SafePharm Laboratories

13F-SFA-MONOMER:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

SPL PROJECT NUMBER: 1458/0064

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	25 April 2005	Standard Test Method Compliance Audit
	02 March 2007	Test Material Preparation
	05 March 2007	Test System Preparation
	07 March 2007	Animal Preparation
	02 March 2007	Dosing
	06 March 2007	Assessment of Response
§	13 April 2007	Draft Report Audit
§	Date of QA Signature	Final Report Audit
§	Evaluation specific to this study	

..... DATE: 11 MAY 2007

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**
Head of Department:
Deputy Head of Department:
Senior Audit Staff:

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: 10/5/07

Study Director

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SUMMARY

Introduction. A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)

- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Directive 2004/73/EC

Methods. Following a preliminary screening test, three groups, each of four animals, were treated with 50 µl (25 µl per ear) of the test material as a solution in dimethyl formamide at concentrations of 25%, 10% or 5% v/v. A further group of four animals was treated with dimethyl formamide alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in dimethyl formamide	Stimulation Index	Result
5	1.06	Negative
10	1.92	Negative
25	1.20	Negative

Conclusion. The test material was considered to be a non-sensitiser under the conditions of the test.

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1. INTRODUCTION

A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)

- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Directive 2004/73/EC

The assay has undergone extensive inter-laboratory validation and has been shown to reliably detect test materials that are moderate to strong sensitisers.

The strain of mouse used in these laboratories has been shown to produce satisfactory responses using known sensitisers and non-sensitisers during the in-house validation. The results of routine positive control studies are shown in Appendix 1 and Appendix 2. The results of the study are believed to be of value in predicting the sensitisation potential of the test material to man.

The study was performed between 19 February 2007 and 27 March 2007.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	13F-SFA-MONOMER
Description	:	clear colourless liquid
Batch number	:	061115
Date received	:	24 November 2006
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

2.2 Preparation of Test Material

For the purpose of the study, the test material was used undiluted and freshly prepared in dimethyl formamide. This vehicle was chosen as it produced the most suitable formulation at the required concentration. The concentrations used are given in the procedure section. The vehicle determination record is included as Appendix 3.

Determination, by analysis, of the concentration, homogeneity and stability of the test material preparations was not appropriate because it was not specified in the Study Plan and is not a requirement of the Test Guideline.

3. METHODS

3.1 Animals and Animal Husbandry

Two female CBA/Ca (CBA/CaBkl) strain mice were supplied by B & K Universal Ltd, Hull, UK and seventeen female CBA/Ca (CBA/Ca CruBR) strain mice were supplied by Charles River UK Limited, Margate, Kent, UK. On receipt the animals were randomly allocated to cages. The animals were nulliparous and non-pregnant. After an acclimatisation period of at least five days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were in the weight range of 15 to 23 g, and were eight to twelve weeks old.

The animals were individually housed in suspended solid-floor polypropylene cages furnished with softwood woodflakes. Free access to mains tap water and food (Certified Rat and Mouse Diet) was allowed throughout the study.

The temperature and relative humidity were controlled to remain within target ranges of 19 to 25°C and 30 to 70%, respectively. Any occasional deviations from these targets were considered not to have affected the purpose or integrity of the study. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled by a time switch to give twelve hours continuous light (06.00 to 18.00) and twelve hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

3.2 Procedure

3.2.1 Preliminary Screening Test

Using available information regarding the systemic irritancy potential of the test material, a preliminary screening test was performed using three mice. The mice were treated by daily application of 25 µl of the undiluted test material or the test material at concentrations of 50% or 25% v/v in dimethyl formamide, to the dorsal surface of each ear for up to three consecutive days (Days 1, 2, 3). The mice were observed twice daily on Days 1 and 2 and pre-dose on Day 3. The surviving mice were observed post dose on Day 3 and once on Day 4. The remaining mouse was observed once daily on Days 5 and 6. Any signs of toxicity or excessive local irritation noted during this period were recorded. The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and of the surviving mouse on Day 6. The bodyweight of each mouse which was killed for humane reasons was recorded at death.

3.2.2 Main Test

3.2.2.1 Test Material Administration

Groups of four mice were treated with the test material at concentrations of 5%, 10% or 25% v/v in dimethyl formamide. The preliminary screening test suggested that the test material would not produce systemic toxicity or excessive local irritation at the highest suitable concentration. The mice were treated by daily application of 25 µl of the appropriate concentration of the test material to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test material formulation was administered using an automatic micropipette and spread over the dorsal surface of the ear using the tip of the pipette.

A further group of four mice received the vehicle alone in the same manner.

3.2.2.2 ^{3}H-Methyl Thymidine Administration

Five days following the first topical application of the test material (Day 6) all mice were injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR: 80 µCi/ml, specific activity 2.0 Ci/mmol, GE Healthcare UK Ltd) giving a total of 20 µCi to each mouse.

