NOA449280

NOA449280 SL (A16003E) - Contact Hypersensitivity in Albino Guinea Pigs, Buehler Test (9-induction)

Final Report

DATA REQUIREMENT(S): OECD [Number 406 “Skin Sensitization”]
EPA [OPPTS 870.2600]
Commission Regulation (EC) No 440/2008
Japanese MAFF [12 NohSan No. 8147]

AUTHOR(S): G. Arcelin

STUDY COMPLETION DATE: 29-Sep-2009

PERFORMING LABORATORY: Harlan Laboratories Ltd.
Wölferstrasse 4
4414 Füllinsdorf, Switzerland

LABORATORY PROJECT ID: Report Number: C26688
Study Number: C26688
Task Number: T002530-07

SPONSOR: Syngenta Ltd
Jealott’s Hill International Research Centre
Bracknell, Berkshire, RG42 6EY, United Kingdom

STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The stability of the test item in purified water is unknown. The formulation trials were performed before the study initiation date. Therefore, they are excluded from this statement.

This study has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted May 18, 2005 [RS 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26, 1997 by decision of the OECD Council [C(97)186/Final].

G. Arcelin
Study Director
Acute Toxicology

29-Sep-2009
Date

Performing Laboratory:

Harlan Laboratories Ltd.,
Wölferstrasse 4
4414 Füllinsdorf / Switzerland

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Harlan Laboratories Ltd., Zelgliweg 1, 4452 Itingen / Switzerland

Harlan Laboratories Study:	C26688
Sponsor Reference Number:	T002530-07
Test Item:	NOA449280 SL (A16003E)
Study Director:	G. Arcelin
Study Title:	NOA449280 SL (A16003E) - Contact Hypersensitivity in Albino Guinea Pigs, Buehler Test (9-induction)

The general facilities and activities are inspected at least once a year and the results are reported to the responsible person and the management.

Study procedures, with exception of the formulation trials, were periodically audited. The study plan and this report were audited by the Quality Assurance. The dates are given below.

Dates and Types of QA Inspections		**Dates of Reports to the Study Director and Test Facility Management**
08-Jan-2009	Study Plan	08-Jan-2009
23-Jan-2009	Study Based (Test System, Test Item, Raw Data, Treatment)	23-Jan-2009
17-Jun-2009	Report	17-Jun-2009

This statement also confirms that this final report reflects the raw data.

Quality Assurance: N. Engelke

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Date: 29-Jun-2009

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Function
Dr. C. Simon	Study Director until 08-Jul-2009
G. Arcelin	Study Director from 09-Jul-2009
G. Arcelin until 08-Jul-2009	Deputy Study Director until 08-Jul-2009
E. Rached from 09-Jul-2009	Deputy Study Director rom 09-Jul-2009
T. Fink	Manager Quality Assurance
R. Doran	Syngenta Study Manager

Study dates

Experimental Starting Date:	14-Jan-2009
Experimental Completion Date	06-Mar-2009
Delivery of Animals:	14-Jan-2009
	25-Feb-2009 (control group 2)
Acclimatization (main study):	14-Jan-2009 to 18-Jan-2009
	25-Feb-2009 to 01-Mar-2009 (control group 2)
Treatment Start (main study):	19-Jan-2009 (induction)
	16-Feb-2009 (first challenge)
	02-Mar-2009 (second challenge)
Observation (main study):	14-Jan-2009 to 06-Mar-2009
Termination:	06-Mar-2009

Deviations from the guidelines

The skin reading was performed approximately 23 and 22 hours (i.e. 24-hour reading) after removal of the patch in the third and sixth induction and approximately 44 hours (i.e. 48 hour reading) after patch removal in the irritation screen. This deviation had no impact on the study.

Retention of samples

See below under Other.

Performing laboratory test substance reference number

216594 A

Other

Harlan Laboratories Ltd. (4452 Itingen / Switzerland) will retain the study plan, study plan amendment, raw data, a sample of test item(s) and the final report of the present study for at least ten years. No data will be discarded without the Sponsor's written consent.

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1.0 EXECUTIVE SUMMARY

1.1 Study design

The purpose of this skin sensitization study was to assess the possible allergenic potential of NOA449280 SL (A16003E) when administered topically to albino Dunkin Hartley guinea pigs.

For this purpose the “Buehler Test” modified by Ritz, H.L. and Buehler, E.V. (1980) was used. Twenty male animals of the test group were treated topically with NOA449280 SL (A16003E) at 100% three times a week for a 3-week induction phase. Ten days after the final induction application the animals were challenged with the test item concentrations of 100% (undiluted) and 75% in purified water. Two weeks after the first challenge the animals of the test group were challenge again with the test item concentrations of 75% and 50% in purified water.

Two control groups each with ten animals were used. The animals of the control group I were treated in the same way as the test group but with purified water only during the induction. They were treated once at challenge with NOA449280 SL (A16003E) at 100% (undiluted) and 75% in purified water. The ten animals of the control group II were not treated during the induction. They were treated once at challenge with NOA449280 SL (A16003E) at 75% and 50% in purified water.

1.2 Results

There were no treatment related deaths and no signs of systemic toxicity observed in the animals.

After the first challenge treatment with the test item at 100% 14 out of the 20 test animals showed discrete/patchy to moderate/confluent erythema at the 24- and 48-hour reading. In 9 out of the 20 test animals discrete/patchy erythema was also noted when the test item was applied at 75% at the 24-hour observation and persisted in 5 animals to 48 hours. Furthermore, 7 out of the 9 control animals showed a discrete/patchy erythema at 24 and 48 hours after challenge with the test item at 100%. When challenged with the test item at 75%, 2 control animals showed discrete/patchy erythema at 24 hours only.

After the second challenge with 75% test item concentration, 14 (at the 24-hour reading) and 12 (at the 48-hour reading) test animals were observed with discrete/patchy erythema. Following treatment with the test item at the 50% concentration, 6 (at the 24-hour reading) and 5 (at the 48-hour reading) test animals were observed with discrete/patchy erythema. In the control animals, 3 out of 10 animals were positive (discrete/patchy erythema) at the 24-hour reading, which persisted in one of these animals up to 48 hours. At the 50% concentration all control animals were negative.

1.3 Conclusion

The test item NOA449280 SL (A16003E) is considered to be a skin sensitizer in the guinea pig.

2.0 INTRODUCTION

2.1 Purpose

The purpose of this skin sensitization study was to determine if the test item under the conditions described in the study plan and this report caused an increased reaction in the skin of guinea pigs at challenge when compared to appropriate controls.

This study provides a rational basis to assess the sensitizing potential of the test item in man.

2.2 Guidelines

The study was done according to the following guidelines:

OECD Guidelines for Testing of Chemicals, Number 406 "Skin Sensitization", adopted by the Council on July 17, 1992 (reported Paris, April 29, 1993).

United States Environmental Protection Agency, Health Effects Test Guidelines, OPPTS 870.2600 Skin Sensitization EPA 712-C-03-197, March 2003.

Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), B.6. Skin sensitisation (Official Journal No L 142, 31/05/2008 p. 0202-0209).

Japanese guideline Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), Guidelines for Preparation of Study Results, Skin sensitization studies. Guideline 2-1-6. Notification 12 NohSan No. 8147, as partly revised in 16-Shouan-9260, on 16 March 2005. English translation by ACIS on 17 Oct 2005.

2.3 Justification of Test System

The sensitivity and reliability of the experimental technique employed was assessed by use of alpha-hexylcinnamaldehyde which is recommended by the OECD 406 Guidelines and is known to have moderate skin sensitization properties in the guinea pig. The results from the most recent test run (Harlan Laboratories study C16250, performed from 01-Oct-2008 to 21-Nov-2008) are included in this report under the Appendix 1.

