RTC

RESEARCH TOXICOLOGY CENTRE - ROMA

[redacted]7850

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS IN VITRO

FINAL REPORT

RTC Study No.: 52420

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COMPLIANCE STATEMENT

We, the undersigned, hereby declare that the following report constitutes a true and faithful account of the procedures adopted, and the results obtained in the performance of the study. The aspects of the study conducted by Research Toxicology Centre S.p.A. were performed in accordance with:

A. *Good laboratory practice for non clinical laboratory studies*, U.S. Food and Drug Administration, Code of Federal Regulations, 21 Part 58, 22 December 1978 and subsequent revisions.

B. Decreto Legislativo 27 Gennaio 1992 n. 120, *Adoption of 88/320/EEC and 90/18/EEC Directives on the inspection and verification of good laboratory practice (G.U. 18 Febbraio 1992 n. 40)* and subsequent revisions.

C. Directive 2004/10/EC of European Parliament and of the Council of 11 February 2004. *On the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances.*

D. ENV/MC/CHEM(98)17 *OECD principles on Good Laboratory Practice (as revised in 1997).*

[redacted]

Date [redacted]

[redacted]

Date [redacted]

QUALITY ASSURANCE STATEMENT

(Relevant to the aspects of the study conducted by Research Toxicology Centre S.p.A.)

Study phases monitored by RTC's QAU according to current relevant Standard Operating Procedures	Quality Assurance Inspections (Day Month Year)		
	Inspection	Report to Study Director	Report to Company Management
PROTOCOL CHECK	13.02.2006	13.02.2006	13.02.2006
PROCESS-BASED INSPECTIONS RELATED TO THIS TYPE OF STUDY			
Dose preparation	04.05.2006	-	11.05.2006
Cell treatment	30.05.2006	-	31.05.2006
Cell harvesting	05.05.2006	-	24.05.2006
Slide preparation	05.05.2006	-	18.05.2006
Slide staining	22.06.2006	-	26.06.2006
Slide coding	19.01.2006	-	20.01.2006
Slide scoring	16.03.2006	-	22.03.2006
Other process-based inspections were carried out on routine activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here. Associated laboratories and support functions are subject to regular facility inspections.			
FINAL REPORT Review of this report by RTC's QAU found the reported methods and procedures to describe those used and the results to constitute an accurate representation of the recorded raw data.		Review completed [redacted]	

[redacted]

[redacted]

Date

Contents

1. SUMMARY

1.1 The test item [redacted] 7850 was assayed for the ability to cause chromosomal damage in Chinese hamster ovary cells, following *in vitro* treatment in the absence and presence of S9 metabolic activation.

1.2 Two assays for chromosomal damage were performed.
The first assay employed dose-levels of 5000, 2500, 1250, 625, 313, 156, 78.1, 39.1, 19.5, 9.77 and 4.88 μg/ml both in the absence and presence of S9 metabolism.
For the second assay, dose-levels of 450, 300, 200, 133, 88.9, 59.3, 39.5 and 26.3 μg/ml were employed. Solutions of the test item were prepared in ethanol.

In the first assay, both in the absence and presence of S9, the cells were treated for 3 hours and the harvest time of 20 hours, corresponding to approximately 1.5 cell cycle, was used. As negative results were obtained, a second assay in the absence of S9 metabolism was performed using a continuous treatment until harvest at 20 hours.

Each experiment included appropriate negative and positive controls. Two cell cultures were prepared at each test point.

1.3 Dose-levels were selected for the scoring of chromosomal aberrations on the basis of the cytotoxicity of the test item treatments as determined by the reduction of cell counts at the time of harvesting.

The treatment-levels selected for scoring were the following:

Experiment	S9	Treatment time (hours)	Harvest time (hours)	Dose-level (μg/ml)
1	- +	3	20	313, 156 and 78.1
2	-	20	20	300, 200 and 133

One hundred metaphase spreads were scored for chromosomal aberrations from each culture.

1.4 Following treatment with [redacted] 7850, for the first experiment, increases in the incidences of cells bearing gaps over the control were observed at all dose-levels selected for scoring in the presence of S9 metabolism.

An increase in the incidence of cells bearing aberrations excluding gaps, reaching statistical significance ($p<0.05$) was observed at the highest dose selected for scoring. The incidence was within the range of historical values observed in our laboratory for negative controls, therefore was not considered biologically meaningful.

For both experiments, no statistically significant increase in the incidence of cells bearing aberrations including or excluding gaps was observed following treatment with the test item in the absence of S9 metabolism.

Statistically significant increases in the number of cells bearing aberrations (including and excluding gaps) were observed following treatments with the positive controls Cyclophosphamide and Mitomycin-C, indicating the correct functioning of the test system.

1.5 It is concluded that [redacted] 7850 does not induce chromosomal aberrations in Chinese hamster ovary cells after *in vitro* treatment in the presence or absence of metabolic activation, under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes the experiment performed to assess the clastogenic activity of [redacted] 7850 in Chinese hamster ovary cells following *in vitro* treatment in the absence or presence of S9 metabolic activation.

The study was designed to comply with the experimental methods indicated in:

- EEC Council Directive 2000/32, Annex 4A.
- OECD Guideline No. 473 (Adopted: 21st July 1997).

2.2 Study organisation

Sponsor

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Location of study

Genetic and Cellular Toxicology Department
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Principal dates

Study protocol approved by Study Director: 18-Jan-2006
Study commenced: 07-Mar-2006 (Main assay I treatment)
Study completed: 19-Jun-2006 (Main Assay II completion of scoring)

Personnel involved in the study

Study Monitor:

[redacted]

Study Director:

[redacted]

Scorers of slides

[redacted]

Archiving:

The original data arising from this study, prepared microscope slides, and a copy of the consigned final report will be stored in the archives of Research Toxicology Centre S.p.A. for a period of three years from the date of consignment of the report. At the completion of this period the Sponsor will be contacted for despatch or disposal of the material, or further archiving. An aliquot of the test item will be retained within the archives of the testing facility for a period of ten years after which it will be destroyed.

3. MATERIALS AND METHODS

3.1 Test item

Details of the test item received at RTC were as follows:

Name	:	[redacted] 7850
Batch	:	3223 ON
C.A.S. No.	:	329238-24-6
Received from	:	SOLVAY SOLEXIS
Date received	:	20-Feb-2006
Amount received	:	100 g
Description	:	Colourless liquid
Expiry date	:	31-Dec-2012
Container	:	Opaque plastic bottle
Storage at RTC	:	Room temperature
RTC reference number	:	10022

On 27-Feb-2006 a 12g sub-sample of the test item was transferred from the Formulation Unit to the Department of Genetic and Cellular Toxicology and stored under the same conditions.

Solutions of the test item, as received, were prepared immediately before use in ethanol. Solutions were prepared on a weight/volume basis without correction for the displacement due to the volume of the test item. Concentrations were expressed in terms of material as received. All test item solutions were used within 25 minutes of the initial formulation. No assay of test item stability, nor its concentration and homogeneity in solvent were undertaken. All dose-levels in this report are expressed to three significant figures.

3.2 Control substances

The solvent used in this study was ethanol, batch no. 4G297304I obtained from Carlo Erba, Italy.
Solutions of Mitomycin-C (batch 104K0331 obtained from Sigma and batch 1149026 10605077 obtained from Fluka) and Cyclophosphamide (batch 075K1661 obtained from Sigma) were prepared in sterile distilled water immediately prior to use and served as positive controls. Injectable grade distilled water (batch 03H28-02) was obtained from Bieffe, Trieste, Italy.

Untreated cultures were included in the experimental scheme and acted as reference control for the positive control cultures.

