RESEARCH TOXICOLOGY CENTRE - ROMA

[redacted]7850

MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

FINAL REPORT

RTC Study no.: 52410

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COMPLIANCE STATEMENT

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

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

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

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

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

[redacted]

(Study Director) Date : [redacted]

[redacted]

(Scientific Director) Date : [redacted]

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	06.02.2006	07.02.2006	07.02.2006
PROCESS-BASED INSPECTIONS RELATED TO THIS TYPE OF STUDY			
Dose preparation	04.05.2006	-	11.05.2006
Cell treatment	15.03.2006	-	17.03.2006
Count and plating out	03.05.2006	-	06.07.2006
Plate scoring	04.05.2006	-	04.05.2006

Other process-based inspections were carried out on routine activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here.

Associated laboratories and support functions are subject to regular facility inspections.

FINAL REPORT Review of this report by RTC's QAU found the reported methods and procedures to describe those used and the results to constitute an accurate representation of the recorded raw data.	Review completed [redacted]

[redacted]

[redacted]
Date

Contents

1. SUMMARY

The test item [redacted] 7850 was examined for mutagenic activity by assaying for the induction of 5-trifluorothymidine resistant mutants in mouse lymphoma L5178Y cells after *in vitro* treatment (in the absence and presence of S9 metabolic activation) using a fluctuation method. Solutions of the test item were prepared in ethanol.

A preliminary cytotoxicity assay was performed. Both in the absence and presence of S9 metabolic activation, the test item was assayed at a maximum dose-level of 5000 µg/ml and at a wide range of lower dose-levels: 2500, 1250, 625, 313, 156, 78.1, 39.1 and 19.5 µg/ml. Following treatment in the absence of S9 metabolic activation, using a 3-hour treatment time, no cells survived at dose levels between 156 and 5000 µg/ml. Slight toxicity was observed over the remaining dose levels reducing survival to 74% of the concurrent negative control at 78.1 µg/ml. Using a long treatment time, no cell survived at concentrations between 313 and 5000 µg/ml, while at the next lower dose-level (156 µg/ml) survival was reduced to 44% of the negative control value. Following treatment in the presence of S9 metabolic activation, no cells survived at concentrations between 313 and 5000 µg/ml. Dose-related toxicity was observed over the remaining dose levels, reducing survival to 8% of the negative control value at 156 µg/ml.

Two independent assays for mutation at the TK locus were performed using dose levels described in the following table:

Assay No.:	S9	Treatment Time (hours)	Dose-level (µg/ml)
1	–	3	125, 100, 80.0, 40.0, 20.0, 10.0 and 5.00
1	+	3	150, 100, 75.0, 37.5, 18.8 and 9.38,
2	–	24	160, 120, 80.0, 40.0, 20.0 and 10.0
2	+	3	120, 100, 83.3, 69.4 and 57.9

No mutant frequency above those seen in the concurrent vehicle control or historical background ranges was observed following treatment with the test item, in the absence or presence of S9 metabolism.

Untreated, vehicle and positive control treatments were included in each mutation experiment in the absence and presence of S9 metabolism. The mutant frequencies in the untreated control cultures fell within the normal range. Marked increases were obtained with the positive control treatments indicating the correct functioning of the assay system.

It is concluded that [redacted] 7850 does not induce mutation in mouse lymphoma L5178Y cells after *in vitro* treatment in the absence or presence of S9 metabolic activation, under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of the test item by assaying for the induction of 5-trifluorothymidine resistant mutants in mouse lymphoma L5178Y cells after *in vitro* treatment (in the absence and presence of S9 metabolic activation) using a fluctuation method. This method may detect gene mutation, clastogenic and aneugenic effects.

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

- EEC Council Directive 2000/32 Annex 4E.
- OECD Guideline for the testing of chemicals No. 476 (adopted July 1997).
- ICH, Topic S2A, Genotoxicity: Specific Aspects of Regulatory Tests, Step 4 (Document, July 1995).
- ICH, Topic S2B, A Standard Battery for Genotoxicity Testing of Pharmaceuticals, Step 4 (Document, July 1997).

2.2 Principles of the method

The mutation assay method used in this study is based on the identification of L5178Y colonies which have become resistant to a toxic thymidine analogue trifluorothymidine (TFT). This analogue can be metabolised by the enzyme thymidine kinase (TK) into nucleosides, which are used in nucleic acid synthesis resulting in the death of TK-competent cells.

TK-deficient cells, which are presumed to arise through mutations in the TK gene, cannot metabolise trifluorothymidine and thus survive and grow in its presence.

In the L5178Y mouse lymphoma cells, the gene which codes for the TK enzyme is located on chromosome 11. Cells which are heterozygous at the TK locus ($TK^{+/-}$) may undergo a single step forward mutation to the $TK^{-/-}$ genotype in which little or no TK activity remains.

The cells used, L5178Y $TK^{+/-}$, are derived from one of the two clones originated from a thymic tumour induced in a DBA/2 mouse by methylcholanthrene. The use of the TK mutation system in L5178Y mouse lymphoma cells has been well characterised and validated (D. Clive *et al.*, 1979) and is accepted by most of the regulatory authorities.

The mouse lymphoma assay often produces a bimodal size distribution of TFT resistant colonies designated as small or large. It has been valuated that point mutations and deletions within the active allele (intragenic event) produce large colonies. Small colonies result in part from lesions that affect not only the active TK allele but also a flanking gene whose expression modulates the growth rate of cells.

2.3 Study organisation

Sponsor

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Test facility

Genetic and Cellular Toxicology Department
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Principal dates

Study protocol approved by Study Director: 18-Jan-2006
Experimental starting date: 06-Mar-2006 (Treatment - Toxicity test)
Experimental completion date: 04-May-2006 (End of scoring - Main Assay 2)

Personnel involved in the study

Study Monitor:

[redacted]

Study Director:

[redacted]

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 3 years from the date of consignment of the report. At the completion of this period the Sponsor will be contacted for despatch or disposal of the material, or further archiving. An aliquot of the test item will be retained within the archives of the testing facility for a period of 10 years after which it will be destroyed.

3. MATERIALS

3.1 Test item

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

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

On 27-Feb-2006 a 12g sub-sample of the test item was transferred from the Formulation Unit to the Department of Genetic and Cellular Toxicology and stored under the same conditions.
A certificate of analysis, supplied by the Sponsor, can be found in Appendix IV of this report.

Solutions of [redacted] 7850, as received, were prepared immediately before use in ethanol on a weight/volume basis, without correction for the displacement due to the volume of the test item. Concentrations were expressed in terms of material as received. All test item solutions were used within 30 minutes of the initial formulation. No assay of test item stability, nor its concentration and homogeneity in solvent were undertaken. All dose levels in this report are expressed to three significant figures.

3.2 Control items

The solvent used in this study was ethanol obtained from [redacted] (batch nos.: 4G297304I and 4D03334D).

Fresh solutions of methylmethanesulphonate (MMS) (batch no.: 359316/153696 obtained from Fluka AG) were prepared in sterile distilled water and served as positive controls in the absence of S9 metabolism. Fresh solutions of benzo(a)pyrene (B(a)P) (batch no.: 18H3486 obtained from Sigma Chemical Co.) were prepared in dimethylsulfoxide (DMSO) and served as positive controls in the presence of S9 metabolism.
Injectable grade distilled water (batch nos.: 03H2801 and 03H2802) was obtained from Bieffe, Trieste, Italy. DMSO (batch nos.: 1060564 41204083) was obtained from Fluka AG.
Untreated cultures were included for each treatment series and acted as concurrent controls for the positive control treatments.

3.3 S9 Tissue homogenate

One batch of S9 tissue homogenate (designated 2006/1) was 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)
2006/1	36.5 ± 2.42	4.59 ± 0.15

The 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.

Induced *Salmonella typhimurium* revertants:		
TA100	2-Aminoanthracene (1 µg/plate)	1557 ± 197
	Benzo(a)pyrene (2.5 µg/plate)	514 ± 27

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

S9 tissue fraction	0.408	ml
NADP (0.03 M)	0.204	ml
G-6-P (0.59 M)	0.204	ml
KCl (150 mM)	0.204	ml
Complete medium (5%)	8.98	ml
	10.0	ml

3.4 L5178Y TK$^{+/-}$ mouse lymphoma cells

L5178Y TK$^{+/-}$ (Clone 3.7.2C) mouse lymphoma cells were obtained from American Type Culture Collection, Rockville, Maryland (ATCC code: CRL 9518). The generation time and mutation rates (spontaneous and induced) have been checked in this laboratory. The cells are checked at regular intervals for the absence of mycoplasmal contamination.

Permanent stocks of the L5178Y TK$^{+/-}$ cells are stored in liquid nitrogen, and subcultures are prepared from the frozen stocks for experimental use. Prior to use cells were cleansed of pre-existing mutants.

3.5 Culture media

The following culture media were used:

Minimal medium A

RPMI 1640 (1X)	516.1 ml
L-glutamine (200 mM)	5.4 ml
Sodium pyruvate (100 mM)	6.0 ml
Non-essential amino acids (100X)	5.4 ml
Streptomycin sulphate 50.000 IU/ml	
Penicillin G 50.000 units/ml	1.1 ml
F 68 Pluronic	6.0 ml

Minimal medium B

RPMI 1640 (1X)	522.1 ml
L-glutamine (200 mM)	5.4 ml
Sodium pyruvate (100 mM)	6.0 ml
Non-essential amino acids (100X)	5.4 ml
Streptomycin sulphate 50.000 units/ml	
Penicillin G 50.000 units/ml	1.1 ml

Complete medium (5%)

Minimal medium A	950 ml
Horse serum (heat-inactivated)	50 ml

Complete medium (10%)

Minimal medium A	900 ml
Horse serum (heat-inactivated)	100 ml

Complete medium A (20%)

Minimal medium A	800 ml
Horse serum (heat-inactivated)	200 ml

Complete medium B (20%)

Minimal medium B	800 ml
Horse serum (heat-inactivated)	200 ml

4. METHODS

4.1 Preparation of test cell cultures

A cell suspension (1 x 10^6 cells/ml) in complete medium was prepared. A common pool was used for each experiment to prepare the test cultures in appropriately labelled conical screw-cap tissue culture tubes.
The treatment media were prepared as follows:

Without S9 metabolism – 3-hour treatment time

Cell suspension (1 x 10^6 cells/ml in complete medium 5%)	10.0 ml
Complete medium (5%)	9.8 ml
Control or Test item solution	0.2 ml
	20.0 ml

Without S9 metabolism – 24-hour treatment time

Cell suspension (1 x 10^6 cells/ml in complete medium 10%)	3.0 ml
Complete medium (10%)	16.8 ml
Control or Test item solution	0.2 ml
	20.0 ml

With S9 metabolism – 3-hour treatment time

Cell suspension (1 x 10^6 cells/ml in complete medium 5%)	10.0 ml
S9 mix	9.8 ml
Control or Test item solution	0.2 ml
	20.0 ml

The cultures were incubated at 37°C. At the end of the incubation period, the treatment medium was removed and the cultures centrifuged and washed twice with Phosphate Buffered Saline (PBS).

