TNO Quality of Life

Nederlandse Organisatie voor
toegepast-natuurwetenschappelijk
onderzoek / Netherlands Organisation
for Applied Scientific Research

Business Unit
Physiological Sciences
Location Zeist
Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

www.tno.nl

T +31 30 694 41 44
F +31 30 695 72 24

TNO report

V6202/18 | Final |

Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in cultured Chinese Hamster Ovary (CHO) cells

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Author	N. de Vogel
At request of	Asahi Glass Co., Ltd. 10 Goikaigan Ichiharashi Chiba 290-8566 Japan
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Summary

1. The test substance **3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol** was examined for its potential to induce structural chromosomal aberrations in Chinese Hamster Ovary (CHO) cells, in both the absence and presence of a metabolic activation system (S9-mix). Two chromosomal aberration tests were conducted. In the first chromosomal aberration test, in both the absence and presence of a metabolic activation system (S9-mix), the treatment/harvesting time was 4/18 hours (pulse treatment). In the second chromosomal aberration test, in the presence of S9-mix, the treatment/harvesting time was 4/18 hours (pulse treatment). In the absence of S9-mix, the treatment/harvesting time was 18/18 hours (continuous treatment).

   The dose levels for the first chromosomal aberration test were selected based on results toxicity obtained in a mammalian gene mutation test (TNO report V6203/14). Based on the results of the first chromosomal aberration test, the dose levels for the second chromosomal aberration test were selected.

2. In the first chromosomal aberration test, in the presence of S9-mix, three concentrations of the test substance (3.9, 15.7 and 31.3 μg/ml) were analysed for chromosomal aberrations. The test substance was too toxic for the cells at the concentrations of 62.5 μg/ml and above. In the absence of S9-mix, three concentrations of the test substance (15.7, 31.3 and 62.5 μg/ml) were analysed for chromosomal aberrations. The test substance was too toxic for the cells at the concentration of 125 μg/ml and above. Dose levels, selected for chromosomal aberration analysis, were based on the results of the mitotic index analysis.

3. In the second chromosomal aberration test, in the presence of S9-mix, three concentrations of the test substance (10, 30 and 60 μg/ml) were analysed for chromosomal aberrations. The test substance was too toxic at the concentration of 80 μg/ml. In the absence of S9-mix, three concentrations of the test substance (30, 60 and 80 μg/ml) were analysed for chromosomal aberrations. Dose levels, selected for chromosomal aberration analysis, were based on the results of the mitotic index analysis.

4. In the first chromosomal aberration test, the test substance did not induce a statistically significant increase in the number of aberrant cells, at any of the concentrations and treatment periods analysed, when compared to the number of aberrant cells found in the negative control (DMSO) cultures.

5. In the second chromosomal aberration test, in the pulse treatment group with metabolic activation, the test substance induced a slight statistically significant ($p<0.05$) increase in the percentage of aberrant cells, at the concentration of 30 μg/ml, when compared to the number of aberrant cells found in the concurrent negative (DMSO) control. This slight statistically significant increase in the percentage of aberrant cells was within the historical range and only observed at the moderate concentration of 30 μg/ml. This increase was not dose related and not observed at the same dose level in the first chromosomal aberration test. Therefore, this observation was judged as not biologically relevant.

6. In both chromosomal aberration tests, the numbers of aberrant cells, observed in the negative control (DMSO) cultures, were within the historical range and the positive control substances mitomycin C (in the absence of the S9-mix) and cyclophosphamide (in the presence of the S9-mix) induced the expected statistically significant increases in the incidence of structural chromosomal aberrations. This demonstrates the validity of the chromosomal aberration test.

7. These data support the conclusion that, under the conditions used in this study, the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol was cytotoxic, but **not clastogenic** for Chinese Hamster Ovary (CHO) cells.

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Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results. All study activities performed by TNO Quality of Life were carried out in compliance with the current OECD Principles of Good Laboratory Practice.

The OECD Principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan.

TNO makes no GLP compliance claim for characterisation and verification of the test substance identity and properties; this is the responsibility of the sponsor.

N. de Vogel
Study Director

Date: 9 December 2005

Approved by:

Dr. Ir. A.F.M. Kardinaal
Management, Business Unit Physiological Sciences

Date: 9 December, 200

Quality Assurance Statement

On:	Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in cultured Chinese Hamster Ovary (CHO) cells
TNO project number:	010.31438/01.42.05
TNO study code:	6202/18
TNO report number:	V6202/18
Status and date:	Final report; 9 December 2005

The study plan was audited as follows:

Date of audit:	Date of report:
31 August 2005	31 August 2005

The experimental phase of this study was audited as follows:

Date of audit:	Date of report:
14 September 2005 (cell harvesting)	14 September 2005

This report was audited as follows:

Date of audit:	Date of report:
6 December 2005 (draft report)	6 December 2005
12 December 2005 (final report)	12 December 2005

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all audits were reported to the study director and the management on the dates indicated.

M. Th. A. Wolters, BSc.
(Quality Assurance Auditor)

Date: 12 December 2005

GLP compliance monitoring unit statement

voedsel en waren autoriteit

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 7-11 June 2004 at

TNO Nutrition and Food Research
Utrechtseweg 48, P.O. Box 360
3700 AJ ZEIST

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, biodegradation, residues, analytical and clinical chemistry, kinetics and metabolism, and occupational toxicity.

