Bayer HealthCare
Bayer Schering Pharma

Report No.: AT05593

o-Dichlorbenzol

LOCAL LYMPH NODE ASSAY IN MICE (LLNA/IMDS)

Report of Study T2080266
by
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Performing Laboratory:

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Sponsor:

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Study Completion Date: October 26, 2009

GLP COMPLIANCE STATEMENT

This study was conducted in compliance with the OECD Principles of Good Laboratory Practice as revised in 1997 (ENV/MC/CHEM(98)17) and with the revised German Principles of Good Laboratory Practice according to Annex I German Chemicals Act (Bundesgesetzblatt, Volume 2008, Part I, No 28, 1173-1184, issued July 11, 2008).

Prof. Dr. H.-W. Vohr
(Study Director)

Sept. 22, 2009
Date

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Quality Assurance Statement

Study No.: T2080266

Test Item: o-Dichlorbenzol

On the dates given below inspections were conducted by the Quality Assurance to ensure that no deviations exist that are likely to affect the integrity of this study.
The Quality Assurance Unit monitors the conduct of each study by study-based inspections or by process-based inspections of a similar type of study if the short-term nature of a study precludes inspection while it is in progress. Routine procedures and the equipment used in the relevant laboratory areas are inspected regularly and reports are made in accordance with current SOPs.

*(study plan amendments, if any, were duly audited and reported to the Study Director and Management)

Date of Audits / Inspections	Phases Audited / Inspected		Date of Report to Study Director and Management
Aug-20-2009	Study Plan *		Aug-20-2009
Oct-08-2009	process based	Sampling of Specimens, Weighing, Raw Data / Documentation	Oct-08-2009
Oct-08-2009	Main Report	1. Draft	Oct-08-2009
Oct-14-2009	Main Report	Final Draft	Oct-14-2009

The results of this study including the methods used have been checked on the basis of the current SOPs. They have been correctly reported and the report reflects the raw data.
In case of a multi-site study audits at the test sites are presented in the QA Statement of the Principal Investigator's report (see appendix).

Quality Assurance Unit
Global R&D Quality, GLP-Mgmt.

Date: Oct-14-2009

Signature: 
Christina Kiedrowski

2 SIGNATURES

Study Director: Prof. Dr. H.-W. Vohr — Date: Oct. 26, 2009

Head of Special Toxicology: Dr. Dr. H.J. Ahr — Date: Oct 15, 2009

3 SUMMARY

The modified Local Lymph Node Assay (IMDS) was performed in 2009 on 24 female NMRI mice of the strain Hsd Win:NMRI (6 animals/test item group and 6 control animals) to determine if there is any specific (sensitizing) or non-specific (irritant) stimulating potential of the test item o-Dichlorbenzol.

The study was conducted according to OECD Guidelines No. 429 and No. 406, EC Guideline 2004/73/EC (29th Adaptation of Guideline 67/548/EEC, B.42)/Health Effects Test Guideline and OPPTS 870.2600 (EPA) with the following test item concentrations:

0 (vehicle control), 2, 10 and 50%.

The test item was formulated in acetone/olive oil (4:1) (A/OO) to yield a solution.

Compared to vehicle treated animals there were clear increases regarding the weights of the draining lymph nodes and the cell counts in the high dose group. These increases are of statistical significance. The "positive level" of index 1.4 [6, 8, 9] for the cell counts was clearly exceeded at the high dose group.

The "positive level" of ear swelling, which is $2x10^{-2}$ mm increase [8, 9], i.e. about 10% of the control values, has not been reached or exceeded in any dose group.
No substance specific effects were determined for ear weight either.

In conclusion, these results show that the test item o-Dichlorbenzol has at least a weak sensitizing potential in mice after dermal application of a 50% concentration.
Therefore, the concentration of 10% turned out to be the NOEL for the parameters investigated in this study with respect to skin sensitization.

4 INTRODUCTION

A modified Local Lymph Node Assay (IMDS) was carried out in mice with the following test item:

o-Dichlorbenzol.

The modifications refer to the measurement of cell proliferation by cell counting instead of radioactive labeling. In addition, the acute inflammatory skin reaction is determined to discriminate specific from non-specific activation of immune competent cells in the draining lymph nodes [cf. 4, 6, 8, 9, 10, 23-25].

