

Section 4)

**Attachment no. 6**

LSR-RTC S.P.A.:

FDP: Unscheduled DNA synthesis in HeLa S3 cells in vitro.

BF file,

1988



LIFE SCIENCE RESEARCH  
ROMA TOXICOLOGY CENTRE S.P.A.

(6)

UNSCHEDULED DNA SYNTHESIS (UDS)  
in HeLa S3 cells in vitro

Test substance: Fruttosio-1,6-difosfato

LSR-RTC Report No.: 003005-M-04788

FINAL REPORT

Seen and approved by:

A. Nunziata Pharm.D, Chem.D.  
Responsible to Ministry of  
Health for Experimentation.

R.K. Haroz Ph.D.  
Managing Director

LSR-RTC Report No.: 003005-M-04788

Q.A. STATEMENT

Quality Assurance Inspections  
(Day Month Year)

	Inspection	Report to Study Director	Report to Head of Responsible Department	Report to Company Management
<u>PROTOCOL</u>				
Inspection of the study protocol was made in accordance with LSR-RTC Standard Operating Procedure QAU/010	20.06.88	20.06.88	20.06.88	26.07.88
<u>DATA</u>				
Inspection of data generated on this type of study was made in accordance with LSR-RTC Standard Operating Procedure QAU/030, QAU/031 and QAU/032.	29.07.88	-	29.07.88	-
	21.09.88	-	21.09.88	-
<u>PROCEDURES</u>				
Inspection of Procedures on this study was made in accordance with LSR-RTC Standard Operating Procedure QAU/020.	20.07.88	21.07.88	21.07.88	-
	28.07.88	29.07.88	29.07.88	-
Other routine procedures performed in this type of study, and facilities were inspected regularly and reports were made in accordance with LSR-RTC Standard Operating Procedure QAU/020.	31.05.88	-	06.06.88	26.07.88
	06.07.88	-	07.07.88	-
	08.07.88	-	08.07.88	-
	26.08.88	-	30.08.88	-
	06.09.88	-	07.09.88	-
	06.09.88	-	07.09.88	-
	09.09.88	-	12.09.88	-
	20.09.88	-	21.09.88	-

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LSR-RTC Report No.: 097033-M-00488

This report has been reviewed by the LSR-RTC Quality Assurance Unit employing methods laid down in LSR-RTC Standard Operating Procedure QAU/040. The reported methods and procedures were found to describe those used and the results to constitute an accurate representation of the data recorded.

This review was completed on: 29 September, 1988

V. Sforza, B.Sc.  
(Quality Assurance Manager)

Valentia 30/9/88

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(Dr. ~~ALFREDO NUNZIATA~~)

LSR-RTC Report No.: 003005-M-04788

**UNSCHEDULED DNA SYNTHESIS (UDS)**  
in HeLa S3 cells in vitro

TEST SUBSTANCE: Fruttosio-1,6-difosfato

FINAL REPORT

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 this study. The aspects of the study conducted by Life Science Research Roma Toxicology Centre were performed essentially in accordance with:

- A. "Good Laboratory Practice" regulations of the U.S. Food and Drug Administration, 21 CFR Part 58, 22-Dec-1978, and sections revised in Fed. Reg. 4-Sep-1987.
- B. "Principles of Good Laboratory Practice relating to the conduct of nonclinical laboratory studies" OECD Guidelines for the testing of chemicals, Annex 2, (81) 30 (Final) 1981.
- C. "Applicazione dei principi di buone pratiche di laboratorio sulle sostanze chimiche e criteri per il rilascio delle autorizzazioni previste dal decreto del Presidente della Repubblica n. 927/81, art.6." Rome, Italy, D.M. No. 76 Gazzetta Ufficiale del 27 Agosto 1986.

A. Seeberg  
Dr. rer. nat. A.H. Seeberg  
(Study Director)

3 Oct 88  
Date

R. Forstel  
R. Forstel M.A. (Cantab) Ph.D.  
(Head of Genetic Toxicology)

3 Oct 88  
Date

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(Dr. LEONARDO NONZIATA)

LSR  
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### SUMMARY

- 1.1 The test material fruttosio-1,6-difosfato was examined for genotoxic properties by assay for the induction of unscheduled DNA synthesis (UDS) in cultured human cells (HeLa S3) both in the absence and presence of S9 metabolic activation.
- 1.2 A preliminary cytotoxicity test was performed. The test substance was assayed at a maximum dose-level of 10000 ug/ml (the highest concentration indicated for testing in the Study Protocol) and a wide range of lower dose-levels spaced by two-fold dilutions. Treatment with the test substance was not markedly toxic either in the absence or presence of S9 metabolism. Therefore the same concentration (10000 ug/ml) was selected at the maximum dose-level to be used in the first UDS assay.
- 1.3 Two independent assays for the induction of UDS were performed. The first UDS assay was performed using dose-levels of 10000, 3160, 1000, 316 and 100 ug/ml. In the second assay the dose-range was modified slightly and concentrations of 1000, 316, 100, 316 and 10.0 ug/ml (absence of S9) and 3160, 1000, 316, 100 and 31.6 ug/ml (presence of S9) were used.

Cultures of HeLa S3 cells were pre-treated with low-serum arginine-free medium and prior to treatment replicative DNA synthesis was blocked with hydroxyurea. After treatment in the presence of tritiated thymidine, DNA was extracted from the cultures, and radiolabel incorporation was measured by scintillation counting. The values for incorporation were corrected for the amount of DNA recovered, and unscheduled DNA synthesis was expressed as tritiated thymidine incorporation per microgram DNA.
- 1.4 Treatment with the test substance did not induce increases in UDS to values 50% greater than the controls at any dose-level in either experiment. There were indications of toxicity following treatment with the test material. Marked increases in UDS were observed following treatment with the positive control compounds, indicating the correct functioning of the assay system.
- 1.5 It is concluded that fruttosio-1,6-difosfato does not induce unscheduled DNA synthesis in HeLa S3 cells in vitro, either in the absence or presence of S9 metabolic activation, under the reported experimental conditions.

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## INTRODUCTION

### 2.1 Purpose

This report describes experiments performed to assess the genotoxic activity of fruttosio-1,6-difosfato by assay for the ability to induce unscheduled DNA synthesis in cultured human cells (HeLa S3) in vitro.

The study was performed to comply with the principles of Good Laboratory Practice as set forth by the U.S. Food and Drug Administration. In addition the study was designed to comply with the experimental methods indicated in the guidelines of:

- OECD Guideline for the Testing of Chemicals No. 482.
- EEC Council Directive 79/831 Annex V Part B.
- TSCA Test Guidelines issued by the US EPA in 40 CFR part 798 on 27-Sept-1985 (Section 798. 5550 Unscheduled DNA synthesis in mammalian cells in culture).

