CYTOGENETIC TEST ON CHINESE HAMSTER CELLS IN VITRO

(EC-CONFORM)

Test Number: 943013

Test Substance: CGA 329 351 tech.

REPORT

Author: Dr. 5.1.2.e Woo

Test Guidelines: OECD 473 (May 26, 1983)
EPA § 798.5375 (May 20, 1987)
EEC B.10 (December 29, 1992)
MAFF Japan (January 1985)

Performing Laboratory: Genetic Toxicology
CIBA-GEIGY Limited
Basle, Switzerland

Study completed: June 29, 1994

Sponsor: Plant Protection Division
CIBA-GEIGY Limited
Basle, Switzerland

Volume 1 of 1 of study

This report contains 37 numbered pages

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CERTIFICATION OF GOOD LABORATORY PRACTICE AND VERIFICATION OF A COMPLETE AND UNALTERED COPY OF THE REPORT BY THE STUDY SPONSOR

The Statement of Compliance with Good Laboratory Practice found on page 4 of this report, and signed by the Study Director is truthful and accurate, and this report as provided by the testing facility is complete and unaltered.

5.1.2.e Woo

Signature of the Sponsor Date

Address of the Sponsor: CIBA-GEIGY Limited
Plant Protection Division
Basle, Switzerland

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

This study has been performed in compliance with "Verfahren und Grundsätze der Guten Laborpraxis (GLP) in der Schweiz" (Good Laboratory Practice (GLP) in Switzerland, Procedures and Principles, March 1986), issued by the Swiss Federal Department of the Interior which recognize the OECD Principles of Good Laboratory Practice (Council Decision 81/30, adopted on May 12th 1981, and the OECD Recommendation 83/95 concerning the 'Mutual Recognition of Compliance with Good Laboratory Practice', adopted on July 26th 1983).

The competent Swiss Federal Authorities have signed Memoranda of Understanding concerning the mutual recognition of compliance with Good Laboratory Practice with the following agencies:

- the US Food and Drug Administration (April 20, 1985)
- the US Environmental Protection Agency (June 22, 1988)
- the Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, Plant Protection Division (January 18, 1993)

Study Director: Date: June 28, 1994

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QUALITY ASSURANCE STATEMENT

Project	943013
Test Substance	CGA 329 351 tech.
Study Title	CYTOGENETIC TEST ON CHINESE HAMSTER CELLS IN VITRO (EC-CONFORM)
Study Director	Dr. 5.1.2.e Woo
QA-Inspector	Mr.

I hereby certify that the following Quality Assurance activities were performed:

Activity	Performed	Reported
Facility Inspection	October 20, 1993	December 20, 1993
Protocol Audit	March 10, 1994	March 10, 1994
Facility Inspection	April 14, 1994	April 18, 1994
Final Report Audit	June 27, 1994	June 27, 1994

July 6, 1994

Date

5.1.2.e Woo

Mr. 5.1.2.e Woo
Inspector Quality Assurance

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COMPOUND INFORMATION

Test material :	CGA 329 351 tech.
Batch No.:	KGL 4634/6
Purity:	97.3 %
Physical appearance:	Liquid
Reanalysis date:	February 1996
Stability (in the vehicle used, under the conditions of the test):	Stable
Material submitted by (sponsor):	CIBA-GEIGY Limited, Plant Protection Division, Basle, Switzerland.

GENERAL

Test No.:	943013
Type of study:	Chromosome studies on Chinese hamster ovary cell line in vitro with and without metabolic activation.
SOP No. (Ciba-Geigy):	305108
Test Guidelines:	OECD 473 (May 26, 1983 / Lit. 1) EPA § 798.5375 (May 20, 1987 / Lit. 2) EEC B.10 (December 29, 1992 / Lit. 3) MAFF Japan (January 1985 / Lit. 10)
Purpose:	Evaluation of any clastogenic property of the substance in vitro as manifested by an increased incidence of specific chromosomal aberrations.
Test organism:	Cell line: ATCC (American Type Culture Collection) CCL 61 (ovary, Chinese hamster, CHO K1).
Extrinsic metabolic activation system:	Post mitochondrial supernatant (S9 fraction) from Aroclor 1254 induced rat liver.
Solvent:	DMSO
Positive controls:	**without metabolic activation:** Mitomycin C (KYOWA HAKKO KOGYO Co. Ltd., Japan), 0.2 µg/ml. **with metabolic activation:** Cyclophosphamide (ASTA-WERKE, Germany), 20.0 µg/ml.
Number of independent experiments:	Three experiments without metabolic activation and three trials with metabolic activation (twice 18 hours and once 42 hours each).
Number of replicate cultures evaluated per concentration:	2
Number of cells evaluated per concentration:	200 (whenever possible; except positive controls)

Testing facility:	CIBA-GEIGY Limited, Basle, Switzerland, Laboratories of Genetic Toxicology.
Location of archives:	CIBA-GEIGY Limited, Basle, Switzerland, Archives of Genetic Toxicology.
Responsible for flow cytometry:	Mr. 5.1.2.e Woo
Responsible for analytical study:	Mr. CIBA-GEIGY Limited, Basle, Switzerland, Laboratories of Ecotoxicology, Analytical Services.
Study director:	Dr. 5.1.2.e Woo
Personnel:	Technical conduct: Mrs. 5.1.2.e Woo Mrs. Investigations of the slides: Mrs. 5.1.2.e Woo Mrs.
Sponsor monitor:	Dr. 5.1.2.e Woo CIBA-GEIGY Limited, Plant Protection Division, Basle, Switzerland
Study initiation date:	March 01, 1994
Experimental starting date:	March 07,1994
Experimental termination date:	April 28, 1994
Study completion date:	June 29, 1994

