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MDS Pharma Services

STUDY SPONSOR :

STUDY TITLE : Lehmann-blau - Local lymph node assay.

PHOENIX INTERNATIONAL STUDY NUMBER : 762/001D

DATE: 20 April 2001

Siege Social :
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TABLE OF CONTENTS

Pages

GLP COMPLIANCE STATEMENT 4

QUALITY ASSURANCE 5

1. SUMMARY 6

2. INTRODUCTION 8

3. TEST/CONTROL ARTICLES AND VEHICLE INFORMATION 10

3.1. TEST ARTICLE 10

3.2. POSITIVE CONTROL ARTICLE 10

3.3. NEGATIVE CONTROL/VEHICLE FOR TEST ARTICLE AND POSITIVE CONTROL ARTICLE 11

3.4. FORMULATION OF THE TEST AND POSITIVE CONTROL ARTICLES 11

4. METHODS AND EXPERIMENTAL DESIGN 12

4.1. TEST SYSTEM 12

4.2. ANIMAL HUSBANDRY 12

4.3. PRE-TREATMENT PROCEDURES 13

4.4. EXPERIMENTAL DESIGN 14

4.5. ADMINISTRATION OF THE TEST/CONTROL ARTICLES 14

5. OBSERVATIONS 15

5.1. MORBIDITY/MORTALITY 15

5.2. CLINICAL SIGNS 15

5.3. EVALUATION OF CELL PROLIFERATION 15

6. DATA EVALUATION 16

7. ARCHIVES 17

8. STUDY TIMETABLE 18

9. PROTOCOL ADHERENCE 19

TABLE OF CONTENTS (cont'd)

Pages

10. RESULTS 20

10.1. MORTALITY 20

10.2. CLINICAL SIGNS 20

10.3. EVALUATION OF CELL PROLIFERATION 20

11. CONCLUSION 20

12. TABLE 21

EVALUATION OF CELL PROLIFERATION 21

13. ADDENDA 24

ADDENDUM 1 25

PROTOCOL NO. 762/001-D 25

ADDENDUM 2 39

CERTIFICATES OF ANALYSIS 39

GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that, unless otherwise stated, the work described in this report was performed in accordance with the following :

- "Good Laboratory Practice" described in the U.S. Federal Register (Food and Drug Administration) dated 22 December 1978 with subsequent revisions with any applicable amendments.
- "OECD Principles of Good Laboratory Practice" concerning Mutual Acceptance of Data in the Assessment of Chemicals dated 26 November 1997 (C (97) 186 Final).

This report is a true and accurate record of the results obtained.

Signature : 

Name : M. Christ

Title : Study Director

Date : 20 April 2001

QUALITY ASSURANCE

TITLE : Lehmann-blau - Local lymph node assay

Inspection of the protocol was made in accordance with Standard Operating Procedure AQ PROT 1. Dates for inspection of any protocol amendments, in accordance with this SOP, are not quoted

Dates (day - month - year)		
Inspection	Report to Study Director	Report to Management
11.03.99	11.03.99	11.03.99

Inspection(s) of procedures on this study was made in accordance with Standard Operating Procedure AQ-INSP 1

Inspected phase(s)	Dates (day - month - year)		
	Inspection	Report to Study Director	Report to Management
Formulation	17.03.99	18.03.99	18.03.99
Administration	19.03.99	24.03.99	24.03.99
^{3}H methyl thymidine preparation	24.03.99	29.03.99	29.03.99
Preparation of samples into scintillation fluid and radioactivity counting	23.03.99	26.03.99	26.03.99
Preparation of cell suspensions	24.03.99	29.03.99	29.03.99
Sacrifice of animals	24.03.99	29.03.99	29.03.99

Other routine procedures used in this type of study were inspected regularly and reports made in accordance with Standard Operating Procedure AQ-INSP 1.

This report has been reviewed by the Quality Assurance Department, employing methods detailed in Standard Operating Procedure AQ-RAP 1. The reported methods and procedures were found to describe those used, and the results constituted an accurate representation of recorded data. Any data supplied by or under the responsibility of the Sponsor were not subjected to review.

The dates mentioned in the tables correspond to Quality Assurance inspections performed during the whole study 762/001 (reports 762/001A to 762/001G), and not exclusively to the set described in this report.

P. Withers, B. Sc.
(Director of International Compliance) P. Withers

Date : 23 April 2001

1. SUMMARY

1.1. The objective of the study was to determine if the test article Lehmann-blau can induce a hypersensitivity response in CBA/Ca mice after topical application on ears for 3 days, as measured by cell proliferation in the draining lymph nodes.

