RTC
RESEARCH TOXICOLOGY CENTRE - ROMA

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C6O4 CYCLIC
CHROMOSOME ABERRATIONS IN
CHINESE HAMSTER OVARY CELLS *IN VITRO*

FINAL REPORT

RTC Study No.: 76240

Sponsor:
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Total number of pages: 57

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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 2 March 2007 n. 50, *Attuazione delle direttive 2004/9/CE e 2004/10/CE, concernenti l'ispezione e la verifica della buona pratica di laboratorio (BPL) e il ravvicinamento delle disposizioni legislative, regolamentari ed amministrative relative all'applicazione dei principi di buona pratica di laboratorio e al controllo della loro applicazione per le prove sulle sostanze chimiche (G.U. 13 April 2007, Serie generale n. 86)* 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] [redacted] Date: [redacted]
(Study Director)

[redacted] [redacted] Date: [redacted]
Spec. Toxicology (Scientific Director)

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	17.03.2009	17.03.2009	17.03.2009
PROTOCOL AMENDMENT (1) CHECK	19.05.2009	19.05.2009	19.05.2009
PROCESS-BASED INSPECTIONS RELATED TO THIS TYPE OF STUDY			
Dose preparation	04.08.2009	-	19.08.2009
Test execution	05.08.2009	-	19.08.2009
Slide coding	04.06.2009	-	13.06.2009
Slide scoring	22.05.2009	-	22.05.2009
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]

Head of Quality Assurance

[redacted]

Date

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1. SUMMARY

1.1 The test item C6O4 CYCLIC 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 main experiments for chromosomal damage were performed.

In the first experiment, the cells were treated for 3 hours with C6O4 CYCLIC in the presence and absence of S9 metabolism. The cells were then harvested after 20 hours, corresponding to approximately 1.5 cell cycle.
Dose levels of 5000, 2500, 1250, 625, 313, 156, 78.1, and 39.1 µg/ml were used in the absence and presence of S9 metabolism. Solutions of the test item were prepared in sterile distilled water of injectable grade.

Since no induction of chromosomal aberrations were obtained in the first main assay, in agreement with the study protocol, a second main experiment was performed where cells were treated with C6O4 CYCLIC in the absence of S9 metabolism and harvested after 20 hours. A continuous treatment until harvest was used.

Besides, in the first main experiment, following treatment with the test item in the presence of S9 metabolism, marked increases in the number of endoreduplicated cells over the concurrent negative control were observed.
Since no adequate toxicity was observed at any dose level for the evaluation of genotoxic effects, an additional treatment was performed in order to confirm the observed results following the approval of a protocol amendment. A modified dose range was adopted for this treatment in order to investigate more closely those doses most likely to exhibit endoreduplication.

For the second main experiment, the dose levels selected for treatment were 2000, 1330, 889, 593, 395, 263, 176 and 117 µg/ml.

Both experiments 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 as determined by the reduction of population doubling.

On the basis of the above described results, the dose levels referred to active ingredient selected for scoring were the following:

Experiment No.	S9	Treatment time (hours)	Harvest time (hours)	Dose level (µg/ml)
1	+ -	3	20	1250, 625 and 313
2	+ -	3 20	20	2000, 1330 and 889 889, 593 and 395

One hundred metaphase spreads were scored for chromosomal aberrations from each culture, with the exception of one replicate culture treated with Cyclophosphamide (first main experiment), where 75 metaphases were examined. In addition, for the first main experiment, due to the high frequency of aberrant cells (excluding gaps), found for replicate cultures treated with Mitomycin-C, scoring was terminated at 50 metaphases.

1.4 For the first experiment, following treatment with the test item C6O4 CYCLIC, no statistically significant increase in the incidence of cells bearing aberrations including or excluding gaps was observed in the absence or presence of S9 metabolism.

In the presence of S9 metabolism, marked increases in the number of endoreduplicated cells over the control were observed. An increase in the number of endoreduplicated cells was also observed in the absence of S9 metabolism at the highest dose selected for scoring.

For the second experiment, following treatment with the test item in the absence of S9 metabolism, slight increases but not statistically significant in the incidence of cells bearing aberrations, excluding gaps, over the control values were observed at all dose levels selected for scoring. More remarkable increases of aberrant cells were observed when including gaps.

In the presence of S9 metabolism, statistically significant increases in the incidence of aberrant cells including and excluding gaps were observed at the highest dose selected for scoring (2000 µg/ml). The incidences exceeded the range of our historical values for negative controls when excluding gaps.
Marked increases in the number of endoreduplicated cells over the controls were seen at the intermediate and high dose levels selected for scoring.

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 On the basis of these results it is concluded that C6O4 CYCLIC induces chromosomal aberrations in Chinese hamster ovary cells after *in vitro* treatment under the reported experimental conditions.

It is also concluded that the test item inhibits cell cycle progression and chromosome segregation under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes the experiment performed to assess the clastogenic activity of C6O4 CYCLIC 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:

- Test method B.10 "*in vitro* mammalian chromosome aberration test" described in Council Regulation (EC) No. 440/2008.
- OECD Guideline No. 473 (Adopted: 21st July 1997).