ABSTRACT

CGA 329 351 tech., identified as a liquid, 97.3% purity, batch no. KGL 4634/6, was investigated for clastogenic (chromosome-damaging) effects on Chinese hamster ovary cells in vitro with and without extrinsic metabolic activation (S9). The compound was dissolved in DMSO and tested at each of the following conditions:

Experiments without metabolic activation:
- 18 hours treatment time:
  original experiment: 126.88, 253.75 and 507.50 µg/ml
  confirmatory experiment: 253.75, 507.50 and 1015.0 µg/ml
- 42 hours treatment time: 253.75, 507.50 and 1015.0 µg/ml

Higher concentrations were not scored due to cytotoxicity. Mitomycin C (0.2 µg/ml) was used as a positive control in the 18 hours experiments.

Experiments with metabolic activation:
- 3 hours treatment followed by 15 hours recovery period:
  original experiment: 253.75, 507.50 and 1015.0 µg/ml
  confirmatory experiment: 507.50, 1015.0 and 2030.0 µg/ml
- 3 hours treatment followed by 39 hours recovery period: 507.50, 1015.0 and 2030.0 µg/ml

Final concentrations higher than 2030 µg/ml of culture medium could not be achieved due to solubility limitations. Cyclophosphamide (20.0 µg/ml) was used as a positive control in the 3 hours/15 hours experiments.

In addition, DNA distribution of cultures treated under the above described conditions (18 hours only) was determined by flow cytometry. These measurements allow to analyse the influence of the test substance on the cell cycle of CHO cells.

In both the experiments performed without and with metabolic activation no meaningful increase in the number of metaphases containing specific chromosomal aberrations was observed.

Flow cytometry experiments revealed a weak cell cycle arresting activity of the test substance in the absence of metabolic activation. This effect however is considered not to affect to validity of the chromosome analyses.

CONCLUSIONS

It is concluded that under the given experimental conditions no evidence of clastogenic effects was obtained in Chinese hamster ovary cells in vitro treated with CGA 329 351 tech..

The test was performed under Good Laboratory Practice conditions and was subjected to a periodical quality assurance evaluation.

No circumstances, which may have affected the quality or integrity of the data, have been noted.

Study Director: 5.1.2.e Woo

Date: June 29, 1994

Report reviewed and approved by: 5.1.2.e Woo

(Dr. 5.1.2.e Woo)
(Head of Genetic Toxicology)

Date: June 29, 1994

RATIONALE

This test system permits the detection of structural chromosome aberrations in Chinese hamster ovary cells in vitro induced by the test substance or by its metabolites (Lit. 4,5,7). To ensure that any clastogenic effects of metabolites of the test substance formed in mammals are also detected, an experiment is performed, in which the metabolic turnover of the test material is simulated in vitro by the addition of an activation mixture containing rat liver post mitochondrial fraction S9 and co-factors to the cell culture (Lit. 6,7). Metaphase cells were examined from the cultures treated with the various concentrations of CGA 329 351 tech. and from the respective control cultures for the presence of structural chromosomal aberrations.

PROCEDURE

Maintenance of the cell line

The cell line CCL 61 (Chinese hamster ovary cells, CHO) was maintained in culture medium consisting of Nutrient Mixture F-12 supplemented with 10% fetal calf serum + Penicillin/Streptomycin 100 units/ml/100 µg/ml (Gibco AG, Basle, Switzerland) in 75 cm² tissue-culture (plastic) flasks. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were passaged twice weekly.

The duration of a normal cell cycle determined in our laboratory was 12 - 13 hours.

The cell cultures were periodically checked for mycoplasma contamination.

Genome stability of the cell line

The cell line CHO CCL 61 has been used for cytogenetic studies for several years. The stability of the genome of these cells can be assessed on the basis of the regular (cytogenetic) analysis of control cultures in the course of the cytogenetic studies. It is judged to be adequate for the particular purpose of cytogenetic studies.

Solubility of the test substance

CGA 329 351 tech. was dissolved in DMSO at room temperature. The highest soluble concentration soluble in DMSO was 744.3 mg/ml. This solution caused the formation of strong precipitates after 100 fold dilution with culture medium. The highest concentration of CGA 329 351 tech. in DMSO (stock solution) which was soluble after 100 fold dilution with culture medium was 203.0 mg/ml. Lower concentrations were prepared by appropriate dilution of the stock solution with DMSO. The respective solutions were added (1:100) to the cell cultures. The final concentration of the vehicle DMSO in the culture medium was 1%.

