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RTC

RESEARCH TOXICOLOGY CENTRE - ROMA

[redacted]

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

FINAL REPORT

RTC Study No.: 8837-008

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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 Regulations"* of the U.S. Food and Drug Administration, Code of Federal Regulations, 21 Part 58, 22 December 1978 and subsequent revisions.

B. Commission Directive 1999/11/EC of 8 March 1999 adapting to technical progress the principles of good laboratory practice as specified in Council Directive 87/18/EEC 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 (adoption of the *"OECD principles on Good Laboratory Practice – as revised in 1997"*) and subsequent revisions.

C. Decreto Legislativo 27 Gennaio 1992, n. 120 published in the Gazzetta Ufficiale della Repubblica Italiana 18 Febbraio 1992 (adoption of the Commission Directive of 18 December 1989 adapting to technical progress the Annex to Council Directive 88/320/EEC on the inspection and verification of Good Laboratory Practice (90/18/EEC)) and subsequent revisions.

O. Scarcella, Biol.D.
(Study Director) : Date : 09 Jan 2003

J. Brightwell, Ph.D.
(Scientific Director) : Date : 9.01.03

QUALITY ASSURANCE STATEMENT
(Relevant to the aspects of the study conducted by RTC)

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	08.08.2001	09.08.2001	09.08.2001
PROCESS-BASED INSPECTIONS			
Dose preparation	15.01.2002		19.01.2002
Treatment	22.03.2002		29.03.2002
Plating out	22.03.2002		29.03.2002
Plate scoring	08.02.2002		15.02.2002
Other routine inspections of a procedural nature were carried out on activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here.			
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 07 Jan 2003

M.M. Brunetti, Biol.D.
(Head of Quality Assurance)

07/01/2003
Date

Contents

1. SUMMARY

1.1 The test item [redacted] was examined for the ability to induce gene mutations in tester strains of *Salmonella typhimurium* and *Escherichia coli,* as measured by reversion of auxotrophic strains to prototrophy. The five tester strains TA1535, TA1537, TA98, TA100 and WP2 *uvr*A were used. Experiments were performed both in the absence and presence of metabolic activation, using liver S9 fraction from rats pre-treated with phenobarbitone and betanaphthoflavone.
Test item solutions were prepared using sterile distilled water.

1.2 In the toxicity test, the test item was assayed at a maximum dose-level of 5000 µg/plate and four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. No signs of toxicity were observed at any dose-level tested, in any tester strain, in the absence or presence of S9 metabolic activation.

1.3 Two main experiments were performed.
In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels, separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate.
As no increases in revertant numbers were observed, all treatments of Main Assay II included a pre-incubation step and used the same dose-range employed in Main Assay I.

1.4 The test item did not induce two fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism.

1.5 It is concluded that the test item [redacted] does not induce reverse mutation in *Salmonella typhimurium* and *Escherichia coli* under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of the test item to *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and to *Escherichia coli* strain WP2 *uvr*A using the procedures developed by Ames *et al.*, 1975 and revised by Maron and Ames, 1983.

The study was designed to comply with the experimental methods indicated in:
- EEC Council Directive 2000/32, Annex 4D.
- OECD Guideline for the testing of chemicals No. 471 (Adopted July 1997).

2.2 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2.3 Study organisation

Sponsor:

AUSIMONT S.p.A.
Via Lombardia, 20
20021 Bollate (MI)
Italy

Location of Study:

Research Toxicology Centre S.p.A.
Genetic Toxicology Department
Via Tito Speri, 12
00040 Pomezia (Roma)
Italy

Principal dates:

Study protocol approved by Study Director: 20-Jul-2001
Study commenced: 22-Feb-2002 (Toxicity assay treatment)
Study completed: 08-Mar-2002 (Completion of scoring Main Assay II)

Study Director:

O. Scarcella, Biol.D.

Archiving:

The original data arising from this study 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 five 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]
Lot or Batch Number	: 90215/91
Concentration of active ingredient	: 5% w/w
Expiry date	: 01-02-2004
Received from	: AUSIMONT S.p.A.
Date received	: 11-02-2002
Amount received	: 2000 grams
Description	: Colourless liquid
Container	: Opaque plastic tank
Storage at RTC	: Ambient conditions
RTC reference number	: 6535

On 20-Feb-2002 the test item was transferred from the Formulation Unit to the Department of Genetic and Cellular Toxicology and stored under the same conditions. A certificate of analysis, supplied by the Sponsor, can be found in Appendix III of this report.

Solutions of the test item, as received, were prepared, immediately before use, on a weight/volume basis without correction for the displacement due to the volume of the test item. All test item solutions were used within 3 hours and 10 minutes of the initial formulation. Concentrations are expressed in terms of active ingredient. 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 items

The solvents used in this study were:
sterile distilled water (Bieffe Medital, batch 01C02-01).
dimethylsulphoxide (DMSO) (Fluka AG, batch 421649/1 13001).

Positive control treatments used solutions prepared as follows:

Sodium azide (Fluka AG, batch 221999 1081) in distilled water.
9-Aminoacridine (ICN K&K Laboratories, batch 12058-A) in DMSO.
2-Nitrofluorene (EGA Chemie, batch 12532) in DMSO.
2-Aminoanthracene (Sigma, batch 58F-3462) in DMSO.
Methylmethanesulphonate (MMS) (Fluka AG, batch 359316/153696) in distilled water.

3.3 Media

The following growth media were used:

Nutrient Broth: Oxoid Nutrient Broth No 2 was prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This was used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) were added to distilled water (1 litre) and autoclaved.
The solutions were then poured into 9 cm plastic Petri dishes and allowed to solidify and dry before use. These plates were used for the non-selective growth of the tester strains.

Minimal Agar: Minimal medium agar was prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into 9 cm plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) was prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) was added to the top agar (100 ml).

3.4 S9 tissue homogenate

Two batches of S9 tissue homogenate (designated 2002/1 and 2002/2) were used in this study and had the following characteristics:

S9 Batch	Protein content (mg/ml)	Aminopyrine demethylase activity (µM/g liver/5 min, formaldehyde production)
2002/1	33.1 ± 1.69	4.14 ± 0.09
2002/2	35.8 ± 2.66	4.04 ± 0.07

Each S9 tissue fraction was prepared from the livers of five young male Sprague-Dawley rats which had received prior treatment with phenobarbital and betanaphthoflavone to induce high levels of xenobiotic metabolising enzymes. The efficacy of the S9 tissue fraction was previously checked in an Ames test and produced acceptable responses with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using *S. typhimurium* tester strain TA100.

The mixture of S9 tissue fraction and cofactors (S9 mix) was prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	10.0 ml

3.5 Bacterial strains

Four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and a strain of *Escherichia coli* (WP2 *uvr*A) were used in this study. Permanent stocks of these strains are kept at -80°C in RTC. Overnight subcultures of these stocks were prepared for each day's work.
Bacteria were taken from vials of frozen cultures, which had been checked for the presence of the appropriate genetic markers, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Bacterial cultures in liquid and on agar were clearly identified with their identity.

3.6 Methods

3.6.1 Preliminary toxicity test

A preliminary toxicity test was undertaken in order to select the concentrations of the test item to be used in the main assays. In this test a wide range of dose-levels of the test item, set at half-log intervals, was used. Treatments were performed both in the absence and presence of S9 metabolism using the plate incorporation method; a single plate was used at each test point and positive controls were not included.

3.6.2 Main experiments

Two experiments were performed including negative and positive controls in the absence and presence of an S9 metabolising system. Three replicate plates were used at each test point.

In addition, plates were prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures were plated on nutrient agar plates to establish the number of bacteria in the cultures.

The first experiment was performed using a plate-incorporation method. The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) were added to molten overlay agar and vortexed. The mixture was then poured onto the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture was composed as follows:

(i)	Overlay agar (held at 45°C)	2 ml
(ii)	Test or control item solution	0.1 ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml
(iv)	Bacterial suspension	0.1 ml

The second experiment was performed using a pre-incubation method.
The components were added in turn to an empty test-tube:

(i)	Bacterial suspension	0.1 ml
(ii)	Test item solution or solvent control	0.1 ml
(iii)	DMSO or positive control solution	0.05 ml
(iv)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml

The incubate was vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar was then added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

3.6.3 Incubation and scoring

The prepared plates were inverted and incubated for approximately 72 hours at 37°C. After this period of incubation, the scoring was effected by counting the number of revertant colonies on each plate.

4. RESULTS

4.1 Solubility test

As indicated by the Sponsor, the test item is an aqueous solution at a concentration of 5% w/w. Since 100 µl of the test item solution are used in the preparation of each plate, this permitted a maximum concentration of 5000 µg/plate to be used in the toxicity test.

4.2 Toxicity test

The test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. Results are presented in Tables 1 and 2.

No signs of toxicity were observed at any dose-level tested, in any tester strain, in the absence or presence of S9 metabolic activation. On the basis of these results a maximum concentration of 5000 µg/plate was selected for the Main Assay with all tester strains.

4.3 Assay for reverse mutation

Two experiments were performed; individual plate counts for these tests, and the mean and standard error of the mean for each test point, together with statistical analysis are presented in Tables 3 to 12.

In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels, separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate. No signs of toxicity were observed.

As no increases in revertant numbers were observed, all treatments of Main Assay II included a pre-incubation step and used the same dose-range employed in Main Assay I. Toxicity, as indicated by thinning of the background lawn and/or reduction in revertant numbers, was observed both in the absence and presence of S9 metabolic activation, at the two higher dose-levels, with TA1537 and TA100 tester strains.

The test item did not induce two-fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism.

The sterility of the S9 mix and the test item solutions was confirmed by the absence of colonies on additional agar plates spread separately with these solutions. Marked increases in revertant numbers were obtained in these tests following treatment with the positive control items, indicating that the assay system was functioning correctly.

5. ANALYSIS OF RESULTS

5.1 Criteria for outcome of the assays

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition, there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose-levels.

5.2 Evaluation

The test item does not induce increases in the number of revertant colonies, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism. On the basis of the stated criteria it must be concluded that the test item [redacted] is not mutagenic to *S. typhimurium* and *E. coli* under the reported experimental conditions.

6. CONCLUSION

It is concluded that the test item [redacted] does not induce reverse mutation in *Salmonella typhimurium* and *Escherichia coli* under the reported experimental conditions.

7. KEY TO TABLES 1-12

7.1 Structure of Tables 3-12

These tables show, for each *Salmonella typhimurium* or *Escherichia coli* tester strain, the individual plate counts obtained for the negative and positive controls, and at each dose-level of the test item. The mean number of revertant colonies and standard error of the mean are also presented. The "untreated" plates receive no treatment. The titre of the bacterial cultures is given (million cells/plate).

7.2 Regression line

i) The regression analysis fits a regression line to the data by the least squares method, after square root transformation of the plate counts to satisfy normal distribution and homoscedasticity assumptions. The regression equation is expressed as:

$y = a + bx$

where y = transformed revertant numbers
a = intercept
b = slope value
x = dose-level (in the units given).

ii) Regression lines are calculated using a minimum of the three lowest dose-levels, and then including the further dose-levels in turn. The correlation co-efficient (r), the value of students "t" statistic, and the p-value for the regression lines are also given.

8. TABLES 1 TO 12

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 1 - WITHOUT METABOLIC ACTIVATION

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 *uvrA* Rev/pl.
Untreated	19	18	30	142	23
50.0	17	16	34	148	24
158	20	18	31	139	30
500	23	17	28	142	26
1580	17	15	32	128	30
5000	19	18	35	134	22

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 2 - WITH METABOLIC ACTIVATION

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 *uvrA* Rev/pl.
Untreated	17	20	42	139	37
50.0	20	23	40	157	38
158	19	19	43	156	38
500	17	25	44	139	34
1580	21	21	44	143	31
5000	15	20	43	148	27

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 3 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA1535 Titre: 225

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	22	27	20	23	2.1	14	18	16	16	1.2
313	25	19	23	22	1.8	14	19	20	18	1.9
625	23	20	24	22	1.2	20	15	19	18	1.5
1250	18	18	17	18	0.3	13	19	16	16	1.7
2500	20	19	22	20	0.9	17	13	19	16	1.8
5000	23	18	21	21	1.5	17	11	13	14	1.8

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.774	-0.0001	-0.10127	0.2693	0.79545
1 - 4	-	4.863	-0.0005	-0.66582	2.8219	0.01810
1 - 5	-	4.721	-0.0001	-0.42030	1.6701	0.11879
1 - 6	-	4.645	0.0000	-0.24023	0.9899	0.33695
1 - 3	+	4.020	0.0004	0.34279	0.9654	0.36650
1 - 4	+	4.123	0.0000	-0.05764	0.1826	0.85879
1 - 5	+	4.123	0.0000	-0.10873	0.3944	0.69970
1 - 6	+	4.159	-0.0001	-0.42949	1.9023	0.07528

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	22	27	20	23	2.1
Sodium Azide	1 µg/pl	-	612	578	606	599	10.5
DMSO	100 µl/pl	+	12	16	15	14	1.2
2-Aminoanthracene	1 µg/pl	+	120	132	114	122	5.3

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 4 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA1537 Titre: 222

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	17	18	15	17	0.9	22	24	22	23	0.7
313	15	12	17	15	1.5	19	24	18	20	1.9
625	15	12	19	15	2.0	20	23	19	21	1.2
1250	16	12	14	14	1.2	18	23	22	21	1.5
2500	17	15	14	15	0.9	17	17	21	18	1.3
5000	12	11	17	13	1.9	18	20	16	18	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.023	-0.0003	-0.24796	0.6772	0.52003
1 - 4	-	4.009	-0.0002	-0.36062	1.2227	0.24949
1 - 5	-	3.925	0.0000	-0.12471	0.4532	0.65788
1 - 6	-	3.939	-0.0001	-0.32570	1.3779	0.18719
1 - 3	+	4.710	-0.0003	-0.37963	1.0857	0.31358
1 - 4	+	4.649	-0.0001	-0.19561	0.6308	0.54234
1 - 5	+	4.671	-0.0001	-0.49804	2.0708	0.05884
1 - 6	+	4.626	-0.0001	-0.55270	2.6528	0.01737

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	20	15	12	16	2.3
9-Aminoacridine	50 µg/pl	-	149	138	106	131	12.9
DMSO	100 µl/pl	+	19	24	21	21	1.5
2-Aminoanthracene	1 µg/pl	+	107	113	90	103	6.9

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 5 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: WP2 *uvrA* Titre: 276

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	24	25	33	27	2.8	31	30	38	33	2.5
313	24	22	24	23	0.7	30	35	39	35	2.6
625	21	27	28	25	2.2	34	36	32	34	1.2
1250	26	23	21	23	1.5	36	31	30	32	1.9
2500	28	26	19	24	2.7	32	39	34	35	2.1
5000	18	25	21	21	2.0	25	32	30	29	2.1