2.2 Study organisation

Sponsor

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

Location of study

Genetic Toxicology, *in vitro* Toxicology and Immunology Department
Research Toxicology Centre S.p.A. (RTC)
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Principal dates

Study protocol approved by Study Director: 09-Mar-2009
Study commenced: 17-Mar-2009 (Main assay I treatment)
Study completed: 08-Jul-2009 (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, microscope slides, the original final protocol and a copy of the final report consigned will be stored in the archives of Research Toxicology Centre S.p.A. for a period of 3 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. An aliquot of the test item will be retained within the archives of the testing facility for a period of 10 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	:	C6O4 CYCLIC
Label name	:	cC6O4 (solution 16%)
Batch	:	150/28
Expiry date	:	31-Dec-2020
Received from	:	Solvay Solexis S.p.A.
Date received	:	05-Mar-2009
Amount received	:	337.94 ml
Description from the Sponsor	:	yellowish solution
Description at first use	:	yellowish liquid
Container	:	colourless glass bottle
Storage at RTC	:	room temperature
RTC reference number	:	11663

On 16 March 2009 and 27 March 2009 two sub-samples of 7 ml and 5ml, respectively, of the test item were transferred from the Formulation Unit to the Department of Genetic Toxicology, *in vitro* Toxicology and Immunology and stored under the same conditions.

Solutions of the test item, as received, were prepared immediately before use in sterile distilled water. Solutions were prepared on a weight/volume basis. Concentrations were expressed in terms of active ingredient. All test item solutions were used within 20 minutes of the preparation. 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.
A copy of the certificate of analysis of the test item can be found in Appendix II of this report.

3.2 Control substances

The solvent used in this study was sterile distilled water of injectable grade, batch no. 08G2803, obtained from Bieffe, Italy.

Solutions of Mitomycin-C, batch 117K1683, and Cyclophosphamide, batch 097K1311, both obtained from Sigma were prepared in sterile distilled water immediately prior to use and served as positive controls.

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

3.3 S9 tissue homogenate

Two [redacted] rat S9 liver tissue fractions: lot numbers 2273 and 2378 were used for main experiments 1 and 2 respectively. The S9 liver tissue fractions had the following characteristics:

Lot Number	:	2273
Inducing Agents	:	Phenobarbital – 5,6-Benzoflavone
Preparation date	:	01-May-2008
Expiry date	:	01-May-2010
Received from	:	[redacted]
Date received	:	29-Jul-2008
Storage at RTC	:	Approximately -80°C
Strain	:	Sprague Dawley
Sex of donors	:	Male
Protein content	:	37.6 mg/ml

Lot Number	:	2378
Inducing Agents	:	Phenobarbital – 5,6-Benzoflavone
Preparation date	:	18-Feb-2009
Expiry date	:	18-Feb-2011
Received from	:	[redacted].
Date received	:	24-Mar-2009
Storage at RTC	:	Approximately -80°C
Strain	:	Sprague Dawley
Sex of donors	:	Male
Protein content	:	37.8 mg/ml

Production and quality control certificates can be found in Appendix IV of this report.

3.4 Methods

The methods used were in compliance with the attached Study Protocol and First amendment to the Protocol (Appendix I), with the exception that, due to a technical oversight, 75 metaphases and not 100 were scored for culture no. 21 treated with Cyclophosphamide. This deviation from the protocol was not considered to have affected the integrity of the study since the expected response of the test system to the positive control was demonstrated.

4. RESULTS

4.1 Sterility test

Since the test item is an aqueous sample, a preliminary sterility test was performed. An aliquot of 500 µl of the test item, as supplied, was added to 4.5 ml of nutrient broth and incubated at 37°C for about 24 hours before evaluating the outcome of the test. Based on the results obtained it was not considered necessary to filter the test item before use in any experimental procedure.

4.2 Assay for chromosomal aberrations

For the first main assay, dose levels of 5000, 2500, 1250, 625, 313, 156, 78.1 and 39.1 µg/ml were employed both in the absence and presence of S9 metabolism.

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.

Since no induction of chromosomal aberrations was obtained in the first main assay, in agreement with the study protocol, a second main experiment was performed where cells were treated with C6O4 CYCLIC in the absence of S9 metabolism and harvested after 20 hours. A continuous treatment until harvest was used.

For the first main experiment, following treatment with the test item in the presence of S9 metabolism, marked increases in the number of endoreduplicated cells over the control were observed.
Since no adequate toxicity was observed at any dose level for the evaluation of genotoxic effects, following the approval of a protocol amendment, for the second main experiment an additional treatment in the presence of S9 metabolism was performed in order to confirm the observed results. For this experiment a modified dose range was adopted in order to investigate more closely those doses most likely to exhibit endoreduplication.

