Substance: Glyphosate
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REPORT

EVALUATION OF THE ABILITY OF GLYFOSAAT TO INDUCE CHROMOSOME ABERRATIONS IN CULTURED PERIPHERAL HUMAN LYMPHOCYTES (WITH INDEPENDENT REPEAT)

NOTOX Project 141918
NOTOX Substance 48033

STATEMENT OF GLP COMPLIANCE

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

The study described in this report was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director Ing. [redacted]

[redacted]

Date: 30/06/1995

QUALITY ASSURANCE STATEMENT

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

Study procedures were subject to periodic inspections and general non study specific processes were also inspected at periodic intervals.

This report was audited by the NOTOX Quality Assurance Unit and the methods and results accurately reflect the raw data.

DATES OF QAU INSPECTIONS/ AUDITS	REPORTING DATES
10-03-1995 27-04-1995 29-06-1995	10-03-1995 27-04-1995 29-06-1995

Quality Assurance Manager [redacted]

[redacted]

Date: 30-6-95

REPORT APPROVAL

STUDY DIRECTOR: Ing. [redacted]

[redacted]

Date: 30/06/1995

MANAGEMENT: Dr. [redacted]
Technical Director

[redacted]

SUMMARY

This report describes the effect of GLYFOSAAT on the induction of chromosome aberrations in cultured peripheral human lymphocytes in the presence and absence of a metabolic activation system (Aroclor-1254 induced rat liver S9-mix).

In the absence of S9-mix GLYFOSAAT was tested up to 237 µg/ml for a 24 h and 48 h fixation time in the first experiment. In the second experiment GLYFOSAAT was tested up to 333 µg/ml for a 24 h fixation time.
In the presence of 1.8% (v/v) S9-fraction GLYFOSAAT was tested up to 562 µg/ml for a 24 h and 48 h fixation time in the first experiment. In the second experiment GLYFOSAAT was tested up to 562 µg/ml for a 24 h fixation time.

None of the tested concentrations induced a statistically and biologically significant increase in the number of cells with chromosome aberrations, neither in the absence nor in the presence of S9-mix.

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.

It is concluded that GLYFOSAAT is not clastogenic in human lymphocytes under the experimental conditions described in this report.

PREFACE

Sponsor	Agrichem B.V. P.O. Box 295 4900 AG OOSTERHOUT The Netherlands
Study Monitor	
Testing Facility	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
Study Director	
Technical Head	
Study Plan	Start : 15 March 1995 Completed : 28 May 1995

TEST SUBSTANCE

Identification	GLYFOSAAT
Description	White powder
Batch	22021
Purity	96%
Instructions for test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	01 January 1998
Stable for at least 4 hours in vehicle	Dimethylsulphoxide : not indicated

The sponsor is responsible for the completeness and GLP Compliance of all test substance data.

VEHICLE

The test substance was suspended in dimethylsulphoxide (DMSO) of spectroscopic quality (Merck). Test substance concentrations were prepared directly prior to use.
The final concentration of the solvent in the culture medium amounted to 0.9 % (v/v).

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: "Genetic Toxicology: In Vitro Mammalian Cytogenetic Test", (adopted May 26, 1983).
- European Economic Community (EEC), Directive 92/69/EEC. Annex V of the EEC Directive 67/548/EEC, Part B: Methods for the Determination of Toxicity; B.10: "Other Effects-Mutagenicity: In Vitro Mammalian Cytogenetic Test". EEC Publication no. L383 (adopted December, 1992).

ARCHIVING

NOTOX B.V. will archive the protocol, report, test article reference sample, all specimens and raw data for at least 10 years.

OBJECTIVE

Purpose of the study

The objective of this study was to evaluate the test substance for its ability to induce structural chromosome aberrations in cultured human lymphocytes, either in the presence or absence of a metabolic system (S9-mix).

