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Study Title

Dodigen 4022

CHROMOSOME ABERRATIONS

IN VITRO

IN V79 CHINESE HAMSTER CELLS

Author

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Performing laboratory

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Laboratory Project ID: Study No. 92.0337

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STATEMENT OF COMPLIANCE

To the best of my knowledge and belief, this study was conducted in compliance with Good Laboratory Practice regulations. No unforeseen circumstances were observed which might have affected the quality or integrity of the study.

Study Director

Testing facility management:

Hoechst

Quality Assurance Statement

Hoechst Aktiengesellschaft
Quality Assurance(GLP)

14.12.1992

Title : Dodigen 4022
CHROMOSOMEN ABERRATIONS
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STUDY NO. : 92.0337

This study was periodically inspected and properly signed records of these inspections were submitted to testing facility management and the study director as shown below :

Inspection	Report
30.07.1992	31.07.1992
05.08.1992	05.08.1992
14.12.1992	14.12.1992
14.12.1992	14.12.1992

[redacted] 14.12.1992
Quality Assurance(GLP)

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1. SUMMARY

The study was performed to investigate the potential of Dodigen 4022 to induce chromosome aberrations in V 79 cells of the Chinese hamster <u>in vitro</u>.

The assay was performed in two parallel cultures, using identical procedures, both with and without rat liver microsomal activation.

The test article was tested with the following concentrations:

<u>without S9-mix:</u>

7 h: 6000* ug/ml
18 h: 6000*, 3000, 600 ug/ml
28 h: 6000* ug/ml

<u>with S9-mix:</u>

7 h: 6000* ug/ml
18 h: 6000*, 3000, 600 ug/ml
28 h: 6000* ug/ml

* = 10 mM

According to the preliminary experiment for solubility and toxicity the concentration ranges were selected. 6000 ug/ml were chosen as the highest test concentration because of 10mM limitation.

This concentration produced a distinct decrease of the mitotic index at the 7 hour fixation interval with and without metabolic activation. In contrast a dose-dependent increase of the mitotic index was observed at the 18 hour fixation interval. These effects can be explained as a consequence of a reversible block of the cell cycle.

This increase seems to be caused by some unspecific effects as indicated by the inhibition of the cell cycle .
Because of the absence of any increased chromosomal aberrations as compared to the controls after completion of the cell cycle (18h and 28h fixation intervals) this effect was considered as not sufficient to evaluate the compound as distinct genotoxic agent.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in the chromosome aberrations indicating the sensitivity of the assay.

In conclusion as described under the conditions above, Dodigen 4022 is considered to be non-mutagenic in this chromosome aberration assay.

2. Introduction

The in vitro cytogenetic test is a mutagenicity test system for the detection of chromosomal aberrations in cultured mammalian cells (1). This system offers several advantages. When modern and adequate techniques are employed cell cultures show only minor variations between a series of passages with respect to the cell cycle, viability, plating efficiency, medium requirements and karyotype. Furthermore the cells can be stored frozen as stock for many experiments and for reference.

Chromosomal aberrations may be either structural or numerical. However, because cytogenetic assays are usually designed to analyse cells at their first post-treatment mitosis and numerical aberrations require at least one cell division to be visualized, this type of aberration is generally not observed in a routine cytogenetic assay. Structural aberrations may be of two types: chromosome or chromatid aberrations.

Chromosome-type aberrations are induced when a compound acts in the G_1 phase of the cell cycle. Chromatid-type aberrations are induced when a chemical acts in the S or G_2 phase of the cell cycle.

- Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same locus
- Chromatid-type aberrations result from damage expressed as breakage of a single chromatid or breakage and/or reunion between chromatids
- Numerical aberrations are variations of the normal chromosome number characteristic of the cells used in the assay

The V79 cell line has been used successfully for many years in *in vitro* experiments. Especially the high proliferation rate (doubling time 12-16 hours in stock cultures) and a high plating efficiency of untreated cells, both necessary for the appropriate performance of the study, recommend the use of this cell line (2,3,4).

