ORIGINAL

# TSCA NON-CONFIDENTIAL BUSINESS INFORMATION

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COMMENTS:

DOES NOT CONTAIN CBI

ENVIRON

MR 326802



May 7, 2010


Office of Pollution Prevention and Toxics
Document Processing Center (Mail Code 7407M)
U.S. Environmental Protection Agency
1201 Constitution Ave., NW
EPA East Room 6428
Washington, DC 20004
Attention: 8(e) Coordinator

**RE: Notification of Substantial Risk**


Dear Sir or Madam:

On behalf of the Alkylamidoamine glycinates (amphoacetates) consortium "the Consortium," and in accordance with Section 8(e) of the Toxic Substances Control Act (TSCA), the Consortium is submitting the following information from a laboratory report entitled:

> Evaluation of the Ability of Alkylamidoamine Glycinate[1], Majority in C12 & 14 (Amphoacetate) to Induce Chromosome Aberrations in Cultured Peripheral Human Lymphocytes[2]

In this in-vitro chromosomal aberration study, the testing laboratory did not observe structural chromosomal aberrations at any culture condition or dose level. However, polyploidy was observed in some of the treated human lymphocyte cell cultures used in the study at higher test article concentrations.

Although polyploidy is not considered a positive finding in this in-vitro chromosomal aberration study, the observed polyploidy occurred in human lymphocyte cell cultures. This effect occurred at a greater frequency in cells treated with the test material than the study's positive control. However, it should be noted that the positive control is not specific for the induction of aneuploidy and was tested at a concentration that is customarily used to elicit chromosomal aberrations, and not aneuploidy. A copy of the study report is appended for your review.

This information is being provided to USEPA in accordance with TSCA Section 8(e). The Consortium has made no determination if the polyploidy findings in this study actually constitute a risk to human health. When properly manufactured and used in its intended applications the Consortium does not believe that the subject substance presents an unreasonable risk to workers, the public, or the environment.

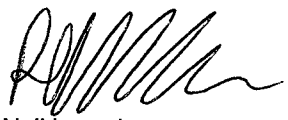
The results of this study were communicated to a consortium member on April 9, 2010.

The Consortium asserts that none of the information contained within this notice constitutes confidential business information (CBI) under TSCA.

[1] Imidazolium compounds, 1-[2-(carboxymethoxy)ethyl]-1-(carboxymethyl)-4,5-dihydro-2-norcoco alkyl, hydroxides, sodium salts, CAS 68650-39-5
[2] Draft report dated April 15, 2010

Should you have any questions, or require further information, please do not hesitate to contact the undersigned. Thank you.

Very truly yours,



Neil Loescher
Senior Consultant

ENVIRON UK Ltd
Consortium Administration Manager/Trustee on behalf of the Alkylamidoamine glycinates (amphoacetates) Consortium (data submitter)

Atts

TNT Ref Number: 1700682833

# DRAFT REPORT APPROVAL STATEMENT

| | |
|---|---|
| **Study Title** | Evaluation of the ability of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) to induce chromosome aberrations in cultured peripheral human lymphocytes |
| **NOTOX Substance** | 200817/B |
| **NOTOX Project** | 493183 |

**STUDY DIRECTOR STATEMENT**

The undersigned declares that this draft report constitutes a true account of the procedures adopted and the results obtained in the performance of this study.

**Study director**

Buskens

.....
Drs. C.A.F. Buskens

15 April 2010
date:

**APPROVAL**
**Management**

i.A.T.M. Heertje
Section Head

.....
Ing. E.J. van de Waart, M.Sc.
Head of In Vitro & Environmental Toxicology

16 April 2010
date:

# DRAFT REPORT

Version 1

Study Title

# EVALUATION OF THE ABILITY OF ALKYLAMIDOAMINE GLYCINATE, MAJORITY IN C12 & 14 (AMPHOACETATE) TO INDUCE CHROMOSOME ABERRATIONS IN CULTURED PERIPHERAL HUMAN LYMPHOCYTES (WITH REPEAT

Author

Drs. C.A.F. Buskens

Study completion date

15 April 2010

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

**NOTOX Project 493183**
**NOTOX Substance 200817/B**

## 1. CONTENTS



TABLES

## 2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

Analysis of stability, homogeneity and concentration of the test substance under test conditions was not performed as part of this study.

NOTOX B.V.

Drs. C.A.F. Buskens
Study Director

Ing. E.J. van de Waart, M.Sc.
Head of In Vitro & Environmental Toxicology

Date: .....

Date: .....

## 3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

The reporting date is the date of reporting to the Study Director. The QAU report was then forwarded to the Test Facility Management.

| Type of Inspections | Phase/Process | Start Inspection date | End Inspection date | Reporting date |
|---|---|---|---|---|
| **Study** | Protocol | 01-Feb-10 | 01-Feb-10 | 01-Feb-10 |
| | Report | 14-Apr-10 | 14-Apr-10 | 14-Apr-10 |
| **Process** | **Genetic and In Vitro Toxicology**<br>Test Substance Handling<br>Exposure<br>Observations/Measurements | 14-Dec-09 | 16-Dec-09 | 22-Dec-09 |

Head of Quality Assurance
C.J. Mitchell B.Sc.

Date: .....

## 4. SUMMARY

Evaluation of the ability of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) to induce chromosome aberrations in cultured peripheral human lymphocytes (with repeat experiment).

This report describes the effect of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) on the number of chromosome aberrations in cultured peripheral human lymphocytes in the presence and absence of a metabolic activation system (phenobarbital and ß-naphthoflavone induced rat liver S9-mix). The possible clastogenicity of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was tested in two independent experiments.

The study procedures described in this report were based on the most recent OECD and EC guidelines.

Batch LK0923 of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was a clear yellow liquid with a actives content of 38.18%. The concentration of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was corrected for the actives content. A correction factor of 2.62 was used. The test substance was dissolved in RPMI 1640 medium.

In the first cytogenetic assay, Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was tested up to 500 µg/ml for a 3 h exposure time with a 24 h fixation time in the absence and presence of 1.8% (v/v) S9-fraction. Appropriate toxicity was reached at this dose level.