Justification and rationale of the test system

Stimulated cultured human lymphocytes were used because they are sensitive indicators of clastogenic activity of a broad range of chemical classes. In combination with a mammalian metabolizing system (S9-mix) also indirect chemical mutagens, i.e. those requiring metabolic transformation into reactive intermediates, could be tested for clastogenic effects in vitro.
Following treatment, cell division was arrested in the metaphase stage of the cell cycle by addition of the spindle poison colchicine. Structural chromosome changes such as breaks, gaps, minutes, dicentrics and exchange figures were examined microscopically in cultures treated with the test substance and the results were compared with those of the control (vehicle-treated) cultures. Chromosome aberrations were generally evaluated in the first post-treatment mitosis. The appearance of the first post-treatment mitosis could be considerably delayed, due to toxic insult of the cells. Therefore, cells were harvested at 24 h and 48 h after beginning of treatment to cover the interval in which maximum aberration frequency was expected.
A test article which induced a positive response in this assay was presumed to be a potential mammalian cell clastogenic agent.

MATERIALS AND METHODS

TEST SYSTEM

Test System	Cultured peripheral human lymphocytes
Rationale	Recognized by the international guidelines as the recommended test system (e.g. EPA, OECD, EEC).
Source	Healthy adult male volunteers pilot study : age 35, AGT = 15.0 h (Dec. 1994) experiment 1: age 28, AGT = 15.0 h (Dec. 1994) experiment 2: age 28, AGT = 14.9 h (Dec. 1994) (AGT= Average Generation Time of the cells)

CELL CULTURE

Blood samples	Blood samples were taken from a healthy adult male volunteer by venapuncture using the Venoject multiple sample blood collecting system with a suitable size sterile vessel containing sodium heparin. The blood samples were stored at a temperature between 4 and 25°C. Within 4 h after withdrawal lymphocyte cultures were started.
F10 complete culture medium	F10 complete culture medium consisted of Ham's F10 medium without thymidine and hypoxanthine (Gibco), supplemented with 20% (v/v) heat-inactivated (56°C; 30 min) foetal calf serum (Gibco), L-glutamine (2 mM), penicillin/streptomycin (50 U/ml and 50 µg/ml respectively), sodium bicarbonate (1.2 g/l) and 30 U/ml heparin.
Cell culture conditions	Whole blood was cultured in F10 complete culture medium with Phytohaemagglutinin (Murex). Per culture (5 ml F10 complete culture medium and 0.4 ml whole blood) 0.1 ml (9 mg/ml) Phytohaemagglutinin was added.
Environmental conditions	All incubations were carried out in a humid atmosphere (80-95%) containing 5% CO_2 in air in the dark at 37°C. The temperature, humidity and CO_2-percentage were monitored during the experiment.

REFERENCE SUBSTANCES

Negative control:
The vehicle of the test article, being dimethylsulphoxide (DMSO).

Positive controls:

Solvent for reference substances
Hank's Balanced Salt Solution (HBSS) without calcium and magnesium.

Without metabolic activation (-S9-mix):
Mitomycin C (MMC-C; CAS no. 50-07-7, Sigma, U.S.A.) was used as a direct acting mutagen at a final concentration of 0.2 µg/ml (solvent: HBSS) for a 24 h treatment period and 0.1 µg/ml (solvent: HBSS) for a 48 h treatment period.

With metabolic activation (+S9-mix):
Cyclophosphamide (CP; CAS no. 50-18-0. Endoxan-Asta, Asta-Werke, F.R.G.) was used as an indirect acting mutagen, requiring metabolic activation, at a final concentration of 15 µg/ml (solvent: HBSS) for a 3 h treatment period (24 h fixation time).

METABOLIC ACTIVATION SYSTEM

Preparation of S9-homogenate:

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats, which were obtained from BRL, Switzerland, for the dose range finding test and the first experiment and for the second experiment adult male Wistar rats, were obtained from Charles River Wiga, Sulzfeld, Germany.

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the SOP's. The rats were injected intraperitoneally with a solution (20% w/v) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days later, they were killed by decapitation; (they were denied access to food for at least 12 hours preceding 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. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. 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).