4.2 Cytotoxicity assay

A preliminary cytotoxicity test was performed in order to select appropriate dose levels for the mutation assays. In this test a wide range of dose levels of the test item was used and the survival of the cells was subsequently determined.

Treatments were performed in the absence and in the presence of S9 metabolic activation for 3 hours and for 24 hours only in the absence of S9 metabolic activation. A single culture was used at each test point. After washing in Phosphate Buffered Saline (PBS), cells were resuspended in 20 ml RPMI minimal medium A. Cell concentrations were adjusted to 8 cells/ml using complete medium (20%) and, for each dose level, 0.2 ml was plated into 96 microtitre wells. The plates were incubated at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) for 8-9 days. Wells containing viable clones were identified by the eye using background illumination and then counted.

4.3 Mutation assay

4.3.1 Treatment of cell cultures

Experiments were performed including untreated, vehicle and positive controls, in the absence and presence of S9 metabolising system.

Preparation of test cell cultures was performed as described in section 4.1. Duplicate cultures were prepared at each test point, with the exception of the positive controls which were prepared in a single culture.

In the first experiment, the cells were exposed to the test item for a short treatment time (3 hours). Since negative results were obtained in the first experiment without metabolic activation, the second experiment in the absence of S9 metabolism, was performed using a long treatment time (24 hours).

After washing in Phosphate Buffered Saline (PBS), cells were resuspended in fresh complete medium (10%) and cell densities were determined. The number of cells was adjusted to give 2 x 10^5 cells/ml.
The cultures were incubated at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) to allow for expression of the mutant phenotype.

4.3.2 Determination of survival

Following adjustment of the cell densities, samples of the cultures were diluted to 8 cell/ml using complete medium A (20%). A 0.2 ml aliquot of each diluted culture was placed into each well of two 96-well plates. The plates were incubated at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) for 8 days. After incubation, wells containing viable clones were identified by the eye using background illumination and then counted.

4.3.3 Expression period

During the expression period (two days after treatment) the cell populations were subcultured in order to maintain them in exponential growth. At the end of this period the cell densities of each culture were determined and adjusted to give 2 x 10^5 cells/ml.

4.3.4 Plating for 5-trifluorothymidine resistance

After dilution, the cell suspensions in complete medium B (20%) were supplemented with trifluorothymidine (final concentration 3.0 µg/ml) and an estimated 2 x 10^3 cells was plated in each well of four 96-well plates.

Plates were incubated at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) for 14 days and wells containing clones were identified as described in section 4.3.2 and counted. In addition, the number of wells containing large colonies and the number containing small colonies were scored.

4.3.5 Plating for viability

After dilution, in complete medium A (20%), an estimated 1.6 cells/well was plated in each well of two 96-well plates. These plates were incubated at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) for 14 days and wells containing clones were identified as above and counted.

5. ANALYSIS OF RESULTS

5.1 Survival or viability

The following formula was used to determine the plating efficiency (PE):

$$PE = \frac{-\ln (ys/ns)}{fs}$$

where:

ys = Number of empty wells (without clones)
ns = Total number of wells
fs = Number of cells plated per well (1.6 cells/well)

Day 1 plating efficiencies in test and control cultures were compared to give the relative plating efficiency (RPE). Since reduction in post-treatment cell count was observed, relative survival calculation was corrected as follows:

%RS = RPE x cell count factor

where:

$$\text{cell count factor} = \frac{\text{treated post-treatment cell count}}{\text{control post-treatment cell count}}$$

5.2 Total suspension growth and relative total growth

In order to aid toxicity data interpretation the relative total growth (RTG), expressed as a percentage of the concurrent negative control, was also calculated. This is a product of the relative suspension growth (RSG) and the Day 2 relative plating efficiency (RPE), expressed as a percentage of the concurrent negative control, for each culture, as follows:

RTG = RSG x RPE (Day 2)

Relative suspension growth (RSG) is given by: [TSG(test) / TSG (control)] x 100

where TSG (total suspension growth) is calculated as follows:

$$TSG = \frac{[\text{post-treatment cell count}]}{[\text{pre-treatment cell count}]} \times \frac{[\text{Day 1 cell count}]}{[2 \times 10^5 *]} \times \frac{[\text{Day 2 cell count}]}{[2 \times 10^5 *]}$$

* Or appropriate cell concentration if lower

5.3 Growth factor over two days

The suspension growth factor (SG) over two days is a parameter to evaluate the number of generations during the phenotypic expression time and is calculated as follows:

$$SG = \frac{[\text{Day 1 cell count}]}{[2 \times 10^5 *]} \times \frac{[\text{Day 2 cell count}]}{[2 \times 10^5 *]}$$

* Or appropriate cell concentration if lower

5.4 Mutation frequency

Mutant frequencies (MF) for each treatment were calculated using Poisson statistics.
The following formula was used:

$MF = [PE(mutant)/PE(viable)] \times 10^6$

Therefore:

$$MF = \left\{ \frac{-\ln (ym/nm)}{fm} / \frac{-\ln (ys/ns)}{fs} \right\} \times 10^6$$

where:

ym = Number of empty wells (mutant plates)
nm = Total number of wells (mutant plates)
fm = Number of cells plated per well (2×10^3 cell/well)

ys = Number of empty wells (viability plates)
ns = Total number of wells (viability plates)
fs = Number of cells plated per well (1.6 cells/well)

5.5 Statistical analysis

Statistical analysis was performed according to UKEMS guidelines (Robinson W.D., 1990).

5.5.1 Test for consistency between plates

Results of individual plates within a replicate treatment were checked for consistency by calculation of the term χ^2 as follows:

$$\chi^2 = \frac{N(N\Sigma[yi^2/ni]-Y^2)}{Y(N-Y)}$$

where:

N = Total number of wells
Y = Total number of empty wells
ni = Total number of wells in the *i*th plate
yi = Total number of empty wells in the *i*th plate

χ^2 was compared with the critical values of the chi-squared distribution ($\alpha = 0.001$) with M-1 degrees of freedom, where M is the number of plates used.

5.5.2 Heterogeneity factors for replicate cultures

For negative control and test item treatments, the consistency between replicate cultures was evaluated by calculation of the heterogeneity factor (H) from a table constructed as follows:

	Empty wells	Total wells	Proportion
Culture A	y_A	n_A	$p_A = y_A / n_A$
Culture B	y_B	n_B	$p_B = y_B / n_B$
Sum	$Y = y_A + y_B$	$N = n_A + n_B$	$P = Y / N$

Where:

$d = p_A - p_B$

$V = P(1 - P)(1/ n_A + 1/ n_B)$

and $H = \frac{d^2}{V}$

The heterogeneity factors (H) were calculated for survival (Hs), viability (Hv) and mutation (Hm). Values obtained should not exceed 10.8 times the current heterogeneity factor where 10.8 is the one-sided 0.1% level of the F-distribution with 1 and infinite degrees of freedom.

5.5.3 Test for overall consistency

The overall consistency was evaluated by the calculation of the following ratio:

H experiment / current heterogeneity factor

where H experiment are the mean values for Hs, Hv and Hm.

This ratio should not exceed the one-sided 1% critical values from the F-distribution (the number of degrees of freedom at the numerator is equal to the number of pairs of cultures, whereas the number of degrees of freedom at the denominator is infinite).

5.5.4 Updated heterogeneity factors

The estimated H experiment values were combined with the current heterogeneity factors to define the updated estimate factors as follows:

Updated heterogeneity = 1/20 H experiment + 19/20 current heterogeneity

5.5.5 Comparison of each treatment with the control

The control log mutant frequency (LMF*c*) was compared with the log mutant frequency from each treatment dose (LMF*i*) by calculation of:

$$\frac{Di^2}{var(Di)}$$

where:

$Di = LMFi - LMFc$

$var(Di) = Vi + Vc$

V*i* and V*c* are the variances of the treatments and control LMF. The heterogeneity factors were used to modify the estimate of variances.
For each comparison of treatment with control, the ratio $Di^2/var(Di)$ was compared to the critical values for the one tailed Dunnett's test.

5.5.6 Test for linear trend

The evaluation of a linear trend in mutant frequency with treatment dose was performed using weighted regression.
The weights were obtained from the variances of LMF (V) as follows:

$$W = \frac{1}{V\,(MF)^2}$$

The slope *b* and its variance *var(b)* were calculated to form the test statistic $b^2/var(b)$ which was compared with tabulated critical values of χ^2 with 1 degree of freedom.

5.6 Acceptance criteria

The assay was considered valid if the following criteria were met:

(i) The cloning efficiencies at Day 2 in the untreated control cultures in the absence of S9 metabolic activation fell within the range of 65-120%.

(ii) The untreated control growth factor in the absence of S9 metabolic activation over 2 days fell within the range of 8 – 32.

(iii) The mutant frequencies in the untreated control cultures fell within the range of 50-200 x 10^6 viable cells.

(iv) The positive control chemicals induced a clear increase in mutant frequency (the difference between the positive and negative control mutant frequencies was greater than half the historical mean value).

5.7 Criteria for outcome of assay

For a test item to be considered mutagenic in this assay, it is required that:

(i) The mutant frequency at one or more doses is statistically significantly greater than that of the negative control.

(ii) There is a significant dose-relationship as indicated by the linear trend analysis.

Any increase in mutant frequency should lie outside the historical control range to have biological relevance.

6. RESULTS

6.1 Solubility test

As indicated by the Sponsor, the test item was found to be miscible in ethanol at a concentration of 500 mg/ml. This solvent was selected since it is compatible with the survival of the cells and the S9 metabolic activity.

A dilution series in ethanol was prepared and aliquots were added to RPMI complete medium (10%) in the ratio 1 : 100. The addition of a stock solution at 500 mg/ml generated precipitation, while an aliquot at 250 mg/ml generated a clear solution. On the basis of this result a concentration of 5000 µg/ml was selected as the highest dose level to be used in the cytotoxicity test.