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.118	-0.0003	-0.23754	0.6470	0.53827
1 - 4	-	5.100	-0.0002	-0.33943	1.1411	0.28040
1 - 5	-	5.031	-0.0001	-0.19348	0.7110	0.48962
1 - 6	-	5.039	-0.0001	-0.40424	1.7678	0.09615
1 - 3	+	5.769	0.0001	0.13942	0.3725	0.72053
1 - 4	+	5.823	-0.0001	-0.13066	0.4168	0.68566
1 - 5	+	5.775	0.0000	0.11542	0.4189	0.68210
1 - 6	+	5.856	-0.0001	-0.40724	1.7836	0.09347

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
UNTREATED		-	24	25	23	24	0.6
MMS	500 µg/pl	-	208	205	202	205	1.7
DMSO	100 µl/pl	+	32	33	34	33	0.6
2-Aminoanthracene	10 µg/pl	+	232	218	225	225	4.0

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 6 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA98 Titre: 243

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	30	32	35	32	1.5	41	45	47	44	1.8
313	30	33	34	32	1.2	37	35	40	37	1.5
625	30	28	34	31	1.8	36	42	38	39	1.8
1250	27	34	35	32	2.5	37	36	40	38	1.2
2500	27	26	34	29	2.5	41	38	37	39	1.2
5000	28	33	26	29	2.1	34	37	39	37	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.709	-0.0002	-0.30596	0.8503	0.42330
1 - 4	-	5.661	0.0000	-0.08461	0.2685	0.79375
1 - 5	-	5.692	-0.0001	-0.36428	1.4103	0.18192
1 - 6	-	5.655	-0.0001	-0.38821	1.6850	0.11140
1 - 3	+	6.547	-0.0007	-0.59783	1.9731	0.08908
1 - 4	+	6.453	-0.0003	-0.52107	1.9306	0.08236
1 - 5	+	6.355	-0.0001	-0.31967	1.2164	0.24545
1 - 6	+	6.330	-0.0001	-0.40689	1.7817	0.09378

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	33	33	28	31	1.7
2-Nitrofluorene	2 µg/pl	-	196	204	195	198	2.8
DMSO	100 µl/pl	+	41	48	40	43	2.5
2-Aminoanthracene	1 µg/pl	+	599	528	541	556	21.8

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 7 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA100 Titre: 224

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	150	146	155	150	2.6	164	159	169	164	2.9
313	142	143	157	147	4.8	157	167	163	162	2.9
625	149	138	151	146	4.0	168	154	162	161	4.1
1250	146	152	139	146	3.8	165	170	159	165	3.2
2500	158	151	144	151	4.0	162	163	154	160	2.8
5000	155	150	148	151	2.1	164	170	161	165	2.6

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	12.248	-0.0003	-0.30384	0.8438	0.42668
1 - 4	-	12.213	-0.0001	-0.27184	0.8933	0.39270
1 - 5	-	12.138	0.0000	0.10554	0.3827	0.70816
1 - 6	-	12.138	0.0000	0.21900	0.8978	0.38261
1 - 3	+	12.801	-0.0002	-0.22682	0.6162	0.55728
1 - 4	+	12.753	0.0000	0.07174	0.2274	0.82467
1 - 5	+	12.786	0.0000	-0.21732	0.8028	0.43654
1 - 6	+	12.744	0.0000	0.08322	0.3341	0.74268

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	150	146	155	150	2.6
Sodium Azide	1 µg/pl	-	985	1043	965	998	23.4
DMSO	100 µl/pl	+	167	151	158	159	4.6
2-Aminoanthracene	1 µg/pl	+	1146	1121	1167	1145	13.3

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 8 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA1535 Titre: 225

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	21	19	15	18	1.8	19	20	16	18	1.2
313	17	22	18	19	1.5	17	14	16	16	0.9
625	19	19	23	20	1.3	15	18	15	16	1.0
1250	19	19	17	18	0.7	19	16	14	16	1.5
2500	18	18	19	18	0.3	17	20	14	17	1.7
5000	10	18	16	15	2.4	15	15	14	15	0.3

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.259	0.0004	0.35306	0.9984	0.35134
1 - 4	-	4.349	0.0000	0.01228	0.0388	0.96980
1 - 5	-	4.363	0.0000	-0.10749	0.3898	0.70297
1 - 6	-	4.424	-0.0001	-0.54707	2.6141	0.01879
1 - 3	+	4.217	-0.0004	-0.50056	1.5298	0.16992
1 - 4	+	4.141	-0.0001	-0.26970	0.8857	0.39659
1 - 5	+	4.085	0.0000	-0.04016	0.1449	0.88700
1 - 6	+	4.114	0.0000	-0.34367	1.4638	0.16261

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	19	20	16	18	1.2
Sodium Azide	1 µg/pl	-	566	581	592	580	7.5
DMSO	50 µl/pl	+	13	19	17	16	1.8
2-Aminoanthracene	1 µg/pl	+	106	99	102	102	2.0

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 9 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA1537 Titre: 223

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	16	15	19	17	1.2	20	22	16	19	1.8
313	17	20	16	18	1.2	20	18	23	20	1.5
625	16	18	21	18	1.5	19	22	20	20	0.9
1250	15	18	19	17	1.2	20	21	19	20	0.6
2500	19*	17*	15*	17	1.2	20*	16*	17*	18	1.2
5000	15*	14*	16*	15	0.6	11*	16*	16*	14	1.7

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.085	0.0003	0.35043	0.9899	0.35519
1 - 4	-	4.149	0.0001	0.10560	0.3358	0.74395
1 - 5	-	4.177	0.0000	-0.04918	0.1775	0.86183
1 - 6	-	4.212	-0.0001	-0.43343	1.9238	0.07235
1 - 3	+	4.406	0.0002	0.20982	0.5678	0.58792
1 - 4	+	4.441	0.0000	0.10729	0.3413	0.73997
1 - 5	+	4.505	-0.0001	-0.37000	1.4359	0.17464
1 - 6	+	4.540	-0.0001	-0.71753	4.1206	0.00080

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	20	18	19	19	0.6
9-Aminoacridine	50 µg/pl	-	168	175	129	157	14.3
DMSO	50 µl/pl	+	26	28	20	25	2.4
2-Aminoanthracene	1 µg/pl	+	97	108	99	101	3.4

* = Thinning of the background lawn

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 10 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: WP2 *uvrA* Titre: 281

Dose-level (µg/pl)	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	23	24	31	26	2.5	35	30	35	33	1.7
313	32	30	26	29	1.8	35	39	32	35	2.0
625	28	25	22	25	1.7	34	34	30	33	1.3
1250	27	31	22	27	2.6	31	32	27	30	1.5
2500	20	24	28	24	2.3	33	32	36	34	1.2
5000	26	27	20	24	2.2	30	33	31	31	0.9

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.211	-0.0001	-0.11516	0.3067	0.76797
1 - 4	-	5.185	0.0000	-0.06095	0.1931	0.85076
1 - 5	-	5.218	-0.0001	-0.29702	1.1216	0.28234
1 - 6	-	5.175	-0.0001	-0.29703	1.2443	0.23132
1 - 3	+	5.836	-0.0001	-0.10115	0.2690	0.79569
1 - 4	+	5.886	-0.0003	-0.51628	1.9064	0.08572
1 - 5	+	5.771	0.0000	-0.12152	0.4414	0.66616
1 - 6	+	5.772	0.0000	-0.25330	1.0474	0.31050

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
UNTREATED		-	23	24	31	26	2.5
MMS	500 µg/pl	-	348	363	321	344	12.3
DMSO	50 µl/pl	+	33	31	27	30	1.8
2-Aminoanthracene	20 µg/pl	+	303	277	276	285	8.8

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 11 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA98 Titre: 243

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	35	29	34	33	1.9	42	39	44	42	1.5
313	31	33	27	30	1.8	39	44	40	41	1.5
625	30	28	34	31	1.8	43	37	41	40	1.8
1250	27	31	35	31	2.3	38	43	40	40	1.5
2500	28	32	30	30	1.2	44	41	39	41	1.5
5000	30	29	27	29	0.9	35	38	36	36	0.9

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.671	-0.0003	-0.29465	0.8158	0.44150
1 - 4	-	5.622	-0.0001	-0.14858	0.4751	0.64491
1 - 5	-	5.612	-0.0001	-0.21260	0.7845	0.44682
1 - 6	-	5.606	-0.0001	-0.37105	1.5983	0.12953
1 - 3	+	6.453	-0.0002	-0.23747	0.6468	0.53840
1 - 4	+	6.432	-0.0001	-0.21097	0.6825	0.51041
1 - 5	+	6.397	0.0000	-0.00979	0.0353	0.97239
1 - 6	+	6.449	-0.0001	-0.56795	2.7601	0.01394

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	36	31	34	34	1.5
2-Nitrofluorene	2 µg/pl	-	174	171	190	178	5.9
DMSO	50 µl/pl	+	38	40	37	38	0.9
2-Aminoanthracene	2 µg/pl	+	556	586	552	565	10.7

[redacted]: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 12 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-008

SOLVENT: Sterile distilled water

Strain: TA100 Titre: 225

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	139	140	132	137	2.5	150	146	156	151	2.9
313	145	138	142	142	2.0	161	156	157	158	1.5
625	130	143	137	137	3.8	139	140	151	143	3.8
1250	133	124	130	129	2.6	136	144	145	142	2.8
2500	125*	118*	132*	125	4.0	131*	137*	143*	137	3.5
5000	95*	84*	80*	86	4.5	113*	128*	118*	120	4.4

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	11.772	0.0000	-0.03138	0.0831	0.93613
1 - 4	-	11.849	-0.0003	-0.60687	2.4146	0.03640
1 - 5	-	11.818	-0.0003	-0.73372	3.8936	0.00185
1 - 6	-	11.987	-0.0005	-0.93590	10.6274	0.00000
1 - 3	+	12.423	-0.0005	-0.41553	1.2087	0.26603
1 - 4	+	12.406	-0.0004	-0.60882	2.4269	0.03564
1 - 5	+	12.347	-0.0003	-0.70437	3.5778	0.00337
1 - 6	+	12.348	-0.0003	-0.88384	7.5576	0.00000

Positive and negative controls Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	139	140	132	137	2.5
Sodium Azide	1 µg/pl	-	954	970	901	942	20.9
DMSO	50 µl/pl	+	136	135	148	140	4.2
2-Aminoanthracene	2 µg/pl	+	944	1012	998	985	20.7

* = Thinning of the background lawn

9. APPENDIX I - Historical Control Data

WITHOUT METABOLIC ACTIVATION

	Untreated *Plate incorporation*	**Untreated** *Pre-incubation*	**Positive control** *Plate incorporation*	**Positive control** *Pre-incubation*
TA1535				
Mean value	19	19	520	516
SD	2.8	2.7	74.5	83.8
n	222	105	222	105
TA1537				
Mean value	17	18	148	123
SD	2.3	1.8	50.3	37.8
n	227	105	227	105
TA98				
Mean value	31	30	224	211
SD	3.1	2.3	31.2	27.0
n	227	102	227	102
TA100				
Mean value	152	135	720	739
SD	18.8	13.7	112.2	128.6
n	228	104	228	104
WP2 *uvr*A				
Mean value	29	30	159	191
SD	5.2	7.0	49.6	111.5
n	6	8	6	8

SD : standard deviation
n : number of experiments

WITH METABOLIC ACTIVATION

	Untreated *Plate incorporation*	**Untreated** *Pre-incubation*	**Positive control** *Plate incorporation*	**Positive control** *Pre-incubation*
TA1535				
Mean value	17	16	154	96
SD	2.4	2.0	32.0	15.5
n	220	105	220	105
TA1537				
Mean value	22	23	120	87
SD	2.8	2.0	25.1	13.6
n	224	103	224	103
TA98				
Mean value	44	42	1079	1008
SD	5.4	4.7	226.2	194.3
n	232	98	232	98
TA100				
Mean value	166	150	1276	1144
SD	18.7	14.9	265.0	181.6
n	235	99	235	99
WP2 *uvrA*				
Mean value	36	37	295	284
SD	8.9	9.4	75.4	91.9
n	6	8	6	8

SD : standard deviation
n : number of experiments

10. APPENDIX II - Study Protocol

RESEARCH TOXICOLOGY CENTRE - ROMA

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

Final protocol
prepared for

AUSIMONT S.p.A.
Via Lombardia, 20
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Italy

by

RESEARCH TOXICOLOGY CENTRE S.p.A.
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RTC Study No.: 8837-008

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RTC Enquiry Number: 8837

July2001

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Version 01/1UMB.

BACTERIAL MUTATION ASSAY
(S. typhimurium and E. coli)

MANAGEMENT OF STUDY

Scientific Director	:	J. Brightwell, Ph.D.
Head of Genetic and Cellular Toxicology	:	S. Cinelli, Biol.D.
Study Director	:	O. Scarcella, Biol. D.
Sponsor	:	AUSIMONT S.p.A. Via Lombardia, 20 20021 Bollate (MI) Italy
Monitor	:	To be appointed by the Sponsor

QUALITY ASSURANCE

Quality Assurance Manager	:	M. M. Brunetti, Biol.D.

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.

Version 01/1UMB.

BACTERIAL MUTATION ASSAY
(S. typhimurium and E. coli)

1. INTRODUCTION

1.1 Objective

To assay a number of test items for the ability to induce gene mutations in *Salmonella typhimurium* and *Escherichia coli*, as measured by reversion of auxotrophic strains to prototrophy.

1.2 Regulatory requirements

The study will be conducted in compliance with the GLP regulations of the US FDA [21 CRF part 58, 22 December 1978] and subsequent revisions; with Commission Directive 1999/11/EC of 8 March 1999 (adoption of the "OECD principles on Good Laboratory Practice – as revised in 1997") and subsequent revisions and with Decreto Legislativo 27 Gennaio 1992, no. 120 and subsequent revisions. In addition, the study is designed to comply with the experimental methods indicated in the guidelines of:

- EEC Council Directive 2000/32, Annex 4D.
- OECD Guidelines for the testing of chemicals No. 471 (Adopted July 1997)

1.3 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2. TEST ITEM

2.1 A number of test items will be supplied for routine testing, each identified by name and relevant univocal identity feature. Documentation of the identity and derivation of each test item will be the responsibility of the Sponsor.

2.2 A study number will be assigned to each test item supplied for investigation. This will consist of a fixed part, identifying the protocol, followed by a sequential number differing for each test item.

2.3 After completion of the study and submission of the final report, all unused samples of each test item will be returned to the Sponsor. An aliquot of each test item will be retained within the archives of the testing facility for a period of ten years after which they will be destroyed.

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

2.5 The test items will be treated with precautions appropriate for potential carcinogens.

2.6 The amount of each 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. Concentrations of solutions will be expressed in terms 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 test item stability, nor its concentration and homogeneity in 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

3.1 Bacterial strains

Stocks of *Salmonella* tester strains (TA 1535, TA 1537, TA 1538, TA 98, TA 100, TA 97 and TA 102 and some other related strains) were obtained from Dr. B.N.Ames, University of California. Stocks of *E. coli* tester strains (WP2, WP2 *uvr*A and WP2 *uvr*A pKM101) were obtained from Life Science Research, Occold, Suffolk, UK. Permanent stocks are kept at -80°C, and overnight subcultures of these stocks are prepared for each day's work.
The presence of the appropriate genetic markers in these strains is checked on a monthly basis for those in regular use, and as necessary for other strains, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Strain identity is also confirmed by reference to the spontaneous reversion levels and responses to mutagens during use. Bacterial cultures in liquid and on agar are clearly identified with their identity.