### 2.2 Study organisation

#### Location of Study:

Genetic Toxicology Department  
Life Science Research - Roma Toxicology Centre  
Via Tito Sperti, 14  
00040 Pomezia (Roma) Italy

#### Principal dates

Study commenced: 12-Jul-1988  
Study completed: 11-Aug-1988

#### Study Director

Dr.rer.nat. A.H. Seeberg, Dipl.Biol.

#### Archiving:

The original data arising from this study, a sample of the test material and a copy of the final report consigned will be stored in the archives of Life Science Research - Roma Toxicology Centre for a minimum period of five years from the date of consignment of the report.

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## MATERIALS AND METHODS

### 3.1 Test substance

Five vials of the test material fruttosio-1,6-difosfato (synonym = Esafosfina batch 393/B/APR/88) each containing 5 grams were received from Biomedica Foscama/IRFI on 9-June-1988. The test material, which was a fine white powder, was contained in clear glass septum-cap vials labelled with the identity, composition, net weight, batch number and instructions for administration. The test material was stored at 4°C in the dark. Solutions of the test material were prepared in EMEM arginine-free immediately before use and filtered to ensure sterility. All dose-levels in this report are expressed to three significant figures only.

### 3.2 Control substances

Since no solvent vehicle was employed in this study, the negative controls consisted of untreated cultures prepared in Minimal Eagle Essential Medium (EMEM arginine-free) obtained from Flow Laboratories, UK. Solutions of 4-nitroquinoline-N-oxide (4-NQO) obtained from Fluka AG, Buchs Switzerland, and benzo(a)pyrene (B(a)P) obtained from Sigma Chemical Co. were prepared in DMSO and served as positive controls in the absence and presence of S9 metabolism respectively.

### 3.3 S9 Tissue Homogenate

The preliminary cytotoxicity test and the first UDS assay were performed using a batch of S9 homogenate (designated 88/11) with the following characteristics:

Protein content : 33.6 ± 2.60 mg/ml

Aminopyrine demethylase activity : 3.28 ± 0.08 uM/g.liver/5 min formaldehyde production.

The second UDS assay was performed using a further batch of S9 homogenate (designated 88/12) with the following characteristics:

Protein content : 34.5 ± 1.04 mg/ml

Aminopyrine demethylase activity : 3.16 ± 0.14 uM/g.liver/5 min formaldehyde production.

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The tissue homogenates were prepared from the livers of young male Sprague Dawley rats which had received prior treatment with phenobarbital and betanaphthoflavone to induce high levels of xenobiotic metabolising enzymes. Five livers were used for the preparation of each homogenates.

The efficacy of the tissue homogenate was checked in an Ames test; both batches produced acceptable responses with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using S. typhimurium tester strain TA 100.

#### 3.4 Methods

The methods used were in compliance with the appended Study Protocol. In the presence of S9 metabolism one preliminary experiment was abandoned since the control values were unacceptable. Although not reported, all data from this experiment will be retained and archived with the other study data.

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## RESULTS

### 4.1 Solubility test

The test substance was directly soluble in arginine-free EMEM medium at a maximum concentration of 200 mg/ml. Therefore the highest concentration indicated for testing in the Study Protocol (10000 ug/ml) could be selected as the maximum dose-level to be used in the cytotoxicity test.

### 4.2 Cytotoxicity test

In the cytotoxicity assay the test substance was assayed at a maximum concentration of 10000 ug/ml and nine lower dose-levels spaced at two-fold intervals. Cytotoxicity was assessed on the basis of cell viability (measured by Trypan blue exclusion), cell detachment (measured as the loss of cells in the treated cultures relative to the negative controls) and signs of gross toxicity.

The results obtained in the cytotoxicity test are shown in Tables 1 and 2 (values obtained at lower dose-levels have been omitted). Only mild toxicity was observed following treatment with the test substance. On the basis of this finding the same concentration (10000 ug/ml) was selected as the maximum dose-level to be used in the first UDS assay.

### 4.3 Unscheduled DNA synthesis

Two independent assays for the induction of UDS were performed. In the first experiment dose-levels of 10000, 3160, 1000, 316, and 100 ug/ml were used in the absence and presence of S9 metabolism. On the basis of the results of the first experiment the dose-levels to be used in the second experiment were modified and the following dose-levels were used: 1000, 316, 100, 31.6 and 10.0 ug/ml (absence of S9) and 3160, 1000, 316, 100 and 31.6 ug/ml (presence of S9). The results of the first experiment are presented in Tables 3 and 4. The calculated values shown in these and all subsequent tables are computer generated, using intermediate values to a greater precision than displayed. Thus verification by hand calculation may give values which differ slightly in the last significant figures.

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Absence of S9 metabolic activation: In the first experiment incorporation of radiolabel decreased in a dose-dependent manner over the entire dose-range. At the maximum dose-level incorporation was only 45% of the controls. This reduction in incorporation indicated a toxic effect of test material. In the second experiment (the dose-range had been lowered by a factor of 10) incorporation was no longer depressed and incorporation values similar to the controls were observed over the entire dose-range.

Presence of S9 metabolic activation: In the first experiment incorporation of radiolabel was depressed at all treatment levels. The reduction followed a dose-dependent pattern and incorporation was reduced to approximately 80% of the controls after treatment at the lowest dose-level and to 55% at the highest treatment level. In the second experiment, in which the dose-range has been lowered, incorporation was similar to the controls after treatment at the two lower dose-levels. At higher dose-levels a dose-dependent reduction in incorporation was observed.

The positive control treatments produced marked increases in UDS in both experiments indicating the correct functioning of the test system. A sterility test was performed with all cultures immediately after the treatment in each experiment. All cultures were found to be sterile.

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## ANALYSIS OF RESULTS

### 5.1 Statistical analysis of results

For the statistical analysis, the tritiated thymidine incorporation values were first square root transformed, to ensure normal distribution and constant variance. An analysis of variance was then performed.

Statistically significant effects were observed as follows:

Experiment (both in the absence and presence of S9):  
Incorporation was generally higher in the second experiment.

Dose-level (both in the absence and presence of S9):  
Incorporation decreased in a dose-dependent manner, indicating a toxic effect of the test material.

The F-values and significance levels obtained in the analysis of variance were:

EXPERIMENT: Absence of S9 :	1.03 (1,33)	N.S.
Presence of S9 :	37.6 (1,33)	P<0.001
DOSE-LEVEL: Absence of S9 :	11.4 (1,33)	P<0.01
Presence of S9 :	41.9 (1,33)	P<0.001

### 5.2 Criterion for outcome of the assay

In this assay a test substance is considered to induce UDS if it causes an increase in tritiated thymidine incorporation to a value 50% greater than the control values at two consecutive dose-levels or at the highest practicable dose-level. This effect must be reproduced in an independent experiment.