1.2. The study was conducted according to the following design :

Sub-group/treatment	Dose (%)	Number of females
1c. Negative control article	-	5
Positive control article[1]		
2a. Low dose	0.25	5
2b. Intermediate dose 1	0.5	5
2c. Intermediate dose 2	1.0	5
2d. High dose	2.0	5
Test article		
6a. Low dose	0.25	5
6b. Intermediate dose 1	0.5	5
6c. Intermediate dose 2	1.0	5
6d. High dose	2.0	5

[1] : The assay of the positive control article (p-phenylenediamine (PPD)) with 5 animals per sub-group was performed in the study number 762/001A. The results are also reported here in this report in order to compare with the effects obtained with the test article.

Group 1 animals received the negative control article (DMSO).

1.3. Morbidity/mortality checks were performed at least once daily. Clinical examinations were performed daily.
All animals were sacrificed on day 6 for evaluation of cell proliferation.

1.4. Results

- No mortality was observed during the study.
- No treatment-related clinical signs were observed.
- The test article Lehmann-blau did not induce a positive response, as it elicited less than a 3-fold increase in isotope incorporation relative to the negative control article. The stimulation indices were 1.29, 1.03, 1.12 and 1.42 at the concentrations of 0.25 %, 0.5 %, 1.0 % and 2.0 % respectively.

1.5 Conclusion

The test article Lehmann-blau is not a skin sensitizer under the defined experimental conditions.

2. INTRODUCTION

2.1. STUDY TITLE

Lehmann-blau - Local lymph node assay.

2.2. PURPOSE

To determine if the test article can induce a hypersensitivity response in mice after topical application on the ear, as measured by cell proliferation in the draining lymph nodes.

2.3. GUIDELINES

The local Lymph node assay is mentioned as an appropriate test in OECD guideline 406. This study was conducted in general compliance with the methodology published in Methods in Immunotoxicology, Vol. 2, pages 279-290, 1995 (Eds Wiley - Liss, Inc.).

2.4. PHOENIX INTERNATIONAL STUDY NUMBER

762/001D.

2.5. TESTING FACILITY

Phoenix International Preclinical Services Europe
Les Oncins - BP 0118
69593 L'Arbresle Cedex
France.

Study Director : M. Christ, Ph. D.

2.6. STUDY SPONSOR

2.7. SCHEDULE OF THE STUDY

- Study initiation date (protocol signed by Study Director) : 10 March 1999.
- Study completion date (final report signed by Study Director) : 20 April 2001.

2.8. PROTOCOL

Protocol no. 762/001-D, dated 9 March 1999, accepted 24 March 1999.
The protocol is presented in Addendum 1.

2.9. RESPONSIBLE PERSONNEL

- Study Director :
  M. Christ, Ph. D.

- Radio-isotopes unit:
  L. Routledge, Ph. D.

- Quality Assurance :
  P. Withers, B.Sc.

3. TEST/CONTROL ARTICLES AND VEHICLE INFORMATION

3.1. TEST ARTICLE

- Denomination : Lehmann-blau.
- Supplier :
- Appearance : grey powder.
- Batch number : 57
- Analytical documentation : presented in Addendum 2.
- Purity : 97.7 % (considered to be 100 % for dose calculation).
- Storage : at room temperature.

3.2. POSITIVE CONTROL ARTICLE

- Denomination : p-phenylenediamine (PPD).
- Supplier :
- Appearance : light brown powder.
- Batch number : R97005275.
- Analytical documentation : presented in Addendum 2.
- Purity : considered to be 100 % for dose calculation.
- Storage : at room temperature.

3.3. NEGATIVE CONTROL/VEHICLE FOR TEST ARTICLE AND POSITIVE CONTROL ARTICLE

- Denomination : DMSO.
- Supplier : Sigma, l'Isle d'Abeau Chesnes, BP701, 38297 La Verpillière cedex, France.
- Batch number : 68H3464.
- Storage : at room temperature.

3.4. FORMULATION OF THE TEST AND POSITIVE CONTROL ARTICLES

- Preparation : the test and positive control articles were prepared as a solution in the vehicle at concentrations of 0.25, 0.50, 1 and 2 % (w/v).
- Stability of the test article in the vehicle : under the responsibility of the Study Sponsor.
- Storage : at room temperature.
- Frequency of preparation : daily. The formulations of test and positive control articles were used within 4 hours of preparation.