In the second cytogenetic assay, Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was tested up to 300 µg/ml for a 24 h continuous exposure time with a 24 h fixation time and up to 240 µg/ml for a 48 h continuous exposure time with a 48 h fixation time in the absence of S9-mix. In the presence of S9-mix Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was tested up to 400 µg/ml for a 3 h exposure time with a 48 h fixation time. Appropriate toxicity was reached at these dose levels.

The number of cells with chromosome aberrations found in the solvent control cultures was within the laboratory historical control data range. Positive control chemicals, mitomycin C and cyclophosphamide, both produced a statistically significant increase in the incidence of cells with chromosome aberrations, indicating that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations in the absence and presence of S9-mix, in either of the two independently repeated experiments.

It was noted that Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) increased the number of polyploid cells in the first cytogenetic assay both in the absence and presence of S9-mix in a dose dependent manner. In the second cytogenetic assay in the presence of S9-mix the number of polyploid cells was increased at the highest concentration tested. This may indicate that Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) has the potential to disturb mitotic processes.

No effects of the test substance on the number of cells with endoreduplicated chromosomes were observed both in the absence and presence of S9-mix.

Finally, it is concluded that this test is valid and that Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) is not clastogenic in human lymphocytes under the experimental conditions described in this report. Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) may have the potential to disturb mitotic processes.

# 5. INTRODUCTION

## 5.1. Preface

| | |
|---|---|
| Sponsor | Rhodia Operations SAS<br>(on behalf of the Amphoacetate consortium)<br>40 rue de la Haie-Coq<br>93306 AUBERVILLIERS CEDEX<br>France |
| Study Monitor | Mrs A. Buard<br>RHODIA Inc.<br>8 Cedar Brook Drive<br>Cranbury, NJ 08512-7500<br>USA |
| Test Facility | NOTOX B.V.<br>Hambakenwetering 7<br>5231 DD 's-Hertogenbosch<br>The Netherlands |
| Study Director | Drs. C.A.F. Buskens |
| Technical Coordinator | M.A.H. van Seggelen |
| Study Plan | Start : 29 January 2010<br>Completion : 23 March 2010 |

## 5.2. Aims of the study

The objective of this study was to evaluate Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) for its ability to induce structural chromosome aberrations in cultured human lymphocytes (1), either in the presence or absence of a metabolic activation system (S9-mix).

Background of the test system
Whole blood samples obtained from healthy male subjects were treated with an anti-coagulant (heparin) and cultured in the presence of a mitogen (phytohaemagglutinin). These stimulated human lymphocytes were used because they are sensitive indicators of clastogenic activity of a broad range of chemicals (1-5).

The stimulated lymphocytes were exposed to Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) both in the absence and presence of a metabolic activation system (S9-mix). In combination with this metabolic activation system indirect chemical mutagens, i.e. those requiring metabolic transformation into reactive intermediates, can be tested for possible clastogenic effects *in vitro*.

At predetermined intervals after exposure of the stimulated human lymphocytes to Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate), cell division was arrested in the metaphase stage of the cell cycle by addition of the metaphase-arresting chemical colchicine. Cells were harvested, stained and metaphase cells were analysed for the presence of structural chromosome aberrations such as breaks, gaps, minutes, dicentrics and exchange figures. Results from cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) were compared with control (vehicle) treated cultures.

Chromosome aberrations were generally evaluated in the first post-exposure mitosis (i.e. 24 hours after exposure). However, since the appearance of the first post-exposure mitosis could be considerably delayed due to toxic insult to the cells, cells were also harvested 48 hours after exposure to cover the interval in which maximum aberration frequency was expected.

A test substance that induces a positive response in this assay is presumed to be a potential mammalian cell clastogenic agent.

### 5.3. Guidelines

The study procedures described in this report were based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Guideline no. 473: *In Vitro* Mammalian Chromosome Aberration Test (adopted July 21, 1997).
- European Community (EC). Commission regulation (EC) No. 440/2008, Part B: Methods for the Determination of Toxicity and other health effects, Guideline B.10: "Mutagenicity - *In Vitro* Mammalian Chromosome Aberration Test". Official Journal of the European Union No. L142, 31 May 2008.

### 5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

## 6. MATERIALS AND METHODS

### 6.1. Test substance

#### 6.1.1. Test substance information

| | |
|---|---|
| Identification | Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) |
| Molecular formula and weight | For two representative structures:<br>$C_{20}H_{36}N_2O_6Na_2$: 446<br>$C_{18}H_{35}N_2O_4Na$ : 389 |
| CAS Number | 68650-39-5 |
| Description | Clear yellow liquid |
| Batch | LK0923 |
| Composition | 49.72% (solids content)<br>11.54% (sodium chloride content)<br>38.18% (actives content) |
| Test substance storage | At room temperature in the dark |
| Stability under storage conditions | Stable |
| Expiry date | 10 November 2010 |

### 6.1.2. Study specific test substance information

| | |
|---|---|
| Stability at higher temperatures | No |
| Stability in vehicle | Water: Not indicated<br>RPMI: Not indicated |
| Solubility in vehicle | Water: Miscible in all proportions<br>RPMI: Not indicated |

### 6.1.3. Test substance preparation

The concentrations of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) were corrected for the actives content (38.18%). A correction factor of 2.62 was used.

Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was dissolved in RPMI 1640 medium (Invitrogen Corporation, Breda, The Netherlands). Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentrations were used within 2.5 hours after preparation and filter (0.22 µm)-sterilized. The pH and the osmolarity of the culture medium containing the highest tested concentration were recorded.

## 6.2. Reference substances

### 6.2.1. Negative control

The vehicle for the test substance was RPMI 1640 medium.

### 6.2.2. Positive controls

Without metabolic activation (-S9-mix):
Mitomycin C (MMC-C; CAS no. 50-07-7, Sigma, Zwijndrecht, The Netherlands) was used as a direct acting mutagen at a final concentration of 0.5 µg/ml for a 3 h exposure period, 0.2 µg/ml for a 24 h exposure period and 0.1 µg/ml for a 48 h exposure period.

With metabolic activation (+S9-mix):
Cyclophosphamide (CP; CAS no. 50-18-0. Endoxan-Asta, Asta-Werke, Germany) was used as an indirect acting mutagen, requiring metabolic activation, at a final concentration of 10 µg/ml for a 3 h exposure period (24 h fixation time).

Solvent for positive controls
Hanks' Balanced Salt Solution (HBSS) (Invitrogen Corporation, Breda, The Netherlands), without calcium and magnesium.