3.3 S9 tissue homogenate

The rat S9 liver tissue fraction used in this study had the following characteristics:

Lot Number	:	1876
Inducing Agents	:	Phenobarbital – 5,6-Benzoflavone
Preparation date	:	09-Jun-2005
Expiry date	:	09-Jun-2007
Received from	:	MOLTOX, Molecular Toxicology, Inc.
Date received	:	14-Jul-2005
Storage at RTC	:	Approximately -80°C
Strain	:	Sprague Dawley
Sex of donors	:	Male
Protein content	:	37.2 mg/ml

A production and quality control certificate can be found in Addendum IV of this report.

3.4 Methods

The methods used were in compliance with the attached Study Protocol with the exception that an additional experiment was performed with the continuous treatment in the absence of S9 metabolism where no dose-level showed an adequate cytotoxicity for the scoring of chromosomal aberrations.
Data concerning this experiment are not presented in the report and will be archived as indicated in the Study Protocol.

4. RESULTS

4.1 Solubility test

The test item was found to be miscible with ethanol at 500 mg/ml. Aliquots of stock solutions exceeding 62.5 mg/ml added to supplemented Ham's F10 medium gave opacity or few particles in suspension in the medium. On the basis of the above mentioned result, the cytogenetic experiments were performed using a maximum dose-level of 5000 µg/ml.

During the first main assay, particles in suspension were observed at the beginning of treatment at the dose levels of 5000 and 2500 µg/ml both in the absence and presence of S9 metabolism. Slight opacity was observed at the dose of 1250 µg/ml both in the absence and presence of S9 metabolism
At the end of treatment, dose-related opacity and precipitation were observed at the dose-levels of 5000, 2500, 1250 and 625 µg/ml both in the absence and presence of S9.

During the second assay slight opacity of the medium was observed at the beginning of treatment at the dose-level of 450 µg/ml. At the end of treatment slight precipitation was also observed.

4.2 Assay for chromosomal aberrations

The first assay employed dose-levels of 5000, 2500, 1250, 625, 313, 156, 78.1, 39.1, 19.5, 9.77 and 4.88 µg/ml both in the absence and presence of S9 metabolism.
For the second assay, dose-levels of 450, 300, 200, 133, 88.9, 59.3, 39.5 and 26.3 µg/ml were employed.

In the first experiment, both in the absence and presence of S9 metabolic activation, the treatment time was 3 hours after which the cells were allowed to recover prior to harvesting. The harvest time of 20 hours, corresponding to approximately 1.5 cell cycle, was used. As negative results were obtained, a second experiment without S9 metabolic activation, was performed with continuous treatment until sampling at 20 hours.

Appropriate negative and positive control cultures were included in each experiment. Positive control treated cultures received Mitomycin-C 0.30 and 0.45 µg/ml for 3 hours treatment and 0.15 and 0.10 µg/ml for the continuous treatment in the absence of S9 metabolism or Cyclophosphamide 15.0 and 23.0 µg/ml in the presence of S9.

Two cultures were prepared at each test point. Air-dried slides were prepared from each culture and stained with 3% Giemsa.

Following treatment, the pH and osmolality of the treatment media at the higher dose-levels were determined for both experiments.
Slight dose-related reductions of these parameters over the control values were observed in the first main assay at the higher dose-levels of 5000 and 2500 µg/ml both in the absence and presence of S9 metabolism. These results are not presented in this report but, are retained in the study file and archived as indicated in the study protocol.

4.3 Selection of dose-levels for scoring

Cell counts were performed at harvesting time for each culture of each treatment series, and the results are presented in Tables 1 to 3.

In the first main assay, following treatment in the absence of S9 metabolism, severe toxicity was observed at the dose-levels of 5000, 2500, 1250 and 625 μg/ml, where no cells or few cells were recovered. Mild toxicity was observed at the dose-level of 313 μg/ml reducing the cell count to 75% of the control. Slight toxicity was observed at the dose-levels of 156 and 78.1 μg/ml (cell counts approximately 80% of the control). No remarkable toxicity was observed over the remaining dose-range.

Following treatment in the presence of S9 metabolism, severe toxicity was observed at the higher dose-levels of 5000, 2500 and 1250 μg/ml, where no cells were recovered. Marked toxicity was observed at the dose-level of 625 μg/ml reducing the cell count to 34% of the control. Mild and slight toxicity was observed at the dose-levels of 313 and 156 μg/ml (cell counts 77% and 85% of the control respectively). No toxicity was observed over the remaining dose-range.

In the second main assay, after the continuous treatment until sampling at 20 hours, severe toxicity was observed at the highest dose-level of 450 μg/ml, where few cells were recovered. Moderate toxicity was observed at the dose-level of 300 μg/ml where cell count was reduced to 58% of the control. Slight toxicity was observed at the dose-level of 200 μg/ml (cell count 82% of the control). No remarkable toxicity was observed over the remaining dose-range.

The highest dose-level selected for the scoring of aberrations should be a concentration causing moderate toxicity (ideally the reduction of cell count should be approximately 50%) and treatments reducing the cell count value to below 20% of the relevant control should not be scored. If no toxicity is observed then the highest practicable dose-level should be selected.
On the basis of the above results the dose-levels selected for scoring were:

Experiment	S9	Treatment time (hours)	Harvest time (hours)	Dose-level (μg/ml)
1	-/+	3	20	313, 156 and 78.1
2	-	20	20	300, 200 and 133

For the first main assay, at the dose-level of 625 (μg/ml) cell count was reduced to 34% of the control, however, due to the toxicity of treatment, an insufficient number of eligible metaphases were recovered for the scoring of aberrations.

4.4 Assay results

One hundred metaphase spreads were scored for chromosomal aberrations from each culture.

The results are presented in Tables 4, 5 and 6. In these tables the numbers and types of aberrations are presented, together with the total number of aberrations (chromatid type and chromosome type) including and excluding gaps. The total number of aberrant metaphases including and excluding gaps are also shown.

For the first experiment, following treatment with the test item in the presence of S9 metabolism, increases in the incidences of cells bearing gaps over the controls were observed at all dose-levels selected for scoring The incidences reached statistical significance for one replicate culture at the lowest dose.

A slight increase in the incidence of cells bearing aberrations excluding gaps, reaching statistical significance ($p<0.05$) for one replicate culture and for the pooled cultures, was observed at the highest dose selected for scoring. The incidence was within the range of historical values observed in our laboratory when considering the pooled cultures, therefore was not considered biologically meaningful.
An increase in the number of endoreduplicated cells over the control was mainly observed at the highest dose selected for scoring.

Following treatment in the absence of S9 metabolism, slight increases in the incidence of cells bearing gaps over the control values were observed at the highest and intermediate dose-levels selected for scoring after the short treatment.
A slight increase in the incidences of cells bearing gaps was also observed at the highest dose selected for scoring after the continuous treatment.
No relevant increase in the incidence of cells bearing aberrations excluding gaps was observed at any dose-level selected for scoring.

A few endoreduplicated cells and polyploid cells were noted for the short and continuous treatment respectively.

Marked increases in the frequency of cells bearing aberrations (including and excluding gaps) were seen in the cultures treated with the positive control substances, indicating the correct functioning of the assay system.

The modal number of chromosomes observed in the cells of untreated cultures (without S9 metabolism) was 21 (72.5% in the first experiment and 89.0% in the second experiment).
The frequency of cells containing 20 and 22 chromosomes was 1.0% and 26.5% in the first experiment, 0.0%, and 11.0% in the second experiment. For both experiments no metaphases were found with 19 or 23 chromosomes.

5. ANALYSIS OF RESULTS

5.1 Statistical analysis

For the statistical analysis, Fisher's Exact Test is used to compare the number of cells bearing aberrations (assumed to be Poisson distributed) in control and treated cultures. The analysis is performed using sets of data either including or excluding gaps. The results of the statistical analysis are presented in Tables 7, 8 and 9.