During the toxicity test, upon addition of [redacted] 7850 to the cultures a gel formation was observed at the dose levels of 5000 and 2500 µg/ml, both in the absence and presence of S9 metabolism. At the end of the treatment incubation period, precipitation was observed at the dose levels of 5000, 2500, 1250 and 625 µg/ml in the presence of S9.

During the first experiment no precipitation was observed at the beginning of treatment, while precipitation was observed at the end of the treatment incubation period at concentration levels between 80.0 and 150 µg/ml.

During the second experiment precipitation was observed at the end of the treatment incubation period at the three highest dose levels, in the presence of S9 metabolic activation.

6.2 Cytotoxicity test

Both in the absence and presence of S9 metabolism, the test item was assayed at a maximum concentration of 5000 µg/ml and at a wide range of lower dose levels: 2500, 1250, 625, 313, 156, 78.1, 39.1 and 19.5 µg/ml. The cell count factors, the raw plate counts and relative survival values are presented in Tables 1 to 3.
Following treatment in the absence of S9 metabolic activation, using a 3-hour treatment time, no cells survived at dose levels between 156 and 5000 µg/ml. Slight toxicity was observed over the remaining concentrations reducing survival to 74% of the concurrent negative control at 78.1 µg/ml. Using a long treatment time, no cell survived at concentrations between 313 and 5000 µg/ml, while at the next lower dose level (156 µg/ml) survival was reduced to 44% of the negative control value. Following treatment in the presence of S9 metabolic activation, no cells survived at concentrations between 313 and 5000 µg/ml. Dose-related toxicity was observed over the remaining dose levels, reducing survival to 8% of the negative control value at 156 µg/ml.

6.3 Mutation assays

6.3.1 Experimental design

Two independent assays for mutation to trifluorothymidine resistance were performed using dose levels described in the following table:

Assay No.:	S9	Treatment Time (hours)	Dose-level (μg/ml)
1	–	3	125, 100, 80.0, 40.0, 20.0, 10.0 and 5.00
1	+	3	150, 100, 75.0, 37.5, 18.8 and 9.38,
2	–	24	160, 120, 80.0, 40.0, 20.0 and 10.0
2	+	3	120, 100, 83.3, 69.4 and 57.9

The results of the mutation assays are presented in Tables 4-9 (without S9) and 10-15 (with S9) and summarised in Tables 17-18.

Untreated, solvent and positive control cultures were included in each mutation experiment in the absence and presence of S9 metabolism. From the data it can be seen that the mutant frequencies in the negative control cultures fell within the normal range (50-200 x 10^{-6} viable cells). The positive control chemicals induced clear increases in mutant frequency (the difference between the positive and negative control mutant frequencies was greater than half the historical mean value).
The cloning efficiencies at Day 2 in the untreated control cultures fell within the range of 65-120% and the control growth factor over 2 days fell within the range of 8 – 32 in both experiments.

Results obtained are presented in the following table:

Assay No.:	**S9**	**Growth Factor**	**Cloning efficiency (%)**
1	–	21	116
2	–	31	102

The study was accepted as valid.

6.3.2 Survival after treatment

Results of relative survival are presented in Tables 4, 7, 10 and 13.
Additional data of toxicity (RTG values) are presented in Tables 5, 8, 11 and 14.
In the absence of S9 metabolic activation, using the 3-hour treatment time, very few cells survived at the highest dose level (125 μg/ml). Marked toxicity was observed at the next lower concentration (100 μg/ml) reducing survival to 23% of the concurrent negative control value. The relative total growth was reduced to 31% at the same concentration. In the second experiment, using a long treatment time, no cells survived at the highest concentration tested (160 μg/ml), while at the next lower dose level (120 μg/ml) the %RS and RTG values were reduced to 47% and 30% of the concurrent negative control value, respectively.

In the presence of S9 metabolic activation, in the first experiment, very few cells survived at the highest dose level (150 μg/ml). Dose-related toxicity was observed over the remaining concentrations reducing the %RS and RTG values to 47% and 27% of the concurrent negative control value at 100 μg/ml. In the second experiment, at the highest concentration tested (120 μg/ml) no cells survived at the end of the phenotypic expression period, while at the next lower dose level (100 μg/ml) the %RS and RTG values were reduced to 18% and 15% of the concurrent negative control value, respectively.

It may be noted that in Experiment 1 in the presence of S9 metabolic activation the heterogeneity between replicate cultures for survival at the end of treatment, at the concentration of 100 and 75.0 μg/ml was higher than usual. This has in no way affected the validity of the study.

6.3.3 Mutation results

In the absence of S9 metabolic activation, a statistically significant increase in mutant frequency was observed at the lowest concentration tested in the second experiment. However, the mutant frequency observed at this dose level was within the historical control range at RTC. In addition, no statistically significant dose-relationship was observed. Hence, the slight increases observed were considered to be attributable to a chance event not related to the action of [redacted] 7850 and of no biological significance.

In the presence of S9 metabolic activation, no statistically significant increases in mutant frequency were observed at any dose level, in any experiment.

It may be noted that a higher than usual heterogeneity was observed for plating efficiency results between replicate cultures at the top dose tested in Experiment 2 in the absence of S9 metabolism. This has in no way affected the validity of the study.

For the untreated, vehicle and positive controls, the number of wells containing small colonies and the number containing large colonies were scored. The small and large colony mutant frequencies were estimated and the proportion of small mutant colonies was calculated. Results are presented in Table 16. Good recovery of small colony mutants was observed following treatment with the positive controls.

6.4 Osmolality and pH

The pH values and osmolality of the post-treatment media were determined and results are presented in Table 19. The addition of the test item solution did not have any obvious effect on the osmolality or pH of the treatment medium.

7. CONCLUSIONS

It is concluded that [redacted] 7850 does not induce mutation at the TK locus of L5178Y mouse lymphoma cells *in vitro* in the absence or presence of S9 metabolic activation, under the reported experimental conditions.

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9. TABLES 1 TO 19

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 1 - CYTOTOXICITY TEST

STUDY NO.: 52410

SOLVENT: Ethanol

TREATMENT: 3 HOURS - WITHOUT S9

Dose-level (μg/ml)	Cells x 10^6 per ml Post treatment	Cell count factor	Total wells	Plate counts#		Cloning efficiency	Survival (%)
Ethanol 1%	0.65	1.00	192	75	72	0.91	100
19.5	0.56	0.86	192	72	74	0.89	85
39.1	0.49	0.75	192	68	77	0.88	73
78.1	0.56	0.86	192	68	69	0.78	74
156	0.06	0.09	-	§	§	§	0
313	0	0.00	-	§	§	§	0
625	0	0.00	-	§	§	§	0
1250	0	0.00	-	§	§	§	0
2500	0	0.00	-	§	§	§	0
5000	0	0.00	-	§	§	§	0

= Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for survival
§ = Insufficient viable cells recovered after treatment incubation period

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 2 - CYTOTOXICITY TEST

STUDY NO.: 52410

SOLVENT: Ethanol

TREATMENT: 24 HOURS - WITHOUT S9

Dose-level (μg/ml)	Cells x 10^6 per ml Post treatment	Cell count factor	Total wells	Plate counts#		Cloning efficiency	Survival (%)
Ethanol 1%	0.54	1.00	192	70	64	0.75	100
19.5	0.56	1.04	192	69	63	0.73	101
39.1	0.48	0.89	192	75	75	0.95	113
78.1	0.43	0.80	192	70	71	0.83	88
156	0.23	0.43	192	68	69	0.78	44
313	0.01	0.02	-	§	§	§	0
625	0	0.00	-	§	§	§	0
1250	0	0.00	-	§	§	§	0
2500	0	0.00	-	§	§	§	0
5000	0	0.00	-	§	§	§	0

= Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for survival
§ = Insufficient viable cells recovered after treatment incubation period

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 3 - CYTOTOXICITY TEST

STUDY NO.: 52410

SOLVENT: Ethanol

TREATMENT: 3 HOURS - WITH S9

Dose-level (μg/ml)	Cells x 10^6 per ml Post treatment	Cell count factor	Total wells	Plate counts#		Cloning efficiency	Survival (%)
Ethanol 1%	0.49	1.00	192	65	60	0.66	100
19.5	0.34	0.69	192	70	68	0.79	84
39.1	0.36	0.73	192	62	63	0.66	73
78.1	0.32	0.65	192	60	69	0.70	69
156	0.10	0.20	192	32	36	0.27	8
313	0.02	0.04	-	§	§	§	0
625	0	0.00	-	§	§	§	0
1250	0	0.00	-	§	§	§	0
2500	0	0.00	-	§	§	§	0
5000	0	0.00	-	§	§	§	0

= Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for survival
§ = Insufficient viable cells recovered after treatment incubation period

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 4 - DAY 0 - SURVIVAL

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITHOUT S9

Dose-level (μg/ml)		Cells x 10^6 per ml Post treatment	Cell count factor	Total wells	Plate counts# (Day 0)		Cloning efficiency	Survival (%)
CM	A	0.36	0.74	192	83	85	1.19	85
	B	0.39		192	78	81		
Ethanol	A	0.47	1.00	192	74	78	1.03	100
1%	B	0.55		192	82	76		
5.00	A	0.44	0.84	192	71	75	0.86	70
	B	0.42		192	68	73		
10.0	A	0.39	0.70	192	80	84	1.27	86
	B	0.33		192	82	88		
20.0	A	0.43	0.75	192	76	81	1.05	77
	B	0.33		192	76	80		
40.0	A	0.50	0.92	192	67	68	0.80	72
	B	0.45		192	73	70		
80.0	A	0.34	0.64	192	77	77	1.10	68
	B	0.31		192	80	84		
100	A	0.21	0.39	192	70	66	0.61	23
	B	0.19		192	50	54		
125	A	0.06	0.10	-	§	§	§	0
	B	0.05		-	§	§		
MMS 10.0	A	0.37	0.97	192	80	83	1.18	96

# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for survival
CM = Culture medium
A and B = Replicate cultures
§ = Insufficient viable cells recovered after treatment incubation period

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 5 - DAY 2 - TOTAL GROWTH AND VIABILITY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITHOUT S9