All reference stock solutions were stored in aliquots at ≤-15°C in the dark. These solutions were thawed immediately before use.

## 6.3. Test system

Cultured peripheral human lymphocytes were used as test system. Peripheral human lymphocytes are recommended in international guidelines (e.g. OECD, EC).

Blood was collected from healthy adult, non-smoking, male volunteers. The Average Generation Time (AGT) of the cells and the age of the donor at the time the AGT was determined (December 2009) are presented below:

| | |
|---|---|
| Dose range finding study: | age 34, AGT = 14.4 h |
| First cytogenetic assay: | age 40, AGT = 14.5 h |
| Second cytogenetic assay: | age 31, AGT = 14.4 h |
| Cytogenetic assay 2A: | age 31, AGT = 14.1 h |

### 6.4. Cell culture

Blood samples
Blood samples were collected by venapuncture using the Venoject multiple sample blood collecting system with a suitable size sterile vessel containing sodium heparin (Vacuette, Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Immediately after blood collection lymphocyte cultures were started.

Culture medium
Culture medium consisted of RPMI 1640 medium (Invitrogen Corporation), supplemented with 20% (v/v) heat-inactivated (56°C; 30 min) foetal calf serum (Invitrogen Corporation), L-glutamine (2 mM) (Invitrogen Corporation), penicillin/streptomycin (50 U/ml and 50 µg/ml respectively) (Invitrogen Corporation) and 30 U/ml heparin (Sigma, Zwijndrecht, The Netherlands).

Lymphocyte cultures
Whole blood (0.4 ml) treated with heparin was added to 5 ml or 4.8 ml culture medium (in the absence and presence of S9-mix, respectively). Per culture 0.1 ml (9 mg/ml) phytohaemagglutinin (Remel, Europe Ltd., United Kingdom) was added.

Environmental conditions
All incubations were carried out in a controlled environment in the dark, in which optimal conditions were a humid atmosphere of 80 - 100% (actual range 76 - 90%), containing 5.0 ± 0.5% $CO_2$ in air, at a temperature of 37.0 ± 1.0°C (actual range 36.3 - 37.6°C). Temperature and humidity were continuously monitored throughout the experiment. The $CO_2$ percentage was monitored once on each working day. Temporary deviations from the humidity (with a maximum of 4%) occurred that were caused by opening and closing of the incubator door, but the time of these deviations did not exceed 1 hour. Based on laboratory historical data these deviations are considered not to affect the study integrity.

### 6.5. Metabolic activation system

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats (6), which were obtained from Charles River (Sulzfeld, Germany).

#### 6.5.1. Preparation of S9-fraction

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the Standard Operating Procedures, and allowed to acclimatise for at least 5 days. The rats were orally dosed at three consecutive days with a suspension of phenobarbital (80 mg/kg body weight) and ß-naphthoflavone (100 mg/kg body weight) in corn oil (they were denied access to food for 3 to 4 hours preceding each dosing). One day after the final exposure (24 h), the rats were sedated using oxygen/carbon dioxide and then killed by decapitation. The rats received a limited quantity of food during the night before sacrifice. The livers of the rats were removed aseptically, and washed in cold (0°C), sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM $Na_2$-EDTA (Merck). The livers were minced in a blender and homogenised in 3 volumes of phosphate buffer with a Potter homogeniser. The homogenate was centrifuged for 15 min at 9000 *g*. The supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C) for a maximum of 1 year.

The S9 batch was characterised in a bacterial reverse mutation assay in *Salmonella typhimurium* tester strain TA98 with the mutagens Benzo-(a)-pyrene and 2-aminoanthracene at concentrations of 5 µg/plate and 1 µg/plate, respectively. These mutagens require metabolic activation.

### 6.5.2. Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice. S9-mix components contained per ml: 1.63 mg $MgCl_2.6H_2O$ (Merck); 2.46 mg KCl (Merck); 1.7 mg glucose-6-phosphate (Roche, Mannheim, Germany); 3.4 mg NADP (Randox Laboratories Ltd., Crumlin, United Kingdom); 4 µmol HEPES (Invitrogen Corporation).
The above solution was filter (0.22 µm)-sterilized. To 0.5 ml S9-mix components 0.5 ml S9-fraction (batches 09-5 and 09-6) was added (50% (v/v) S9-fraction) to complete the S9-mix.

Metabolic activation was achieved by adding 0.2 ml S9-mix to 5.3 ml of a lymphocyte culture (containing 4.8 ml culture medium, 0.4 ml blood and 0.1 ml (9 mg/ml) phytohaemagglutinin). The concentration of the S9-fraction in the exposure medium was 1.8% (v/v).

## 6.6. Study design

### 6.6.1. Dose range finding test

In order to select the appropriate dose levels for the chromosome aberration test cytotoxicity data were obtained in a dose range finding test. Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was tested in the absence and in the presence of 1.8% (v/v) S9-fraction.

Lymphocytes (0.4 ml blood of a healthy male donor was added to 5 ml or 4.8 ml culture medium, without and with metabolic activation respectively and 0.1 ml (9 mg/ml) Phytohaemagglutinin) were cultured for 48 h and thereafter exposed to selected doses of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) for 3 h, 24 h and 48 h in the absence of S9-mix or for 3 h in the presence of S9-mix.

The highest tested concentration was 5000 µg/ml.

After 3 h exposure to Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the absence or presence of S9-mix, the cells were separated from the exposure medium by centrifugation (5 min, 365 *g*). The supernatant was removed and cells were rinsed with 5 ml HBSS. After a second centrifugation step, HBSS was removed and cells were resuspended in 5 ml culture medium and incubated for another 20 - 22 h (24 h fixation time). The cells that were exposed for 24 h and 48 h in the absence of S9-mix were not rinsed after exposure but were fixed immediately (24 h and 48 h fixation time).

Cytotoxicity of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the lymphocyte cultures was determined using the mitotic index.

Based on the results of the dose range finding test an appropriate range of dose levels was chosen for the cytogenetic assays considering the highest dose level had an inhibition of the mitotic index of 50% or greater whereas the mitotic index of the lowest dose level was approximately the same as the mitotic index of the solvent control.

### 6.6.2. Cytogenetic assay

The cytogenetic assay was carried out as described by Evans, 1984 (2) with minor modifications. Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) was tested in the absence and presence of 1.8% (v/v) S9-fraction in duplicate in two independent experiments. To be able to select appropriate dose levels for scoring of chromosome aberrations a repeat experiment had to be performed in the second cytogenetic assay.