For the first experiment, following treatment with the test item in the presence of S9 metabolism, a statistically significant increase in the incidence of cells bearing gaps was observed for one replicate culture at the lowest dose selected for scoring.
A slight but significant increase ($p<0.05$) in the incidence of cells bearing aberrations excluding gaps was observed for one replicate at the highest dose selected for scoring.
A statistically significant increase in the incidence of cells bearing aberrations excluding gaps was also observed for the pooled cultures from the highest dose selected for scoring. The incidence of aberrant cells, however, was within the range of historical values observed in our laboratory for negative controls, therefore was not considered biologically meaningful.

For both experiments, no statistically significant increase in the incidence of cells bearing aberrations including or excluding gaps was observed following treatment with the test item in the absence of S9 metabolism.

5.2 Criterion for outcome

In this assay, the test item is considered to have clastogenic properties if the following criteria are all fulfilled:

(i) Statistically significant increases in the incidence of cells bearing aberrations are observed at any dose-level over the concurrent control.

(ii) The increases must exceed the historical control values.

(iii) The increases are reproduced in both replicate cultures.

The evaluation is based on the set of results, which excludes gaps. A more detailed explanation of the criteria for evaluation of the results is given in the Study Protocol.

5.3 Evaluation

On the basis of the above mentioned results and in accordance with the criteria for the outcome of the study the test item was not considered to induce chromosomal aberrations in Chinese hamster ovary cells *in vitro*.

Statistically significant increases in aberrant cells compared with the relevant control values were seen in cultures treated with the positive controls Mitomycin-C and Cyclophosphamide, indicating the correct functioning of the assay system.

6. CONCLUSIONS

A summary of the results is presented in Tables 10 and 11 giving the incidence of cells bearing aberrations (excluding gaps) and the relative cell count for each test point. The statistical significance of the recorded numbers of cells bearing aberrations is also shown.

On the basis of these results it is concluded that FLUOROLINK 7850 does not induce chromosomal aberrations in Chinese hamster ovary cells after ***in vitro*** treatment in the absence or presence of S9 metabolic activation, under the reported experimental conditions.

7. TABLES 1-11

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 1 - Cell growth results - Without metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 1

SOLVENT: ETHANOL

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose-level (µg/ml)	Culture No.	N° viable cellsx10^6/ml	Mean	Relative cell growth (%)
Untreated	-	1	0.63	0.63	107
		2	0.63		
Solvent	1%	3	0.59	0.59	100
		4	0.59		
Test Item	4.88	25	0.62	0.61	103
		26	0.60		
Test Item	9.77	23	0.61	0.60	102
		24	0.59		
Test Item	19.5	21	0.55	0.56	94
		22	0.56		
Test Item	39.1	19	0.51	0.56	95
		20	0.61		
Test Item	78.1	17	0.47	0.49	83
		18	0.51		
Test Item	156	15	0.48	0.48	81
		16	0.47		
Test Item	313	13	0.43	0.44	75
		14	0.45		
Test Item	625	11	0.02	0.02	3
		12	0.02		
Test Item	1250	9	0.00	0.00	0
		10	0.00		
Test Item	2500	7	0.00	0.00	0
		8	0.00		
Test Item	5000	5	0.00	0.00	0
		6	0.00		
Mitomycin-C	0.30	27	0.47	0.48	75
		28	0.48		
Mitomycin-C	0.45	29	0.41	0.42	67
		30	0.43		

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 2 - Cell growth results - With metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 1

SOLVENT: ETHANOL

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose-level (µg/ml)	Culture No.	N° viable cells*10**6/ml	Mean	Relative cell growth (%)
Untreated	-	31	0.62	0.62	106
		32	0.62		
Solvent	1%	33	0.61	0.59	100
		34	0.56		
Test Item	4.88	55	0.59	0.60	103
		56	0.61		
Test Item	9.77	53	0.59	0.61	103
		54	0.62		
Test Item	19.5	51	0.62	0.61	103
		52	0.59		
Test Item	39.1	49	0.60	0.61	103
		50	0.61		
Test Item	78.1	47	0.61	0.59	101
		48	0.57		
Test Item	156	45	0.51	0.50	85
		46	0.48		
Test Item	313	43	0.44	0.45	77
		44	0.46		
Test Item	625	41	0.17	0.20	34
		42	0.23		
Test Item	1250	39	0.00	0.00	0
		40	0.00		
Test Item	2500	37	0.00	0.00	0
		38	0.00		
Test Item	5000	35	0.00	0.00	0
		36	0.00		
Cyclophosphamide	15.0	57	0.44	0.43	69
		58	0.42		
Cyclophosphamide	23.0	59	0.35	0.37	59
		60	0.38		

[redacted]7850: CHROMOSOME ABERRATIONS

TABLE 3 - Cell growth results - Without metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 2

SOLVENT: ETHANOL

TREATMENT TIME: 20 hours

SAMPLING TIME: 20 hours

Treatment	Dose-level (µg/ml)	Culture No.	N° viable cells*10**6/ml	Mean	Relative cell growth (%)
Untreated	-	85	0.79	0.78	142
		86	0.76		
Solvent	1%	87	0.50	0.55	100
		88	0.59		
Test Item	26.3	103	0.60	0.60	110
		104	0.60		
Test Item	39.5	101	0.58	0.59	107
		102	0.59		
Test Item	59.3	99	0.57	0.56	102
		100	0.54		
Test Item	88.9	97	0.52	0.54	99
		98	0.56		
Test Item	133	95	0.53	0.51	93
		96	0.48		
Test Item	200	93	0.47	0.45	82
		94	0.42		
Test Item	300	91	0.31	0.32	58
		92	0.32		
Test Item	450	89	0.02	0.02	3
		90	0.01		
Mitomycin-C	0.10	105	0.40	0.39	50
		106	0.38		
Mitomycin-C	0.15	107	0.37	0.39	50
		108	0.41		

8. KEY TO TABLES 4-6

This table shows, for each test culture used in the main assay and by treatment group totals, the types and numbers of aberrations, identified as follows:

Gaps - This refers to either chromatid or chromosome gaps.
Del - Interstitial or terminal deletions.
Exch - Exchanges:

a) Chromatid exchanges
Includes symmetrical and asymmetrical exchanges; intra- and inter-chromosome exchanges.
b) Chromosome exchanges
Includes both dicentric and ring types.

Other - These include
H : Heavily damaged cells
(more than 5 aberrations/cell)
ER: Endoreduplicated cells
PP: Polyploid cells

Isolocus - Includes isochromatid and isolocus breaks when these cannot be distinguished.

Tot.abs - Total number of aberrations observed.

Cells with abs. - Cells with aberrations.

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 4 - Scoring of Aberrations - Without metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 1

SOLVENT: ETHANOL

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose-level (µg/ml)	Culture No.	Cells Scored	Gaps	Chromatid Del	Chromatid Exch	Chromosome Del	Chromosome Exch	Isolocus	Other	Tot.abs (+gaps)	Tot.abs (-gaps)	Cells with abs (+gaps)	Cells with abs (-gaps)
Untreated	-	1	100	6	1	0	0	0	0	0	7	1	7	1
		2	100	2	0	0	0	0	0	0	2	0	1	0
			200	8	1	0	0	0	0	0	9	1	8	1
Solvent	1%	3	100	3	0	0	0	1	0	0	4	1	4	1
		4	100	3	0	0	0	0	0	0	3	0	3	0
			200	6	0	0	0	1	0	0	7	1	7	1
Test Item	78.1	17	100	6	0	0	0	0	0	0	6	0	4	0
		18	100	1	0	0	0	0	0	0	1	0	1	0
			200	7	0	0	0	0	0	0	7	0	5	0
Test Item	156	15	100	5	1	0	0	0	0	0	6	1	6	1
		16	100	6	1	0	0	0	0	0	7	1	6	1
			200	11	2	0	0	0	0	0	13	2	12	2
Test Item	313	13	100	6	0	0	0	1	0	0	7	1	5	1
		14	100	3	1	0	0	0	0	2ER	4	1	4	1
			200	9	1	0	0	1	0	2ER	11	2	9	2
Mitomycin-C	0.30	27	100	8	29	30	0	0	4	0	71	63	47	45
		28	100	11	26	25	4	1	1	0	68	57	40	38
			200	19	55	55	4	1	5	0	139	120	87	83

