

LRI Workshop

*Applicability of sensitization testing methods
for regulatory purposes*

Brussels, February 2nd – 3rd, 2010

Dr. Reinhard Kreiling
CPS Toxicology
Clariant Produkte (DE) GmbH
January 25, 2010

Comparative Sensitization Data based on LLNA vs Guinea Pig Testing

Experience Report from EFfCI



Available online at www.sciencedirect.com



Food and Chemical Toxicology 46 (2008) 1896–1904



www.elsevier.com/locate/foodchemtox

Comparison of the skin sensitizing potential of unsaturated compounds
as assessed by the murine local lymph node assay (LLNA)
and the guinea pig maximization test (GPMT)

R. Kreiling^a, H.M. Hollnagel^{b,1}, L. Hareng^b, D. Eigler^c, M.S. Lee^a, P. Griem^a, B. Dreeßen^d,
M. Kleber^e, A. Albrecht^f, C. Garcia^g, A. Wendel^{h,*}

^a Clariant Produkte (Deutschland) GmbH, D-65840 Sulzbach, Germany

^b BASF AG, D-67056 Ludwigshafen, Germany

^c Goldschmidt GmbH, Goldschmidtstraße 100, D-45127 Essen, Germany

^d Sasol Germany GmbH, Paul-Baumann-Straße 1, D-45764 Marl, Germany

^e Cognis Deutschland GmbH & Co. KG, Rheinpromenade 1, D-40789 Monheim, Germany

^f BSL Bioservice Scientific Laboratories GmbH, Behringstraße 6, D-82152 Planegg, Germany

^g SEPPIC, 127 chemin de la Poudrière, F-81100 Castres, France

^h University of Konstanz, Biochemical Pharmacology, P.O. Box M447, D-78457 Konstanz, Germany

Received 26 September 2007; accepted 12 January 2008



Exactly your chemistry.

Comparative Sensitization Data: LLNA vs GPMT Experience Report from EFfCI



Exactly your chemistry.



European Federation for Cosmetic Ingredients

*Experience Report on discordant sensitization
results from LLNA versus GPMT studies*

■ Background Information on the EFfCI "LLNA Project"

- First indications of unexplainable findings in the mouse local lymph node assay (LLNA) with fatty acid derived surfactant raw materials in 2004
- Virtually no allergic responses were seen in guinea pig maximization tests (GPMT), but unexplainable high stimulation indices (SI) indicating skin sensitization potential were revealed in the LLNA
- Initial series of experiments started in 2005 to clarify whether these unexpected positive results in the LLNA may reflect *false positive* findings in terms of sensitization

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI




Exactly your chemistry.

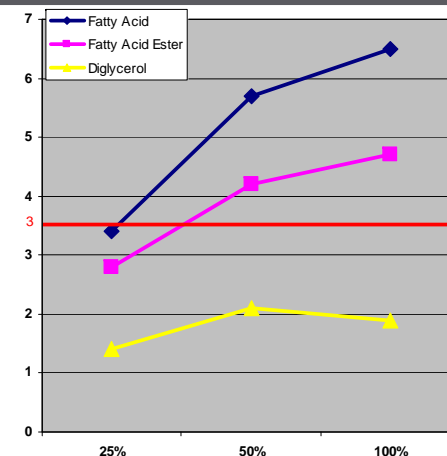
■ Initial data set 1

- Testing of a Fatty Acid Ester which was used for decades without any indication of skin sensitizing properties based on human experience reveals positive findings in the LLNA but negative responses in the GPMT

Results of a comparative screening study using a fatty acid ester and its reactants (Kreiling & Lee 2007)

Compounds		LLNA	GPMT
Fatty Acid-Diglycerol Ester	yes	sensitizing	not sensitizing
Fatty Acid	yes	sensitizing	not sensitizing
Diglycerol	no	not sensitizing	not sensitizing

- A comparison of the results on the basis of the obtained SI values reveals a gradation (Fatty Acid > Ester > Diglycerol) indicative of the fatty acid being a potential driver of the overall positive response
- Based hereupon a series of tests were initiated focussing on common functional / structural features which might be responsible for these unexpected findings