Preparation of the metabolic activation mixture

Rat-liver post mitochondrial supernatant (S9 fraction) was prepared in advance from male RAI rats (Tif: RAIf[SPF]), reared at the Animal Farm of CIBA-GEIGY, Sisseln, Switzerland. The animals were treated with Aroclor 1254 (Analabs Inc., North Haven, Connecticut, U.S.A.; 500 mg/kg, i.p.) 5 days prior to sacrifice. The livers were homogenized with 3 volumes of 150 mM KCl. The homogenate was centrifuged at 9000x g for 15 minutes and the resulting supernatant (S9 fraction) was stored at approximately -80°C for no longer than one year (Lit. 6).

The S9 fraction was thawed immediately before use, mixed with NADP and isocitric acid and added to culture medium to give the following final concentrations (Lit. 7):

Rat liver S9 fraction	15 µl/ml (1.5%)
NADP	3.14 µmol/ml
Isocitric acid (trisodium salt)	15.3 µmol/ml

Cytotoxicity / Mutagenicity test

The cytotoxicity test was performed as an integral part of the mutagenicity test. A series of glass slides in quadruple culture dishes (Quadriperm) was seeded with Chinese hamster ovary cells (passage number: 54/18, 54/20) at a density of at least $1x10^4$ cells/ml (18 hours experiments) or $4x10^3$ cells/ml (42 hours experiments). The preincubation time before treatment was about 24 hours. The substance in DMSO was added (1:100) to the cells in culture medium. In the experiments in which the substance was metabolically activated, 0.5 ml of an activation mixture (Lit. 7) was added to 4.5 ml of Nutrient Mixture F-12. Mitomycin C (KYOWA HAKKO KOGYO Co. Ltd., Japan) 0.2 µg/ml, a mutagen not requiring S9 activation, and cyclophosphamide (CPA, ASTA-WERKE,

Germany) 20.0 µg/ml, which requires metabolic activation, were used as positive controls. In addition a negative control was set in each experiment, supplemented with the respective volume of the vehicle. Quadruplicate cultures were prepared for each group in each assay.

To ensure analysis of first post treatment mitoses a major sampling time of 1.5 times cell cycle was selected. This corresponds to about 18 hours for the CHO cells used. To ensure the above requirement even in case of a delayed cell cycle, which may be caused by the test substance, an additional sampling time was chosen, 24 hours after the first one. Treatment was performed throughout the whole period in the absence of metabolic activation. In the presence of metabolic activation treatment was shortened to three hours because prolonged exposure to S9-fraction would result in cytotoxicity. The detailed treatment conditions were as follows:

	Original Study		Confirmatory Study			
Experiment no.	1	2	1	2	3	4
Metabolic activation (S9)	-	+	-	+	-	+
Treatment (h)	18	3	18	3	42	3
Recovery after treatment (h)	/	15	/	15	/	39
Number of concentrations	8	8	6	5	8	8
Lowest conc. (µg/ml)	15.86	15.86	63.44	126.88	15.86	15.86
Highest conc. (µg/ml)	2030.0	2030.0	2030.0	2030.0	2030.0	2030.0

Two hours prior to harvesting, the cultures were treated with Colcemide (GIBCO) 0.4 µg/ml to arrest cells in metaphase. The experiment was terminated by hypotonic treatment (0.075 M KCl solution) of the cells, followed by fixation (methanol:acetic acid, 3:1). Slides were air-dried and stained with orcein.

Selection of concentrations for analysis

The highest concentration used or the lowest concentration which suppresses mitotic activity by approximately 50 - 80% compared to the control group was selected as the highest for the analysis of chromosome aberrations together with two lower concentrations in succession. For the determination of the mitotic index (M.I.) the preparations from the various cultures were examined first, uncoded. The percentages of mitotic suppression in comparison with the controls were evaluated by counting at least 2000 cells from one slide each of the treatment groups and the negative control group (Table 1). The determination of the mitotic coefficient was performed for each separately. From the results of corresponding original run, five suitable concentrations were determined for the first and second experiment of the confirmatory study.

Scoring of the slides

Prior to analysis the selected slides were coded, likewise the cultures treated with the vehicle alone as well as the positive control. Whenever possible two hundred well spread metaphase figures with 19 to 21 centromeres from two cultures (100 metaphases per replicate culture) in the vehicle control and in the treated groups were scored. At least fifty metaphases were scored in the positive controls (25 per replicate culture). The slides were examined for the following structural aberrations (for description of aberrations see e.g. Lit. 9):

- specific aberrations:
  chromatid and chromosome deletions (including breaks, deletions and fragments),
  chromatid exchanges (including triradials, quadriradials, endfusions, acentric rings),
  chromosome exchanges (including dicentrics, polycentrics, centric and acentric rings),
- multiple aberrations: metaphases containing more than 10 aberrations of different types or more than 5 aberrations of one particular type (excluding gaps),
- unspecific aberrations: gaps (chromatid- and chromosome-),

In addition to the required number of metaphases having 19-21 centromeres the following types of numerical alterations (metaphases with >21 centromeres) were recorded: polyploid metaphases (multiples of '2n'), endoreduplication figures.

Using a computerized coordinate reading system (Microptic system, Microptic Ltd., UK) attached to the vernier scale on the microscope stage, the coordinates of all metaphases were recorded.

