[redacted]
Huntingdon
Life Sciences

[redacted]

MOUSE MICRONUCLEUS TEST

Company Sanitized. Does not contain TSCA CBI

[redacted]

MOUSE MICRONUCLEUS TEST

Sponsor

DuPont Specialty Chemicals,
Jackson Laboratory,
Chambers Works,
Deepwater,
NJ 08023,
USA.

Research Laboratory

Huntingdon Life Sciences Ltd.,
Eye,
Suffolk,
IP23 7PX,
ENGLAND.

Report issued 2 March 1999

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The United Kingdom Good Laboratory Practice Regulations 1997 (Statutory Instrument No. 654).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Council Directive 87/18/EEC of 18 December 1986 (Official Journal No. L15/29).

C. E. Mason
Christine E. Mason, B.Sc.,
Study Director,
Huntingdon Life Sciences Ltd.

2nd March 1999
Date

QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	24.11.98	25.11.98
Process Based Inspections		
Formulation of test compound	07.09.98	07.09.98
Slide scoring	01.10.98	01.10.98
Bodyweights	21.10.98	22.10.98
Animal dosing	04.11.98	04.11.98
Bone marrow extraction and slide preparation	08.12.98	08.12.98
Report Audit	09.02.99	09.02.99

Protocol: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process based inspections: At or about the time this study was in progress inspections of other routine and repetitive procedures employed on this type of study were carried out. These were promptly reported to appropriate Company Management.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

Helen Comb

Helen Comb, B.Sc.,
Principal Auditor,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

1 MARCH 1999
Date

SUMMARY

This study was designed to assess the potential induction of micronuclei by [redacted] in bone marrow cells of mice. Mice were treated with a single intraperitoneal administration of the test substance at dose levels of 50, 100 and 200 mg/kg bodyweight. A preliminary toxicity test had previously shown that a dose of 200 mg/kg was expected to be approximately the maximum tolerated; this level was therefore selected as an appropriate maximum for use in the micronucleus test.

The test substance and negative control were administered by intraperitoneal injection. The negative control group received the vehicle, purified water. A positive control group was dosed orally, by intragastric gavage, with mitomycin C at 12 mg/kg bodyweight.

Bone marrow smears were obtained from five male and five female animals in the negative control, each of the test substance groups and the positive control group 24 hours after dosing. In addition bone marrow smears were obtained from five male and five female animals in the negative control and high level treatment groups 48 hours after dosing. One smear from each animal was examined for the presence of micronuclei in 2000 immature erythrocytes. The proportion of immature erythrocytes was assessed by examination of at least 1000 erythrocytes from each animal. A record of the incidence of micronucleated mature erythrocytes was also kept.

Mice treated with the test substance did not show any significant increase in the frequency of micronucleated immature erythrocytes at either sampling time.

There was no significant decrease in the proportion of immature erythrocytes after treatment of the animals with the test substance.

No statistically significant increases in the frequency of micronucleated immature erythrocytes and no substantial decrease in the proportion of immature erythrocytes were observed in mice treated with [redacted] and killed 24 or 48 hours later, compared to vehicle control values ($p>0.01$ in each case).

The positive control compound, mitomycin C, produced large, highly significant increases in the frequency of micronucleated immature erythrocytes.

It is concluded that [redacted] did not show any evidence of causing chromosome damage or bone marrow cell toxicity when administered by intraperitoneal injection in this *in vivo* test procedure.

INTRODUCTION

The purpose of this study was to assess the potential of [redacted] to induce mutagenic effects in mice following acute intraperitoneal administration using an *in vivo* cytogenetic system (Boller and Schmid 1970, MacGregor *et al* 1987, Mavournin *et al* 1990). The intraperitoneal route was chosen for this particular study to maximise potential absorption of the test substance.