Preparation of S9-mix:

S9-mix was prepared immediately before use and kept on ice during the test. S9-mix contained per ml: 1.02 mg $MgCl_2.6H_2O$; 2.46 mg KCl; 1.7 mg glucose-6-phosphate; 3.4 mg NADP; 4 µmol HEPES and 0.5 ml S9-fraction. 0.5 ml S9-fraction was added to 0.5 ml filter (0.22 µm)-sterilized S9-mix components (50% v/v S9-fraction).

The S9-batch used in the dose range finding test was no. 94-6 (final concentration of Cytochrome P-450 in the S9-mix was 22.3 nmol/ml).
The S9-batch used in experiment 1 was no. 94-7 (final concentration of Cytochrome P-450 in the S9-mix was 23.6 nmol/ml).
The S9-batch used in experiment 2 was no. 95-1 (final concentration of Cytochrome P-450 in the S9-mix was 25.9 nmol/ml).

Metabolic activation was achieved by adding 0.2 ml liver S9-mix to 5.3 ml exposition medium (4.8 ml F10 complete culture medium, 0.4 ml blood and 0.1 ml (9 mg/ml) Phytohaemagglutinin). The concentration of the S9-fraction in the exposition medium was 1.8% (v/v).

EXPERIMENTAL PROCEDURE

Dose range finding test

In order to obtain the appropriate dose levels for the chromosome aberration test a dose range finding test was performed. The test substance was tested in the absence of S9-mix and in the presence of S9-mix.

Lymphocyte cultures (0.4 ml blood of a healthy male donor was added to 5 ml culture medium and 0.1 ml (9 mg/ml) Phytohaemagglutinin) were cultured for 48 h and thereafter exposed to selected doses of the test substance for 24 h and 48 h without S9-mix or for 3 h with S9-mix.

The highest concentration was determined by the solubility of the test substance in the culture medium.

After 3 h treatment, the cells exposed to the test substance in the presence of S9-mix were rinsed once with 5 ml of HBSS and incubated in 5 ml culture medium for another 20-22 h (24 h fixation time).
The cells which were treated for 24 h and 48 h in the absence of S9-mix were not rinsed after treatment but were worked up immediately after 24 h and 48 h (24 h and 48 h fixation time).

During the last 3 h of the culture period, cell division was arrested by addition of the spindle inhibitor colchicine (0.5 µg/ml medium). Thereafter the cell cultures were centrifuged for 5 min at 1300 rpm (150 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 solution at 37°C. After hypotonic treatment, cells were fixed with 3 changes of methanol: acetic acid fixative (3:1 v/v). Slides were prepared and the mitotic index of each culture was determined by counting the number of metaphases per 1000 cells.

Based on the results of the dose range finding test an appropriate range of dose levels was chosen for the cytogenetic assay.

Cytogenetic assay

The test is carried out with minor modifications as described by Evans (1984).
The test substance was tested in the absence of S9-mix and in the presence of S9-mix in duplicate in two independent experiments.

Lymphocyte cultures (0.4 ml blood of a healthy male donor was added to 5 ml culture medium and 0.1 ml (9 mg/ml) Phytohaemagglutinin) were cultured for 48 h and thereafter exposed in duplicate to selected doses of the test substance for 24 h and 48 h without S9-mix or for 3 h with S9-mix.

After 3 h treatment, the cells exposed to the test substance in the presence of S9-mix were rinsed once with 5 ml of HBSS and incubated in 5 ml culture medium for another 20-22 h (24 h fixation time) or for 44-46 h (48 h fixation time).

The cells which were treated for 24 h and 48 h in the absence of S9-mix were not rinsed after treatment but were worked up immediately after 24 h and 48 h (24 h and 48 h fixation time).

During the last 3 h of the culture period, cell division was arrested by addition of the spindle inhibitor colchicine (0.5 µg/ml medium). Thereafter the cell cultures were centrifuged for 5 min at 1300 rpm (150 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 solution at 37°C. After hypotonic treatment, cells were fixed with 3 changes of methanol: acetic acid fixative (3:1 v/v).