The aim of these investigations was to establish whether there is any specific (sensitizing) or non-specific (irritant) stimulating potential of the test item o-Dichlorbenzol.

The investigations were carried out at the Bayer Schering Pharma AG, GDD-GED-GTOX-Special Toxicology in 42096 Wuppertal, Germany.

The study plan, raw data and the final report are retained in the archives specified by Bayer Schering Pharma AG, Wuppertal, GDD-GED-IC-PM.
A retention sample of the test item is archived in the test item storage, Bayer Schering Pharma AG, Wuppertal, GDD-GED-IC-AHPM.

5 STUDY IDENTIFICATION AND RESPONSIBILITIES

5.1 Study identification

The laboratory study was carried out under number LLN09.077.

Study number:	T2080266
Experimental starting date:	August 25, 2009
Experimental completion date:	August 28, 2009
Study completion date:	see signature page
Sponsor:	LANXESS Deutschland GmbH, 51369 Leverkusen, Germany

5.2 Responsibilities

Head of test facility:	Dr. F.-W. Jekat
Head of Special Toxicology:	Dr. Dr. H.J. Ahr
Study Director:	Prof. Dr. H.-W. Vohr
Head of Quality Assurance Unit:	Dr. A. Paeßens
Archiving of study data:	R. Zils

6 MATERIAL AND METHODS

The methods used in this study are in principle specified in guidelines (OECD 406, 1992; EPA guideline OPPTS 870.2600, Skin Sensitization, March 2003; CPMP/SWP/2145/00, 2001; OECD 429, 2002). According to these guidelines the so-called Local Lymph Node Assay (LLNA) is recommended for the assessment of skin sensitization as first-stage screening study or as a stand-alone test (OECD 429; OPPTS 870.2600).

The principle of the method had been published in 1989 [1], and a first collaborative validation study in 1991 [2]. In these first trials the stimulation of the lymph nodes, i.e. cell proliferation, was measured by ^{3}H-Thymidin incorporation. In 1999 the principle of the LLNA had been stated as valid alternative to guinea pig assays by the ICCVAM [3], although the need for further modifications was also noted.

A modification of the assay by measuring the cell counts instead of radioactive labeling provides comparable sensitivity [4, 5, 24, 25, 28], and has the advantage that the cell suspension can be further analyzed by different methods (flow cytometry, chemiluminescence responses, immunofluorescence) to gain an insight into mechanistic events [4-7]. A further modification was done by including the measurement of the ear swelling after treatment leading to a much more simplified and reliable assay (Integrated Model for the Differentiation of Skin reactions (IMDS), [8]). By comparing the specific immune reaction induced by the test item in the draining lymph nodes (LN; cell counts / LN weights) with the immediate unspecific acute skin reaction (ear swelling / ear weight) it is possible to discriminate the irritant potential from the sensitizing potential of the compound tested. International standards have been successfully determined using this modification [9, 24, 25]. Such modifications are also authorized in the Note of Guidance SWP/2145/00 of the CPMP (2001) and OECD guideline 429.
With respect to this simple discrimination between sensitizing and irritant local reactions comparable findings have been reported in the human patch test system [10].

6.1 Test item

Test item:	o-Dichlorbenzol
Batch No.:	CHH 1470807
CAS No.:	95-50-1
CAS name:	1,2-Dichloro benzene
Content:	99.9%
Approval:	until July 08, 2011
Sum formula:	$C_6 H_4 CL_2$
Molar mass:	147 g/mol
Physical state:	liquid
Appearance:	clear, colourless
Storage*:	room temperature

* This information was taken from the accompanying document

6.2 Animals

6.2.1. Selection of the species

SPF-bred female NMRI mice of the strain Hsd Win:NMRI were used from commercial breeder Harlan Nederland, Kreuzelweg 53, 5960 AD Horst, Netherland. Animals of this strain have been used for years for toxicity studies at Bayer Schering Pharma AG. Mice of this strain have also been used for the intra-laboratory validation of the IMDS [8, 9], as well as an inter-laboratory validation study [24, 25].
Historical data on their physiology and spontaneous alterations are available. The health status of the strains is checked regularly at random for the most important specific infection pathogens, and the results of these tests are stored at Bayer Schering Pharma AG.