Statistical analysis

The evaluated numbers of specific aberrations were subjected to statistical analysis. In the preliminary tests the data were assessed for flask effects (dependence of cells within each culture) using a chi-squared test. The nonsignificant result of this test means there is no substantial evidence to conclude a flask effect (although a flask effect still might exist). Accordingly a chi-squared test for trend was performed modelling all cells in a given experiment as independent (Lit. 8). That is, the individual cell is taken as the experimental unit. Consequently the power of the test is substantially increased, resulting in a rather safe judgement of the observed effects.

Assay evaluation criteria

Criteria for a positive response

Under the standard conditions of our laboratories, the test substance is generally considered to be active in the Chinese Hamster cells if the following conditions are met:

- The percentage of metaphases containing specific aberrations in a treatment group is higher than 6.0 (based on historical negative control range) and differs statistically significant from the respective value of the negative control.
- A concentration-related response should be demonstrable.

Criteria for a negative response

Under the standard conditions of our laboratories, the test substance is generally considered to be inactive in the Chinese Hamster cells if the following conditions are met:

- The percentage of metaphases containing specific aberrations in all treatment groups is less than or equal to 6.0 (based on historical negative control range) and does not differ statistically significant from the respective value of the negative control.

Exceptions

At the limits of the criteria for a positive or for a negative response or if the criteria for a positive response are only partially fulfilled or if effects are obtained at extremely high concentrations or in the toxic range of the test substance only, the Study Director will decide by experience about the interpretation of the results.

Assay acceptance criteria

- The results of the experiments should not be influenced by a technical error, contamination or a recognized artifact.
- The quality of the slides should allow, at least to a large extent, the chromosomes to be easily identifiable.
- In the negative controls the percentage of metaphases showing specific chromosomal aberrations should be less than 6.0 (based on historical negative control range).
- The results of the positive control experiments should meet the criteria for a positive response.
- The highest concentration to which cells were exposed in the mutagenicity test should exert sufficient toxicity (suppression of mitotic activity by 50% or more), represent the limit of solubility of the test material, or be at least 5 mg/ml (or 10 mMol/l).

Historical negative controls

The summarized negative control data from studies reported between 1989 and 1993 are shown on Table 8.

Flow cytometry

The DNA distribution of cell cultures was determined by flow cytometry. Cultures treated with the test substance or with the vehicle alone were fixed and stained with DAPI (4',6-diamino-2-phenylindole, Serva). Fluorescence of DAPI stained DNA was measured with a Partec PAS-II flow cytophotometer. A substantial shift in the DNA distribution pattern of cell cultures in comparison with the pattern of the vehicle control would indicate a disturbance of the cell cycle induced by the test substance.

Analytical control

To confirm that the cells were actually exposed to the intended test concentrations and to confirm the stability of the test substance in the vehicle used, determination of the concentration of the test substance in solution was performed by the analytical unit. This determination was performed with the lowest concentration of the stock solution used in the first and last segment of the mutagenicity test. The analytical report is stored with the raw data of this study.

RESULTS AND DISCUSSION

Toxicity test / Selection of concentrations

The results obtained from the toxicity test (mitotic index) are listed on Table 1. The highest concentration of 507.50 µg/ml selected for analysis in the first experiment of the original study (without metabolic activation, 18 hours treatment) caused 55.28% suppression of mitotic activity. The highest concentration of 1015.0 µg/ml selected for analysis in the second experiment of the original study (with metabolic activation, 3 hours treatment/15 hours recovery) caused 28.97% suppression of mitotic activity. At the next higher concentration only cell debris were present due to toxicity. In the first experiment of the confirmatory study (without metabolic activation, 18 hours treatment) the highest concentration of 1015.0 µg/ml selected for analysis caused 35.43% suppression of mitotic activity. The next higher concentration caused a mitotic suppression by 88.19%. In the second experiment (with metabolic activation, 3 hours treatment/15 hours recovery) the highest concentration of 2030.0 µg/ml selected for analysis caused 29.27% suppression of mitotic activity. The highest concentration of 1015.0 µg/ml selected for analysis in the third experiment of the confirmatory study (without metabolic activation, 42 hours treatment) caused 59.50% suppression of mitotic activity. In the fourth experiment (with metabolic activation, 3 hours treatment/39 hours recovery) the highest concentration of 2030.0 µg/ml selected for analysis caused 14.43% suppression of mitotic activity.

Original mutagenicity study

In the experiment performed **without metabolic activation** (experiment 1, **18 hours treatment**; Table 2), 0.5% of metaphases with specific chromosomal aberrations were detected in the negative control. At the concentrations of 126.88 µg/ml, 253.75 µg/ml and 507.50 µg/ml 6.5%, 2.5% and 3.5% of cells with specific chromosomal aberrations were found.

The value obtained with the lowest concentration showed a statistically significant difference when compared with the negative control.

In the experiment performed **with metabolic activation** (experiment 2, **3 hours treatment/15 hours recovery**; Table 3), 4.0% of metaphases with specific chromosomal aberrations were seen in

the negative control. At the concentrations of 253.75 µg/ml, 507.50 µg/ml and 1015.0 µg/ml the respective values were 2.5%, 5.0% and 2.0%.

None of these values showed a statistically significant difference when compared with the negative control.

Confirmatory mutagenicity study

In the experiment performed **without metabolic activation** after **18 hours treatment** (experiment 1; Table 4), 0.5% of metaphases with specific chromosomal aberrations were detected in the negative control. At the concentrations of 253.75 µg/ml, 507.50 µg/ml and 1015.0 µg/ml 0%, 3.0% and 4.5% of cells with specific chromosomal aberrations were registered.