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3. GENERAL

Study-No.	:	92.0337
Test compound	:	Dodigen 4022
Code	:	Hoe CG 0351 OE ZD82 0001
Ordered by	:	Hoechst AG, Abt. MTH-Entwicklung TVS, Herr [redacted]
Test system	:	in vitro mammalian cytogenetic test
Test organism	:	cells of Chinese hamster cell line V79
Initiation of the study	:	July 22nd, 1992
Termination of the study	:	November 3rd, 1992

Responsibility

Head of Genetic Toxicology: [redacted]

Head of Toxicology :

Quality assurance unit :

Testing facilities and archives: Pharma Development Central Toxicology
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4. MATERIAL AND METHODS

4.1. Test compound

Name	:	Dodigen 4022
Code	:	Hoe CG 0351 OE ZD 0001
Chemical nomenclature	:	Trimethyl-ethoxypolyoxpropylammoniumchloride
Purity	:	82 %
Stability	:	stable until April 1993 guaranteed by the sponsor
Stability in solvent	:	stable for four hours in water Analytical Laboratory; Oekochemie/Analytik-E dated December 4th, 1991
Appearance	:	viscous yellow liquid
Certificate of analysis	:	No. 04701 dated August 20th, 1991
Boiling point	:	> 250 °C
Molecular weight	:	ca 600
Batch No.	:	E 06159865
Storage conditions	:	dark at approx. 0 °C
Cell culture medium	:	MEM (Minimal essential medium) with Hanks-salts and 25 mM Hepes-buffer
Concentration of stock solution	:	0.61 %

At the day of the experiment the test substance was dissolved as a solution in cell culture medium at appropriate concentrations. Two independent cell cultures (No. 1 and 2) were used.

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4.2 Preparation and storage of a liver homogenate fraction (S9)

Male Sprague Dawley rats (200-300 g) received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bodyweight) 5 days before sacrifice. Preparation is performed at 0 to 4 °C using cold sterile solutions and glassware. The livers from at least 5-6 animals are removed, pooled and washed in approx. 150 mM KCl (approx. 1 ml/g wet liver). The washed livers are cut into small pieces and homogenized in three volumes of KCl. The homogenate is centrifuged at 9000 g for 10 minutes. The supernatant is the S9 fraction. It is divided into small portions, rapidly frozen and stored at approx. -80 °C.

4.3 Preparation of S9-mix

Sufficient S9 fraction is thawed immediately before each test at room temperature. One volume of S9 fraction is mixed with 9 volumes of the S9 cofactor solution and kept on ice until its use. This preparation is termed S9-mix. The concentrations of the different compounds in the S9-mix are:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM $NADP^+$
100 mM phosphate buffer pH 7.4

4.4 Mammalian cells

Large stocks of the V79 cell line stored in liquid nitrogen in the Laboratory of Genetic Toxicology of Hoechst AG allow the repeated use of the same cell culture batch in experiments. Consequently, the cellular parameters of the experiments remain similar because of the reproducible characteristics of the cells.
The tawed stock cultures were propagated at approx. 37 °C in 175 cm^2 plastic flasks. Seeding was done with about 8-10 x 10^5 cells per flask in 30 ml of MEM-medium supplemented with approx. 10 % fetal calf serum (FCS). The cells were subcultured twice a week.

4.5 Procedure of the assay

Two days old, exponentially growing stock cultures which were over 50 % confluent were trypsinised and a single-cell suspension was prepared. The trypsin concentration was approx. 0.25 % in Ca-Mg-free salt solution. 1-3 x 10^6 cells/flask were seeded into four 75 cm^2 plastic flasks containing 15 ml MEM with approx. 10 % FCS (7 h preparation). 6-9 x 10^5 cells/flask were seeded into four 25 cm^2 plastic flasks containing 5 ml MEM with approx. 10 % FCS (18 h preparation). 3-6 x 10^5 cells/flask were seeded into four 25 cm^2 plastic flasks containing 5 ml MEM with approx. 10 % FCS (28 h preparation).
After 24 h, the medium was replaced with medium containing approx. 5 % FCS and the test substance, both without S9-mix and with S9-mix.
After 4 h this medium was replaced with normal medium after rinsing once with physiological saline solution.