For the second main experiment, the dose levels selected for treatment were 2000, 1330, 889, 593, 395, 263, 176 and 117 µg/ml.

Appropriate negative and positive control cultures were included in the experiment. Positive control treated cultures received Mitomycin-C 0.30 and 0.45 µg/ml for 3 hours treatment or 0.10 and 0.15 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. No relevant effect on pH of the treatment media was observed at any dose level tested.

Due to the presence of water in the solvent control cultures, a slight increase in osmolality over the control was observed for cultures treated at the highest dose level selected for treatment. The values however were comparable to those observed in untreated control cultures.
These results are not presented in this report but are retained in the study file and archived as indicated in the study protocol.

During the main experiments, no precipitation or opacity of the media was observed at the beginning or end of treatment.

4.3 Selection of dose levels for scoring

At the time of harvesting, cell counts were performed for each culture and cytotoxicity was evaluated as determined by the reduction of population doubling (PD) over the relevant control.

Population doubling is the log of the ratio of the final cell count at harvesting (N) to the starting (baseline) count (X_0) divided by the log of 2:

$PD = [\log (N/ X_0)]/\log 2$

Results are presented in Tables 1 to 4.

In the first main experiment, following treatment in the absence or presence of S9 metabolism, a severe toxicity was observed at the higher dose levels of 5000 and 2500 µg/ml, where no cells or few cells where recovered at harvesting.

In the absence of S9, moderate toxicity was observed at the next lower dose level of 1250 µg/ml where PD was 75% of the control. No cytotoxicity was observed over the remaining dose range.
In the presence of S9 metabolism, no remarkable toxicity was observed over the remaining dose range.

In the second main experiment following treatment in the absence of S9, a severe toxicity was observed at the higher dose levels of 2000 and 1330 µg/ml, where few cells were recovered at harvesting.
Marked toxicity was also observed at the dose levels of 889 and 593 µg/ml where the PD was 31% of the control. No remarkable toxicity was observed over the remaining dose range.
In the presence of S9 metabolism distinct toxicity was observed at the highest dose level of 2000 µg/ml where the PD was 51% of the control. Slight toxicity reducing the PD at approximately 80% of the control 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 PD should be approximately 50%).
If no toxicity is observed then the highest practicable dose level should be selected.

On the basis of the above described results the dose levels selected for scoring were the following:

Experiment No.	S9	Treatment time (hours)	Harvest time (hours)	Dose level (µg/ml)
1	+ -	3	20	1250, 625 and 313
2	+ -	3 20	20	2000, 1330 and 889 889, 593 and 395

The dose levels of 0.30 and 0.10 µg/ml were selected for the scoring of cultures treated with Mitomycin-C (first and second main experiment respectively) and the dose level of 15.0 µg/ml was selected for the scoring of cultures treated with Cyclophosphamide.

4.4 Assay results

One hundred metaphase spreads were scored for chromosomal aberrations from each culture, with the exception of one replicate culture treated with Cyclophsphamide (first main experiment), where 75 metaphases were examined. In addition, for the first main experiment, due to the high frequency of aberrant cells (excluding gaps), found for replicate cultures treated with Mitomycin-C, scoring was terminated at 50 metaphases.

The results are presented in Tables 5 to 8. 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 is also shown.

For the first experiment, following treatment with the test item C6O4 CYCLIC in the absence or presence of S9 metabolism, no remarkable increase over the control values in the number of chromosome aberrations including or excluding gaps was observed.

Following treatment in the presence of S9 metabolism, marked increases in the number of endoreduplicated cells over the control were observe. An increase in the number of endoreduplicated cells was also observed in the absence of S9 metabolism at the highest dose selected for scoring.

For the second experiment, following treatment with the test item in the absence of S9 metabolism, slight increases in the incidence of cells bearing aberrations excluding gaps, over the control values were observed at all dose levels selected for scoring. More remarkable increases of aberrant cells were observed when including gaps.

In the presence of S9 metabolism, increases in the incidence of aberrant cells including and excluding gaps, were observed at the highest dose selected for scoring (2000 µg/ml). The incidences exceeded the range of our historical values for negative controls when excluding gaps.
Additionally the presence of a heavily damaged cell, bearing more than five aberrations was also noted.
In this experiment the induction of endoreduplicated cells was confirmed since marked increases were seen at the intermediate and high dose levels selected for scoring.

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 (64.25%). The frequency of cells containing 20, 22 and 23 chromosomes was 0.75%, 34.75% and 0.25%. No metaphases were found with 19 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 9 to 12.

Following treatment with the test item in the absence of S9 metabolism, no statistically significant increase in the incidence of cells bearing aberrations including or excluding gaps was observed.
In the presence of S9 metabolism, statistically significant increases in the incidence of cells bearing aberrations, including and excluding gaps, exceeding the range of our historical controls, were observed at the highest dose level selected for scoring.

