81	85	83	95
5	0.30	2	0	1	72	72	72	87	81	82	81	94
6	0.50	0	0	0	68	70	69	83	70	72	71	82
7	0.75	n.d.	n.d.	n.d.	52	52	52	63	n.d.	n.d.	n.d.	n.d.
8	1.00	n.d.	n.d.	n.d.	36	38	37	45	n.d.	n.d.	n.d.	n.d.
9	1.25	n.d.	n.d.	n.d.	6	14	10	12	n.d.	n.d.	n.d.	n.d.
10	1.50	n.d.	n.d.	n.d.	4	42	23	28	n.d.	n.d.	n.d.	n.d.
CPA	0.83 µg/mL	1	1	1	92	64	78	94	85	87	86	99

The number of polyploid cells was determined in 100 cells per culture of each test group.
The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the solvent controls.

C: Negative Control (Culture Medium)
S: Solvent Control (DMSO)
EMS: Positive Control (*without* metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (*with* metabolic activation: Cyclophosphamide

Table 3: Main Experiment - Structural Chromosomal Aberrations, *without* metabolic activation: 4 h treatment, 20 h fixation period.

Dose Group	[illegible]	Culture	[illegible]	Polyploid Cells	[illegible]	[illegible]	Gaps		Types of Aberrations found									
									Chromatid types				Chromosome types				Other	
							g	ig	b	f	d	ex	ib	if	id	cx	ma	cd
C	0	1	100	1	4	2	2	0	2	0	0	0	0	0	0	0	0	0
		2	100	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0
		total	200	1	5	3	2	0	3	0	0	0	0	0	0	0	0	0
S	0	1	100	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
		2	100	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
		total	200	1	2	0	2	0	0	0	0	0	0	0	0	0	0	0
7	0.40	1	100	1	8	7	1	0	6	0	0	3	0	0	0	0	0	0
		2	100	1	12	8	4	1	6	1	1	1	0	0	0	0	0	0
		total	200	2	20	15	5	1	12	1	1	4	0	0	0	0	0	0
8	0.60	1	100	0	13	8	6	1	14	0	0	1	0	0	0	0	0	0
		2	100	1	10	9	1	0	8	1	0	0	0	1	0	0	0	0
		total	200	1	23	17	7	1	22	1	0	1	0	1	0	0	0	0
9	0.80	1	100	0	15	14	4	0	21	1	0	1	1	0	0	0	0	0
		2	100	0	12	10	2	0	7	0	0	3	0	0	0	0	0	0
		total	200	0	27	24	6	0	28	1	0	4	1	0	0	0	0	0
EMS	900 µg/mL	1	100	0	14	8	7	0	4	0	1	3	0	0	0	2	0	0
		2	100	1	11	11	0	1	2	0	2	5	0	0	0	4	0	0
		total	200	1	25	19	7	1	6	0	3	8	0	0	0	6	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (DMSO)
EMS: Ethylmethanesulfonate

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

Table 4: Main Experiment - Structural Chromosomal Aberrations, *with* metabolic activation: 4 h treatment, 20 h fixation period.

Dose Group	Concentration [mM]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells without Gaps	Aberrant Cells with Gaps	Gaps g	Gaps ig	Types of Aberrations found: Chromatid types b	f	d	ex	Chromosome types ib	if	id	cx	Other ma	cd
C	0	1	100	1	7	4	3	0	1	0	0	2	0	0	0	1	0	0
		2	100	0	2	2	0	0	2	0	0	0	0	0	0	0	0	0
		total	200	1	9	6	3	0	3	0	0	2	0	0	0	1	0	0
S	0	1	100	0	7	2	5	0	2	0	0	0	0	0	0	0	0	0
		2	100	1	3	2	1	1	2	0	0	0	0	0	0	0	0	0
		total	200	1	10	4	6	1	4	0	0	0	0	0	0	0	0	0
4	0.15	1	100	0	9	8	1	0	6	0	0	0	0	2	0	0	0	0
		2	100	0	8	4	7	0	4	0	0	0	0	0	0	0	0	0
		total	200	0	17	12	8	0	10	0	0	0	0	2	0	0	0	0
5	0.30	1	100	2	8	8	0	0	7	0	0	1	0	0	0	0	0	0
		2	100	0	12	10	3	0	8	1	0	2	1	2	0	0	0	0
		total	200	2	20	18	3	0	15	1	0	3	1	2	0	0	0	0
6	0.50	1	100	0	16	15	1	0	16	0	0	3	0	1	0	0	0	0
		2	100	0	14	14	3	0	15	0	1	3	0	0	0	0	0	0
		total	200	0	30	29	4	0	31	0	1	6	0	1	0	0	0	0
CPA	0.83 µg/mL	1	100	1	12	11	1	0	7	0	0	4	0	0	0	0	0	0
		2	100	1	10	8	6	0	6	0	0	2	1	1	0	0	0	0
		total	200	2	22	19	7	0	13	0	0	6	1	1	0	0	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (DMSO)
CPA: Positive Control (Cyclophosphamide)

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

13.4. Summary

Table 5: Main Experiment – Summary of Aberration Rates

Dose Group	[illegible]	Treatment Time	[illegible]	mean % aberrant cells	
				incl. Gaps	excl. Gaps
without metabolic activation					
C	0	4 h	20 h	**2.5**	**1.5**
S	0	4 h	20 h	**1.0**	**0.0**
7	0.40	4 h	20 h	**10.0**	**7.5**
8	0.60	4 h	20 h	**11.5**	**8.5**
9	0.80	4 h	20 h	**13.5**	**12.0**
EMS	900 µg/mL	4 h	20 h	**12.5**	**9.5**
with metabolic activation					
C	0	4 h	20 h	**4.5**	**3.0**
S	0	4 h	20 h	**5.0**	**2.0**
4	0.15	4 h	20 h	**8.5**	**6.0**
5	0.30	4 h	20 h	**10.0**	**9.0**
6	0.50	4 h	20 h	**15.0**	**14.5**
CPA	0.83 µg/mL	4 h	20 h	**11.0**	**9.5**