Dose-level (μg/ml)		Cells x 10^6 per ml Day 1	Cells x 10^6 per ml Day 2	Total wells	Plate counts# (Day 2)		Cloning Efficiency Day 2	TSG	RTG
CM	A	1.11	0.69	192	80	88	1.16	15.3	121
	B	1.00	0.86	192	79	76			
Ethanol	A	0.61	0.88	192	83	78	1.11	13.2	100
1%	B	0.60	0.84	192	77	81			
5.00	A	0.69	1.03	192	71	74	1.00	13.8	94
	B	0.66	0.87	192	81	79			
10.0	A	0.77	0.80	192	81	75	1.07	11.9	87
	B	0.91	0.79	192	82	77			
20.0	A	0.79	0.74	192	83	84	1.20	13.7	111
	B	1.03	0.87	192	82	78			
40.0	A	0.71	0.57	192	87	79	1.33	10.5	95
	B	0.76	0.64	192	86	86			
80.0	A	0.82	0.65	192	83	78	1.19	9.8	80
	B	0.92	0.74	192	85	81			
100	A	0.60	0.68	192	82	81	1.33	3.4	31
	B	0.50	0.54	192	90	84			
MMS 10.0	A	0.81	0.89	192	85	79	1.20	13.3	90

# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for viability
CM = Culture medium
A and B = Replicate cultures

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 6 - MUTANT FREQUENCY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITHOUT S9

Dose-level (μg/ml)		Total wells	Resistant mutants# after day 2				Mean mutant frequency (Per 10^6 viable cells)	
CM	A	384	23	26	24	19	90.99	-
	B	384	16	11	16	10		
Ethanol	A	384	24	23	23	26	124.1	-
1%	B	384	22	20	22	25		
5.00	A	384	6	10	8	6	60.83	NS
	B	384	13	17	17	10		
10.0	A	384	10	9	7	11	52.62	NS
	B	384	15	6	11	13		
20.0	A	384	19	14	13	11	66.75	NS
	B	384	14	18	11	13		
40.0	A	384	18	16	17	16	65.80	NS
	B	384	12	14	17	13		
80.0	A	384	14	17	16	14	71.90	NS
	B	384	14	16	16	14		
100	A	384	15	15	16	22	68.81	NS
	B	384	22	20	15	15		
MMS 10.0	A	384	56	55	48	50	326.5	
Linear trend								NS

\# = Wells with clones/plate - 2 x 10^3 cells in each well of four 96-well plates plated for 5-TFT resistance
A and B = Replicate cultures
CM = Culture medium

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 7 - DAY 0 - SURVIVAL

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 24 HOURS - WITHOUT S9

Dose-level (μg/ml)		Cells x 10^6 per ml Post treatment	Cell count factor	Total wells	Plate counts# (Day 0)		Cloning efficiency	Survival (%)
CM	A	0.29	1.33	192	80	80	1.11	135
	B	0.35		192	80	79		
Ethanol 1%	A	0.24	1.00	192	81	75	1.10	100
	B	0.24		192	82	80		
10.0	A	0.31	1.05	192	74	79	0.95	90
	B	0.20		192	75	72		
20.0	A	0.21	1.11	192	80	76	0.96	97
	B	0.33		192	75	70		
40.0	A	0.27	1.01	192	80	82	1.00	92
	B	0.22		192	74	70		
80.0	A	0.17	0.78	192	80	80	0.98	70
	B	0.21		192	71	73		
120	A	0.13	0.52	192	70	79	1.00	47
	B	0.12		192	77	80		
160	A	0.01	0.01	-	§	§	§	0
	B	0.00		-	§	§		
MMS 5.00	A	0.28	0.88	192	68	70	0.79	62

\# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for survival
CM = Culture medium
A and B = Replicate cultures
§ = Insufficient viable cells recovered after treatment incubation period

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 8 - DAY 2 - TOTAL GROWTH AND VIABILITY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 24 HOURS - WITHOUT S9

Dose-level (μg/ml)		Cells x 10^6 per ml Day 1	Cells x 10^6 per ml Day 2	Total wells	Plate counts# (Day 2)		Cloning Efficiency Day 2	TSG	RTG
CM	A	1.19	1.18	192	70	69	1.02	65.5	149
	B	1.32	0.82	192	81	84			
Ethanol 1%	A	1.25	0.63	192	84	84	1.27	35.0	100
	B	1.28	0.75	192	81	85			
10.0	A	0.70	0.80	192	83	85	1.08	25.2	61
	B	0.62	1.04	192	70	73			
20.0	A	1.23	0.80	192	76	80	1.07	34.8	84
	B	0.81	0.79	192	77	82			
40.0	A	0.78	0.84	192	81	79	1.17	26.1	69
	B	0.95	0.65	192	81	84			
80.0	A	0.91	0.66	192	74	76	1.04	22.2	52
	B	1.09	0.64	192	79	82			
120	A	0.42	0.65	192	81	76	1.53	8.9	30
	B	0.37	0.70	192	93	91			
MMS 5.00	A	1.35	0.67	192	79	76	1.03	42.2	65

# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for viability
CM = Culture medium
A and B = Replicate cultures

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 9 - MUTANT FREQUENCY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 24 HOURS - WITHOUT S9

Dose-level (μg/ml)		Total wells	Resistant mutants# after day 2				Mean mutant frequency (Per 10^6 viable cells)	
CM	A	384	12	24	14	16	66.59	-
	B	384	8	5	11	15		
Ethanol 1%	A	384	27	27	15	20	82.74	-
	B	384	17	17	11	12		
10.0	A	384	30	28	30	34	142.0	*
	B	384	20	16	22	16		
20.0	A	384	14	17	15	18	105.1	NS
	B	384	27	25	18	21		
40.0	A	384	14	11	19	11	73.88	NS
	B	384	13	17	18	19		
80.0	A	384	18	13	9	14	82.61	NS
	B	384	19	19	14	15		
120	A	384	12	19	10	12	66.05	NS
	B	384	22	18	16	18		
MMS 5.00	A	384	59	53	45	54	381.8	-
Linear trend								NS

#	= Wells with clones/plate - 2 x 10^3 cells in each well of four 96-well plates plated for 5-TFT resistance
CM	= Culture medium
A and B	= Replicate cultures
NS	= Not statistically significant
*	= Statistically significant at P<5%

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 10 - DAY 0 - SURVIVAL

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITH S9

Dose-level (µg/ml)		Cells x 10^6 per ml Post treatment	Cell count factor	Total wells	Plate counts# (Day 0)		Cloning efficiency	Survival (%)
CM	A	0.25	1.04	192	75	76	1.16	133
	B	0.25		192	86	87		
Ethanol 1%	A	0.18	1.00	192	74	79	0.91	100
	B	0.30		192	68	73		
9.38	A	0.27	1.10	192	72	77	1.01	124
	B	0.26		192	77	82		
18.8	A	0.23	1.01	192	74	76	0.91	101
	B	0.26		192	72	72		
37.5	A	0.20	1.01	192	72	75	0.79	88
	B	0.29		192	67	61		
75.0	A	0.10	0.63	192	89	86	1.05	72
	B	0.20		192	67	70		
100	A	0.18	0.60	192	53	57	0.70	47
	B	0.11		192	75	73		
150	A	0.04	0.17	-	§	§	§	0
	B	0.04		-	§	§		
B(a)P 2.00	A	0.38	1.50	192	35	41	0.31	41

\# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for survival
CM = Culture medium
A and B = Replicate cultures
§ = Insufficient viable cells recovered after treatment incubation period

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 11 - DAY 2 - TOTAL GROWTH AND VIABILITY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITH S9

Dose-level (µg/ml)		Cells x 10^6 per ml Day 1	Cells x 10^6 per ml Day 2	Total wells	Plate counts# (Day 2)		Cloning Efficiency Day 2	TSG	RTG
CM	A	0.84	0.62	192	81	78	1.23	7.3	171
	B	1.00	0.65	192	85	85			
Ethanol 1%	A	0.32	1.06	192	74	77	1.07	4.9	100
	B	0.67	0.67	192	79	84			
9.38	A	0.77	0.76	192	81	74	1.01	8.0	155
	B	0.73	0.86	192	78	75			
18.8	A	0.66	0.75	192	81	80	1.25	5.5	131
	B	0.58	0.71	192	86	84			
37.5	A	0.74	0.80	192	81	80	1.12	6.6	141
	B	0.58	0.87	192	80	79			
75.0	A	0.44	0.76	192	86	78	1.06	3.1	63
	B	0.83	0.55	192	74	74			
100	A	0.18	0.64	192	87	85	1.29	1.1	27
	B	0.24	0.82	192	84	78			
B(a)P 2.00	A	0.41	1.18	192	48	47	0.43	9.2	44

\# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for viability
CM = Culture medium
A and B = Replicate cultures

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 12 - MUTANT FREQUENCY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITH S9

Dose-level (μg/ml)		Total wells	Resistant mutants# after day 2				Mean mutant frequency (Per 10^6 viable cells)	
CM	A	384	17	14	12	12	67.41	-
	B	384	10	14	20	17		
Ethanol 1%	A	384	13	9	9	17	83.49	-
	B	384	24	13	19	21		
9.38	A	384	15	17	9	19	81.62	NS
	B	384	15	14	14	14		
18.8	A	384	19	25	26	20	105.8	NS
	B	384	24	22	28	13		
37.5	A	384	12	14	8	14	83.50	NS
	B	384	19	25	14	25		
75.0	A	384	20	14	11	18	71.70	NS
	B	384	14	10	9	11		
100	A	384	14	20	17	12	80.23	NS
	B	384	19	21	16	23		
B(a)P 2.00	A	384	69	60	59	48	1117	-
Linear trend								NS

# = Wells with clones/plate - 2 x 10^3 cells in each well of four 96-well plates plated for 5-TFT resistance
CM = Culture medium
A and B = Replicate cultures
NS = Not statistically significant

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 13 - DAY 0 - SURVIVAL

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 3 HOURS - WITH S9

Dose-level (μg/ml)		Cells x 10^6 per ml Post treatment	Cell count factor	Total wells	Plate counts# (Day 0)		Cloning efficiency	Survival (%)
CM	A	0.47	1.06	192	71	75	0.91	111
	B	0.49		192	74	75		
Ethanol 1%	A	0.46	1.00	192	73	73	0.87	100
	B	0.44		192	70	72		
57.9	A	0.25	0.68	192	67	69	0.79	62
	B	0.36		192	71	68		
69.4	A	0.30	0.72	192	52	59	0.52	43
	B	0.35		192	55	51		
83.3	A	0.26	0.55	192	44	46	0.46	29
	B	0.23		192	57	53		
100	A	0.13	0.37	192	47	50	0.42	18
	B	0.21		192	47	44		
120	A	0.17	0.32	192	16	26	0.21	8
	B	0.13		192	38	31		
B(a)P 2.00	A	0.38	0.54	192	57	53	0.53	31

# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for survival
CM = Culture medium
A and B = Replicate cultures