4. METHODS AND EXPERIMENTAL DESIGN

4.1. TEST SYSTEM

- Species/strain : mouse : CBA/Ca.
- Supplier : Harlan UK.
- Number of animals in the study : 25 females.
- Age at initiation of treatment : 8 weeks.
- Justification : This strain was recommended as the most sensitive strain used in the local lymph node assay (I Kimber et al, Arch. Toxicol. (1989) 63 : 274 - 282). No known contra-indication to its use.

4.2. ANIMAL HUSBANDRY

- Housing : one air-conditioned room in a barrier protected unit (building G4) :
  - temperature : 22 ± 2°C,
  - relative humidity : 55 ± 15 %,
  - air changes : minimum 12 air changes per hour,
  - lighting cycle : 12 hours light (artificial)/12 hours dark.
- Caging : animals were housed in groups of 5 of the same dose group in plastic cages (265 x 160 x 140 mm).
- Bedding : dust-free sawdust made from spruce tree wood, analysed at least twice a year for chemical and bacterial contaminants.
- Diet : Rat & Mouse pelleted complete diet *ad libitum* (Diet A04 C-10, Usine d'Alimentation Rationnelle, Villemoisson, 91360 Epinay S/Orge, France) sterilised by irradiation and analysed for chemical and bacterial contaminants.

- Water : filtered (0.2 µm) mains drinking water *ad libitum* (via bottles) analysed at least once a year for chemical contaminants and at least twice a year for bacterial contaminants (Laboratoire de l'Environnement du Département d'Ecologie Urbaine de la Ville de Lyon, France).

- Contaminants : no known contaminants were present in bedding, diet or water at levels which might have interfered with achieving the objective of the study.

Certificates of analysis for the diet, the water and the bedding materials are maintained in the archives of the testing facility.

4.3. PRE-TREATMENT PROCEDURES

- Animal health procedure : all animals received a clinical inspection for ill-health on arrival.

- Acclimatisation period : 10 days between animal arrival and start of treatment.

- Animals identification : using microchip implants : Electronic Laboratory Animal Monitoring System, ELAMS (Bio Medic Data Systems), implanted in the lower dorsal region.

- Animal numbers :

Group number	Animal numbers
1	91 to 95
6	96 to 115

- Identification of the cages : group-related coloured cards with study number, group, sub-group and animal numbers.

4.4. EXPERIMENTAL DESIGN

- Allocation to treatment groups : performed during the acclimatisation period using a computer generated randomisation.

- Animals were assigned to the following groups :

Sub-group/treatment	Dose (%)	Number of females
1c. Negative control article	-	5
Positive control article[1]		
2a. Low dose	0.25	5
2b. Intermediate dose 1	0.5	5
2c. Intermediate dose 2	1.0	5
2d. High dose	2.0	5
Test article		
6a. Low dose	0.25	5
6b. Intermediate dose 1	0.5	5
6c. Intermediate dose 2	1.0	5
6d. High dose	2.0	5

[1] : The assay of the positive control article (p-phenylenediamine (PPD)) was performed with 5 animals per sub-group in the study number 762/001A. The results are also reported here in this report in order to compare with the effects obtained with the test article.

Group 1 animals received the negative control article (DMSO).

4.5. ADMINISTRATION OF THE TEST/CONTROL ARTICLES

- Route of administration : on the dorsum of both ears.

- Frequency : on days 1, 2 and 3.

- Duration : 3 administrations.

- Method of administration : 5 mice from the same cage were treated at the same time then placed in a cage. A hair dryer was used for about 5 minutes to dry mice ears.

- Volume of administration : 25 µl.

5. OBSERVATIONS

5.1. MORBIDITY/MORTALITY

All animals were observed at least once daily.

5.2. CLINICAL SIGNS

Animals were observed daily. During the treatment period, animals were observed before and at least once after dosing to detect any clinical signs or reaction to treatment.

5.3. EVALUATION OF CELL PROLIFERATION

- Animals examined and frequency : all animals on day 6 (five days after initiation of treatment).

- Method and material : all mice received an intravenous injection of 250 µl of phosphate buffered saline (PBS) containing 20 µCi of [^{3}H] methyl thymidine. Five hours later the mice were sacrificed by carbon dioxide inhalation and the draining auricular lymph node excised. A single cell suspension was prepared for each sub-group. Cells were washed twice with PBS and precipitated with ice cold 5 % trichloro-acetic acid (TCA). Approximately 18 hours later pellets were resuspended in 1 ml of TCA and transferred to 10 ml of scintillation cocktail. Incorporation was evaluated by a liquid scintillation technique using a TRI-CARB 2700TR Packard liquid scintillation analyser.