First cytogenetic assay
Lymphocytes were cultured for 48 h and thereafter exposed in duplicate to selected doses of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) for 3 h in the absence and presence of S9-mix. After 3 h exposure, the cells were separated from the exposure medium by centrifugation (5 min, 365 *g*). The supernatant was removed and the cells were rinsed once with 5 ml HBSS. After a second centrifugation step, HBSS was removed and cells were resuspended in 5 ml culture medium and incubated for another 20 - 22 h (24 h fixation time). Appropriate negative and positive controls were included in the first cytogenetic assay.

Based on the mitotic index of the dose range finding test and the first cytogenetic assay appropriate dose levels were selected for the second cytogenetic assay. The independent repeat was performed with the following modifications of experimental conditions.

Second cytogenetic assay
Lymphocytes were cultured for 48 h and thereafter exposed in duplicate to selected doses of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) for 24 h and 48 h in the absence of S9-mix or for 3 h in the presence of S9-mix.

After 3 h exposure, the cells exposed to Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the presence of S9-mix were separated from the exposure medium by centrifugation (5 min, 365 *g*). The supernatant was removed and the cells were rinsed once with 5 ml of HBSS and incubated in 5 ml culture medium for another 44 - 46 h (48 h fixation time).

The cells that were treated for 24 h and 48 h in the absence of S9-mix were not rinsed after exposure but were fixed immediately after 24 h and 48 h (24 h and 48 h fixation time). Appropriate negative and positive controls were included in the second cytogenetic assay.

### 6.6.3. Chromosome preparation

During the last 2.5 - 3 h of the culture period, cell division was arrested by the addition of the spindle inhibitor colchicine (0.5 µg/ml medium) (Acros Organics, Belgium). Thereafter the cell cultures were centrifuged for 5 min at 1300 rpm (365 *g*) and the supernatant was removed. Cells in the remaining cell pellet were swollen by a 5 min treatment with hypotonic 0.56% (w/v) potassium chloride (Merck) solution at 37°C. After hypotonic treatment, cells were fixed with 3 changes of methanol (Merck): acetic acid (Merck) fixative (3:1 v/v).

### 6.6.4. Preparation of slides

Fixed cells were dropped onto cleaned slides, which were immersed in a 1:1 mixture of 96% (v/v) ethanol (Merck)/ether (Merck) and cleaned with a tissue. The slides were marked with the NOTOX study identification number and group number. At least two slides were prepared per culture. Slides were allowed to dry and thereafter stained for 10 - 30 min with 5% (v/v) Giemsa (Merck) solution in tap water.
Thereafter slides were rinsed in tap-water and allowed to dry. The dry slides were cleared by dipping them in xylene (Klinipath, Duiven, The Netherlands) before they were embedded in Pertex (Klinipath) and mounted with a coverslip.

### 6.6.5. Mitotic index/dose selection for scoring of the cytogenetic assay

The mitotic index of each culture was determined by counting the number of metaphases per 1000 cells. At least three analysable concentrations were used for scoring of the cytogenetic assay. Chromosomes of metaphase spreads were analysed from those cultures with an inhibition of the mitotic index of about 50% or above whereas the mitotic index of the lowest dose level was approximately the same as the mitotic index of the solvent control. Also cultures treated with an intermediate dose were examined for chromosome aberrations.

### 6.6.6. Analysis of slides for chromosome aberrations

To prevent bias, all slides were randomly coded before examination of chromosome aberrations and scored. An adhesive label with NOTOX study identification number and code was placed over the marked slide. One hundred metaphase chromosome spreads per culture were examined by light microscopy for chromosome aberrations. In case the number of aberrant cells, gaps excluded, was ≥ 25 in 50 metaphases, no more metaphases were examined. Only metaphases containing 46 ± 2 centromeres (chromosomes) were analysed. The number of cells with aberrations and the number of aberrations were calculated.

## 6.7. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programme(s):
REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): temperature and humidity.

## 6.8. Interpretation

### 6.8.1. Acceptability of the assay

A chromosome aberration test is considered acceptable if it meets the following criteria:
a) The number of chromosome aberrations found in the solvent control cultures should reasonably be within the laboratory historical control data range (see Appendix III).
b) The positive control substances should produce a statistically significant (Chi-square test, one-sided, $p < 0.05$) increase in the number of cells with chromosome aberrations.
c) A homogeneous response between the replicate cultures is observed.
d) A possible precipitate present on the slides should not interfere with the scoring of chromosome aberrations.

### 6.8.2. Data evaluation and statistical procedures

A test substance was considered positive (clastogenic) in the chromosome aberration test if:
a) It induced a dose-related statistically significant (Chi-square test, one-sided, $p < 0.05$) increase in the number of cells with chromosome aberrations.
b) A statistically significant and biologically relevant increase in the frequencies of the number of cells with chromosome aberrations was observed in the absence of a clear dose-response relationship.

A test substance was considered negative (not clastogenic) in the chromosome aberration test if none of the tested concentrations induced a statistically significant (Chi-square test, one-sided, $p < 0.05$) increase in the number of cells with chromosome aberrations.

The preceding criteria are not absolute and other modifying factors might enter into the final evaluation decision.

The incidence of aberrant cells (cells with one or more chromosome aberrations, gaps included or excluded) for each exposure group outside the laboratory historical control data range was compared to that of the solvent control using Chi-square statistics:

$$X^2 = \frac{(N-1)\,(ad-bc)^2}{(a+b)\,(c+d)\,(a+c)\,(b+d)}$$

where b = the total number of aberrant cells in the control cultures.
d = the total number of non aberrant cells in the control cultures.
$n_0$ = the total number of cells scored in the control cultures.
a = the total number of aberrant cells in treated cultures to be compared with the control.
c = the total number of non aberrant cells in treated cultures to be compared with the control.
$n_1$ = the total number of cells scored in the treated cultures.
N = sum of $n_0$ and $n_1$

If P $\left[ X^2 > \frac{(N-1)(ad-bc)^2}{(a+b)(c+d)(a+c)(b+d)} \right]$ (one-tailed) is small ($p < 0.05$) the hypothesis that the incidence of cells with chromosome aberrations is the same for both the treated and the solvent control group is rejected and the number of aberrant cells in the test group is considered to be significantly different from the control group at the 95% confidence level.