The Hague, 19 August 2004

Dr Th. Helder
GLP Compliance Monitoring Department

Inspectorate for Health Protection and Veterinary Public Health
Food and Consumer Product Safety Authority

Testing facility

TNO Quality of Life
Business Unit Physiological Sciences
P.O. Box 360, 3700 AJ ZEIST, the Netherlands
Telephone +31 30 69 44 144
Telefax +31 30 69 44 986
Visitors address: Utrechtseweg 48, Zeist, the Netherlands

Contributors

Study director	: N. de Vogel
Deputy study director	: Dr. C.A.M. Krul
Technician	: Ing. B. Usta
Management	: Dr. Ir. A.F.M. Kardinaal

Sponsor

Sponsor: Asahi Glass Co., Ltd.
10 Goikaigan Ichiharashi
Chiba 290-8566
Japan

Study monitor: Mr. Katsuji Ito

1 Objective

The objective of this study was to provide data on the ability of the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol to induce structural chromosomal aberrations in cultured Chinese Hamster Ovary (CHO) cells, in both the absence and presence of a metabolic activation system (S9-mix).

The study was conducted according to a study plan, entitled: "Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in cultured Chinese Hamster Ovary (CHO) cells", which was approved by the study director on 29 August 2005.

The study plan had been drafted in accordance with:
- the OECD guideline 473 Genetic Toxicology: *In vitro* mammalian chromosome aberration test; adopted 21 July 1997.

2 Experimental procedures

2.1 Test substance

Test substance	: 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol
Other name	: C6AL
Appearance	: transparent liquid
Molecular formula	: $C_8H_5OF_{13}$
Molecular weight	: 364
CAS Reg. Nr	: 647-42-7
Batch number	: re-AL-27, 28
User sample ID	: 0939-02-0279
Purity	: 98.5 %
Total quantity	: 200 g
Storage conditions	: ambient temperature
Date received	: 18 July 2005
Expiry date	: 26 April 2006
Supplier	: Asahi Glass Co. Ltd., Japan.
TNO Dispense no.	: 050104

A Test Material Information Sheet (TMIS) and a Certificate of Analysis (CoA) with respect to the physico-chemical properties of the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol were provided by the sponsor. The Certificate of Analysis (CoA) is included as Annex 5 of this report.

2.2 Tissue culture media and other chemicals

Ham's F-12 (with Glutamax-I), penicillin-streptomycin and foetal calf serum were purchased from Life Technologies (Gibco) B.V., Breda, The Netherlands; nicotinamide-adenine dinucleotide phosphate disodium salt (NADP) and D-glucose-6-phosphate disodium salt (G-6-P) from Roche Diagnostics, Almere, The Netherlands; Aroclor 1254 from Monsanto Chemical Company, St. Louis, USA; glacial acetic acid, methyl alcohol and Giemsa stain from Merck-Darmstadt, Darmstadt, Germany; mitomycin C, dimethylsulfoxide (DMSO) and Demecolcine (Colcemid) from Sigma-Aldrich Chemie GmbH, Germany; cyclophosphamide from Asta Werke, Bielefeld, Germany.

2.3 Chinese hamster ovary (CHO) cells

The CHO cells (CHO K-1 line) were obtained from Prof. Dr. A.T. Natarajan, University of Leiden, The Netherlands. This cell line derives from the CHO cells originally isolated from an explant of the ovary of the Chinese hamster (*Cricetulus griseus*, 2n = 22) by Kao and Puck (1968). The modal chromosome number of these cells is 20-22 (stable aneuploid karyotype). The cell-cycle time is 12-14 h. The cells are stored as frozen stock cultures in liquid nitrogen. Subcultures were prepared from these stocks (passage 16) for experimental use. Each passage CHO cells in the liquid nitrogen is checked for mycoplasma contamination and karyotype stability, which were absent and stable, respectively (see Annex 1).

2.4 Culture medium for CHO cells

The medium for culturing the CHO cells consisted of: Ham's F-12 medium (with Glutamax-I), supplemented with heat-inactivated (45 min, 56 °C) foetal calf serum (10 %), penicillin (100 IU/ml medium) and streptomycin (100 µg/ml medium).

2.5 Metabolic activation system

The S9-mix consisted of a liver homogenate fraction (S9) and cofactors as described by Ames et al. (1975) and Maron and Ames (1983). The S9, used in this study, was part of a batch prepared on 29 September 2004. The preparation and characterization of the batch is described in Annex 2. Immediately before use, a S9-mix was prepared by mixing the thawed S9 with a NADPH-generating system. The final concentrations of the various ingredients in the S9-mix were: $MgCl_2$ 8 mM; KCl 33 mM; G-6-P 5 mM; NADP 4 mM; sodium phosphate 100 mM (pH 7.4) and S9 40 % (v/v). For both the first and second chromosomal aberration test, 40 % (v/v) S9 was used. The final concentration of S9 in the culture medium was 4 %.

2.6 Solubility and preparation of the test substance dose levels

DMSO (99.9 %) was used as vehicle for the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol. The selection of dose levels for the first chromosomal aberration test was based on results, obtained in a mammalian gene mutation test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol (TNO report V6203/14).

For the first chromosomal aberration test, 1000 µg/ml (final concentration in he culture medium) was used as highest concentration of the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol.