The Instrument Performance Assessment (IPA) was tested by counting special background, ^{3}H and ^{14}C IPA standards.

- The counting protocol parameters used for the direct radioactivity measurement were as follows :
  - Packard scintillation cocktail : 10 ml ULTIMA-GOLD.
  - Maximum counting time : 10 min.
  - Count termination based on 2 Sigma % = 2.00 %.
  - Quench set : ^{3}H - Toluene Packard sealed standards.
  - Energy windows : 0-18.6 keV.
  - Quench indicator : SIS.

The background samples were prepared for each series using 1 ml TCA.

6. DATA EVALUATION

As the lymph nodes were pooled for each group, no statistical analysis was performed (one value per group only).

In order to homogenize results obtained in the different studies (762/001A, 762/001B, 762/001C, 762/001D, 762/001E, 762/001F, 762/001G), all the DPM values were divided by the DPM/ml value obtained when measuring the radioactivity in the solution of ^{3}H methyl thymidine administered, leading to a corrected DPM value. The solution was freshly made for each day of injection, thus the DPM/ml value was different for each solution (see DPM/ml of PBS-^{3}H methyl thymidine, Table).

Each cell proliferation value (i.e. corrected DPM value) obtained for each sub-group was compared to the cell proliferation value obtained with the negative control article, leading to the calculation of a stimulation index

$$\left(\frac{\text{corrected DPM test article}}{\text{corrected DPM negative control article}} \right)$$

The stimulation index reflects the increase in isotope incorporation relative to the negative control article.

The criterion for a positive response is that the test or positive control articles elicit a 3-fold or greater increase in isotope incorporation relative to the negative control article (stimulation index of 3 or greater than 3).

7. ARCHIVES

All raw data, supporting documents and materials are maintained in the archives of the testing facility for 5 years. The archive period will start 3 months after the dispatch of the draft report.

At the end of the study or utilisation all remaining test and/or control article (excluding the archive samples) are returned to the Study Sponsor unless otherwise requested by the Study Sponsor.

The archive sample of the test/control articles will be destroyed at the end of the above mentioned archive period.

At the end of the 5-year archive period, the Study Sponsor will be contacted and asked if they wish the testing facility to continue archival storage, destroy the materials or have the materials returned to them (costs not included).

All Quality Assurance documents, an original signed protocol and the administrative copy of the final report with any subsequent amendments or addenda are retained by the testing facility as their property and will not be returned to the Study Sponsor or destroyed at the end of the archive period.

8. STUDY TIMETABLE

- Animal arrival : 9 March 1999 (Day –10).

- Allocation to treatment groups : 12 March 1999 (day –7).

FIRST DAY OF TREATMENT (Day 1) : 19 March 1999.

- Sacrifice for evaluation of cell proliferation : 24 March 1999 (Day 6).

9. PROTOCOL ADHERENCE

The study was performed in accordance with the protocol no. 762/001-D with the following deviations :

- The body weight range at animal arrival and not at initiation of treatment was 18 to 23 g.
- Allocation to treatment groups was not performed at the completion of the acclimatisation period but during the acclimatisation period.
- Animals were observed for morbidity/mortality at least once daily instead of at least twice daily.
- A single cell suspension was prepared for each sub-group and not for each group (error in the protocol).
- Since the issue of the draft report the name of the testing facility has changed and is now MDS Pharma Services.

These deviations were not considered to have affected the outcome or the achievement of the study objectives.

10. RESULTS

10.1. MORTALITY

No mortality was observed during the course of the study.

10.2. CLINICAL SIGNS

No treatment-related clinical signs were observed.

10.3. EVALUATION OF CELL PROLIFERATION

(see Table on pages 21 to 23)

The test article Lehmann-blau induced stimulation indices of 1.29, 1.03, 1.12 and 1.42 at the concentrations of 0.25 %, 0.5 %, 1.0 % and 2.0 % respectively. These stimulations indices were not greater than 3, the response was considered as negative.

11. CONCLUSION

The test article Lehmann-blau is not a skin sensitizer under the defined experimental conditions.