### 6.9. List of deviations

#### 6.9.1. List of protocol deviations

There were no deviations from the protocol.

#### 6.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

## 7. RESULTS

### 7.1. Dose range finding test

A concentration of 5000 µg/ml showed no precipitation in the culture medium and was used as the highest concentration of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate).

The pH and osmolarity of a concentration of 5000 µg/ml were 7.98 and 365 mOsm/kg respectively (compared to 7.84 and 294 mOsm/kg in the solvent control).

In the dose range finding test blood cultures were treated with 100, 333, 1000, 3330 and 5000 µg Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate)/ml culture medium with and without S9-mix.

Table 1 shows the mitotic index of cultures treated with various Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentrations or with the negative control substance.

### 7.2. First cytogenetic assay

Based on the results of the dose range finding test the following dose levels were selected for the cytogenetic assay:

Without S9-mix : 10, 30, 100, 300, 350, 400, 450, 500 and 750 µg/ml culture medium (3 h exposure time, 24 h fixation time).

With S9-mix : 10, 30, 100, 300, 350, 400, 450 and 500 µg/ml culture medium
(3 h exposure time, 24 h fixation time).

Table 2 shows the mitotic index of cultures treated with various Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentrations or with the positive or negative control substances.

The following dose levels were selected for scoring of chromosome aberrations:

Without and with S9-mix: 100, 450 and 500 µg/ml culture medium
(3 h exposure time, 24 h fixation time).

Both in the absence and presence of S9-mix, Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations (Tables 3, 4).

Both in the absence and presence of S9-mix, Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) did not increase the number of cells with endoreduplicated chromosomes.

Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) increased the number of polyploid cells both in the absence and presence of S9-mix in a dose dependent manner.

**7.3. Second cytogenetic assay**

To obtain more information about the possible clastogenicity of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate), a second cytogenetic assay was performed in which human lymphocytes were continuously exposed to Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the absence of S9-mix for 24 or 48 hours. In the presence of S9-mix, cells were fixed after 48 hours following a 3 hour exposure to Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate). The following dose levels were selected for the second cytogenetic assay:

Without S9-mix : 10, 30, 100, 150, 200, 250, 300, 350 and 400 µg/ml culture medium
(24 h exposure time, 24 h fixation time).
10, 30, 50, 100, 150, 200, 250 and 300 µg/ml culture medium
(48 h exposure time, 48 h fixation time).
With S9-mix : 100, 300, 400, 500, 600, 700 and 800 µg/ml culture medium
(3 h exposure time, 48 h fixation time).

Table 5 shows the mitotic index of cultures treated with various Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentrations or with the positive or negative control substances.

In the absence of S9-mix, at the 48 h continuous exposure time, no appropriate dose levels could be selected for scoring of chromosome aberrations since at the concentration of 200 µg/ml not enough cytotoxicity was observed (43%), whereas the next higher concentration of 250 µg/ml was too toxic for scoring (66%). This part of the experiment was repeated.

Cytogenetic assay 2A:
Based on the results of the second cytogenetic assay the following dose levels were selected for experiment 2A:

Without S9-mix : 10, 100, 150, 200, 220, 240, 260 and 280 µg/ml culture medium
(48 h exposure time, 48 h fixation time).

Table 6 shows the mitotic index of cultures treated with various Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentrations or with the positive or negative control substance.

Based on these observations the following doses were selected for scoring of chromosome aberrations:

| | | |
|---|---|---|
| Without S9-mix | : | 100, 200 and 300 µg/ml culture medium (24 h exposure time, 24 h fixation time). 10, 200 and 240 µg/ml culture medium (48 h exposure time, 48 h fixation time). |
| With S9-mix | : | 100, 300 and 400 µg/ml culture medium (3 h exposure time, 48 h fixation time). |

Both in the absence and presence of S9-mix, Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations (Tables 7 - 9).

Both in the absence and presence of S9-mix, Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) did not increase the number of cells with endoreduplicated chromosomes.

Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) did not increase the number of polyploid cells in the absence of S9-mix. In the presence of S9-mix Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) increased the number of polyploid cells at the highest tested concentration of 400 µg/ml.

**7.4. Evaluation of the results**

The ability of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) to induce chromosome aberrations in human peripheral lymphocytes was investigated in two independent experiments. The highest concentration analysed was selected based on toxicity, inhibition of the mitotic index of about 50% or greater.

The mitotic indices of cultures treated with various Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentrations or with the negative control substances are presented in Tables 1, 2, 5 and 6. The scores for the number of aberrant cells (gaps included and excluded) and the number of the various types of chromosome aberrations at the various concentrations of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) are presented in Tables 3, 4 and 7 - 9. Duplicate cultures are indicated by A and B. The criteria according to which the aberrations were classified are outlined in Appendix I. Appendix II presents the statistical evaluations of the test results.

The number of cells with chromosome aberrations found in the solvent control cultures was within the laboratory historical control data range (see Appendix III). The number of polyploid cells and cells with endoreduplicated chromosomes in the solvent control cultures was within the laboratory historical control data range (see Appendix IV). The positive control chemicals (MMC-C and CP) both produced statistically significant increases in the frequency of aberrant cells. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

Both in the absence and presence of S9-mix Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations in two independent experiments.

It was noted that Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) increased the number of polyploid cells in the first cytogenetic assay both in the absence and presence of S9-mix in a dose dependent manner. In the second cytogenetic assay in the presence of S9-mix the number of polyploid cells was increased at the highest concentration tested. This may indicate that Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) has the potential to disturb mitotic processes.

No effects of Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) on the number of cells with endoreduplicated chromosomes were observed both in the absence and presence of S9-mix.

## 8. CONCLUSION

Finally, it is concluded that this test is valid and that Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) is not clastogenic in human lymphocytes under the experimental conditions described in this report. Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) may have the potential to disturb mitotic processes.