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 5 - Scoring of Aberrations - With metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 1

SOLVENT: ETHANOL

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose-level (µg/ml)	Culture No.	Cells Scored	Gaps	Chromatid Del	Chromatid Exch	Chromosome Del	Chromosome Exch	Isolocus	Other	Tot.abs (+gaps)	Tot.abs (-gaps)	Cells with abs (+gaps)	Cells with abs (-gaps)
Untreated	-	31	100	5	0	0	0	0	0	0	5	0	5	0
		32	100	4	0	0	0	0	0	1ER	4	0	3	0
			200	9	0	0	0	0	0	1ER	9	0	8	0
Solvent	1%	33	100	10	0	0	0	0	0	0	10	0	9	0
		34	100	11	0	0	0	0	0	0	11	0	8	0
			200	21	0	0	0	0	0	0	21	0	17	0
Test Item	78.1	47	100	9	0	0	0	0	0	0	9	0	9	0
		48	100	28	3	0	0	0	0	2ER	31	3	19	3
			200	37	3	0	0	0	0	2ER	40	3	28	3
Test Item	156	45	100	9	2	0	0	0	0	0	11	2	10	2
		46	100	18	0	1	0	0	0	3ER	19	1	15	1
			200	27	2	1	0	0	0	3ER	30	3	25	3
Test Item	313	43	100	12	0	0	0	1	0	5ER	13	1	10	1
		44	100	13	5	0	0	0	0	6ER	18	5	16	5
			200	25	5	0	0	1	0	11ER	31	6	26	6
Cyclophosphamide	15.0	57	100	15	25	38	0	0	8	0	86	71	46	42
		58	100	19	23	26	0	0	11	0	79	60	43	40
			200	34	48	64	0	0	19	0	165	131	89	82

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 6 - Scoring of Aberrations - Without metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 2

SOLVENT: ETHANOL

TREATMENT TIME: 20 hours

SAMPLING TIME: 20 hours

Treatment	Dose-level (µg/ml)	Culture No.	Cells Scored	Gaps	Chromatid Del	Chromatid Exch	Chromosome Del	Chromosome Exch	Isolocus	Other	Tot.abs (+gaps)	Tot.abs (-gaps)	Cells with abs (+gaps)	Cells with abs (-gaps)
Untreated	-	85	100	2	0	0	0	0	0	0	2	0	2	0
		86	100	0	0	0	0	0	0	0	0	0	0	0
			200	2	0	0	0	0	0	0	2	0	2	0
Solvent	1%	87	100	2	0	0	0	0	0	0	2	0	2	0
		88	100	3	0	0	0	0	0	0	3	0	3	0
			200	5	0	0	0	0	0	0	5	0	5	0
Test Item	133	95	100	2	0	0	0	0	0	0	2	0	2	0
		96	100	0	0	0	0	0	0	0	0	0	0	0
			200	2	0	0	0	0	0	0	2	0	2	0
Test Item	200	93	100	2	0	0	0	0	0	1PP	2	0	2	0
		94	100	4	0	0	0	0	0	1PP	4	0	4	0
			200	6	0	0	0	0	0	2PP	6	0	6	0
Test Item	300	91	100	1	0	0	0	0	0	1PP	1	0	1	0
		92	100	7	0	0	0	0	0	0	7	0	7	0
			200	8	0	0	0	0	0	1PP	8	0	8	0
Mitomycin-C	0.10	105	100	9	1	15	0	0	0	0	25	16	17	13
		106	100	2	1	16	0	0	0	1H	19	17	19	18
			200	11	2	31	0	0	0	1H	44	33	36	31

9. KEY TO TABLES 7-9

This table summarises the statistical analyses for the individual treated cultures and for the treatment groups using the pooled data. The analyses are presented both including and excluding gaps from the data-sets. The following abbreviations are used:

P. Value — The calculated probability value obtained from the comparison of the treated with the controls, using Fisher's Exact Test.

The test item treatments are compared with the relevant solvent or untreated controls as appropriate (according to the vehicle used for the test material). Positive control treatments are compared with the untreated controls.

Sig. — Significance level.

The significance level of the achieved P-value.
For the test item treatments, correction is made for multiple comparisons (as indicated at the foot of each table).
For positive control treatments and for all dose-levels combined significance is indicated as:

* Statistically significant at $P<0.05$
** Statistically significant at $P<0.01$
*** Statistically significant at $P<0.001$

\# — No statistic was calculated since the proportion of cells bearing aberrations was identical for the treated and relevant negative control cultures.

NS — Not significant.

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 7 - Statistical Analysis - Without metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 1

SOLVENT: ETHANOL

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Comparison with Negative control	Dose µg/ml	CULT.	Cells with aberrations (+ gaps) P. values	Sig.	Cells with aberrations (- gaps) P. values	Sig.
Test Item	78.1	17	0.5292	N.S.	0.6668	N.S.
		18	0.1914	N.S.	0.6668	N.S.
	Pooled		0.3856	N.S.	0.5000	N.S.
Test Item	156	15	0.2371	N.S.	0.5564	N.S.
		16	0.2371	N.S.	0.5564	N.S.
	Pooled		0.1738	N.S.	0.5000	N.S.
Test Item	313	13	0.3669	N.S.	0.5564	N.S.
		14	0.5292	N.S.	0.5564	N.S.
	Pooled		0.3998	N.S.	0.5000	N.S.
All dose levels			0.3909	N.S.	0.6328	N.S.
Mitomycin-C	0.30	27	0.0000	***	0.0000	***
		28	0.0000	***	0.0000	***
	Pooled		0.0000	***	0.0000	***

In this table correction is made for the multiple comparisons of the test item treatments with the negative controls. For individual cultures, since six comparisons are made, the required "p" values for significance are 0.009(*), 0.002(**) and 0.0002(***).
For treatment levels, since three comparisons are made the required "p" values for significance are 0.017(*), 0.003(**) and 0.0003(***).

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 8 - Statistical Analysis - With metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 1

SOLVENT: ETHANOL

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Comparison with Negative control	Dose µg/ml	CULT.	Cells with aberrations (+ gaps) P. values	Sig.	Cells with aberrations (- gaps) P. values	Sig.
Test Item	78.1	47	0.5208	N.S.	#	N.S.
		48	0.0083	*	0.0363	N.S.
	Pooled		0.0564	N.S.	0.1241	N.S.
Test Item	156	45	0.4082	N.S.	0.1104	N.S.
		46	0.0663	N.S.	0.3334	N.S.
	Pooled		0.1267	N.S.	0.1241	N.S.
Test Item	313	43	0.4082	N.S.	0.3334	N.S.
		44	0.0413	N.S.	0.0038	*
	Pooled		0.0981	N.S.	0.0150	*
All dose levels			0.0480	*	0.0308	*
Cyclophosphamide	15.0	57	0.0000	***	0.0000	***
		58	0.0000	***	0.0000	***
	Pooled		0.0000	***	0.0000	***

In this table correction is made for the multiple comparisons of the test item treatments with the negative controls. For individual cultures, since six comparisons are made, the required "p" values for significance are 0.009(*), 0.002(**) and 0.0002(***).
For treatment levels, since three comparisons are made the required "p" values for significance are 0.017(*), 0.003(**) and 0.0003(***).