2.4 Test Facility

This study was performed in an AAALAC-accredited laboratory in accordance with the Swiss Animal Protection Law under license no. 63.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The following information was provided by the sponsor:

Identification:	NOA449280 SL (A16003E)
Description:	Liquid; dark brown red to black
Batch Number:	J8308/145
Density:	1.06 g/mL
Stability of Test Item:	Stable under specified storage conditions.
Reanalysis Date:	05/2011
Storage Conditions:	At a temperature < 30°C (room temperature, range of 20 ± 5 °C, provided by Harlan Laboratories), light protected.
Safety Precautions:	Routine hygienic procedures were used to ensure the health and safety of the personnel.

3.2 Vehicle

The following information was provided by Harlan Laboratories Ltd.:

Purified water prepared at Harlan Laboratories Ltd (deionised water which was processed and treated by the Purelab Option-R unit. This latter links four purification technologies: reverse osmosis, adsorption, ion-exchange and photo oxidation).

The vehicle was selected based on preliminary solubility testing which was performed before the study initiation date. Therefore, the formulation trials were excluded from the statement of GLP compliance. Purified water was a suitable vehicle to be used for the study.

3.3 Experimental Design

The viability/mortality and clinical signs were recorded daily from delivery of the animals to the termination of the test.

The body weights were recorded at acclimatization and treatment start, and at the termination of the study.

Skin response scores were graded during the irritation screens, induction and challenge period.

3.3.1 Animals

Animals:	Albino Dunkin Hartley Guinea Pig, CRL:(HA)BR, SPF
Rationale:	Skin reactions in the guinea pig are classically used for determining the potential of test items to induce delayed contact hypersensitivity. No valid non-animal model (*in-vitro*) is available at present for the test of contact sensitization.
Breeder:	Charles River Deutschland GmbH Stolzenseeweg 32-36 D-88353 Kisslegg / Germany
Number of Animals for Main Study / Irritation Screen:	40 males / 3 females First challenge: - 20 test animals - 10 control animals Second challenge: - A further 10 untreated control animals Irritation Screen: - 3 animals
Age at Delivery / Acclimatization Start:	3 to 6 weeks
Body weight at Delivery / Acclimatization start:	Test and control animals: 248 to 380 g Animals used for irritation screen: 338 to 367 g
Identification:	By unique cage number and individual animal number.
Randomization:	Randomly selected by hand at time of delivery. No computer randomization.
Acclimatization:	Under laboratory conditions after health examination. Five days for the control and test group. One day for the animals used in the irritation screen for induction and challenge. Only animals without any visible signs of illness were used for the study.

3.3.2 Allocation

The animals were distributed as follows:

		NUMBER OF ANIMALS PER GROUP	ANIMAL NUMBERS PER GROUP
1	Irritation Screen for Induction and Challenge	3	986 - 988
2	Control Group I	10	989 - 998
3	Test Group	20	999 - 1018
4	Control Group II	10	1019 - 1028

3.3.3 Husbandry

Room Number:	0114 / Harlan Laboratories Ltd., Füllinsdorf
Conditions:	**Standard Laboratory Conditions:** Air-conditioned with ranges for room temperature 22 ± 3 °C, relative humidity 30-70% and approximately 10-15 air changes per hour. Room temperature and humidity were monitored continuously and values outside of these ranges occasionally occurred, usually following room cleaning. These transient variations are considered not to have any influence on the study and, therefore, these data are not reported but are retained at Harlan Laboratories. The animals were provided with an automatically controlled light cycle of 12 hours light / 12 hours dark. Music was played during the daytime light period.
Accommodation:	Individually in Makrolon type-4 cages with standard softwood bedding ("Lignocel", Schill AG, 4132 Muttenz / Switzerland).
Diet:	Pelleted standard Provimi Kliba 3418, batch no. 55/08 and 72/08 guinea pig breeding / maintenance diet, containing Vitamin C (Provimi Kliba AG, 4303 Kaiseraugst / Switzerland), *ad libitum*. Results of analyses for contaminants are archived at Harlan Laboratories Ltd.
Water:	Community tap water from Füllinsdorf, *ad libitum*. Results of bacteriological, chemical and contaminant analyses are archived at Harlan Laboratories Ltd.

3.3.4 Study conduct – treatment procedure

The test item and vehicle were placed into a glass beaker on a tared Mettler balance and weight/weight dilutions were prepared. Homogeneity of the test item in purified water was ensured and maintained during treatment using a magnetic stirrer. The preparations were made immediately prior to each dosing.

Dose levels were in terms of material as supplied by the Sponsor.

Patching method: The same patching method was used for irritation screen, induction, and challenge.

The animal's fur was shaved with a fine clipper blade just prior to the exposure. Closed patches were applied to the animals as follows:
0.5 mL of the undiluted test item or freshly prepared test item solution in a 25 mm Hill Top Chamber.

The 25 mm Hill Top Chamber was firmly secured by an elastic plaster wrapped around the trunk of the animal and secured with impervious adhesive tape. The occlusive dressing was left in place for six hours (± 15 minutes).

Grading Method
The test item skin area of the animals used for irritation screen and challenge were depilated approximately 21 hours after the patches had been removed, using an approved depilatory cream (VEET Cream, Reckitt & Colman AG, CH-4123 Allschwil). The depilatory cream was placed on the patch sites and surrounding areas, and left on for up to 3-5 minutes. It was then thoroughly washed off with a stream of warm, running water. The animals were then dried with a disposable towel, and returned to their cages.

The scoring system was performed by visual assessment of erythema, oedema and other clinical changes in skin conditions. They were assessed as follows:

0	=	no visible change
1	=	discrete or patchy erythema
2	=	moderate and confluent erythema
3	=	intense erythema and swelling

Grading of all animals was done by positioning each animal under true-light (Philips TLD 36W/84 or Osram 36W/31 830).

The grading method used for the irritation screen, induction and challenge was identical. It was performed 24 ± 2 hours after removal of the patches for irritation screen, induction and challenge and repeated 24 ± 2 hours later (48-hour grades) for the irritation screen and the challenge.

Note: At challenge, control animals were graded before the test animals.

3.3.5 Selection of concentration of test item for main study

A number of factors contributed to the selection of the concentrations of test item including irritancy, slope of dose response curve and experience with similar test items. Selection was based on the following criteria:

Epidermal Induction:
Concentration that produced some irritation but not adversely affected the animals (determined at the irritation screen). In this study, no irritating concentration was selected since no skin effects were observed with the test item applied undiluted during the irritation screen. Therefore, the highest test item concentration of 100% was applied for the induction phase.

Epidermal Challenge:
Concentration that was the highest tested non-irritant concentration.
The test item concentrations of 100% and 75% were selected

3.3.6 Diagrammatic study plan

Acclimatization		Study day					
-5	-4	1,3,5	8,10,12	15,17,19	22	29	43
	IS	I	I	I		C1	C2

IS = Irritation screen to determine the minimal irritating concentration used in the induction period and the highest non-irritating concentration used for the challenge.
I = Induction
C1 = First Challenge
C2 = Second Challenge

3.3.7 Irritation screen for induction and challenge

The test item concentrations described below were selected during a preliminary solubility testing which was done before the study initiation date.

The fur was removed from both flanks of the animals.

For patch placements, the format described below was used on 4 guinea pigs. Four different concentrations were used on each animal for a 6-hour exposure period.