Treatment was performed with three concentrations of the test substance.

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The mitotic index was determined in samples of 1000 cells. The toxicity of the test substance was determined in a preliminary experiment by establishing the concentration-related plating efficiency. According to these data the concentration range was chosen.

4.5, 15.5 and 25.5 h after the start of the treatment colcemide was added (approx. 0.04 ug/ml culture medium) to the cultures. 2.5 h later (7 h, 18 h and 28 h preparation) the cells were trypsinised.

For hypotonic treatment, approximately 5 ml of approx. 0.075 M potassium chloride solution at approx. 37 °C was quickly added and suspended. This suspension was then allowed to incubate for 10 minutes in a water bath at approx. 37 °C. Addition of 1.5 ml fixative and flow through with air.

After re-centrifuging for five minutes at 1000 rpm, all but one drop of the supernatant was drawn off by pipette. The sediment was carefully covered with a layer composed of 2.5 ml fixative (methanol : glacial acetic acid 3 + 1). After at least 20 minutes, the fixation was carefully removed with a pipette and suspended in 2.5 ml fixative. The mixture was centrifuged when needed after another 30 minutes, after which the liquid was removed by pipette and fresh fixative added. The tubes were covered and kept for at least 12 hours (overnight) in a refrigerator at approx 4 °C.

After re-centrifuging for 5 minutes at 1000 rpm, all but one drop of the liquid was removed by pipette and a new suspension formed with a small quantity of freshly prepared fixative. A few drops of this suspension were placed with a pasteur pipette onto clean microscopic slides which had been stored in distilled water at approx. 4 °C, the drops were then briefly passed through a Bunsen flame and air-dried for 24 hours. Staining was performed as follows:

- staining for 10 minutes in approx. 2 % orcein solution
- rinsing 3 times in distilled water
- rinsing twice in acetone
- brief rinsing in acetone/xylene
- 2 minutes in acetone/xylene
- 5 minutes in xylene
- 10 minutes in xylene
- embedding in Entellan® or Eukitt®

2-5 slides were prepared from each flask.

In the same way both negative and positive controls were prepared 18 h after medium change or treatment respectively.

4.6 Controls

Negative controls:

Untreated cultures and cultures treated with the solvent (medium).

Positive controls:

Without metabolic activation:

EMS (Ethylmethanesulfonate) dissolved in nutrient medium in a concentration of 1750 ug/ml. The solution was prepared on the day of the experiment.

With metabolic activation:

CPA (Cyclophosphamide) = Endoxan final concentration in nutrient medium was 5.0 ug/ml. The solution was prepared on the day of the experiment.

The stability of the positive control substances in solution is unknown but a mutagenic response in the expected range is proof of biological stability.

4.7 Experimental groups

Preparation time 18 h

Negative control:	untreated cells	
Negative control:	untreated cells + S9-mix	
Solvent control :	cells + solvent	
Solvent control :	cells + solvent + S9-mix	
Positive control:	cells + EMS	
Positive control:	cells treated with CPA + S9-mix	
Test group 1:	cells + test substance	600 ug/ml
Test group 1:	cells + test substance + S9-mix	600 ug/ml
Test group 2:	cells + test substance	3000 ug/ml
Test group 2:	cells + test substance + S9-mix	3000 ug/ml
Test group 3:	cells + test substance	6000 ug/ml
Test group 3:	cells + test substance + S9-mix	6000 ug/ml

Preparation time 7 and 28 h

Solvent control :	cells + solvent	
Solvent control :	cells + solvent + S9-mix	
Test group 3:	cells + test substance	6000 ug/ml
Test group 3:	cells + test substance + S9-mix	6000 ug/ml