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 9 - Statistical Analysis - Without metabolic activation

STUDY NO.: 52420

MAIN ASSAY: 2

SOLVENT: ETHANOL

TREATMENT TIME: 20 hours

SAMPLING TIME: 20 hours

Comparison with Negative control	Dose µg/ml	CULT.	Cells with aberrations (+ gaps) P. values	Sig.	Cells with aberrations (- gaps) P. values	Sig.
Test Item	133	95	0.5707	N.S.	#	N.S.
		96	0.1295	N.S.	#	N.S.
	Pooled		0.2245	N.S.	#	N.S.
Test Item	200	93	0.5707	N.S.	#	N.S.
		94	0.3484	N.S.	#	N.S.
	Pooled		0.5000	N.S.	#	N.S.
Test Item	300	91	0.3490	N.S.	#	N.S.
		92	0.0625	N.S.	#	N.S.
	Pooled		0.2873	N.S.	#	N.S.
All dose levels			0.5670	N.S.	#	N.S.
Mitomycin-C	0.10	105	0.0000	***	0.0000	***
		106	0.0000	***	0.0000	***
	Pooled		0.0000	***	0.0000	***

In this table correction is made for the multiple comparisons of the test item treatments with the negative controls. For individual cultures, since six comparisons are made, the required "p" values for significance are 0.009(*), 0.002(**) and 0.0002(***).
For treatment levels, since three comparisons are made the required "p" values for significance are 0.017(*), 0.003(**) and 0.0003(***).

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 10 - Summary Table

STUDY NO.: 52420

MAIN ASSAY: 1

SOLVENT: ETHANOL

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose µg/ml	Presence of S9 metabolism			Absence of S9 metabolism		
		%CA		(Rel.cell growth)	%CA		(Rel.cell growth)
Untreated	-	0.0		(106)	0.5		(107)
Solvent	1%	0.0		(100)	0.5		(100)
Test Item	78.1	1.5	N.S.	(101)	0.0	N.S.	(83)
Test Item	156	1.5	N.S.	(85)	1.0	N.S.	(81)
Test Item	313	3.0	*	(77)	1.0	N.S.	(75)
Mitomycin-C	0.30	-			41.5	***	(75)
Cyclophosphamide	15.0	41.0	***	(69)	-		

Key:
% CA : Percentage of cells bearing aberrations (excluding gaps)
Rel.cell growth: Relative to solvent controls (percent)
- : Not tested or not selected for the scoring of aberrations
* : Statistically significant at P<0.05
** : Statistically significant at P<0.01
*** : Statistically significant at P<0.001

[redacted] 7850: CHROMOSOME ABERRATIONS

TABLE 11 - Summary Table

STUDY NO.: 52420

MAIN ASSAY: 2

SOLVENT: ETHANOL

TREATMENT TIME: 20 hours

SAMPLING TIME: 20 hours

Treatment	Dose µg/ml	Presence of S9 metabolism		Absence of S9 metabolism	
		%CA	(Rel.cell growth)	%CA	(Rel.cell growth)
Untreated	-	-		0.0	(142)
Solvent	1%	-		0.0	(100)
Test Item	133	-		0.0 N.S.	(93)
Test Item	200	-		0.0 N.S.	(82)
Test Item	300	-		0.0 N.S.	(58)
Mitomycin-C	0.10	-		15.5 ***	(50)

Key:
% CA : Percentage of cells bearing aberrations (excluding gaps)
Rel.cell growth: Relative to solvent controls (percent)
- : Not tested or not selected for the scoring of aberrations
* : Statistically significant at P<0.05
** : Statistically significant at P<0.01
*** : Statistically significant at P<0.001

10. APPENDIX I - Historical background incidences of aberrant cells

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Background incidences (%) of aberrant cells (Years 1990-2005)

	Absence of S9		Presence of S9 metabolism	
	20 hours sampling time		20 hour sampling time	
	+ gaps	- gaps	+ gaps	- gaps
UNTREATED				
Mean	1.6	0.5	2.0	0.5
SD (σ_{n-1})	1.5	0.7	1.7	0.6
n	68	68	47	47
Minimum	0.0	0.0	0.0	0.0
Maximum	6.0	3.0	7.0	2.5
SOLVENT				
Mean	1.8	0.6	1.9	0.7
SD (σ_{n-1})	1.6	0.8	1.7	0.9
n	51	51	35	35
Minimum	0.0	0.0	0.0	0.0
Maximum	8.0	4.0	8.0	4.0
POSITIVE				
Mean	41.1	39.1	37.6	35.4
SD (σ_{n-1})	15.2	14.4	14.8	14.7
n	71	71	49	49
Minimum	15.0	14.5	15.5	15.5
Maximum	77.0	76.0	67.0	66.0

SD = standard deviation
n = number of experiments

11. APPENDIX II - Study Protocol

RESEARCH TOXICOLOGY CENTRE - ROMA

[redacted] 7850

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS *IN VITRO*

Final Protocol
prepared for

SOLVAY SOLEXIS S.p.A.
Viale Lombardia, 20
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by

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RTC Enquiry No. 52420

January 2006

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Cod. Fisc.: 00853120584
Partita IVA: 00920611001

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS *IN VITRO*

MANAGEMENT OF STUDY

Scientific Director : [redacted]

Head of Genetic and Cellular Toxicology : [redacted]

Study Director : [redacted]

Sponsor : SOLVAY SOLEXIS S.p.A.
Viale Lombardia, 20
20021 Bollate (MI)
Italy

Monitor : [redacted]

QUALITY ASSURANCE

Quality Assurance Manager : [redacted]

LOCATION OF STUDY

The study will be performed at : Research Toxicology Centre, S.p.A.
Via Tito Speri, 12
00040 Pomezia, ROMA.
Italy

The laboratory facilities, archives and administration are located at this site.

TIME SCHEDULE OF STUDY

The study will be conducted with a time schedule agreed between the Sponsor and RTC.

TEST ITEM IDENTITY

The test item will be : [redacted]7850

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS *IN VITRO*

1. INTRODUCTION

1.1 Objective

To assay the test item for the ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells, after treatment in the presence and absence of S9 metabolism.

1.2 Regulatory requirements

This study will be conducted in compliance with the GLP regulations of:

- US FDA [21 CFR part 58, 22 December 1978] and subsequent revisions;
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004;
- ENV/MC/CHEM(98)17 "OECD principles on Good Laboratory Practice – as revised in 1997";
- Decreto Legislativo no. 120 of 27 January 1992 and subsequent revisions.

In addition, the study is designed following the experimental methods indicated in the guidelines of:

- EEC Council Directive 2000/32, Annex 4A.
- OECD Guideline No. 473 (adopted July 1997).

1.3 Principles of the assay

This assay detects structural changes in the chromosomes of cultured mammalian cells induced by test agents. The Chinese hamster ovary cell line is useful for this work because of its stable aneuploid karyotype (modal number is 21 chromosomes), short cell cycle (12-14 hours) and its high plating efficiency. The cell line is derived from an explant of the ovary of the Chinese hamster (Cricetulus griseus, 2n = 22). In the assay method, exponentially growing cultures of these cells are exposed to the test substance. Since the cell line has lost much of its ability to metabolise indirect mutagens to reactive forms, the test is performed both in the presence and in the absence of an S9 metabolising system.

The cells are exposed to the test item both in the absence and presence of metabolic activation for a short treatment time (3 hours) and post treatment mitosis are harvested for analysis at a time corresponding to approximately 1.5 cell cycle lengths (20 hours) to allow detection of chemicals which may cause cell cycle delays.

The spindle poison colcemid is used to arrest the cells in metaphase. The cells are subsequently brought into suspension and air-dried slide preparations are made and stained. The chromosomes in the metaphase spreads are examined by microscopy for evidence of chromosomal damage.

Clear positive results with and/or without S9 metabolism will not be confirmed in further experiments.

If negative or equivocal results both with and/or without S9 metabolism are obtained, an additional experiment will be performed with continuous treatment in the absence of metabolic activation and harvest time at approximately 1.5 cell cycle lengths.

Any additional experiment will be performed following discussion with the Sponsor.