3.2.2.3 Observations

Clinical Observations: All animals were observed twice daily on Days 1, 2 and 3 and on a daily basis on Days 4, 5 and 6. Any signs of toxicity or signs of ill health during the test were recorded.

Bodyweights: The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and Day 6 (prior to termination).

3.2.2.4 Terminal Procedures

Termination: Five hours following the administration of ^{3}HTdR all mice were killed by carbon dioxide asphyxiation. The draining auricular lymph nodes from the four mice were excised and pooled for each experimental group. For each group 1 ml of PBS was added to the pooled lymph nodes.

Preparation of Single Cell Suspension: A single cell suspension of pooled lymph node cells was prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells were rinsed through the gauze with 4 ml of PBS into a petri dish labelled with the project number and dose concentration. The lymph node cell suspension was transferred to a centrifuge tube. The petri dish was washed with an additional 5 ml of PBS to remove all remaining lymph node cells and these were added to the centrifuge tube. The pooled lymph node cells were pelleted at 1400 rpm (approximately 190 *g*) for ten minutes. The pellet was resuspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet was resuspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation: After approximately eighteen hours incubation at approximately 4°C, the precipitates were recovered by centrifugation at 2100 rpm (approximately 450 *g*) for ten minutes, resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Trisafe'). ^{3}HTdR incorporation was measured by β-scintillation counting. The "Poly Q^{TM}" vials containing the samples and scintillation fluid were placed in the sample changer of the scintillator and left for approximately twenty minutes. The purpose of this period of time in darkness was to reduce the risk of luminescence, which has been shown to affect the reliability of the results. After approximately twenty minutes, the vials were shaken vigorously. The number of radioactive disintegrations per minute was then measured using the Beckman LS6500 scintillation system (Beckman Instruments Inc, Fullerton, CA).

3.3 Interpretation of Results

The proliferation response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph node (disintegrations per minute/node) and as the ratio of ^{3}HTdR incorporation into lymph node cells of test nodes relative to that recorded for the control nodes (Stimulation Index).

The test material will be regarded as a sensitiser if at least one concentration of the test material results in a threefold or greater increase in ^{3}HTdR incorporation compared to control values. Any test material failing to produce a threefold or greater increase in ^{3}HTdR incorporation will be classified as a "non-sensitiser".

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Preliminary Screening Test

Clinical observations, bodyweight and mortality data are given in Table 1.

The animals treated with the undiluted test material or the test material at a concentration of 50% v/v were killed for humane reasons on Days 3 or 4, due to the approach of the moderate severity limit. Signs of systemic toxicity noted were hunched posture, lethargy, ataxia, pilo-erection, decreased respiratory rate, tiptoe gait and bodyweight loss. No signs of systemic toxicity were noted in the animal treated with the test material at a concentration of 25% v/v.

Based on this information the dose levels selected for the main test were 25%, 10% and 5% v/v in dimethyl formamide.

5.2 Main Test

5.2.1 Estimation of the Proliferative Response of Lymph Node Cells

The radioactive disintegrations per minute per lymph node and the stimulation index are given in Table 2.

A stimulation index of less than 3 was recorded for the three concentrations of the test material (25%, 10% and 5% v/v in dimethyl formamide).

5.2.2 Clinical Observations and Mortality Data

Individual clinical observations and mortality data for test and control animals are given in Table 3.

There were no deaths. No signs of systemic toxicity were noted in the test or control animals during the test.

5.2.3 Bodyweight

Individual bodyweights and bodyweight changes for test and control animals are given in Table 4.

Bodyweight changes of the test animals between Day 1 and Day 6 were comparable to those observed in the corresponding control group animals over the same period.

6. CONCLUSION

The test material was considered to be a non-sensitiser under the conditions of the test.

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Table 1 Clinical Observations, Bodyweight and Mortality Data – Preliminary Screening Test

Concentration (% v/v) in dimethyl formamide	Animal Number	Bodyweight (g)		Day								
				1		2		3		4	5	6
		Day 1	Day 6	Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
100	S-1	17	-	0	0	0	0	HLA PRd ⊗ X*	-	-	-	-
50	S-2	20	-	0	0	0	0	0	0	HLP Wt • X*	-	-
25	S-3	17	18	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity
H = Hunched posture
L = Lethargy
A = Ataxia
P = Pilo-erection
Rd = Decreased respiratory rate
Wt = Tiptoe gait
⊗ = Bodyweight loss noted (3 g) animal weighed 14 g
• = Bodyweight loss noted (3 g) animal weighed 17 g
- = Animal dead
X* = Animal killed for humane reasons due to the approach of the moderate severity limit

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Table 2 Disintegrations per Minute, Disintegrations per Minute/Node and Stimulation Index

Concentration (% v/v) in dimethyl formamide	dpm	dpm/Node [a]	Stimulation Index [b]	Result
Vehicle	3765.09	470.64	na	na
5	3976.13	497.02	1.06	Negative
10	7231.61	903.95	1.92	Negative
25	4523.35	565.42	1.20	Negative

a = Disintegrations per minute/node obtained by dividing the disintegrations per minute value by 8 (total number of lymph nodes)
b = Stimulation Index of 3.0 or greater indicates a positive result
na = Not applicable
dpm = Disintegrations per minute