The procedures used were based on the recommendations of the following guidelines:

- OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test, Guideline 474.
- EEC Annex to Directive 92/69/EEC (1992) Part B : Methods for determination of toxicity, B.12. Mutagenicity (Micronucleus test). *O.J.* No. L 383 A, **35**, 154.
- US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5395 Mammalian erythrocyte micronucleus test. EPA 712-C-98-226.

The bone marrow micronucleus test, originally developed by Matter and Schmid (1971), is a widely employed and internationally accepted short-term assay for identification of genotoxic effects (chromosome damage and aneuploidy) associated with mutagens and carcinogens (Mavournin *et al* 1990). This *in vivo* system allows consideration of various factors including pharmacokinetics, metabolism and DNA repair which cannot be accurately modelled in an *in vitro* system. Young adult mice are chosen for use because of the high rate of cell division in the bone marrow, the wealth of background data on this species, and their general suitability for toxicological investigations.

In mitotic cells in which chromosomal breakage has been caused by the test substance or its metabolites, acentric fragments of the chromosomes do not separate at the anaphase stage of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and hence will form single or multiple micronuclei (Howell-Jolly bodies) in the cytoplasm of these cells. Micronuclei are seen in a wide variety of cells, but erythrocytes are chosen for examination since micronuclei are not obscured by the main nucleus and are therefore easily detected in this cell type (Boller and Schmid 1970).

A few hours after the last mitosis is completed, erythroblasts expel their nucleus. Young erythrocytes, less than 24 hours old, stain blue with Giemsa due to the presence of ribonucleic acid and are termed polychromatic (immature). This ribonucleic acid gradually disappears so that more mature erythrocytes stain orange/pink (*ie* they show normochromatic staining). Virtually all the chromosome damage detected in immature erythrocytes will have been caused during the recent exposure to the test substance. Mature erythrocytes may also be examined for the presence of micronuclei. No substantial increases in the incidence of micronuclei in mature erythrocytes would usually be expected 24 hours after administration of a chromosome-damaging agent; any micronucleus-like artefacts (which could otherwise possibly give a false positive result) are therefore readily distinguishable in this cell type (Schmid 1976).

Substances which interfere with the mitotic spindle apparatus will cause non-disjunction (unequal separation of the chromosomes at anaphase resulting in aneuploidy) or lagging chromosomes at anaphase which may not be incorporated into the daughter nuclei. These lagging chromosomes are not excluded from the erythroblast with the main nucleus and hence also give rise to micronuclei.

Any toxic effects of the test substance on the nucleated cells may lead either to a reduction in cell division or to cell death. These effects in turn lead to a reduction in the number of nucleated cells and immature erythrocytes; to compensate for this, peripheral blood is shunted into the bone marrow (von Ledebur and Schmid 1973). If the proportion of immature erythrocytes is found to be significantly less than the control value, this is taken as being indicative of toxicity. A very large decrease in the proportion would be indicative of a cytostatic effect.

The bone marrow is sampled twice after treatment to allow for variations in the rate of absorption and metabolism of test substances and to allow for any delay in erythrocyte production as a result of cytostatic or cytotoxic effects (MacGregor *et al* 1987).

The protocol was approved by Huntingdon Life Sciences Management on 8 October 1998, by the Sponsor on 21 October 1998 and by the Study Director on 20 November 1998.

The study was performed between 30 November 1998 and 5 January 1999 at the Department of Genetic Toxicology, Huntingdon Life Sciences, Eye, Suffolk, IP23 7PX, England. Subsequently statistical analysis was performed by the Department of Statistics, Huntingdon Life Sciences, Huntingdon, Cambridgeshire, PE18 6ES, England.

TEST SUBSTANCE

Identity:	[redacted]
Chemical name:	[redacted]
Intended use:	[redacted]
Appearance:	Pale yellow slurry
Storage conditions:	Room temperature
Batch number:	3
Expiry date:	2 years from date of receipt
Purity:	[redacted]
Date received:	23 June 1998

The above information with regard to the physical characterisation of the test substance is the responsibility of the Sponsor.