2. TEST ITEM

2.1 It is the responsibility of the Sponsor to supply the test item, accompanied by analytical data confirming the identity, purity, stability, strength and composition of the item, the solubility and stability in the proposed vehicle and details of any known hazards to laboratory staff. The test item should be accompanied by a certificate of analysis.

2.2 Approximately one year after the submission of the final report, remaining amounts of the test item will be destroyed by incineration. An aliquot of the item will be retained within the archives of the testing facility for a period of ten years after which it will be destroyed.

2.3 The test item identity is indicated on previous pages of this protocol.

2.4 Unless otherwise indicated by the Sponsor, the storage conditions for the test item will be room temperature.

2.5 The precautions necessary when handling either the test item or prepared formulations of the test item are based on information supplied by the Sponsor. The minimum safety precautions necessary are detailed under the RTC Hazard Classification System, according to RTC standard procedures.

2.6 The amount of test item received and used will be recorded according to standard procedures.

2.7 Fresh solutions of the test item will be prepared for each day's work; solutions will be prepared on a weight/volume basis without correction for the displacement due to the volume occupied by the test item. Unless specified by the Sponsor, concentrations of solutions will be expressed in terms of material as received, and not of active constituents. Preferred solvents will be sterile distilled water, culture medium, DMSO, ethanol, acetone. Other solvents may be used as necessary.

2.8 No assay of the test item stability, nor its concentration and homogeneity in the solvent/vehicle will be undertaken, nor samples of formulated test item consigned to the Sponsor, without express instructions from the Sponsor. No determination of the absorption of the test item in the test system will be made without express instructions from the Sponsor.

3. MATERIALS AND METHODS

3.1 Chinese hamster ovary (CHO) cells

Chinese hamster ovary cells were obtained from Dr. A.T. Natarajan (State University of Leiden). This cell line derives from the CHO isolate originally described by Kao and Puck (1968).

The karyotype, generation time and plating efficiency have been checked in this laboratory. The cells are checked at regular intervals for the absence of mycoplasmal contamination.

Permanent stocks of CHO cells are stored in liquid nitrogen, and subcultures are prepared from these stocks for experimental use. Cultures are grown in Ham's F10 medium supplemented with 15% Newborn Calf serum. All incubations are at 37°C in a 5% carbon dioxide atmosphere (100% humidity nominal). Culture flasks are labelled during subculturing and experimental use with their identity and appropriate codices.

3.2 Culture medium

The medium used for the growth of the cells has the following composition:

Ham's F.10	499.0 ml
Streptomycin sulphate 50 mg/ml Penicillin G 50,000 IU/ml	1.0 ml
Newborn Calf Serum	88.2 ml

3.3 S9 mix

The S9 liver tissue fraction will be prepared according to RTC standard procedures or will be obtained from an appropriate supplier (MOLTOX, Molecular Toxicology, Inc., USA). Induction of drug metabolising enzyme-levels is routinely performed using phenobarbitone and betanaphtoflavone (mixed induction). Induction with Aroclor 1254 will be performed if specifically requested by the Sponsor. Records pertaining to the preparation of the S9 tissue fraction are kept on file at RTC.

The mixture of S9 tissue fraction and cofactors (S9 mix) will be prepared in the following proportions:

S9 tissue fraction	3.0 ml
NADP (0.1M)	0.4 ml
G-6-P (0.1M)	0.5 ml
KCl (0.33M)	1.0 ml
MgCl2 (0.1M)	0.4 ml
Hepes (0.2M)	1.0 ml
H.B.S.S.	3.7 ml
	10.0 ml

3.4 Control items

Positive control treatments are included in every experiment. The positive control items, Mitomycin-C and Cyclophosphamide, are obtained commercially and characterised by their labelling. Fresh solutions in sterile distilled water will be prepared for each day's work. Determination of the stability and concentration of solutions of these agents will not be undertaken since it is sufficient to provide evidence for the correct expected response of the test system to them.

4. SELECTION OF DOSE LEVELS

4.1 Selection of dose levels for treatment

The highest dose-level to be used is determined according to the solubility of the test item in the culture medium and solvent vehicle, but will not exceed a maximum concentration of 5 mg/ml. Up to two dose-levels at which precipitation is observed may be included in the treatment series. Seven lower dose-levels will be used, spaced at approximately equal intervals.

If an additional experiment is performed, dose-levels will be selected on the basis of the results obtained in the first experiment.
Space intervals will be selected according to the results obtained (e.g. toxicity) in the first experiment.

Positive control treatments will be Mitomycin-C in the absence of S9 metabolism and Cyclophosphamide in the presence of S9 metabolism, both at the appropriate concentrations to give a clear positive response.

Each experiment will include also solvent vehicle and untreated control cultures.
Duplicate cultures are prepared for each experimental test point. When it seems advisable, further test points or controls may be included in the experiment.

5. ASSAY PROCEDURE

5.1 Preparation of the test cultures

Approximately 20 hours before treatment an appropriate number of flasks for the experiment are prepared from a single pool of cells. Each 25 sq.cm. flask is seeded with 300,000 cells in supplemented Ham's F10. These cells should be in exponential growth phase at the time of treatment.

The following section describes treatment procedures which will be followed in the first experiment.

5.2 Treatment of the cultures

Treatment medium without metabolic activation is prepared for each test point in the following proportions:

Test item or control solution	0.05 ml
Supplemented Ham's F10 medium	4.95 ml

Treatment medium with metabolic activation is prepared containing the test item and S9 mix in the following proportions:

Test item or control solution	0.05 ml
S9 mix	0.50 ml
Supplemented Ham's F10 medium	4.45 ml

Both in the absence and presence of S9 metabolism, the cultures are incubated in this treatment media for three hours. The medium is then removed and the flasks are washed twice with Ca/Mg-free Phosphate Buffered Saline (PBS). Fresh medium is added and the cultures are incubated for a further 17 hours (Recovery Period). Colcemid (0.2 µg/ml final concentration) is added for the last three hours of the recovery period, leading up to harvesting. In this way cultures are prepared for harvesting at 20 hours after treatment commenced.
Where it is necessary to adjust the composition of the treatment medium (for example, where culture medium is used as the solvent), the final composition of the treatment medium will be indicated in the Final Report.

5.3 Additional experiment

If negative or equivocal results both with and/or without S9 metabolism are obtained, an additional experiment is performed with continuous treatment and a harvest time at approximately 1.5 cell cycle lengths.

This experiment is conducted only in the absence of S9 and treatment medium is prepared for each test point as described in section 5.2. The treatment media are added to the tubes and the cultures are incubated for twenty hours. Colcemid is added (0.2 µg/ml final concentration) for the last three hours of the treatment period, leading up to harvesting. Cells are harvested at twenty hours after beginning of treatment.

Negative results with metabolic activation need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is considered necessary, an additional experiment will be carried out following approval of an amendment to the protocol.

If there is evidence of a cell cycle delay, the clastogenic potential may be readily detected by treatment/sampling times longer than 1.5 cell cycle lengths. In this case an additional experiment may be performed after discussion with the Sponsor. The performance of this experiment will be carried out following approval of an amendment to the protocol.

5.4 Osmolality and pH measurement

For each experiment, the osmolality and pH of treatment media (at the higher dose-levels of treatment), with and without S9 metabolic activation will be checked. If considered appropriate, data will be presented in the Final Report.

5.5 Harvesting and slide preparation

The medium is removed from the flasks, and the cells are brought into suspension by trypsinization. The cell suspension is centrifuged and the cell pellet is resuspended in hypotonic solution.

The cells are then fixed in freshly prepared methanol:acetic acid fixative and washed two times with fixative. A few drops of the cell suspension obtained in this way are dropped onto clean, wet, grease-free, glass slides and air-dried to produce metaphase chromosome spreads. At least three slides will usually be prepared, each labelled with the identity of the culture. The slides are stained in 3% Giemsa in tap water and rinsed in tap and distilled water. The slides are then made permanent with Eukitt.