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

Based on the positive results obtained in the presence of S9 metabolism, together with increases in the numbers of endoreduplicated cells and in accordance with the criteria for the outcome of the study, the test item was considered to induce chromosomal aberrations and inhibition of cell cycle progression 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 are presented in Tables 13 and 14 giving the incidence of cells bearing aberrations (excluding gaps), the incidence of endoreduplicated cells and the relative population doubling 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 C6O4 CYCLIC induces chromosomal aberrations in Chinese hamster ovary cells after *in vitro* treatment under the reported experimental conditions.

It is also concluded that the test item inhibits cell cycle progression and chromosome segregation under the reported experimental conditions.

7. TABLES 1-4

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 1 – Population doubling results - Without metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 1

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose level (µg/ml)	Culture No.	No.viable cells$x10^6$/ml	Mean	Relative PD (%)
Untreated	-	1	0.76	0.73	106
		2	0.70		
Solvent	10%	3	0.68	0.70	100
		4	0.71		
Test Item	39.1	19	0.71	0.71	102
		20	0.71		
Test Item	78.1	17	0.71	0.70	100
		18	0.69		
Test Item	156	15	0.71	0.71	102
		16	0.70		
Test Item	313	13	0.71	0.70	100
		14	0.69		
Test Item	625	11	0.73	0.72	104
		12	0.71		
Test Item	1250	9	0.56	0.58	75
		10	0.59		
Test Item	2500	7	0.33	0.33	0
		8	0.33		
Test Item	5000	5	0.00	0.00	0
		6	0.00		
Mitomycin-C	0.30	21	0.48	0.46	42
		22	0.43		
Mitomycin-C	0.45	23	0.56	0.52	57
		24	0.48		

Note: Baseline cell count: 0.33 cells x 10^6/ml

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 2 - Population doubling results - With metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 1

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose level (µg/ml)	Culture No.	No.viable cellsx10^6/ml	Mean	Relative PD (%)
Untreated	-	25	0.66	0.66	116
		26	0.66		
Solvent	10%	27	0.62	0.60	100
		28	0.58		
Test Item	39.1	43	0.64	0.62	106
		44	0.60		
Test Item	78.1	41	0.65	0.62	106
		42	0.59		
Test Item	156	39	0.66	0.64	111
		40	0.62		
Test Item	313	37	0.63	0.62	106
		38	0.61		
Test Item	625	35	0.61	0.62	106
		36	0.63		
Test Item	1250	33	0.61	0.59	97
		34	0.56		
Test Item	2500	31	0.25	0.26	0
		32	0.26		
Test Item	5000	29	0.00	0.00	0
		30	0.00		
Cyclophosphamide	15.0	45	0.44	0.46	48
		46	0.48		
Cyclophosphamide	23.0	47	0.38	0.38	20
		48	0.37		

Note: Baseline cell count: 0.33 cells x 10^6/ml

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 3 - Population doubling results - Without metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 2

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 20 hours

SAMPLING TIME: 20 hours

Treatment	Dose level (µg/ml)	Culture No.	No.viable cells$x10^6$/ml	Mean	Relative PD (%)
Untreated	-	49	0.57	0.55	102
		50	0.53		
Solvent	10%	51	0.54	0.54	100
		52	0.54		
Test Item	117	67	0.47	0.49	89
		68	0.51		
Test Item	176	65	0.47	0.50	91
		66	0.52		
Test Item	263	63	0.50	0.50	91
		64	0.49		
Test Item	395	61	0.51	0.50	91
		62	0.49		
Test Item	593	59	0.24	0.30	31
		60	0.36		
Test Item	889	57	0.29	0.30	31
		58	0.31		
Test Item	1330	55	0.14	0.15	0
		56	0.16		
Test Item	2000	53	0.04	0.05	0
		54	0.06		
Mitomycin-C	0.10	69	0.34	0.34	45
		70	0.33		
Mitomycin-C	0.15	71	0.29	0.28	23
		72	0.27		

Note: Baseline cell count: 0.23 cells x 10^6/ml

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 4 - Population doubling results - With metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 2

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose level (µg/ml)	Culture No.	No.viable cellsx10^6/ml	Mean	Relative PD (%)
Untreated	-	73	0.64	0.61	94
		74	0.57		
Solvent	10%	75	0.67	0.65	100
		76	0.63		
Test Item	117	91	0.55	0.55	84
		92	0.54		
Test Item	176	89	0.53	0.54	82
		90	0.54		
Test Item	263	87	0.52	0.52	79
		88	0.52		
Test Item	395	85	0.52	0.53	80
		86	0.54		
Test Item	593	83	0.55	0.55	84
		84	0.55		
Test Item	889	81	0.54	0.55	84
		82	0.55		
Test Item	1330	79	0.53	0.54	82
		80	0.54		
Test Item	2000	77	0.46	0.39	51
		78	0.31		
Cyclophosphamide	15.0	93	0.43	0.44	67
		94	0.45		
Cyclophosphamide	23.0	95	0.44	0.43	64
		96	0.42		

Note: Baseline cell count: 0.23 cells x 10^6/ml

8. KEY TO TABLES 5-8

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.