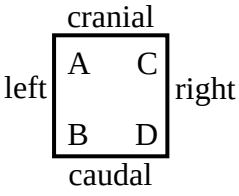
Test item: NOA449280 SL (A16003E)

Vehicle: Purified water

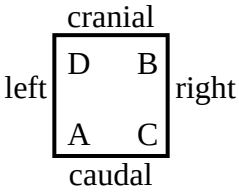
Formulation: weight/weight

Concentrations

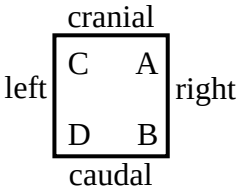
A = 100%
B = 75%
C = 50%
D = 25 %



Animal no. 986



Animal no. 987



Animal no. 988

3.3.8 Induction – 9 exposures,

The fur was clipped from the left shoulder of each test animal and the patches applied on 9 occasions over a total period of 3 weeks. Each animal received three patches per week with the undiluted test item which remained in place for approximately 6 hours each. A total of 9 applications were made during the induction phase. Repeated applications were made to the same application site.

As the test item was selected to be applied undiluted during the induction, the control animals remained untreated but were covered with dressing in the same way as the test animals.

After the last induction exposure the animals were left untreated for 9 days.

The skin responses were graded approximately 24 hours after the patches had been removed.

Any gross skin reactions were recorded without depilation.

3.3.9 First Challenge – on test day 29

The animals previously exposed during the induction period (i.e. test group) as well as the previously not treated control animals (i.e. control group I) were challenged 10 days after the last induction exposure using the test item at 100% (undiluted) and 75% in purified water.

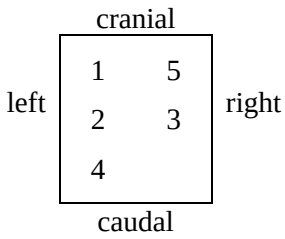
Patch sites for challenge are indicated in section 3.3.11. The exposure period was 6 hours on a naive skin site. The skin responses were graded approximately 24 and 48 hours after the patches had been removed.

3.3.10 Second Challenge – on test day 43

The animals of the test group and 10 additional untreated control animals (i.e. control group II) were challenged two weeks after the first challenge using the test item at 75% and 50% in purified water.

Patch sites for challenge are indicated below. The exposure period was 6 hours on a naive skin site. The skin responses were graded approximately 24 and 48 hours after the patches had been removed.

3.3.11 Format for induction and challenge patch application



1 = Induction (test group only)
2 = Primary Challenge (100%; control group I and test group)
3 = Primary Challenge (75%; control group I and test group)
4 = Second Challenge (75%; control group II and test group)
5 = Second Challenge (50%; control group II and test group)

3.4 *Post Mortem* Investigations

Necropsy was performed in one animal of the control group that died on the third day of the main study.

No necropsies were performed on the surviving animals of the control and test group sacrificed at termination of their observation period or on the animals of the irritation screen sacrificed on test day 1.

The surviving animals were euthanized by intraperitoneal injection of pentobarbitonel at a dose of at least 2.0 mL/kg body weight (324 mg sodium pentobarbitone/kg body weight) and discarded.

3.5 Data Evaluation

Viability/mortality was recorded daily from delivery of the animals to the termination of the test. Clinical signs were recorded daily from delivery of the animals to the termination of the test. Body weights were recorded at acclimatization and treatment start, and at the termination of the study. Skin response score were graded during the irritation screens, induction and challenge period.

The following data were compiled into the RCC Tox Computer System during recording: macroscopic findings.

The following data were recorded on data sheets and transcribed for compilation and analysis: clinical signs (local/systemic), mortality/viability and skin reactions.

The following data were recorded on-line: body weights.

The RCC Tox Computer System (RCC-Tox-Lims) has been validated with respect to data collection, storage and retrievability.

For evaluation, two parameters were used: the incidence index and the severity index, for both test and control animals. The incidence index is an expression of the number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals in the group, while the severity index is calculated from the total sum of 24- and 48 hour response readings divided by the number of animals exposed.

4.0 RESULTS AND DISCUSSION

4.1 Viability / Mortality / Macroscopic Findings

One animal of the control group died on the third day of the main study. At necropsy, a haemorrhagic congestion of the lungs as well as petechiae at the mouth of the guinea pig was observed.

4.2 Clinical Signs

No signs of systemic toxicity were observed in the animals.

4.3 Skin effects during the Induction Phase

Discrete/patchy to moderate/confluent erythema with or without scaling was observed in the animals of the test group at the end of the induction period after the treatment with NOA449280 SL (A16003E) at the concentration of 100% (see Tables Section table 2). No skin effect was observed in the control animals.

4.4 Skin Effects at the First Challenge

The highest non-irritating concentration of NOA449280 SL (A16003E) used for the first challenge was 100% (undiluted). The incidence of positive erythema reactions after topical challenge is described as follows:

ERYTHEMA SCORE	TEST GROUP 20 animals		CONTROL GROUP 9 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	6	6	2	2
1	12	14	7	7
2	2	0	0	0
3	0	0	0	0
No. with grades ≥ 1	14	14	7	7
No. tested	20	20	9	9
INCIDENCE*	14/20		7/9	
SEVERITY**	0.7 - 0.8		0.78	

* Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.

** Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

The second concentration of NOA449280 SL (A16003E) used in the first challenge was 75% in purified water. The incidence of positive erythema reactions after topical challenge is described as follows:

ERYTHEMA SCORE	TEST GROUP 20 animals		CONTROL GROUP 9 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	11	15	7	9
1	9	5	2	0
2	0	0	0	0
3	0	0	0	0
No. with grades ≥ 1	9	5	2	0
No. tested	20	20	9	9
INCIDENCE*	9/20		2/9	
SEVERITY**	0.25 - 0.45		0.00 - 0.22	

* Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.

** Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

(see Tables 3)

4.5 Skin Effects at the Second Challenge

The concentrations of NOA449280 SL (A16003E) used for the second challenge were 75% and 50% in purified water. The incidence of positive erythema reactions after topical challenge with 75% concentration is described as follows:

ERYTHEMA SCORE	TEST GROUP 20 animals		CONTROL GROUP 10 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	6	8	7	9
1	14	12	3	1
2	0	0	0	0
3	0	0	0	0
No. with grades ≥ 1	14	12	3	1
No. tested	20	20	10	10
INCIDENCE*	14/20		3/10	
SEVERITY**	0.6 – 0.7		0.1 - 0.3	

* Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.

** Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

The incidence of positive erythema reactions after topical challenge with 50% concentration is described as follows:

ERYTHEMA SCORE	TEST GROUP 20 animals		CONTROL GROUP 10 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	14	15	10	10
1	6	5	0	0
2	0	0	0	0
3	0	0	0	0
No. with grades ≥ 1	6	5	0	0
No. tested	20	20	10	10
INCIDENCE*	6/20		0/10	
SEVERITY**	0.25 - 0.30		0	

* Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.

** Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

(see Tables 4)

4.6 Body Weights

One animal of the control group I lost body weight (11.66%) between the start of acclimatization and the day of treatment. The animal died on the third day of the main study. Body weight loss between the start of acclimatization and the day of treatment was also observed in one animal from the control group II (0.8%) and one test animal (23.15%). The animals recovered by the end of the study. Furthermore, two animals of control group II did not gain body weight from acclimatisation to treatment and from treatment to the end of the study, respectively. Otherwise, the body weight of the animals was considered to be within the range commonly recorded for animals of this strain and age.

(see Tables 5)

4.7 Discussion

In this study, 70 % (at the 24- and 48-hour reading) of the animals of the test group were observed with discrete/patchy to moderate/confluent erythema (in 2 animals) after the first challenge treatment performed with NOA449280 SL (A16003E) at the concentration of 100%. Discrete/patchy erythema was also noted in 45% (at the 24-hour reading) and 25% (at the 48-hour reading) of the test animals treated with NOA449280 SL (A16003E) at 75% concentration in purified water.