6.2.2. Adaptation

After their arrival, the animals intended for the study were allowed to adapt to the conditions of the animal room for at least 6 days and their state of health was monitored.

6.2.3. Health status

Only healthy animals showing no signs of disease were used in the study. The animals were not vaccinated or treated with antiinfectives either before their arrival or during the adaptation or study period. The females were nulliparous and nonpregnant.

6.2.4. Age and Body weight

The mice exhibited a weight of 27 – 33 grams at the beginning of the study. The age of the animals was 8 weeks.

6.3 Housing of the animals

6.3.1. Housing conditions

During the adaptation period up to 8 mice were housed together in conventional Makrolon® type III cages [11, 12], cages were changed at least twice a week. While during the study period the animals were single-housed in type II cages.

Low-dust wood shavings named Lignocel BK 8-15 supplied by Rettenmaier & Söhne, GmbH & Co, 73494 Rosenberg, Germany were used as bedding. At the instigation of the Laboratory Animal Services (LAS) the wood granulate was analyzed at random for contaminants. The relevant documents have been retained. The analytical results have not yielded evidence of any influence on the study objective.

6.3.2. Animal rooms

All the animals used in this study were kept in the same room. At times animals taking part in other toxicological studies were kept in the same room, but adequate spatial separation and appropriate organization of the working procedures ensured that animals could not be confused.

6.3.3. Cleaning, disinfection, and pest control

The animal room was cleaned once a week and disinfected at least once a month with Terralin (20 g benzalkonium chloride and 35 g Phenoxypropanol/100 g; diluted to 1% for use, i.e. 10 ml in 1 litre of water). Contamination of the feed and contact with the animals were excluded.

Pest control was not carried out in the animal room, but Killgerm Roach Traps which use no pesticides were placed in the animal room for cockroach control.

6.3.4. Environmental conditions

The environmental conditions in the animal room were standardized as follows:

Room temperature:	22 ± 2° C
Relative humidity:	40%-70%
Light/dark cycle:	12 h/12 h, with artificial illumination
Air throughput:	About 10 changes per hour

Occasional deviations from these conditions occurred e.g. as a result of the cleaning of the animal room, but they had no apparent effect on the course of the study.

6.3.5. Diet

The feed, PROVIMI KLIBA SA 3883 maintenance diet for rats and mice (from Provimi Kliba SA, CH-4303 Kaiseraugst), and tap water (drinking bottles) were provided ad libitum.

The nutritive composition of PROVIMI KLIBA SA 3883 and the contaminant content of the standard diet were checked and analyzed routinely in random samples. The tap water was of drinking water quality [13].

The results of the feed and water analyses have been stored at Bayer Schering Pharma AG. The available data do not show evidence of any effects on the study objective.

Polycarbonate bottles with a capacity of about 300 ml (study period) or 700 ml (adaptation period) were used for drinking water [11, 12].

6.4 Methods

The method used has been described in the literature (see above). Unless internationally recognized standardized reference values or tests are available, the method used here must be viewed with this in mind. On the other hand there are enough peer reviewed data available confirming the validity of this method [reviewed in 3, 4-9], and an international catch-up validation study verified the reliability of this method (24, 25).

6.4.1. Methodological Reliability

The Local Lymph Node Assay Test methodology was checked for reliability in a test on female NMRI mice using Alpha Hexyl Cinnamic Aldehyde formulated in different vehicles (PEG 400, DAE 433, DMF, MEK, acetone/olive oil (4:1) and Cremophor EL/ physiological saline solution 2% v/v) at concentrations of 3%, 10% and 30%.
The sensitivity as well as the reliability of the experimental technique is thus confirmed by this study [26].
A similar check is done in regular intervals using one of the above mentioned vehicles in order to confirm the reliability of the method. The last reliability test using Alpha Hexyl Cinnamic Aldehyde formulated in acetone/olive oil (4:1) at concentrations of 3%, 10% and 30% clearly showed the sensitizing potential of the test item [27].

6.4.2. Procedure

6.4.2.1. Grouping and identification of the animals

Six animals were placed in each group.
The animals were identified by cage labels giving the test item, the animal number, dose, sex, and the study number and marking of the tail immediately before autopsy.