### 5.3 Evaluation

The test substance did not induce any increase in tritiated thymidine incorporation to a value 50% greater than the control values. Therefore fruttosio-1,6-difosfato cannot be considered to have induced unscheduled DNA synthesis under the reported experimental conditions.

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CONCLUSIONS

It is concluded that fruttosio-1,6-difosfato does not induce unscheduled DNA synthesis in HeLa S3 cells in vitro, either in the absence or presence of S9 metabolic activation, under the reported experimental conditions.

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Schedule No. : 003-005

Table 1

HeLa S3 CELLS: CYTOTOXICITY

Test Substance: Fruttosio-1,6-difosfato  
Solvent : EMEM arginine-free

Treatment	Dose-level (ug/ml)	S9 mix	% Cell loss	% Viable cells
Solvent control	/	-	0	96
Fruttosio- 1,6-difosfato	156	-	0	96
Fruttosio- 1,6-difosfato	313	-	3	95
Fruttosio- 1,6-difosfato	625	-	2	97
Fruttosio- 1,6-difosfato	1250	-	17	93
Fruttosio- 1,6-difosfato	2500	-	25	93
Fruttosio- 1,6-difosfato	5000	-	15	83
Fruttosio- 1,6-difosfato	10000	-	37	88

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Schedule No.: 003-005

Table 2

HeLa S3 CELLS: CYTOTOXICITY

Test Substance: Fruttosio-1,6-difosfato  
Solvent : EMEM arginine-free

Treatment	Dose-level (ug/ml)	S9 mix	% Cell loss	% Viable cells
Solvent control	/	+	0	95
Fruttosio- 1,6-difosfato	156	+	0	96
Fruttosio- 1,6-difosfato	313	+	0	95
Fruttosio- 1,6-difosfato	625	+	0	96
Fruttosio- 1,6-difosfato	1250	+	0	95
Fruttosio- 1,6-difosfato	2500	+	18	89
Fruttosio- 1,6-difosfato	5000	+	27	71
Fruttosio- 1,6-difosfato	10000	+	18	77

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Table 3

UNSCHEDULED DNA SYNTHESIS IN HeLa S3 CELLS  
WITHOUT METABOLIC ACTIVATION  
EXPERIMENT I

Test substance: Fruttosio-1,6-difosfato  
Solvent : EMEM arginine-free

Dose (ug/ml)	Replicate	DNA recovered (ug/ml)	DPM*	DPM/ug DNA	Mean	S.D.	Increase over solvent control
0.00	1	12.60	2432	193.0	187.1	9.07	
	2	16.78	3216	191.7			
	3	17.35	3065	176.7			
100	1	16.59	2935	176.9	205.8	27.03	1.10
	2	12.41	2861	230.6			
	3	13.74	2883	209.8			
316	1	18.87	3413	180.9	171.3	12.69	0.92
	2	16.97	2989	176.2			
	3	15.83	2484	156.9			
1000	1	16.21	2825	174.3	157.2	14.85	0.84
	2	13.36	1982	148.4			
	3	12.60	1875	148.8			
3160	1	14.12	2079	147.3	134.0	11.64	0.72
	2	14.31	1850	129.3			
	3	13.17	1652	125.4			
10000	1	11.65	832	71.4	84.4	12.89	0.45
	2	9.37	794	84.7			
	3	4.05	394	97.2			

Positive control: 4-Nitroquinoline-N-Oxide

5.00	1	13.55	40449	2985.4	2794.7	184.12	14.93
	2	15.07	39448	2618.0			
	3	11.27	31338	2780.7			

\* = Disintegrations per minute

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Table 4

UNSCHEDULED DNA SYNTHESIS IN HeLa S3 CELLS  
WITH METABOLIC ACTIVATION  
EXPERIMENT I

Test substance: Fruttosio-1,6-difosfato  
Solvent : EMEM arginine-free

Dose (ug/ml)	Replicate	DNA recovered (ug/ml)	DPM*	DPM/ug DNA	Mean	S.D.	Increase over solvent control
0.00	1	21.02	4492	213.7	257.6	58.74	
	2	9.97	3234	324.3			
	3	17.57	4126	234.8			
100	1	19.30	4725	244.8	208.5	34.33	0.81
	2	24.13	4263	176.6			
	3	18.95	3865	203.9			
316	1	21.37	4794	224.3	203.0	25.61	0.79
	2	23.44	4922	210.0			
	3	21.72	3791	174.6			
1000	1	23.10	3450	149.4	166.8	25.99	0.65
	2	21.72	3353	154.4			
	3	17.57	3456	196.7			
3160	1	19.99	2810	140.6	144.1	31.94	0.56
	2	22.41	2556	114.1			
	3	17.92	3183	177.7			
10000	1	19.64	2168	110.4	141.9	35.35	0.55
	2	15.84	2144	135.3			
	3	9.63	1734	180.1			
Positive control: Benzo (a) pyrene							
2.50	1	22.75	6171	271.2	398.6	113.57	1.55
	2	13.77	5992	435.1			
	3	12.04	5894	489.4			

\* = Disintegrations per minute

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Table 5

UNSCHEDULED DNA SYNTHESIS IN HeLa S3 CELLS  
WITHOUT METABOLIC ACTIVATION  
EXPERIMENT II

Test substance: Fruttosio-1,6-difosfato  
Solvent : EMEM arginine-free

Dose (ug/ml)	Replicate	DNA recovered (ug/ml)	DPM*	DPM/ug DNA	Mean	S.D.	Increase over solvent control
0.00	1	16.53	2436	147.3	151.7	6.73	
	2	16.19	2582	159.5			
	3	16.88	2505	148.4			
10.0	1	16.53	2601	157.3	135.6	43.91	0.89
	2	10.32	878	85.1			
	3	14.12	2322	164.5			
31.6	1	21.02	2605	123.9	151.1	27.94	1.00
	2	16.53	2473	149.6			
	3	13.77	2475	179.7			
100	1	19.30	2589	134.2	130.4	3.27	0.86
	2	19.64	2519	128.2			
	3	18.26	2352	128.8			
316	1	18.61	2556	137.4	146.4	32.68	0.96
	2	17.57	2095	119.2			
	3	11.35	2074	182.7			
1000	1	16.53	2249	136.0	148.7	11.46	0.98
	2	10.32	1633	158.3			
	3	12.73	1934	151.9			

Positive control: 4-Nitroquinoline-N-Oxide

5.00	1	14.81	38311	2587.3	2381.1	353.35	15.69
	2	14.12	36463	2583.0			
	3	15.50	30580	1973.1			

\* = Disintegrations per minute

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(D. AFRÉDO NUNZIATA)