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**Table 1 Mitotic index of human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the dose range finding test**

Period of treatment: From: 03-02-2010 To: 05-02-2010

| Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentration (µg/ml) | Number of metaphases per 1000 cells | |
|---|---|---|
| | Absolute | Percentage of control |
| Without metabolic activation (-S9-mix) | | |
| 3 h exposure time, 24 h fixation time | | |
| Control [a] | 36 | 100 |
| 100 | 25 | 69 |
| 333 | 23 | 64 |
| 1000 | -[b] | -[b] |
| 3330 | -[b] | -[b] |
| 5000 | -[b] | -[b] |
| 24 h exposure time, 24 h fixation time | | |
| Control [a] | 28 | 100 |
| 100 | 23 | 82 |
| 333 | 11 | 39 |
| 1000 | -[b] | -[b] |
| 3330 | -[b] | -[b] |
| 5000 | -[b] | -[b] |
| 48 h exposure time, 48 h fixation time | | |
| Control [a] | 41 | 100 |
| 100 | 26 | 63 |
| 333 | 4 | 10 |
| 1000 | -[b] | -[b] |
| 3330 | -[b] | -[b] |
| 5000 | -[b] | -[b] |
| With metabolic activation (+S9-mix) | | |
| 3 h exposure time, 24 h fixation time | | |
| Control [a] | 34 | 100 |
| 100 | 32 | 94 |
| 333 | 15 | 44 |
| 1000 | -[b] | -[b] |
| 3330 | -[b] | -[b] |
| 5000 | -[b] | -[b] |

[a] RPMI 1640 medium
[b] Cell lysis

**Table 2 Mitotic index of human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the first cytogenetic assay**

Period of treatment: From: 10-02-2010 To: 11-02-2010

| Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentration (µg/ml) | Number of metaphases per 1000 cells [a] | |
|---|---|---|
| | Absolute | Percentage of control |
| Without metabolic activation (-S9-mix) | | |
| 3 h exposure time, 24 h fixation time | | |
| Control [b] | 45 - 39 | 100 |
| 10 | 44 - 42 | 102 |
| 30 | 46 - 40 | 102 |
| 100 | 45 - 38 | 99 |
| 300 | 39 - 36 | 89 |
| 350 | 42 - 37 | 94 |
| 400 | 40 - 35 | 89 |
| 450 | 33 - 26 | 70 |
| 500 | 22 - 19 | 49 |
| 750 | 11 - 13 | 29 |
| MMC-C; 0.5 µg/ml | 41 - 36 | 92 |
| With metabolic activation (+S9-mix) | | |
| 3 h exposure time, 24 h fixation time | | |
| Control [b] | 40 - 42 | 100 |
| 10 | 42 - 39 | 99 |
| 30 | 40 - 39 | 96 |
| 100 | 44 - 39 | 101 |
| 300 | 46 - 34 | 98 |
| 350 | 33 - 38 | 87 |
| 400 | 36 - 36 | 88 |
| 450 | 26 - 23 | 60 |
| 500 | 19 - 19 | 46 |
| CP; 10 µg/ml | 18 - 24 | 51 |

[a] Duplicate cultures
[b] RPMI 1640 medium

**Table 3** **Chromosome aberrations in human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the absence of S9-mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)**

| Conc | RPMI 1640 medium | | | 100 µg/ml | | | 450 µg/ml | | | 500 µg/ml | | | MMC-C 0.5 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 99 | | | 70 | | | 49 | | | 92 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 50 | 150 |
| No. of Cells with aberrations (+ gaps) [a)] | 2 | 1 | 3 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 47 | 25 | 72 ***) |
| No. of Cells with aberrations (- gaps) | 2 | 1 | 3 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 47 | 25 | 72 ***) |
| g' | | | | | | | | | | | | | | | |
| g" | | | | | | | | | | | | | | | |
| b' | 2 | 1 | | | | | 1 | | | 1 | | | 31 | 13 | |
| b" | | | | | | | | | | | | | 12 | 3 | |
| m' | | | | | | | | | | | | | | | |
| m" | | | | | | | | | | | | | 1 | 2 | |
| exch. | | | | | | | | | | | | | 11 | 11 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | 3poly | 2poly 2endo | | 6poly | 3poly endo | | 2intra | tric | |
| total aberr (+ gaps) | 2 | 1 | | 0 | 0 | | 0 | 1 | | 0 | 1 | | 57 | 30 | |
| total aberr (- gaps) | 2 | 1 | | 0 | 0 | | 0 | 1 | | 0 | 1 | | 57 | 30 | |

a) Abbreviations used for various types of aberrations are listed in Appendix I.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.

*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

**Table 4** **Chromosome aberrations in human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the presence of S9-mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)**

| Conc | RPMI 1640 medium | | | 100 µg/ml | | | 450 µg/ml | | | 500 µg/ml | | | CP 10 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 101 | | | 60 | | | 46 | | | 51 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [a] | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 2 | 2 | 2 | 0 | 2 | 40 | 31 | 71 *** |
| No. of Cells with aberrations (- gaps) | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 2 | 2 | 2 | 0 | 2 | 40 | 31 | 71 *** |
| g' | | | | | | | | | | | | | 1 | | |
| g" | | | | | | | | | | | | | | | |
| b' | | | | | 1 | | | | | 1 | | | 30 | 28 | |
| b" | | | | | | | | 1 | | | | | 10 | 2 | |
| m' | | | | | | | | | | 1 | | | | | |
| m" | | | | | | | | | | 2 | | | 1 | 2 | |
| exch. | | | | | | | | | | | | | 5 | 5 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | endo | | | endo | | | | 3poly p | | 8poly endo | | 2poly 2endo | 3intra | | intra |
| total aberr (+ gaps) | 0 | 0 | | 0 | 1 | | 0 | 2 | | 4 | 0 | | 50 | 38 | |
| total aberr (- gaps) | 0 | 0 | | 0 | 1 | | 0 | 2 | | 4 | 0 | | 49 | 38 | |

[a] Abbreviations used for various types of aberrations are listed in Appendix I.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.