200 cells evaluated for each concentration

C: Negative Control (Culture Medium)
S: Solvent Control (DMSO)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

14. Discussion

In an *in vitro* chromosome aberration assay, the test item Triethoxy(3-thiocyanatopropyl)silane was investigated for the potential to induce structural chromosomal aberrations in Chinese hamster V79 cells in the absence and presence of metabolic activation with S9 homogenate.

The selection of the concentrations used in the main experiment was based on data from the solubility test and the pre-experiment which were performed according to the guidelines.

In the main experiment **without** metabolic activation 0.80 mM and **with** metabolic activation 0.50 mM were selected as highest dose groups for the microscopic analysis of chromosomal aberrations.

The chromosomes were prepared 20 h after start of treatment with the test item. The treatment intervals were 4 h **with** and **without** metabolic activation. Two parallel cultures were set up. 100 metaphases per culture were scored for structural chromosomal aberrations.

The following concentrations were evaluated for microscopic analysis:

without metabolic activation: 0.40, 0.60 and 0.80 mM

with metabolic activation: 0.15, 0.30 and 0.50 mM

Precipitation:

In the main experiment precipitation of the test item was noted after incubation with the test item **without** metabolic activation at a concentration of 0.80 mM, **with** metabolic activation no precipitation was observed.

Toxicity:

In the main experiment **without** metabolic activation a biologically relevant decrease of the relative mitotic index (decrease below 70% rel. mitotic index) was noted at concentrations of 0.60 mM and higher (48% at 0.60 mM, 32% at 80 mM, Table 2). The cell density was decreased at a concentration of 0.80 mM (48%). **With** metabolic activation no biologically relevant decrease of the relative mitotic index and the cell density was observed at the concentrations evaluated. At a concentration of 0.75 mM the mitotic index was decreased down to 63%. However, it was not possible to evaluate the metaphases, as most of the chromosomes seemed to be in disintegration.

Clastogenicity:

In the main experiment **without** metabolic activation the aberration rates of the negative control (1.5%) and the solvent control (0.0%) were within the historical control data of the testing facility (0.0% – 4.0%, Table 6). A clear increase of aberrant cells was noted at all concentrations evaluated (7.5% (0.40 mM), 8.5% (0.60 mM) and 12.0% (0.80 mM), Table 5). In addition, a dose response relationship was indicated. **With** metabolic activation the aberration rates of the negative control (3.0%) and the solvent control (2.0%) were within the historical control data of the testing facility (0.0% – 4.0%, Table 6). A distinct increase of aberrant cells was noted at all concentrations evaluated (6.0% (0.15 mM), 9.0% (0.30 mM) and 14.5% (0.50 mM). Furthermore, a dose-response relationship was observed.

Polyploid cells:

Tables 2 shows the occurrence of polyploid metaphases. No biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item.

EMS (900 µg/mL) and CPA (0.83 µg/mL) were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations.

14.1. Conclusion

In conclusion, it can be stated that during the described *in vitro* chromosomal aberration test and under the experimental conditions reported, the test item Triethoxy(3-thiocyanatopropyl)silane induced structural chromosomal aberrations in the V79 Chinese hamster cell line.

Therefore, the test item Triethoxy(3-thiocyanatopropyl)silane is considered to be clastogenic in this chromosome aberration test.

15. Distribution of the Report

1 original (paper):	Study Monitor
1 copy (paper):	BSL BIOSERVICE
1 copy (electronic):	Study Monitor

16. References

16.1. Guidelines

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16.3. Internal BSL BIOSERVICE SOPs

Gentoxikologische Prüfungen: Verwendung von S9 Mix bei in-vitro-Systemen (SOP 15-1-2)

Gentoxikologische Prüfungen: Chromosomenaberrationen *in vitro* (SOP 15-2-6)

Gentoxikologische Prüfungen: Auswertung Chromosomenaberrationen (SOP 15-2-8)

17. Appendix

17.1. Historical Laboratory Control Data

Table 6: Historical Laboratory Control Data of the negative control (2006 - 2010)

	NC Number of aberrant cells *metabolic activation*			
	with		without	
	+gaps	-gaps	+gaps	-gaps
mean [%]	4.3	2.0	3.6	1.5
SD	1.43	0.81	1.55	0.82
RSD [%]	33.5	41.4	42.4	56.6
min [%]	1.0	0.0	0.5	0.0
max [%]	8.5	4.0	9.0	4.0
n	181	181	181	181

NC: Negative Control
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.: minimum number of aberrant cells
max.: maximum number of aberrant cells
n: Number of assays

Table 7: Historical Laboratory Control Data of the positive control (2006 - 2010)

	PC Number of aberrant cells *metabolic activation*			
	with		without	
	+gaps	-gaps	+gaps	-gaps
mean [%]	13.1	9.7	13.1	9.7
SD	2.29	1.78	2.70	1.96
RSD [%]	17.4	18.3	20.7	20.3
min [%]	8.5	1.5	8.5	8.0
max [%]	23.0	16.0	26.5	20.5
n	172	172	172	172

PC: Positive Control
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.: minimum number of aberrant cells
max.: maximum number of aberrant cells
n: Number of assays

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