The concentrations were chosen from the data of the cytotoxicity assay in the preliminary experiment as follows:

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The survival rate of cells treated with the test substance.

ug/ml	without S9-mix	with S9-mix
Negative control	85.4 %	85.2 %
Solvent control	95.5 % = 100 %	82.2 % = 100 %
100	85.1 %	92.9 %
250	91.5 %	74.7 %
500	85.5 %	79.5 %
1000	82.4 %	80.6 %
2000	77.9 %	76.3 %
3000	62.4 %	84.5 %
4000	62.1 %	38.5 %
5000	59.0 %	76.9 %
6000	58.2 %	86.2 %

4.8 Analysis of metaphases

After the slides had been coded (Coding Scheme 552/92), 100 metaphases per experimental group were examined. The set of chromosomes was examined for completeness and the various chromosomal aberrations were assessed. The chromosomal aberrations were classified as shown in chapter 6.1. Only metaphases with 22 +/- 1 chromosomes are included in the analysis. The metaphases were examined for the following aberrations: gap (g), isogap (ig), break (b), isobreak (ib), fragment (f), isofragment (if), minute (m), isominute (im), deletion (d), isodeletion (id), exchanges including intrachanges (ex), dicentrics (di), chromosome disintegration (cd) and ring formation (ri). In addition, metaphases with 5 or more aberrations were classified separately as multiple aberrations (ma).

After the metaphases had been evaluated, the code was lifted. The values for the control group were compared with the results from the dose group and the positive control at each preparation time.

4.9 Evaluation

The evaluation of the results was performed as follows:

- The test substance is classified as mutagenic if it induces a statistically significant increased aberration rate(without gaps) as compared with the solvent controls
with one of the concentrations tested.
- the test substance is classified as mutagenic if there is a reproducible concentration related increase in the aberration rate(without gaps).
- the test substance is classified as non mutagenic when it tests negatively both with and without metabolic activation.

4.10 Biometry

The Biometry of the results was performed with a one-sided Fisher - Exact test (see tables page 31 - 34).

5. RESULTS

5.1 Solubility and toxicity

In a preliminary experiment **Dodigen 4022** was solved in cell culture medium. At a concentration of 6000 ug/ml (= 10 mM, which is the highest dose level tolerated for the test system) no visible precipitation was observed.

Accordingly, the preliminary study was carried out using a maximum concentration of 6000 ug/ml and a wide range of lower dose levels. Results are presented on page 13.

In the cytotoxicity experiment **Dodigen 4022** was slightly cytotoxic without S9-mix from 3000 ug/ml up to the concentration of 6000 ug/ml.
In the presence of S9-mix no indication of cytotoxicity was observed.

5.2 Mutagenicity

5.2.1 Experimental design

Preparation of chromosomes was carried out 7 h (high dose), 18 h (low, medium and high dose) and 28 h (high dose) after start of the treatment with the test article. The treatment interval was 4 h.
In each experimental group two parallel cultures were used. Per culture 100 metaphases were scored.
The following dose levels were evaluated:

without S9-mix:	with S9-mix:
7 h: 6000 ug/ml	7 h: 6000 ug/ml
18 h: 6000, 3000, 600 ug/ml	18 h: 6000, 3000, 600 ug/ml
28 h: 6000 ug/ml	28 h: 6000 ug/ml

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5.2.2 Toxicity and mutation results

In the main experiment the mitotic index was reduced after treatment with the highest dose levels at fixation intervals 7 h with and without S9-mix (page 20). After the 18h fixation interval a distinct increase of the mitotic index occured in the medium and high concentration with and without S9-mix.
In contrast the mitotic index showed no remarkable changes compared to the controls after the 28 hour fixation interval.

Table 2 shows the number of polyploid cells. After treatment with the test article there was no relevant increase in the number of polyploid cells as compared with the solvent controls.