*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

**Table 5 Mitotic index of human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the second cytogenetic assay**

Period of treatment: From: 24-02-2010 To: 26-02-2010

| Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentration (µg/ml) | Number of metaphases per 1000 cells [a] | |
|---|---|---|
| | Absolute | Percentage of control |
| Without metabolic activation (-S9-mix) | | |
| 24 h exposure time, 24 h fixation time | | |
| Control [b] | 48 - 40 | 100 |
| 10 | 46 - 44 | 102 |
| 30 | 47 - 42 | 101 |
| 100 | 50 - 43 | 106 |
| 150 | 37 - 33 | 80 |
| 200 | 32 - 34 | 75 |
| 250 | 22 - 27 | 56 |
| 300 | 20 - 19 | 44 |
| 350 | 24 - 18 | 48 |
| 400 | 10 - 13 | 26 |
| MMC-C; 0.2 µg/ml | 17 - 19 | 41 |
| 48 h exposure time, 48 h fixation time | | |
| Control [b] | 37 - 33 | 100 |
| 10 | 37 - 32 | 99 |
| 30 | 27 - 27 | 77 |
| 50 | 29 - 28 | 81 |
| 100 | 34 - 24 | 83 |
| 150 | 24 - 23 | 67 |
| 200 | 20 - 20 | 57 |
| 250 | 12 - 12 | 34 |
| 300 | 12 - 9 | 30 |
| MMC-C; 0.1 µg/ml | 15 - 17 | 46 |

[a] Duplicate cultures
[b] RPMI 1640 medium

**Table 5 - Continued**

| Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentration (µg/ml) | Number of metaphases per 1000 cells [a] | |
|---|---|---|
| | Absolute | Percentage of control |
| With metabolic activation (+S9-mix) | | |
| 3 h exposure time, 48 h fixation time | | |
| Control [b] | 32 - 30 | 100 |
| 100 | 31 - 34 | 105 |
| 300 | 20 - 22 | 68 |
| 400 | 13 - 13 | 42 |
| 500 | 13 - 16 | 47 |
| 600 | 9 - 13 | 35 |
| 700 | 14 - 13 | 44 |
| 800 | 8 - 7 | 24 |
| CP; 10 µg/ml | 27 - 29 | – [c] |

[a] Duplicate cultures
[b] RPMI 1640 medium
[c] CP was fixed after 24 hours. Therefore, the mitotic index could not be calculated as percentage of control.

**Table 6 Mitotic index of human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in cytogenetic assay 2A**

Period of treatment: From: 03-03-2010 To: 05-03-2010

| Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) concentration (µg/ml) | Number of metaphases per 1000 cells [a] | |
|---|---|---|
| | Absolute | Percentage of control |
| Without metabolic activation (-S9-mix) | | |
| 48 h exposure time, 48 h fixation time | | |
| Control [b] | 66 - 64 | 100 |
| 10 | 62 - 60 | 94 |
| 100 | 49 - 51 | 77 |
| 150 | 47 - 50 | 75 |
| 200 | 42 - 47 | 68 |
| 220 | 35 - 31 | 51 |
| 240 | 27 - 29 | 43 |
| 260 | 28 - 25 | 41 |
| 280 | 22 - 26 | 37 |
| MMC-C; 0.1 µg/ml | 36 - 39 | 58 |

[a] Duplicate cultures
[b] RPMI 1640 medium

**Table 7** **Chromosome aberrations in human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the absence of S9-mix in the second cytogenetic assay (24 h exposure time, 24 h fixation time)**

| Conc | RPMI 1640 medium | | | 100 µg/ml | | | 200 µg/ml | | | 300 µg/ml | | | MMC-C 0.2 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | | 100 | | | 106 | | | 75 | | | 44 | | | 41 | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [a)] | 1 | 3 | 4 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 2 | 3 | 20 | 16 | ***) 36 |
| No. of Cells with aberrations (- gaps) | 1 | 2 | 3 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 2 | 3 | 19 | 15 | ***) 34 |
| g' | | 1 | | | | | | | | | | | 1 | 1 | |
| g" | | | | | | | | | | | | | 2 | 2 | |
| b' | 1 | 2 | | | | | | 1 | | | 1 | | 5 | 9 | |
| b" | | | | 1 | | | 1 | | | 1 | | | 13 | 9 | |
| m' | | | | | | | | | | | 1 | | 2 | | |
| m" | | | | | | | | | | | | | | | |
| exch. | | | | | | | | | | | | | 2 | | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | | | | | endo | | | poly | |
| total aberr (+ gaps) | 1 | 3 | | 1 | 0 | | 1 | 1 | | 1 | 2 | | 25 | 21 | |
| total aberr (- gaps) | 1 | 2 | | 1 | 0 | | 1 | 1 | | 1 | 2 | | 22 | 18 | |

a) Abbreviations used for various types of aberrations are listed in Appendix I.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.

*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

**Table 8** **Chromosome aberrations in human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the absence of S9-mix in cytogenetic assay 2A (48 h exposure time, 48 h fixation time)**

| Conc | RPMI 1640 medium | | | 10 µg/ml | | | 200 µg/ml | | | 240 µg/ml | | | MMC-C 0.1 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 94 | | | 68 | | | 43 | | | 58 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [a] | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 2 | 3 | 1 | 4 | 19 | 20 | ***) 39 |
| No. of Cells with aberrations (- gaps) | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 3 | 18 | 19 | ***) 37 |
| g' | | | | | | | | | | 1 | | | 1 | 1 | |
| g" | | | | | | | | | | | | | 1 | 4 | |
| b' | 1 | | | | | | 1 | | | 2 | 1 | | 4 | 4 | |
| b" | | | | | 1 | | | | | | | | 11 | 11 | |
| m' | | | | | | | 1 | | | | | | 3 | | |
| m" | | | | | | | | 1 | | | | | | 3 | |
| exch. | | | | | | | | | | | | | 2 | 4 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | | | | | poly | | 2p poly | p | |
| total aberr (+ gaps) | 1 | 0 | | 0 | 1 | | 2 | 1 | | 3 | 1 | | 24 | 28 | |
| total aberr (- gaps) | 1 | 0 | | 0 | 1 | | 2 | 1 | | 2 | 1 | | 22 | 23 | |

[a] Abbreviations used for various types of aberrations are listed in Appendix I.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
The numerical variation polyploidy (poly) was not counted as an aberration.