6.4.2.2. Test item formulation

The test item was formulated once at day 1 of the study in A/OO.
The formulations were visually described as solutions.
The stability of the test item in the vehicle was analytically verified for up to 4 days.

6.4.2.3. Results of the Stability of the test item in the formulation

nominal value in %	content in%			content as % of nominal value		
	start	after 2 hours	after 4 days	start	after 2 hours	after 4 days
1	1.0383	1.0595	1.0265	104	106	103
50	53.9774	56.3847	52.3391	108	113	105

Data taken from Study number: F5011940

6.4.2.4. Route of administration and dosage

The test item (described in Section 6.1) in the formulation (described in Section 6.4.2.2) or the vehicle were applied epicutaneously onto the dorsal part of both ears of the animals. This treatment was repeated on three consecutive days (d1, d2 and d3).

The volume administered was 25µl/ear.

Based on our experiences with this test system and the known properties of the test items the following concentrations were used: 0 (vehicle control), 2%, 10% and 50%.

6.4.3. Investigations

6.4.3.1. Autopsies

The animals were anaesthetized by inhalation of carbon dioxide and sacrificed one day after the last application (day 4). The appropriate organs were then removed. Lymphatic organs (the auricular lymph nodes) were transferred into physiological saline (PBS).

6.4.3.2. LLN Weight and cell count determinations

The weight and cell count determinations were carried out by appropriate laboratory procedures. The weights of the lymph nodes were determined on a Mettler semiautomatic balance and stored in an IBM compatible PC. After crushing the lymph nodes through a sieve in a 12-well plate, the cell counts per ml were determined using a Multisizer 3® from Coulter Electronics. These data were also directly collected and processed by computer (Multisizer 3 software and Excel). Means, indices and standard deviations were calculated by an Excel data sheet.
A special BASIC program (GWBASIC compiler) was used to calculate means and standard deviations of the Lymphnodes' weights. Indices were calculated manually.

The so-called stimulation (or LLN-) index is calculated by dividing the absolute number of weight or cell counts of the substance treated lymph nodes by the vehicle treated ones. Thus, in case of no stimulating effect the index is always about 1.00 (+/- standard deviation), and the indices of vehicle treated animals are set to 1.00 (+/- standard deviation).
The samples (cell suspensions) of this study have been analyzed by flow cytometry (FACScan) in addition. These analyzes are not part of the GLP-study (routine lymph node assay (IMDS/LLNA)). The results serve to collect more data of the different subpopulations involved in skin reactions.

6.4.3.3. Ear Swelling

Before the first treatment and before sacrifice the thickness of both auricles of the animals was measured using a spring-loaded micrometer (Oditest, Dyer Company or Fa. Kroeplin). Means, indices and standard deviations of the ear swelling were calculated by an Excel data sheet.

6.4.3.4. Ear weight

On day 4 of the study the ear weight of the sacrificed animals was measured using a punch to take of a piece of every ear with a diameter of 8 mm. The weights were determined on a Mettler semiautomatic balance. Means, indices and standard deviations of the ear weights were calculated by an Excel data sheet.

6.4.3.5. Body weights

The body weights of the animals were recorded at the start and the end of the study (day 1 and day 4); cf. Appendix 11.4.

6.4.4. Statistics

When it was statistically reasonable, the values from treated groups were compared with those from the control group(s; vehicle) by a one-way analysis of variance (ANOVA) [15, 16] when the variances are considered homogeneous according to a homogeneity testing like Cochran's test [17]. Alternatively, if the variances are considered to be heterogenous ($p \leq 0.05$), a non-parametric Kruskal-Wallis test has been used (Kruskal-Wallis ANOVA) at significance levels of 5%. Two sided multiple test procedures were done according to Dunnett [18, 19] or Bonferroni-Holm [20], respectively. Outlying values in the LN weights were eliminated at a probability level of 99% by Nalimov's method [21]. In addition, for the LLNA/IMDS the smallest significant differences in the means were calculated by Scheffe's method [22], which according to Sachs [17] can be used for both equal and unequal sample sizes.
In this method of statistical processing of measurements a large number of comparisons is made, and as a result of the multiple tests the overall probability of error is considerably greater than the p values suggest (increased number of false-positive results). On the other hand, the known methods of adjusting p values lead to an excessive increase in the number of false negatives. In view of these problems the biological and toxicological relevance is also taken into consideration in the evaluation of statistical significance.