For the first chromosomal aberration test, serial stock dilutions of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2 and 0.1 mg/ml were freshly prepared in DMSO. Fifty µl of each stock dilutions was added to the cells. This resulted in final concentrations in the culture medium of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0 and 1.0 µg/ml. The dose levels for the second chromosomal aberration test were based on the results of the first chromosomal aberration test.

For the second chromosomal aberration test, serial stock dilutions of 12.5, 10, 8, 6, 5, 3, 2, 1, 0.5, 0.25, 0.125, 0.062 and 0.031 mg/ml were freshly prepared in DMSO. Fifty µl of each stock dilution was added to the cells. This resulted in final concentrations in the culture medium of 125, 100, 80, 60, 50, 30, 20, 10, 5, 2.5, 1.25, 0.62 and 0.31 µg/ml.

For both chromosomal aberration tests, DMSO (1 % in the final culture medium) was used as vehicle control.

2.7 The first chromosomal aberration test

Exponentially growing cells were seeded in sterile, screw-capped tissue culture flasks (surface area 25 cm^2; 120,000 cells per flask) containing 5 ml culture medium (see paragraph 2.4) and then incubated at ca. 37 °C in humidified air containing 5 % CO_2. On the next day (ca. 24 hours after seeding), the cells were exposed to the test substance, in both the absence and presence of the S9-mix. In all instances duplicate cultures were used.

In the absence of the S9-mix, 50 µl of the vehicle control (DMSO), 50 µl of each of the dilutions of the test substance and 50 µl of the positive control substance mitomycin C was added to the tissue culture medium in the flasks and the culture medium was checked visually. The total volume in the flasks was 5 ml. The cultures were incubated at ca. 37 °C in humidified air containing ca. 5 % CO_2 and treated for 4 hours (pulse treatment). After 4 hours, the cells and culture medium were checked again. The medium with the test substance was removed, the cells were washed twice with phosphate-buffered saline (pH 7.4) and supplied with 5 ml freshly prepared culture medium. Thereafter, the cells were incubated for an additional 14 hours at ca. 37 °C in humidified air containing ca. 5 % CO_2. Two hours before the end of the culture period (18 hours), the cells and culture medium were checked visually.

In the presence of the S9-mix, the culture medium with foetal calf serum was replaced by culture medium with penicilline and streptomycine but without foetal calf serum. Thereafter, 50 µl of the vehicle control (DMSO), 50 µl of each of the dilutions of the test substance and 50 µl of the positive control substance cyclophosphamide was added to the tissue culture medium in the flasks and the culture medium was checked visually. Thereafter, 0.5 ml of S9-mix (see paragraph 2.5) was added to all cultures. The total volume in the flasks was 5 ml. After

4 hours, the culture medium and the cells were checked visually. The medium with the test substance was removed, the cells were washed twice with phosphate-buffered saline (pH 7.4) and supplied with 5 ml freshly prepared culture medium with foetal calf serum. The cells were incubated for an additional 14 hours at ca. 37 °C in humidified air containing ca. 5 % CO_2. Two hours before the end of the culture period (18 hours), the cells and culture medium were checked visually. The dose levels of the test substance and the positive controls as well as the observations made during the performance of the first chromosomal aberration test are given in the following schemes:

Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the presence of S9-mix: (Test 1)				
treatment time: harvesting time:		4 h 18 h		
dose (µg/ml)	observation[1)] directly after start of treatment	observation[1)] at the end of treatment	observation[1)] 2 h prior to fixation	selection for mitotic index scoring
0 (DMSO)				+
1000	test substance flocculated as small oily droplets	all cells dead; cultures discarded	-	-
500		± 70 % of the cells dead and rounded	all cells dead	-
250		± 60 % of the cells rounded and shrivelled	all cells dead	-
125		± 60 % of the cells rounded and shrivelled	all cells dead	-
62.5		cells affected; less cell growth	cells affected	+
31.3				+
15.7				+
7.8				+
3.9				+
2				+
1				+
cyclophos-phamide 5.0				+

1) no entry: no aberrant findings
\+ selected cultures
\- discarded cultures

Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the absence of S9-mix: (Test 1)				
treatment time: harvesting time:		4 h 18 h		
dose (µg/ml)	observation[1] directly after start of treatment	observation[1] at the end of treatment	observation[1] 2 h prior to fixation	selection for mitotic index scoring
0 (DMSO)				+
1000	test substance flocculated as small oily droplets	± 50 % rounded cells	all cells dead	-
500		cells affected; less cell growth	all cells dead	-
250		cells affected; less cell growth	all cells dead	-
125		cells slightly affected; less cell growth	all cells dead	-
62.5			cells affected	+
31.3				+
15.7				+
7.8				+
3.9				+
2				+
1				+
mitomycin C 0.1				+

1) no entry: no aberrant findings
\+ selected cultures
\- discarded cultures

2.8 The second (independent) chromosomal aberration test

The dose levels, used in the second chromosomal aberration test, were based on the results obtained in the first chromosomal aberration test. The second (independent) chromosomal aberration test was carried out essentially as described in paragraph 2.7. In the presence of S9-mix, the cells were pulse-treated for 4 hours. In the absence of S9-mix, the cells were treated continuously for 18 hours. The cells of both treatment groups were harvested 18 hours after onset of the treatment. The dose levels of the test substance and the positive controls used, as well as the observations made during the performance of the second chromosomal aberration test are given in the following schemes.

Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the presence of S9-mix: (Test 2)				
treatment time: harvesting time:		4 h 18 h		
dose (µg/ml)	observation[1] directly after start of treatment	observation[1] at the end of treatment	observation[1] 2 h prior to fixation	selection for mitotic index scoring
0 (DMSO)				+
80			± 20 % rounded cells; cells affected	+
60			± 20 % rounded cells; cells affected; less cell growth	+
50				+
30				+
20				+
10				+
5				+
2.5				-
1.25				-
cyclophospha-mide 5.0				+

1) no entry: no aberrant findings

+ selected cultures

- discarded cultures

Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the absence of S9-mix: (Test 2)			
treatment time: harvesting time:		18 h 18 h	
Dose (µg/ml)	observation[1)] directly after start of treatment	observation[1)] 2 h prior to fixation	selection for mitotic index scoring
0 (DMSO)			+
125		all cells dead	-
100		all cells dead	-
80		± 60 % of the cells rounded; less cell growth	+
60			+
50			+
30			+
20			+
10			+
5			+
2.5			-
1.25			-
0.62			-
0.31			-
mitomycin C 0.05			+

1) no entry: no aberrant findings
\+ selected cultures
\- discarded cultures

2.9 Cell fixation, preparation of slides and scoring

Two hours before the end of the total incubation period, the cells of the remaining cultures were arrested in the metaphase stage of mitosis by the addition of colcemid (final concentration: 0.1 μg/ml). At the end of the total incubation period the cells were harvested by trypsinization, treated for 15 min at ca. 37 °C with a hypotonic solution (1 % sodium citrate), fixed with a 3:1 mixture of methanol: glacial acetic acid (two refreshments of the fixative), and transferred to clean microscope slides. Two slides were prepared from each culture. The slides were stained in a 2 % solution of Giemsa, rinsed in water, air-dried and embedded. The slides were coded by a qualified person not involved in scoring the slides, to enable "blind" scoring. At least 1000 nuclei in each culture were examined (500 on each slide) to determine the mitotic index (percentage of cells in mitosis). After the results of the mitotic index scoring (see Tables 1-4) and the observations with respect to the quality of the metaphases had been obtained, a selection of the concentrations of the test substance, to be analysed for chromosomal aberrations, was carried out.

In all treatment groups, in both the first and second (independent) chromosomal aberration test, three concentrations of the test substance together with the negative and positive controls were selected for the analysis of chromosomal aberrations. If possible, the highest concentration should reduce the mitotic index with at least 50 % (but not more than 70 %), when compared to the negative control value or exhibit some other clear indication of cytotoxicity. Subsequently, the cultures of the selected concentrations of the test substance, together with the negative and positive control cultures, were analysed for the induction of structural chromosomal aberrations (see Tables 5-8). For each treatment group, 200 well-spread metaphases per concentration (100 metaphases per culture), each containing 20-22 centromeres, were analysed by microscopic examination for chromatid-type aberrations (gaps, breaks, fragments, interchanges), chromosome-type aberrations (gaps, breaks, minutes, rings, dicentrics) and other anomalies, such as interstitial deletions, endoreduplication, polyploidy and multiple aberrations (>10 aberrations per cell, excluding gaps), according to the criteria recommended by Savage (1975). If heavily damaged or endoreduplicated cells were observed, these cells were recorded but the cells were not counted and included in the 200 analysed cells. The Vernier readings of all aberrant metaphases were recorded

2.10 Statistical analysis

Data were analysed statistically by Fisher's exact probability test (two-sided) to determine significant differences between treated and control cultures.

2.11 Evaluation of test results

The study was considered valid because the positive controls gave the statistically significant increases in the number of aberrant cells and the negative controls were within the historical range.

A response is considered to be positive if a concentration-related increase or a reproducible increase in the number of cells with structural chromosomal aberrations is observed.

A response is considered to be equivocal if the percentage of cells with structural chromosomal aberrations is statistically marginal higher than that of the negative control ($0.05<p<0.1$).

A test substance is considered to be clastogenic if a concentration-related increase in the percentage of cells with structural chromosomal aberrations over the concurrent control frequencies is observed, or if a single positive test point is observed in both tests.

A test substance is considered to be negative in the chromosomal aberration test if it produces neither a dose-related increase in the number of structural chromosomal aberrations nor a reproducible positive response at any of the test points.

Cells with only gaps (achromatic lesions), heavily damaged cells (cells with multiple aberrations) and cells with polyploidy and endoreduplication were recorded separately and not included in the final assessment of clastogenic activity.

Both statistical significance and biological relevance are considered together in the evaluation of the results.

2.12 Time schedule

The study was conducted between 13 September 2005 (start date of the first chromosomal aberration test) and 21 October 2005 (last day of slide analysis of the second chromosomal aberration test).

2.13 Retention of records, samples and specimens

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study, including microscopic slides, will be retained in the archives of the TNO Quality of Life, Zeist, the Netherlands, for a period of at least 15 years after reporting of the study. At the end of the fifteen year storage period these will be discarded, unless the sponsor had indicated otherwise. Remaining test substance will be retained for at least six months after submission of the final report.

2.14 Deviations from the study plan

No deviation from the study plan occurred during the performance of both chromosomal aberration tests.