5.6 Selection of dose-levels for scoring of chromosomal aberrations

At the time of harvesting, cell counts will be performed for each culture.

The highest dose-level selected for scoring should show a significant reduction in cell count. Ideally the reduction should be approximately 50% of the control and treatments reducing cell count values to below 20% of the control should not be used. If the test item does not induce toxicity at any dose-level, then the highest treatment level will be selected as the highest dose-level for scoring.

Two lower dose-levels will be also selected for the scoring of chromosomal aberrations. The lowest dose-level for scoring should be on the borderline of cytotoxicity. The intermediate dose-level will be evenly spaced between the two.

Where it seems advisable, the mitotic index (number of metaphases per 1000 cells) will also be determined in order to have additional information on the cytotoxicity of treatment.

5.7 Metaphase analysis

The slides are randomly assigned code numbers by a person not subsequently involved in slide evaluation, and any other identification marks are concealed.

Metaphase spreads judged to be of sufficient quality to permit scoring are examined at high magnification. Metaphases that differ from the modal chromosomal complement by more than two centromeres are not scored.

Polyploid and endoreduplicated cells encountered will be recorded, but not included in the count of eligible metaphases. From 100 eligible metaphases per test culture, the number of chromosomes, the specific types and numbers of aberrations are recorded. The Vernier readings of aberrant or equivocal metaphases are recorded.

If more than 50% of the cells are found to contain aberrations (other than gaps), then at the discretion of the Study Director, scoring for that culture may be terminated at 50 metaphase spreads only.

Where it seems advisable, additional metaphases may be scored using microscope slides that have not previously been examined. These slides will be re-coded prior to scoring.

6. REPORTING

6.1 Presentation of data

The data is presented in the form of tables giving frequencies of chromosome exchanges and deletions, chromatid exchanges and deletions, gaps, and other anomalies (polyploid cells, endoreduplication, seriously damaged cells etc.). The total number of aberrant metaphases (including and excluding gaps), the relative cell counts, and the number of metaphases analysed for each test culture are also shown. These are all tabulated by individual treated cultures, and by dose-level groups. The modal number of chromosomes in the cell line will be recorded together with the distribution of chromosome numbers.

6.2 Statistical evaluation of the data

The numbers of cells bearing aberrations in the control and treated cultures are compared using Fisher's exact test. The comparison is performed both including and excluding gaps from the aberration counts. The values obtained at all the treatment dose-levels combined are compared with the control values. Since multiple dose-levels are compared with the negative controls, the problem of Type I error (chance 'positive' results) arises. Accordingly, significance levels for each treatment-level will also be presented after application of Bonferroni's correction.
The solvent/vehicle controls will be used as the reference point for comparison in the statistical evaluation and the evaluation of the results.

6.3 Evaluation of the results

For a test item to be considered clastogenic, four criteria must be met:

(i) Increases over the concurrent controls

If any dose-level shows a statistically significant increase in aberration-bearing cells, this will be considered as evidence of a clastogenic effect.

(ii) Increase over historical controls

If the increases fall within the range of values normally observed in the negative control cultures, the test item cannot be judged clastogenic. Any significant increases over the concurrent solvent/vehicle controls will therefore be compared with historical control values derived from recent studies. The historical data-base will be included as an appendix in the Study Report.

(iii) Reproducibility

Any increases observed must be present in both replicate cultures.

(iv) Biological significance

The conclusions must be consistent with the underlying biology of the assay. Specifically:

- It is not required that an increased response is observed at increasing dose-levels, but dose-related activity will be taken as further evidence of a clastogenic effect.
- The types of aberrations observed will be taken into consideration.
- An increased incidence of an aberration type with an exceedingly low background frequency will be considered as evidence of a clastogenic effect.

The variability inherent in biological material may inevitably lead to the generation of equivocal data sets [eg. (i) dose-related increases over concurrent controls that do not exceed historical values or (ii) increases over historical values which are not significantly greater than concurrent controls].
In such cases it is strongly recommended that further evaluation should be undertaken in the form of a further experimental treatment of the relevant treatment series and scoring.

6.4 Reporting procedure

A Draft Report will be supplied, and a Final Report issued subsequently to include any agreed changes or amendments.
If any corrections or additions are required to the Final Report, these will be in the form of an amendment by the Study Director. The amendment will clearly identify that part of the Final report that is being added to or corrected, and the reasons for the changes, and will be signed and dated by the person responsible.

6.5 Final Report

The following information and data will be included in the final report:

- name and address of the facility performing the study and the dates on which the study was initiated and completed;
- objective and procedures stated in the approved protocol, including approved changes to the original protocol;
- the test item, identified by name, chemical name or chemical number;
- method used;
- data generated while conducting the study;
- statistical methods employed for analysing the data;

- a summary of the data, an analysis of the data and a statement of the conclusions drawn from the analysis;
- any unforeseen circumstances that may have affected the quality or integrity of the study;
- historical solvent/vehicle control data;
- the name and signature of the Study Director;
- the location where all raw data, specimens and final report are to be stored;
- Quality assurance statement.

One original unbound, one copy bound and a PDF version will be supplied.

6.6 Records kept

Full records will be maintained of all aspects of study conduct, along with the results of all measurements and observations. Prior to final archiving of the study data a full list will be prepared of all records associated with the study.

6.7 Archiving

All raw data, records and documentation arising from this study and a copy of the final report consigned, generated during the course of this study will be retained at RTC. Archiving will be provided for a period of 3 years after which the Sponsor will be contacted for instructions regarding despatch or disposal of the material. As a further option, archiving space can be rented for an additional time. The signed Final Protocol and the top copy of the Final Report will be despatched to and archived by the Sponsor.

7. STUDY CONDUCT

7.1 Language

English language and Italian language version of the study protocol, Standard Operating Procedures and other study documents may be used interchangeably. Similarly, English and Italian renderings of chemical names, including that of the test item will be considered to be equivalent.

7.2 Scientific decisions

The procedures described in this protocol may not comprehensively cover all the circumstances that can arise in the assay of test item. When the study director considers it advisable to modify the procedures described for the selection of a solvent, selection of dose-levels, interpretation of the outcome of the study or other aspects of the study conduct, he/she will record carefully the decision he/she has reached and the reasoning which led to it.

7.3 Quality assurance

The study is subjected to the procedure for quality assurance as defined by the relevant GLP regulations. Specifically:

- the protocol is inspected for compliance;
- procedures of the laboratories concerned will be inspected at intervals adequate to assure the integrity of the study;
- the final report is reviewed to ensure that it accurately describes the methods and relevant Standard Operating Procedures and that the results are in agreement with the raw data;
- periodic reports on these activities are made to management and the Study Director.

All raw data pertaining to the study are available for inspection by the Study Monitor (for scientific monitoring) or the Quality Assurance Unit of the Sponsor (compliance monitoring).

8. DEPARTURES FROM REGULATORY REQUIREMENTS

Items which are the responsibility of the Sponsor are indicated in sections 2.1, 2.4, 2.7, 2.8 and 3.3 of this protocol. Since full compliance with regulatory requirements may depend on the performance of some of these items, the Sponsor should ensure that appropriate actions are initiated or undertaken.