The values obtained with the two higher concentrations showed a statistically significant difference when compared with their respective negative control.

In the experiment performed **with metabolic activation** after **3 hours treatment/15 hours recovery** (experiment 2; Table 5), 3.5% of metaphases with specific chromosomal aberrations were seen in the negative control. At the concentrations of 507.50 µg/ml, 1015.0 µg/ml and 2030.0 µg/ml 2.5%, 3.5% and 0.8% of cells showed specific chromosomal aberrations.

None of these values showed a statistically significant difference when compared with the negative control.

In the experiment performed **without metabolic activation** after **42 hours treatment** (experiment 3; Table 6), 1.5% of metaphases with specific chromosomal aberrations were detected in the negative control cultures. At the concentrations of 253.75 µg/ml, 507.50 µg/ml and 1015.0 µg/ml the corresponding values were 0%, 0.5% and 3.5%.

The value obtained with the highest concentration showed a statistically significant difference when compared with the negative control.

In the experiment performed **with metabolic activation** after **3 hours treatment/39 hours recovery** (experiment 4; Table 7), 1.0% of metaphases with specific chromosomal aberrations were registered in the negative control cultures. At the concentrations of 507.50 µg/ml, 1015.0 µg/ml and 2030.0 µg/ml 3.0, 2.5% and 1.5% of cells with specific chromosomal aberrations were found.

None of these values showed a statistically significant difference when compared with the negative control.

Unspecific chromosomal aberrations in the form of chromatid gaps found in all experiments were within the frequency generally observed.

In the experiments performed without metabolic activation statistically significant effects were occasionally obtained. However, the respective values of the confirmatory study were all clearly below the critical value of 6.0% required for a positive response and are within the range of the historical negative controls. They are therefore considered to be of no biological relevance. In the original experiment a frequency of cells with aberrations of 6.5% revealed a statistical significance. This value is slightly above the critical value of 6.0% required for a positive response. The effect however was obtained with the lowest concentration only. Furthermore chromatid exchanges, which are usually induced by clastogenic compounds, were not obtained. Again, this effect is therefore considered to be of no biological relevance.

Positive controls

The treatment of the cultures with mitomycin-C, 0.2 µg/ml and cyclophosphamide, 20.0 µg/ml, respectively, was followed by a high incidence of specific chromosomal aberrations in the experiments one and two of the original study (60.0% and 26.0%) and in the experiments one and two of the confirmatory study (40.0% and 15.0%).

Flow cytometry

(see attached report)

The influence of the test substance on the cell cycle of CHO cells was tested at the concentrations selected for chromosome analysis. The DNA distribution was determined by flow cytometry and compared with the profile of the respective control culture. In the presence of metabolic activation a shift in the DNA distribution profile could not be detected. In the absence of metabolic activation a slight, concentration dependent shift in the DNA distribution profile was obtained, indicating a weak cell cycle arresting activity by the test substance in the CHO cells. The effect however is considered no to affect the validity of the chromosome analyses.

Analytical results

The test material in solution was analysed by HPLC with UV detection to confirm the intended concentrations to be used in the mutagenicity tests and the stability of the test substance in the vehicle used. The concentration values found were 137.3% and 115.8% of the calculated concentrations, thus indicating a sufficient stability of the test substance in the vehicle.

LITERATURE

[1]OECD (May 26, 1983) Genetic Toxicology: In vitro Mammalian Cytogenetic Test. OECD Guideline for Testing of Chemicals 473

[2]EPA (May 20, 1987) In vitro Mammalian Cytogenetics. Environmental Protection Agency Health Effects Testing Guidelines. § 798.5375

[3]EEC Directive 92/69, Annex (July 31, 1992). B.10 Mutagenicity (In vitro Mammalian Cytogenetic Test). Official Journal of the European Communities, L383A, Vol. 35, December 29, 1992

[4]GALLOWAY, S.M. and WOLFF, S.: The relation between chemically induced sister-chromatid exchanges and chromatid breakage. Mutation Res. 61, 297-307 (1979).

[5]NATARAJAN, A.T., TATES, A.D., VAN BUUL, P.P.W., MEIJERS, M. and DE VOGEL, N.: Cytogenetic effects of mutagens/carcinogens after activation in a microsomal system in vitro. I. Induction of chromosome aberrations and sister chromatid exchanges by diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) in CHO cells in the presence of rat-liver microsomes. Mutation Res. 37, 83-90 (1976).

[6]AMES, B.N., McCANN, J. and YAMASAKI, E.: Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Res. 31, 347-364 (1975).

[7]GALLOWAY, S.M., BLOOM, A.D., RESNICK, M., MARGOLIN, B.H., NAKAMURA, F., ARCHER, P. and ZEIGER, E.: Development of a Standard Protocol for In Vitro Cytogenetic Testing With Chinese Hamster Ovary Cells: Comparison of Results for 22 Compounds in Two Laboratories. Environ. Mutagen. 7, 1-51 (1985).

[8]SNEDECOR, G.W. and COCHRAN, W.G., 1980, Statistical Methods, 7th Ed., pp. 206-208.