Based on the mitotic index of the dose range finding test and the first experiment, appropriate dose levels were chosen for the second experiment. For the independent repeat the 24 h fixation time is needed only.

Preparation of slides

Fixed cells were dropped onto slides which were immersed for 24 hours in a 1:1 mixture of 96% (v/v) ethanol/ether and cleaned with a tissue. The slides were marked with the NOTOX study identification number and group number. Two slides were prepared per culture. Slides were allowed to dry and thereafter stained for 10-30 min with 5% (v/v) Giemsa 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 before they were embedded in DePeX and mounted with a coverslip.

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. For the first fixation time (24 h harvest) chromosomes of metaphase spreads were analysed of those cultures with an inhibition of the mitotic index of about 50% or greater whereas the mitotic index of the lowest dose level was approximately the same as the solvent control. Also cultures treated with an intermediate dose were examined for chromosome aberrations. For the second fixation time (48 h harvest) one appropriate dose level was selected for scoring of chromosome aberrations.

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 stuck over the marked slide. At least 100 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 chromosomes were analysed. The number of cells with aberrations and the number of aberrations were calculated.

ACCEPTABILITY OF ASSAY

A chromosome aberration test was considered acceptable if it met the following criteria:

a) The numbers of chromosome aberrations found in the solvent control cultures should reasonably be within the laboratory historical control data range.
b) The positive control substances should produce a statistically significant (Chi-square test, $P < 0.05$) increase in the number of cells with chromosome aberrations.

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, $P < 0.05$) increase in the number of cells with chromosome aberrations.
b) A statistically significant increase in the frequency of 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:

a) None of the tested concentrations induced a statistically significant (Chi-square test, $P < 0.05$) increase in the number of cells with chromosome aberrations.

The preceding criteria were 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, inclusive or exclusive gaps) for each treatment group was compared to that of the solvent control using Chi-square statistics:

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

where b = the total number of aberrant cells in the control cultures.
d = the total number of nonaberrant 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 nonaberrant 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

$$\text{If } P \left[X^2 > \frac{(N-1) \times (ad-bc)^2}{(a+b)\ (c+d)\ (a+c)\ (b+d)} \right] \qquad \text{(two-tailed)}$$

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.

RESULTS

Dose range finding test

At a concentration of 1000 µg/ml GLYFOSAAT precipitated in the culture medium. Therefore, a concentration of 1000 µg/ml was used as the highest concentration of GLYFOSAAT.

In the dose range finding test blood cultures were treated with 10, 33, 100, 333 and 1000 µg/ml culture medium with and without S9-mix (Table 1).

TABLE 1 MITOTIC INDEX OF DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT
DOSE RANGE FINDING TEST

Test substance concentration (µg/ml)	Number of metaphases per 1000 cells Absolute	Percentage of control
Without metabolic activation (-S9-mix)		
24 h fixation time		
Control[a)]	36	100
10	27	75
33	32	89
100	25	69
333	0	0
1000[b)]	0	0
48 h fixation time		
Control[a)]	44	100
10	40	91
33	31	70
100	28	64
333	15	34
1000[b)]	0	0
With metabolic activation (+S9-mix)		
24 h fixation time		
Control[a)]	63	100
10	68	108
33	61	97
100	55	87
333	12	19
1000[b)]	58	92

a) DMSO
b) GLYFOSAAT precipitated in the exposition medium

Further investigation showed that a concentration of 562 µg/ml GLYFOSAAT already precipitated in the F10 complete culture medium. Therefore, this dose was selected as higesht dose in the presence of S9-mix, all other dose groups did not precipitate in the F10 complete culture medium.

Based on the results of the dose range finding test the following dose levels were selected for the chromosome aberration test:

Experiment 1

Without S9-mix : 33, 56, 100, 133, 178 and 237 µg/ml culture medium (24 h fixation time)
56, 100, 133, 178, 237 and 333 µg/ml culture medium (48 h fixation time)

With S9-mix : 33, 100, 133, 178, 237, 333 and 562 µg/ml culture medium (24 h fixation time)
100, 133, 178, 237, 333 and 562 µg/ml culture medium (48 h fixation time)

Table 2 shows the mitotic index of cultures (from blood of a healthy male donor) treated with various test substance concentrations or with the positive or negative control substances.