Schedule No.:003-005

Table 6

UNSCHEDULED DNA SYNTHESIS IN HeLa S3 CELLS  
WITH METABOLIC ACTIVATION  
EXPERIMENT II

Test substance: Fruttosio-1,6-difosfato  
Solvent : EMEM arginine-free

Dose (ug/ml)	Replicate	DNA recovered (ug/ml)	DPM*	DPM/ug DNA	Mean	S.D.	Increase over solvent control
0.00	1	6.28	2095	333.3	313.6	40.55	
	2	8.42	2869	340.6			
	3	8.92	2381	267.0			
31.6	1	8.75	2833	323.7	298.6	43.76	0.95
	2	9.90	2457	248.1			
	3	9.08	2943	324.1			
100	1	7.93	2419	305.0	302.2	38.59	0.96
	2	8.26	2802	339.3			
	3	8.42	2209	262.2			
316	1	8.75	2231	254.9	255.2	32.55	0.81
	2	9.41	2097	222.8			
	3	6.45	1857	287.9			
1000	1	9.41	2380	252.9	249.9	23.97	0.80
	2	11.06	2483	224.6			
	3	7.11	1935	272.2			
3160	1	13.19	1989	150.7	165.9	23.05	0.53
	2	5.63	1083	192.5			
	3	8.59	1328	154.6			
<b>Positive control: Benzo (a) pyrene</b>							
5.00	1	12.54	4572	364.7	469.6	106.89	1.50
	2	8.42	4872	578.4			
	3	8.92	4152	465.6			

\* = Disintegrations per minute

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Table 7

UNSCHEDULED DNA SYNTHESIS IN HeLa S3 CELLS

Test Substance: Fruttosio-1,6-difosfato

Summary table

Mean incorporation of tritiated thymidine (DPM/ug DNA)

Absence of S9			Presence of S9		
Dose-level (ug/ml)	Expt 1	Expt 2	Dose-level (ug/ml)	Expt 1	Expt 2
0.00	187.1	151.7	0.00	257.6	313.6
10.0	NT	135.6	10.0	NT	NT
31.6	NT	151.1	31.6	NT	298.6
100	205.8	130.4	100	208.5	302.2
316	171.3	146.4	316	203.0	255.2
1000	157.2	148.7	1000	166.8	249.9
3160	134.0	NT	3160	144.1	165.9
10000	84.4	NT	10000	141.9	NT
<b>Positive controls</b>					
4-NQO	2794.7	2381.1		NT	NT
B(a)P	NT	NT		398.6	469.6

Mean historical values for solvent control cultures in this laboratory based on the ten preceding studies (Mean  $\pm$  S.D.):

98.36  $\pm$  30.5142.4  $\pm$  31.2

NT: Not tested

4-NQO: 5.00 ug/ml 4-Nitroquinoline-N-oxide

B(a)P: 2.5 ug/ml Benzo(a)pyrene (Expt I) and 5.00 ug/ml (Expt II)

The values given for the zero dose-level were obtained after treatment with the vehicle only.

APPENDIX I  
STUDY PROTOCOL

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RICEVUTO



LIFE SCIENCE RESEARCH  
ROMA TOXICOLOGY CENTRE S.P.A. 003-005

LSR-RTC Enquiry no. 1472

**UNSCHEDULED DNA SYNTHESIS (UDS)  
in HeLa S3 cells in vitro  
Test Substance: ESAFOSFINA**

Protocol prepared for

BIOMEDICA FOSCAMA  
IRFI.  
Via Morolese, 87  
03013 Ferentino (FR)

by

Life Science Research  
Roma Toxicology Centre  
Via Tito Speri 14  
Pomezia (Roma)

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ROMA TOXICOLOGY CENTRE S.p.A.  
(Dr. ALBERTO NUNZIATA)



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ROMA TOXICOLOGY CENTRE S.P.A.

**UNSCHEDULED DNA SYNTHESIS (UDS)  
in HeLa S3 cells in vitro  
PROTOCOL APPROVAL**

For LIFE SCIENCE RESEARCH - ROMA TOXICOLOGY CENTRE

Issued by : *A. L. S.* . . . . . date: *13.10.88* . . . . .

Released by: *R. Z.* . . . . . date: *19. May '88* . . . . .

For BIOMEDICA FOSCAMA

This protocol is accepted without revision and my signature authorizes the study to proceed as described in this document. The document becomes the FINAL PROTOCOL for the study, and will be reproduced in the final report.

Approved by: *[Signature]* . . . . . date: *9.6.88* . . . . .

STUDY DIRECTOR

The Sponsor has approved the initiation of this study according to the procedures described in this document. My signature below denotes that I have read and agreed the contents of this document.

Agreed by : *A. L. S.* . . . . . date: *14. June 88* . . . . .  
(Dr. rer. nat. A.H. Seeberg Dip1. Biol., Study Director)

LIFE SCIENCE RESEARCH  
ROMA TOXICOLOGY CENTRE S.P.A.  
*(Dr. ALFREDO NUNZIATA)*

**UNSCHEDULED DNA SYNTHESIS (UDS)  
in HeLa S3 cells in vitro**

**MANAGEMENT OF STUDY**

Head Department of Genetic Toxicology : R. Forster, M.A.(Cantab.), Ph.D.  
Person Responsible to Ministry of Health : A. Nunziata, Pharm.D., Chem.D.  
Study Director : Dr.rer.nat. A.H. Seeberg Dipl.Biol.  
Sponsor : BIOMEDICA FOSCAMA  
IRFI.  
Via Morolese, 87  
03013 Ferentino (FR)

Monitor : to be appointed by the Sponsor.

**QUALITY ASSURANCE**

Quality Assurance Manager : V.Sforza B.Sc.

**LOCATION OF STUDY**

The study will be performed at:

Life Science Research Roma Toxicology Centre  
Via Tito Spéri, 14  
00040 Pomezia, ROMA.

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 LSR-RTC.

**TEST SUBSTANCE IDENTITY**

The test substance will be : ESAFOSFINA

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(D. ALFREDO NUNZIATA)

**UNSCHEDULED DNA SYNTHESIS (UDS)  
in HeLa S3 cells in vitro**

1. INTRODUCTION

1.1 Objective

To assay the test substance for the ability to induce Unscheduled DNA Synthesis (UDS) in HeLa S3 cells after in vitro treatment in the absence and presence of S9 metabolism.

1.2 Regulatory requirements

The study will be performed to comply with the principles of Good Laboratory Practice as set forth by the U.S. Food and Drug Administration. In addition the study is designed to comply with the experimental methods indicated in the guidelines of:

- OECD Guideline for the Testing of Chemicals No. 482.
- EEC Council Directive 79/831 Annex V Part B.
- TSCA Test Guidelines issued by the US EPA in 40 CFR part 798 on 27-Sept-1985 (Section 798. 5550 Unscheduled DNA synthesis in mammalian cells in culture).