EXPERIMENTAL PROCEDURE

ANIMALS

All animals in this study were Specific Pathogen Free CD-1 outbred albino mice of Swiss origin. Males weighed between 28 and 30 grams and females weighed between 22 and 24 grams on despatch from Charles River UK Limited, Margate, Kent, England.

On arrival the weight of the animals was checked and found to be acceptable. The animals were randomly assigned to groups and tail marked. Each group was kept, with the sexes separated, in plastic cages and maintained in a controlled environment, with the thermostat set at 22°C and relative humidity set at 50%. The room was illuminated by artificial light for 12 hours per day. All animals were allowed free access to pelleted expanded rat and mouse No.1 maintenance diet (SQC grade obtained from Special Diets Services Ltd, Witham, Essex, UK) and tap water *ad libitum*. Food and tap water are routinely analysed for quality at source. Dietary contaminants are not suspected of having any significant effect on parameters measured in this test in this laboratory at any time over the last ten years. All animals were acclimatised for a minimum of five days, examined daily and weighed prior to dosing.

TEST SUBSTANCE FORMULATION AND ADMINISTRATION

Suspensions of the test substance were individually prepared on the day of the test, using identical methods for each phase of the test, in purified water (prepared by reverse osmosis of tap water). All dosages cited in this report are stated in terms of the purity of the test substance [redacted]

Stability and homogeneity of the test substance and of the test substance in the vehicle were not determined in this test and remain the responsibility of the Sponsor. Chemical analysis of dosing formulations for achieved concentration or stability was not performed in this study.

Mitomycin C, obtained from Sigma Chemical Company, batch numbers 48H2510 and 48H2511 were used as the positive control compound. They were prepared as a solution in purified water, at a concentration of 0.6 mg/ml and pooled, just prior to administration.

All animals in all groups were dosed with the standard volume of 20 ml/kg bodyweight. The test substance and negative control were dosed by intraperitoneal injection, and mitomycin C, the positive control compound, was administered orally by intragastric gavage.

DATES OF DOSING

Preliminary toxicity test:	Group 1 - 2: 30 November 1998 Group 3 - 4: 2 December 1998
Micronucleus test:	7 December 1998

PRELIMINARY TOXICITY TEST

The purpose of this test was to determine a suitable dose level for use in the micronucleus test. The dosages employed were used to give an approximate indication of the maximum tolerated dose, *ie* the highest dosage which would be expected to elicit signs of toxicity without producing extreme clinical signs or having a significant effect on survival. The experimental design is shown below:

Group	Treatment	Concentration (mg/ml)	Dosage (mg/kg)*	Number of mice	
				Male	Female
1	[redacted]	12.5	250	2	2
2		6.25	125	2	2
3		10	200	2	2
4		11.25	225	2	2

* Dosage expressed in terms of purity of test substance [redacted]

Following dosing, the animals were observed regularly during the working day for a period of 48 hours and any mortalities or clinical signs of reaction during the experiment were recorded. At the end of this observation period, surviving animals were sacrificed and discarded.

MICRONUCLEUS TEST

From the results obtained in the preliminary toxicity study (see RESULTS), dose levels of 50, 100 and 200 mg/kg bodyweight were chosen for the micronucleus test. The experimental design is shown below:

Group	Treatment	Concentration (mg/ml)	Dose (mg/kg)*	Number of mice	
				Male	Female
1	Vehicle control	-	-	10	10
2	[redacted]	2.5	50	5	5
3		5	100	5	5
4		10	200	10	10
5	Mitomycin C	0.6	12	5	5

* Dosage expressed in terms of purity of test substance [redacted]

Following dosing, the animals were examined regularly and any mortalities or clinical signs of reaction were recorded. Five males and five females from the negative control, each of the test substance groups and the positive control group were sacrificed 24 hours after dosing. In addition five male and five female animals in the negative control and high level treatment groups were sacrificed 48 hours after dosing.