For this reason, in the case of indices only the standard deviations between groups and difference analysis of the mean values were used in the evaluation of the biological relevance.

7 RESULTS

7.1 Stimulation indices (weight and cell counts; ear swelling and ear weight)

Based on results obtained in validation studies and general experiences with this test system (see Section 6 and 6.4) groups of mice were treated with vehicle, 2, 10 or 50% o-Dichlorbenzol in A/OO.

The NMRI mice showed a clear increase in the weights of the draining lymph nodes and in the stimulation indices for cell counts in the high dose group, which is of statistical significance (Appendix 11.1 and 11.2, 1.), compared to control animals after application of the test item o-Dichlorbenzol. The "positive level", which is 1.4 for cell count indices, has been exceeded in the high dose group.

The "positive level" of ear swelling, which is $2x10^{-2}$ mm increase [8, 9], i.e. about 10% of the control values, has not been reached or exceeded (Appendix 11.2, 2.) in any dose group.
No substance specific effects were determined for ear weight either.

It has to be clarified that the "positive levels" mentioned above are exclusively defined for the NMRI outbreed mice used for this study [8, 9]. Such positive limits have to be calculated for each strain of mice individually [24, 25].

7.2 Body weights

The body weights of the animals were not affected by any treatment (Appendix 11.4).

8 DISCUSSION AND EVALUATION

A LLNA/IMDS was carried out in female NMRI mice after epicutaneous application of a formulation containing 0, 2, 10 or 50% of the test item o-Dichlorbenzol for 3 consecutive days onto both ears of the animals.

This study does point to a specific immunostimulating (skin sensitizing) potential of the test item.

This applies to NMRI mice, for weight and cell counts of the draining lymph nodes as well as ear swelling and ear weight indices evaluated after application of o-Dichlorbenzol.

After treatment with o-Dichlorbenzol there was a clear increase compared to control animals regarding the weights of the draining lymph nodes which is of statistical significance in the high dose group. The "positive level" which is 1.4 for cell counts has been exceeded in the high dose group.

A sensitizing potential can be assumed from the increases in cell proliferation in the draining lymph nodes. On the basis of our experiences using this method the "positive level" had been set to an increase in cell count index by 0.4 (i.e. index $\geq$ 1.4), which has been exceeded in the high dose group. Differentiation indices (DI) calculated according to our publications [8, 9] which is the quotient of the relative lymph node reaction divided by the relative acute skin reaction were > 1 for the high dose group of the test item, i. e. 2.92. This DI value does also point to a skin sensitizing potential of the test item.

The "positive level" of ear swelling which is $2x10^{-2}$ mm increase [8, 9], i.e. more than 10% increase in index, has not been reached or exceeded.

The EC 1.4 value calculated is 32.09% for this test item. In accordance with the classification proposed in the Technical Report No. 78 of the ECETOC [30] this value corresponds to a weak skin sensitizer.

Taken together, a specific activation of the cells of the immune system via dermal route was determined after application of 50% o-Dichlorbenzol by the method used. Thus, o-**Dichlorbenzol** has to be classified as a weak skin sensitizer.
Therefore, the concentration of 10% turned out to be the NOEL for the parameters investigated in this study.

9 REFERENCES

1. Kimber, I. and Weisenberger, C.: A murine local lymph node assay for the identification of contact allergens. Assay development and result of an initial validation study.
Arch. Toxicol. **63** (1989), 274-282

2. Basketter, D. A., Scholes, E. W., Kimber, I., Botham, P. A., Hilton, J., Miller, K., Robbins, M. C., Harrison, P. T. C. and Waite, S. J.:Interlaboratory Evaluation of the Local Lymph Node Assay with 25 Chemicals and Comparison with Guinea Pig Test Data.
Toxicology Methods **1**, 30-43 (1991)

3. ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) (1999). [website online]. The Murine Local Lymph Node Assay: A Test for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds. The Results of an Independent Peer Review Evaluation Coordinated by the ICCVAM. NIH Publication No. 99-4494. Available on the Internet, http://iccvam.niehs.nih.gov/

4. Vohr, H.-W. et al.: Detection of photoreactivity demonstrated in a modified local lymph node assay in mice.
Photoderm. Photoimm. & Photomed. **10**, 57 (1994).