1.3 Principles of the method

The induction of DNA repair synthesis in cultured mammalian cells is commonly accepted as evidence for the mutagenic and carcinogenic potential of chemical agents. The detection of repair synthesis has been developed as a short-term screening assay. Unscheduled (or non-semiconservative) DNA synthesis (UDS), which is presumed to result from DNA repair, may be detected either by autoradiography or scintillation counting after the uptake of tritium-labelled thymidine into cells in which semiconservative (replicative) synthesis has been blocked. Incubation with hydroxyurea, or in arginine-free medium, or growth to confluence, will help to inhibit semiconservative synthesis. This protocol describes a method for the detection of UDS by scintillation counting in cells which have had semiconservative synthesis inhibited.

Since cultured cells generally lose their capacity for metabolic activation, it is necessary to provide a metabolising system (S9 mix) to detect indirect mutagens. Accordingly, this test is performed both in the absence and the presence of an S9 metabolising system.

The HeLa S3 cell line is selected for this assay since it is robust and simple to culture, of human origin, and has been used extensively for UDS studies. (Martin et al 1978).

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2. TEST SUBSTANCE

- 2.1 It is the responsibility of the Sponsor to supply the test substance, accompanied by analytical data confirming the identity, purity, stability, strength and composition of the substance, the solubility and stability in the proposed vehicle and details of any known hazards to laboratory staff.
- 2.2 To comply with the requirements of the Italian Ministry of Health, the test substance should be accompanied by a certificate of analysis, and a sample will be retained in the archives for a period of five years after the completion of the study.
- 2.3 The test substance identity is indicated on previous pages of this protocol.
- 2.4 Unless otherwise indicated by the Sponsor, the storage conditions for the test substance will be 4°C in the dark.
- 2.5 The test substance will be treated with precautions appropriate for a potential carcinogen.
- 2.6 The amount of test substance received and used will be recorded according to standard procedures.
- 2.7 Fresh solutions of the test substance 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 substance. Unless specified by the Sponsor, concentrations of solutions will be expressed in terms of material as received, and not of active constituents. Preferred solvents will be sterile distilled water, culture medium, DMSO, ethanol, acetone. Other solvents may be used as necessary.
- 2.8 No assay of test substance stability, nor its concentration and homogeneity in vehicle will be undertaken, nor samples of formulated test substance consigned to the Sponsor, without express instructions from the Sponsor. No determination of the absorption of the test substance in the test system will be made without express instructions from the Sponsor.

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3. MATERIALS AND METHODS

3.1 HeLa S3 cells

HeLa S3 cells obtained from Flow Laboratories Ltd are used in this assay. The karyotype, generation time and control thymidine-incorporation rates are monitored in this laboratory. The cells are checked at regular intervals for the absence of mycoplasma contamination.

Permanent stocks of the HeLa S3 cells are kept stored at -80°C, and subcultures are prepared from the frozen stocks for experimental use. Cultures of the cells are grown in Minimal Eagle Medium with Earle's salts (E.M.E.M.), supplemented with 10% foetal calf serum. Incubation are at 37°C in a 5% carbon dioxide atmosphere (100% humidity nominal).

3.2 Media

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

E.M.E.M.	(10 x)	
(Minimal Eagle Medium with Earles salts)		45.0 ml
Sterile Water		383.1 ml
Sodium Bicarbonate	(7.5%)	12.0 ml
Non-essential amino acids	(100 x)	4.5 ml
Glutamine	(200 mM)	4.5 ml
Antibiotic solution		0.9 ml
Foetal Calf Serum		50.0 ml
		<hr/>
		500.0 ml

The medium used to limit replicative DNA synthesis (arginine-free medium) has the following composition:

Arginine-free E.M.E.M.	489.0 ml
Antibiotic solution	1.0 ml
L-Glutamine (200 mM)	5.0 ml
Non Essential Amino Acids (100x)	5.0 ml
Dialysed Foetal Calf Serum	12.8 ml
	<hr/>
	512.8 ml

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### 3.3 S9 Mix

The S9 liver tissue fraction will be prepared according to the attached standard method. The mixture of S9 tissue fraction and co-factors (S9 Mix) will be prepared in the following proportions:

S9 tissue fraction	4.0 ml
NADP (0.1M)	0.64 ml
G-6-P (0.1M)	1.28 ml
KCl (330 mM)	1.0 ml
MgCl <sub>2</sub> (200 mM)	1.5 ml
Hepes Buffer (200 mM)	1.0 ml
Hank's saline	0.58 ml

This S9 mix is added to treatment media at a final concentration of 50 ul/ml.

### 3.4 Control substances

Positive control treatments will be prepared in every experiment. The positive control agents will be characterised by their labelling, and their stability determined from the scientific literature. Solutions of 4-nitroquinoline-N-oxide and benzo(a)pyrene will usually be prepared in DMSO. Determination of the stability and concentration of solutions of these agents will not be undertaken without express instructions from the Sponsor.

## 4. CYTOTOXICITY ASSAY

### 4.1 Experimental design

A preliminary cytotoxicity assay is conducted in order to set appropriate dose-levels for the UDS assays. In this test a wide range of dose-levels of test substance, evenly spaced over several log-cycles, are used; cell cultures are treated using the same treatment conditions as the UDS assays, and the survival of the cells is subsequently determined. The test includes the following treatments:

Solvent controls : The final concentration of organic solvents will not exceed 1%.

Test substance : The highest dose-level will be determined by the solubility of the test material, up to a maximum of 10 mg/ml.

Treatments are performed both in the absence and presence of S9 metabolism; a single culture is used at each test point. Where it seems advisable, further test points may be included in the cytotoxicity test.

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#### 4.2 Test procedure

Culture vessels are seeded with HeLa S3 cells and grown to near-confluence. A wide range of concentrations of the test substance are then added to the cultures in the presence of hydroxyurea, both with and without S9 metabolism. The cultures are incubated for three hours, after which the treatment media are removed. The cells are washed with PBS (Phosphate Buffered Saline) and stained with trypan blue.

#### 4.3 Scoring and selection of dose-levels for main assay

The cells are examined by microscope for evidence of gross toxicity, loss of cells by detachment or lysis, morphological alterations and trypan blue exclusion index; cell counts are also performed. These parameters are used to detect different mechanisms of toxicity; for example, dye exclusion is an index of increased membrane permeability. Cell detachment is often a precursor of death. The highest dose-level for the principal assay is selected as the lowest concentration to reduce cell viability (as measured by the trypan blue exclusion index) to less than 50% of control values. However, where the loss of cells exceeds 50%, a lower concentration must be selected to ensure that there will be an adequate yield of treated cells in the main assay. If the test substance is apparently non-toxic up to the maximum dose-level employed, then this concentration will be used as the highest dose-level in the principal assay. Four lower dose-levels will be selected, separated by half-logarithmic intervals.