The animals were killed by cervical dislocation following carbon dioxide inhalation and both femurs dissected out from each animal. The femurs were cleared of tissue and the proximal epiphysis removed from each bone. The bone marrow of both femurs from each animal was flushed out and pooled in a total volume of 2 ml of pre-filtered foetal calf serum using a 2 ml disposable syringe fitted with a 21 gauge needle. The cells were sedimented by centrifugation, the supernatant was discarded and the cells were resuspended in a small volume of fresh serum. A small drop of the cell suspension was transferred to a glass microscope slide and a smear was prepared in the conventional manner (Schmid 1976). At least three smears were made from each animal. The prepared smears were fixed in methanol (> 10 minutes). After air-drying the smears were stained for 10 minutes in 10% Giemsa (prepared by 1 : 9 dilution of Gurr's improved R66 Giemsa (BDH) with purified water). Following rinsing in purified water and differentiation in buffered purified water, the smears were air-dried and mounted with coverslips using DPX.

The stained smears were examined (under code) by light microscopy to determine the incidence of micronucleated cells per 2000 polychromatic erythrocytes per animal. Usually only one smear per animal was examined. The remaining smears were held temporarily in reserve in case of technical problems with the first smear.

Micronuclei are identified by the following criteria:

- Large enough to discern morphological characteristics
- Should possess a generally rounded shape with a clearly defined outline
- Should be deeply stained and similar in colour to the nuclei of other cells - not black
- Should lie in the same focal plane as the cell
- Lack internal structure, *ie* they are pyknotic
- There should be no micronucleus-like debris in the area surrounding the cell.

The proportion of immature erythrocytes for each animal was assessed by examination of at least 1000 erythrocytes. A record of the number of micronucleated mature erythrocytes observed during assessment of this proportion was also kept as recommended by Schmid (1976).

ASSESSMENT OF RESULTS

The results for each treatment group were compared with the results for the concurrent control group using non-parametric statistics. Non-parametric statistical methods were chosen for analysis of results because:

- They are suited to analysis of data consisting of discrete/integer values with ties such as the incidence of micronucleated immature erythrocytes
- The methods make few assumptions about the underlying distribution of data and therefore the values do not require transformation to fit a theoretical distribution (where data can be approximately fitted to a normal distribution, the results of non-parametric analysis and classical analysis of variance are very similar)
- 'Outliers' are frequently found in the proportion of immature erythrocytes for both control and treated animals; non-parametric analysis based on rank does not give these values an undue weighting.

Unless there is a substantial difference in response between sexes (which occurs only rarely) results for the two sexes are combined to facilitate interpretation and maximise the power of statistical analysis.

For incidences of micronucleated immature erythrocytes, exact one-sided p-values are calculated by permutation (StatXact, CYTEL Software Corporation, Cambridge, Massachussetts). Comparison of several dose levels are made with the concurrent control using the Linear by Linear Association test for trend in a step-down fashion if significance is detected (Agresti *et al.* 1990); for individual inter-group comparisons (*ie* the positive control group) this procedure simplifies to a straightforward permutation test (Gibbons 1985). For assessment of effects on the proportion of immature erythrocytes, equivalent permutation tests based on rank scores are used, *ie* exact versions of Wilcoxon's sum of ranks test and Jonckheere's test for trend.

A positive response is normally indicated by a statistically significant dose-related increase in the incidence of micronucleated immature erythrocytes for the treatment group compared with the concurrent control group ($P<0.01$); individual and/or group mean values should exceed the laboratory historical control range (Morrison and Ashby 1995). A negative result is indicated where individual and group mean incidences of micronucleated immature erythrocytes for the group treated with the test substance are not significantly greater than incidences for the concurrent control group ($P>0.01$) and where these values fall within the historical control range. An equivocal response is obtained when the results do not meet the criteria specified for a positive or negative response.