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 5 - Scoring of Aberrations - Without metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 1

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

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	0	0	0	0	0	0	0	0	0	0	0
		2	100	0	0	0	0	0	0	0	0	0	0	0
			200	0	0	0	0	0	0	0	0	0	0	0
Solvent	10%	3	100	3	0	0	0	0	0	0	3	0	3	0
		4	100	1	0	0	0	0	0	0	1	0	1	0
			200	4	0	0	0	0	0	0	4	0	4	0
Test Item	313	13	100	2	0	0	0	0	0	0	2	0	2	0
		14	100	2	2	0	1	0	0	0	5	3	3	1
			200	4	2	0	1	0	0	0	7	3	5	1
Test Item	625	11	100	0	0	0	0	0	0	0	0	0	0	0
		12	100	4	0	0	0	0	0	0	4	0	4	0
			200	4	0	0	0	0	0	0	4	0	4	0
Test Item	1250	9	100	0	0	0	0	0	0	3ER	0	0	0	0
		10	100	1	0	0	0	0	0	5ER	1	0	1	0
			200	1	0	0	0	0	0	8ER	1	0	1	0
Mitomycin-C	0.30	21	75	1	4	51	3	1	10	0	70	69	41	40
		22	100	0	0	40	8	2	2	2H	52	52	33	33
			175	1	4	91	11	3	12	2H	122	121	74	73

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 6 - Scoring of Aberrations - With metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 1

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

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	-	25	100	4	0	0	0	0	0	1ER,1PP	4	0	4	0
		26	100	4	2	0	0	0	0	0	6	2	5	2
			200	8	2	0	0	0	0	1ER,1PP	10	2	9	2
Solvent	10%	27	100	1	0	0	0	0	0	0	1	0	1	0
		28	100	2	0	0	0	0	0	0	2	0	2	0
			200	3	0	0	0	0	0	0	3	0	3	0
Test Item	313	37	100	2	1	0	0	0	0	4ER	3	1	3	1
		38	100	2	0	0	0	1	0	7ER	3	1	3	1
			200	4	1	0	0	1	0	11ER	6	2	6	2
Test Item	625	35	100	4	0	0	0	0	0	18ER	4	0	4	0
		36	100	0	0	0	0	0	0	10ER	0	0	0	0
			200	4	0	0	0	0	0	28ER	4	0	4	0
Test Item	1250	33	100	4	0	0	0	0	0	14ER	4	0	4	0
		34	100	0	0	0	0	0	0	12ER	0	0	0	0
			200	4	0	0	0	0	0	26ER	4	0	4	0
Cyclophosphamide	15.0	45	50	5	13	31	0	1	3	1H	53	48	35	35
		46	50	4	20	25	0	0	1	1H	50	46	34	33
			100	9	33	56	0	1	4	2H	103	94	69	68

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 7 - Scoring of Aberrations - Without metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 2

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

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	-	49	100	1	0	0	0	0	0	0	1	0	1	0
		50	100	2	0	0	0	0	0	0	2	0	2	0
			200	3	0	0	0	0	0	0	3	0	3	0
Solvent	10%	51	100	0	1	0	0	0	0	1PP	1	1	1	1
		52	100	1	0	0	0	0	0	0	1	0	1	0
			200	1	1	0	0	0	0	1PP	2	1	2	1
Test Item	395	61	100	0	0	0	0	4	0	0	4	4	4	4
		62	100	2	0	0	0	1	1	1ER	4	2	4	2
			200	2	0	0	0	5	1	1ER	8	6	8	6
Test Item	593	59	100	3	1	0	0	1	0	0	5	2	4	1
		60	100	3	4	0	0	0	0	2PP,1ER	7	4	6	3
			200	6	5	0	0	1	0	2PP,1ER	12	6	10	4
Test Item	889	57	100	0	2	0	0	0	1	0	3	3	3	3
		58	100	2	1	0	0	0	0	0	3	1	3	1
			200	2	3	0	0	0	1	0	6	4	6	4
Mitomycin-C	0.10	69	100	3	6	19	0	0	9	1PP	37	34	24	23
		70	100	2	5	18	0	2	2	0	29	27	19	18
			200	5	11	37	0	2	11	1PP	66	61	43	41

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 8 - Scoring of Aberrations - With metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 2

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

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	-	73	100	5	1	0	0	0	0	4ER	6	1	5	1
		74	100	5	1	0	0	0	0	2ER	6	1	5	1
			200	10	2	0	0	0	0	6ER	12	2	10	2
Solvent	10%	75	100	2	0	0	0	0	0	1ER	2	0	2	0
		76	100	3	1	0	0	0	0	1ER	4	1	4	1
			200	5	1	0	0	0	0	2ER	6	1	6	1
Test Item	889	81	100	2	0	0	0	0	0	3ER	2	0	2	0
		82	100	7	0	0	0	0	0	2ER	7	0	7	0
			200	9	0	0	0	0	0	5ER	9	0	9	0
Test Item	1330	79	100	2	0	0	0	0	0	11ER	2	0	2	0
		80	100	1	0	0	0	0	0	11ER	1	0	1	0
			200	3	0	0	0	0	0	22ER	3	0	3	0
Test Item	2000	77	100	4	0	1	0	1	2	19ER	8	4	7	3
		78	100	6	2	1	0	1	3	11ER,1H	13	7	10	6
			200	10	2	2	0	2	5	30ER,1H	21	11	17	9
Cyclophosphamide	15.0	93	100	2	13	32	0	1	5	1H	53	51	36	36
		94	100	5	23	42	0	1	13	1H	84	79	45	45
			200	7	36	74	0	2	18	2H	137	130	81	81