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Table 3 Individual Clinical Observations and Mortality Data

Concentration (% v/v) in dimethyl formamide	Animal Number	Day 1		Day 2		Day 3		Day 4	Day 5	Day 6
		Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
Vehicle	1-1	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0
5	2-1	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0
10	3-1	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0
25	4-1	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

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Table 4 Individual Bodyweights and Bodyweight Changes

Concentration (% v/v) in dimethyl formamide	Animal Number	Bodyweight (g)		Bodyweight Change (g)
		Day 1	Day 6	
Vehicle	1-1	19	20	1
	1-2	19	20	1
	1-3	18	18	0
	1-4	21	21	0
5	2-1	20	19	-1
	2-2	19	20	1
	2-3	16	17	1
	2-4	17	18	1
10	3-1	16	18	2
	3-2	20	21	1
	3-3	18	17	-1
	3-4	21	20	-1
25	4-1	22	20	-2
	4-2	20	19	-1
	4-3	19	19	0
	4-4	19	21	2

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Appendix 1 Current Positive Control Study for the Local Lymph Node Assay

Introduction. A study was performed to assess the sensitivity of the strain of mouse used at these laboratories to a known sensitiser. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Directive 2004/73/EC

Test Material:	α-Hexylcinnamaldehyde, Tech, 85%
Safepharm Laboratories Project number:	0039/0868
Study dates:	30 June 2006 to 06 July 2006

Methods. Three groups, each of five animals, were treated with 50 µl (25 µl per ear) of α-Hexylcinnamaldehyde, Tech, 85% as a solution in dimethyl formamide at concentrations of 5%, 10% and 25% v/v. A further control group of five animals was treated with dimethyl formamide alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in dimethyl formamide	Stimulation Index	Result
5	2.81	Negative
10	4.20	Positive
25	13.15	Positive

Conclusion. α-Hexylcinnamaldehyde, Tech, 85% was considered to be a sensitiser under the conditions of the test.

Appendix 2 Summary of Positive Control Data for the Local Lymph Node Assay

Project Number	Start Date	Finish Date	Test Material	Concentration	Vehicle	Stimulation Index [a]	Classification [b]
039/747•	04/03/05	10/03/05	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	acetone/olive oil (4:1 v/v)	2.76, 3.34, 8.91	Positive
039/750*	20/04/05	26/04/05	α-Hexylcinnamaldehyde	5%, 10%, 25% w/v	ethanol/distilled water (7:3 v/v)	2.64, 8.36, 12.94	Positive
0039/0766*⊕	29/06/05	05/07/05	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	acetone/olive oil (4:1 v/v)	2.24, 1.94, 4.76	Positive
0039/0821•	05/04/06	11/04/06	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	acetone/olive oil (4:1 v/v)	2.50, 4.03, 9.13	Positive
0039/0867•	16/06/06	22/06/06	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	butanone	3.08, 4.54, 8.06	Positive
0039/0868•	30/06/06	06/07/06	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	dimethyl formamide	2.81, 4.20, 13.15	Positive
0039/0869•	06/07/06	12/07/06	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	dimethyl sulphoxide	1.39, 3.81, 5.84	Positive
0039/0870•	07/07/06	13/07/06	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	acetone	3.53, 5.39, 8.23	Positive
0039/0880•	28/09/06	04/10/06	2,4-Dinitrobenzenesulfonic acid, sodium salt	1%, 10%, 20% w/w	1% pluronic L92 in distilled water	1.39, 11.33, 19.34	Positive
0039/0881•	29/09/06	05/10/06	2,4-Dinitrobenzenesulfonic acid, sodium salt	5%, 10%, 25% w/w	propylene glycol	10.68, 23.80, 40.25	Positive
0039/0911•	17/01/07	07/02/07	Phenylacetaldehyde (90%)	5%, 10%, 25% w/w	propylene glycol	11.25, 20.00, 29.49	Positive

a = Ratio of test to control lymphocyte proliferation
b = Stimulation index greater than 3.0 indicates a positive result
* = Standard Test Method 595 ('Pooled' nodes)
• = Standard Test Method 599 ('Individual' nodes)
⊕ = B6CBAF1/Crl strain mice were used instead of CBA/Ca strain mice

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Appendix 3 Vehicle Determination Record

Vehicle	Concentration	Method of Preparation	Description of Formulation	Suitability*
acetone/olive oil (4:1)	50% 0.5 ml test material + 0.5 ml vehicle	Vortex mixer	-	not suitable for dosing
dimethyl formamide	50% 0.5 ml test material + 0.5 ml vehicle	Vortex mixer	solution	suitable for dosing

* = Suitable for dosing if formulation is a solution or fine homogenous suspension which can be administered via a micropipette

Appendix 4 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **London Road** **Shardlow** **Derby** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

30th August 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

105.

Head, UK GLP Monitoring Authority