*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

**Table 9** **Chromosome aberrations in human lymphocyte cultures treated with Alkylamidoamine glycinate, majority in C12 & 14 (amphoacetate) in the presence of S9-mix in the second cytogenetic assay (3 h exposure time, 48 h fixation time)**

| Conc | RPMI 1640 medium | | | 100 µg/ml | | | 300 µg/ml | | | 400 µg/ml | | | CP 10 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 105 | | | 68 | | | 42 | | | - [b] | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [a] | 0 | 1 | 1 | 0 | 3 | 3 | 1 | 0 | 1 | 1 | 2 | 3 | 24 | 21 | 45 *** |
| No. of Cells with aberrations (- gaps) | 0 | 1 | 1 | 0 | 2 | 2 | 1 | 0 | 1 | 1 | 2 | 3 | 22 | 17 | 39 *** |
| g' | | | | | | | | | | | | | 3 | 1 | |
| g" | | | | | 1 | | | | | | | | | 3 | |
| b' | | 1 | | | | | | | | | 1 | | 13 | 11 | |
| b" | | | | | 1 | | | | | 1 | 1 | | 11 | 6 | |
| m' | | | | | 1 | | 1 | | | | | | | | |
| m" | | | | | | | | | | | | | | | |
| exch. | | | | | | | | | | | | | 1 | 4 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | | | | 6poly | | | | | |
| total aberr (+ gaps) | 0 | 1 | | 0 | 3 | | 1 | 0 | | 1 | 2 | | 28 | 25 | |
| total aberr (- gaps) | 0 | 1 | | 0 | 2 | | 1 | 0 | | 1 | 2 | | 25 | 21 | |

a) Abbreviations used for various types of aberrations are listed in Appendix I.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
The numerical variation polyploidy (poly) was not counted as an aberration.

b) CP was fixed after 24 hours. Therefore, the mitotic index could not be calculated as percentage of control.

*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

## APPENDIX I DEFINITIONS OF CHROMOSOME ABERRATIONS SCORED IN METAPHASE PORTRAITS

| Aberration | Abbreviation | Description |
|---|---|---|
| Chromatid gap | g' | An achromatic lesion which appears as an unstained region in the chromatid arm, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatid arm are in alignment. |
| Chromosome gap | g" | An achromatic lesion which appears as an unstained region in both chromatids at the same position, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatids are in alignment. |
| Chromatid break | b' | An achromatic lesion in a chromatid arm, the size of which is larger than the width of the chromatid. The broken segments of the chromatid arm are aligned or unaligned. |
| Chromosome break | b" | An achromatic lesion in both chromatids at the same position, the size of which is larger than the width of the chromatid. The broken segments of the chromatids are aligned or unaligned. |
| Chromatid deletion | d' | Deleted material at the end of a chromatid arm. |
| Minute | m' | A single, usually circular, part of a chromatid lacking a centromere. |
| Double minutes | m" | Two, usually circular, parts of a chromatid lacking a centromere. |
| Dicentric chromosome | dic | A chromosome containing two centromeres. |
| Tricentric chromosome | tric | A chromosome containing three centromeres. |

**APPENDIX I – continued –**

| Aberration | Abbreviation | Description |
|---|---|---|
| Ring chromosome | r | A ring structure with a distinct lumen. |
| Exchange figure | exch. | An exchange(s) between two or more chromosomes resulting in the formation of a tri- or more-armed configuration. |
| Chromosome intrachange | intra | A chromosome intrachange is scored after rejoining of a lesion within one chromosome. |
| Pulverized chromosomes | p | A fragmented or pulverized chromosome |
| Multiple aberrations | ma | A metaphase spread containing ten or more of the above mentioned aberrations (chromatid and chromosome gaps not included). ma is counted as 10 aberrations. |
| Polyploidy | poly | A chromosome number that is a multiple of the normal diploid number. |
| Endoreduplication | endo | A form of polyploidy in which each centromere connects two or four pairs of chromatids instead of the normal one pair. |

## APPENDIX II STATISTICAL EVALUATION OF THE TEST RESULTS

Chi-square Test

TOTAL NUMBER OF CELLS WITH ABERRATIONS; TREATMENT/CONTROL COMPARISON, (INCLUSIVE/EXCLUSIVE GAPS) [1].

| EXPOSURE DOSE (µg/ml) LEVEL | S9-MIX | GAPS | P-VALUE one-sided | DECISION AT 95% CONFIDENCE |
|---|---|---|---|---|
| **First cytogenetic assay** | | | | |
| 24 h fixation time | | | | |
| *Positive controls* | | | | |
| MMC-C (0.5) | - | + | ≤0.0001 | significant |
| | - | - | ≤0.0001 | significant |
| CP (10) | + | + | ≤0.0001 | significant |
| | + | - | ≤0.0001 | significant |
| **Second cytogenetic assay** | | | | |
| 24 h fixation time | | | | |
| *Positive controls* | | | | |
| MMC-C (0.2) | - | + | ≤0.0001 | significant |
| | - | - | ≤0.0001 | significant |
| CP (10) | + | + | ≤0.0001 | significant |
| | + | - | ≤0.0001 | significant |
| 48 h fixation time | | | | |
| *Positive control* | | | | |
| MMC-C (0.1) | - | + | ≤0.0001 | significant |
| | - | - | ≤0.0001 | significant |

[1] Only statistically significant results are presented.

## APPENDIX III HISTORICAL CONTROL DATA FOR LYMPHOCYTE CHROMOSOME ABERRATION STUDIES

| | Aberrant cells per 100 metaphases | | | |
|---|---|---|---|---|
| | Gaps included | | Gaps excluded | |
| | + S9-mix | - S9-mix | + S9-mix | - S9-mix |
| Range | 0 - 6 | 0 - 6 | 0 - 5 | 0 - 5 |
| Mean | 0.6 | 0.8 | 0.5 | 0.6 |
| SD | 0.9 | 1.0 | 0.8 | 0.8 |
| n | 438 | 652 | 441 | 652 |

SD = Standard deviation
n = Number of observations

Historical control data from experiments performed between January 2007 and December 2009.

## APPENDIX IV HISTORICAL CONTROL DATA FOR NUMERICAL ABERRATIONS FOR LYMPHOCYTE CHROMOSOME ABERRATION STUDIES

| | Polyploid cells and endoreduplicated chromosomes per 100 metaphases | | | |
|---|---|---|---|---|
| | polyploidy | | endoreduplication | |
| | + S9-mix | - S9-mix | + S9-mix | - S9-mix |
| Range | 0 - 2 | 0 - 2 | 0 - 2 | 0 - 1 |
| Mean | 0.04 | 0.04 | 0.02 | 0.01 |
| SD | 0.22 | 0.21 | 0.15 | 0.10 |
| n | 437 | 652 | 436 | 653 |

SD = Standard deviation
n = Number of observations

Historical control data from experiments performed between January 2007 and December 2009.

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