Detailed information about the genetic constitution of the tester strains may be found in the cited publications of Dr. B.N.Ames and Drs. M.H.L. Green and W.J. Muriel.

3.2 Media

The following growth media will be used:

Nutrient Broth: Oxoid Nutrient Broth No 2 will be prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This will be used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) will be added to one litre of distilled water and autoclaved.
The solution will then be poured into 9 cm plastic Petri dishes and allowed to solidify and dry before use. These plates will be used for the non-selective growth of the tester strains. Incubations on Nutrient Agar will be for approximately 48 or 72 hours.

Minimal Agar: Minimal medium agar will be prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into 9 cm plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) will be prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. This solution will be autoclaved, and stored. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) will be added to 100 ml of the top agar.

All incubations will be at 37°C.

3.3 S9 mix

The S9 liver tissue fraction will be prepared according to RTC standard procedures. Induction of drug metabolising enzyme-levels is routinely performed using phenobarbitone and betanaphthoflavone (Mixed Induction); induction with Aroclor 1254 will be performed if specifically requested by the Sponsor. Records pertaining to the preparation of the S9 fraction are kept in file at RTC. The mixture of S9 tissue fraction and cofactors (S9 mix) will be prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	=====
	10.0 ml

3.4 Control substances

Positive control treatments will be used in each experiment. The positive control agents are obtained commercially and characterised by their labelling, and their stability determined from the scientific literature. Sodium azide and methylmethanesulphonate will usually be dissolved in distilled water; 9-aminoacridine, 2-nitrofluorene and 2-aminoanthracene will usually be dissolved in DMSO. The frequency of preparation of stock solutions will be determined by their stability.

4. PRELIMINARY TOXICITY TEST

4.1 Experimental design

In order to establish the concentrations of test item to be used in the main assay, a preliminary toxicity test will be performed.
This test follows the method described in section 6.1, using only one plate per dose level, a single S9 mix concentration (10%) and covering a wide range of concentrations of the test item.

The highest dose-level for this preliminary test, unless limited by the solubility of the test item, will be 5 mg/plate, and the lower dose-levels will be spaced at approximately half-log intervals.

4.2 Selection of dose-levels

The toxicity will be assessed on the basis of a decline in the number of spontaneous revertants or a thinning of the background lawn. The highest dose-level for the mutation assays will be selected as a concentration which elicits moderate toxicity. If there is no evidence of toxicity following treatment with the test item, then the highest dose-level will be 5 mg/plate.

5. EXPERIMENTAL DESIGN

Each experiment will include negative and positive controls, and at least five doses of the test item, tested in the absence and presence of an S9 metabolising system. Three replicate plates will be used at each test point, and two independent experiments will be performed. If a positive result is obtained in any tester strain, a confirmatory experiment will be performed under the same experimental conditions. If, however, negative results are obtained in the first experiment, the confirmatory experiment will be performed using the pre-incubation method. A further experiment may be undertaken if inconsistent results are obtained.

The five bacterial strains *S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2 *uvr*A will be used in this study.

Negative controls: untreated and solvent vehicle controls will be prepared for each experiment; when the solvent is distilled water, these will be considered to be equivalent and only one set of controls is performed.

Positive controls: treatments are indicated in the following table:

Tester strain	***Absence of S9***	***Presence of S9***
TA1535	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate
TA100	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate (2 µg/plate)
TA1537	9-amino-acridine 50 µg/plate	2-aminoanthracene 1 µg/plate
TA98	2-nitrofluorene 2 µg/plate	2- aminoanthracene 1 µg/plate (2 µg/plate)
WP2 *uvr*A	methylmethanesulphonate 500 µg/plate	2-aminoanthracene 10 µg/plate (20 µg/plate)

Concentrations refer to both treatment methods. When two values are given, the figures in brackets refer to the pre-incubation method assay.

Test item: the highest dose-level of the test item to be used will be selected as described above. Further dose levels will be selected at intervals of a factor of two.

Where it seems advisable, further test points or controls may be included in experiments.

In addition, plates will be prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures will be plated on nutrient agar plates to establish the number of bacteria in the cultures.

6. ASSAY PROCEDURE

6.1 Plate-incorporation

The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) will be added to molten overlay agar and vortexed. The mixture will then be poured on the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture will be composed as follows:

(i)	Overlay agar (held at 45°C)	2 ml
(ii)	Test or control substance solution	0.1 ml
(iii)	S9 mix or phosphate buffer	0.5 ml
(iv)	Bacterial suspension	0.1 ml

The volume of test item solution, as indicated, will usually be 0.1 ml; in the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

6.2 Pre-incubation

The components will be added in turn to an empty test-tube:

(i) Bacterial suspension	0.1 ml
(ii) Test or control substance solution	0.05 ml
(iii) S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml

The volume of test item solution, as indicated, will usually be 0.05 ml. Where control or test items are dissolved in aqueous solvents, the volume used may be 0.1 ml. In the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

The incubate will be vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar will then be added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

6.3 Incubation and scoring

The prepared plates will be inverted and incubated for approximately 72 hours at 37°C. When the test item is a liquid at ambient temperature, the plates will be incubated in separate closed containers for each dose-level. After this period of incubation, the plates may be held at 4°C prior to scoring. Scoring is effected by counting the number of revertant colonies on each plate, either manually, or using a Cardinal - Automatic colony counting system (Perceptive Instruments). Contaminated plates will be considered on a case-by-case basis.

7. REPORTING

7.1 Presentation of data

The data will be presented in tabular form. The individual plate counts for each experiment will be given, together with the means and standard errors of the means, and regression analyses.

7.2 Evaluation of data

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose-levels.

Evaluation of Ames test data based on a 'doubling rate' has been shown to be as effective as statistical techniques in allowing the correct interpretation of test results (Chu et al. 1981).

7.3 Historical Data

In any case of unexpected results or analytical findings in treated or untreated plates historical data shall be included for comparison and interpretation.

7.4 Reporting procedure

A draft report will be despatched for comment before finalisation.

7.5 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;
- data generated while conducting the study;
- statistical methods employed for analysing the data;
- the test item identified by name;
- method used;
- any unforeseen circumstances that may have affected the quality or integrity of the study;
- the name and signature of the Study Director;
- a summary of the data, an analysis of the data and a statement of the conclusions drawn from the analysis;
- the location where all raw data, specimens and final report are to be stored;
- Quality Assurance statement.

Three copies of the final report (2 bound, 1 unbound) will be supplied.

7.6 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.

7.7 Archiving

All raw data, records and documentation arising from this study 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 five years from the date of consignment of the Final Report. At the end of this period, the Sponsor will be contacted for despatch or disposal of the material.

8. STUDY CONDUCT

8.1 Language

English language and Italian language versions 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 material will be considered to be equivalent.

8.2 Scientific decisions

The procedures described in this protocol may not comprehensively cover all the circumstances that can arise in the assay of test items. 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 will record carefully the decision he has reached and the reasoning which led to it.
Each scientific decision has to be discussed with the Sponsor before application.

8.3 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 will be available for inspection by the study monitor (for scientific monitoring) or the Quality Assurance Unit of the Sponsor (compliance monitoring).

9. REFERENCES

Ames,BN, J. McCann and E. Yamasaki (1975)
Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test.
Mutation Research 31, 347-364.

Chu K.C. et al. (1981)
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Guide for the *Salmonella typhimurium*/mammalian microsome tests for bacterial mutagenicity.
Mutation Research 189, 83-91.

Gatehouse D. et al. (1994)
Recommendations for the performance of bacterial mutation assays.
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Green M.H.L. and W.J. Muriel (1976)
Mutagen testing using TRP^+ reversion in *Escherichia coli.*
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Mutation Research 113, 173-215.

Venitt S., R. Forster and E. Longstaff (1983).
Bacterial Mutation Assays
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U.K.E.M.S., Swansea, 1983.

Venitt S., C. Croften-Sleigh and R. Forster (1984)
Bacterial mutation assays using reverse mutation
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S. Venitt and J.M. Parry (eds.)
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Version 01/1UMB.

PROTOCOL APPROVAL PAGE

STUDY TITLE : BACTERIAL MUTATION ASSAY (*S. typhimurium and E. coli*)

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

RTC ENQUIRY NO 8837

APPROVED BY : Ornella Scarcella 20 July 2001
O. Scarcella, Biol. D. Date
Study Director

RELEASED BY : Elena Cinelli 20 Jul 2001
S. Cinelli, Biol. D. Date
Head of Genetic and Cellular Toxicology

SPONSOR : AUSIMONT S.p.A.
Via Lombardia, 20
20021 Bollate (MI)
Italy

AUTHORISED BY SPONSOR* : Gianella Rhodia 1 Aug 2001
* GIUSEPPE MANNUCCI Date

Name and Title : Head of Regulatory Affairs and Industrial Toxicology

11. APPENDIX III – Certificate of analysis

AUSIMONT S.p.A.

Bollate, 30 gennaio 2002

Certificato di analisi

Prodotto:	[redacted]
Batch:	90215/91
Concentrazione della soluzione:	5 % peso
PH della soluzione:	6.5

Caratteristiche del precursore acido:

Peso equivalente:	560
Metodo:	titolazione acidimetrica

REDACTED AS TO TRADE NAMES

RESEARCH TOXICOLOGY CENTRE - ROMA

[redacted]

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

FINAL REPORT

RTC Study No.: 8837-007

Sponsor:
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Via Lombardia, 20
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Capitale sociale Euro 5.164.000
C.C.I.A.A. n. 375376
Reg. Soc. Trib. di Roma n. 2828/72
Cod. Fisc.: 00653120584
Partita IVA 00920611001

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 Regulations"* of the U.S. Food and Drug Administration, Code of Federal Regulations, 21 Part 58, 22 December 1978 and subsequent revisions.

B. Commission Directive 1999/11/EC of 8 March 1999 adapting to technical progress the principles of good laboratory practice as specified in Council Directive 87/18/EEC 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 (adoption of the *"OECD principles on Good Laboratory Practice – as revised in 1997"*) and subsequent revisions.

C. Decreto Legislativo 27 Gennaio 1992, n. 120 published in the Gazzetta Ufficiale della Repubblica Italiana 18 Febbraio 1992 (adoption of the Commission Directive of 18 December 1989 adapting to technical progress the Annex to Council Directive 88/320/EEC on the inspection and verification of Good Laboratory Practice (90/18/EEC)) and subsequent revisions.

O. Scarcella, Biol.D.
(Study Director) : Date : 09 JAN 2003

J. Brightwell, Ph.D.
(Scientific Director) : Date : 9.01.03

QUALITY ASSURANCE STATEMENT

(Relevant to the aspects of the study conducted by RTC)

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	08.08.2001	09.08.2001	09.08.2001
PROCESS-BASED INSPECTIONS			
Dose preparation	15.01.2002		19.01.2002
Treatment	22.03.2002		29.03.2002
Plating out	22.03.2002		29.03.2002
Plate scoring	08.02.2002		15.02.2002
Other routine inspections of a procedural nature were carried out on activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here.			

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 07 Jan 2003

M.M. Brunetti, Biol.D.
(Head of Quality Assurance)

07/01/2003
Date

Contents

1. SUMMARY

1.1 The test item [redacted] was examined for the ability to induce gene mutations in tester strains of *Salmonella typhimurium* and *Escherichia coli,* as measured by reversion of auxotrophic strains to prototrophy. The five tester strains TA1535, TA1537, TA98, TA100 and WP2 *uvr*A were used. Experiments were performed both in the absence and presence of metabolic activation, using liver S9 fraction from rats pre-treated with phenobarbitone and betanaphthoflavone.
Test item solutions were prepared using sterile distilled water.

1.2 In the toxicity test, the test item was assayed at a maximum dose-level of 5000 µg/plate and four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. No signs of toxicity were observed at any dose-level tested, in any tester strain, in the absence or presence of S9 metabolic activation.

1.3 Two main experiments were performed.
In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels, separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate.
As no increases in revertant numbers were observed, all treatments of Main Assay II included a pre-incubation step and used the same dose-range employed in Main Assay I.

1.4 The test item did not induce two fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism.

1.5 It is concluded that the test item [redacted] does not induce reverse mutation in *Salmonella typhimurium* and *Escherichia coli* under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of the test item to *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and to *Escherichia coli* strain WP2 *uvr*A using the procedures developed by Ames *et al.*, 1975 and revised by Maron and Ames, 1983.

The study was designed to comply with the experimental methods indicated in:
- EEC Council Directive 2000/32, Annex 4D.
- OECD Guideline for the testing of chemicals No. 471 (Adopted July 1997).

2.2 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2.3 Study organisation

Sponsor:

AUSIMONT S.p.A.
Via Lombardia, 20
20021 Bollate (MI)
Italy

Location of Study:

Research Toxicology Centre S.p.A.
Genetic Toxicology Department
Via Tito Speri, 12
00040 Pomezia (Roma)
Italy

Principal dates:

Study protocol approved by Study Director: 20-Jul-2001
Study commenced: 22-Feb-2002 (Toxicity assay treatment)
Study completed: 08-Mar-2002 (Completion of scoring Main Assay II)

Study Director:

O. Scarcella, Biol.D.

Archiving:

The original data arising from this study 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 five 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]
Lot or Batch Number	: 90215/92
Concentration of active ingredient	: 20% w/w
Expiry date	: 28-02-2004
Received from	: AUSIMONT S.p.A.
Date received	: 11-02-2002
Amount received	: 500 grams
Description	: Colourless liquid
Container	: Opaque plastic tank
Storage at RTC	: Ambient conditions
RTC reference number	: 6533

On 20-Feb-2002 the test item was transferred from the Formulation Unit to the Department of Genetic and Cellular Toxicology and stored under the same conditions. A certificate of analysis, supplied by the Sponsor, can be found in Appendix III of this report.

Solutions of the test item, as received, were prepared, immediately before use, on a weight/volume basis without correction for the displacement due to the volume of the test item. All test item solutions were used within 2 hours and 30 minutes of the initial formulation. Concentrations are expressed in terms of active ingredient. 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 items

The solvents used in this study were:
sterile distilled water (Bieffe Medital, batch 01C02-01).
dimethylsulphoxide (DMSO) (Fluka AG, batch 421649/1 13001).

Positive control treatments used solutions prepared as follows:

Sodium azide (Fluka AG, batch 221999 1081) in distilled water.
9-Aminoacridine (ICN K&K Laboratories, batch 12058-A) in DMSO.
2-Nitrofluorene (EGA Chemie, batch 12532) in DMSO.
2-Aminoanthracene (Sigma, batch 58F-3462) in DMSO.
Methylmethanesulphonate (MMS) (Fluka AG, batch 359316/153696)
in distilled water.