9. KEY TO TABLES 9-14

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 between the treated and 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.

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 9 - Statistical Analysis - Without metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 1

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Comparison with Negative control			Cells with aberrations			
			(+ gaps)		(- gaps)	
	Dose µg/ml	CULT.	P. values	Sig.	P. values	Sig.
Test Item	313	13	#	N.S.	#	N.S.
		14	0.4294	N.S.	0.3334	N.S.
	Pooled		0.5002	N.S.	0.5000	N.S.
Test Item	625	11	0.1956	N.S.	#	N.S.
		12	0.2564	N.S.	#	N.S.
	Pooled		#	N.S.	#	N.S.
Test Item	1250	9	0.1956	N.S.	#	N.S.
		10	0.4599	N.S.	#	N.S.
	Pooled		0.1860	N.S.	#	N.S.
All dose levels			0.4792	N.S.	0.7501	N.S.
Mitomycin-C	0.30	21	0.0000	***	0.0000	***
		22	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(***).

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 10 - Statistical Analysis - With metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 1

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Comparison with Negative control			Cells with aberrations			
			(+ gaps)		(- gaps)	
	Dose µg/ml	CULT.	P. values	Sig.	P. values	Sig.
Test Item	313	37	0.3186	N.S.	0.3334	N.S.
		38	0.3186	N.S.	0.3334	N.S.
	Pooled		0.2515	N.S.	0.2493	N.S.
Test Item	625	35	0.1707	N.S.	#	N.S.
		36	0.2948	N.S.	#	N.S.
	Pooled		0.5000	N.S.	#	N.S.
Test Item	1250	33	0.1707	N.S.	#	N.S.
		34	0.2948	N.S.	#	N.S.
	Pooled		0.5000	N.S.	#	N.S.
All dose levels			0.3506	N.S.	0.5623	N.S.
Cyclophosphamide	15.0	45	0.0000	***	0.0000	***
		46	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(***).

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 11 - Statistical Analysis - Without metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 2

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 20 hours

SAMPLING TIME: 20 hours

Comparison with Negative control			Cells with aberrations			
			(+ gaps)		(- gaps)	
	Dose µg/ml	CULT.	P. values	Sig.	P. values	Sig.
Test Item	395	61	0.0979	N.S.	0.0439	N.S.
		62	0.0979	N.S.	0.2585	N.S.
	Pooled		0.0525	N.S.	0.0609	N.S.
Test Item	593	59	0.0979	N.S.	0.5564	N.S.
		60	0.0183	N.S.	0.1096	N.S.
	Pooled		0.0179	N.S.	0.1859	N.S.
Test Item	889	57	0.2082	N.S.	0.1096	N.S.
		58	0.2082	N.S.	0.5564	N.S.
	Pooled		0.1421	N.S.	0.1859	N.S.
All dose levels			0.0241	*	0.0782	N.S.
Mitomycin-C	0.10	69	0.0000	***	0.0000	***
		70	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(***).

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 12 - Statistical Analysis - With metabolic activation

STUDY NO.: 76240

MAIN ASSAY: 2

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 20 hours

SAMPLING TIME: 20 hours

Comparison with Negative control			Cells with aberrations			
			(+ gaps)		(- gaps)	
	Dose µg/ml	CULT.	P. values	Sig.	P. values	Sig.
Test Item	889	81	0.4668	N.S.	0.6668	N.S.
		82	0.0986	N.S.	0.6668	N.S.
	Pooled		0.3001	N.S.	0.5000	N.S.
Test Item	1330	79	0.4668	N.S.	0.6668	N.S.
		80	0.2603	N.S.	0.6668	N.S.
	Pooled		0.2514	N.S.	0.5000	N.S.
Test Item	2000	77	0.0986	N.S.	0.1096	N.S.
		78	0.0136	N.S.	0.0063	*
	Pooled		0.0148	*	0.0100	*
All dose levels			0.1863	N.S.	0.2422	N.S.
Cyclophosphamide	15.0	93	0.0000	***	0.0000	***
		94	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(***).