Seventy eight percent of the animals of control group I showed discrete/patchy erythema when treated with NOA449280 SL (A16003E) at the concentration of 100% at the 24- and

48-hour reading and 22% were positive at the 24-hour observation after treatment with the test item at 75% concentration in purified water.

These results indicate an irritating potential of the test item due to reactions also in the control animals, which are similar to those observed in the test group. However, two animals of the test group (10%) showed a higher grade of reactions (moderate/confluent erythema). Furthermore, a higher incidence of positive reaction was observed in the test animals versus the control animals after treatment with the test item at the 75% concentration. This suggests that there might be also a sensitising potential of the test item that was covered by the irritating reactions at these two concentrations.

To clarify this and to distinguish between irritation and sensitization, a second challenge with the same concentration of 75% as well as a lower concentration of 50% was performed with a new, naive control group II.

After the second challenge, there were also positive reactions in control group II (30%) when the test item was applied at 75% concentration. In the test animals, all animals that had reacted in the first challenge and 5 additional animals (14 animals, 70%) showed discrete/patchy erythema at the 24-hour reading, which persisted in 60% of the test animals up to the 48-hour observation. Discrete/patchy erythema was noted in 30% (at the 24-hour reading) and 25% (at the 48-hour reading) of the test animals treated with NOA449280 SL (A16003E) at 50% concentration in purified water whereas the control group II animals at this concentration showed no reactions.

Thus, after the second challenge treatment, on the one hand, the irritation at 75% was confirmed. But furthermore, at this concentration a higher incidence of positive reactions was determined. Re-challenge normally acts as a booster that increases the incidence and/or severity of the allergic reactions. The presence of allergic reaction was additionally confirmed by the results of the treatment at 50%. In the control animals, no reactions were observed, hence no irritation was determined at this lower concentration. Whereas 30% and 25% of the test animals (> 15% mentioned in the Commission Directive) showed positive reactions at this concentration.

In summary, beside a concentration dependent irritating reaction, a sensitising potential of the test item was determined in this study.

5.0 CONCLUSIONS

Based on the results of this study, NOA449280 SL (A16003E) is considered to be a skin sensitizer in the guinea pig.

6.0 REFERENCES

Literature references listed are available upon request.

External references

Ritz, H.L. and Buehler, E.V.
Current Concepts Cutaneous Toxicity, ed. Drill, V.A. and Lazar, T. (Academic Press, 1980) pp. 25-40: Planning, Conduct and Interpretation of Guinea Pig Sensitization Patch Tests.

TABLES SECTION

TABLE 1 Skin Reactions during Irritation Screen for Induction and Challenge

Test item: NOA449280 SL (A16003E)
Vehicle: Purified water

Animal No.986: male

	Skin reactions after	
	24 Hours	48 Hours
A = 100%	0	0
B = 75%	0	0

	Skin reactions after	
	24 Hours	48 Hours
C = 50%	0	0
D = 25%	0	0

Animal No.987: male

	Skin reactions after	
	24 Hours	48 Hours
D = 25%	0	0
A = 100%	0	0

	Skin reactions after	
	24 Hours	48 Hours
B = 75%	0	0
C = 50%	0	0

Animal No.988: male

	Skin reactions after	
	24 Hours	48 Hours
C = 50%	0	0
D = 25%	0	0

	Skin reactions after	
	24 Hours	48 Hours
A = 100%	0	0
B = 75%	0	0

Three hours prior to the 24-hour reading both flanks were depilated.

TABLE 2 Skin Reactions Observed during Induction

Test item: **NOA449280 SL (A16003E)**
Test item concentration: **100%**

TEST GROUP

INDUCTION WEEK 1 to 3

Animal number male / Application	999	1000	1001	1002	1003	1004	1005	1006	1007	1008
Test day 1	0	0	0	0	0	0	0	0	0	0
Test day 3	0	0	0	0	0	0	0	0	0	0
Test day 5	0	0	**1**	**1**	**1**	0	0	0	**1**	**1**
Test day 8	**1**	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Test day 10	**1**	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Test day 12	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**2**	**1**
Test day 15	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**2**	**1**
Test day 17	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**2**	**1**
Test day 19	**1***	**1**	**1***	**1**	**2***	**1***	**1**	**1**	**2***	**1**

Animal number male / Application	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018
Test day 1	0	0	0	0	0	0	0	0	0	0
Test day 3	0	0	0	0	0	0	0	0	0	0
Test day 5	0	**1**	0	**1**	0	0	0	**1**	0	0
Test day 8	0	**1**	**1**	**1**	0	0	0	**1**	**1**	**1**
Test day 10	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Test day 12	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Test day 15	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Test day 17	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Test day 19	**1**	**2***	**2**	**1***	**1***	**1**	**1**	**1**	**1**	**1**

*Scaling present

TABLE 3 Skin Reactions after the First Challenge

Test item: **NOA449280 SL (A16003E)**

CONTROL GROUP

Test item concentration: **100%**

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours	Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
989	**1**	**1**	994	**1**	**1**
990	0	0	995	**1**	**1**
991	0	0	996	**1**	**1**
992	**1**	**1**	997	**1**	**1**
993	**1**	**1**	998	+	+

+ No assessment, the animal was found dead during the induction phase.

Test item concentration: **75%**
Vehicle: **purified water**

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours	Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
989	**1**	0	994	0	0
990	0	0	995	0	0
991	0	0	996	0	0
992	0	0	997	**1**	0
993	0	0	998	+	+

+ No assessment, the animal was found dead during the induction phase.

TABLE 3 Skin Reactions after First Challenge (continued)

TEST GROUP

Test item concentration: 100%

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours	Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
999	**2**	**1**	1009	**1**	**1**
1000	0	0	1010	**1**	**1**
1001	**1**	**1**	1011	**1**	**1**
1002	0	0	1012	0	0
1003	0	0	1013	**1**	**1**
1004	**1**	**1**	1014	**1**	**1**
1005	0	0	1015	**1**	**1**
1006	0	0	1016	**1**	**1**
1007	**1**	**1**	1017	**1**	**1**
1008	**1**	**1**	1018	**2**	**1**

Test item concentration: 75%
Vehicle: purified water

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours	Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
999	**1**	0	1009	**1**	0
1000	0	0	1010	0	0
1001	**1**	0	1011	**1**	0
1002	0	0	1012	0	0
1003	0	0	1013	0	0
1004	**1**	**1**	1014	**1**	**1**
1005	0	0	1015	0	0
1006	0	0	1016	**1**	**1**
1007	**1**	**1**	1017	0	0
1008	0	0	1018	**1**	**1**

TABLE 4 Skin Reactions after the Second Challenge

Test item: **NOA449280 SL (A16003E)**

CONTROL GROUP

Test item concentration: **75%**
Vehicle: **purified water**

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours	Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
1019	0	0	1024	0	0
1020	0	0	1025	**1**	**1**
1021	**1**	0	1026	0	0
1022	**1**	0	1027	0	0
1023	0	0	1028	0	0

Test item concentration: **50%**
Vehicle: **purified water**

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours	Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
1019	0	0	1024	0	0
1020	0	0	1025	0	0
1021	0	0	1026	0	0
1022	0	0	1027	0	0
1023	0	0	1028	0	0

TABLE 4 Skin Reactions after Second Challenge (continued)

TEST GROUP

Test item concentration: 75%
Vehicle: purified water

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
999	**1**	**1**
1000	0	0
1001	**1**	**1**
1002	0	0
1003	**1**	**1**
1004	**1**	**1**
1005	**1**	**1**
1006	0	0
1007	**1**	**1**
1008	0	0

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
1009	**1**	**1**
1010	**1**	0
1011	**1**	**1**
1012	**1**	**1**
1013	**1**	0
1014	**1**	**1**
1015	0	0
1016	**1**	**1**
1017	0	0
1018	**1**	**1**