12. TABLE

EVALUATION OF CELL PROLIFERATION

Abbreviation

DPM : Disintegrations per minute

EVALUATION OF THE CELL PROLIFERATION

PRINTED: 08-Apr-99

DPM/ml of PBS + (^{3}H) methylthymidine : 30968801

STUDY NUMBER: 762001D

	DOSE (%)	DPM	CORRECTED DPM[1] (x10^{-4})	STIMULATION INDEX (2)
NEGATIVE CONTROL ARTICLE				
1c.		13448.2	4.34	-
TEST ARTICLE - Lehmann-blau				
6a.	0.25	17373.5	5.61	1.29
6b.	0.5	13898.6	4.49	1.03
6c.	1.0	15087.3	4.87	1.12
6d.	2.0	19073.2	6.16	1.42

[1] : DPM/(DPM/ml)

(2) corrected DPM of test or positive control articles/corrected DPM of negative control article

Table (cont'd)

EVALUATION OF THE CELL PROLIFERATION

PRINTED: 08-Apr-99

DPM/ml of PBS + (^{3}H) methylthymidine : 28306636

STUDY NUMBER: 762001D

	DOSE (%)	DPM	CORRECTED DPM[1] (x10^{-4})	STIMULATION INDEX (2)
NEGATIVE CONTROL ARTICLE				
1a.		3775.6	1.33	-
POSITIVE CONTROL ARTICLE				
2a.	0.25	20568.5	7.27	5.47
2b.	0.5	46652.5	16.48	12.39
2c.	1.0	71978.4	25.43	19.12
2d.	2.0	26597.7	9.40	7.07

[1] : DPM/(DPM/ml)

(2) corrected DPM of test or positive control articles/corrected DPM of negative control article

Table (cont'd)

13. ADDENDA

Addendum 1

PROTOCOL NO. 762/001-D

Chrysalis
Pre-Clinical Services - Europe
Les Oncins, B.P. 118
69593 L'ARBRESLE Cedex, France
Tel: +33 (0)4 74 01 63 63
Fax: +33 (0)4 74 01 63 99
E-mail: preclin.eu@chrysalisintl.com

Addendum 1 (cont'd)

Protocol: 762/001-D - 9 March 1999

Study : Local lymph node assay

Chrysalis study number : 762/001

Study Sponsor :

1. INTRODUCTION

1.1. STUDY TITLE

Local lymph node assay

1.2. PURPOSE

To determine if the test articles can induce a hypersensitivity response in mice after topical application on ears as measured by cell proliferation in the draining lymph nodes.

1.3. GUIDELINES

There are no published guidelines but this study will be conducted in general compliance with the methodology published in Methods in Immunotoxicology, Vol. 2, pages 279-290, 1995 (Eds Wiley - Liss, Inc.).

1.4. CHRYSALIS STUDY NUMBER

762/001.

1.5. TESTING FACILITY

CHRYSALIS Preclinical Services - Europe
Les Oncins - BP 0118
69593 L'ARBRESLE CEDEX
FRANCE.

Study Director : M. CHRIST, PhD.
Deputy Study Director : L. FAURE, PhD.

1.6. STUDY SPONSOR

Study monitor : H. SCHEFFLER.

1.7. SCHEDULE OF THE STUDY

Start of treatment : 17, 18, 19 and 20 March 1999.
Necropsy : 22, 23, 24 and 25 March 1999.
Draft report : July 1999.

2. TEST/CONTROL ARTICLES AND VEHICLE INFORMATION

(see Sponsor documentation, if supplied)

The Study Sponsor is responsible for sending a certificate of conformity to the Study Director for each batch of test articles supplied to Chrysalis Preclinical Services - Europe.
This certificate documents that appropriate checking procedures have been used to ensure that the test or positive control articles conform to established specifications and is that intended for use in the study.

2.1. TEST ARTICLE 1

- Denomination : Ammonium thioglycolate 70 %.
- Batch number : B99S4154.
- Appearance : colorless liquid.
- Purity : between 59 and 61 g/100 ml (consider 60 % for dose calculation).
- Storage : room temperature, protected from light.
- Hazards : standard precautions.

2.2. TEST ARTICLE 2

- Denomination : GED 1230.
- Batch number : 7-16069.
- Appearance : clear liquid at room temperature, white solid at refrigerated temperature.
- Purity : 96 % (consider 100 % for dose calculation).
- Storage : refrigerated temperature.
- Hazards : standard precautions.

2.3. TEST ARTICLE 3

- Denomination : A010766.
- Batch number : MET0200/3.
- Appearance : light brown powder.
- Purity : consider 100 % for dose calculation.
- Storage : room temperature.
- Hazards : standard precautions.

2.4. TEST ARTICLE 4

- Denomination : Lehmann-blau.
- Batch number : 57.
- Appearance : grey powder.
- Purity : 97.7 % (consider 100 % for dose calculation).
- Storage : room temperature.
- Hazards : standard precautions.