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 14 - DAY 2 - TOTAL GROWTH AND VIABILITY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 3 HOURS - WITH S9

Dose-level (µg/ml)		Cells x 10^6 per ml Day 1	Cells x 10^6 per ml Day 2	Total wells	Plate counts# (Day 2)		Cloning Efficiency Day 2	TSG	RTG
CM	A	0.60	0.90	192	77	71	0.86	13.6	130
	B	0.57	1.04	192	70	69			
Ethanol 1%	A	0.43	0.71	192	82	82	1.16	7.8	100
	B	0.52	0.75	192	78	82			
57.9	A	0.60	0.67	192	76	81	1.05	5.7	66
	B	0.59	0.60	192	78	77			
69.4	A	0.37	0.72	192	76	79	1.06	3.9	45
	B	0.32	0.68	192	78	80			
83.3	A	0.29	0.70	192	80	81	1.14	2.4	30
	B	0.35	0.52	192	82	79			
100	A	0.15	0.55	192	77	83	1.02	1.3	15
	B	0.17	0.93	192	74	74			
120	A	0.03	§	-	§	§	§	0	0
	B	0.04	§	-	§	§			
B(a)P 2.00	A	0.43	0.66	192	76	69	0.88	5.4	40

\# = Wells with clones/plate - 1.6 cells in each well of two 96-well plates plated for viability
CM = Culture medium
A and B = Replicate cultures
§ = Insufficient viable cells recovered at Day 1

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 15 - MUTANT FREQUENCY

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 3 HOURS - WITH S9

Dose-level (μg/ml)		Total wells	Resistant mutants# after day 2				Mean mutant frequency (Per 10^6 viable cells)	
CM	A	384	11	8	17	16	74.19	-
	B	384	8	11	11	10		
Ethanol 1%	A	384	23	16	16	20	92.26	-
	B	384	22	19	16	16		
57.9	A	384	9	13	22	16	78.99	NS
	B	384	17	14	17	9		
69.4	A	384	22	11	17	12	90.87	NS
	B	384	15	17	22	18		
83.3	A	384	23	11	21	11	84.81	NS
	B	384	16	21	13	19		
100	A	384	18	16	15	11	77.85	NS
	B	384	13	13	12	14		
B(a)P 2.00	A	384	75	73	72	76	837.5	-
Linear trend								NS

\# = Wells with clones/plate - 2 x 10^3 cells in each well of four 96-well plates plated for 5-TFT resistance
CM = Culture medium
A and B = Replicate cultures
NS = Not statistically significant

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 16 - SMALL AND LARGE COLONY MUTANT FREQUENCIES

STUDY NO.: 52410

EXPERIMENT NO.: 1

Dose-level μg/ml	S9	Mutant frequency° Small colony	Large colony	Proportion small colony mutants
CM	-	37.2	49.7	0.43
Ethanol 1%	-	65.5	50.9	0.56
MMS 10.0	-	183	95.7	0.66
CM	+	31.2	34.1	0.48
Ethanol 1%	+	33.6	47.6	0.41
B(a)P 2.00	+	417	484	0.46

EXPERIMENT NO.: 2

Dose-level μg/ml	S9	Mutant frequency° Small colony	Large colony	Proportion small colony mutants
CM	-	25.9	38.6	0.40
Ethanol 1%	-	36.4	43.2	0.46
MMS 5.00	-	152	169	0.47
CM	+	35.9	36.7	0.49
Ethanol 1%	+	40.0	48.0	0.45
B(a)P 2.00	+	289	296	0.49

° = Figures displayed are mutation frequencies per 10^6 viable cells

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 17 - SUMMARY TABLE

STUDY NO.: 52410

SOLVENT: Ethanol

Dose-level (μg/ml)	%RS	RTG	MF°	P	Proportion small colony mutants
		MAIN ASSAY 1 - WITHOUT S9 (Treatment time: 3 hours)			
CM	85	121	90.99	-	0.43
Ethanol 1%	100	100	124.1	-	0.56
5.00	70	94	60.83	NS	-
10.0	86	87	52.62	NS	-
20.0	77	111	66.75	NS	-
40.0	72	95	65.80	NS	-
80.0	68	80	71.90	NS	-
100	23	31	68.81	NS	-
125	0	§	§	-	-
MMS 10.0	96	90	326.5	-	0.66
Linear trend				NS	

Dose-level (μg/ml)	%RS	RTG	MF°	P	Proportion small colony mutants
		MAIN ASSAY 2 - WITHOUT S9 (Treatment time: 24 hours)			
CM	135	149	66.59	-	0.46
Ethanol 1%	100	100	82.74	-	0.40
10.0	90	61	142.0	*	-
20.0	97	84	105.1	NS	-
40.0	92	69	73.88	NS	-
80.0	70	52	82.61	NS	-
120	47	30	66.05	NS	-
160	0	§	§	-	-
MMS 5.00	62	65	381.18	-	0.47
Linear trend				NS	

° =Figures displayed are mutation frequencies per million surviving cells
NS = Not statistically significant
§ = Insufficient viable cells recovered after treatment incubation period
* = Statistically significant at $p < 5\%$

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 18 - SUMMARY TABLE

STUDY NO.: 52410

SOLVENT: Ethanol

Dose-level (μg/ml)	MAIN ASSAY 1 - WITH S9 (Treatment time: 3 hours) %RS	RTG	MF°	P	Proportion small colony mutants
CM	133	171	67.41	-	0.48
Ethanol 1%	100	100	83.49	-	0.41
9.38	124	155	81.62	NS	-
18.8	101	131	105.8	NS	-
37.5	88	141	83.50	NS	-
75.0	72	63	71.70	NS	-
100	47	27	80.23	NS	-
150	0	§	§	-	
B(a)P 2.00	41	44	1117	-	0.46
Linear trend				NS	

Dose-level (μg/ml)	MAIN ASSAY 2 - WITH S9 (Treatment time: 3 hours) %RS	RTG	MF°	P	Proportion small colony mutants
CM	111	130	74.19	-	0.49
Ethanol 1%	100	100	92.26	-	0.45
57.9	62	66	78.99	NS	-
69.4	43	45	90.87	NS	-
83.3	29	30	84.81	NS	-
100	18	15	77.85	NS	-
120	8	§§	§§	-	-
B(a)P 2.00	31	40	837.5	-	0.49
Linear trend				NS	

° = Figures displayed are mutation frequencies per million surviving cells
NS = Not statistically significant
§ = Insufficient viable cells recovered after treatment incubation period
§§ = Insufficient viable cells recovered at Day 1

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 19 - pH AND OSMOLALITY OF TREATMENT MEDIUM

STUDY NO.: 52410

EXPERIMENT NO.: 1

SOLVENT: Ethanol

Dose-level (µg/ml)		WITHOUT S9 pH	WITHOUT S9 mOsm/kg	Dose-level (µg/ml)		WITH S9 pH	WITH S9 mOsm/kg
Culture medium	A	7.63	281	Culture medium	A	7.52	289
	B	7.64	281		B	7.53	289
Ethanol 1%	A	7.68	462	Ethanol 1%	A	7.47	475
	B	7.63	455		B	7.51	465
5.00	A	7.69	465	9.38	A	7.53	474
	B	7.72	459		B	7.53	463
10.0	A	7.71	484	18.8	A	7.42	473
	B	7.64	461		B	7.48	466
20.0	A	7.65	463	37.5	A	7.41	473
	B	7.63	458		B	7.39	467
40.0	A	7.65	462	75.0	A	7.40	475
	B	7.63	458		B	7.46	465
80.0	A	7.67	460	100	A	7.44	470
	B	7.66	454		B	7.46	466
100	A	7.69	459	150	A	7.42	471
	B	7.61	454		B	7.52	463
125	A	7.68	463				
	B	7.74	453				

The solvent, ethanol, causes an increase in the apparent osmolality of the treatment solution, since it depresses the freezing point. However, since it passes freely in and out of cells, this increase is only apparent. A small but real increase in osmotic pressure may be caused only during the period of equilibration after the addition of the solvent. The increase due to the solvent (approx. 150 mOsm/kg) can therefore be subtracted from the treatment medium values, to assess the effects of the test substance. Since the test item displaces the solvent in the test item solution, lower apparent osmotic pressures may be observed at higher dose-levels.

10. APPENDIX I - Statistical analysis

SYMBOLS USED IN THE STATISTICAL ANALYSIS

Hs	Survival heterogeneity factor
Hv	Viability heterogeneity factor
Hm	Mutation heterogeneity factor
LMF	Log mutant frequency
W	Weight for regression
V	Variance
Di	LMF treatment- LMF control
Var(d)	Variance of d
b^2 /Var(b)	Test for linear trend/slope b and its variance Var(b)
E	Extention
NA	Not applicable

[redacted] 7850: MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 1 - CONSISTENCY TEST

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITHOUT METABOLIC ACTIVATION

Test for consistency between plates

Dose-level	Survival		Viability		Mutants	
(µg/ml)	A	B	A	B	A	B
CM	0.190	0.329	3.048	0.301	1.487	2.692
Ethanol 1%	0.505	1.287	0.962	0.572	0.333	0.746
5.00	0.457	0.668	0.254	0.150	1.591	2.864
10.0	0.669	1.848	1.231	0.915	1.047	4.506
20.0	0.874	0.547	0.046	0.600	2.864	2.174
40.0	0.025	0.247	2.847	0.000	0.199	1.171
80.0	0.000	0.669	0.962	0.712	0.526	0.316
100	0.403	0.336	0.041	2.087	2.430	2.598

A and B = Replicate culture

Test for overall consistency

Dose-level (µg/ml)	Survival (Hs)	Viability (Hv)	Mutants (Hm)
Ethanol 1%	0.603	0.167	0.349
5.00	0.345	3.586	9.450
10.0	0.828	0.159	0.874
20.0	0.017	1.009	0.010
40.0	0.834	0.889	1.171
80.0	1.830	0.515	0.010
100	11.378	13.190	0.140
H experiment	2.262	2.788	1.715
Current heterogeneity	1.801	1.705	1.771
Updated heterogeneity	1.824	1.759	1.768

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 2 - CONSISTENCY TEST

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 24 HOURS - WITHOUT METABOLIC ACTIVATION

Test for consistency between plates

Dose-level	Survival		Viability		Mutants	
(µg/ml)	A	B	A	B	A	B
CM	0.000	0.037	0.026	0.388	1.228	1.424
Ethanol 1%	1.231	0.158	0.000	0.712	6.011	2.534
10.0	0.804	0.261	0.190	0.247	0.913	1.808
20.0	0.547	0.704	0.547	0.915	0.750	2.808
40.0	0.158	0.444	0.150	0.388	3.629	1.501
80.0	0.000	0.111	0.122	0.346	3.534	1.501
120	2.427	0.314	0.874	0.522	4.093	1.272