3.3 Media

The following growth media were used:

Nutrient Broth: Oxoid Nutrient Broth No 2 was prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This was used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) were added to distilled water (1 litre) and autoclaved.
The solutions were then poured into 9 cm plastic Petri dishes and allowed to solidify and dry before use. These plates were used for the non-selective growth of the tester strains.

Minimal Agar: Minimal medium agar was prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into 9 cm plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) was prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) was added to the top agar (100 ml).

3.4 S9 tissue homogenate

Two batches of S9 tissue homogenate (designated 2002/1 and 2002/2) were used in this study and had the following characteristics:

S9 Batch	Protein content (mg/ml)	Aminopyrine demethylase activity (μM/g liver/5 min, formaldehyde production)
2002/1	33.1 ± 1.69	4.14 ± 0.09
2002/2	35.8 ± 2.66	4.04 ± 0.07

Each S9 tissue fraction was prepared from the livers of five young male Sprague-Dawley rats which had received prior treatment with phenobarbital and betanaphthoflavone to induce high levels of xenobiotic metabolising enzymes. The efficacy of the S9 tissue fraction was previously checked in an Ames test and produced acceptable responses with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using *S. typhimurium* tester strain TA100.

The mixture of S9 tissue fraction and cofactors (S9 mix) was prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	10.0 ml

3.5 Bacterial strains

Four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and a strain of *Escherichia coli* (WP2 *uvr*A) were used in this study. Permanent stocks of these strains are kept at -80°C in RTC. Overnight subcultures of these stocks were prepared for each day's work.
Bacteria were taken from vials of frozen cultures, which had been checked for the presence of the appropriate genetic markers, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Bacterial cultures in liquid and on agar were clearly identified with their identity.

3.6 Methods

3.6.1 Preliminary toxicity test

A preliminary toxicity test was undertaken in order to select the concentrations of the test item to be used in the main assays. Due to an oversight, an additional toxicity test was performed. The results regarding this experiment were consistent with these presented and are retained in the study file and archived with all other data. In the toxicity test a wide range of dose-levels of the test item, set at half-log intervals, was used. Treatments were performed both in the absence and presence of S9 metabolism using the plate incorporation method; a single plate was used at each test point and positive controls were not included.

3.6.2 Main experiments

Two experiments were performed including negative and positive controls in the absence and presence of an S9 metabolising system. Three replicate plates were used at each test point.

In addition, plates were prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures were plated on nutrient agar plates to establish the number of bacteria in the cultures.

The first experiment was performed using a plate-incorporation method. The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) were added to molten overlay agar and vortexed. The mixture was then poured onto the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture was composed as follows:

(i)	Overlay agar (held at 45°C)	2 ml
(ii)	Test or control item solution	0.1 ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml
(iv)	Bacterial suspension	0.1 ml

The second experiment was performed using a pre-incubation method.
The components were added in turn to an empty test-tube:

(i)	Bacterial suspension	0.1 ml
(ii)	Test item solution or solvent control	0.1 ml
(iii)	DMSO or positive control solution	0.05 ml
(iv)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml

The incubate was vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar was then added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

3.6.3 Incubation and scoring

The prepared plates were inverted and incubated for approximately 72 hours at 37°C. After this period of incubation, the scoring was effected by counting the number of revertant colonies on each plate.

4. RESULTS

4.1 Solubility test

As indicated by the Sponsor, the test item is an aqueous solution at a concentration of 20% w/w. Since 100 µl of the test item solution are used in the preparation of each plate, this permitted a maximum concentration of 5000 µg/plate to be used in the toxicity test.

4.2 Toxicity test

The test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. Results are presented in Tables 1 and 2.

No signs of toxicity were observed at any dose-level tested, in any tester strain, in the absence or presence of S9 metabolic activation. On the basis of these results a maximum concentration of 5000 µg/plate was selected for the Main Assay with all tester strains.

4.3 Assay for reverse mutation

Two experiments were performed; individual plate counts for these tests, and the mean and standard error of the mean for each test point, together with statistical analysis are presented in Tables 3 to 12.

In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels, separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate. No signs of toxicity were observed.

As no increases in revertant numbers were observed, all treatments of Main Assay II included a pre-incubation step and used the same dose-range employed in Main Assay I. Toxicity, as indicated by thinning of the background lawn and/or reduction in revertant numbers, was observed both in the absence and presence of S9 metabolic activation, at the two higher dose-levels with TA1537 and TA100 tester strains. In the absence of S9 metabolism, toxicity was also observed with TA1535 and TA98 at the highest dose-level.

The test item did not induce two-fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism.

The sterility of the S9 mix and the test item solutions was confirmed by the absence of colonies on additional agar plates spread separately with these solutions. Marked increases in revertant numbers were obtained in these tests following treatment with the positive control items, indicating that the assay system was functioning correctly.

5. ANALYSIS OF RESULTS

5.1 Criteria for outcome of the assays

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition, there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose-levels.

5.2 Evaluation

The test item does not induce increases in the number of revertant colonies, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism. On the basis of the stated criteria it must be concluded that the test item [redacted] is not mutagenic to *S. typhimurium* and *E. coli* under the reported experimental conditions.

6. CONCLUSION

It is concluded that the test item [redacted] does not induce reverse mutation in *Salmonella typhimurium* and *Escherichia coli* under the reported experimental conditions.

7. KEY TO TABLES 1-12

7.1 Structure of Tables 3-12

These tables show, for each *Salmonella typhimurium* or *Escherichia coli* tester strain, the individual plate counts obtained for the negative and positive controls, and at each dose-level of the test item. The mean number of revertant colonies and standard error of the mean are also presented. The "untreated" plates receive no treatment. The titre of the bacterial cultures is given (million cells/plate).

7.2 Regression line

i) The regression analysis fits a regression line to the data by the least squares method, after square root transformation of the plate counts to satisfy normal distribution and homoscedasticity assumptions. The regression equation is expressed as:

$y = a + bx$

where y = transformed revertant numbers
a = intercept
b = slope value
x = dose-level (in the units given).

ii) Regression lines are calculated using a minimum of the three lowest dose-levels, and then including the further dose-levels in turn. The correlation co-efficient (r), the value of students "t" statistic, and the p-value for the regression lines are also given.

8. TABLES 1 TO 12

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 1 - WITHOUT METABOLIC ACTIVATION

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 *uvrA* Rev/pl.
Untreated	19	18	30	142	23
50.0	22	18	36	126	26
158	17	18	32	124	22
500	20	14	32	129	24
1580	22	11	31	136	23
5000	17	12	29	113	19

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 2 - WITH METABOLIC ACTIVATION

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 *uvrA* Rev/pl.
Untreated	17	20	42	139	37
50.0	21	19	42	142	30
158	20	24	45	133	32
500	16	21	36	138	30
1580	14	20	35	142	32
5000	11	21	41	137	21

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 3 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA1535 Titre: 225

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	22	27	20	23	2.1	14	18	16	16	1.2
313	18	20	21	20	0.9	17	19	14	17	1.5
625	17	21	22	20	1.5	20	17	13	17	2.0
1250	19	23	21	21	1.2	15	13	14	14	0.6
2500	21	19	22	21	0.9	16	17	15	16	0.6
5000	14	16	19	16	1.5	15	19	20	18	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.722	-0.0005	-0.45506	1.3521	0.21840
1 - 4	-	4.622	-0.0001	-0.17933	0.5764	0.57706
1 - 5	-	4.591	0.0000	-0.11259	0.4086	0.68952
1 - 6	-	4.648	-0.0001	-0.58814	2.9089	0.01025
1 - 3	+	4.009	0.0001	0.10486	0.2790	0.78834
1 - 4	+	4.092	-0.0002	-0.36799	1.2515	0.23923
1 - 5	+	4.010	0.0000	-0.12842	0.4669	0.64830
1 - 6	+	3.951	0.0000	0.25901	1.0727	0.29933

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	22	27	20	23	2.1
Sodium Azide	1 µg/pl	-	612	578	606	599	10.5
DMSO	100 µl/pl	+	12	16	15	14	1.2
2-Aminoanthracene	1 µg/pl	+	120	132	114	122	5.3

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 4 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA1537 Titre: 222

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	17	18	15	17	0.9	22	24	22	23	0.7
313	14	15	19	16	1.5	21	21	18	20	1.0
625	16	14	11	14	1.5	24	22	22	23	0.7
1250	14	20	15	16	1.9	19	22	18	20	1.2
2500	13	15	16	15	0.9	21	19	22	21	0.9
5000	13	17	11	14	1.8	21	19	17	19	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.116	-0.0006	-0.54675	1.7277	0.12769
1 - 4	-	3.976	-0.0001	-0.08125	0.2578	0.80180
1 - 5	-	3.978	-0.0001	-0.18508	0.6791	0.50901
1 - 6	-	3.978	-0.0001	-0.33121	1.4041	0.17941
1 - 3	+	4.663	0.0000	-0.00069	0.0018	0.99860
1 - 4	+	4.710	-0.0002	-0.41940	1.4610	0.17472
1 - 5	+	4.655	-0.0001	-0.29285	1.1043	0.28948
1 - 6	+	4.650	-0.0001	-0.47665	2.1689	0.04551

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	20	15	12	16	2.3
9-Aminoacridine	50 µg/pl	-	149	138	106	131	12.9
DMSO	100 µl/pl	+	19	24	21	21	1.5
2-Aminoanthracene	1 µg/pl	+	107	113	90	103	6.9

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 5 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: WP2 *uvrA* Titre: 276

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	24	25	33	27	2.8	31	30	38	33	2.5
313	20	29	24	24	2.6	33	30	29	31	1.2
625	28	24	27	26	1.2	30	34	36	33	1.8
1250	29	24	23	25	1.9	30	37	38	35	2.5
2500	20	24	26	23	1.8	32	28	30	30	1.2
5000	20	19	22	20	0.9	28	29	34	30	1.9

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.130	-0.0001	-0.10187	0.2709	0.79426
1 - 4	-	5.117	-0.0001	-0.11549	0.3677	0.72078
1 - 5	-	5.132	-0.0001	-0.31492	1.1963	0.25293
1 - 6	-	5.138	-0.0001	-0.59260	2.9428	0.00955
1 - 3	+	5.664	0.0001	0.05227	0.1385	0.89377
1 - 4	+	5.628	0.0002	0.32139	1.0733	0.30837
1 - 5	+	5.742	-0.0001	-0.19022	0.6986	0.49710
1 - 6	+	5.733	0.0000	-0.29171	1.2199	0.24018

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
UNTREATED		-	24	25	33	27	2.8
MMS	500 µg/pl	-	208	205	202	205	1.7
DMSO	100 µl/pl	+	32	33	34	33	0.6
2-Aminoanthracene	10 µg/pl	+	232	218	225	225	4.0

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 6 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA98 Titre: 243

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	30	32	35	32	1.5	41	47	45	44	1.8
313	32	28	34	31	1.8	41	37	40	39	1.2
625	33	30	37	33	2.0	39	43	37	40	1.8
1250	30	31	30	30	0.3	39	42	40	40	0.9
2500	30	27	34	30	2.0	40	37	36	38	1.2
5000	27	30	27	28	1.0	38	35	40	38	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.639	0.0001	0.14979	0.4008	0.70049
1 - 4	-	5.699	-0.0001	-0.24189	0.7883	0.44878
1 - 5	-	5.685	-0.0001	-0.30316	1.1470	0.27205
1 - 6	-	5.685	-0.0001	-0.54599	2.6068	0.01908
1 - 3	+	6.587	-0.0006	-0.59158	1.9413	0.09335
1 - 4	+	6.491	-0.0002	-0.37136	1.2648	0.23463
1 - 5	+	6.474	-0.0001	-0.53992	2.3128	0.03775
1 - 6	+	6.422	-0.0001	-0.51620	2.4108	0.02830

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	33	33	28	31	1.7
2-Nitrofluorene	2 µg/pl	-	196	204	195	198	2.8
DMSO	100 µl/pl	+	41	48	40	43	2.5
2-Aminoanthracene	1 µg/pl	+	599	528	541	556	21.8

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 7 - Experiment 1 - Plate incorporation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA100 Titre: 224

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	150	146	155	150	2.6	164	159	169	164	2.9
313	139	148	151	146	3.6	170	162	158	163	3.5
625	147	154	140	147	4.0	156	170	162	163	4.1
1250	153	142	150	148	3.3	166	172	161	166	3.2
2500	140	138	152	143	4.4	163	170	156	163	4.0
5000	150	132	156	146	7.2	174	166	164	168	3.1

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	12.224	-0.0002	-0.26064	0.7143	0.49818
1 - 4	-	12.177	0.0000	-0.06890	0.2184	0.83150
1 - 5	-	12.200	-0.0001	-0.31504	1.1969	0.25273
1 - 6	-	12.158	0.0000	-0.17009	0.6904	0.49982
1 - 3	+	12.805	-0.0001	-0.11078	0.2949	0.77661
1 - 4	+	12.766	0.0001	0.18034	0.5798	0.57488
1 - 5	+	12.800	0.0000	-0.00125	0.0045	0.99649
1 - 6	+	12.777	0.0000	0.25560	1.0575	0.30597

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	150	146	155	150	2.6
Sodium Azide	1 µg/pl	-	985	1043	965	998	23.4
DMSO	100 µl/pl	+	167	151	158	159	4.6
2-Aminoanthracene	1 µg/pl	+	1146	1121	1167	1145	13.3

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 8 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA1535 Titre: 225

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	21	19	15	18	1.8	19	20	16	18	1.2
313	17	21	16	18	1.5	15	15	16	15	0.3
625	13	16	19	16	1.7	18	16	19	18	0.9
1250	17	15	14	15	0.9	16	18	15	16	0.9
2500	14	20	16	17	1.8	20	15	17	17	1.5
5000	15*	12*	17*	15	1.5	17	15	19	17	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.307	-0.0005	-0.37011	1.0541	0.32686
1 - 4	-	4.272	-0.0003	-0.47583	1.7108	0.11790
1 - 5	-	4.171	-0.0001	-0.23462	0.8702	0.39997
1 - 6	-	4.164	-0.0001	-0.38473	1.6672	0.11492
1 - 3	+	4.169	-0.0001	-0.14568	0.3896	0.70842
1 - 4	+	4.165	-0.0001	-0.23414	0.7616	0.46388
1 - 5	+	4.121	0.0000	-0.01433	0.0517	0.95957
1 - 6	+	4.119	0.0000	-0.00494	0.0197	0.98449

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	19	20	16	18	1.2
Sodium Azide	1 µg/pl	-	566	581	592	580	7.5
DMSO	50 µl/pl	+	13	19	17	16	1.8
2-Aminoanthracene	1 µg/pl	+	106	99	102	102	2.0

* = Thinning of the background lawn

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 9 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA1537 Titre: 223