Comparative Sensitization Data: LLNA vs GPMT

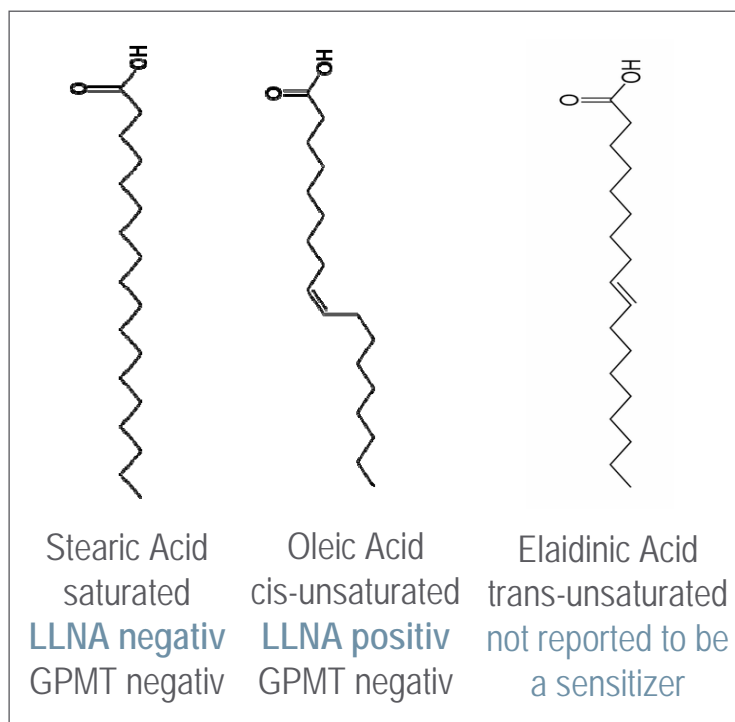
Experience Report from EFfCI



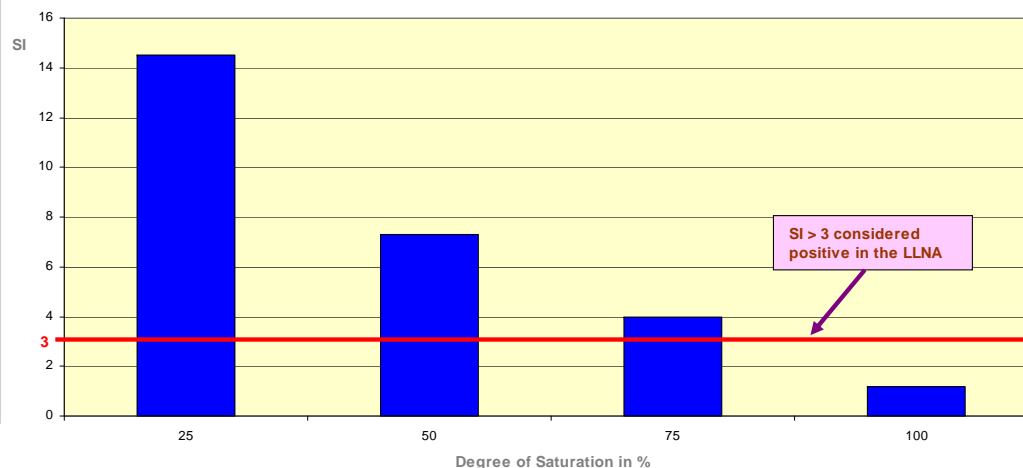
Exactly your chemistry.

■ Initial data set 2

- Testing of saturated versus unsaturated fatty acids (e.g. stearic vs oleic acid) reveal different findings in the LLNA indicating that carbon-carbon double-bonds as “structural / functional element” may have an influence in the LLNA response



- Differences in the degree of hydrogenation of the same basic fatty acid have an influence on LLNA responses, leading to a significant decrease in the SI value when increasing the saturation degree of the fatty acid



Comparative Sensitization Data: LLNA vs GPMT




Experience Report from EFfCI



Exactly your chemistry.

■ EFfCI Project “Significance of LLNA Data for Cosmetic Raw Materials”

- Based on the identified uncertainties in LLNA data, EFfCI started a project using LLNA and GPMT assays to comparatively analyse the data focussing on common structural / functional features which may be responsible for the observed unexpected positive findings in the LLNA
- A literature research identified conspicuous clusters of positively tested classes of compounds which apparently were not adequately represented in available validation trials for the LLNA

Identified Compounds	Double bond structure	LLNA 	Buehler 	GPMT 	HR IPT
Aromatic compound	yes	sensitizing		Not sensitizing	
Quaternary ammonium compound	yes	sensitizing		Not sensitizing	
High molecular weight polymer	yes	sensitizing	Not sensitizing	Not sensitizing	
lip amino acid	yes	sensitizing		Not sensitizing	Not sensitizing
Surfactant type material	yes	sensitizing	Not sensitizing	Not sensitizing	Not sensitizing