2.5. TEST ARTICLE 5

- Denomination : A10771.
- Batch number : PIR0087/3.
- Appearance : white powder.
- Purity : consider 100 % for dose calculation.
- Storage : room temperature.
- Hazards : standard precautions

2.6. TEST ARTICLE 6

- Denomination : A011071.
- Batch number : HAY0252.
- Appearance : white powder.
- Purity : consider 100 % for dose calculation.
- Storage : room temperature.
- Hazards : standard precautions.

2.7. TEST ARTICLE 7

- Denomination : A007317.
- Batch number : DOU229612.
- Appearance : grey powder.
- Purity : consider 100 % for dose calculation.
- Storage : room temperature.
- Hazards : standard precautions.

2.8. POSITIVE CONTROL ARTICLE

- Denomination : p-phenylenediamine (PPD).
- Batch number : R97005275.
- Appearence : light brown powder.
- Purity : consider 100 % for dose calculation.
- Supplier : Study Sponsor.
- Storage : at room temperature.
- Hazards : standard precautions.

2.9. NEGATIVE CONTROL/VEHICLE FOR TEST ARTICLES 1, 2, 3, 4 AND POSITIVE CONTROL ARTICLE

- Denomination : DMSO.
- Supplier : SIGMA, l'Isle d'Abeau Chesnes, BP701, 38297 La Verpillière cedex, France.
- Batch number : will be indicated in the report.
- Storage : room temperature.
- Hazards : standard precautions.

2.10. VEHICLE FOR TEST ARTICLES 5, 6, 7

- Denomination : DMSO/1N NaOH.
- Supplier for DMSO : SIGMA, l'Isle d'Abeau Chesnes, BP701, 38297 La Verpillière cedex, France.
- Supplier for NaOH : E. MERCK, 64271 Darmstadt, Germany.
- Batch number : will be indicated in the final report.
- Preparation : will be documented in the final report.

2.11. FORMULATION OF THE TEST AND CONTROL ARTICLES

- The test and control articles will be prepared daily in the vehicule at 0.25, 0.50, 1.0, and 2.0 % (w/v).
- The stability of test article in the vehicule is the responsibility of the Study Sponsor.
- The formulations of test or control articles will be used within 4 hours following the preparation.

3. METHODS AND EXPERIMENTAL DESIGN

3.1. TEST SYSTEM

- Species/strain : mouse : CBA/Ca.
- Supplier : Harlan UK.
- Justification : This strain was recommended as the most sensitive strain used in the local lymph node assay (I Kimber et al, Arch. Toxicol. (1989) 63 : 274 - 282). No known contra-indication to its use.
- Number of animals in the study : **180 females.**
- Age at initiation of treatment : 8 to 12 weeks.
- Body weight range at initiation of treatment : 18 to 30 g.

3.2. ANIMAL HUSBANDRY

- Housing : in the same air - conditioned room as other animals of the same species.
  - temperature : 22 ± 2°C (target range),
  - relative humidity : 55 ± 15 % (target range),
  - air changes : minimum 8 air changes per hour,
  - lighting cycle : 12 hours light (artificial)/12 hours dark.
- Caging : animals housed 5 per cage in plastic cages (265 x 160 x 140 mm).
- Bedding : dust-free sawdust made from spruce tree wood, analysed at least twice a year for chemical and bacterial contaminants.
- Diet : pelleted complete diet *ad libitum* (Diet reference A04-C10, Usine d'Alimentation Rationnelle, Villemoisson, 91360 Epinay s/Orge, France), sterilised by irradiation and analysed for the absence of chemical and bacteriological contaminants.
- Water : filtered (0.2 µm) mains drinking water, *ad libitum* analysed at least once a year for chemical contaminants and at least twice a year for bacterial contaminants (Laboratoire de l'Environnement du Département d'Ecologie Urbaine de la ville de Lyon).
- Contaminants : no contaminants are known to be present in the diet or water at levels which might interfere with achieving the objective of the study.
  Certificates of analysis for the diet, the water and the bedding will be maintained in the archives of the testing facility.

3.3. PRE-TREATMENT PROCEDURES

- Animal health procedure : clinical inspection for ill-health on arrival.
- Acclimatisation period : 7 days minimum between animal arrival and start of treatment.
- Allocation to treatment group : performed at the completion of the acclimatisation period, using a computer generated randomisation.
- Identification of the animals :
  - using microchip implants : Electronic Laboratory Animal Monitoring System, ELAMS (Bio Medic Data Systems),
  - implanted in the lower dorsal region.
- Identification numbers :

Group number	Colour code	Animal number
1	White	01 to 05, 46 to 50 91 to 95, 136 to 140
2	Green	06 to 25
3	Blue	26 to 45
4	Red	51 to 70
5	Yellow	71 to 90
6	Salmon	96 to 115
7	Green with one stripe	116 to 135
8	Blue with one stripe	141 to 160
9	Red with one stripe	161 to 180

- **Identification of the cages : group related coloured card with study number, group and sub-group numbers and animal numbers.**

3.4. EXPERIMENTAL DESIGN

Animals will be divided in 4 sub-sets (A, B, C and D), each sub-set being treated independently on a different day.