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	16	15	19	17	1.2	20	22	16	19	1.8
313	16	13	17	15	1.2	21	20	16	19	1.5
625	12	19	16	16	2.0	19	23	25	22	1.8
1250	12	18	18	16	2.0	19	20	21	20	0.6
2500	14*	17*	17*	16	1.0	12*	19*	14*	15	2.1
5000	7*	11*	8*	9	1.2	14*	16*	12*	14	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.044	-0.0002	-0.19600	0.5288	0.61328
1 - 4	-	4.002	0.0000	-0.06623	0.2099	0.83796
1 - 5	-	3.984	0.0000	-0.00721	0.0260	0.97965
1 - 6	-	4.132	-0.0002	-0.71486	4.0892	0.00086
1 - 3	+	4.320	0.0005	0.42664	1.2481	0.25213
1 - 4	+	4.423	0.0001	0.18122	0.5827	0.57299
1 - 5	+	4.570	-0.0002	-0.51375	2.1591	0.05012
1 - 6	+	4.527	-0.0002	-0.66868	3.5972	0.00241

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	20	18	19	19	0.6
9-Aminoacridine	50 µg/pl	-	168	175	129	157	14.3
DMSO	50 µl/pl	+	26	28	20	25	2.4
2-Aminoanthracene	1 µg/pl	+	97	108	99	101	3.4

* = Thinning of the background lawn

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 10 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: WP2 *uvrA* Titre: 281

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	23	24	31	26	2.5	35	30	35	33	1.7
313	23	29	29	27	2.0	29	34	31	31	1.5
625	29	26	32	29	1.7	30	33	27	30	1.7
1250	27	29	24	27	1.5	31	36	34	34	1.5
2500	31	30	29	30	0.6	30	33	35	33	1.5
5000	29	20	21	23	2.8	32	27	28	29	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.072	0.0005	0.38417	1.1009	0.30735
1 - 4	-	5.172	0.0001	0.09408	0.2988	0.77118
1 - 5	-	5.144	0.0001	0.38179	1.4894	0.16024
1 - 6	-	5.279	-0.0001	-0.28760	1.2011	0.24718
1 - 3	+	5.761	-0.0005	-0.50810	1.5608	0.16254
1 - 4	+	5.634	0.0000	0.08761	0.2781	0.78659
1 - 5	+	5.640	0.0000	0.12045	0.4375	0.66894
1 - 6	+	5.700	0.0000	-0.33291	1.4122	0.17705

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
UNTREATED		-	23	24	31	26	2.5
MMS	500 µg/pl	-	348	363	321	344	12.3
DMSO	50 µl/pl	+	33	31	27	30	1.8
2-Aminoanthracene	20 µg/pl	+	303	277	276	285	8.8

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 11 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA98 Titre: 243

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	35	29	34	33	1.9	42	39	44	42	1.5
313	31	34	28	31	1.7	38	44	41	41	1.7
625	33	31	27	30	1.8	40	39	43	41	1.2
1250	36	32	30	33	1.8	42	36	39	39	1.7
2500	27	33	29	30	1.8	43	41	38	41	1.5
5000	24*	27*	25*	25	0.9	37	35	39	37	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.696	-0.0003	-0.34871	0.9844	0.35772
1 - 4	-	5.610	0.0000	0.04169	0.1320	0.89763
1 - 5	-	5.649	-0.0001	-0.23754	0.8817	0.39395
1 - 6	-	5.689	-0.0001	-0.66591	3.5704	0.00255
1 - 3	+	6.448	-0.0001	-0.19000	0.5120	0.62438
1 - 4	+	6.459	-0.0002	-0.40727	1.4101	0.18884
1 - 5	+	6.401	0.0000	-0.16105	0.5884	0.56638
1 - 6	+	6.422	-0.0001	-0.52008	2.4357	0.02693

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	36	31	34	34	1.5
2-Nitrofluorene	2 µg/pl	-	174	171	190	178	5.9
DMSO	50 µl/pl	+	38	40	37	38	0.9
2-Aminoanthracene	2 µg/pl	+	556	586	552	565	10.7

* = Thinning of the background lawn

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 12 - Experiment 2 - Preincubation method -

STUDY NO.: 8837-007

SOLVENT: Sterile distilled water

Strain: TA100 Titre: 225

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	139	140	132	137	2.5	150	146	156	151	2.9
313	132	123	138	131	4.4	142	138	155	145	5.1
625	125	121	127	124	1.8	134	137	140	137	1.7
1250	136	130	132	133	1.8	144	135	141	140	2.6
2500	126*	136*	120*	127	4.7	130*	136*	123*	130	3.8
5000	92*	81*	93*	89	3.8	101*	89*	90*	93	3.8

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	11.709	-0.0009	-0.76434	3.1362	0.01647
1 - 4	-	11.526	-0.0001	-0.23093	0.7506	0.47021
1 - 5	-	11.511	-0.0001	-0.31102	1.1799	0.25917
1 - 6	-	11.741	-0.0004	-0.87272	7.1502	0.00000
1 - 3	+	12.290	-0.0009	-0.74459	2.9512	0.02137
1 - 4	+	12.154	-0.0004	-0.55715	2.1217	0.05985
1 - 5	+	12.135	-0.0003	-0.74797	4.0631	0.00134
1 - 6	+	12.269	-0.0005	-0.94218	11.2465	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	139	140	132	137	2.5
Sodium Azide	1 µg/pl	-	954	970	901	942	20.9
DMSO	50 µl/pl	+	136	135	148	140	4.2
2-Aminoanthracene	2 µg/pl	+	944	1012	998	985	20.7

* = Thinning of the background lawn

9. APPENDIX I - Historical Control Data

WITHOUT METABOLIC ACTIVATION

	Untreated	**Untreated**	**Positive control**	**Positive control**
	Plate incorporation	*Pre-incubation*	*Plate incorporation*	*Pre-incubation*
TA1535				
Mean value	19	19	520	516
SD	2.8	2.7	74.5	83.8
n	222	105	222	105
TA1537				
Mean value	17	18	148	123
SD	2.3	1.8	50.3	37.8
n	227	105	227	105
TA98				
Mean value	31	30	224	211
SD	3.1	2.3	31.2	27.0
n	227	102	227	102
TA100				
Mean value	152	135	720	739
SD	18.8	13.7	112.2	128.6
n	228	104	228	104
WP2 *uvr*A				
Mean value	29	30	159	191
SD	5.2	7.0	49.6	111.5
n	6	8	6	8

SD : standard deviation
n : number of experiments

WITH METABOLIC ACTIVATION

	Untreated	**Untreated**	**Positive control**	**Positive control**
	Plate incorporation	*Pre-incubation*	*Plate incorporation*	*Pre-incubation*
TA1535				
Mean value	17	16	154	96
SD	2.4	2.0	32.0	15.5
n	220	105	220	105
TA1537				
Mean value	22	23	120	87
SD	2.8	2.0	25.1	13.6
n	224	103	224	103
TA98				
Mean value	44	42	1079	1008
SD	5.4	4.7	226.2	194.3
n	232	98	232	98
TA100				
Mean value	166	150	1276	1144
SD	18.7	14.9	265.0	181.6
n	235	99	235	99
WP2 *uvrA*				
Mean value	36	37	295	284
SD	8.9	9.4	75.4	91.9
n	6	8	6	8

SD : standard deviation
n : number of experiments

10. APPENDIX II - Study Protocol

RESEARCH TOXICOLOGY CENTRE - ROMA

Version 01/1UMB.

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

Final protocol
prepared for

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Via Lombardia, 20
20021 Bollate (MI)
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by

RESEARCH TOXICOLOGY CENTRE S.p.A.
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RTC Study No.: 8837-007

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RTC Enquiry Number: 8837

July2001

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Version 01/1UMB.

BACTERIAL MUTATION ASSAY
(S. typhimurium and E. coli)

MANAGEMENT OF STUDY

Scientific Director	:	J. Brightwell, Ph.D.
Head of Genetic and Cellular Toxicology	:	S. Cinelli, Biol.D.
Study Director	:	O. Scarcella, Biol. D.
Sponsor	:	AUSIMONT S.p.A. Via Lombardia, 20 20021 Bollate (MI) Italy
Monitor	:	To be appointed by the Sponsor

QUALITY ASSURANCE

Quality Assurance Manager	:	M. M. Brunetti, Biol.D.

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.

Version 01/1UMB.

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

1. INTRODUCTION

1.1 Objective

To assay a number of test items for the ability to induce gene mutations in *Salmonella typhimurium* and *Escherichia coli*, as measured by reversion of auxotrophic strains to prototrophy.

1.2 Regulatory requirements

The study will be conducted in compliance with the GLP regulations of the US FDA [21 CRF part 58, 22 December 1978] and subsequent revisions; with Commission Directive 1999/11/EC of 8 March 1999 (adoption of the "OECD principles on Good Laboratory Practice – as revised in 1997") and subsequent revisions and with Decreto Legislativo 27 Gennaio 1992, no. 120 and subsequent revisions. In addition, the study is designed to comply with the experimental methods indicated in the guidelines of:

- EEC Council Directive 2000/32, Annex 4D.
- OECD Guidelines for the testing of chemicals No. 471 (Adopted July 1997)

1.3 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2. TEST ITEM

2.1 A number of test items will be supplied for routine testing, each identified by name and relevant univocal identity feature. Documentation of the identity and derivation of each test item will be the responsibility of the Sponsor.

2.2 A study number will be assigned to each test item supplied for investigation. This will consist of a fixed part, identifying the protocol, followed by a sequential number differing for each test item.

2.3 After completion of the study and submission of the final report, all unused samples of each test item will be returned to the Sponsor. An aliquot of each test item will be retained within the archives of the testing facility for a period of ten years after which they will be destroyed.

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

2.5 The test items will be treated with precautions appropriate for potential carcinogens.

2.6 The amount of each 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. Concentrations of solutions will be expressed in terms 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 test item stability, nor its concentration and homogeneity in 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

3.1 Bacterial strains

Stocks of *Salmonella* tester strains (TA 1535, TA 1537, TA 1538, TA 98, TA 100, TA 97 and TA 102 and some other related strains) were obtained from Dr. B.N.Ames, University of California. Stocks of *E. coli* tester strains (WP2, WP2 *uvr*A and WP2 *uvr*A pKM101) were obtained from Life Science Research, Occold, Suffolk, UK. Permanent stocks are kept at -80°C, and overnight subcultures of these stocks are prepared for each day's work.
The presence of the appropriate genetic markers in these strains is checked on a monthly basis for those in regular use, and as necessary for other strains, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Strain identity is also confirmed by reference to the spontaneous reversion levels and responses to mutagens during use. Bacterial cultures in liquid and on agar are clearly identified with their identity.

Detailed information about the genetic constitution of the tester strains may be found in the cited publications of Dr. B.N.Ames and Drs. M.H.L. Green and W.J. Muriel.

3.2 Media

The following growth media will be used:

Nutrient Broth: Oxoid Nutrient Broth No 2 will be prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This will be used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) will be added to one litre of distilled water and autoclaved.
The solution will then be poured into 9 cm plastic Petri dishes and allowed to solidify and dry before use. These plates will be used for the non-selective growth of the tester strains. Incubations on Nutrient Agar will be for approximately 48 or 72 hours.

Minimal Agar: Minimal medium agar will be prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into 9 cm plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) will be prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. This solution will be autoclaved, and stored. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) will be added to 100 ml of the top agar.

All incubations will be at 37°C.

3.3 S9 mix

The S9 liver tissue fraction will be prepared according to RTC standard procedures. Induction of drug metabolising enzyme-levels is routinely performed using phenobarbitone and betanaphthoflavone (Mixed Induction); induction with Aroclor 1254 will be performed if specifically requested by the Sponsor. Records pertaining to the preparation of the S9 fraction are kept in file at RTC. The mixture of S9 tissue fraction and cofactors (S9 mix) will be prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	=====
	10.0 ml

3.4 Control substances

Positive control treatments will be used in each experiment. The positive control agents are obtained commercially and characterised by their labelling, and their stability determined from the scientific literature. Sodium azide and methylmethanesulphonate will usually be dissolved in distilled water; 9-aminoacridine, 2-nitrofluorene and 2-aminoanthracene will usually be dissolved in DMSO. The frequency of preparation of stock solutions will be determined by their stability.

4. PRELIMINARY TOXICITY TEST

4.1 Experimental design

In order to establish the concentrations of test item to be used in the main assay, a preliminary toxicity test will be performed.
This test follows the method described in section 6.1, using only one plate per dose level, a single S9 mix concentration (10%) and covering a wide range of concentrations of the test item.

The highest dose-level for this preliminary test, unless limited by the solubility of the test item, will be 5 mg/plate, and the lower dose-levels will be spaced at approximately half-log intervals.

4.2 Selection of dose-levels

The toxicity will be assessed on the basis of a decline in the number of spontaneous revertants or a thinning of the background lawn. The highest dose-level for the mutation assays will be selected as a concentration which elicits moderate toxicity. If there is no evidence of toxicity following treatment with the test item, then the highest dose-level will be 5 mg/plate.

5. EXPERIMENTAL DESIGN

Each experiment will include negative and positive controls, and at least five doses of the test item, tested in the absence and presence of an S9 metabolising system. Three replicate plates will be used at each test point, and two independent experiments will be performed. If a positive result is obtained in any tester strain, a confirmatory experiment will be performed under the same experimental conditions. If, however, negative results are obtained in the first experiment, the confirmatory experiment will be performed using the pre-incubation method. A further experiment may be undertaken if inconsistent results are obtained.

The five bacterial strains *S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2 *uvr*A will be used in this study.

Negative controls: untreated and solvent vehicle controls will be prepared for each experiment; when the solvent is distilled water, these will be considered to be equivalent and only one set of controls is performed.

Positive controls: treatments are indicated in the following table:

Tester strain	*Absence of S9*	*Presence of S9*
TA1535	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate
TA100	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate (2 µg/plate)
TA1537	9-amino-acridine 50 µg/plate	2-aminoanthracene 1 µg/plate
TA98	2-nitrofluorene 2 µg/plate	2- aminoanthracene 1 µg/plate (2 µg/plate)
WP2 *uvr*A	methylmethanesulphonate 500 µg/plate	2-aminoanthracene 10 µg/plate (20 µg/plate)

Concentrations refer to both treatment methods. When two values are given, the figures in brackets refer to the pre-incubation method assay.

Test item: the highest dose-level of the test item to be used will be selected as described above. Further dose levels will be selected at intervals of a factor of two.

Where it seems advisable, further test points or controls may be included in experiments.

In addition, plates will be prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures will be plated on nutrient agar plates to establish the number of bacteria in the cultures.