Test item concentration: 50%
Vehicle: purified water

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
999	0	0
1000	0	0
1001	**1**	**1**
1002	0	0
1003	0	0
1004	0	0
1005	0	0
1006	0	0
1007	**1**	0
1008	0	0

Animal No. male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
1009	**1**	**1**
1010	0	0
1011	0	0
1012	**1**	**1**
1013	0	0
1014	**1**	**1**
1015	0	0
1016	0	0
1017	0	0
1018	**1**	**1**

TABLE 5 Body Weights

BODY WEIGHTS (GRAM)
MALES

	ACCLI./PRETEST	TREATMENT			
DAYS	1	1	38	43	47
WEEKS	1	1	6	7	7
ANIMAL					
GROUP 1 (IRRITATION SCREEN)					
986	367	379	---	---	---
987	355	368	---	---	---
988	338	363	---	---	---
GROUP 2 (CONTROL GROUP)					
989	360	391	548	---	---
990	340	391	605	---	---
991	356	382	548	---	---
992	354	388	517	---	---
993	355	393	553	---	---
994	369	405	635	---	---
995	370	400	605	---	---
996	334	358	506	---	---
997	359	418	644	---	---
998	360	318	---	---	---
GROUP 3 (TEST GROUP)					
999	355	401	---	---	581
1000	336	407	---	---	780
1001	367	404	---	---	612
1002	359	362	---	---	538
1003	362	408	---	---	624
1004	362	408	---	---	679
1005	366	414	---	---	666
1006	373	425	---	---	708
1007	344	383	---	---	622
1008	331	348	---	---	595
1009	363	408	---	---	711
1010	330	365	---	---	657
1011	353	404	---	---	668
1012	368	413	---	---	629
1013	349	406	---	---	584
1014	337	367	---	---	581
1015	347	369	---	---	495
1016	349	398	---	---	620
1017	380	292	---	---	636
1018	362	429	---	---	657
GROUP 4 (CONTROL GROUP II)					
1019	---	---	261	322	337
1020	---	---	255	308	309
1021	---	---	255	301	311
1022	---	---	258	306	321
1023	---	---	275	316	315
1024	---	---	271	319	338
1025	---	---	248	293	305
1026	---	---	248	246	256
1027	---	---	265	265	292
1028	---	---	265	332	343

APPENDICES SECTION

APPENDIX I Results of Positive Control

Harlan Laboratory Study C16250

ALPHA-HEXYLCINNAMALDEHYDE:

Contact Hypersensitivity in Albino Guinea Pigs, Buehler Test

POSITIVE CONTROL

Performed from 01-Oct-2008 to 21-Nov-2008

RESULTS OF POSITIVE CONTROL (CONTINUED)

SUMMARY

General

The purpose of this skin sensitizing study was to assess the possible allergenic potential of alpha-hexylcinnamaldehyde when administered topically to albino Dunkin Hartley guinea pigs.

For this purpose the “Buehler Test” modified by Ritz, H.L. and Buehler, E.V. (1980) was used. Twenty male animals of the test group were treated topically with alpha-hexylcinnamaldehydeat 50% in PEG 300 once a week for a 3-week induction phase. Two weeks after the final induction application the animals were challenged with the same test item concentration of 5% in PEG 300.

The ten animals of the control group I were not treated during the induction. They were treated once at challenge with alpha-hexylcinnamaldehyde at 5% in PEG 300.

Two weeks after the first challenge a second challenge was performed in the same way as the previous challenge using the test group and a naive control group, a naive skin site and the test item concentration of 3% and 1% in PEG 300.

- First Challenge

The highest tested non-irritating concentration of alpha-hexylcinnamaldehyde used for the first challenge was 5% in PEG 300. The incidence of positive erythema reactions after topical challenge is described as follows:

ERYTHEMA SCORE	TEST GROUP 20 animals		CONTROL GROUP I 10 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	9	10	5	4
1	8	9	5	6
2	3	1	0	0
3	0	0	0	0
No. with grades ≥ 1	11	10	5	6
No. tested	20	20	10	10
INCIDENCE*	12/20 (60%)		6/10 (60%)	
SEVERITY**	0.55 – 0.7		0.5 – 0.6	

*Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.
**Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

RESULTS OF POSITIVE CONTROL (CONTINUED)

Due to equivocal findings after the first challenge results, a second challenge was performed in the same test group and a new control group with the test item concentrations of 3% and 1% in PEG 300.

- Second challenge

Test item concentration of 3% in PEG 300:

ERYTHEMA SCORE	TEST GROUP 20 animals		CONTROL GROUP II 10 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	10	13	10	10
1	10	7	0	0
2	0	0	0	0
3	0	0	0	0
No. with grades ≥ 1	10	7	0	0
No. tested	20	20	10	10
INCIDENCE*	10/20 (50%)		0/10	
SEVERITY**	0.35 – 0.5		0	

*Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.
**Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

Test item concentration of 1% in PEG 300:

ERYTHEMA SCORE	TEST GROUP 20 animals		CONTROL GROUP II 10 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	20	20	10	10
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
No. with grades ≥ 1	0	0	0	0
No. tested	20	20	10	10
INCIDENCE*	0/20		0/10	
SEVERITY**	0		0	

* Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.

** Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

RESULTS OF POSITIVE CONTROL (CONTINUED)

PURPOSE

The purpose of this skin sensitization study was to confirm if the test item under the conditions described in the study plan and this report causes an increased reaction in the skin of guinea pigs at challenge when compared to appropriate controls.

This study should provide a rational basis for risk assessment of the sensitizing potential of the test item in man.

MATERIALS AND METHODS

Test System

Animals:	Albino Dunkin Hartley Guinea Pig, HsdPoc: DH, SPF Albino Dunkin Hartley Guinea Pig, CRL:(HA)BR, SPF
Rationale:	Skin reactions in the guinea pig are classically used for determining the potential of test items to induce delayed contact hypersensitivity. No valid non-animal model (in-vitro) is available at present for the test of contact sensitization.
Breeder:	Charles River Deutschland GmbH Stolzenseeweg 32-36 88353 Kisslegg / Germany
Number of Animals for Main Study / Irritation Screen:	40 males / 3 males First challenge: 20 test animals 10 control animals Second challenge: Further 10 untreated control animals Irritation Screen: 3 animals
Age at Delivery / Acclimatization Start:	5 - 6 weeks
Body Weight at Delivery / Acclimatization Start:	Test and control animals: 355 g - 546 g Animals used for irritation screen: 370 g - 379 g
Identification:	By unique cage number and individual animal number.
Randomization:	Randomly selected by hand at time of delivery. No computer randomization.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Acclimatization: Under laboratory conditions after health examination. Only animals without any visible signs of illness were used for the study.

Allocation

The animals were distributed as follows:

		NUMBER OF ANIMALS PER GROUP	ANIMAL NUMBERS PER GROUP
1	Irritation Screen for Induction and Challenge	3	896 - 898
2	Control Group I	10	899 - 908
3	Test Group	20	909 - 928
4	Control Group II	10	929 - 938

Husbandry

Room Number: 0108 / Harlan Laboratories Ltd., Füllinsdorf

Conditions: **Standard Laboratory Conditions:** Air-conditioned with ranges for room temperature 22 ± 3 °C, relative humidity 30-70% and approximately 10-15 air changes per hour. Room temperature and humidity were monitored continuously and values outside of these ranges occasionally occurred, usually following room cleaning. These transient variations are considered not to have any influence on the study and, therefore, these data are not reported but are retained at Harlan Laboratories. The animals were provided with an automatically controlled light cycle of 12 hours light and 12 hours dark. Music was played during the daytime light period.