9. REFERENCES

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Report from working group on in vitro tests for chromosomal aberrations
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Hsu T.C. *et al.* (1977)
Cytogenetic assays of chemical clastogens using mammalian cells in culture.
Mutation Research 45 233-247

Kao F.T. and Puck T.T. (1968)
Genetics of Somatic mammalian cells VII.
Proceedings of the National Academy of Science (USA) 60 1275-1281

Preston R.J. *et al.* (1981).
Mammalian *in vivo* and *in vitro* cytogenetic assays:
A report of the US EPA's Gene-tox program
Mutation Research 87 143-188

Scott D. *et al.* (1990)
Metaphase chromosome aberration assay *in vitro*
in: Basic mutagenicity tests: UKEMS recommended procedures
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Seeberg A.H. and R. Forster (1988).
Testing above the limit of solubility.
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PROTOCOL APPROVAL PAGE

STUDY TITLE : CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS *IN VITRO*

TEST FACILITY : RESEARCH TOXICOLOGY CENTRE S.p.A.
Via Tito Speri, 12
00040 Pomezia (RM)
Italy

RTC ENQUIRY NO. : 52420

TEST ITEM : [redacted] 7850

APPROVED BY : [redacted] _____ [redacted]
Study Director

RELEASED BY : [redacted] _____ [redacted]
[redacted]icology

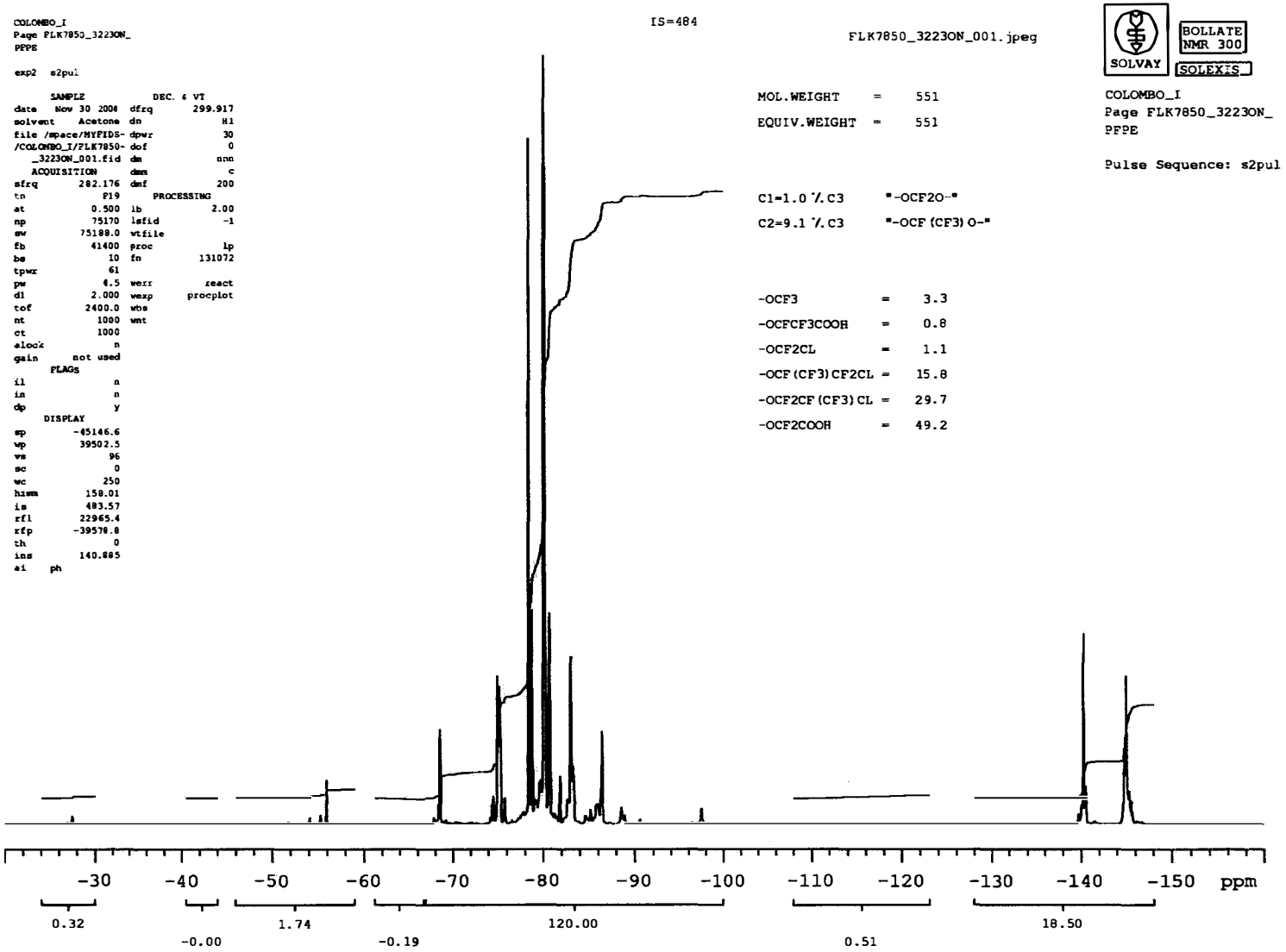
SPONSOR : SOLVAY SOLEXIS S.p.A.
Viale Lombardia, 20
20021 Bollate (MI)
Italy

AUTHORISED BY SPONSOR : [redacted] _____ [redacted]
Date

Name and Title : [redacted]

* Please print or type your name and company status below your signature

12. APPENDIX III - Certificate of analysis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PFPE
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SAMPLE
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solvent Acetone
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/COLOMBO_I/FLK7850-
_3223ON_001.fid
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tn F19
at 0.500
np 75170
sw 75188.0
fb 41400
bs 10
tpwr 61
pw 6.5
d1 2.000
tof 2400.0
nt 1000
ct 1000
alock n
gain not used
FLAGS
il n
in n
dp y
DISPLAY
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wp 39502.5
vs 96
sc 0
wc 250
hzmm 158.01
is 483.57
rf1 22965.4
rfp -39578.8
th 0
ins 140.885
ai ph
DEC. 6 VT
dfrq 299.917
dn H1
dpwr 30
dof 0
dm nnn
dmm c
dmf 200
PROCESSING
lb 2.00
lsfid -1
wtfile
proc lp
fn 131072
werr react
wexp procplot
wbs
wnt
IS=484
FLK7850_3223ON_001.jpeg
SOLVAY
BOLLATE NMR 300
SOLEXIS
COLOMBO_I
Page FLK7850_3223ON_
PFPE
Pulse Sequence: s2pul
MOL.WEIGHT = 551
EQUIV.WEIGHT = 551
C1=1.0 %.C3 "-OCF2O-"
C2=9.1 %.C3 "-OCF(CF3)O-"
-OCF3 = 3.3
-OCFCF3COOH = 0.8
-OCF2CL = 1.1
-OCF(CF3)CF2CL = 15.8
-OCF2CF(CF3)CL = 29.7
-OCF2COOH = 49.2
-30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 ppm
0.32 -0.00 1.74 -0.19 120.00 0.51 18.50

13. APPENDIX IV - S9 production of quality control certificate

MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9)
PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 1876 | SPECIES: Rat | PREPARATION DATE: June 09, 2005
PART NO.: 11-105 | STRAIN: Sprague Dawley | EXPIRATION DATE: June 09, 2007
VOLUME: 5 ml | SEX: Male | BUFFER: 0.154 M KCl
TISSUE: Liver | INDUCING AGENT(s): Phenobarbital - 5,6-Benzoflavone

REFERENCE: Matsushima, et. al., In: In Vitro Metabolic Activation in Mutagenesis Testing (F.J. de Serres, Ed.), Elsevier, 1976, p. 85.

STORAGE: At or below -70°C

BIOCHEMISTRY:

- PROTEIN

37.2 mg/ml

Assayed according to the method of Lowry et al., JBC 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-O-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	101.3
PROD	2B1	21.0
BROD	2B1	35.6
MROD	1A2	20.3

Assays for ethoxyresorufin-O-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-O-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 43.8, 20.6, 64.4, & 15.5 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. *his*+ Revertants	
EtBr/ TA98	CPA/ TA1535
302.4	2222

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.*113:173, 1983).

	µl S9 per plate/number *his*+ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	116	158	279	406	527	608
2-AA (2.5 µg)	128	200	426	1621	1613	864

MOLECULAR TOXICOLOGY, INC.
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Boone, NC 28604 [redacted]