6. ASSAY PROCEDURE

6.1 Plate-incorporation

The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) will be added to molten overlay agar and vortexed. The mixture will then be poured on the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture will be composed as follows:

(i)	Overlay agar (held at 45°C)	2 ml
(ii)	Test or control substance solution	0.1 ml
(iii)	S9 mix or phosphate buffer	0.5 ml
(iv)	Bacterial suspension	0.1 ml

The volume of test item solution, as indicated, will usually be 0.1 ml; in the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

6.2 Pre-incubation

The components will be added in turn to an empty test-tube:

(i) Bacterial suspension	0.1 ml
(ii) Test or control substance solution	0.05 ml
(iii) S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml

The volume of test item solution, as indicated, will usually be 0.05 ml. Where control or test items are dissolved in aqueous solvents, the volume used may be 0.1 ml. In the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

The incubate will be vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar will then be added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

6.3 Incubation and scoring

The prepared plates will be inverted and incubated for approximately 72 hours at 37°C. When the test item is a liquid at ambient temperature, the plates will be incubated in separate closed containers for each dose-level. After this period of incubation, the plates may be held at 4°C prior to scoring. Scoring is effected by counting the number of revertant colonies on each plate, either manually, or using a Cardinal - Automatic colony counting system (Perceptive Instruments). Contaminated plates will be considered on a case-by-case basis.

7. REPORTING

7.1 Presentation of data

The data will be presented in tabular form. The individual plate counts for each experiment will be given, together with the means and standard errors of the means, and regression analyses.

7.2 Evaluation of data

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose-levels.

Evaluation of Ames test data based on a 'doubling rate' has been shown to be as effective as statistical techniques in allowing the correct interpretation of test results (Chu et al. 1981).

7.3 Historical Data

In any case of unexpected results or analytical findings in treated or untreated plates historical data shall be included for comparison and interpretation.

7.4 Reporting procedure

A draft report will be despatched for comment before finalisation.

7.5 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;
- data generated while conducting the study;
- statistical methods employed for analysing the data;
- the test item identified by name;
- method used;
- any unforeseen circumstances that may have affected the quality or integrity of the study;
- the name and signature of the Study Director;
- a summary of the data, an analysis of the data and a statement of the conclusions drawn from the analysis;
- the location where all raw data, specimens and final report are to be stored;
- Quality Assurance statement.

Three copies of the final report (2 bound, 1 unbound) will be supplied.

7.6 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.

7.7 Archiving

All raw data, records and documentation arising from this study 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 five years from the date of consignment of the Final Report. At the end of this period, the Sponsor will be contacted for despatch or disposal of the material.

8. STUDY CONDUCT

8.1 Language

English language and Italian language versions 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 material will be considered to be equivalent.

8.2 Scientific decisions

The procedures described in this protocol may not comprehensively cover all the circumstances that can arise in the assay of test items. 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 will record carefully the decision he has reached and the reasoning which led to it.
Each scientific decision has to be discussed with the Sponsor before application.

8.3 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 will be available for inspection by the study monitor (for scientific monitoring) or the Quality Assurance Unit of the Sponsor (compliance monitoring).

9. REFERENCES

Ames,BN, J. McCann and E. Yamasaki (1975)
Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test.
Mutation Research 31, 347-364.

Chu K.C. et al. (1981)
Evaluating statistical analyses and reproducibility of microbial mutagenicity assays.
Mutation Research 85, 119-132.

Claxton L.D. et al. (1987)
Guide for the *Salmonella typhimurium*/mammalian microsome tests for bacterial mutagenicity.
Mutation Research 189, 83-91.

Gatehouse D. et al. (1994)
Recommendations for the performance of bacterial mutation assays.
Mutation Research 312, 217-233

Green M.H.L. and W.J. Muriel (1976)
Mutagen testing using TRP^+ reversion in *Escherichia coli.*
Mutation Research 38, 3-32.

Maron D.M. and B.N. Ames (1983).
Revised methods for the *Salmonella* mutagenicity test.
Mutation Research 113, 173-215.

Venitt S., R. Forster and E. Longstaff (1983).
Bacterial Mutation Assays
in: Report of the UKEMS Subcommittee on Guidelines for Mutagenicity testing.
U.K.E.M.S., Swansea, 1983.

Venitt S., C. Croften-Sleigh and R. Forster (1984)
Bacterial mutation assays using reverse mutation
in: Mutagenicity Testing - a practical approach
S. Venitt and J.M. Parry (eds.)
IRL Press, Oxford, 1984.

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SC

Version 01/1UMB.

PROTOCOL APPROVAL PAGE

STUDY TITLE : BACTERIAL MUTATION ASSAY (*S. typhimurium and E. coli*)

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

RTC ENQUIRY NO 8837

APPROVED BY : Orsella Scarcella 20 July 2001
O. Scarcella, Biol. D. Date
Study Director

RELEASED BY : Elena Cinelli 20 Jul 2001
S. Cinelli, Biol. D. Date
Head of Genetic and Cellular Toxicology

SPONSOR : AUSIMONT S.p.A.
Via Lombardia, 20
20021 Bollate (MI)
Italy

AUTHORISED BY SPONSOR* : Graziella Rhodes 1 Aug 2001
* GIUSEPPE MANNUCCI Date

Name and Title : Head of Regulatory Affairs and Industrial Toxicology

11. APPENDIX III – Certificate of analysis

AUSIMONT S.p.A.

Bollate, 30 gennaio 2002

Certificato di analisi

Prodotto:	[redacted]
Batch:	90215/92
Concentrazione della soluzione:	20 % peso
PH della soluzione:	6.6

Caratteristiche del precursore acido:

Peso equivalente:	534
Metodo:	titolazione acidimetrica

REDACTED AS TO TRADE NAMES

LABORATORIES & CLINICS GROUP
BIOSCIENCE

RBM Exp. No. 970591

RBM Via Ribes 1
10010 Colleretto Giacosa (TO)
Italy

Tel: 0125 222111
Fax: 0125 222599

Study on the ability of the test article

[redacted]

to induce gene mutations in strains of
Salmonella typhimurium and Escherichia coli

RBM EXP. No. 970591

Issued on April 6, 1998

SPONSOR

AUSIMONT S.p.A.
Via S. Pietro, 50/A
20021 Bollate (Milano)
Italy

PERFORMING LABORATORY

Istituto di Ricerche Biomediche
"Antoine Marxer" RBM S.p.A.
Via Ribes, 1
10010 - Colleretto Giacosa (Torino)
Italy

LABORATORIES & CLINICS GROUP BIOSCIENCE

RBM Exp. No. 970591

INDEX

This report consists of 35 pages.

Dr. Franca Fassio
RBM Study Director

Ivrea, April 6, 1998

LABORATORIES & CLINICS GROUP
BIOSCIENCE

RBM Exp. No. 970591

FOREWORD

On behalf AUSIMONT S.p.A., Via S. Pietro, 50/A - 20021 Bollate (Milano) Italy, of Istituto di Ricerche Biomediche "Antoine Marxer" RBM S.p.A., authorized by the Italian Health Authorities (1-2) to conduct toxicological studies, has performed a study of the possible mutagenic activity of the test article

[redacted]

with Salmonella typhimurium and Escherichia coli strains.

A sample of the substance used, along with pertinent documentation is held in sufficient quantity in the RBM archives at the disposal of the Ministero della Sanità.

The undersigned declare that the experiment was conducted using the same batch of substance as that of the sample held on file.

For verification by the Ministero della Sanità, the undersigned moreover guarantee the identification and classification of all those materials, documents and recordings used in conducting the experiment held on file for a period of at least 10 years from the date of this report. Following this time, they will be placed at the disposal of the Sponsor.

Dr. Domenico Barone

Scientific Director
Recognized by the Italian Health Authorities as Responsible for Experimentation of Mutagenesis

Dr. Angelo Conz

General Manager of the Istituto di Ricerche Biomediche "Antoine Marxer", RBM S.p.A.

Ivrea, April 6, 1998

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(1): **Pharmaceuticals:**
Authorization dated March 12, 1976 in accordance with "Circolare 73", May 16, 1974.

(2): **Chemicals:**
Authorization in accordance with DPR 927/81 (D.M. dated January 7, 1988 published in G.U. no. 12, dated January 16, 1988).

LABORATORIES & CLINICS GROUP
BIOSCIENCE

RBM Exp. No. 970591

QUALITY ASSURANCE STATEMENT

RBM Experiment number: 970591

Study title:

"Study on the ability of the test article [redacted] to induce gene mutations in strains of Salmonella typhimurium and Escherichia coli".

Studies of the type described in this report are conducted in a manner which involves frequent repetition of identical or similar procedures.

In compliance with the Principles of Good Laboratory Practice, at the time of this study, procedure-based inspections were made by the Q.A.U. of critical phases and procedures relevant to this type of study. For the inspection of any given procedure, studies were selected at random. All such inspections were reported promptly to the Study Director and to facility management.

This study was inspected on:

Dates of inspections/Audit	Dates of report to Study Director and Management
January 26, 1998	January 26, 1998
March 5, 6 and 9, 1998	March 9, 1998
March 31, 1998	March 31, 1998

This report has been audited by the Q.A.U. and was found to be an accurate description of such methods and procedures as were used during the conduct of the study and an accurate reflection of the raw data.

Date of final report audit: April 16, 1998

Enrico Invernizzi
Head of Quality Assurance Unit

Date: April 16, 1998

LABORATORIES & CLINICS GROUP
BIOSCIENCE

RBM Exp. No. 970591

RBM MANAGEMENT DECLARATION OF GLP COMPLIANCE

RBM Experiment number: 970591

Study title:

"Study on the ability of the test article [redacted] to induce gene mutations in strains of Salmonella typhimurium and Escherichia coli"

was performed in compliance with the OECD-GLP in the testing of chemicals, [C(81) 30 (final)], regulations enforced by the Italian Health Authority (D.M. dated June 26, 1986 as published in G.U. No. 198, August 27, 1986 and D.L. January 27, 1992, No. 120 as published in G.U. (Supplement) No. 40, February 18, 1992).

Dr. Franca Fassio

RBM Study Director

Dr. Angelo Conz

General Manager of the Istituto di Ricerche Biomediche "Antoine Marxer" S.p.A.

Ivrea, April 16, 1998

RBM Exp. No. 970591

SCIENTISTS INVOLVED IN THE STUDY

RBM Study Director — Dr. Franca Fassio

Head of Biotechnology Unit — Dr. Domenico Barone

PURPOSE OF THE STUDY

Appraisal of the possible genotoxic activity exerted by the test article [redacted]

TEST METHOD

The test method is in accordance with the "Organization for Economic Cooperation and Development Guidelines", Section 4, Subparts 471-472, Paris 1981, subsequent revisions, with "Annex to Commission Directive 92/69/EEC of July 31, 1992 adapting to technical progress for the seventeenth time Council Directive 67/548/EEC on approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (Test methods B.13 - B.14)" and with the Japanese Guidelines - Testing Methods for New Chemical Susbtances enacted July 13, 1974, amended April 6, 1987.

TEST PRINCIPLE

Bacterial tests are widely used in mutagenicity studies for their simplicity, speed and sensitivity.
The Ames test is used to identify point mutations (reversions) in mutant strains of Salmonella typhimurium (1-2) and Escherichia coli (3).

In the present experiment four different strains of histidine-requiring Salmonella typhimurium and a strain of tryptophan-requiring Escherichia coli, are used to identify histidine (His^+) or tryptophan-independent (Trp^+) mutants, respectively.

REDACTED AS TO TRADE NAMES

The bacterial cells, in early stationary growth phase, are exposed to different concentrations of the test agent, both in the presence and in the absence of a liver microsomal enzyme preparation (in order to reveal possible indirect activity of the compound). Mutagenic activity is determined by the capacity of the test substance to induce a significant increase in the number of reverted colonies in comparison to the spontaneous reversions occurring in the control cultures.

Concerning Salmonella bacterial cells, reversions can be induced by chemical agents causing base substitutions or frameshift mutations. Strains TA 1535 and TA 100 are specific testers for mutagens causing base substitutions; the sensitivity of TA 100 is greatly enhanced by the introduction of an R factor, pkM101 plasmid, which confers ampicillin resistance. The frameshift tester strains used are TA 1537 and TA 98; TA 98, like TA 100, is ampicillin resistant.

All S. typhimurium strains carry, along with the defect in the gene of histidine (His^-), a deep rough (rfa) character and an uvrB deletion ($uvrB^-$). This latter, extending to bio gene is responsible also for biotin requirement.

The Escherichia coli strain used carries a defect in one of the genes for tryptophan biosynthesis and the reversions (prototrophy colonies) can arise by a base change. An additional genetic feature of the bacteria used as test system is a defect in the repair system ($uvrA^-$).

RBM Exp. No. 970591

SUMMARY

The mutagenic potential of [redacted] was investigated using Salmonella typhimurium TA 1535, TA 1537, TA 98 and TA 100 and Escherichia coli WP2 uvrA⁻ as tester strains.

The study was performed with and without metabolic activation (S9 Mix).
S9 fraction (9000 g supernatant) was prepared from adult male Sprague Dawley rats pretreated by intraperitoneal route at 80 mg/kg (5 ml/kg) of a mixture of Phenobarbital Na and β-Naphthoflavone.
S9 Mix consisted of S9 plus cofactors.

Two independent experiments were performed, setting up triplicate plates for each experimental point.

The preliminary toxicity test was performed as part of the first study, using the plate incorporation assay (Exp. No. 970591/1). In this trial seven test article concentrations, spaced approximately at half-log intervals, ranging from 5 to 5000 µg/plate, were tested, both with and without metabolic activation.

Hydrazine sulfate, 9-Aminoacridine HCl monohydrate, Doxorubicine HCl, Methylmethanesulfonate, 2-Aminofluorene and 2-Aminoanthracene served as positive controls to test the mutagenicity of the S. typhimurium and E. coli bacterial strains as well as the activity of the metabolizing system.

The negative control was the test article solvent, i.e. ethanol.

In the first experiment at the highest dose tested, 5000 µg/plate, the test article proved to be cytotoxic on the test system, either with and without metabolic activation. This was demonstrated by zero to low colony growth with severe thinning of the background lawn.
There was also less marked toxicity in all strains tested at 1500 µg/plate, causing a decrease in the number of revertant colonies and of the background lawn.
At the other test article dosage levels tested no significant cytotoxic effects were observed. either with or without metabolic activation.

On the basis of the results obtained in the first experiment, 1500 µg/plate was chosen as the highest dosage level to be tested in the second run.
Moreover four additional doses were tested. (see Tables 3 and 4).
The second trial (Exp. No. 970591/2) was performed using the plate incorporation assay without metabolic activation and the pre-incubation method with metabolic activation.

In the second trial the results obtained at 1500 µg/plate confirmed the slight toxicity observed in the first experiment, either in the test with or without metabolic activation.
Moreover, at the other test article doses tested, no significant cytotoxic effects were observed, either with or without S9 Mix.

In both trials, in the concentration ranges investigated, the test article did not show any mutagenic activity with or without the addition of S9 liver homogenate fractions.

The known reversion properties were determined for the tester strains with the control substances; the positive responses confirmed the good metabolic activity of the liver homogenate fractions.

MATERIALS AND METHODS

Two independent experiments were performed.

Experiment No. 970591/1
Started on March 5, 1998 (experimental part)
Ended on March 9, 1998 (experimental part)

Experiment No. 970591/2
Started on March 23, 1998 (experimental part)
Ended on March 27, 1998 (experimental part)

Materials and methods were the same in both experiments, unless otherwise specified.