The test substance Dodigen 4022 was assessed for its mutagenic potential in vitro in the chromosome-aberration-test with two independent cell cultures without metabolic activation and two independent cell cultures with metabolic activation. The results of these experiments are presented in tables 1 - 8.
The sensitivity of the test system was demonstrated by the enhanced mutation frequency in the cell cultures treated with the positive control substances.

There was an enhancement of the aberration rates 7 h after the start of the treatment with 6000 ug/ml with S9-mix inclusive and exclusive gaps. Also at the same concentration at preparation time 7h without metabolic activation the number of cells with aberrations and the aberration rate were enhanced, both inclusive gaps.
These data were found significantly enhanced in the Fisher's exact-test. Although this statistical test did not indicate significance for other data.

5.2.3 Interpretation and discussion of the results

As mentianed above Dodigen 4022 produced a distinct decrease of the mitotic index after the 7h fixation interval as an expression of an inhibition of the cell cycle. This effect was reversible because af the dose-dependent increase of the mitotic index after the 18h fixation interval.
After removal of the Dodigen 4022 induced inhibition and synchronisation of the cell cycle an enlarged number of cells went in mitosis.
A slight increase of the aberration rates was observed only during the inhibition of the cell cycle. After the completion of the cell cycle (18 and 28 hour fixation intervals) no increase of chromosomal aberrations ocurred. The inhibition of the cell cycle indicates the same unspecific effect of Dodigen 4022 induced an increased aberration rate after the 7 hour fixation interval.

However, these results should not be used for the definitive ranking of this substance as a mutagenic compound because after competion of the cell cycle no signs of mutagenicity occured.

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Therefore it is concluded that the test compound is not mutagenic in the chromosome aberration test system in vitro with cells of the V79 Chinese hamster cell line as described under the conditions above.

Dr. IST/AB

Quality assurance unit
3., 14.12.92

Pharma Development Central Toxicology
HOECHST AKTIENGESELLSCHAFT

[redacted]

Study Director

[redacted]

Head of Toxicology

Hoechst

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6. APPENDIX

6.1 Examples of aberrations

1. Structural aberrations

Gap: Non-stained segment (achromatic gap) of chromatid without dislocation of the apparently separate part, irrespective of size of the non-stained area.

Break: A visible fracture of the chromatid structure where the broken piece is laterally dislocated or shifted in the longitudinal axis but can still be assigned to the corresponding centric part.

Fragment: Acentric part of a chromosome which may appear individually, regardless of its size.

Minute: Small chromatide body with a diameter smaller than the width of the chromatide.

Deletion: Terminal or interstitial loss of part of the chromatide.

Exchange: These are exchange aberrations, subdivided into intrachanges (the union of parts that can combine within a chromosome) and interchanges (the union of parts that can combine from two or more chromosomes). Dicentric chromosomes and ring chromosomes are included in this group.

The chromatide aberrations specified above can also occur as iso-chromatide aberrations (e.g. isochromatide break)

2. Numerical aberrations

Aneuploidy: A deviation from the typical number of individual chromosomes in a set of chromosomes; a decrease in the number is known as hypoploidy and an increase as hyperploidy.

Polyploidy: More than two sets of chromosomes.

3. Additional criterion:

Chromosomal disintegration: Where all or most of the chromosomes are irregular particles. If exchange figures occur in the metaphases, they are only included in this aberration group.