5. Ikarashi, Y., Tsuchiya, T. and Nakamura, A.: A sensitive mouse lymph node assay with two application phases for detection of contact allergens.
Arch. Toxicol. **67**, 629-636 (1993)

6. Vohr, H.-W. et al.: The UV-irradiated local lymph node assay: a new screening test for photoreactive compounds.
Arch. Derm. Res. **284**, 41 (1992)

7. Gerberick, G. F., Cruse, L. W., Ryan, C. A., Sikorsky, E. E., Miller, C. M. and Ridder, G. M.: Differential expression of T cell activation markers (CD62L, CD44, CD45RB, CD25) in draining lymph nodes of mice following exposure to allergens and irritants.
The Toxicologist **30**, 95 (1996)

8. Homey, B., von Schilling, C., Blümel, J., Schuppe, H.-C., Ruzicka, T., Ahr, H.-J., Lehmann, P. and Vohr, H.-W.: An integrated Model for the Differentiation of Chemical-Induced Allergic and Irritant Skin Reactions (IMDS).
Toxicol. and Appl. Pharmacol. **153**, 83-94 (1998)

9. Vohr, H.-W., Blümel, J., Blotz, A., Homey, B. and Ahr, H.J.
An intra-laboratory validation of IMDS: Discrimination Between (Photo)Allergic and (Photo)Irritant Skin Reactions in Mice.
Arch. Toxicol., **73**, 501-509 (2000)

10. Arnstrop, C., Balsler, E. and Thomsen H.K.
The Occurence of Different Morphological Parameters in Allergic and Irritant Patch Test Reactions.
In: Current Topics in Contact Dermatitis, Frosch P.G. et al.
[Eds], Springer-Verlag, 38-41 (1989)

11. Spiegel, A. und Gönnert, R.: Neue Käfige für die Haltung und Zucht von Kleinnagern.
Zschr. Versuchstierkunde **1** (1961), 38-46

12. Meister, G: Versuchstierkäfige für die Haltung und Zucht von Kleinnagern.
Zschr. Versuchstierkunde **7** (1965), 144-153

13. Trinkwasser-Verordnung vom 21.05.2001, Bundesgesetzblatt, I Nr. 24 vom 28.5.2001, S.959

14. Maurer Th.: Experimental photocontact allergenicity: guinea pig models.
Photodermatology 1984; 1:221-231

15. Mann, H.B. and Whitney, D.R.: On a test of whether one of two random variables is statistically larger than the other.
Ann. Math. Stat. **18** (1947), 50-60.

16. Wilcoxon, F.: Individual comparisons by ranking methods.
Biometrics **1** (1945), 80-83.

17. Sachs , L .: Angewandte Statistik 6^{th} and 10^{th} edition.
Springer Verlag, Berlin (1978/2002)

18. Dunett, C. W.: A multiple comparison procedure for comparing several treatments with a control.
Amer. Statist. Ass. J. **50** (1955), 1096-1121

19. Dunett, C. W.: New tables for multiple comparisons with a control.
Biometrics **20** (1964), 482-491

20. Holm, S.: A simple sequentially rejective multiple test procedure.
Scand. J. Statist. **6** (1979), 65-70

21. Keller, F.: Ausreißertest nach Nalimov,
Statistik f. naturwissenschaftliche Berufe (1982), 88-89

22. Scheffe, H. : A method of judging all contrasts in the analysis of variance.
Biometrica **40** (1953), 87-104

23. Ulrich, P., Homey, B. and Vohr, H.-W.: A Modified Murine Local Lymph Node Assay for the differentiation of contact photoallergy from phototoxicity by analysis of cytokine expression in skin-draining lymph node cells.
Toxicology **125** (1998), 149-168

24. Ehling G., Hecht M., Heusener A., Huesler J., Gamer A.O., v. Loveren H., Maurer Th., Riecke K., Ullmann L., Ulrich P., Vandebriel R., Vohr H.-W.
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Local Lymph Node Assay. 1st ROUND.
Toxicology **212** (2005), 60-68