- Interestingly also here a high proportion of materials having double-bonds in their structures are reported to react positively in the LLNA but not in other well standardized test systems, like Guinea Pig assays or in humans

Comparative Sensitization Data: LLNA vs GPMT

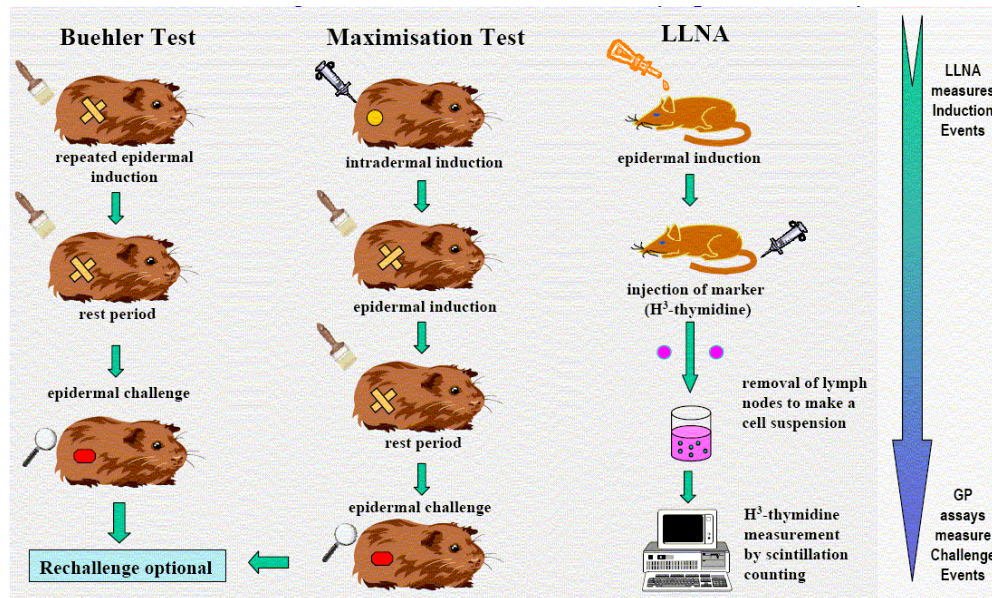
Experience Report from EFfCI



Exactly your chemistry.

■ EFfCI Project: Purpose of the project

- Aim of the study was
 - to extend the scientific basis on possible limitations of the LLNA for substance classes which apparently were not adequately represented in previous validation trials
 - to address the hypothesis that chemicals with carbon-carbon double bonds may result in a higher number of unspecific (false positive) results in the LLNA versus the GPMT



- The study compared the activity of selected cosmetic raw materials in the LLNA and the GPMT
- The study was performed under supervision of Prof. A. Wendel (University of Konstanz, Biochemical Pharmacology, Germany)
- The experimental work was conducted at Bioservice Scientific Laboratories (BSL, Planegg / Munich, Germany)

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ EFfCI Project: Test Substances for comparative testing

Test Substance	Structure	Purity	Occurrence / Use
Oleic acid CAS 112-80-1		99%	Natural food ingredient
Linoleic acid CAS 60-33-3		99%	Natural food ingredient
Linolenic acid CAS 463-40-1		90%	Natural food ingredient
Undecylenic acid CAS 112-38-9		98%	Cosmetic ingredient
Fumaric acid CAS 110-17-8		99%	Endogenous substance
Maleic acid CAS 110-16-7		99%	Endogenous substance
Succinic acid CAS 110-15-6		99%	Endogenous substance
Squalene CAS 111-02-4		98%	Endogenous substance
1-Octin-3-ol CAS 818-72-4		96%	Ingredient in flavours and fragrances

- Mainly unsaturated aliphatic and fatty acids including also some other related substances
- All test substances are either endogeneous physiological components of the human body and/or natural constituents of food and/or cosmetics
- All test substances have a long history of widespread and safe consumer use without exhibiting any indication of skin sensitizing potential

Comparative Sensitization Data: LLNA vs GPMT



Experience Report from EFfCI



Exactly your chemistry.