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7. REFERENCES

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Office of Pesticides and Toxic Substances
U.S. Environmental Protection Agency
Washington, D.C. 20460

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8. TABLES

Table 1: Mitotic index

Test group	Dose ug/ml	S9 mix	fixation interval (h)	mitotic index per cent* slide 1	slide 2	abs.	rel.
Solvent control	0	-	7	8.2	9.1	8.7	100.0
Test article	6000		7	3.6	2.5	3.1	35.6
Solvent control	0	+	7	11.5	8.6	10.1	100.0
Test article	6000	+	7	5.2	4.8	5.0	49.5
Solvent control	0	-	18	9.7	8.0	8.9	100.0
Positive control EMS	1750	-	18	9.8	7.8	8.8	98.9
Test article	600	-	18	9.6	8.2	8.9	100.0
Test article	3000	-	18	11.6	12.3	11.9	133.7
Test article	6000	-	18	13.0	9.8	11.4	128.1
Solvent control	0	+	18	8.9	9.6	9.3	100.0
Positive control CPA	5	+	18	4.3	6.0	5.2	55.9
Test article	600	+	18	10.2	7.6	8.9	95.7
Test article	3000	+	18	12.5	10.9	11.7	125.8
Test article	6000	+	18	12.9	15.3	14.1	151.6
Solvent control	0	-	28	12.5	11.4	12.0	100.0
Test article	6000	-	28	8.3	7.0	7.7	64.2
Solvent control	0	+	28	11.0	12.0	11.5	100.0
Test article	6000	+	28	13.1	11.3	12.2	106.1

* The mitotic index was determined in 1000 cells from each of two slides per test group

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Table 2: Number of polyploid cells

Test group	Dose ug/ml	S9 mix	fixation interval (h)	polyploid cells* slide 1	slide 2	total	mean
Solvent control	0	-	7	6	4	10.0	5.0
Test article	6000	-	7	0	0	0.0	0.0
Solvent control	0	+	7	3	1	4.0	2.0
Test article	6000	+	7	0	1	1.0	0.5
Solvent control	0	-	18	4	4	8.0	4.0
Positive control EMS	1750	-	18	2	1	3.0	1.5
Test article	600	-	18	2	4	6.0	3.0
Test article	3000	-	18	4	6	10.0	5.0
Test article	6000	-	18	1	0	1.0	0.5
Solvent control	0	+	18	1	5	6.0	3.0
Positive control CPA	5	+	18	4	1	5.0	2.5
Test article	600	+	18	3	1	4.0	2.0
Test article	3000	+	18	3	5	8.0	4.0
Test article	6000	+	18	1	3	4.0	2.0
Solvent control	0	-	28	4	4	8.0	4.0
Test article	6000	-	28	1	4	5.0	2.5
Solvent control	0	+	28	1	2	3.0	1.5
Test article	6000	+	28	4	3	7.0	3.5

* = the number of polyploid cells was determined in 100 cells from each of the two slides per test group.

Table 3: Chromosome aberrations in V79 cells
Test substance: Dodigen 4022
Preparation: 7 h after administration (100 metaphases were analysed)

Dose ug/ml	Culture without S9 mix	No. of phases with aberrations incl. Gaps	excl.	No. of aberrations incl. Gaps	excl.	g	ig	b	ib	f	if	d	id	ma	ex	cd	others	MI %
0	S/1	1	1	1	1												In	8.2
0	S/2	0	0	0	0													9.1
Total		1	1	1	1												1	
6000	3/1	3	1	3	1	2				1								3.6
6000	3/2	6	5	6	5	1		1		2					1		1ri	2.5
Total		9*	6	9*	6	3		1		3					1		1	

S = solvent control

* = $p < 0.05$

Table 4: Chromosome aberrations in V79 cells
Test substance: Dodigen 4022
Preparation: 7 h after administration (100 metaphases were analysed)

Dose ug/ml	Culture with S9 mix	No. of phases with aberrations incl.	excl. Gaps	No. of aberrations incl.	excl. Gaps	g	ig	b	ib	f	if	d	id	ma	ex	cd	others	MI %
0	S/1	3	2	3	2	1											1m,1di	11.5
0	S/2	0	0	0	0													8.6
Total		3	2	3	2	1											2	
6000	3/1	3	2	7	5	2				2			1	3			1im,2m	5.2
6000	3/2	3	2	5	4	1												4.8
Total		6	4	12*	9*	3				2			1	3			3	