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

Without S9-mix : 33, 100 and 237 µg/ml culture medium (24 h fixation time)
237 µg/ml (48 h fixation time)

With S9-mix : 237, 333 and 562 µg/ml culture medium (24 h fixation time)
562 µg/ml (48 h fixation time).

The data of the dose range finding test and the first cytogenetic assay were used to determine the dose levels for the second cytogenetic assay.

Experiment 2

Without S9-mix : 33, 100, 133, 178, 237 and 333 µg/ml culture medium (24 h fixation time)

With S9-mix : 100, 333, 422 and 562 µg/ml culture medium (24 h fixation time)

Table 3 shows the mitotic index of cultures (from blood of a healthy male donor) treated with various test substance concentrations or with the positive or negative control substances.

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

Without S9-mix : 33, 237 and 333 µg/ml culture medium
(24 h fixation time)
With S9-mix : 333, 422 and 562 µg/ml culture medium
(24 h fixation time)

Cytogenetic assay

The ability of GLYFOSAAT to induce chromosome aberrations in human peripheral lymphocytes was investigated. The test was carried out in duplicate in two independent experiments. The results of duplicate cultures are indicated by A and B. The scores for the numbers of aberrant cells (inclusive and exclusive gaps) and the numbers of the various types of chromosome aberrations at the various concentrations of the test substance are presented in Tables 4-9. The criteria according to which the aberrations were classified are outlined in Appendix 1.

Both in the absence and presence of S9-mix GLYFOSAAT did not induce a statistically and biologically significant increase in the number of cells with chromosome aberrations.

The number of cells with chromosome aberrations found in the solvent control cultures were within the laboratory historical control data range {i.e. 1.0 ± 1.1 (mean ± standard deviation) aberrant cells per 100 metaphases (without S9-mix; gaps excluded) and 0.8 ± 0.9 aberrant cells per 100 metaphases (with S9-mix; gaps excluded)}. 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 appropriate and that the metabolic activation system (S9-mix) functioned properly.

Finally, it is concluded that this test should be considered valid and that GLYFOSAAT is not clastogenic under the experimental conditions of this test.

TABLE 2 MITOTIC INDEX OF DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT
Experiment 1

Test substance concentration (µg/ml)	Number of metaphases per 1000 cells b) Absolute	Percentage of control
Without metabolic activation (-S9-mix)		
24 h fixation time		
Control[a)]	27 - 18	100
33	26 - 17	96
56	17 - 22	87
100	19 - 16	78
133	12 - 5	38
178	9 - 10	42
237	9 - 12	47
MMC-C; 0.2 µg/ml	10 - 10	44
48 h fixation time		
Control[a)]	20 - 34	100
56	36 - 45	150
100	28 - 26	100
133	20 - 23	80
178	20 - 29	91
237	19 - 16	65[d)]
333	8 - 21	54
MMC-C; 0.1 µg/ml	25 - 20	83
With metabolic activation (+S9-mix)		
24 h fixation time		
Control[a)]	46 - 37	100
33	66 - 49	139
100	49 - 42	110
133	66 - 43	131
178	44 - 44	106
237	44 - 40	101
333	38 - 36	89
562[c)]	33 - 13	55
CP; 15 µg/ml	15 - 12	33
48 h fixation time		
Control[a)]	52 - 55	100
100	66 - 63	121
133	74 - 69	134
178	60 - 67	119
237	66 - 67	124
333	62 - 68	121
562[c)]	62 - 67	121

a) DMSO
b) duplicate cultures
c) GLYFOSAAT precipitated in the exposition medium
d) This concentration was selected for scoring of chromosome aberrations because it showed a clear indication of cytotoxicity at the 24 h fixation period (mitotic index was reduced by 53%).