3 Results and discussion

The results of the first chromosomal aberration test are summarized in the Tables 1 and 2 (mitotic index scoring) and in the Tables 5 and 6 (chromosomal analysis of the selected cultures). The results of the second chromosomal aberration test are summarized in the Tables 3 and 4 (mitotic index scoring) and in the Tables 7 and 8 (chromosomal analysis of the selected cultures).

In the first chromosomal aberration test, in the pulse treatment group with metabolic activation (S9-mix), the mitotic indices of the three concentrations analysed (3.9, 15.7 and 31.3 μg/ml) were reduced to 61 %, 35 % and 41 %, respectively of that of the concurrent (DMSO) control. At higher concentrations (62.5 to 1000 μg/ml), the test substance was too toxic for the cells. It was concluded that the test substance was cytotoxic for the cells, but did not induce a statistically significant increase in the number of aberrant cells, when compared to the number of aberrant cells found in the negative (DMSO) control cultures. In this treatment group, a dose-related increase in the number of gaps was observed. This observation was not included in the final assessment of clastogenic activity (see Table 1 and 5).

In the first chromosomal aberration test, in the pulse treatment group without metabolic activation (S9-mix), only the mitotic index of the moderate concentration analysed (31.3 μg/ml) was reduced to 88 % of that of the concurrent (DMSO) control. At higher concentrations (125 to 1000 μg/ml), the test substance was too toxic for the cells. It was concluded that the test substance was cytotoxic for the cells, but did not induce a statistically significant increase in the number of aberrant cells, when compared to the number of aberrant cells found in the negative (DMSO) control cultures (see Table 2 and 6).

In the second chromosomal aberration test, in the pulse treatment group with metabolic activation (S9-mix), the mitotic indices of the three concentrations analysed (10, 30 and 60 μg/ml) were reduced to 74 %, 59 % and 41 %, respectively of that of the concurrent (DMSO) control. At higher concentrations (80 μg/ml), the test substance was too toxic for the cells. At the concentration of 30 μg/ml, the test substance induced a slight statistically significant ($p<0.05$) increase in the percentage of aberrant cells, when compared to the number of aberrant cells found in the concurrent negative (DMSO) control. This slight statistically significant increase in the percentage of aberrant cells was within the historical range and was only observed at the moderate concentration of 30 μg/ml. Therefore, this observation was judged as not biologically relevant. The observed positive response was within acceptable toxicity levels (see Table 3 and 7).

In the second chromosomal aberration test, in the continuous treatment group of 18 hours without metabolic activation (S9-mix), the mitotic index of the highest (80 μg/ml) concentration analysed was reduced to 49 %, when compared to the concurrent (DMSO) control. At the two lower concentrations analysed (60 and 30 μg/ml), the mitotic index was not reduced.

At higher concentrations (100 and 125 µg/ml), the test substance was too toxic for the cells. It was concluded that the test substance was cytotoxic for the cells, but did not induce a statistically significant increase in the number of aberrant cells, when compared to the number of aberrant cells found in the negative (DMSO) control cultures (see Table 4 and 8).

In both chromosomal aberration tests, the positive control substances mitomycin C (in the absence of a metabolic activation system) and cyclophosphamide (in the presence of a metabolic activation system), induced the expected statistically significant increases in the incidence of structural chromosomal aberrations (Table 5-8).

4 Conclusion

The data obtained in both chromosomal aberration tests, support the conclusion that, under the conditions used in this study, the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol was cytotoxic, but **not clastogenic** for CHO cells.

5 References

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Tables

Table 1: Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the presence of S9-mix: mitotic index (Test 1)

treatment time: harvesting time:			4 h 18 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
DMSO	0	1000	8.7	100	+
		1000	8.4		+
test substance	62.5	1000	1.6	19	-
		1000	1.7		-
	31.3	1000	3.3	41	+
		1000	3.7		+
	15.7	1000	3.1	35	+
		1000	2.9		+
	7.8	1000	5.0	58	-
		1000	4.9		-
	3.9	1000	5.4	61	+
		1000	5.0		+
	2.0	1000	6.2	75	-
		1000	6.7		-
cyclophosphamide	5.0	1000	4.8	58	+
		1000	5.2		+

Table 2: Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the absence of S9-mix: mitotic index (Test 1)

treatment	dose (µg/ml)	number of cells scored	mitotic index		
treatment time: harvesting time:			4 h 18 h		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
DMSO	0	1000	12.9	100	+
		1000	11.9		+
test substance	62.5	1000	12.1	100	+
		1000	12.6		+
	31.3	1000	11.3	88	+
		1000	10.4		+
	15.7	1000	12.8	104	+
		1000	12.9		+
	7.8	1000	14.0	106	-
		1000	12.2		-
mitomycin C	0.1	1000	10.1	79	+
		1000	9.5		+

Table 3: Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the presence of S9-mix: mitotic index (Test 2)

treatment time: 4 h
harvesting time: 18 h

treatment	dose (µg/ml)	number of cells scored	mitotic index: percentage of cells in mitosis	mitotic index: percentage of treated versus negative control value	mitotic index: selection for chromosomal aberration scoring
DMSO	0	1000	8.8	100	+
		1000	8.2		+
test substance	80	1000	2.7	30	-
		1000	2.4		-
	60	1000	3.3	41	+
		1000	3.7		+
	50	1000	4.3	51	-
		1000	4.4		-
	30	1000	5.1	59	+
		1000	5.0		+
	20	1000	5.1	60	-
		1000	5.1		-
	10	1000	6.2	74	+
		1000	6.3		+
	5.0	1000	8.3	98	-
		1000	8.4		-
cyclophos-phamide	5.0	1000	5.3	59	+
		1000	4.8		+