### 5. EXPERIMENTAL DESIGN (MAIN ASSAY)

Each experiment will include negative and positive controls, and at least five dose-levels of the test substance, tested in the absence and presence of an S9 metabolising system. Triplicate cultures are prepared at each test point. Two independent experiments will be performed. A further experiment may be undertaken if inconsistent results are obtained.

**Negative controls:** Treated with the maximum amount of solvent vehicle used in any test substance treatment.

**Positive controls:** In the absence of S9 metabolism, 4-nitroquinoline-N-oxide is used, at a concentration of 5.00 ug/ml. In the presence of S9 metabolism benzo(a)pyrene is used, at a concentration of 2.50 ug/ml. Alternative appropriate positive control treatments may be substituted.

**Test substance :** The selection of test substance dose-levels is described in the preceding section.

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(Dr. A. SPINELLI) (1981)

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

## 6. ASSAY PROCEDURE

### 6.1 Preparation of test cultures

Cultures of near confluent monolayers of HeLa S3 cells are prepared for the UDS assay by treatment for approximately 24 hours with arginine-free medium, and then for a further approximately 48 hours with fresh arginine-free medium (see section 3.2). This treatment helps to limit replicative DNA synthesis.

### 6.2 Treatment of test cultures

Hydroxyurea is added to the medium of all test cultures, at a final concentration of 10 mM. After incubation for one hour, tritiated thymidine is added (final concentration 5 uCi/ml), together with the solutions of test substance, vehicle or positive control substance, and S9 mix as required. The cultures are incubated for three hours at 37°C. The medium is then removed, and the cell layers are washed with cold Phosphate Buffered Saline. The cells are subsequently removed and pelleted by centrifugation at 800 rpm for five minutes, and the pellets are stored frozen until DNA extraction is performed.

### 6.3 DNA extraction

For the extraction of DNA, the cell pellets are thawed and kept on ice in labelled plastic centrifuge tubes. Each tube receives 2 ml of chilled 10% trichloroacetic acid (TCA), and the precipitate is centrifuged. The supernatant is discarded, and the pellet is suspended in 0.3 M KOH (2 ml) and incubated at 37°C for one hour. After neutralization with cold 1M HCl, 2.5 ml of 20% TCA (optionally containing 1% Bovine Serum Albumin) is added, and the pellet collected by centrifugation as before. The supernatant is discarded, and the pellet is resuspended in 5% TCA (3.5 ml) and heated at 90°C for 20 minutes. After a final centrifugation the supernatant is decanted and retained as a solution of extracted DNA.

### 6.4 Liquid Scintillation Counting

The uptake of tritiated thymidine is determined by liquid scintillation counting. One ml is taken from each of the extracted DNA solutions, and added to 10 ml of scintillation fluid, and is counted in a Packard Series 4000 Liquid Scintillation counter.

Disintegrations per minute (dpm) are estimated automatically by an internal computer using an external standard, and previously constructed quench curve.

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## 6.5 DNA Assay

The amount of DNA extracted is estimated using the Burton colorimetric assay. A further 1 ml is taken from each solution of extracted DNA, and added to 2 ml of freshly prepared Burton's reagent. These samples are incubated for 16-20 hours at 32°C, and the absorbance is measured at 600 nm in a spectrophotometer. A standard plot is prepared using known concentrations of DNA; the standards are prepared freshly for each experiment in 5% TCA and incubated at 90°C at the same time as the UDS test culture extracts. Absolute values for the DNA concentration are then determined using the regression equation obtained from the standards.

## 7. REPORTING

### 7.1 Presentation of results

The data will be presented in tabular form; for each treatment, the extent of tritiated thymidine incorporation will be expressed as disintegrations per minute (dpm) per microgram of DNA. The mean and standard deviation of tritium incorporation will be presented for each test point, together with the results of statistical analysis. The current historical mean values for negative control cultures in this laboratory will also be presented.

### 7.2 Evaluation of results

The test substance will be considered to induce Unscheduled DNA Synthesis if a 50% increase in tritiated thymidine incorporation (per microgram of DNA) compared with the solvent control values is observed:

- (i) at two consecutive dose-levels.
- (ii) at a single dose-level if that is the highest dose-level which can be tested (as the result of solubility, toxicity, formulation, etc.).

The increases must be reproduced in an independent experiment.

Evidence of a dose-response relationship will be considered as supportive evidence for genotoxic activity but will not be a requirement for a positive result.

### 7.3 Reporting procedure

Unless specified by the Sponsor, a Final Report will be issued after the completion of the study. If any corrections or additions are required to the Final Report, these will be in the form of an addendum by the Study Director.

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The addendum will clearly identify that part of the final report that is being added to or corrected, and the reasons for the changes, and will be signed and dated by the person responsible. If previously specified by the Sponsor, a Draft Report may be supplied, and a Final Report issued subsequently to include any agreed changes or amendments.

#### 7.4 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 any approved changes to the original protocol;
- data generated while conducting the study;
- statistical methods employed for analysing the data;
- the test article, identified by name, chemical name or chemical number;
- 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.

#### 7.5 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.

#### 7.6 Archiving

The original data arising from this study, a sample of the test material and a copy of the final report consigned will be stored in the archives of Life Science Research - Roma Toxicology Centre for a period of five years from the date of consignment of the report.

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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 substances. 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.

8.3 Quality assurance

The study is subjected to the procedure for quality assurance specified in relevant sections of the regulations pertaining to the conduct of Non Clinical Laboratory Studies, published by the U.S. Food and Drug Administration. Specifically:

- the protocol is inspected for compliance;
- procedures and data of the laboratories concerned are periodically inspected;
- 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 this study are available for inspection by the Study Monitor (for scientific monitoring).

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9. REFERENCES

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Version No. 87/1

APPENDIX II  
CERTIFICATE OF ANALYSIS

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(Dr. ~~ALFREDO NONZATI~~)

**LABORATORI DI CONTROLLO**

Richiesta N.

per il Laboratorio

C. Chimico

C. Biologico

del 29-04-88

Prodotto ESAFOSFINA SGR JEMIO Codice N. 60-6062 Lotto N. 1513938 21 27

Fornitore/Rep. F. W. POWELL N. di Lotto del fornitore 80100

Firma del richiedente A. B. ...

Campionato il 2/5/88 da D. ...