[9]SCOTT, D., DEAN, B.J., DANFORD, N.D. and KIRKLAND, D.J.: Metaphase chromosome aberration assays in vitro. In: Basic Mutagenicity Tests: UKEMS Recommended Procedures. Edited by D.J. Kirkland; Cambridge University Press, 1990, pp. 62-86.

[10]MAFF (January, 1985). English translation: SHIRASU Y.: The Japanese mutagenicity studies guidelines for pesticide registration.
Mutation Res. 205, 393-395 (1988).

Legend to Table 1

Remarks:

a) When three subsequent concentrations with a frequency of 70% mitosis or more in relation to the solvent control are found, the evaluation of the lower concentrations is omitted.

b) When the three highest concentrations show a frequency of 70% mitosis or more in relation to the solvent control, the evaluation of the lower concentrations, with the exception of the last one, is omitted.

c) No cells scored due to toxicity

Cell density:

+++ similar to solvent control

++ about 50% of solvent control

+ less than 50% of solvent control

+/- few cells present only

- cell debris present only

-- no cell and no cells debris

TABLE 1 MITOTIC INDEX VALUES / CYTOTOXICITY

Original study, experiment 1 **18 h treatment without metabolic activation**

	Cells scored	Mitosis	M.I. %	Frequency % of control	Cell density
Solvent control	2000	123	6.15	100.00	+++
CGA 329 351 tech.					
2030.00 µg/ml	2000	0	0.00	0.00	+
1015.00 µg/ml	2000	51	2.55	41.46	++
507.50 µg/ml	2000	55	2.75	44.72	+++
253.75 µg/ml	2000	71	3.55	57.72	+++
126.88 µg/ml	2000	93	4.65	75.61	+++
63.44 µg/ml	2000	97	4.85	78.86	+++
31.72 µg/ml	2000	133	6.65	108.13	+++
15.86 µg/ml	a				

Original study, experiment 2 **3 h treatment with metabolic activation / 15 h recovery**

	Cells scored	Mitosis	M.I. %	Frequency % of control	Cell density
Solvent control	2000	107	5.35	100.00	+++
CGA 329 351 tech.					
2030.00 µg/ml	c				--
1015.00 µg/ml	2000	76	3.80	71.03	+++
507.50 µg/ml	2000	76	3.80	71.03	+++
253.75 µg/ml	2000	92	4.60	85.98	+++
126.88 µg/ml	a				
63.44 µg/ml	a				
31.72 µg/ml	a				
15.86 µg/ml	a				

TABLE 1 MITOTIC INDEX VALUES / CYTOTOXICITY (CONT.)

Confirmatory study, experiment 1 18 h treatment without metabolic activation

	Cells scored	Mitosis	M.I. %	Frequency % of control	Cell density
Solvent control	2000	127	6.35	100.00	+++
CGA 329 351 tech.					
2030.00 µg/ml	2000	15	0.75	11.81	+
1015.00 µg/ml	2000	82	4.10	64.57	+++
507.50 µg/ml	2000	142	7.10	111.81	+++
253.75 µg/ml	2000	143	7.15	112.60	+++
126.88 µg/ml	2000	165	8.25	129.92	+++
63.44 µg/ml	a				

Confirmatory study, experiment 2 3 h treatment with metabolic activation / 15 h recovery

	Cells scored	Mitosis	M.I. %	Frequency % of control	Cell density
Solvent control	2000	164	8.20	100.00	+++
CGA 329 351 tech.					
2030.00 µg/ml	2000	116	5.80	70.73	++
1015.00 µg/ml	2000	145	7.25	88.41	+++
507.50 µg/ml	2000	184	9.20	112.20	+++
253.75 µg/ml	b				
126.88 µg/ml	2000	159	7.95	96.95	+++

TABLE 1 MITOTIC INDEX VALUES / CYTOTOXICITY (CONT.)

Confirmatory study, experiment 1 42 h treatment without metabolic activation

	Cells scored	Mitosis	M.I. %	Frequency % of control	Cell density
Solvent control	2000	121	6.05	100.00	+++
CGA 329 351 tech.					
2030.00 µg/ml	2000	28	1.40	23.14	+
1015.00 µg/ml	2000	49	2.45	40.50	++
507.50 µg/ml	2000	70	3.50	57.85	++
253.75 µg/ml	2000	99	4.95	81.82	+++
126.88 µg/ml	2000	131	6.55	108.26	+++
63.44 µg/ml	2000	117	5.85	96.69	+++
31.72 µg/ml	a				
15.86 µg/ml	a				

Confirmatory study, experiment 2 3 h treatment with metabolic activation / 39 h recovery

	Cells scored	Mitosis	M.I. %	Frequency % of control	Cell density
Solvent control	2000	194	9.70	100.00	+++
CGA 329 351 tech.					
2030.00 µg/ml	2000	166	8.30	85.57	+++
1015.00 µg/ml	2000	191	9.55	98.45	+++
507.50 µg/ml	2000	184	9.20	94.85	+++
253.75 µg/ml	b				
126.88 µg/ml	b				
63.44 µg/ml	b				
31.72 µg/ml	b				
15.86 µg/ml	2000	187	9.35	96.39	+++