A and B = Replicate culture

Test for overall consistency

Dose-level (µg/ml)	Survival (Hs)	Viability (Hv)	Mutants (Hm)
Ethanol 1%	0.659	0.092	8.660
10.0	0.549	10.571	15.783
20.0	1.860	0.159	5.892
40.0	5.213	0.501	1.403
80.0	4.042	2.047	1.658
120	1.030	(19.091)	4.160
H experiment	2.225	2.674	6.260
Current heterogeneity	1.801	1.581	2.347
Updated heterogeneity	1.822	1.636	2.543

Values in parentheses are not included in the calculation of “H experiment”

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 3 - CONSISTENCY TEST

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1

TREATMENT: 3 HOURS - WITH METABOLIC ACTIVATION

Test for consistency between plates

Dose-level	Survival		Viability		Mutants	
(µg/ml)	A	B	A	B	A	B
CM	0.031	0.058	0.329	0.000	1.422	4.268
Ethanol 1%	0.804	0.668	0.279	1.015	4.190	4.207
9.38	0.749	0.915	1.640	0.290	4.425	0.062
18.8	0.122	0.000	0.038	0.205	2.148	7.178
37.5	0.261	0.844	0.038	0.037	2.286	5.211
75	0.581	0.229	2.676	0.000	3.703	1.437
100	0.341	0.118	0.223	1.422	2.791	1.705

A and B = Replicate culture

Test for overall consistency

Dose-level (µg/ml)	Survival (Hs)	Viability (Hv)	Mutants (Hm)
Ethanol 1%	2.090	2.516	8.036
9.38	1.640	0.066	0.091
18.8	0.522	1.773	0.066
37.5	4.625	0.075	11.274
75	(24.684)	4.376	3.920
100	(17.057)	2.299	2.212
H experiment	3.688	1.851	4.266
Current heterogeneity	1.563	1.564	2.186
Updated heterogeneity	1.669	1.578	2.290

Values in parentheses are not included in the calculation of “H experiment”

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 4 - CONSISTENCY TEST

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 2

TREATMENT: 3 HOURS - WITH METABOLIC ACTIVATION

Test for consistency between plates

Dose-level	Survival		Viability		Mutants	
(µg/ml)	A	B	A	B	A	B
CM	0.457	0.030	1.061	0.026	4.804	0.670
Ethanol 1%	0.000	0.108	0.000	0.600	2.303	1.674
57.9	0.101	0.235	0.874	0.033	7.111	3.523
69.4	1.046	0.337	0.301	0.143	5.924	1.778
83.3	0.084	0.341	0.038	0.346	9.002	2.597
100	0.188	0.188	1.350	0.000	2.054	0.178
120	3.048	1.109				

A and B = Replicate culture

Test for overall consistency

Dose-level (µg/ml)	Survival (Hs)	Viability (Hv)	Mutants (Hm)
Ethanol 1%	0.222	0.316	0.033
57.9	0.115	0.068	0.091
69.4	0.265	0.156	0.904
83.3	4.174	0.000	0.081
100	0.375	2.362	0.669
120	9.238		
H experiment	2.074	0.580	0.356
Current heterogeneity	1.669	1.578	2.290
Updated heterogeneity	1.689	1.528	2.193

Values in parentheses are not included in the calculation of “H experiment”

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 5 - MUTATION ASSAY - WITHOUT METABOLIC ACTIVATION

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1 TREATMENT TIME: 3 HOURS

Dose-level (µg/ml)	LMF	W	V	Di	Var(d)	D^2/Var(d)
Ethanol 1%	-8.994	3.876E+09	0.0167	0.000	NA	NA
5.00	-9.707	9.855E+09	0.0274	-0.713	0.044	-
10.0	-9.852	1.259E+10	0.0287	-0.858	0.045	-
20.0	-9.615	9.801E+09	0.0229	-0.620	0.040	-
40.0	-9.629	1.055E+10	0.0219	-0.635	0.039	-
80.0	-9.540	8.845E+09	0.0219	-0.546	0.039	-
100	-9.584	1.029E+10	0.0205	-0.590	0.037	-

Test for linear trend	Slope (b)	:	1.94E-08
	Var (b)	:	1.18E-14
	B^2/Var(b)	:	0.03

EXPERIMENT NO.: 2 TREATMENT TIME: 24 HOURS

Dose-level (µg/ml)	LMF	W	V	Di	Var(d)	D^2/Var(d)
Ethanol 1%	-9.400	5.760E+09	0.0254	0.000	NA	NA
10.0	-8.860	2.410E+09	0.0206	0.540	0.046	6.35E+00
20.0	-9.161	3.765E+09	0.0241	0.239	0.049	1.15E+00
40.0	-9.513	6.393E+09	0.0287	-0.113	0.054	-
80.0	-9.401	5.102E+09	0.0287	-0.002	0.054	-
120	-9.625	8.123E+09	0.0282	-0.225	0.054	-

Test for linear trend	Slope (b)	:	-1.49E-07
	Var (b)	:	1.22E-14
	B^2/Var(b)	:	-

– = Not calculated for negative Di or Slope(b) values

[redacted] 7850: MUTATION IN L5178Y $TK^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

TABLE 6 - MUTATION ASSAY - WITH METABOLIC ACTIVATION

STUDY NO.: 52410

SOLVENT: Ethanol

EXPERIMENT NO.: 1 TREATMENT TIME: 3 HOURS

Dose-level (µg/ml)	LMF	W	V	Di	Var(d)	D^2/Var(d)
Ethanol 1%	-9.391	5.800E+09	0.0247	0.000	NA	NA
9.38	-9.413	5.781E+09	0.0260	-0.023	0.051	-
18.80	-9.154	4.565E+09	0.0196	0.237	0.044	1.27E+00
37.50	-9.391	5.992E+09	0.0239	0.000	0.049	3.02E-07
75.00	-9.543	6.997E+09	0.0278	-0.152	0.053	-
100.00	-9.431	6.817E+09	0.0228	-0.040	0.048	-

Test for linear trend	Slope (b)	:	-1.22E-07
	Var (b)	:	2.02E-14
	B^2/Var(b)	:	-

EXPERIMENT NO.: 2 TREATMENT TIME: 3 HOURS

Dose-level (µg/ml)	LMF	W	V	Di	Var(d)	D^2/Var(d)
Ethanol 1%	-9.291	5.539E+09	0.0212	0.000	NA	NA
57.90	-9.446	6.395E+09	0.0251	-0.155	0.046	-
69.40	-9.306	5.339E+09	0.0227	-0.015	0.044	-
83.30	-9.375	6.146E+09	0.0226	-0.084	0.044	-
100.00	-9.461	6.373E+09	0.0259	-0.170	0.047	-

Test for linear trend	Slope (b)	:	-1.03E-08
	Var (b)	:	1.86E-14
	B^2/Var(b)	:	-

– = Not calculated for negative Di or Slope(b) values

11. APPENDIX II - Historical data

HISTORICAL DATA OF NEGATIVE/SOLVENT AND POSITIVE CONTROLS
(Mutation frequencies per million surviving cells)

WITHOUT METABOLIC ACTIVATION (3 hour treatment)		WITH METABOLIC ACTIVATION (3 hour treatment)		WITHOUT METABOLIC ACTIVATION (24 hour treatment)	
Negative control	**Positive control**	**Negative control**	**Positive control**	**Negative control**	**Positive control**
		Mean Value			
90.2	467	93.9	648	87.8	566
		Standard deviation			
30.1	150	31.8	254	32.1	190
		Upper confidence limit (P<1%)			
168	NC	176	NC	171	NC
		Observed range			
51 - [redacted]	[redacted]	44.9 - [redacted]	[redacted]	53.4 - 217	278 - 933
		n			
76	76	117	117	43	43

NC = Not calculated
n = number of experiment

12. APPENDIX III - Certificate of analysis

SOLVAY SOLEXIS

Centro Ricerche e Svilup
Viale Lombardia, 20
20021 Bollate (MI)

Pag.

Richiesta analisi Numero:

Data 23/11/2004

Risposta Numero:

Data

C.D.C.	Commessa	Richiedente	Destinatario
0316	N751	[redacted]	[redacted]

Prodotto, suoi riferimenti e precauzioni antinfortunistiche

[redacted] 7850 Acido Batch: 3223 ON

Tossico e corrosivo: utilizzare guanti e occhiali, lavorare sotto cappa

Lavoro richiesto

Caratterizzazione NMR: analisi terminali, peso molecolare

Risultati

Si allega spettro 19F-NMR con caratterizzazione del prodotto
Si nota la presenza di piccolissime quantità di estere

Distribuzione

Riferimenti 11542

Analitica e Strutturistica

Firme [redacted]

[redacted]

Page FLK7850_3223ON_
PFPE

exp2 s2pul

SAMPLE		DEC. & VT	
date	Nov 30 2004	dfrq	299.917
solvent	Acetone	dn	H1
file	/space/MYPIDS~	dpwr	30
	[redacted]/FLK7850~	dof	0
	_3223ON_001.fid	dm	nnn
ACQUISITION		dmm	c
sfrq	282.176	dmf	200
tn	F19	PROCESSING	
at	0.500	lb	2.00
np	75178	lsfid	-1
sw	75188.0	wtfile	
fb	41400	proc	lp
bs	10	fn	131072
tpwr	61		
pw	4.5	werr	reset
dl	2.000	wexp	procplot
tof	2400.0	wbs	
nt	1000	wnt	
ct	1000		
alock	n		
gain	not used		
FLAGS			
il	n		
in	n		
dp	y		
DISPLAY			
sp	-45146.6		
wp	39502.5		
vs	96		
sc	0		
wc	250		
hzmm	158.01		
is	483.57		
rfl	22965.4		
rfp	-39578.8		
th	0		
ins	140.885		
ai ph			

IS=484

FLK7850_3223ON_001.jpeg

MOL.WEIGHT = 551

EQUIV.WEIGHT = 551

C1=1.0 %.C3 "-OCF2O-"

C2=9.1 %.C3 "-OCF(CF3)O-"