Table 4: Chromosomal aberration test with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the absence of S9-mix: mitotic index (Test 2)

treatment time: harvesting time:			18 h 18 h		
treatment	dose (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	percentage of treated versus negative control value	selection for chromosomal aberration scoring
DMSO	0	1000	9.4	100	+
		1000	9.3		+
test substance	80	1000	4.7	49	+
		1000	4.4		+
	60	1000	9.8	105	+
		1000	9.9		+
	50	1000	9.3	98	-
		1000	9.1		-
	30	1000	9.3	95	+
		1000	8.5		+
	20	1000	8.9	96	-
		1000	9.0		-
	10	1000	9.4	101	-
		1000	9.4		-
mitomycin C	0.05	1000	8.3	87	+
		1000	7.9		+

Table 5:
Results of Chromosomal Aberration Test 1 (pulse treatment method with S9-mix) with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol

Treatment / harvest time (h)	Dose level of test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing numerical chromosome aberrations			
		Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics [2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
4/18	neg. control (DMSO)	100	0	2	0	0	0	2		3	100	100	0	0	0
		100	1	0	0	0	0	1		4		100	0	0	0
		200	1	2	0	0	0	3 (1.5)		7		200	0	0	0 (0.0)
	3.9	100	1	0	0	0	0	1	-	4	61	100	0	0	0
		100	1	0	0	0	0	1		3		100	0	0	0
		200	2	0	0	0	0	2 (1.0)		7		200	0	0	0 (0.0)
	15.7	100	0	0	0	0	0	0	-	6	35	100	0	0	0
		100	2	0	0	0	1	3		7		100	0	0	0
		200	2	0	0	0	1	3 (1.5)		13		200	0	0	0 (0.0)
	31.3	100	1	1	0	0	0	1	-	10	41	100	0	0	0
		100	0	3	0	0	0	3		12		100	0	0	0
		200	1	4	0	0	0	4 (2.0)		22		200	0	0	0 (0.0)
	pos.control cyclophos-phamide (5.0)	100	14	17	0	0	0	31	***	0	58	100	0	0	0
		100	16	12	1	0	0	30		1		100	0	0	0
		200	30	29	1	0	0	61 (30.5)		1		200	0	0	0 (0.0)

1) Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps.
2) Fisher's exact probability test (two-sided), - p>0.05, * p≤0.05, ** p≤0.01, *** p≤0.001

Table 6:

Results of Chromosomal Aberration Test 1 (pulse treatment method without S9-mix) with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol

Treatment / harvest time (h)	Dose level of test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing numerical chromosome aberrations			
		Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics[2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
4/18	neg. control (DMSO)	100	0	0	0	0	0	0		4	100	100	0	0	0
		100	0	0	0	0	0	0		7		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		11		200	0	0	0 (0.0)
	15.7	100	0	0	0	0	0	0	-	3	104	100	0	0	0
		100	1	0	0	0	0	1		5		100	0	0	0
		200	1	0	0	0	0	1 (0.5)		8		200	0	0	0 (0.0)
	31.3	100	0	1	0	0	0	1	-	5	88	100	0	0	0
		100	1	0	0	0	0	1		7		100	0	0	0
		200	1	1	0	0	0	2 (1.0)		12		200	0	0	0 (0.0)
	62.5	100	0	0	0	0	0	0	-	5	100	100	0	0	0
		100	1	0	0	0	0	1		6		100	0	0	0
		200	1	0	0	0	0	1 (0.5)		11		200	0	0	0 (0.0)
	pos.control: mitomycin C (0.1)	100	17	15	2	0	0	30	***	0	79	100	0	0	0
		100	22	22	2	0	0	36		0		100	0	0	0
		200	39	27	4	0	0	66 (33.0)		0		200	0	0	0 (0.0)

[1] Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps.

[2] Fisher's exact probability test (two-sided), - p>0.05, * p≤0.05, ** p≤0.01, *** p≤0.001

Table 7:

Results of Chromosomal Aberration Test 2 (short time treatment method with S9-mix) with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol

Treatment / harvest time (h)	Dose level of Test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing numerical chromosome aberrations			
		Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics [2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
4/18	neg. control (DMSO)	100	0	0	0	0	0	0		2	100	100	0	0	0
		100	0	0	0	0	0	0		2		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		4		200	0	0	0 (0.0)
	10	100	0	0	0	0	0	0	-	2	74	100	0	0	0
		100	0	0	0	0	0	0		5		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		7		200	0	0	0 (0.0)
	30	100	1	1	1	0	0	2	*	4	59	100	0	0	0
		100	3	0	1	0	0	4		3		100	0	0	0
		200	4	1	2	0	0	6 (3.0)		7		200	0	0	0 (0.0)
	60	100	1	0	0	0	0	1	-	0	41	100	0	0	0
		100	0	0	0	0	0	0		3		100	0	0	0
		200	1	0	0	0	0	1 (0.5)		3		200	0	0	0 (0.0)
	pos. control: cyclophos-phamide (5.0)	100	6	9	10	0	0	25	***	0	59	100	0	0	0
		100	10	12	9	0	0	27		0		100	0	0	0
		200	16	21	19	0	0	52 (26.0)		0		200	0	0	0 (0.0)

1) Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps.