Accommodation: Individually in Makrolon type-4 cages with standard softwood bedding ("Lignocel", Schill AG, 4132 Muttenz / Switzerland).

Diet: Pelleted standard Provimi Kliba 3418, batch no. 46/08 guinea pig breeding / maintenance diet, containing Vitamin C (Provimi Kliba AG, 4303 Kaiseraugst / Switzerland), *ad libitum*. Results of analyses for contaminants are archived at Harlan Laboratories Ltd.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Water:	Community tap water from Füllinsdorf, *ad libitum*. Results of bacteriological, chemical and contaminant analyses are archived at Harlan Laboratories Ltd.

Test Item and Vehicle

Test Item
The following information was provided by the Sponsor:

Identification:	Alpha-Hexylcinnamaldehyde
Description:	Yellow to yellow-green liquid
Lot Number:	04012JE
Source	Aldrich Chemicals Company, inc. 3050 Spruce Street Saint Louis, Missouri 63105 / USA
Purity:	99.0%
Stability of Test Item:	Stable under storage conditions.
Expiry Date:	24-Oct-2012
Stability of Test Item Dilution:	Stable in PEG 300 and in a 1:1 (v/v) mixture of FCA/physiological saline for at least 2 hours at room temperature; was determined at Harlan Laboratories Ltd., under a non-GLP study.
Storage Conditions:	At room temperature (range of 20 ± 5 °C, provided by Harlan Laboratories Ltd.), light protected.
Safety Precautions:	Routine hygienic procedures were used to ensure the health and safety of the personnel.

Vehicle
The following information was provided by Harlan Laboratories Ltd.:

Identification:	Polyethylene glycol 300 (PEG 300)
Description:	Colorless viscous liquid
Lot Number:	1349048
Source:	FLUKA Chemie GmbH, 9471 Buchs / Switzerland
Stability of the Vehicle:	Stable under storage conditions.
Expiry Date:	Apr-2010
Storage Conditions:	At room temperature (range of 20 ± 5 °C), light protected.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Safety Precautions:	Routine hygienic procedures were used to ensure the health and safety of the personnel.

Preparation of Dose Formulation

The test item and vehicle were placed into a glass beaker on a tared Mettler balance and weight/weight dilutions were prepared. Homogeneity of the test item in PEG 300 was ensured and maintained during treatment using a magnetic stirrer. The preparations were made immediately prior to each dosing.

Dose levels were in terms of material as supplied by the Sponsor.

Test Item Administration

A number of factors contributed to the selection of the concentrations of test item including irritancy, slope of dose response curve and experience with similar test items. Selection was based on the following criteria:

Epidermal Induction: Concentration that produced some irritation but not adversely affected the animals (determined at the irritation screen).

Epidermal Challenge: Concentration that was the maximum tested non-irritant concentration.

Rationale

Dermal administration has historically been used as the route of choice for determining delayed contact hypersensitivity.

Observation

Viability / Mortality:	Daily from delivery of the animals to the termination of the test.
Clinical Signs / Grading of Skin Response:	Daily from delivery of the animals to the termination of test. Skin responses were graded during the irritation screens, induction and challenges periods.
Body Weights:	At delivery/acclimatization start, at the end of the irritation screen, at test day 1 (day of treatment) and at the termination of the study.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Pathology

Necropsy
No necropsy was performed on the animals euthanized at termination of the observation.

The animals were euthanized by intraperitoneal injection of pentobarbitone at a dose of at least 2.0 mL/kg body weight (equivalent to 324 mg sodium pentobarbitone/kg body weight) and discarded.

Treatment Methods

The diagrammatic study plan is as follows:

Identical patching method was used for the irritation screen, induction, challenge and rechallenge.

Acclimatization		Study day					
-6	-5	1	8	15	22	29	43
	IS	I	I	I		C 1	C 2

IS= Irritation screen to determine the minimal irritating concentration used in the induction period and the highest non-irritating concentration used for the challenge.

I= Induction (test group only)

C 1= First Challenge (control group I and test group)

C 2= Second Challenge (control group II and test group)

The animal's fur was shaved with a fine clipper blade just prior to the exposure. Closed patches were applied to the animals as follows:

0.5 mL of the undiluted test item or freshly prepared test item solution in a 25 mm Hill Top Chamber.

The 25 mm Hill Top Chamber was firmly secured by an elastic plaster wrapped around the trunk of the animal and secured with impervious adhesive tape. The occlusive dressing was left in place for six hours (± 15 minutes).

RESULTS OF POSITIVE CONTROL (CONTINUED)

Irritation screen for induction and challenge

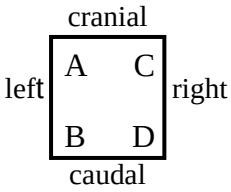
- Performed during the acclimatization period of the main animals.

The test item concentrations described below and vehicle were based on previous RCC historical data.

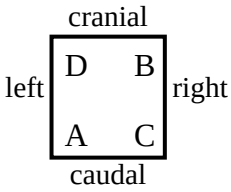
This investigation identified the test item concentration required for the induction and challenge phase of the main study.

The fur was removed from both flanks of the animals. For patch placements, the format described below was used on 3 guinea pigs. Four different concentrations were used on each animal for a 6-hour exposure period.

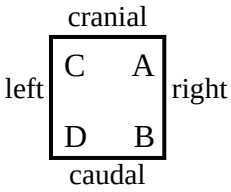
Test Item	Concentrations		Vehicle	Formulation
Alpha-Hexylcinnamaldehyde	A = 50% B = 25%	C = 15% D = 5%	PEG 300	weight/weight



Animal no. 896



Animal no. 897



Animal no. 898

The allocation of the different test item dilutions to the sites (A, B, C, D) on the three animals was alternated in order to minimize site-to-site variation in responsiveness.

The results are described on page 27 and are summarized as follows:

RESULTS OF POSITIVE CONTROL (CONTINUED)

IRRITATION SCREEN	Irritancy Results after the 24-hour reading concentration (%) of alpha-hexylcinnamaldehyde				after the 48-hour reading concentration (%) of alpha-hexylcinnamaldehyde			
	number of grade-related skin response							
Response Grade	50%	25%	15%	5%	50%	25%	15%	5%
0	0	0	0	3	0	0	3	3
1	0	1	3	0	1	3	0	0
2	3	2	0	0	2	0	0	0
3	0	0	0	0	0	0	0	0

The test item at 50% in PEG 300 was used for the 3-week induction and the 5% in PEG 300 was selected for the first challenge. The second challenge was performed with the test item at 3% and 1% in PEG 300.

Induction – Performed on Test Days 1, 8 and 15

The fur was clipped from the left shoulder of each test animal and the patches applied, over a period of 3 weeks. Each animal received one patch per week with the test item at 50% in PEG 300 which remained in place for approximately 6 hours each. The repeated application was performed at the same site. The interval between exposure was one week. The control animals remained untreated.

After the last induction exposure the test animals were left untreated for 2 weeks before the challenge.

Any gross skin reactions were recorded without depilation.

First Challenge – Performed on Test Days 29

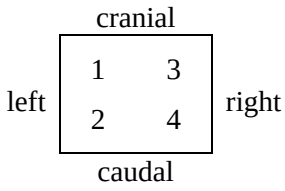
The animals previously exposed during the induction period (i.e. test group) as well as the previously untreated control animals were challenged two weeks after the last induction exposure using the test item at 5% in PEG 300. The fur was clipped from the left posterior quadrant of the side and back of the animals. Patch sites for challenge are indicated below. The exposure period was 6 hours on a naive skin site.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Rechallenge

The test group was rechallenged 14 days following the first challenge. All animals in the test group were included in the rechallenge, together with 10 previously untreated controls. Both groups were treated with the test item at 3% and 1% in PEG 300 applied on the right shoulder and right flank, respectively.