Bone marrow cell toxicity (or depression) is normally indicated by a substantial and statistically significant dose-related decrease in the proportion of immature erythrocytes ($P<0.01$). This decrease would normally be evident at the 48 hour sampling time; a decrease at the 24 hour sampling time is not necessarily expected because of the relatively long transition time of erythroid cells [late normoblast → immature erythrocyte (approximately 6 hours) → mature erythrocyte (approximately 30 hours)].

MAINTENANCE OF RECORDS

All specimens, raw data and study related documents generated during the course of the study at Huntingdon Life Sciences, together with a copy of the final report, are lodged in the Huntingdon Life Sciences Archives.

Such specimens and records will be retained for a minimum period of five years from the date of issue of the final report. At the end of the five year retention period the Sponsor will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the Sponsor's knowledge. Huntingdon Life Sciences will also retain a copy of the final report in its Archives indefinitely.

RESULTS

Mortality data for the entire experiment are presented in Appendix 1.

PRELIMINARY TOXICITY TEST

The details of any toxic reactions observed are given in Appendix 2.

Results showed that a dose level of 200 mg/kg was approximately the maximum tolerated, and this level was considered to be an appropriate maximum for use in the micronucleus test. Dose levels of 50, 100 and 200 mg/kg bodyweight were chosen for use in the main test.

MICRONUCLEUS TEST

Table 1 gives a summary of the results of the micronucleus test and the results of statistical analysis. The results for individual animals at the 24 and 48 hour sampling times are presented in Tables 2 and 3 respectively. Appendix 4 summarises the vehicle control values for micronucleated polychromatic erythrocytes obtained in previous, unrelated experiments. Appendix 5 summarises the corresponding values obtained for the positive control in previous, unrelated experiments.

Clinical signs and mortalities

No mortalities were obtained in the micronucleus test.

Clinical signs for animals treated with the test substance are detailed in Appendix 3. Clinical signs for the high level group were consistent with the maximum tolerated dose having effectively been achieved. No adverse clinical signs were obtained for the vehicle control or positive control treated animals over the duration of the test.

Micronucleated immature erythrocyte counts (mie)

The test substance did not cause any statistically significant increases in the number of micronucleated immature erythrocytes at either sampling time ($P>0.01$).

Mitomycin C caused large, highly significant increases ($P<0.001$) in the frequency of micronucleated immature erythrocytes.

Micronucleated mature erythrocytes (mme)

The test substance did not cause any substantial increases in the incidence of micronucleated mature erythrocytes at either sampling time.

Proportion of immature erythrocytes (% ie/ie + me)

The test substance failed to cause any significant decreases in the proportion of immature erythrocytes ($P>0.01$).

Mitomycin C caused a statistically significant decrease in the proportion ($P<0.01$).

CONCLUSION

No statistically significant increases in the frequency of micronucleated immature erythrocytes and no substantial decrease in the proportion of immature erythrocytes were observed in mice treated with [redacted] and killed 24 or 48 hours later, compared to vehicle control values ($p>0.01$ in each case).

It is concluded that [redacted] did not show any evidence of causing chromosome damage or bone marrow cell toxicity when administered by intraperitoneal injection in this *in vivo* test procedure.

TABLE 1

Summary of results and statistical analysis

Sampling time	Treatment	Dose (mg/kg)	% ie/(ie+me) †	Incidence mie (mean)	Incidence mme (total)
24 Hours	Vehicle control	-	44	0.5	0.3
	[redacted]	50	43	0.9	0.6
		100	45	0.5	1.6
		200	42	0.9	0.0
	Mitomycin C	12	38**	65.6***	2.3
48 Hours	Vehicle control	-	43	0.4	0.0
	[redacted]	200	42	0.2	0.3

% ie/(ie+me) Proportion of immature erythrocytes
mie Number of micronucleated cells observed per 2000 immature erythrocytes examined
mme Number of micronucleated cells calculated per 2000 mature erythrocytes examined

Results of statistical analysis using the appropriate nonparametric method of analysis based on permutation (one-sided probabilities):