2) Fisher's exact probability test (two-sided), - $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$

Table 8:

Results of Chromosomal Aberration Test 2 (continuous treatment method without S9-mix) with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol

Treatment / harvest time (h)	Dose level of test substance (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)	Number of cells showing numerical chromosome aberrations			
		Cells observed	chromatid break	chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics[2]			Cells observed	Polyploids	Others	Number of cells showing aberrations (%)
18/18	neg. control (DMSO)	100	0	0	0	0	0	0		2	100	100	0	0	0
		100	0	0	0	0	0	0		1		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		3		200	0	0	0 (0.0)
	30	100	0	0	0	0	0	0	-	3	95	100	0	0	0
		100	0	0	0	0	0	0		4		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		7		200	0	0	0 (0.0)
	60	100	0	0	0	0	0	0	-	2	105	100	0	0	0
		100	0	1	0	0	0	1		4		100	0	0	0
		200	0	1	0	0	0	1 (0.5)		6		200	0	0	0 (0.0)
	80	100	0	0	0	0	0	0	-	4	49	100	0	0	0
		100	0	0	0	0	0	0		2		100	0	0	0
		200	0	0	0	0	0	0 (0.0)		6		200	0	0	0 (0.0)
	pos.control: mitomycin C (0.05)	100	10	9	5	0	0	22	***	0	87	100	0	0	0
		100	9	9	5	0	0	20		1		100	0	0	0
		200	19	18	10	0	0	42 (21.0)		1		200	0	0	0 (0.0)

1) Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps.

2) Fisher's exact probability test (two-sided), - $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$

Annexes

Annex 1
Check for mycoplasma, karyotype and cell cycle kinetics of Chinese hamster ovary cells (passage 16)

1. Determination of the average generation time (AGT).

CHO cells with passage number 16, used for the present assay, were cultured for 22.5 h in Ham's F-12 medium (with Glutamax-I), supplemented with heat-inactivated (30 min at 56 °C) foetal calf serum (10 %), penicillin (100 IU/ml medium), streptomycin (100 µg/ml medium) and 5-bromodeoxyuridine at 10 µM (BrdUrd; Sigma Chemical Company, St. Louis, USA) in a humidified atmosphere of 5 % CO_2 in air, at 37 °C. Two h before harvest, colcemid at 10 µg/ml (Fluka AG, Buchs, Switzerland) was added and accumulated mitotic cells were collected by trypsinization, treated with hypotonic sodium citrate (1 %) and fixed in 3:1 methanol:glacial acetic acid. Air-dried chromosome preparations were stained by the fluorescence-plus-Giemsa technique of Perry and Wolff (1974).
To estimate the average generation time (AGT), a replicative index (RI) was calculated from the proportions of cells that had completed 1 (= M1), 2 (= M2) or between 1 and 2 (= M1+) cycles in BrdUrd. RI was calculated as (M1 x 1) + (M1+ x 1.5) + (M2 x 2). The AGT (h) was calculated as the number of h in BrdUrd divided by the RI and multiplied by 100. The AGT represents a rough estimate of the cell cycle time (Armstrong, Bean and Galloway, 1992). The experiment was carried out in September 1994.
Results: One hundred cells of passage 16 of the CHO cells used in the present assay were analysed for the proportion M1, M2 and M1+. The number of cells completed M1 was 25, completed M2 was 75 and completed M1+ was 0. The RI is 175 and the AGT is 12.9 h (see above).

2. Determination of the modal chromosome number.

The modal chromosome number of passage 16 of the CHO cells used in the present assay was determined in 100 metaphases by counting the number of chromosomes per metaphase. The experiment was carried out in September 1994.
Results: Three metaphases consisted of 20 chromosomes, 97 metaphases consisted of 21 chromosomes. The modal chromosome number of these CHO cells (passage 16) is 21.03.

3. Check for the absence of mycoplasma contamination.

The mycoplasma determination in the CHO cells, used in the present assay, was carried out by the Business Unit Microbiology of TNO Quality of Life on 28 September 1994. The determination was carried out with the Gen-Probe MYCOPLASMA T.C. Rapid Detection System (Gen-Probe incorporated, San Diego, USA).
Results: The CHO cells used in the present assay were mycoplasma-negative.

References

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Annex 2 Preparation and characterization of Aroclor 1254-induced rat liver homogenate (batch of 6 July 2005)

The batch of S9 dated 6 July 2005 was prepared according to Ames et al. (1975) and Maron and Ames (1983) as follows.

Methods
Male Wistar rats (n =12; obtained from Charles River Deutschland, Sulzfeld, Germany) were injected intraperitoneally with a single dose of Aroclor 1254 (nominal dose of 500 mg/kg body weight) in soy bean oil (20% w/v). The rats were provided with tap water and the Institute's stock diet ad libitum. Five days after the injection of Aroclor 1254 the rats were killed by CO_2 asphyxiation. The livers were removed aseptically and immediately put into a cold, sterile 0.15 M KCl solution. After washing in the KCl solution, the livers were weighed, cut into pieces and homogenized in 3 volumes of 0.15 M KCl solution in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for 10 minutes at 9,000 g. The supernatant, which is called S9, was collected and divided into small aliquots in sterile polypropylene vials. The vials were quickly frozen on dry ice and subsequently stored in a freezer at <-60 °C.