■ EFfCI Project: Study Details

- Selected cosmetic raw materials were comparatively tested both in the LLNA and the GPMT following the principles of Good Laboratory Practice (GLP)
- All test materials were of analytical grade purity
- Testing was performed in strict adherence to OECD test guideline 429 (LLNA) and OECD test guideline 406 (GPMT)
- Additional measurements included
 - Ear thickness in LLNA studies (day 1 prior to first treatment and day 5 prior to ^3H -methyl thymidine injection)
 - Lymph Node weight Index calculation in LLNA studies
 - Obligatory re-challenge in GPMT studies
 - Skin reaction readings after 24 h, 48 h and also after 72 h (1st and 2nd challenge)
 - Statistical analysis using a one-sided Student's *t*-test

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ EFfCI Project: LLNA Data

Stimulation indices (SI) obtained in the LLNA and overall test result						Result
Substance	Vehicle	Test Concentration				
		5%	10%	25%	50%	
Oleic acid	AOO	-	2,6	14,9	6,9	positiv
Linolic acid	AOO	-	1,5	7,0	9,1	positiv
Linolenic acid	AOO	-	3,1	9,3	10,3	positiv
Undecylenic acid	AOO	-	2,5	3,3	4,4	positiv
Fumaric acid	DMSO	1,3	2,3	1,4	-	negativ
Maleic acid	DMSO	-	6,7	16,1	16,1	positiv
Succinic acid	DMSO	1,2	1,2	1,3	-	negativ
Squalene	AOO	-	3,8	6,9	8,2	positiv
Octinol	AOO	-	5,6	8,8	11,2	positiv

– no signs of systemic toxicity, no clinical signs of irritation, no significant ear swelling,

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ EFfCI Project: GPMT Data

Number of animals with skin reactions in GPMT and overall test results

Test substance	Test Concentration (Intradermal / Topical / Challenge)	Number of animals with skin-reactions out of 10 (in control out of 5)			Number of animals with reproducible skin-reactions (first and re- challenge)	Overall Result
		24h	48h	72h		
Oleic Acid	5 / 50 / 25	0 (0)	1 (0)	4 (0)	2	negativ
Linoleic Acid	5 / 100 / 50	2 (4)	1 (3)	0 (3)	2	negativ
Linolenic Acid	5 / 100 / 50	2 (0)	1 (1)	1 (0)	no re-challenge for technical reasons	negativ
Undecylenic Acid	1 / 25 / 25	2 (0)	2 (0)	4 (0)	4	positiv
Fumaric Acid	5 / 25 / 10	1 (0)	1 (0)	0 (0)	0	negativ
Maleic Acid	0.5 / 25 / 25	1 (0)	1 (0)	0 (0)	0	negativ
Succinic Acid	0.5 / 25 / 10	1 (1)	0 (0)	1 (0)	0	negativ
Squalene	5 / 100 / 100	1 (0)	1 (0)	3 (0)	2	negativ
Octinol	0.25 / 10 / 5	1 (0)	1 (0)	1 (0)	0	negativ

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ Biochemical properties of test compounds which may trigger cell proliferation based on literature data

- Oleic -, linoleic - and linolenic acid involved in diverse cell proliferative and inflammatory processes, e.g. modulation of cell-mediated immune reactions, morphological alteration of keratinocytes and epidermal Langerhans cells, enhancement of cytokine production
- Squalene is an endogenous intermediate in the bio-synthesis of cholesterol and is known as “immune response booster”, eliciting non-specific immune responses after oral or i.v. application
- Maleic acid, but not fumaric acid or succinic acid, was positive in the LLNA. No relevant literature data could be identified
- Octinol is rarely used as ingredient in cosmetics and no data could be retrieved that would explain the high SI values obtained in the LLNA
- Undecylenic acid is the only substance that was weakly positive both in LLNA and GPMT. Together with the available human case reports regarding skin sensitization, a weak skin sensitizing potential can be therefore reasonably assumed

Comparative Sensitization Data: LLNA vs GPMT

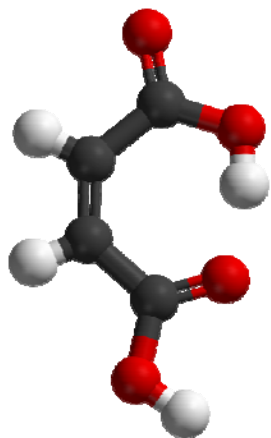
Experience Report from EFfCI



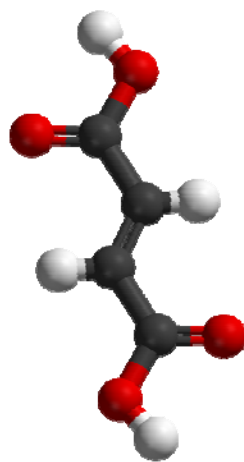
Exactly your chemistry.