A.

Sub-group/treatment	Dose (%)	Number of females
1.a Negative control article	-	5
2a. Positive control article	0.25	5
2b. Positive control article	0.5	5
2c. Positive control article	1.0	5
2d. Positive control article	2.0	5
3a. Test article 1	0.25	5
3b. Test article 1	0.5	5
3c. Test article 1	1.0	5
3d. Test article 1	2.0	5

B.

Sub-group/treatment	Dose (%)	Number of females
1.b Negative control article	-	5
4a. Test article 2	0.25	5
4b. Test article 2	0.5	5
4c. Test article 2	1.0	5
4d. Test article 2	2.0	5
5a. Test article 3	0.25	5
5b. Test article 3	0.5	5
5c. Test article 3	1.0	5
5d. Test article 3	2.0	5

C.

Sub-group/treatment	Dose (%)	Number of females
1.c Negative control article	-	5
6a. Test article 4	0.25	5
6b. Test article 4	0.5	5
6c. Test article 4	1.0	5
6d. Test article 4	2.0	5
7a. Test article 5	0.25	5
7b. Test article 5	0.5	5
7c. Test article 5	1.0	5
7d. Test article 5	2.0	5

D.

Sub-group/treatment	Dose (%)	Number of females
1.c Negative control article	-	5
8a. Test article 6	0.25	5
8b. Test article 6	0.5	5
8c. Test article 6	1.0	5
8d. Test article 6	2.0	5
9a. Test article 7	0.25	5
9b. Test article 7	0.5	5
9c. Test article 7	1.0	5
9d. Test article 7	2.0	5

3.5. ADMINISTRATION OF THE TEST/CONTROL ARTICLES

On days 1, 2 and 3, the animals will receive 25 µl of one of the test articles or the control article on the dorsum of both ears.

4. OBSERVATIONS

4.1. MORBIDITY/MORTALITY

Animals will be observed twice daily. Moribund animals will be killed and submitted to necropsy. Animals found dead will be submitted to necropsy to investigate the cause of death.

4.2. CLINICAL SIGNS

Animals will be observed daily, before and at least once after dosing to detect any clinical signs or reaction to treatment.

4.3. EVALUATION OF THE CELL PROLIFERATION

On day 6 (five days following the initiation of treatment), all mice will receive an intravenous injection of 250 µl of phosphate buffered saline (PBS) containing 20 µCi of [^{3}H] methyl thymidine. Five hours later the mice will be sacrificed by carbon dioxide inhalation and the draining auricular lymph node excised. A single cell suspension will be prepared for each group. Cells will be washed twice with PBS and precipitated with ice cold 5 % trichloro-acetic acid (TCA). Approximately 18 hours later pellets will be resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid. Incorporation will be evaluated using a scintillation counter.

5. DATA EVALUATION

As the lymph nodes will be pooled for each group, no statistical analysis will be performed (one value per group only).

The criterion for a positive response is that the test or positive control articles elicit a 3-fold or greater increase in isotope incorporation relative to the negative control article.

6. QUALITY ASSURANCE

This study will be subjected to quality assurance procedures in compliance with :

- "OECD Principles of Good Laboratory Practice" concerning Mutual Acceptance of Data in the Assessment of Chemicals dated 26 November 1997 (C (97) 186 Final).
- "Good Laboratory Practice" described in the U.S. Federal Register (Food and Drug Administration) dated 22 December 1978 with any applicable amendments.

The protocol will be audited. Procedures similar to those used on this type of study are inspected periodically in the laboratory and animal areas. The final reports will be reviewed to assure that they accurately describes the methods and procedures, and that the results accurately reflect the raw data. Reports on these activities will be made to the Study Director and to Management.

Any analyses performed by or under the responsibility of the Study Sponsor will not be audited by the Quality Assurance Unit of Chrysalis Preclinical Services - Europe.

7. REPORTS

Incidental reports

The Study Sponsor will be informed promptly of any significant findings at any time during the study.