*** $P < 0.001$ (highly significant)
** $P < 0.01$ (significant)
otherwise $P > 0.01$ (not significant)

† Occasional apparent errors of ± 1% may occur due to rounding of values for presentation in the table

TABLE 2

Results for individual animals - 24 hour sampling time

Treatment	Dose (mg/kg)	Animal number	% ie/(ie+me)	Incidence mie	me	Incidence mme
Vehicle control	-	M 201	44	0	640	0
		M 202	46	1	612	0
		M 203	44	1	650	0
		M 204	34	0	794	0
		M 205	44	0	567	0
		F 206	45	0	653	1
		F 207	49	2	555	0
		F 208	50	1	608	0
		F 209	43	0	621	0
		F 210	45	0	683	0
[redacted]	50	M 211	40	2	736	0
		M 212	45	0	630	0
		M 213	44	1	631	0
		M 214	40	2	752	0
		M 215	48	0	788	0
		F 216	34	0	701	0
		F 217	41	2	601	1
		F 218	48	0	645	0
		F 219	46	1	730	0
		F 220	46	1	580	1
[redacted]	100	M 221	45	0	609	0
		M 222	45	0	551	0
		M 223	42	0	666	0
		M 224	40	0	672	0
		M 225	45	1	641	1
		F 226	46	0	640	1
		F 227	45	0	618	1
		F 228	47	2	646	0
		F 229	44	1	639	1
		F 230	48	1	583	1
[redacted]	200	M 231	43	1	644	0
		M 232	41	0	723	0
		M 233	46	3	673	0
		M 234	36	2	703	0
		M 235	42	2	785	0
		F 236	44	0	792	0
		F 237	43	0	793	0
		F 238	43	0	849	0
		F 239	40	1	691	0
		F 240	43	0	590	0
Mitomycin C	12	M 241	34	42	798	1
		M 242	34	69	863	0
		M 243	39	100	638	0
		M 244	44	56	702	0
		M 245	33	97	751	1
		F 246	34	59	702	2
		F 247	50	15	526	0
		F 248	37	75	631	2
		F 249	35	92	674	2
		F 250	38	51	649	0

% ie/(ie+me) Proportion of immature erythrocytes
mie Number of micronucleated cells observed per 2000 immature erythrocytes
me Total number of mature erythrocytes examined for micronuclei
mme Number of micronucleated mature erythrocytes

TABLE 3

Results for individual animals - 48 hour sampling time

Treatment	Dose (mg/kg)	Animal number	% ie/(ie+me)	Incidence mie	me	Incidence mme
Vehicle control	-	M 301	44	2	577	0
		M 302	41	1	611	0
		M 303	47	0	641	0
		M 304	38	0	692	0
		M 305	46	0	787	0
		F 306	43	0	632	0
		F 307	36	0	699	0
		F 308	46	0	543	0
		F 309	43	0	656	0
		F 310	45	1	623	0
[redacted]	200	M 331	48	0	580	0
		M 332	42	1	648	0
		M 333	38	0	890	0
		M 334	43	0	743	0
		M 335	46	0	598	0
		F 336	37	0	693	0
		F 337	45	0	608	0
		F 338	45	1	627	0
		F 339	34	0	769	1
		F 340	38	0	693	0

% ie/(ie+me) Proportion of immature erythrocytes
mie Number of micronucleated cells observed per 2000 immature erythrocytes
me Total number of mature erythrocytes examined for micronuclei
mme Number of micronucleated mature erythrocytes

APPENDIX 1

Mortality data

	Group	Treatment	Dosage (mg/kg)	Mortality ratio = No. of deaths / No. dosed		
				Males	Females	Combined
Preliminary Toxicity Test	1	[redacted]	250	0/2	0/2	0/4
	2		125	0/2	0/2	0/4
	3		200	0/2	0/2	0/4
	4		225	1/2	0/2	1/4
Micronucleus Test	1	Vehicle	-	0/10	0/10	0/20
	2	[redacted]	50	0/5	0/5	0/10
	3		100	0/5	0/5	0/10
	4		200	0/10	0/10	0/20
	5	Mitomycin C	12	0/5	0/5	0/10