Legend to Tables 2 - 7

ct del	Chromatid deletions (including deletions, breaks, fragments)
ct exc	Chromatid exchanges (including triradials, quadriradials, endfusions and acentric rings)
cs del	Chromosome deletions (including deletions, breaks, fragments)
cs exc	Chromosome exchanges (including dicentrics, polycentrics, centric and acentric rings)
mab	Multiple aberrations: metaphases containing more than 10 aberrations of different types or more than 5 aberrations of one particular type (excluding gaps)
gaps	Chromatid and chromosome type gaps
pol	Polyploid metaphases (>30 centromers)
end	Endoreduplications
CPA	Cyclophosphamide
Mito-C	Mitomycin-C
*)	Statistical significance: $0.05 = P > 0.01$
**)	Statistical significance: $0.01 = P > 0.001$
***)	Statistical significance: $P \leq 0.001$
#)	% cells with aberrations excluding gaps and numerical alterations (pol, end)

TABLE 2 ORIGINAL MUTAGENICITY STUDY, EXPERIMENT 1

18 h treatment without metabolic activation

Treatment	total no of cells examined	% cells with specific aberrations#	total number of cells with aberrations: gaps	ct del	ct exc	cs del	cs exc	mab	pol	end
Solvent control	200	0.5	5			1			7	
CGA 329 351 tech.										
126.88 µg/ml	200	6.5**	7	5		7	1		7	
253.75 µg/ml	200	2.5	6	3		2			5	
507.50 µg/ml	200	3.5	4	5	1	1	1		5	
positive control (Mito-C, 0.2 µg/ml)	50	60.0***	8	20	14	4	2		2	

TABLE 3 ORIGINAL MUTAGENICITY STUDY, EXPERIMENT 2

3 h treatment with metabolic activation / 15 h recovery

Treatment	total no of cells examined	% cells with specific aberrations#	total number of cells with aberrations: gaps	ct del	ct exc	cs del	cs exc	mab	pol	end
Solvent control	200	4.0	8	4	1	3			3	1
CGA 329 351 tech.										
253.75 µg/ml	200	2.5	6	1		4			8	3
507.50 µg/ml	200	5.0	9	6	2	2			6	1
1015.00 µg/ml	200	2.0	9	1		3			10	1
positive control (CPA, 20 µg/ml)	50	26.0***	6	9	3	1	1			

TABLE 4 CONFIRMATORY MUTAGENICITY STUDY, EXPERIMENT 1

18 h treatment without metabolic activation

Treatment	total no of cells examined	% cells with specific aberrations#	total number of cells with aberrations							
			gaps	ct del	ct exc	cs del	cs exc	mab	pol	end
Solvent control	200	0.5	1			1			4	
CGA 329 351 tech.										
253.75 µg/ml	200	0.0	1						2	
507.50 µg/ml	200	3.0**	1	2		2	2		1	
1015.00 µg/ml	200	4.5***	5	4	1	5	1			
positive control (Mito-C, 0.2 µg/ml)	50	40.0***	9	16	7	3				

TABLE 5 CONFIRMATORY MUTAGENICITY STUDY, EXPERIMENT 2

3 h treatment with metabolic activation / 15 h recovery

Treatment	total no of cells examined	% cells with specific aberrations#	total number of cells with aberrations							
			gaps	ct del	ct exc	cs del	cs exc	mab	pol	end
Solvent control	200	3.5	2	5			1	1		
CGA 329 351 tech.										
507.50 µg/ml	200	2.5	2	3		2				
1015.00 µg/ml	200	3.5	2	6	1	1	1		4	
2030.00 µg/ml	120	0.8	2	1					2	1
positive control (CPA, 20 µg/ml)	200	15.0***	10	19	8	8	2		3	

TABLE 6 CONFIRMATORY MUTAGENICITY STUDY, EXPERIMENT 3

42 h treatment without metabolic activation

Treatment	total no of cells examined	% cells with specific aberrations#	total number of cells with aberrations							
			gaps	ct del	ct exc	cs del	cs exc	mab	pol	end
Solvent control	200	1.5				2	1		3	
CGA 329 351 tech.										
253.75 µg/ml	200	0	2						2	
507.50 µg/ml	200	0.5	1			1			6	
1015.00 µg/ml	200	3.5*	3	4	1	3			1	

TABLE 7 CONFIRMATORY MUTAGENICITY STUDY, EXPERIMENT 4

3 h treatment with metabolic activation / 39 h recovery

Treatment	total no of cells examined	% cells with specific aberrations#	total number of cells with aberrations							
			gaps	ct del	ct exc	cs del	cs exc	mab	pol	end
Solvent control	200	1.0	4	1			1			
CGA 329 351 tech.										
507.50 µg/ml	200	3.0	1			3	3		3	
1015.00 µg/ml	200	2.5	1	3		3				
2030.00 µg/ml	200	1.5	1	2		1			3	

Table 8 SUMMARIZED HISTORICAL SOLVENT CONTROL DATA (1989 - 1993)