TABLE 3 MITOTIC INDEX OF DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT
Experiment 2

Test substance concentration (µg/ml)	Number of metaphases per 1000 cells b) Absolute	Percentage of control
Without metabolic activation (-S9-mix)		
24 h fixation time		
Control[a)]	40 - 37	100
33	31 - 34	84
100	22 - 26	62
133	23 - 29	68
178	20 - 17	48
237	21 - 26	61
333	16 - 10	34
MMC-C; 0.2 µg/ml	15 - 18	43
With metabolic activation (+S9-mix)		
24 h fixation time		
Control[a)]	64 - 59	100
100	56 - 61	95
333	60 - 55	93
422	44 - 52	78
562[c)]	53 - 52	85
CP; 15 µg/ml	21 - 23	36

a) DMSO
b) duplicate cultures
c) GLYFOSAAT precipitated in the exposition medium

TABLE 4 CHROMOSOME ABERRATIONS IN DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT (Without S9-mix)[a)]
24 h fixation time
Experiment 1

Conc µg/ml	DMSO (0.9% v/v)			33 µg/ml			100 µg/ml			237 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
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)	2	2	4	2	2	4	1	1	2	2	3	5	38	33	71***
No. of cells with aberrations (- gaps)	2	1	3	1	1	2	1	0	1	2	1	3	27	23	50***
g'		1			1			1			2		7	11	
g"				1									10	10	
b'										2			6	11	
b"				2	1		1						11	8	
m'	2	1											3		
m"															
exch.													8	7	
dic															
d'															
misc.	2poly			p poly	endo poly					poly	p		intra		
total aberr (+ gaps)	2	2		4	2		1	1		2	3		46	47	
total aberr (- gaps)	2	1		3	1		1	0		2	1		29	26	

a) Abbreviations used for various types of aberrations are listed in appendix 1. The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
*) Significantly different from control group (Chi-square test),
* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

TABLE 5 CHROMOSOME ABERRATIONS IN DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT (With S9-mix)[a)]
24 h fixation time
Experiment 1

Conc µg/ml	DMSO (0.9% v/v)			237 µg/ml			333 µg/ml			562 µg/ml			CP 15 µg/ml		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+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)	1	5	6	2	0	2	2	1	3	1	3	4	57	32	89***
No. of cells with aberrations (- gaps)	1	3	4	0	0	0	1	1	2	1	3	4	37	16	53***
g'		1		2			1			1			22	13	
g"		1											21	13	
b'		1					1						25	10	
b"		1									2		23	2	
m'		1													
m"	1										1				
exch.										2	1		6	4	
dic															
d'							1								
misc.		poly					poly	poly		4endo	poly endo		poly	p ma	
total aberr (+ gaps)	1	5		2	0		2	1		3	4		97	53	
total aberr (- gaps)	1	3		0	0		1	1		2	4		54	27	

a) Abbreviations used for various types of aberrations are listed in appendix 1. The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.
misc.= (miscellaneous) aberrations not belonging to the ones mentioned above.
*) Significantly different from control group (Chi-square test),
* P < 0.05, ** P < 0.01 or *** P < 0.001.

TABLE 6 CHROMOSOME ABERRATIONS IN DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT (Without S9-mix)[a)]
48 h fixation time
Experiment 1

Conc µg/ml	DMSO (0.9% v/v)			237 µg/ml			MMC-C 0.1 µg/ml		
Culture	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200
No. of cells with aberrations (+ gaps)	1	3	4	0	1	1	36	45	81 ***
No. of cells with aberrations (- gaps)	1	0	1	0	0	0	32	35	67 ***
g'		2			1		16	12	
g"		1					5	9	
b'							9	6	
b"							24	17	
m'									
m"	2								
exch.							8	17	
dic							2		
d'									
misc.				poly				p endo	
total aberr (+ gaps)	2	3		0	1		64	62	
total aberr (- gaps)	2	0		0	0		43	41	

a) Abbreviations used for various types of aberrations are listed in appendix 1. The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.
misc.= (miscellaneous) aberrations not belonging to the ones mentioned above.