APPENDIX 2

Preliminary toxicity test - Clinical signs and mortalities

Treatment	[redacted]							
Dose (mg/kg)	250		125		200		225	
Approx. time after dosing (hr : min)	Male	Female	Male	Female	Male	Female	Male	Female
0 : 15	U	U, SR						
3 : 50	P, U, HP, SR	P, U, HP, SR						
6 : 40	P, U, HP, SR	P, U, HP, SR						
22 : 40	P, U, HP, SR, 1(REPC)	HP, 1(P, U)						
30 : 30	P, U, HP, SR, 1(REPC), 1(EPC)	HP, 1(U, P)						
46 : 05	P, U, HP, SR, 1(EPC)	HP, 1(P)						
0 : 20			U, 1(HP)	U, HP				
2 : 20								
18 : 20			HP, 1(U)					
26 : 10			HP, 1(P, U)					
41 : 45			HP, 1(P)					
47 : 55			HP, 1(P)					
0 : 20					U, 1(P)	U, HP	P, U	
4 : 55					P, U, HP, SR	U, HP	P, U, HP, SR	P, U, HP
21 : 00					P, U, HP, SR, 1(EPC)	HP, 1(P)	P, U, HP, SR, 1(EPC)	P, U, HP, 1(EPC), 1(REC)
22 : 35*							1(P, U, HP, SR, UG, EC, KIE)	
28 : 45					P, U, HP, SR, 1(EPC)	HP, 1(P)	1(P, U, HP, SR, EPC)	P, HP, 1(EPC)
45 : 30					P, HP, 1(U, SR, EPC)	HP, 1(P)	1(P, U, HP, SR, EPC, UG)	HP, 1(P, EPC)
Mortalities	0/2	0/2	0/2	0/2	0/2	0/2	1/2	0/2

* Observations performed on one male animal only.

Type of reaction: EPC Eyes Partially Closed, EC Eyes Closed, HP Hunched Posture, KIE Killed *in Extremis*, P Piloerection, SR Slow Respiration, REC Right Eye Closed, REPC Right Eye Partially Closed, U Underactive, UG Unstable Gait.

Clinical signs shown refer to all animals within that dose group and sex, except where x(...), x denoting the number of animals displaying the clinical sign(s) within the brackets.

APPENDIX 3

Micronucleus test - Clinical signs and mortalities

Treatment	[redacted]					
Dose (mg/kg)	50		100		200	
Approx. time after dosing (hr : min)	Male	Female	Male	Female	Male	Female
1 : 25						
5 : 25		1(P, HP, SJ)				
21 : 55		1(P, HP, SJ)				
1 : 05						
5 : 05			1(HP), 2(P)			
21 : 40			1(P)			
1 : 10a					P, U, HP	U, 4(HP)
5 : 10a					P, U, HP, 2(SR)	P, HP, 3(U)
21 : 45a					P, HP	P, HP,
0 : 30b					U, HP, 2(SR), 3(P)	3(P, U, HP)
4 : 35b					P, U, HP, 3(SR), 1(EC)	1(SR), 3(P, U, HP)
21 : 05b					P, HP	3(P, HP)
28 : 15b					P, HP	3(P, HP)
44 : 50b					P, HP	3(P, HP)
Mortalities	0/5	0/5	0/5	0/5	0/10	0/10

a 24 hour sacrifice
b 48 hour sacrifice

NB No adverse clinical signs were noted for the vehicle or positive control groups throughout the experiment

Type of reaction: EC Left Eye Closed, HP Hunched Posture, P Piloerection, SJ Swollen Jaw, SR Slow Respiration, U Underactive.