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 13 - Summary Table

STUDY NO.: 76240

MAIN ASSAY: 1

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 3 hours

SAMPLING TIME: 20 hours

Treatment	Dose µg/ml	Presence of S9 metabolism %CA	(Rel.PD)	Absence of S9 metabolism %CA	(Rel.PD)
Untreated	-	1.0	(116)	0.0	(106)
Solvent	10%	0.0	(100)	0.0	(100)
Test Item	313	1.0 N.S.	(106)	0.5 N.S.	(100)
Test Item	625	0.0 N.S.	(106)	0.0 N.S.	(104)
Test Item	1250	0.0 N.S.	(97)	0.0 N.S.	(75)
Mitomycin-C	0.30	-		41.7 ***	(42)
Cyclophosphamide	15.0	68.0 ***	(48)	-	

Key:
% CA : Percentage of cells bearing aberrations (excluding gaps)
Rel.PD : Population doubling 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

C6O4 CYCLIC: CHROMOSOME ABERRATIONS

TABLE 14 - Summary Table

STUDY NO.: 76240

MAIN ASSAY: 2

SOLVENT: STERILE DISTILLED WATER OF INJECTABLE GRADE

TREATMENT TIME: 3 hours (+S9)/20 hours (-S9)

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	-	1.0		(94)	0.0		(102)
Solvent	10%	0.5		(100)	0.5		(100)
Test Item	395	-			3.0	N.S.	(91)
Test Item	593	-			2.0	N.S.	(31)
Test Item	889	0.0	N.S.	(84)	2.0	N.S.	(31)
Test Item	1330	0.0	N.S.	(82)	-		
Test Item	2000	4.5	*	(51)	-		
Mitomycin-C	0.10	-			20.5	N.S.	(45)
Cyclophosphamide	15.0	40.5	***	(67)	-		

Key:
% CA : Percentage of cells bearing aberrations (excluding gaps)
Rel.PD : Population doubling 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 - Study Protocol and First Amendment to Protocol

RESEARCH TOXICOLOGY CENTRE - ROMA

C604 CYCLIC CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS *IN VITRO*

Final Protocol
prepared for

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Viale Lombardia, 20
20021 Bollate (MI)
Italy

by

RESEARCH TOXICOLOGY CENTRE S.p.A.
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RTC Enquiry Number: 75841
RTC Study Number: 76240

March 2009

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Cod. Fisc.: 00653120584
Partita IVA: 00920611001

CHROMOSOME ABERRATIONS IN
CHINESE HAMSTER OVARY CELLS *IN VITRO*

MANAGEMENT OF STUDY

Scientific Director : [redacted]

Head of Genetic Toxicology,
in vitro Toxicology and Immunology : [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, 12/14
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: C604 CYCLIC

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. 50 of 2 March 2007 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 dilutions of the test item as supplied will be prepared for each day's work at appropriate concentrations. One sterility test, using nutrient broth, will be performed before commencing all experimental treatments. Concentrations of solutions will be expressed in terms of active ingredient. Preferred solvent will be sterile distilled water.

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 [redacted] 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.
Two additional solvent control cultures will be added to the experimental scheme in order to evaluate the average baseline cell count (see section 5.6).

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 population doubling (PD).

Population doubling is the log of the ratio of the final cell count (N) to the starting (baseline) count (X_0) divided by the log of 2:

$PD = [\log (N/ X_0)]/\log 2$

In order to evaluate baseline count at the beginning of treatment, two additional control cultures are included in the experimental scheme. These two cultures will be trypsinized and cell counts will be performed approximately at time zero. The baseline count is the average of the total numbers of cells from the two flasks.

Ideally the reduction should be approximately 50% of the control and not greatly exceeding this percentage reduction. 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 comments have not been received within 6 months of despatch of the audited draft report, the final report will be issued.
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, microscope slides, documentation arising from this study, including the original final protocol 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. The original 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

According to the RTC quality assurance programme, defined in the RTC QA SOPs, this study will be subjected to the following procedures:

- the protocol will be inspected,

- study/process based inspections of procedures/facilities will be carried out at intervals adequate to assure the integrity of the study,

- the report will be inspected to assure that it accurately describes the methods and Standard Operating Procedures and that the results accurately reflect the raw data.

Periodic reports on these activities will be made to management and the Study Director.
All raw data pertaining to the study will be available for inspection by the Sponsor's representative and regulatory authorities (following authorisation from the Sponsor).

8. DEPARTURES FROM REGULATORY REQUIREMENTS

Items which are the responsibility of the Sponsor are indicated in sections 2.1, 2.4, 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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PROTOCOL APPROVAL PAGE

for

R.T.C. S.p.A.

APPROVED BY : [redacted] [redacted]
Date

RELEASED BY : [redacted]
[redacted]icology, *in vitro*
Toxicology and Immunology
[redacted]
Date

RTC Enquiry Number: 75841
RTC Study Number: 76240

March 2009

Version 09/1_S

PROTOCOL APPROVAL PAGE

for

SOLVAY SOLEXIS S.p.A.