Test system

Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, directly supplied by B. Ames (University of California, Berkeley).

Escherichia coli strain WP2 uvrA⁻, supplied by the National Collection of Industrial Bacteria (NCIMB), Scotland (UK).

Test and control article characterizations

Test article

Identification:	[redacted]
Batch No.:	19387/17-8
Appearance:	colourless liquid
Manufacturing date:	November 1997
Expiry date:	December 2000
Storage conditions:	room temperature

The Sponsor reserves the right to divulge relevant data on test article characterization directly to Regulatory Agency(ies), when appropriate.

RBM Exp. No. 970591

Control articles

Negative control (vehicle):	Ethanol	(Merck, batch No. K24324483739, espiry date August 31, 2002)

Positive controls in the test without metabolic activation (direct test):

S. typhimurium TA 1535:	Hydrazine sulfate (Hyd) (Merck, batch No. A522503)
S. typhimurium TA 1537:	9-Aminoacridine HCl monohydrate (9-AA) (Merck, batch No. 33988926)
S. typhimurium TA 98 and TA 100:	Doxorubicine HCl (Doxo) (Sigma, batch No.150H0093)
E. coli WP2 uvrA⁻:	Methylmethanesulfonate (MMS) (Merck, batch No. 2185941)

Positive controls in the test with metabolic activation (indirect test):

S. typhimurium TA 1535 and TA 1537:	2-Aminoanthracene (2-AA) (Sigma, batch No. 13H3454)
S. typhimurium TA 98 and TA 100:	2-Aminofluorene (2-AF) (Merck, batch No. 41815087)
E. coli WP2 uvrA⁻:	2-Aminoanthracene (2-AA) (Sigma, batch No. 13H3454)

Rationale of control article choice

For the negative control article, it corresponds to the suitable solvent for the test article.

The rationale of the choice of mutagens is:

- to check each strain for sensitivity to an appropriate mutagen (direct test);
- to check metabolic activation system (S9 Mix) for its capability to activate an indirect mutagen.

RBM Exp. No. 970591

Test article dosage levels

The dosage levels, expressed as quantities of the test article per plate, were the following:

Exp. No. 970591/1: 5, 15, 50, 150, 500, 1500 and 5000 µg/plate.
Exp. No. 970591/2: 15, 50, 150, 500 and 1500 µg/plate.

Preparation of the test article solutions

In each experiment, a weighed amount of the test article [redacted] was dissolved and serially diluted in ethanol to obtain the following concentrations:

Exp. No. 970591/1: 50, 15, 5, 1.5, 0.5, 0.15 and 0.05 mg/ml
Exp. No. 970591/2: 15, 5, 1.5, 0.5 and 0.15 mg/ml

All test article solutions were prepared just before use.

Negative and positive control dosage levels

Negative control (vehicle): Ethanol, 100 µl/plate

Positive controls in the test without metabolic activation (direct test):

S. typhimurium TA 1535:	Hydrazine sulphate (Hyd)	500	µg/plate
S. typhimurium TA 1537:	9-Aminoacridine HCl (9-AA)	40	µg/plate
S. typhimurium TA 98 and TA 100:	Doxorubicine HCl (Doxo)	4	µg/plate
E. coli WP2 uvrA⁻:	Methylmethanesulphonate(MMS)	325	µg/plate

Positive controls in the test with metabolic activation (indirect test):

S. typhimurium and TA 1535 and TA 1537:	2-Aminoanthracene (2-AA)	6.25	µg/plate
S. typhimurium TA 98 and TA 100:	2-Aminofluorene (2-AF)	5	µg/plate
E. coli WP2 uvrA⁻:	2-Aminoanthracene (2-AA)	1	µg/plate

Preparation of the positive control solutions

The following solutions will be used:

Test without metabolic activation:

S. typhimurium TA 1535:	Hydrazine sulphate (Hyd)	5	mg/ml in water
S. typhimurium TA 1537:	9-Aminoacridine HCl monohydrate (9-AA)	0.4	mg/ml in DMSO
S. typhimurium TA 98 and TA 100:	Doxorubicine HCl (Doxo)	0.04	mg/ml in DMSO
E. coli WP2 uvrA⁻:	Methylmethanesulphonate MMS)	3.25	mg/ml in DMSO

Test with metabolic activation

S. thyphimurium TA 1535 and TA 1537:	2-Aminoanthracene (2-AA)	0.0625	mg/ml in DMSO
S. typhimuriumTA 98 and TA100:	2-Aminofluorene (2-AF)	0.05	mg/ml in DMSO
E. coli WP2 uvrA⁻:	2-Aminoanthracene (2-AA)	0.01	mg/ml in DMSO

Experimental design

Tester strain storage and control

The S. typhimurium and E. coli strains are stored in growth medium plus 25 µg/ml ampicillin (if ampicillin resistance bacteria) and 8% dimethyl sulphoxide, at -80°C (permanent cultures).
Fresh bacterial cultures are subcultivated on complete medium (Master plate), and stored in a refrigerator for up to 1 month.

Both permanent cultures and Master plates are checked to confirm the tester strain genotype.

The liquid culture used to prepare Master plate or the first overnight culture from fresh Master plate is submitted to the following controls:

a) Check for histidine-requirement (Salmonella typhimurium strains)

Culture (0.1 ml) is added to 2 ml of soft agar or soft agar +His and plated on Minimal Medium plates (MM). After 48 h incubation at 37°C, bacterial growth has to be observed on MM+His plate, but not on MM-His plate.

b) Check for tryptophan-requirement (Escherichia coli strain)

Culture (0.1 ml) is added to 2 ml of soft agar or soft agar +Trp and plated on Minimal Medium plates (MM). After 48 h incubation at 37°C, bacterial growth has to be observed on MM+Trp plate, but not on MM-Trp plate.

c) Check for the rfa mutation (Salmonella typhimurium strains) (Crystal violet sensitivity)

Culture (0.1 ml) is added to 2 ml of soft agar and plated on Complete Medium plates. When the medium is solidified, 10 µl of crystal violet solution (1 mg/ml) are deposited in the centre of agar surface. After 24 h incubation at 37°C, a neat inhibition zone has to be observed for all strains.

d) Check for the UV sensitivity (Salmonella typhimurium and Escherichia coli strains)

Culture (0.1 ml) is added to 2 ml of soft agar and plated on Complete Medium plates. Half plate is exposed to UV rays (15W germicidal lamp, at 33 cm distance) for 6 seconds (TA 1535, TA 1537 S. typhimurium strains and WP2 uvrA⁻ E. coli strain) or 8 seconds (TA 98 and TA 100).
After 24 h incubation at 37°C all strains have to be grown on the un-irradiated side of the plate only.

e) Check for the R factor (Salmonella typhimurium strains)

Culture (0.1 ml) is added to 2 ml of soft agar and plated on Complete Medium plates. When the medium is solidified, 10 µl of ampicillin solution (8 mg in NaOH 0.02N) are deposited in the centre of agar surface. After 24 h incubation at 37°C, no inhibition zone has to be observed for ampicillin resistant strains TA 98 and TA 100 S. typhimurium strains. Inhibition zone must be recorded for strains TA 1535 and TA 1537 S. typhimurium strains.

Culture media

a) Liquid growth medium

Eight g of nutrient broth and 5 g of NaCl were dissolved in one liter of deionized water and sterilized at 1.0 atm., 121 °C, for 15 min.

b) Complete medium

Nutrient medium was prepared by dissolving 8 g of nutrient broth, 5 g of NaCl and 15 g of agar in one liter of deionized water.
The medium was sterilized at 1.0 atm. (121°C) for 15 min and when it had cooled to about 45°C was poured into sterile plastic Petri plates (9-10 cm dia.).
About 25 ml of the medium were poured into each sterile plastic Petri plate.

c) Minimum medium (MM)

The selective medium consisted of 2.5 g (E. coli strain) or 20 g (S. typhimurium strains) of glucose and 15 g of agar, in one litre of deionized water. It was sterilized at 1.0 atm. (121°C) for 15 min.
Thereafter, the medium was allowed to cool to about 45° C and to every liter of medium, 20 ml of the following sterile solution (Vogel-Bonner 50x) pre-warmed at 60-70°C were added:

10 g/l $MgSO_4 .7H_2O$
100 g/l citric acid.H_2O
500 g/l $K2HPO_4$ anhydrous
175 g/l $NaNH_4HPO4.4H_2O$

About 25 ml of the medium were poured into each sterile plastic Petri plate (9-10 cm dia.)

d) Soft agar

Six g of agar and 5 g of NaCl were dissolved in 1 litre of deionized water and sterilized at 1.0 atm. (121°C) for 15 min.

e) Soft agar + His (Salmonella typhimurium strains)

In the preparation of the soft agar 7.7 mg/l of histidine and 12.2 mg/l biotin were added to the agar and NaCl.

f) Soft agar + Trp (Escherichia coli strain)

When the temperature of the soft agar is about 65° C, to every 100 ml of soft agar, 0.5 ml of a filter-sterilized tryptophan solution (2 mg/ml, freshly prepared in water) were added (final tryptophan concentration: 10 µg/ml).

Preparation of the bacterial culture

For the experiment, bacterial cell suspensions were prepared by inoculating two colonies of the Master culture in 5 ml liquid growth medium. The liquid culture was developed for 16.5 hours at 37°C in a horizontally shaking thermostatic bath (overnight culture).

Preparation of S9 Mix

Each day, for 3 consecutive days, adult male Sprague Dawley rats (Charles River CD) supplied by Charles River of Calco weighing 200-250 g received a single intraperitonal dose of 80 mg/kg (5 ml/kg) of a mixture of Phenobarbital Na and β-Naphthoflavone (16 mg/ml solution in commercial corn oil). The day after the last administration the rats were sacrificed and the liver of each removed.

The livers were homogenized for 30 seconds at 4°C with a 0.15M KCl solution (solution to tissue ratio 3:1).

The homogenate was centrifuged for 20 min at 9,000xg in a refrigerated supercentrifuge.

The supernatant was divided into aliquots, deep frozen and stored at -80 deg. C.

The supernatant was assayed for protein concentration by the Biuret (4) method, then it was assayed for its activation capacity versus an indirect mutagen (2- aminofluorene) with Salmonella typhimurium strains TA 98 and TA 100.

The S9 Mix with the following composition/plate was prepared immediately before use in an ice cold bath:

0.05	ml	liver microsomal suspension (S9)
0.25	ml	0.2M phosphate buffer pH 7.4
0.01	ml	0.4M $MgCl_2$ and 1.65M KCl
0.85	mg	G6P
1.53	mg	NADP
0.19	ml	water for injection

Plate test without and with metabolic activation

0.1 ml of the positive and negative control solutions or of the different test article solutions, according to the dosage levels, were introduced into sterile test tubes containing 2 ml of soft agar +His or +Trp, kept liquid in a thermostatic bath at 45°C.

0.1 ml of an overnight culture of Salmonella typhimurium strains TA 1535, TA 1537, TA 98 and TA 100 or of Escherichia coli were rapidly added. 0.5 ml of the S9 Mix (test with metabolic activation) or 0.5 ml of phosphate buffer (test without metabolic activation) were also added. The test tubes were rapidly shaken and the contents poured onto plates containing solid minimum (selective) growth medium.

Three plates per dose were prepared for each bacterial strain, both in the test without and with metabolic activation.

All the plates were incubated at 37°C for 72 hours.

Plates, adequately identified, were examined following incubation for background lawn and revertant colonies visually counted.

Repetition of the assay

The experiment was repeated in an independent assay. Since negative results were obtained in the first trial, the repeat test followed the pre-incubation method with metabolic activation and the plate test method without metabolic activation.

For the test without metabolic activation the same experimental conditions of the first experiment were used, whereas for the test with metabolic activation the method is specified below.

Pre-incubation test with metabolic activation

0.5 ml of S9 Mix were dispensed in sterile test tubes placed in an ice bath.

0.1 ml of bacterial suspension and 0.1 ml of the test or control article solutions according to the dosage levels, were introduced.

The test tubes were gently vortexed and incubated at 37°C for 20 min, in a horizontally shaking thermostatic bath.

At the end of the incubation 2 ml of soft agar (kept liquid in a thermostatic bath at about 45°C) were added to each tube.

The test tubes were rapidly shaken and the contents poured onto plates containing solid selective growth medium.

Three plates per dose were prepared.

The plates were incubated at 37°C for 72 hours, after which the reverted colonies were counted.

Evaluation of the study

For the test to be considered valid, the following criteria must be met:

a) The E. coli strain used in the test must prove to be tryptophan-requiring.

b) The S. typhimurium strains used in the test must prove to be histidine-requiring.

c) The sterility check S9 Mix must prove negative for bacterial growth.

d) The growth of all S. typhimurium strains must be inhibited by crystal violet; the growth of TA 1535 and TA 1537 must be inhibited by ampicillin, while the growth of strains TA 98 and TA 100 must not.
The growth of all strains of S. typhimurium and of WP2 uvrA⁻ E. coli strain must be inhibited by exposure to UV rays.

e) The frequency of spontaneous reversions for each strain must fall within the range reported in the literature and the historical range of our laboratory.

f) The activity of the microsomal preparation must be confirmed by its capability to activate the positive control which requires a metabolic transformation in order to explicate its mutagenic effect.

g) The number of reverted colonies owing to the mutagenic activity of the positive controls must be statistically greater than, and at least double the number of spontaneously reverted colonies (Student's "t" test).

The test article is considered to have elicited a positive response when:

- the number of reverted colonies is significantly higher when compared with the number of revertants in the solvent controls (as determined by Student's t);

and

- ***either***: a dose-response can be verified, that is, a positive correlation between the number of revertants and the dose in an interval of at least 3 doses (linear regression test);

- ***or***: a statistically significant increase is recorded at one dose only, when confirmed in independent assays.

Data evaluation (5)

The mean and standard deviation were calculated for reversions read in each dosage group.

Comparison of the spontaneous reversions (in the negative control-vehicle) with the ones in the test article plates and in the positive control plates were done by Student's "t" test.

Significances were expressed as follows:

*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$

RECORD FILING

The protocol, a reserve sample of the batch of the test article used, the certificates of analysis, the raw data bound in registers numbered 970591/1 and 970591/2, the final report, including records and report of maintenance, cleaning, calibration and inspection of equipment are filed at RBM premises.

They will be stored in the RBM archives for ten years from the study report date and then sent to the Sponsor.

At the end of the ten year archiving period, the Sponsor can request the extension of the storage of all materials or part of them for a further period. An appropriate agreement will be drawn up accordingly.

PROCEDURAL DETAILS

The study was conducted in accordance with the procedures described in the RBM Standard Operating Procedures (SOPs) collection.

Protection of animals used in the experiment is in accordance with Directive 86/609/EEC, enforced by the Italian D.L. No. 116 of January 27, 1992.

Physical facilities and equipment for accomodation and care of animals are in accordance with the provisions of EEC Council Directive 86/609.

The Institute is fully authorized by Competent Veterinary Health Authorities.