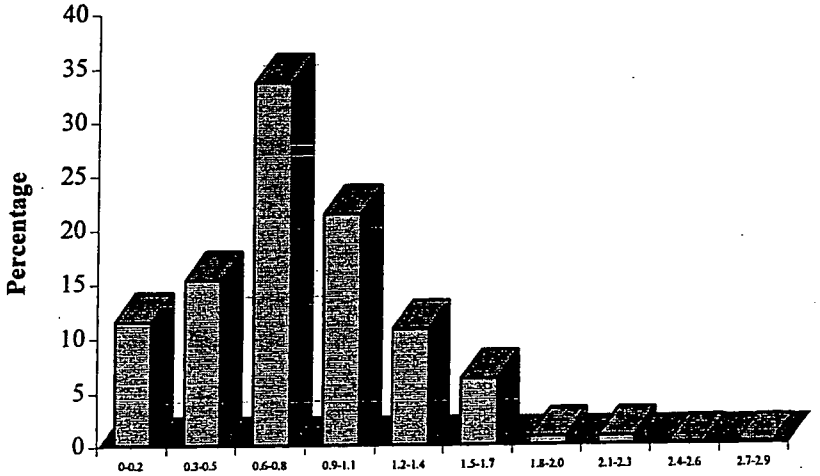
Clinical signs shown refer to all animals within that dose group and sex, except where x(...), x denoting the number of animals displaying the clinical sign(s) within the brackets.

APPENDIX 4

Historical vehicle control data (August 1995 - June 1998)
Percentage of micronucleated immature erythrocytes (individual animals)

Frequency distribution of micronucleated immature erythrocytes per 1000 cells

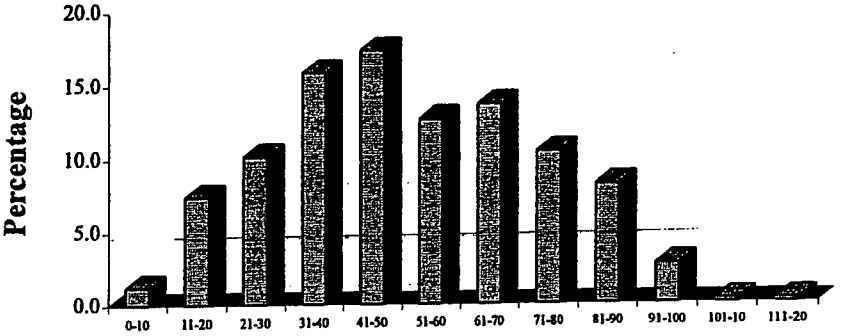
Historical vehicle control data (August 1995 - June 1998)
Percentage of micronucleated immature erythrocytes (group mean values)



Frequency distribution of micronucleated immature erythrocytes per 1000 cells

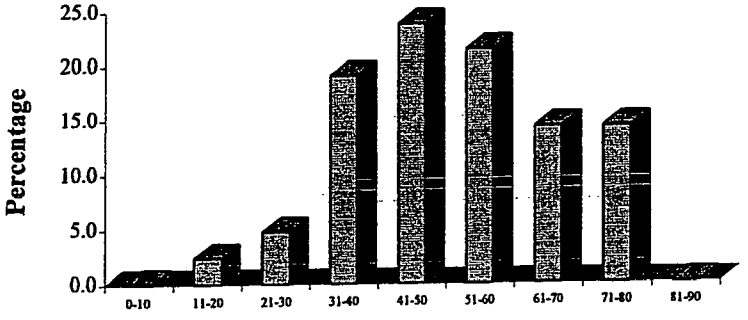
APPENDIX 5

Historical positive control data (August 1995 - June 1998)
Frequency of micronucleated erythrocytes (individual animals)



Frequency distribution of micronucleated immature erythrocytes per 1000 cells

Historical positive control data (August 1995 - June 1998)
Frequency of micronucleated immature erythrocytes (group means)



Frequency distribution of micronucleated immature erythrocytes per 1000 cells

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