Treatment	S9	solvent	N (exp.)	N (cells)	% cells with specific aberrations (excl. gaps)			
					mean	S.D.	minimum	maximum
18h	-	medium/water	49	9800	1.490	1.269	0	4.5
		DMSO	105	18600	1.590	1.317	0	7
		acetone	14	2600	1.893	1.041	0	4
		ethanol	5	1000	1.300	1.151	0	2.5
		all solvents	173	32000	1.578	1.274	0	7
3h/15h	+	medium/water	48	9600	1.531	1.302	0	5.5
		DMSO	103	18110	1.567	1.349	0	6
		acetone	14	2600	2.107	1.389	0	5
		ethanol	5	1000	1.700	1.396	0.5	4
		all solvents	170	31310	1.606	1.337	0	6
42h	-	medium/water	28	5600	1.071	1.095	0	4
		DMSO	54	10300	1.398	1.134	0	6
		acetone	8	1500	2.125	1.026	1	4
		ethanol	2	400	2.500		0.5	4.5
		all solvents	92	17800	1.386	1.175	0	6
3h/39h	+	medium/water	27	5400	1.519	1.458	0	5.5
		DMSO	54	10500	1.764	1.265	0	6.5
		acetone	8	1500	1.375	1.217	0	4
		ethanol	2	400	1.750		0	3.5
		all solvents	91	17800	1.657	1.326	0	6.5
all treatments		all solvents	526	98910	1.567	1.287	0	7

CYTOGENETIC TEST ON CHINESE HAMSTER CELLS IN VITRO (EC-CONFORM)

CGA 329 351 tech.

APPENDIX NO.1 TO REPORT NO. 943013

FLOW CYTOMETRY REPORT

(4 pages)

ANALYSIS OF CELL-CYCLE DISTRIBUTION OF CHINESE HAMSTER CELLS IN VITRO TREATED WITH CGA 329 351 tech.

CGA 329 351 tech. was tested for cytotoxic effects on Chinese hamster ovary cells in vitro by monitoring the cell cycle of the treated cells by flow cytometry. The following concentrations were tested:

Experiment without metabolic activation:
original experiment:
- 18 hours incubation time (Table 1 of Appendix 1):
- solvent control, 126.88, 253.75, 507.5, 1015.0 µg/ml.

Experiment with metabolic activation:
original experiment:
- 3 hours incubation followed by 15 hours recovery period
(Table 1 of Appendix 1):
- solvent control, 253.75, 507.5, 1015.0, 2030.0 µg/ml

Additionally an absolute control was run in both experiments without any treatment in order to check the condition of the cultures.

Cells (passage no.: 55/4) were maintained in tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 and were then detached with trypsin-EDTA. The monodispersed cells were then fixed for at least 2 hours in an ice-cold mixture of water/methanol /ethanol/isopropanol (30/56/7/7 V%). For DNA-staining the cells were washed by centrifugation and the cell pellet was resuspended in DAPI-solution (5 µMol 2,4-diamidino-2-phenylindole in Tris-Puffer at pH 7.4), supplemented with FITC-labeled microbeads (as an internal standard). The proportion of microbeads and cells in the total particle count indicates the growth of the cultured cells.

All samples were resuspended in equal volumes of staining solution.

After one hour of staining at 4°C the cell cycle distribution was analysed in a flow cytometer (Partec PAS II) connected with a HP Vectra computer.

In the experiment without metabolic activation at the concentration of 1015.0 µg/ml treatment with CGA 329 351 tech. led to a weak increase in the proportion of cells in the $G_{1/0}$-stage; simultaneously the proportion of cells in the S-stage reduced (Table 1 of Appendix 1). This effect is interpreted as a weak cell cycle arresting activity of the test substance on cells in the $G_{1/0}$-stage.

In the experiment performed with metabolic activation at the concentration of 2030 µg/ml the number of particles, identified as cells was reduced by about 35% by comparison with the solvent control. This effect indicates some cytotoxic effects of the metabolites of the test substance, resulting in the reduced cell number (Table 1 of Appendix 1).

Responsible for flow-cytometry: 5.1.2.e Woo

Date: June 29, 1994

RESULTS

(Table 1 of Appendix 1)

Cell cycle distribution of the cells.

Experiment without metabolic activation:

Concentration	Total Particle Count*	Particles, identified as cells (=100%)	Cell Cycle Phase (Distribution %) $G_{1/0}$	S	G_2+M
Solvent	20'000	18'778	38.8	45.9	15.3
126.88 µg/ml	20'000	17'789	45.3	39.8	14.9
253.75 µg/ml	20'000	17'990	51.1	35.2	13.7
507.5 µg/ml	20'000	18'030	56.5	28.3	15.2
1015.0 µg/ml	20'000	16'598	60.1	27.1	12.8
abs.Control	20'000	18'105	43.3	41.1	15.6

*) including internal standard beads

Experiment with metabolic activation:

Concentration	Total Particle Count*	Particles, identified as cells (=100%)	Cell Cycle Phase (Distribution %) $G_{1/0}$	S	G_2+M
Solvent	20'000	18'020	45.2	41.9	12.8
253.75 µg/ml	20'000	18'516	44.1	41.7	14.2
507.5 µg/ml	20'000	18'817	41.9	44.6	13.5
1015.0 µg/ml	20'000	18'671	42.9	42.1	15.0
2030.0 µg/ml	20'000	11'607**	38.8**	48.7**	12.5**
abs.Control	20'000	18'648	38.8	46.6	14.6

*) including internal standard beads
**) estimated

Verteiler:

HH. 5.1.2.e Woo (2x)

(1x)