Draft report

A complete draft report in English, containing all procedures and results will be issued for discussion with the Study Sponsor.

Final report

After reciprocal agreement the final report will be issued and 3 copies (2 bound and 1 unbound) in English sent to the Study Sponsor. **Separate reports for each of the seven test articles will be issued (762/001A, 762/001B, 762/001C, 762/001D, 762/001E, 762/001F, 762/001G). Positive and negative control data will be included in each report.**

8. ARCHIVES

All raw data supporting documents and materials will be maintained in the archives of the testing facility for 5 years. The archive period will start 3 months after the dispatch of the draft report.

Labile specimens (e.g. frozen serum, urine or tissue samples) will be destroyed at the start of the above mentioned archive period unless otherwise requested by the Study Sponsor.

At the end of the study or utilisation all remaining test and/or control article (excluding the archive sample(s)) will be returned to the Study Sponsor unless otherwise requested by the Study Sponsor.

The archive sample of the test/control article(s) will be destroyed at the end of the above mentioned archive period.

At the end of the 5-year archive period, the Study Sponsor will be contacted and asked if they wish the testing facility to continue archival storage, destroy the materials or have the materials returned to them (costs not included).

All Quality Assurance documents, an original signed protocol and any amendments and the administrative copy of the final report with any subsequent amendments or addenda will be retained by the testing facility as their property and will not be returned to the Study Sponsor or destroyed at the end of the archive period.

Chrysalis will not be held responsible for the safe keeping or transport of any data or samples removed or dispatched from the testing facility at the request of the Study Sponsor.

Addendum 1 (cont'd)

PROTOCOL : 762/001-D

CHRYSALIS STUDY NUMBER : 762/001

SIGNATURE PAGE

Approved by :

CHRYSALIS Preclinical Services - Europe		STUDY SPONSOR	
Signature :		Signature :	
Name :	M. CHRIST	Name :	
Title :	Study Director	Title :	
Date	10 March 99	Date	15/3

Addendum 2

CERTIFICATES OF ANALYSIS

Addendum 2 (cont'd)

Analysenzertifikat / 762 / 001 D

ondal

Probennr. : **R96900523** Revision der Spezifikation : / 03

Rohst.-Nr. : **23081** —> **LEHMANN BLAU**

Charge : **57** / 01 Eingangsdatum : **08.01.1996**

Aussehen : **Pulver, graublau**

Geruch : **Eigengeruch**

Bemerkung : **Sollmuster: gemäß Analysenzertifikat Nr. 4 vom 18.07.1994**

Bemerkungen :

Prüfpunkt	Einheit	Zusätzliche Informationen	Bedingung des Prüfpunktes	Grenzwert unten	Grenzwert oben	Meßwert
Aussehen			prüfen			i.O.
Geruch			prüfen			i.O.
Ausfärbung			prüfen			ntspricht Standa
Alkali-Test			prüfen			i.O.
Gehalt an Eisen	ppm		prüfen	0	100	82,00
Gehalt nach HPLC	% rel.	HPLC-Methode 16	prüfen	98	100	97,7
Gehalt an 2,4-Diaminoanisol	ppm		prüfen	0	600	ntspricht Grenzw
Gehalt an Schwermetallen (Steighöhe)			prüfen			i.O.
pH-Wert		1 %ige Lsg.	prüfen	1,9	2,3	entfällt

Hünfeld, 09.02.2000

QS-Labor B. Bieber

Dieses Dokument wurde elektronisch erstellt und ist ohne Unterschrift gültig.

Addendum 2 (cont'd)

Certificate of Analysis

ondal

Sample-No. : **R97005275** Revision : KRUSE / 02

Raw material No : **23033** —> **PP-STOFF**

Batch : **978277/886 / 02** Date : **28.07.1997**

Appearance : **Schuppen, beige**

Odour : **Eigengeruch**

Remarks : **Sollmuster: gemäß Analysenzertifikat Nr. 46 vom 21.11.95**

Remarks :

Test criterion	Unit	Additional Informations	Status Test criterion	Limit minimum	Limit maximum	Test Result
Aussehen			prüfen			I.O.
Alkali-Test			prüfen			I.O.
Coloristische Prüfung	%		prüfen	-5	5	1
Gehalt an Eisen	ppm		prüfen	0	100	122,00
Gehalt nach HPLC	% rel.	HPLC-Methode 68	prüfen	98	100	100,0
Gehalt an Schwermetallen (Steighöhe)			prüfen			I.O.

Hünfeld, 05.03.2001

QS-Labor B. Bieber

This document has been made out electronically and is effective unsigned.