Format for Induction and Challenge Patch Application



1	=	Induction (test group only) at 50% in PEG 300
2	=	Primary Challenge (control group I and test group) at 5% in PEG 300
3	=	Rechallenge (control group II and test group) at 3% in PEG 300
4	=	Rechallenge (control group II and test group) at 1% in PEG 300

Determination of Skin Reactions

Observation and Scoring

The test item skin area of the animals used for irritation screen and challenges were depilated approximately 21 hours after the patches had been removed, using an approved depilatory cream (VEET Cream, Reckitt & Colman AG, 4123 Allschwil / Switzerland). The depilation was performed to clean the stratum corneum from the staining produced by the test item and to facilitate the reading of the skin reactions. The depilatory cream was placed on the patch sites and surrounding areas, and left on for up to 3-5 minutes. It was then thoroughly washed off with a stream of warm, running water. The animals were then dried with a disposable towel, and returned to their cages.

The grading method used for irritation screen, induction and challenges was identical. It was performed 24 ± 2 hours after removal of the patches for irritation screen, induction and challenges and repeated 24 ± 2 hours later (48-hour grades) for the irritation screen and the challenges.

RESULTS OF POSITIVE CONTROL (CONTINUED)

The scoring system was performed by visual assessment of erythema, oedema and other clinical changes in skin conditions. They were assessed as follows:

0 = no visible change

1 = discrete or patchy erythema

2 = moderate and confluent erythema

3 = intense erythema and swelling

Grading of all animals was done by positioning each animal under true-light (Philips Master TLS HE 28W/840 or FL-LPE Osram D16 FH 28W/840 EP).

Note: At challenge and rechallenge, control animals were graded before the test animals.

For evaluation, two parameters were used: the incidence index and the severity index, for both test and control animals. The incidence index is an expression of the number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals in the group, while the severity index is calculated from the total sum of 24- and 48 hour response readings divided by the number of animals exposed.

Statistical Analysis

Descriptive statistics (means and standard deviations) were calculated for body weights. No inferential statistics were used.

Data Compilation

The following data were recorded on data sheets and transcribed for compilation and analysis: clinical signs (local/systemic), mortality/viability and skin reactions.

The following data were recorded on-line: body weights.

The RCC Tox Computer System (RCC-Tox-Lims) has been validated with respect to data collection, storage and retrievability.

RESULTS OF POSITIVE CONTROL (CONTINUED)

RESULTS AND DISCUSSION OF THE MAIN STUDY

Observations

Viability / Mortality / Macroscopic Findings
There were no deaths during the course of the study, hence no necropsies were performed.

Clinical Signs
No signs of systemic toxicity were observed in the animals.

Body Weights

The body weight of the animals was within the range commonly recorded for animals of this strain and age.

See page 35 – 37.

Skin Effect in the Induction
In three weeks of induction, discrete/patchy to moderate/confluent erythema was observed in all test animals after treatment with the test item at 50% in PEG 300.

See page 28.

Skin Effect in the Challenge
FIRST CHALLENGE

Control group I:

Discrete/patchy erythema was observed in five animals at the 24-hour reading and in six animals at the 48-hour reading after the challenge treatment with the test item at 5% in PEG 300.

Test group:

Discrete/patchy to moderate/confluent erythema was observed in eleven animals at the 24-hour reading and in ten animals at the 48-hour reading after treatment with the test item at 5% in PEG 300.

See page 30.

RESULTS OF POSITIVE CONTROL (CONTINUED)

SECOND CHALLENGE

Control group II:

No skin reactions were observed in the animals of the control group II after being treated with the test item at 3% in PEG 300 on the right shoulder and at 1% in PEG 300 on the right flank.

Test group:

Discrete/patchy erythema was observed in ten animals at the 24-hour reading and in seven animals at the 48-hour reading after treatment with the test item at 3% in PEG 300 on the right shoulder. No skin effect was observed after treatment with the test item at 1% in PEG 300 on the right flank.

See page 31 – 33.

RESULTS OF POSITIVE CONTROL (CONTINUED)

CONCLUSION

Comparable incidence and severity of local skin reactions were observed in the control and test group after the first challenge performed with alpha-hexylcinnamaldehyde at 5% in PEG 300. The skin reactions consisted of discrete/patchy erythema in both groups up to moderate/confluent erythema in 15% or 5% of the test animals at the 24- and 48-hour reading, respectively. This explained the very slight difference in the severity index with values of 0.05 to 1 point higher than the control group.

As the results were considered to be equivocal and would suggest rather an irritation than an allergy, with a slight doubt on a possible allergy due to the three out of 20 (i.e. 15%) test animals with a moderate/confluent erythema (borderline at 15% in accordance to Commission Directive 2001/59/EC) versus the control animals without moderate/confluent erythema, a second challenge was performed by treating the same test group and a new control group with the test item at 3% and 1% in PEG 300. At this stage of procedure, 50% of the test animals were observed with discrete/patchy erythema when treated at 3% in PEG 300 while no local skin reactions were observed in the new control group. When treated at 1% in PEG 300, neither the test group nor the control group was observed with local skin reactions. With these findings, a clear concentration-dependent was observed in the animals by using alpha-hexylcinnamaldehyde: an irritating potential of the test item at 5% in PEG 300 (with a slight doubt on a possible allergy), an allergenic potential at 3% in PEG 300 without irritating potential (due to the absence of local findings in the new control group) and no irritation and no allergy with the test item at 1% in PEG 300.

Based on the above mentioned findings in a non-adjuvant sensitization test in guinea pigs and in accordance to Commission Directive 2001/59/EC, alpha-hexylcinnamaldehyde has to be classified and labelled as a skin sensitizer.

RESULTS OF POSITIVE CONTROL (CONTINUED)

REFERENCES

1. Ritz, H.L. and Buehler, E.V. Current Concepts Cutaneous Toxicity, ed. Drill, V.A. and Lazar, T. (Academic Press, 1980) pp. 25-40: Planning, Conduct and Interpretation of Guinea Pig Sensitization Patch Tests.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Skin Reactions during Irritation Screen for Induction and Challenge

Test item: **Alpha-Hexylcinnamaldehyde**
Vehicle: **PEG 300**

Animal No.: 896 (Male)

	Skin reactions after	
	24 Hours	48 Hours
A = 50%	2	2
B = 25%	1	1

	Skin reactions after	
	24 Hours	48 Hours
C = 15%	1	0
D = 5%	0	0

Animal No.: 897 (Male)

	Skin reactions after	
	24 Hours	48 Hours
D = 5%	0	0
A = 50%	2	1

	Skin reactions after	
	24 Hours	48 Hours
B = 25%	2	1
C = 15%	1	0

Animal No.: 898 (Male)

	Skin reactions after	
	24 Hours	48 Hours
C = 15%	1	0
D = 5%	0	0

	Skin reactions after	
	24 Hours	48 Hours
A = 50%	2	2
B = 25%	2	1

Three hours prior to the 24-hour reading both flanks were depilated.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Skin Reactions in the Three-Week Induction of the Test Group

Test item: Alpha-Hexylcinnamaldehyde

Test item concentration: 50%

Vehicle: PEG 300

Induction Week I / Application on Test Day 1

Animal number Male	909	910	911	912	913	914	915	916	917	918
Skin reaction	1	1	1	0	0	1	1	1	1	1

Animal number Male	919	920	921	922	923	924	925	926	927	928
Skin reaction	1	1	1	1	1	1	1	1	1	1

Induction Week 2 / Application on Test Day 8

Animal number Male	909	910	911	912	913	914	915	916	917	918
Skin reaction	1	1	1	1	1	1	1	1	1	1