Secondo il P.S.O. N. AF 01406

Campionati N. 22 di contenitori su 10687 su 428415

Aspetto dei contenitori VE 720

**CONTROLLO CHIMICO**

Analisi N. CA 7 048-3 P.S.O. N. AF 01400 Data 4/5/88

SAGGI	RISULTATI	SIGLA	SAGGI	RISULTATI	SIGLA
Descrizione			Sost. ossidabili		
Identità			Solidi totali		
Int. fus. o di ebol.			Fosforo inorganico		
Potere rotatorio			Disgregazione		
Peso spec. (... °C)			Conten. in volume		
Ind. di rifrazione			Colore della sol.		
pH			Nitriti		
Acqua (K.F.)			Nitrati		
Perd. peso t =			Cromatografia		
Peso medio <u>Grammi</u>	<u>5,05</u>	<u>A H</u>	Titolo		
Carat. spettrofot.					
Ceneri			<u>SOLUBILITA'</u>	<u>BUONA</u>	<u>A</u>
Arsenico					
Metalli pesanti			<u>EDNA<sub>3</sub>H. gr./fl.</u>	<u>4,43</u>	<u>A</u>
Ferro					
Cloruri			<u>UNIFORMITA' PESO</u>	<u>Conforme</u>	<u>A</u>
Solfati					
Acido ossalico			<u>CONTENUTO PARTICELLE</u>	<u>NEL LIMITI</u>	<u>A</u>
Ammoniaca					
Calcio					

Approvato  Respinto

25/5/88

Responsabile Laboratorio

M. ...

**CONTROLLO BIOLOGICO**

Data

SAGGI	RISULTATI	ANALISI N.	SIGLA
Sterilità			
Apirogenicità			
Atossicità			
Titolo microbiologico			

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Responsabile Laboratorio

Approvato  Respinto

**LABORATORI DI CONTROLLO**

Richiesta N. \_\_\_\_\_

per il Laboratorio \_\_\_\_\_

- Ric. e Svil.  
 C. Chimico  
 C. Biologico

del 29-04-88

Prodotto ESAFOSFINA Sae

Codice N. 60-6062

Lotto N. 3/3933

del 29-4

Fornitore/Rep. FAL POWER

N. di Lotto del fornitore 80.100 B.T.

Firma del richiedente A. Amico

Campionato il 2/5/88

da Duca

Secondo il P.S.O. N. AMOLG

Campionati N. 24 da 100cc

di contenitori su 10687 su 475415

Aspetto dei contenitori VERO

**CONTROLLO CHIMICO**

Analisi N. 30/061/09

P.S.O. N. 30/0110

Data 24/5/88

SAGGI	RISULTATI	SIGLA	SAGGI	RISULTATI	SIGLA
Descrizione			Sost. ossidabili		
Identità			Solidi totali		
Int. fus. o di ebol.			Fosforo inorganico		
Potere rotatorio			Disgregazione		
Peso spec. (.....°C)			Conten. in volume		
Ind. di rifrazione			Colore della sol.		
pH			Nitriti		
Acqua (K.F.)			Nitrati		
Perd. peso (=			Cromatografia		
Peso medio			Titolo		
Carat. spettrofot.					
Ceneri					
Arsenico					
Metalli pesanti					
Ferro					
Cloruri					
Solfati					
Acido ossalico					
Ammoniacale					
Calcio					

Responsabile Laboratorio

Approvato

Respinto

**CONTROLLO BIOLOGICO**

Data 24/5/88

SAGGI	RISULTATI	ANALISI N.	SIGLA
Sterilità	<u>STERILI</u>	<u>B5/069/04</u>	<u>MA</u>
Apirogenicità	<u>Aptogeno</u>	<u>B2/084/07</u>	<u>ML</u>
Atossicità	<u>ATOSSICO</u>	<u>B72/098/23</u>	<u>IG</u>
Titolo microbiologico			

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 (D/ ALFREDO NUZZI)

Responsabile Laboratorio

DATI DI PRODUZIONE

Tipo di soluzione infusionale: ESAFOSFINA gr 5 semil.  
 N° lotto interno : P8/383 B - liofilizzato 80'100 B.F.  
 Data di preparazione : 23/04/88

DATI DI LABORATORIO C.Q.

Controllo visuale:

Ø PARTICELLE (µm)	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	$\bar{m}$	$\bar{m}/g \text{ o } \bar{m}/l$
50 um-100 um								
100 um-300 um								
>300 um								

Controllo microscopico:

Ø PARTICELLE	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	$\bar{m}$	$\bar{m}/g \text{ o } \bar{m}/l$
<10 µm								
10 um - 25 µm								
25 um - 50 µm								
>50 µm								

Controllo particellare:  
 volume campionato: 5ml

25/5/88

Ø PARTICELLE (µm)	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	$\bar{m}$	$\bar{m}/g \text{ o } \bar{m}/l$
2	32	714	764	722	417	470	585,4	23
5	4	144	157	164	116	117	135,6	5
10	1	17	17	24	22	26	20,2	8
20	0	0	1	0	1	1	0,6	2
25	0	0	0	0	0	0	0	
50	0	0	0	0	0	0	0	

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ADDENDUM I  
PREPARATION OF S9 RAT LIVER  
TISSUE FRACTION

1. INTRODUCTION

Some chemicals are not directly mutagenic, but after entering the body are metabolised to reactive intermediate forms which can damage DNA and cause mutation. Such chemicals are referred to as "indirect" mutagens, in contrast with 'direct' mutagens which do not require metabolism.

Metabolism of foreign-compounds in the body is performed by the mixed function oxygenase system of enzymes (the cytochrome P450 system). At the sub-cellular level, these enzymes are located in the endoplasmic reticulum and nuclear membranes (during the preparation of homogenates these membranes break and close up to form "microsomes"). The liver is the primary organ concerned with xenobiotic metabolism, and is rich in these enzymes; appreciable levels, however, can be found in many other tissues. The enzyme system can be 'induced' to high levels by the treatment of animals with a variety of chemicals.

In order to detect indirect mutagens, in vitro mutagenicity tests are routinely performed using a metabolising system (S9 mix) to simulate in vivo metabolism. The S9 mix contains the microsomal fraction of rat liver tissue homogenate (S9 fraction) and appropriate co-factors.

To prepare S9 fraction, young male rats are treated with inducing agents (Aroclor 1254, or mixed induction with phenobarbitone and betanaphthoflavone). After an appropriate number of days, the animals are sacrificed, and a liver homogenate prepared. The homogenate is centrifuged, and the post-mitochondrial fraction is retained. This fraction is known as the S9 fraction, since it is the Supernatant (S) fraction produced at 9000 g (9). The S9 fraction is submitted to quality control checks and stored at -80°C until used.