25. Ehling G., Hecht M., Heusener A., Huesler J., Gamer A.O., v. Loveren H., Maurer Th., Riecke K., Ullmann L., Ulrich P., Vandebriel R., Vohr H.-W.
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Local Lymph Node Assay. 2nd ROUND.
Toxicology **212** (2005), 69-79

26. Vohr, H.-W.: Confirmation of the function of a Local Lymph Node Assay in mice (LLNA/IMDS) with Alpha Hexyl Cinnamic Aldehyde.
Bayer HealthCare AG, AT00361, April 14, 2003

27. Vohr, H.-W.: Confirmation of the function of a Local Lymph Node Assay in mice (LLNA/IMDS) with Alpha Hexyl Cinnamic Aldehyde.
Bayer HealthCare AG, PH-35826, April 24, 2009

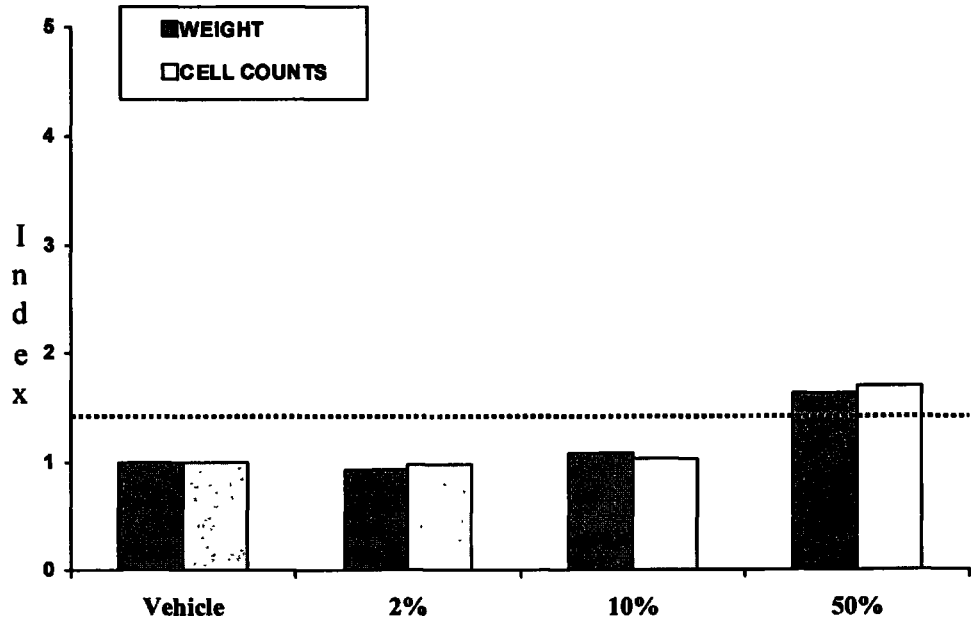
28. Gamer A.O., Nies E., Vohr H.-W.: Local Lymph Node Assay (LLNA): Comparison of different protocols by testing skin-sensitizing epoxy resin system components.
Reg. Tox. Pharmacol. **52** (2008), 290-298

10 ABBREVIATIONS

A/OO	acetone/olive oil (4:1)
B	B cell
CC	cell counts
^{o}C	degrees centigrade
cf	confer
d	day
DAE 433	dimethylacetamide (40%), acetone (30%) and ethanol (30%)
DMF	dimethylformamide
DMSO	dimethylsulfoxide
FACScan	Fluorescence Activated Cell Scanner
h	hour
IMDS	Integrated Model for the Differentiation of chemical-induced Skin reactions
kg	kilogram
LN	lymph node
LLN	local lymph node
LLNA	local lymph node assay
MEK	methyl ethyl ketone
mg	milligram
ml	milliliter
MO	macrophage
NaCl	sodium chloride
no.	number
PBS	phosphate buffered saline
PEG 400	polyethylene glycol 400
Pluronic/ NaCl	Pluronic PE 9200/ 0.9% NaCl solution, 1% v/v
rel.	relative
SD	standard deviation
T	T cell
Veh.	vehicle
v/v	volume/volume
w/w	weight/weight
w/v	weight/volume