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

TABLE 7 CHROMOSOME ABERRATIONS IN DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT (With S9-mix)[a)]
48 h fixation time
Experiment 1

Conc µg/ml	DMSO (0.9% v/v)			562 µg/ml		
Culture	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200
No. of cells with aberrations (+ gaps)	1	0	1	0	0	0
No. of cells with aberrations (- gaps)	0	0	0	0	0	0
g'	1					
g"						
b'						
b"						
m'						
m"						
exch.						
dic						
d'						
misc.						
total aberr (+ gaps)	1	0		0	0	
total aberr (- gaps)	0	0		0	0	

a) Abbreviations used for various types of aberrations are listed in appendix 1. The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.
misc.= (miscellaneous) aberrations not belonging to the ones mentioned above.

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

TABLE 8 CHROMOSOME ABERRATIONS IN DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT (Without S9-mix)[a)]
24 h fixation time
Experiment 2

Conc µg/ml	DMSO (0.9% v/v)			33 µg/ml			237 µg/ml			333 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
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)	0	1	1	0	2	2	4	1	5	2	2	4	25	26	51***
No. of cells with aberrations (- gaps)	0	0	0	0	0	0	3	0	3	1	1	2	25	26	51***
g'		1			2		1	1		1	1		1		
g"														2	
b'							3			1	1		21	16	
b"													8	7	
m'															
m"															
exch.													2	6	
dic															
d'															
misc.											endo				
total aberr (+ gaps)	0	1		0	2		4	1		2	2		32	31	
total aberr (- gaps)	0	0		0	0		3	0		1	1		31	29	

a) Abbreviations used for various types of aberrations are listed in appendix 1. The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
*) Significantly different from control group (Chi-square test),
* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

TABLE 9 CHROMOSOME ABERRATIONS IN DONOR CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF GLYFOSAAT (With S9-mix)[a)]
24 h fixation time
Experiment 2

Conc µg/ml	DMSO (0.9% v/v)			333 µg/ml			422 µg/ml			562 µg/ml			CP 15 µg/ml		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+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)	3	1	4	3	4	7	2	2	4	1	2	3	26	27	53***
No. of cells with aberrations (- gaps)	2	1	3	2	3	5	2	0	2	0	1	1	26	27	53***
g'	1			1	1			2		1	1			1	
g"															
b'	2	1		2	2		2				1		27	25	
b"					1								9	11	
m'															
m"															
exch.													8	7	
dic														1	
d'															
misc.				poly											
total aberr (+ gaps)	3	1		3	4		2	2		1	2		44	45	
total aberr (- gaps)	2	1		2	3		2	0		0	1		44	44	

a) Abbreviations used for various types of aberrations are listed in appendix 1. The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.
misc.= (miscellaneous) aberrations not belonging to the ones mentioned above.
*) Significantly different from control group (Chi-square test),
* P < 0.05, ** P < 0.01 or *** P < 0.001.

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APPENDIX 1

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 1 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 2

STATISTICAL EVALUATION OF TEST RESULTS

Chi-square Test

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

Experiment 1

TREATMENT DOSE (µg/ml)	S9-MIX	GAPS	P-VALUE two-sided	DECISION AT 95% CONFIDENCE LEVEL
24 h fixation time				
MMC-C (0.2)	-	+	≤0.0004	significant
	-	-	≤0.0004	significant
CP (15)	+	+	≤0.0004	significant
	+	-	≤0.0004	significant
48 h fixation time				
MMC-C (0.1)	-	+	≤0.0004	significant
	-	-	≤0.0004	significant

Experiment 2

TREATMENT DOSE (µg/ml)	S9-MIX	GAPS	P-VALUE two-sided	DECISION AT 95% CONFIDENCE LEVEL
24 h fixation time				
MMC-C (0.2)	-	+	≤0.0004	significant
	-	-	≤0.0004	significant
CP (15)	+	+	≤0.0004	significant
	+	-	≤0.0004	significant