2. ANIMALS AND HUSBANDRY

2.1 Animal supply

Male Sprague-Dawley rats are obtained from Charles River, Como, and at the commencement of treatment weigh approximately 200-250 gm. For the preparation of each batch of S9 fraction, the livers from several animals (usually between five and fifteen) are bulked to reduce the effects of between-animal variation. Each batch of S9 is allocated a unique batch number and this number is indicated on the cage labels and all documentation during the preparative steps.

## 2.2 Animal husbandry

The animals are housed at 5 animal/cage, in clear polycarbonate cages measuring 35.5 x 23.5 x 19 cm with a stainless steel mesh lid and floor (Type 2b: Techniplast). Each cage will hold absorbent bedding which will be inspected daily and changed as necessary. The temperature and relative humidity of the animal rooms are monitored daily. The animals will be kept in a 12 hour light/dark cycle.

Food and drinking water will be supplied ad libitum. The animals are maintained on Altromin MT diet. Quality control aspects of the diet and drinking water are detailed in Addendum II.

At least five days are allowed for acclimatisation and quarantine; during this period the health status of the animals will be assessed by daily observations. Animals observed to be unfit prior to treatment will be removed from the study and may be replaced if stocks allow.

Dated and signed records of activities relating to the day to day running and maintenance of the study in the animal accommodation will be recorded in a study daybook.

## 3. PREPARATION OF S9 SUPERNATANT FRACTION

### 3.1 Induction of drug metabolising enzyme-levels

Induction is routinely performed using phenobarbitone and betanaphthoflavone (Mixed Induction); induction with Aroclor 1254 will be performed if specifically requested by the Sponsor.

#### Mixed induction

The required number of animals are starved for 16 hours prior to the onset of induction, which begins on Day 1. The hepatic microsomal drug metabolising enzymes are induced by the mixed induction method, according to the following schedule:

Day 1	ip.	Phenobarbital	30 mg/kg
Day 2	ip.	Phenobarbital	60 mg/kg
Day 3	ip.	Phenobarbital	60 mg/kg
	+ip.	Betanaphthoflavone	80 mg/kg
Day 4	ip.	Phenobarbital	60 mg/kg
Day 5		Sacrifice	

The animals are all given a fixed dose of each agent, calculated on the basis of the daily mean weight of the group of animals. Animals which die during the treatment period are not used and are not replaced.

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The solutions of Betanaphthoflavone (Sigma) and Phenobarbital (Carlo Erba) are prepared in corn oil and sterile distilled water respectively.

#### Induction with Aroclor 1254

The animals are starved for 16 hours prior to induction. On Day 1 a single injection of Aroclor 1254 (Foxborough Analytical) in Corn-oil (200 mg/ml) is administered by intraperitoneal injection at a dose of 500 mg/kg. The dose is calculated on the basis of the mean weight of the group of animals. No further treatment is given, and the animals are sacrificed on Day 6.

#### 3.2 Sacrifice

During the final 16 hours of induction the animals are starved, prior to sacrifice by cervical dislocation.

#### 3.3 Preparation of tissue fraction

The livers are immediately removed aseptically. All subsequent steps are performed using chilled, sterile equipment. The pooled livers are washed in 0.15 M KCl, and then weighed in a tared sterile container. The livers are then chopped finely, and homogenised in 0.15 M KCl (3 ml homogenising solution: 1 gm liver) using a Braun Potter S homogeniser. The homogenate is centrifuged at 9000 g/av for 10 minutes, and the supernatant is collected and distributed to sterile vials for storage at -80°C. The pellet, consisting of cell debris, nuclei and mitochondria, is discarded.

#### 3.4 Quality control

Prior to use, each batch of S9 fraction is characterised using the following assays:

- (i) Total protein content.
- (ii) Aminopyrine demethylase activity.
- (iii) Performance with standard mutagens in the Ames test.
- (iv) Sterility.

Batches of S9 giving inadequate results for any of the above may be rejected for use in mutagenicity assays. Batches giving adequate results are given an expiry date and issued for use.

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## ADDENDUM II

### Quality Control aspects of Diet and Drinking Water

#### 1. DIET

The animals are maintained on Altromin MT diet. Altromin MT is a fixed formula rodent diet manufactured by Altromin-Rieper, Bolzano, Italy. The standards of production adopted by the manufacturers have been approved by the LSR-RTC Quality Assurance Manager. The nutritional content is as shown below:

<u>Nutrients</u>	<u>Typical level (%)</u>
Crude protein	23
Crude lipid	5.5
Crude fibre	5.0
Ash	9
Moisture	13

Analyses are made on all batches of diet used to establish the levels of specified substances and micro-organisms likely to be present in feed components and which, if in excess of specified amounts, might have an undesirable effect on the test animals.

Reject levels are based on those quoted in EPA guidelines for the administration of the Toxic Substances Control Act (USA).

<u>(A) Contaminants</u>	<u>Maximum allowable concentration (ppm)</u>
Total Aflatoxin (B1, B2, G1, G2)	0.005
Lindane	0.02
Heptachlor	0.02
Malathion	2.50
DDT (total)	0.10
Dieldrin	0.02
PCB	0.15
Cadmium	0.48
Arsenic	2.00
Lead	3.00
Mercury	0.20
Selenium	0.60

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ADDENDUM II (continued)

(B) Microbial content

Maximum count, at time of manufacture.

Total viable organisms	20,000/g
<u>E.coli</u>	0 in 10g
<u>Salmonella</u>	0 in 50g

In addition LSR-RTC receive estrogenic activity assay results every three months and will monitor levels.

2. DRINKING WATER

Water is taken from the public supply, and conforms to European Council Standards for potable water intended for human consumption (80/778/EEC). At approximately six monthly intervals, samples of water are tested for the chemical quality of the water by screening for the priority pollutants listed below and the microbiological quality of the water is tested.

(A) CHEMICAL CONTAMINANTS

1. Organic materials

Maximum admissible concentration (ppb)

Persistent organochlorine and organophosphorus pesticides.

- substances considered separately	0.1
- total	0.5
- PCB (total)	0.5
- purgeable organochlorine substances including trihalomethanes	1

2. Metals

Maximum admissible concentration (ppm)

Arsenic	0.05
Cadmium	0.005
Calcium	100 (guide-level)
Copper	3 (guide-level)
Mercury	0.001
Lead	0.05
Selenium	0.01
Zinc	5 (guide-level)

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ADDENDUM II (continued)

<u>3. Inorganic ions</u>	<u>Maximum admissible concentration (ppm)</u>
Nitrate	50
Nitrite	0.1

<u>(B) MICROBIOLOGICAL CONTAMINANTS</u>	<u>Maximum admissible content per 100 ml</u>
Total coliforms	0
Faecal coliforms	0
Salmonella	0

The results of the above analyses of the diet and drinking water will be retained in the archives of LSR-RTC, and referenced where appropriate in the study data.

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