S = solvent control * = $p < 0.05$

Table 5: Chromosome aberrations in V79 cells
Test substance:
Preparation: 18 h after administration (100 metaphases were analysed)

Dose µg/ml	Culture without S9 mix	No. of phases with aberrations incl. Gaps	excl. Gaps	No. of aberrations incl. Gaps	excl. Gaps	g	ig	b	ib	f	if	d	id	ma	ex	cd	others	MI %
0	S/1	0	0	0	0													9.7
0	S/2	1	1	1	1								1					8.0
Total		1	1	1	1								1					
# 1750	P/1	12	12	27	26	1		3		3			2	1	16		1ri	9.8
# 1750	P/2	10	10	16	16			1	1	1				1	12			7.8
Total		22*	22*	43*	42*	1		4	1	4			2	2	28		1	

N = negative control S = solvent control P = positive control * = $p < 0.05$
# = only 50 metaphases were evaluated

Table 5 (cont.): Chromosome aberrations in V79 cells
Test substance: Dodigen 4022
Preparation: 18 h after administration (100 metaphases were analysed)

Dose ug/ml	Culture without S9 mix	No. of phases with aberrations incl. Gaps	excl. Gaps	No. of aberrations incl. Gaps	excl. Gaps	g	ig	b	ib	f	if	d	id	ma	ex	cd	others	MI %
600	1/1	3	2	3	2	1					1		1					9.5
600	1/2	2	2	2	2					1							1m	8.2
Total		5	4	5	4	1				1	1		1				1	
3000	2/1	1	0	1	0	1												11.5
3000	2/2	1	0	1	0	1												12.3
Total		2	0	2	0	2												
6000	3/1	4	1	5	1	4							1					13.0
6000	3/2	1	0	1	0	1												9.8
Total		5	1	6	1	5							1					

Table 6: Chromosome aberrations in V79 cells
Test substance: Dodigen 4022
Preparation: 18 h after administration (100 metaphases were analysed)

Dose ug/ml	Culture with S9 mix	No. of phases with aberrations incl. Gaps	excl. Gaps	No. of aberrations incl. Gaps	excl. Gaps	g	ig	b	ib	f	ff	d	id	ma	ex	cd	others	MI %
0	S/1	1	1	2	2					2								8.9
0	S/2	2	2	2	2					1							1m	9.6
Total		3	3	4	4					3							1	
5	P/1	20	19	29	25	4			1	8			1		10	1	3m,1rl	4.3
5	P/2	10	10	15	13	2		1	1	5					4		2m	6.0
Total		30*	29*	44*	38*	6		1	2	13			1		14	1	6	

N = negative control S = solvent control P = positive control * = $p < 0.05$

Table 6 (cont.): Chromosome aberrations in V79 cells
Test substance: Dodigen 4022
Preparation: 18 h after administration (100 metaphases were analysed)

Dose ug/ml	Culture with S9 mix	No. of phases with aberrations incl. Gaps	excl. Gaps	No. of aberrations incl. Gaps	excl. Gaps	g	ig	b	ib	f	if	d	id	ma	ex	cd	others	MI %
600	1/1	3	1	3	1	1	1								1			10.2
600	1/2	3	2	3	2	1				1			1					7.6
Total		6	3	6	3	2	1			1			1		1			
3000	2/1	2	2	2	2								1				1m	12.5
3000	2/2	2	2	2	2										1		1m	10.9
Total		4	4	4	4								1		1		2	
6000	3/1	8	4	8	4	4		1		1			1		1			12.9
6000	3/2	1	1	1	1										1			15.3
Total		9	5	9	5	4		1		1			1		2			

Table 7: Chromosome aberrations in V79 cells
Test substance: Dodigen 4022
Preparation: 28 h after administration (100 metaphases were analysed)