-OCF3	=	3.3
-OCFCF3COOH	=	0.8
-OCF2CL	=	1.1
-OCF(CF3)CF2CL	=	15.8
-OCF2CF(CF3)CL	=	29.7
-OCF2COOH	=	49.2

SOLVAY

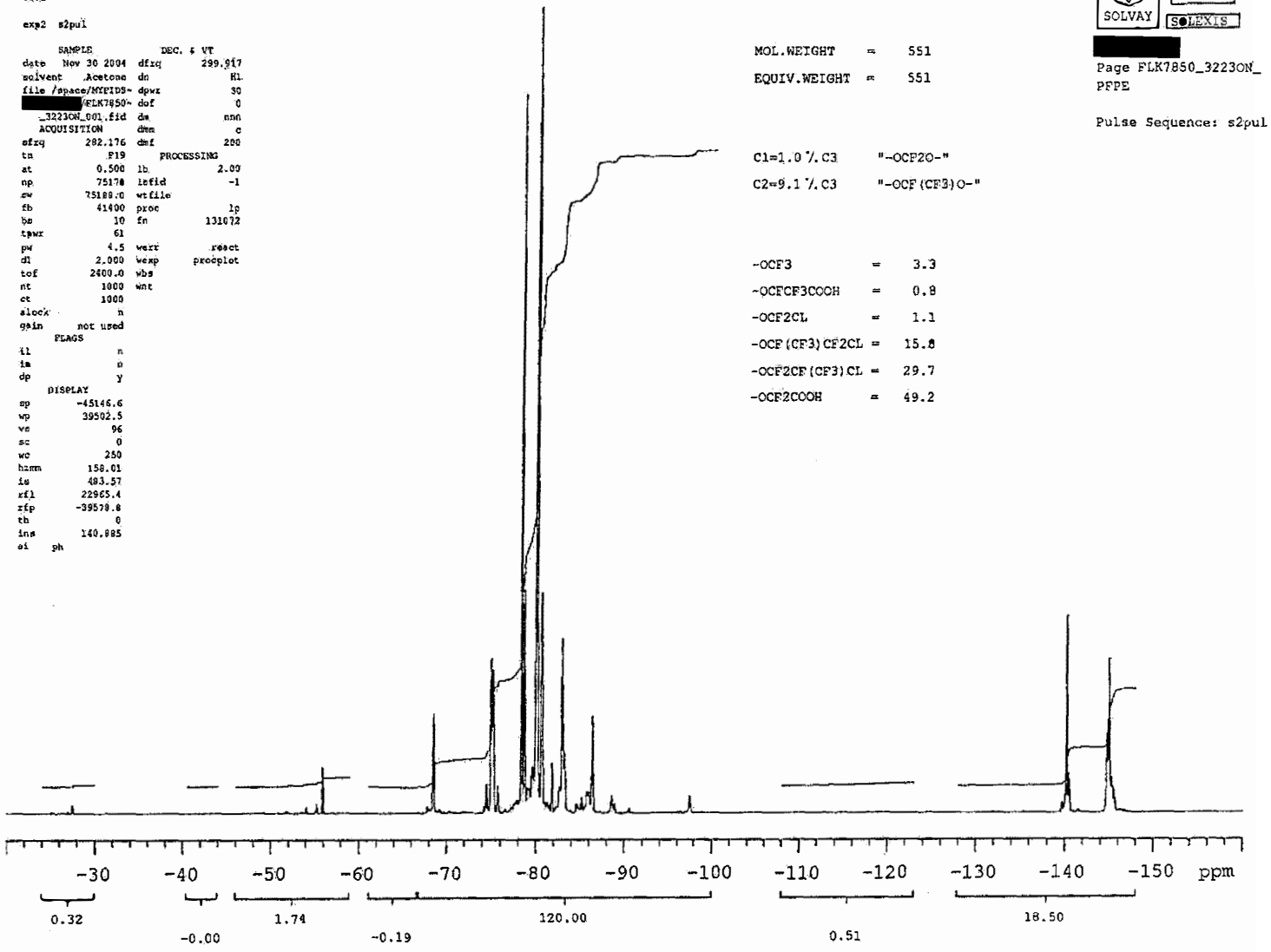
BOLLATE NMR 300

SOLEXIS

[redacted]

Page FLK7850_3223ON_
PFPE

Pulse Sequence: s2pul



13. APPENDIX IV - Study Protocol

RESEARCH TOXICOLOGY CENTRE - ROMA

Version No.: 05/1LT

[redacted] 7850

MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

Final Protocol
prepared for

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

by

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

RTC Enquiry Number: 52410

January 2006

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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: 00920811001

[redacted]7850
MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS
(FLUCTUATION METHOD)

MANAGEMENT OF STUDY

Scientific Director : [redacted]

Head of Genetic and Cellular Toxicology : [redacted]

Study Director : [redacted]

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

Monitor : [redacted]

QUALITY ASSURANCE

Quality Assurance Manager : [redacted]

LOCATION OF STUDY

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

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

TIME SCHEDULE OF STUDY

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

TEST ITEM IDENTITY

The test item will be : [redacted]7850

MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

1. INTRODUCTION

1.1 Objective

To assay the test item for the ability to induce mutations in L5178Y TK$^{+/-}$ mouse lymphoma cells cultured after *in vitro* treatment in the absence and presence of S9 metabolism.

1.2 Regulatory requirements

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

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

In addition, the study is designed to comply with the experimental methods indicated in the guidelines of:

- EEC Council Directive 2000/32, Annex 4E.
- OECD Guidelines for the testing of chemicals No. 476 (Adopted July 1997).
- ICH, Topic S2A, Genotoxicity: Specific Aspects of Regulatory Tests, Step 4 (Document, July 1995).
- ICH, Topic S2B, A Standard Battery for Genotoxicity Testing of Pharmaceuticals, Step 4 (Document, July 1997).

1.3 Principles of the method

The mutation assay method used in this study is based on the identification of L5178Y colonies which have become resistant to a toxic thymidine analogue trifluorothymidine (TFT). This analogue can be metabolised by the enzyme thymidine kinase (TK) into nucleosides, which are used in nucleic acid synthesis resulting in the death of TK-competent cells.

TK-deficient cells, which are presumed to arise through mutations in the TK gene, cannot metabolise trifluorothymidine and thus survive and grow in its presence.

In the L5178Y mouse lymphoma cells, the gene which codes for the TK enzyme is located on chromosome 11. Cells which are heterozygous at the TK locus (TK$^{+/-}$) may undergo a single step forward mutation to the TK$^{-/-}$ genotype in which little or no TK activity remains.

The cells used, L5178Y TK$^{+/-}$, are derived from one of the two clones derived from a thymic tumour induced in a DBA/2 mouse by methylcholanthrene. The use of the TK mutation system in L5178Y mouse lymphoma cells has been well characterised and validated (D. Clive *et al.* 1979) and is accepted by many regulatory authorities.

The mouse lymphoma assay often produces a bimodal size distribution of TFT resistant colonies designated as small or large. It has been proposed that small colonies result in part from lesions that affect not only the active TK allele but also a flanking gene whose expression modulates the growth rate of cells. According to this hypothesis, point mutations and deletions within the active allele (intragenic event) produce large colonies. The assay is performed in the following way: first the cytotoxicity of the test item is determined, and dose-levels are selected for the mutation assays. Two mutation assays are then performed.

The cells are treated with the test item in the absence and presence of S9 metabolism, and are subcultured for a number of generations to allow the phenotypic expression of the induced mutations. At the end of the expression time the cells are seeded in selective medium (in which only mutant cells can grow) and non-selective medium (to determine the surviving proportion).

In the first experiment, the cells are exposed to the test item for a short treatment time (3 hours). If negative or equivocal results are obtained in the first experiment without metabolic activation, a second experiment in the absence of S9 metabolism will be performed using a longer treatment time (24 hours).

If negative or equivocal results are obtained in the first experiment with metabolic activation, a second experiment will be performed and a modified dose-range will be used to focus on the highest concentrations that could be tested.

If, however, the first experiment produces a clear positive response, no further experiments will be undertaken.

2. TEST ITEM

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

2.2 Approximately 1 year after the final report has been issued, remaining amounts of the test item will be destroyed by incineration. An aliquot of the item will be retained within the archives of the testing facility for a period of ten years after which it will be destroyed.

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

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

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

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

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

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

3. MATERIALS AND METHODS

3.1 L5178Y $TK^{+/-}$ mouse lymphoma cells

L5178Y $TK^{+/-}$ mouse lymphoma cells were obtained from American Type Culture Collection, Rockville, Maryland (ATCC code: CRL 9518). The generation time and mutation rates (spontaneous and induced) have been checked in this laboratory. The cells are checked at regular intervals for the absence of mycoplasmal contamination.

Permanent stocks of the L5178Y $TK^{+/-}$ cells are stored in liquid nitrogen, and subcultures are prepared from the frozen stocks for experimental use. Cultures of the cells are grown in RPMI 1640 minimal medium supplemented with 10% horse serum heat-inactivated at 56°C for 20 minutes before use (Complete medium 10%). The incubations are at 37°C in a 5% carbon dioxide atmosphere (100% nominal relative humidity).

3.2 Media

Minimal medium A

RPMI 1640 (1X)	516.1 ml
L-glutamine (200 mM)	5.4 ml
Sodium pyruvate (100 mM)	6.0 ml
Non-essential amino acids (100X)	5.4 ml
Streptomycin sulphate 50.000 IU/ml	
Penicillin G 50.000 UI/ml	1.1 ml
F 68 Pluronic	6.0 ml

Minimal medium B

RPMI 1640 (1X)	522.1 ml
L-glutamine (200 mM)	5.4 ml
Sodium pyruvate (100 mM)	6.0 ml
Non-essential amino acids (100X)	5.4 ml
Streptomycin sulphate 50.000 IU/ml	
Penicillin G 50.000 IU/ml	1.1 ml

Complete medium (5%)

Minimal medium A	950 ml
Horse serum (heat-inactivated)	50 ml

Complete medium (10%)

Minimal medium A	900 ml
Horse serum (heat-inactivated)	100 ml

Complete medium A (20%)

Minimal medium A	800 ml
Horse serum (heat-inactivated)	200 ml

Complete medium B (20%)

Minimal medium B	800 ml
Horse serum (heat-inactivated)	200 ml

3.3 Preparation of S9 Mix

The S9 liver tissue fraction will be prepared according to RTC standard procedures or will be obtained from an appropriate supplier (MOLTOX, Molecular Toxicology, Inc., USA). Induction of drug metabolising enzyme-levels is routinely performed using phenobarbitone and beta-naphthoflavone (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 on file at RTC.