The S9 was checked for sterility. The protein and cytochrome P-450 content of the S9 fraction were determined according to the method published by Rutten et al. (1987).

Results
The protein content of the batch was 21.2 g/litre.
The cytochrome P450 content of the batch was 19.4 μmol/litre.
The batch contained 0.91 μmol cytochrome P450 per gram protein.
The sterility check of the batch resulted in 0 colonies per 100 μl S9.

Conclusion
- The batch of S9 of 6 July 2005 meets all of the in-house quality criteria.

References

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Annex 3 Definition of chromosomal aberrations

Chromatid gap:	An achromatic lesion smaller than the width of one chromatid, and with minimal misalignment of the chromatid.
Chromatid break:	A breakage of one chromatid larger than the width of one chromatid, or a clear misalignment of a chromatid.
Chromatid exchange:	A breakage and reunion between chromatids from different chromosomes (interchange) or within a chromosome (intrachange; including interstitial deletion).
Chromosome gap:	An achromatic lesion at an identical site in both chromatids smaller than the width of one chromatid, and with minimal misalignment of the chromatids.
Chromosome break:	A breakage at an identical site of both chromatids larger than the width of one chromatid, or a clear misalignment of the chromatids (misalignment of the chromatids can result in cases where only the acentric fragment but not the shortened monocentric chromosome can be identified).
Chromosome exchange:	A breakage of both chromatids with a reunion between chromatids from different chromosomes (dicentric) or within a chromosome (ring).
Multiple aberrations:	A cell containing more than 10 chromosomal aberrations.
Polyploidy:	A cell containing a multiple of the haploid chromosome number (n) other than the diploid number (i.e., 3n, etc.).
Endoreduplication:	A cell in which after an S (synthesis) period of DNA replication, the nucleus did not go into mitosis but started another S period. The result is chromosomes with 4, 8, 16 or more chromatids.

References:

- Savage, Annotation: Classification and relationships of induced chromosome structural change. *J. Med. Gen.* **13**, 103-122, 1975.
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Annex 4

Historical data of chromosomal aberrations tests in CHO cells:

Historical negative controls (vehicles): studies from 2000-2003

Treatment/harvest times	% of cells with aberrations (excl. gaps) mean ± standard deviation; range (number of treatment groups performed)					
	culture medium or saline			DMSO		
without S9-mix						
4/18 hours	0.8 ± 0.8	0.0-2.5	(21)	0.8 ± 1.1	0.0-4.5	(20)
18/18 hours	0.6 ± 0.7	0.0-3.0	(30)	0.6 ± 0.8	0.0-3.5	(31)
with S9-mix						
4/18 hours	1.0 ± 0.9	0.0-3.5	(29)	0.8 ± 1.0	0.0-4.5	(33)

Annex 4 (continued)

Historical data of chromosomal aberrations tests in CHO cells:

Historical positive controls: studies from 2000-2003

Treatment/ harvest times	Compound	% of cells with aberrations (excl. gaps) mean ± standard deviation; range (number of treatment groups performed)		
	without S9-mix			
4/18 hours	mitomycin C, 0.1 μg/ml	28.0 ± 7.1	15.0-40.5	(43)
18/18 hours	mitomycin C, 0.05 μg/ml	30.0 ± 6.5	15.5-43.5	(55)
18/18 hours	mitomycin C, 0.025 μg/ml	15.4 ± 5.0	4.0-21.0	(11)
32/32 hours	mitomycin C, 0.05 μg/ml	47.0 ± 6.7	41.5-54.5	(3)
32/32 hours	mitomycin C, 0.025 μg/ml	32.2 ± 11.0	8.0-50.5	(17)
	with S9-mix			
4/18 hours	cyclophosphamide, 3.0 μg/ml	21.0 ± 9.7	5.5-47.0	(56)
	cyclophosphamide, 3.75 μg/ml	29.7 ± 11.4	6.5-45.5	(13)
	cyclophosphamide, 5.0 μg/ml	33.0 ± 2.0	31.5-34.5	(2)
4/32 hours	cyclophosphamide, 3.0 μg/ml	20.1 ± 14.3	4.5-51.0	(16)
	cyclophosphamide, 3.75 μg/ml	25.3 ± 15.1	12.5-42.0	(3)
	cyclophosphamide, 5.0 μg/ml	35.0	35.0	(1)
	cyclophosphamide, 6.0 μg/ml	30.0	30.0	(1)

Annex 5: Certificate of Analysis (provided by the sponsor)

21/10/2005

Shuichiro Sugimoto
Senior Chemist
Business management general division
Research & Development division
Asahi Glass Co., Ltd. Chemicals Company
1150 Hazawa-cho, Kanagawa-ku Yokohama,
Kanagawa Japan 221-8755
Phone: +81-45-374-7270
Fax: +81-45-374-8862

Certificate of Analysis of 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctylalcohol

3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctylalchol was analyzed by Asahi glass Co., Ltd.

1 Sample

3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctylalchol (CAS RN 647-42-7)
(Synthesized by Asahi Glass Co., Ltd. (Japan), lot re-AL-27, 28)
Synthesized date: 24/ 12 / 2004

2 Analysis

Samples were analyzed by gas chromatography with FID detector.

3 Result

Purity: 98.5%
Impurities: 1.2% C5F11-CF=CH-CH2-OH
(0.3% all the rest)