Dose ug/ml	Culture without S9 mix	No. of phases with aberrations incl. Gaps	excl. Gaps	No. of aberrations incl. Gaps	excl. Gaps	g	ig	b	ib	f	if	d	id	ma	ex	cd	others	MI %
0	S/1	0	0	0	0													12.5
0	S/2	2	1	2	1	1											1m	11.4
Total		2	1	2	1	1											1	
6000	3/1	1	1	2	2			1									1m	8.3
6000	3/2	5	4	5	4	1		1				3						7.0
Total		6	5	7	6	1		2				3					1	

S = solvent control

Table 8: Chromosome aberrations in V79 cells
Test substance: Dodigen 4022
Preparation: 28 h after administration (100 metaphases were analysed)

Dose ug/ml	Culture with S9 mix	No. of phases with aberrations incl. Gaps	excl. Gaps	No. of aberrations incl. Gaps	excl. Gaps	g	ig	b	ib	f	if	d	id	ma	ex	cd	others	MI %
0	S/1	5	1	5	1	4						1						11.0
0	S/2	0	0	0	0													12.0
Total		5	1	5	1	4						1						
6000	3/1	2	2	4	4					1							2m,1di	13.1
6000	3/2	1	0	1	0	1												11.3
Total		3	2	5	4	1				1							3	

S = solvent control

Hoechst

Table 9: Summary of results

Test group	Number of cells analysed	Dose ug/ml	S9 mix	fixation interval (h)	per cent aberrant cells incl. gaps	excl. gaps	exchanges
Solvent control	200	0	-	7	0.5	0.5	0.0
Test article	200	6000	-	7	4.5	3.0	0.5
Solvent control	200	0	+	7	1.5	1.0	0.5
Test article	200	6000	+	7	3.0	2.0	0.5
Solvent control	200	0	-	18	0.5	0.5	0.0
Positive control EMS	100	1750	-	18	22.0	22.0	20.0
Test article	200	600	-	18	2.5	2.0	0.0
Test article	200	3000	-	18	1.0	0.0	0.0
Test article	200	6000	-	18	2.5	0.5	0.0
Solvent control	200	0	+	18	1.5	1.5	0.0
Positive control CPA	200	5	+	18	15.0	14.5	7.0
Test article	200	600	+	18	3.0	1.5	0.5
Test article	200	3000	+	18	2.0	2.0	0.5
Test article	200	6000	+	18	4.5	2.5	1.0
Solvent control	200	0	-	28	1.0	0.5	0.0
Test article	200	6000	-	28	3.0	2.5	0.0
Solvent control	200	0	+	28	2.5	0.5	0.0
Test article	200	6000	+	28	1.5	1.0	0.5

Hoechst

9. BIOMETRY

FISHER TEST

7 H -S9 No. of phases with aberrations inclusive gaps

92.0337	Res onse		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	1	199			
6000 ug/ml	9	191	0.0100	*	*

7 H -S9 No. of phases with aberrations exclusive gaps

92.0337	Response		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	1	199			
6000 ug/ml	6	194	0.0609		

FISHER TEST

7 H -S9 No of aberrations inclusive gaps

92.0337	Response		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	1	199			
6000 ug/ml	9	191	0.0100	*	*

7 H -S9 No. of aberrations exclusive gaps

92.0337	Response		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	1	199			
6000 ug/ml	6	194	0.0609		

Hoechst

FISHER TEST

7 H +S9 No. of phases with aberrations inclusive gaps

92.0337	Response		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	3	197			
6000 ug/ml	6	194	0.2514		

7 H +S9 No. of phases with aberrations exclusive gaps

92.0337	Response		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	2	198			
6000 ug/ml	3	197	0.5000		

Hoechst

FISHER TEST

7 H +S9 No. of aberrations inclusive gaps

92.0337	Response		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	3	197	0.0159	*	*
6000 ug/ml	12	188			

7 H +S9 No. of aberrations exclusive gaps

92.0337	Response		P = 0.05		
Group	Yes	No	Prob	(1)	(2)
CONTROL	2	198	0.0309	*	*
6000 ug/ml	9	191			