RESULTS

Both the tests carried out on Salmonella typhimurium (TA 1535, TA 1537, TA 98 and TA 100 strains) and on Escherichia coli WP2 uvrA⁻ strains to investigate any possible mutagenic activity of the test article [redacted] were experimentally valid, as all the conditions to be complied with (bacteria checks, S9 Mix activity, responsiveness to the positive controls) were met.

Table 1 presents the results of the first plate test (Exp. 970591/1) without metabolic activation of the compound [redacted] on Salmonella typhimurium and Escherichia coli strains.

Table 2 presents the results of the first plate test (Exp. 970591/1) with metabolic activation of the compound [redacted] on Salmonella typhimurium and Escherichia coli strains.

Mean and standard deviations are reported.

The results of the negative and positive controls are also given.

In the first experiment at the highest dose tested, 5000 µg/plate, the test article proved to be cytotoxic on the test system, either with and without metabolic activation. This was demonstrated by zero to low colony growth with severe thinning of the background lawn.

There were also less marked citoxicity at 1500 µg/plate, causing a decrease in the number of revertant colonies and of the background lawn.

At the other test article dosage levels tested no significant cytotoxic effects were observed, either with or without metabolic activation.

On the basis of the results obtained in the first experiment, 1500 µg/plate was chosen as the highest dosage level to be tested in the second run.

Moreover four additional doses were tested (see Tables 3 and 4).

In the second trial the results obtained at 1500 µg/plate confirmed the slight toxicity observed in the first experiment, either in the test with or without metabolic activation.

Moreover, at the other test article doses tested, no significant cytotoxic effects were observed, either with or without S9 Mix.

As regards mutagenicity, no appreciable increase in the number of revertants in comparison with the negative control was evident in either experiment at any of the doses of [redacted] on S.typhimurium and E. coli strains, either in the presence or in the absence of metabolic activation.

As expected, the reference mutagens induced a number of mutant clones statistically greater than and at least double the mean number of spontaneous (Student's "t" test).

TABLES

LABORATORIES & CLINICS GROUP
BIOSCIENCE

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/1

TABLE 1. - Test without metabolic activation (p. 1)

Salmonella typhimurium: TA 1535

Compound	Concentr. µg/plate	Reversions/plate			M e a n	S. D.	(P)
Vehicle control (Ethanol)		18	26	21	21.67	4.041	
Test Article	5	20	17	22	19.67	2.517	.507
Test Article	15	21	22	22	21.67	.577	1.000
Test Article	50	26	18	24	22.67	4.163	.780
Test Article	150	22	18	24	21.33	3.055	.915
Test Article	500	17	20	16	17.67	2.082	.202
Test Article	1500	11	11	15	12.33	2.309	.026 *
Test Article	5000	tox	tox	tox			
Positive control (Hydrazine sulphate (Hyd))		74	88	83	81.67	7.095	<0.001 ***

Salmonella typhimurium: TA 1537

Compound	Concentr. µg/plate	Reversions/plate			M e a n	S. D.	(P)
Vehicle control (Ethanol)		7	8	9	8.00	1.000	
Test Article	5	6	8	11	8.33	2.517	.842
Test Article	15	7	9	5	7.00	2.000	.482
Test Article	50	8	6	9	7.67	1.528	.768
Test Article	150	8	10	8	8.67	1.155	.492
Test Article	500	7	7	7	7.00	.000	.158
Test Article	1500	6	3	2	3.67	2.082	.031 *
Test Article	5000	tox	tox	tox			
Positive control (9-Aminoacridine HCl monohydrate (9-AA))		54	60	47	53.67	6.506	<0.001 ***

tox - Toxic

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/1

TABLE 1. - Test without metabolic activation (p. 2)

Salmonella typhimurium: TA 98

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)	
Vehicle control (Ethanol)		44	38	33	38.33	5.508		
Test Article	5	40	39	41	40.00	1.000	.633	
Test Article	15	35	36	42	37.67	3.786	.871	
Test Article	50	44	46	42	44.00	2.000	.169	
Test Article	150	42	46	36	41.33	5.033	.525	
Test Article	500	38	43	39	40.00	2.646	.661	
Test Article	1500	19	21	31	23.67	6.429	.040	*
Test Article	5000	tox	tox	tox				
Positive control (Doxorubicine HCl (Doxo))		926	840	918	894.67	47.511	<0.001	***

Salmonella typhimurium: TA 100

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)	
Vehicle control (Ethanol)		184	179	190	184.33	5.508		
Test Article	5	180	182	193	185.00	7.000	.903	
Test Article	15	184	178	166	176.00	9.165	.248	
Test Article	50	170	197	177	181.33	14.012	.747	
Test Article	150	190	204	186	193.33	9.452	.227	
Test Article	500	178	186	188	184.00	5.292	.943	
Test Article	1500	136	147	150	144.33	7.371	.002	**
Test Article	5000	tox	tox	tox				
Positive control (Doxorubicine HCl (Doxo))		544	470	452	488.67	48.758	<0.001	***

tox - Toxic

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/1

TABLE 1. - Test without metabolic activation (p. 3)

Escherichia Coli: WP2 uvrA-

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		36	46	42	41.33	5.033	
Test Article	5	45	36	35	38.67	5.508	.569
Test Article	15	38	42	31	37.00	5.568	.374
Test Article	50	40	34	41	38.33	3.786	.456
Test Article	150	36	36	42	38.00	3.464	.398
Test Article	500	37	39	37	37.67	1.155	.286
Test Article	1500	26	20	13	19.67	6.506	.010 *
Test Article	5000	tox	tox	tox			
Positive control (Methylmethanesulphonate (MMS))		186	203	193	194.00	8.544	<0.001 ***

tox - Toxic

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/1

TABLE 2. - Test with metabolic activation (p. 1)

Salmonella typhimurium: TA 1535

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)	
Vehicle control (Ethanol)		26	20	26	24.00	3.464		
Test Article	5	23	25	21	23.00	2.000	.687	
Test Article	15	28	20	22	23.33	4.163	.842	
Test Article	50	22	20	25	22.33	2.517	.537	
Test Article	150	28	26	22	25.33	3.055	.643	
Test Article	500	23	24	18	21.67	3.215	.441	
Test Article	1500	20	19	17	18.67	1.528	.071	
Test Article	5000	10	9	11	10.00	1.000	.003	**
Positive control (2-Aminoanthracene (2-AA))		188	170	162	173.33	13.317	<0.001	***

Salmonella typhimurium: TA 1537

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)	
Vehicle control (Ethanol)		9	10	13	10.67	2.082		
Test Article	5	11	10	9	10.00	1.000	.643	
Test Article	15	8	10	8	8.67	1.155	.219	
Test Article	50	13	6	11	10.00	3.606	.795	
Test Article	150	11	8	10	9.67	1.528	.539	
Test Article	500	11	13	7	10.33	3.055	.883	
Test Article	1500	10	7	6	7.67	2.082	.152	
Test Article	5000	9	11	5	8.33	3.055	.336	
Positive control (2-Aminoanthracene (2-AA))		135	118	140	131.00	11.533	<0.001	***

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RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/1

TABLE 2. - Test with metabolic activation (p. 2)

Salmonella typhimurium: TA 98

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		45	41	48	44.67	3.512	
Test Article	5	38	44	38	40.00	3.464	.177
Test Article	15	42	45	47	44.67	2.517	1.000
Test Article	50	50	38	43	43.67	6.028	.816
Test Article	150	44	36	32	37.33	6.110	.146
Test Article	500	32	31	36	33.00	2.646	.010 *
Test Article	1500	31	42	36	36.33	5.508	.092
Test Article	5000	18	9	11	12.67	4.726	<0.001 ***
Positive control (2-Aminofluorene (2-AF))		760	828	864	817.33	52.814	<0.001 ***

Salmonella typhimurium: TA 100

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		170	190	186	182.00	10.583	
Test Article	5	182	177	182	180.33	2.887	.805
Test Article	15	198	176	160	178.00	19.079	.767
Test Article	50	185	190	168	181.00	11.533	.917
Test Article	150	190	184	170	181.33	10.263	.941
Test Article	500	187	173	168	176.00	9.849	.512
Test Article	1500	144	180	176	166.67	19.732	.301
Test Article	5000	118	109	106	111.00	6.245	<0.001 ***
Positive control (2-Aminofluorene (2-AF))		780	740	686	735.33	47.173	<0.001 ***

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RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/1

TABLE 2. - Test with metabolic activation (p. 3)

Escherichia Coli: WP2 uvrA-

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		42	34	39	38.33	4.041	
Test Article	5	44	40	46	43.33	3.055	.163
Test Article	15	50	44	35	43.00	7.550	.399
Test Article	50	30	45	37	37.33	7.506	.849
Test Article	150	44	44	45	44.33	.577	.064
Test Article	500	39	48	37	41.33	5.859	.506
Test Article	1500	38	42	48	42.67	5.033	.310
Test Article	5000	18	30	19	22.33	6.658	.024 *
Positive control (2-Aminoanthracene (2-AA))		274	302	324	300.00	25.060	<0.001 ***

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/2

TABLE 3. - Test without metabolic activation (p. 1)

Salmonella typhimurium: TA 1535

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		26	21	22	23.00	2.646	
Test Article	15	24	26	29	26.33	2.517	.189
Test Article	50	21	27	18	22.00	4.583	.760
Test Article	150	26	21	28	25.00	3.606	.482
Test Article	500	18	28	24	23.33	5.033	.924
Test Article	1500	22	16	21	19.67	3.215	.238
Positive control (Hydrazine sulphate (Hyd))		90	92	96	92.67	3.055	<0.001 ***

Salmonella typhimurium: TA 1537

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		11	7	9	9.00	2.000	
Test Article	15	14	9	11	11.33	2.517	.277
Test Article	50	8	10	6	8.00	2.000	.573
Test Article	150	7	9	7	7.67	1.155	.374
Test Article	500	10	10	6	8.67	2.309	.859
Test Article	1500	6	5	9	6.67	2.082	.234
Positive control (9-Aminoacridine HCl monohydrate (9-AA))		42	61	48	50.33	9.713	.002 **

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/2

TABLE 3. - Test without metabolic activation (p. 2)

Salmonella typhimurium: TA 98

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)	
Vehicle control (Ethanol)		42	45	37	41.33	4.041		
Test Article	15	46	36	36	39.33	5.774	.649	
Test Article	50	38	40	48	42.00	5.292	.871	
Test Article	150	39	33	34	35.33	3.215	.114	
Test Article	500	32	45	32	36.33	7.506	.367	
Test Article	1500	25	36	29	30.00	5.568	.046	*
Positive control (Doxorubicine HCl (Doxo))		1008	940	998	982.00	36.715	<0.001	***

Salmonella typhimurium: TA 100

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)	
Vehicle control (Ethanol)		169	199	183	183.67	15.011		
Test Article	15	192	183	170	181.67	11.060	.862	
Test Article	50	177	207	168	184.00	20.421	.983	
Test Article	150	173	165	173	170.33	4.619	.215	
Test Article	500	168	186	163	172.33	12.097	.366	
Test Article	1500	148	157	152	152.33	4.509	.026	*
Positive control (Doxorubicine HCl (Doxo))		590	520	542	550.67	35.796	<0.001	***

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RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/2

TABLE 3. - Test without metabolic activation (p. 3)

Escherichia Coli: WP2 uvrA-

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		34	41	51	42.00	8.544	
Test Article	15	37	55	45	45.67	9.018	.636
Test Article	50	53	49	45	49.00	4.000	.268
Test Article	150	37	41	36	38.00	2.646	.482
Test Article	500	42	47	40	43.00	3.606	.861
Test Article	1500	38	31	37	35.33	3.786	.284
Positive control (Methylmethanesulphonate (MMS))		212	198	236	215.33	19.218	<0.001 ***

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/2

TABLE 4. - Test with metabolic activation (p. 1)

Salmonella typhimurium: TA 1535

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		25	20	19	21.33	3.215	
Test Article	15	21	24	26	23.67	2.517	.378
Test Article	50	21	17	20	19.33	2.082	.417
Test Article	150	21	22	20	21.00	1.000	.872
Test Article	500	18	21	25	21.33	3.512	1.000
Test Article	1500	14	18	16	16.00	2.000	.071
Positive control (2-Aminoanthracene (2-AA))		192	178	190	186.67	7.572	<0.001 ***

Salmonella typhimurium: TA 1537

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		10	12	10	10.67	1.155	
Test Article	15	9	11	7	9.00	2.000	.279
Test Article	50	9	11	10	10.00	1.000	.492
Test Article	150	7	10	9	8.67	1.528	.145
Test Article	500	9	5	7	7.00	2.000	.051
Test Article	1500	7	4	6	5.67	1.528	.011 *
Positive control (2-Aminoanthracene (2-AA))		139	146	114	133.00	16.823	<0.001 ***

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/2

TABLE 4. - Test with metabolic activation (p. 2)

Salmonella typhimurium: TA 98

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		42	39	45	42.00	3.000	
Test Article	15	34	40	45	39.67	5.508	.554
Test Article	50	48	41	44	44.33	3.512	.431
Test Article	150	47	40	42	43.00	3.606	.731
Test Article	500	38	45	42	41.67	3.512	.907
Test Article	1500	36	32	39	35.67	3.512	.076
Positive control (2-Aminofluorene (2-AF))		880	962	834	892.00	64.838	<0.001 ***

Salmonella typhimurium: TA 100

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		194	178	183	185.00	8.185	
Test Article	15	168	181	191	180.00	11.533	.573
Test Article	50	177	179	156	170.67	12.741	.176
Test Article	150	165	186	189	180.00	13.077	.605
Test Article	500	170	183	193	182.00	11.533	.732
Test Article	1500	153	161	157	157.00	4.000	.006 **
Positive control (2-Aminofluorene (2-AF))		694	688	714	698.67	13.614	<0.001 ***

RBM Exp. No. 970591

Test article: [redacted]
Title : Ames test
RBM exp. : 970591/2

TABLE 4. - Test with metabolic activation (p. 3)

Escherichia Coli: WP2 uvrA-

Compound	Concentr. µg/plate	Reversions/plate			Mean	S. D.	(P)
Vehicle control (Ethanol)		47	45	39	43.67	4.163	
Test Article	15	42	52	38	44.00	7.211	.948
Test Article	50	41	48	41	43.33	4.041	.926
Test Article	150	42	44	49	45.00	3.606	.697
Test Article	500	38	41	46	41.67	4.041	.583
Test Article	1500	36	40	35	37.00	2.646	.079
Positive control (2-Aminoanthracene (2-AA))		286	314	302	300.67	14.048	<0.001 ***

CONCLUSIONS

The test article [redacted] did not induce any significant increase in the number of mutant clones, either in the absence or in the presence of metabolic activation, up to the concentration of 1500 µg/plate in Salmonella typhimurium TA 1535, TA 1537, TA 98 and TA 100 strains or on Escherichia coli WP2 uvrA⁻, in two independent experiments.

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RBM Study Director

April 6, 1998

Dr. Domenico Barone

Scientific Director
Recognized by the Italian Health Authorities as Responsible for Experimentation of Mutagenesis

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