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

S9 tissue fraction	0.408 ml
NADP (0.03 M)	0.204 ml
G-6-P (0.59 M)	0.204 ml
KCl (150 mM)	0.204 ml
Complete medium (5%)	8.98 ml
	10.0 ml

Where it is necessary to change the composition of the S9 mix, all modifications will be indicated in the final report.

3.4 Control items

Positive control treatments are included in every experiment.
The positive control agents, methylmethanesulphonate (MMS), and benzo(a)pyrene (BP) are both obtained commercially, and characterised by their labelling. Fresh solutions of MMS in sterile distilled water are prepared for each day's work. Solutions of BP are prepared in dimethylsulphoxide and may be stored at -20°C.
Determination of the stability and concentration of solutions of these agents will not be undertaken since it is sufficient to provide evidence for the correct expected response of the test system to them.

4. CYTOTOXICITY ASSAY

4.1 Experimental design

A preliminary cytotoxicity test is undertaken in order to select appropriate dose levels for the mutation assays. In this test a wide range of dose-levels of the test item, set at intervals of a factor of two, are used; cell cultures are treated using the same treatment conditions as the mutation assays, and the survival of the cells is subsequently determined.

The test includes the following treatments:

Solvent/vehicle controls	:	The final concentration of organic solvents will not exceed 1%.
Test item	:	The highest dose-level will be determined by the solubility of the test material, up to a maximum of 5 mg/ml. At the discretion of the Study Director, dose-levels where test item precipitation occurs may be employed.

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

4.2 Test procedure

The cultures will be prepared, and the treatment conducted using the methods described in Sections 6.1, 6.2 and 6.3. The plates will be incubated for one to two weeks. Wells containing viable clones will be identified by eye using background illumination and counted.

4.3 Evaluation and selection of doses

Percentage survival relative to the solvent controls will be calculated for each treatment. Dose-levels giving a predicted 10-20% survival will be estimated from the results obtained; the with and without S9 series may be considered separately, if appropriate. The estimated concentrations will be chosen as the highest dose-levels for the mutation assays. If the test item is not sufficiently toxic to reduce survival to 10-20%, the highest practicable dose-level (up to a maximum of 5 mg/ml) will be selected. A minimum of five dose-levels separated by a factor of two will be selected for the mutation assay. Where there is cytotoxicity, these concentrations should cover a range from the maximum to little or no toxicity. Selection of dose-levels for the second experiment will be based on the results obtained in the first experiment as described in Section 1.3.

5. EXPERIMENTAL DESIGN (MUTATION ASSAY)

Each experiment will include solvent/vehicle and positive controls and at least four analysable concentrations of the test item, tested in the absence and presence of S9 metabolising system.

Duplicate cultures will be prepared at each test point, with the exception of the positive controls which will be prepared in a single culture.

Solvent/vehicle controls	:	Treated with the maximum amount of solvent/vehicle used in any test item treatment.
Positive controls	:	Methylmethanesulphonate (experiments in the absence of S9 metabolism) or benzo(a)pyrene (experiments in the presence of S9 metabolism) will be used, both at the appropriate concentration to give a clear positive response.
Test item	:	The selection of test item dose-levels is described in the preceding section.

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

6. MUTATION ASSAY PROCEDURE

6.1 Preparation of the test system

On the day of the experiment, sufficient number of cells will be used to prepare a cell suspension (1 x 10^6 cells/ml) in complete medium (3-hour treatment time: 5%; 24-hour treatment time: 10%). A common pool will be used to prepare the test cultures in appropriately labelled conical screw-cap tissue culture tubes.

6.2 Treatment

On the day of the experiment, treatment media will be prepared as follows:

Without S9 metabolism - 3 hours treatment

Cell suspension (1x10^6 cells/ml)	10.0 ml
Complete medium (5%)	9.8 ml
Control or test item solution	0.2 ml
	20.0 ml

Without S9 metabolism - 24 hours treatment

Cell suspension ($1x10^6$ cells/ml)	3.0 ml
Complete medium (10%)	16.8 ml
Control or test item solution	0.2 ml
	20.0 ml

With S9 metabolism

Cell suspension ($1x10^6$ cells/ml)	10.0 ml
S9 mix	9.8 ml
Control or test item solution	0.2 ml
	20.0 ml

Where it is necessary to adjust the composition of the treatment medium (for example when the culture medium is used as the solvent), the final composition used will be indicated in the final report.

The cultures will be incubated at 37°C. At the end of treatment time the cell cultures will be centrifuged and washed twice with Phosphate Buffered Saline (PBS). Fresh complete medium (10%) will be added and cell densities determined. The number of cells will be adjusted to give $2x10^5$ cells/ml. The cultures will be incubated at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) to allow for expression of the mutant phenotype.

The pH and osmolality of the treatment solutions will be measured during the performance of one of the main experiments.

6.3 Determination of survival

Following adjustment of the cell densities, samples of the cultures will be diluted to 8 cell/ml using complete medium A (20%). 0.2 ml of each diluted culture will be placed into each well of two 96-well plates. The plates will be incubated at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) for one to two weeks. After incubations, wells containing viable clones will be identified by eye using background illumination and counted.

6.4 Determination of mutant frequency

During the expression period (two days after treatment) the cell populations will be subcultured in order to maintain them in exponential growth. At the end of this period the cell densities of each culture will be determined and adjusted to give 2 X 10^5 cells/ml. The following two procedures will be carried out with each culture:

(i) After dilution, the cell suspension in complete medium B (20%) will be supplemented with trifluorothymidine (final concentration 3.0 μg/ml) and an estimated 2 X 10^3 cells will be plated in each well of four 96-well plates. Only $TK^{-/-}$ mutant colonies are able to grow in the presence of trifluorothymidine; these plates will be subsequently scored for the presence of mutants.

(ii) After dilution, in complete medium A (20%), an estimated 1.6 cells/well will be plated in each of two 96-well plates. These plates will be used to estimate Plating Efficiency (P.E.).

6.5 Incubation and scoring

Plating efficiency and mutant plates will be incubated for one to two weeks to ensure adequate colony size. Plates prepared on any one day will receive the same period of incubation.

After incubation, wells containing viable clones will be identified by eye using background illumination and counted.

Small colony and large colony mutant frequencies will be estimated for the solvent/vehicle and positive controls and for the doses of test item showing positive results. The definition of the small and large colonies is as follows:

1) Size: Small: less than ¼ of well's diameter
Large: more than ¼ of well's diameter

2) Morphology: Small: compact
Large: diffused, totally or in periphery

The positive control items should be capable of producing both small and large colonies in the L5178Y $TK^{+/-}$ mouse lymphoma assay.

7. REPORTING

7.1 Calculations

7.1.1 Survival or viability

The following formula will be used to determine the plating efficiency (PE):

$$PE = \frac{-\ln (ys/ns)}{fs}$$

where:

ys = Number of empty wells (without clones)
ns = Total number of wells
fs = Number of cells plated per well (1.6 cells/well)

Plating efficiencies in test and control cultures will be compared to give the percentage relative survival. The survival calculation will take into account the reduction in post-treatment cell-count as follows:

% RS = % PE x cell count factor

where cell count factor = $\frac{\text{treated post-treatment cell count}}{\text{control post-treatment cell count}}$

In order to aid toxicity data interpretation the relative total growth, expressed as a percentage of the concurrent solvent/vehicle control, will also be calculated. This is a product of the relative suspension growth (RSG) and the Day 2 relative plating efficiency (RPE), expressed as a percentage of the concurrent negative control, for each culture, as follows:

RTG = RSG x RPE (Day 2)

Relative suspension growth (RSG) is given by: [TSG(test) / TSG (control)] x 100

where TSG (total suspension growth) is calculated as follows:

$$TSG = \frac{\text{[post treatment cell count]}}{\text{[pre treatment cell count]}} \quad \frac{\text{[Day 1 cell count]}}{[2 \times 10^5 *]} \quad \frac{\text{[Day 2 cell count]}}{[2 \times 10^5 *]}$$

* Or appropriate cell concentration if lower

7.1.2 Mutation frequency

The following formula will be used to determine the mutation frequency (MF):

$$MF = [PE(mutant)/PE(viable)] \times 10^6$$

Therefore:

$$MF = \{ \frac{-\ln (ym/nm)}{fm} / \frac{-\ln (ys/ns)}{fs} \} \times 10^6$$

where:

ym	=	Number of empty wells (mutant plates)
nm	=	Total number of wells (mutant plates)
fm	=	Number of cells plated per well ($2x10^3$ cell/well)
ys	=	Number of empty wells (viability plates)
ns	=	Total number of wells (viability plates)
fs	=	Number of cells plated per well (1.6 cells/well)

Statistical analyses will be performed according to UKEMS guidelines (Robinson W.D., 1990).

7.2 Evaluation

For a test item to be considered mutagenic in this assay, it is required that:

(i) The mutant frequency at one or more doses is statistically significantly greater than that of the solvent/vehicle control.

(ii) There is a significant dose-relationship as indicated by the linear trend analysis.

Results which only partially satisfy the above criteria will be dealt with on a case-by-case basis. Similarly, positive responses seen only at high levels of cytotoxicity will require careful interpretation when assessing their biological significance. Any increase in mutant frequency should lie outside the historical control range to have biological relevance.

7.3 Presentation of data

The results will be presented in the form of tables which will show the individual plate counts in non-selective medium, the calculated plating efficiency, the individual plate counts in selective medium, the total number of mutant colonies and the calculated mutation frequency.

Small and large colony mutant frequencies for negative and positive controls and for doses which show positive results will also be presented.

7.4 Reporting procedure

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

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 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 for the analysis;
- historical solvent/vehicle and positive control data with ranges, means and standard deviations;
- QAU statement;
- the location where all raw data, specimens and final report are to be stored.

One original unbound, one copy bound and a PDF version 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 generated during the course of this study will be retained at RTC. Archiving will be provided for a period of 3 years after which the Sponsor will be contacted for instructions regarding despatch or disposal of the material. As a further option, archiving space can be rented for an additional time. The signed Final Protocol and the top copy of the Final Report will be despatched to and archived by the Sponsor.

8. STUDY CONDUCT

8.1 Langnage

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/she will record carefully the decision he/she has reached and the reasoning which led to it.

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

9. DEPARTURES FROM REGULATORY REQUIREMENTS

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

10. REFERENCES

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PROTOCOL APPROVAL PAGE

STUDY TITLE : MUTATION IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS (FLUCTUATION METHOD)

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RTC ENQUIRY NO. : 52410

TEST ITEM : [redacted]7850

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

RELEASED BY : [redacted] Date [redacted]
Technical & Operational Director

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Name and Title : [redacted]

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