11 APPENDIX

11.1 Bar charts (weight and cell count) for the LLNA



11.2 Tabular summary of the LLNA/IMDS results

1. Direct LLNA (NMRI mice, female, 6 animals/group)

Dose (%)	**Weight index (index of mean +/- SD in %)**		**Cell count index**	
0*	**1.00**	+/- 25.26	**1.00**	+/- 23.13
2	**0.93**	+/- 28.80	**0.98**	+/- 60.70
10	**1.08**	+/- 26.37	**1.03**	+/- 33.20
50	**1.63↑**	+/- 29.08	**1.70↑**	+/- 36.82

2. Ear swelling (NMRI mice, female, 6 animals/group, in 0.01 mm)

Dose (%)	day 1 (**mean** +/- SD in %)		day 4		Index day 4
0*	**17.75**	+/- 4.88	**17.92**	+/- 4.43	1.00
2	**17.58**	+/- 2.93	**17.67**	+/- 2.79	0.99
10	**17.33**	+/- 3.76	**17.67**	+/- 3.69	0.99
50	**17.33**	+/- 4.49	**17.83**	+/- 5.77	1.00

* = A/OO
↑ = statistically significant increase ($p \leq 0.05$)

3. Ear weight (NMRI mice, female, 6 animals/group, in mg per 8 mm diameter punch)

Dose (%)	day 4 (**mean** +/- SD in %)	Index day 4
0*	**11.58** +/- 6.92	1.00
2	**11.39** +/- 5.03	0.98
10	**11.21** +/- 2.96	0.97
50	**11.19** +/- 8.59	0.97

* = A/OO

11.3 Individual cell counts

Cell counts [Thousand cells / mL cell suspension] in auricular lymph nodes* on study day 4 after daily epicutaneous application of a 0, 2, 10 or 50% solution of o-Dichlorbenzol in acetone / olive oil 4:1 for 3 days onto the ears of female NMRI mice (6 / treatment group) in a Local Lymph Node Assay

Treatment group	Animal no.	Cell count [Thousand cells / mL lymph node suspension]* Repeat determination	Arithmetic mean	Group mean	SD	SD [%]	Cell count index**
Vehicle control (Group 1)	1	7719 7938	7828,5	8613,75	1992,04	23,13	1,00
	2	8354 8733	8543,5				
	3	7138 7232	7185				
	4	6281 6368	6324,5				
	5	11660 11861	11760,5				
	6	10049 10032	10040,5				
Group 2	7	8650 8525	8587,5	8399,08	5098,09	60,70	0,98
	8	3488 3419	3453,5				
	9	4407 4540	4473,5				
	10	6028 5993	6010,5				
	11	10430 10649	10539,5				
	12	17383 17277	17330				
Group 3	13	11806 11772	11789	8909,25	2957,80	33,20	1,03
	14	5295 5232	5263,5				
	15	12008 11690	11849				
	16	10249 9794	10021,5				
	17	9179 9063	9121				
	18	5477 5346	5411,5				
Group 4	19	8514 8603	8558,5	14657,00	5397,34	36,82	1,70
	20	19306 19588	19447				
	21	23450 22179	22814,5				
	22	10946 11095	11020,5				
	23	12572 12442	12507				
	24	13638 13551	13594,5				

* : The right and left auricular lymph node of each animal was obtained, crushed trough a sieve and dispersed in 2 mL phosphate buffered saline. An aliquot of this sample was taken for a repeated determination of the cell count per mL of this cell suspension. The cell count of every animal is the calculated mean of these two measurements.

** : Cell count index: mean cell count of the animals of a treatment group divided by the mean cell count of the vehicle control group. The Cell count index for the vehicle control group is 1.

11.4 Body weights

Animal-No.	Body weight in g	
	Day 1	**Day 4**
group 1 control		
1	30	30
2	29	29
3	29	31
4	32	30
5	30	33
6	29	31
Mean	**29.8**	**30.7**
group 2 2%		
7	27	29
8	29	29
9	27	27
10	32	29
11	27	30
12	33	31
Mean	**29.2**	**29.2**
group 3 10%		
13	28	31
14	27	30
15	31	32
16	29	29
17	29	31
18	31	31
Mean	**29.2**	**30.7**
group 4 50%		
19	31	30
20	31	32
21	31	30
22	28	30
23	28	27
24	29	26
Mean	**29.7**	**29.2**