■ Structural differences in LLNA and GPMT responses

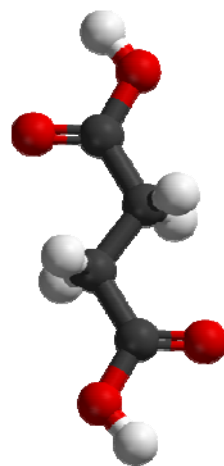
- Based on literature data, all identified biochemical processes seem to apply only for *cis*-unsaturated fatty acids, but not for *trans*-unsaturated nor saturated fatty acids
- Succinic - and fumaric acid, but not maleic acid are intermediates in the citric acid cycle
- Only maleic acid but not fumaric acid or succinic acid, positive in the LLNA.



Maleic Acid
cis / unsaturated
LLNA positive
GPMT negative



Fumaric Acid
trans / unsaturated
LLNA negative
GPMT negative



Succinic Acid
Saturated
LLNA negative
GPMT negative

- These reactions occurred without any significant ear swelling response or clinical signs of local irritation on the ears
- Dermal irritative effects alone cannot explain the high SI value observed for maleic acid since the geometric isomer fumaric acid and the corresponding saturated succinic acid exhibited comparable (or even higher) irritating effects in pre-tests

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ EFfCI Project: Theoretical regulatory consequences

Theoretical Classification based on either LLNA or GPMT data			
Substance	Structure	LLNA	GPMT
Oleic Acid		R43	No labelling
Linoleic Acid		R43	No labelling
Linolenic Acid		R43	No labelling
Undecylenic Acid		R43	R43
Fumaric Acid		No labelling	No labelling
Maleic Acid		R43	No labelling
Succinic Acid		No labelling	No labelling
Squalene		R43	No labelling
Octinol		R43	No labelling

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ Conclusions 1

- The high number of positive responses in the LLNA whereas virtually no or low allergenic responses were revealed in the GPMT was surprising
- In the published literature several reports suggest that cell activation / proliferation is not solely related to sensitization but can be initiated by a number of several specific biochemical mechanisms
- The compounds tested here are considered to have low or no sensitizing properties based on human experience.
- It cannot be decided without further data whether the absence of reports on human cases of allergic contact dermatitis, despite of widespread exposure, indicate the absence of hazard or the absence of risk
- For compounds that bear structural similarity to the substances investigated here the GPMT seem to give results that more adequately reflect the relevance in humans

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ Conclusions 2

- The more relevant point for the European manufacturing, importing and downstream industries is the question whether a substance has to be classified as skin sensitizer and, thus, has to be regarded as a dangerous substance
- It should be noted that the LLNA is considered the "*gold standard*" under REACH and that for animal welfare reasons it is unlawful in the EU to perform both tests with the same substance in order to do a hazard assessment
- EFfCI concludes that the substances tested here and that caused a positive result in the LLNA, but a negative result in the GPMT, possess skin sensitization properties – if at all – to such a limited degree that formal classification and labeling would be inappropriate
- EFfCI further concludes that basing the classification and labeling in this case solely on the LLNA would lead to the scientifically unjustified ban of safe uses

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ Recommendation

- It is acknowledged that the LLNA has advantages over the GPMT in terms of animal welfare, time need, objectivity of readout and quantification of results
- However, for unsaturated compounds that bear structural similarity to the substances investigated here, the suitability of the LLNA should be carefully considered, e.g., by evaluating test results for structurally related substances, before deciding to use the LLNA for skin sensitization hazard testing
- For this group of chemicals, the GPMT may give results that more adequately reflect the relevance in humans
- In light of the identified uncertainties EFfCI propose that as long as unspecific findings cannot be excluded in the LLNA, the GPMT should remain available as a recognized and accepted test method for identification of a skin sensitization hazard
- Further work seems to be necessary before scientific judgements and regulatory decisions should be solely based on results from LLNA studies

Comparative Sensitization Data: LLNA vs GPMT

Experience Report from EFfCI



Exactly your chemistry.

■ Acknowledgements

- Birte Dreeßen, Sasol Germany GmbH
- Dorothea Eigler, Goldschmidt GmbH
- Christine Garcia, Air Liquide
- Peter Griem, Clariant Produkte (Deutschland) GmbH
- Lars Hareng, BASF AG
- Heli Miriam Hollnagel, Dow Europe GmbH
- Marcus Kleber, Cognis Deutschland GmbH & Co. KG
- Mounq Sook Lee, Clariant Produkte (Deutschland) GmbH
- Peter Ungeheuer, EFfCI

- Special thanks to
 - Prof. Albrecht Wendel (University of Konstanz) for supervising the project
 - Dr. Achim Albrecht (BSL Bioservice) for the experimental support