AUTHORISED BY : [redacted] [redacted]
Date

Name and Title* : [redacted]

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

Date of Protocol Approval: [redacted]

RTC Enquiry Number: 75841
RTC Study Number: 76240

March 2009

RESEARCH TOXICOLOGY CENTRE - ROMA

PROTOCOL AMENDMENT (1)

STUDY TITLE	:	C604 CYCLIC CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS IN VITRO
STUDY NO.	:	76240
DATE OF PROTOCOL APPROVAL	:	09 March 2009

AMENDMENT:

In order to further evaluate results obtained in Main assay 1 in the presence of S9 metabolism, treatment was repeated in Main assay 2.
Data will be presented and evaluated following section 6 of the Study Protocol.

Reason	:	Agreed with the Sponsor
Effective date	:	Date of study note 1 (24 Apr 2009).

RTC Enquiry Number: 75841
RTC Study Number: 76240

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PROTOCOL AMENDMENT (1)

APPROVAL PAGE

for

R.T.C. S.p.A.

APPROVED BY : [redacted] Date [redacted]

Study Director

RELEASED BY : [redacted] Date [redacted]

Senior Scientist/Study Director Coordinator

RTC Enquiry Number: 75841
RTC Study Number: 76240

PROTOCOL AMENDMENT (1)

APPROVAL PAGE

for

SOLVAY SOLEXIS S.p.A.

AUTHORISED BY : [redacted] [redacted]
Date

Name and Title : [redacted]

Date of Amendment Approval: [redacted]

RTC Enquiry Number: 75841
RTC Study Number: 76240

11. APPENDIX II - Certificate of analysis

SOLVAY SOLEXIS

R,D&T Analytical & Structural
Characterization Department
Viale Lombardia, 20 – 20021 Bollate (MI) - Italy

Sample Description:
C_6O_4 Ammonium Salt, sample 150/28

Analysis Requests:
Ammonium chloride and fluoride determination
Salt title quantification
Organic impurity determination

Method (Equipment):
Ionic Chromatography
Potentiometry
NMR Spectroscopy

Performance:
MDL: na
MQL: na

Results:
The analyzed sample shows the following weight/volume of:
NH4F =28 mg/l
NH4Cl = 601 mg/l
C6O4 (NH4 titration) = 160 g/l

The organic impurities are dioxolen based and represent 0.8% by mol on total mol of organic phase of sample.

[redacted]

Analytical & Structural Characterization Department

12. APPENDIX III - Historical control data

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Background incidences (%) of aberrant cells (Years 1990-2008)

	Absence of S9 20 h sampling time		Presence of S9 20 h sampling time	
	+ gaps	- gaps	+ gaps	- gaps
	UNTREATED CONTROLS			
Mean	1.8	0.5	2.3	0.6
SD (σ_{n-1})	1.5	0.7	1.7	0.7
n	121	121	92	92
Minimum	0.0	0.0	0.0	0.0
Maximum	7.0	3.0	8.0	3.0
	SOLVENT CONTROLS			
Mean	2.0	0.4	2.4	0.5
SD (σ_{n-1})	1.8	0.7	1.9	0.7
n	93	93	72	72
Minimum	0.0	0.0	0.0	0.0
Maximum	8.0	4.0	8.5	4.0
	POSITIVE CONTROLS			
Mean	37.4	35.4	38.3	36.0
SD (σ_{n-1})	14.8	14.6	13.8	13.8
n	124	124	93	93
Minimum	14.0	12.0	15.5	15.5
Maximum	77.0	76.0	84.0	82.0

SD = standard deviation
n = number of experiments

13. APPENDIX IV - S9 production and quality control certificates

TRINOVA Biochem GmbH

[redacted] POST MITOCHONDRIAL SUPERNATANT (S-9)
PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 2273 **SPECIES:** Rat **PREPARATION DATE:** May 1, 2008
PART NO.: 11-105 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** May 1, 2010
VOLUME: 1 & 2 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

37.6 mg/ml — Assayed according to the method of Lowry et al., JBC **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	55.5
PROD	2B1	19.7
BROD	2B1	19.9
MROD	1A2	11.2

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-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 31.0, 14.2, 57.4, 10.4 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

EtBr/ CPA/	No. *his*+ Revertants	
	TA98	TA1535
	342.8	1528

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)	181	297	387	650	855	1065
2-AA (2.5 µg)	184	365	1075	1727	2026	1283

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[redacted] **POST MITOCHONDRIAL SUPERNATANT (S-9)**
PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 2378 **SPECIES:** Rat **PREPARATION DATE:** February 18, 2009
PART NO.: 11-105 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** February 18, 2011
VOLUME: 1 & 2 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

37.8 mg/ml — Assayed according to the method of Lowry et al., JBC **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	60.7
PROD	2B1	25.9
BROD	2B1	23.5
MROD	1A2	34.8

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-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 14.0, 7.5, 22.6, 2.6 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

EtBr/ CPA/	No. *his*+ Revertants	
	TA98	TA1535
	276.8	868

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)	113	186	281	431	801	1105
2-AA (2.5 µg)	114	196	486	1249	1433	1497

[redacted]

Approved: [redacted] 2/23/09