Animal number Male	919	920	921	922	923	924	925	926	927	928
Skin reaction	1	1	1	1	1	1	1	1	1	1

Induction Week 3 / Application on Test Day 15

Animal number Male	909	910	911	912	913	914	915	916	917	918
Skin reaction	2	2	1	2	2	2	2	2	2	1

Animal number Male	919	920	921	922	923	924	925	926	927	928
Skin reaction	2	2	2	2	2	2	2	2	2	1

RESULTS OF POSITIVE CONTROL (CONTINUED)

Skin Reactions after the First Challenge

Test item: Alpha-Hexylcinnamaldehyde

Test item concentration: 5%

Vehicle: PEG 300

CONTROL GROUP I

Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours
899	1	1
900	1	1
901	1	1
902	0	0
903	0	0

Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours
904	0	1
905	0	0
906	1	1
907	1	1
908	0	0

TEST GROUP

Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours
909	1	0
910	1	1
911	0	0
912	1	1
913	0	0
914	2	1
915	1	0
916	1	2
917	0	0
918	0	0

Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours
919	0	0
920	2	1
921	0	0
922	1	1
923	0	1
924	0	0
925	1	1
926	2	1
927	1	1
928	0	0

Three hours prior to the 24-hour reading, the test item-treated flank was depilated.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Skin Reactions after the Second Challenge

Test item: Alpha-Hexylcinnamaldehyde

Test item concentration: 3%

Vehicle: PEG 300

CONTROL GROUP II (right shoulder)

Animal No.	Skin Reactions after (± 2 Hours)		Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours	Male	24 Hours	48 Hours
929	0	0	934	0	0
930	0	0	935	0	0
931	0	0	936	0	0
932	0	0	937	0	0
933	0	0	938	0	0

Test item: Alpha-Hexylcinnamaldehyde

Test item concentration: 1%

Vehicle: PEG 300

CONTROL GROUP II (right flank)

Animal No.	Skin Reactions after (± 2 Hours)		Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours	Male	24 Hours	48 Hours
929	0	0	934	0	0
930	0	0	935	0	0
931	0	0	936	0	0
932	0	0	937	0	0
933	0	0	938	0	0

RESULTS OF POSITIVE CONTROL (CONTINUED)

Skin Reactions after the Second Challenge (continued)

Test item: Alpha-Hexylcinnamaldehyde

Test item concentration: 3%

Vehicle: PEG 300

TEST GROUP (right shoulder)

Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours
909	1	1
910	1	1
911	0	0
912	1	0
913	1	0
914	0	0
915	1	1
916	0	0
917	1	0
918	0	0

Animal No.	Skin Reactions after (± 2 Hours)	
Male	24 Hours	48 Hours
919	0	0
920	0	0
921	0	0
922	0	0
923	1	1
924	0	0
925	1	1
926	0	0
927	1	1
928	1	1

Three hours prior to the 24-hour reading, the test sites were depilated.

RESULTS OF POSITIVE CONTROL (CONTINUED)

Skin Reactions after the Second Challenge (continued)

Test item: Alpha-Hexylcinnamaldehyde

Test item concentration: 1%

Vehicle: PEG 300

TEST GROUP (right flank)

Animal No. Male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
909	0	0
910	0	0
911	0	0
912	0	0
913	0	0
914	0	0
915	0	0
916	0	0
917	0	0
918	0	0

Animal No. Male	Skin Reactions after (± 2 Hours) 24 Hours	48 Hours
919	0	0
920	0	0
921	0	0
922	0	0
923	0	0
924	0	0
925	0	0
926	0	0
927	0	0
928	0	0

Three hours prior to the 24-hour reading, the test sites were depilated.

APPENDIX 2 Certificate of Analysis

syngenta

GLP Testing Facility WMU
Analytical Development &
Product Chemistry GS2131

Syngenta Crop Protection Münchwilen AG
Breitenloh 5
CH-4333 Münchwilen

Certificate of Analysis

A16003E
NOA449280 SL (200)
J8308/145

Batch Identification	**J8308/145**
Product Code	**A16003E**
Other Product Code(s)	---

Chemical Analysis
(Active Ingredient Content)

– **Identity of the Active Ingredient(s)***	**confirmed**
– **Content of NOA449280***	**18.3 % w/w corresponding to 197 g/l**
Methodology used for Characterization	HPLC

The Active Ingredient(s) content is within the FAO limits.

Physical Analysis

– **Appearance**	**brown liquid**
– **Density ***	**1079 kg/m^3**

Stability:

– **Storage Temperature**	**< 30°C**
– **Reanalysis date**	**End of May 2011**

The stability of this test substance will be controlled by reanalysis of material held in the inventory at Syngenta Crop Protection Muenchwilen AG at the appropriate time.

This Certificate of Analysis summarizes data which originates either from a single study or from several individual studies. Tests marked with an asterisk (*) have been conducted in compliance with GLP. Raw data, documentation, study plans, any amendments to study plans and reports pertaining to this/these study/studies are stored under the study number(s) referenced below within the archives of the GLP Testing Facility WMU at Syngenta Crop Protection Muenchwilen AG.

Characterisation: 119332 Reanalysis:

Authorisation: Andrew McIntyre 14th January 2009

Dr. A. McIntyre
Analytical Development & Product Chemistry

APPENDIX 3 GLP-Certificate

The Swiss GLP Monitoring Authorities

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Home Affairs DHA
Federal Office of Public Health FOPH

Federal Department of the Environment, Transport, Energy and Communications DETEC
Federal Office for the Environment FOEN

swissmedic
Swiss Agency for Therapeutic Products

Statement of GLP Compliance

According to Article 14 paragraph 3 Ordinance on Good Laboratory Practice [OGLP, SR 813.112.1]

The notification authority for chemicals confirms that the following test facility was inspected with respect to the compliance with the Swiss Ordinance on Good Laboratory Practice, adopted on 18th May 2005 [OGLP, SR 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted on 26th November 1997 by decision of the OECD Council [C(97)186/Final].

Unequivocal name and address of the test facility:	Areas of expertise according to article 3 paragraph 1 letter d OGLP:
Harlan Laboratories Ltd. Zelgliweg 1 4452 Itingen, Switzerland.	1./ Physical-chemical testing, 2./ Toxicity studies, 4./ Environmental toxicity studies on aquatic and terrestrial organisms, 5./ Studies on behaviour in water, soil and air; bioaccumulation, 6./ Residue studies, 7./ Studies on effects on mesocosms and natural ecosystems, 8./ Analytical and clinical chemistry testing, 9./ Other studies (safety pharmacology and animal metabolism).

Inspection authority: Federal Office for the Environment (FOEN) / Federal Office of Public Health (FOPH) / Swiss Agency for Therapeutic Products (Swissmedic)

Date of inspection: 05th to 09th and 26th to 30th November 2007

Date of decision: 30th April 2008

Based on the above mentioned decision it can be confirmed that the above mentioned test facility is able to conduct studies according to the aforementioned areas of expertise in compliance with the principles of GLP. The above mentioned test facility is listed in the register and GLP list according to the Article 14 OGLP and is inspected on a regular basis according to Article 6 paragraph 2 OGLP.

Swiss Federal Office of Public Health
Consumer protection directorate
Notification authority for chemicals
CH-3003 Bern

FEDERAL OFFICE OF PUBLIC HEALTH

Bern, 12th November 2008, The Head, Dr. Dag Kappes.

The notification authority for chemicals is the coordination and decision authority for the good laboratory practice (GLP) for the FOEN, the FOPH and Swissmedic.

Swiss Federal Office of Public Health, Consumer protection directorate, Notification authority for chemicals, CH-3003 Bern.

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