KeratinoSens test submission - Attachment 12c - PC-extended list_new

Test report

Further evaluation of the predictivity of the KeratinoSens assay to detect skin sensitizers

Study period: **August 2010 – April, 2011**

Study laboratory: **Givaudan *in vitro* toxicology laboratory**

Report date: **8. April 2011**

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1. Goal of the study and study set-up

The predictivity of the KeratinoSens assay had previously been tested on a list of 67 chemicals (Emter et al., 2010) (see Attachment 12a). This chemical list, also referred to as the 'Silver list', had been mainly compiled from chemicals for which the available evidence from the LLNA, guinea pig tests and human data give congruent results. In addition, it included all the chemicals from the draft ICCVAM performance standards (ICCVAM, 2008) and from the ECVAM/COLIPA list (Casati et al., 2009) and therefore chemicals for which, in the literature, there is sufficient agreement on whether they are *bona fide* skin–sensitizers or non-sensitizers, and which therefore can be used to evaluate the predictivity of a novel *in vitro* assay.
Here, this original Silver list is extended based on literature data to further evaluate the predictivity. The rationale for selecting chemicals is described below in detail. The predictivity of the KeratinoSens against the weight of evidence *in vivo* data was then evaluated. All chemicals were tested according to the SOP.

It is generally assumed, that skin sensitization is best predicted by combining several *in vitro* assays. In particular, the determination of a chemical's potential to covalently modify peptide/proteins is considered a key step in the sensitization cascade, and a peptide reactivity test is likely to be included in any integrated testing strategy (Jowsey et al., 2006; Basketter and Kimber, 2009). As already done for the Silver-list previously (Emter et al., 2010), all chemicals were tested in parallel in a direct peptide reactivity assay (Natsch and Gfeller, 2008). This assay is a modification of the direct peptide reactivity assay (DPRA) currently under prevalidation at ECVAM. The main differences are (i) the use of a highly reactive test peptide containing both cysteine and lysine residues and (ii) determination of peptide depletion, peptide adduct formation and peptide oxidation in parallel. It is not the goal of the current prevalidation of the KeratinoSens to perform any pre-validation of this approach. Nevertheless, we present the assessment from this particular peptide reactivity test in parallel, because in the assessment of the predictivity of an assay and in the discussion on false-negatives, it is important to consider whether the assay may fit into an integrated testing strategy and whether other assays of an integrated testing strategy could yield complementary information / a complementary applicability domain.

2. Test chemicals

The test chemicals were all selected from the ICCVAM publication on the validation of the LLNA (further refered to as the 'ICCVAM validation paper' (Haneke et al., 2001) and from the ICCVAM database available online (further refered to as the 'ICCVAM database' (ICCVAM, 2008). The following criteria were applied to select the chemicals:

1.) Chemicals from the ICCVAM validation paper which gave congruent results in the Guinea pig tests and the LLNA. These chemicals and their animal data are summarized in the Appendix, **Table A**
2.) Additional chemicals from the ICCVAM database which gave congruent results in the Guinea pig tests and the LLNA These chemicals and their animal data are summarized in the Appendix, **Table B**
3.) Additional chemicals from the ICCVAM database which gave congruent results in the human maximization tests and the LLNA These chemicals and their animal/human data are summarized in the Appendix, **Table C**
4.) Chemicals which formed part of the original 'Silver list' (Emter et al., 2010) and which were not included in the above three selection criteria. These chemicals and their animal/human data are summarized in the Appendix, **Table D**.

The original selections were reduced to those chemicals which were commercially available. The ICCVAM validation paper also included a number of proprietary chemicals. Attachment 11b only

contains the chemicals which were successfully purchased and tested. In total these are 114 chemicals, and thus 47 additional chemicals as compared to the original Silver list.

Excluded chemicals:
The original selection also contained two chemicals which could not be tested for physicochemical reasons: 1-Bromohexadecane, 112-82-3, cLogP = 9.01, not soluble in DMSO and 3,5,5-Trimethylhexanoyl chloride, 36727-29-4, unstable in DMSO and water.

3. Test results

Table 1 lists all the results from the KeratinoSens assay sorted for sensitizers and non-sensitizers. Chemicals are listed as sensitizers or non-sensitizers based on the WoE analysis presented in Tables A-D in the Appendix. Table 1 also indicates whether the chemicals are rated as directly peptide-reactive based on adduct formation in the LC-MS assay.
The following parameters are given in Table 1:

- I_{max} Maximal fold-gene induction of the luciferase gene over the full dose-response
- EC 1.5 Concentration in µM for 1.5 fold gene induction
- Pos / Neg Rating of chemical according to prediction model (1 or 0)
- reps. Positive number of independent repetitions positive / number of repetitions done
- IC50 Concentration in µM for 50% reduction of cell viability

Table 2 lists the Cooper statistics for both the KeratinoSens result alone as well as the peptide reactivity results. Table 2 also summarises the results from a combined assessment taking evidence from both assays, i.e. from rating any chemical positive if it is either directly peptide reactive (adduct-forming) or/and positive in the KeratinoSens assay.
The accuracy of the KeratinoSens assay alone is 78.1%, the accuracy of the peptide reactivity assay is 69.3 % and the combined accuracy is 83.3%.

Table 1. Results in the KeratinoSens assay for 114 chemicals selected based on a WoE assessment

Name	CAS-Number	LLNA EC3	KeratinoSens result					Adduct peptide reactivity
			I_{max}	EC 1.5	Pos / Neg	reps. Positive	IC50	
Non-sensitizers								
4-hydroxybenzoic acid	99-96-7	>25	1.1	>2000	0	0 of 2	>2000	0
Lactic acid	50-21-5	>25	1.3	>2000	0	1 of 4	>2000	0
Tween 80	9005-65-6	NC [1)]	2.7	19.3	1	2 of 2	399.8	0
Salicylic acid	69-72-7	>25	1.1	>2000	0	0 of 2	>2000	0
Chlorobenzene	108-90-7	>25	1.2	>2000	0	0 of 2	>2000	0
Tartaric acid	87-69-4	>25	1.2	>2000	0	0 of 2	>2000	0
Sulphanilamide	63-74-1	NC	1.4	>2000	0	0 of 2	>2000	0
4-Aminobenzoic acid *	150-13-0	>10	1.2	>2000	0	0 of 2	>2000	0
Diethyl phthalate	84-66-2	>100	1.1	>2000	0	0 of 2	>2000	0
Isopropanol	67-63-0	>50	1.2	>2000	0	0 of 2	>2000	0
Methyl salicylate	119-36-8	>20	1.2	>2000	0	0 of 2	>2000	0
Propylene glycol	57-55-6	>100	1.2	>2000	0	0 of 2	>2000	0
6-methyl coumarin	92-48-8	>25	4.6	239.3	1	2 of 2	>2000	0
Propylparaben	94-13-3	>50	9.7	14.5	1	2 of 2	813.1	0
Benzalkonium chloride *			1.5	>2000	0	1 of 4	4.0	0
Dextran	9004-54-0	NC [1)]	1.5	>2000	0	0 of 2	>2000	0
Dimethyl isophthalate	1459-93-4	NC[1)]	1.95	773	1	5 of 8	>2000	0
Benzoic acid	65-85-0	>20	1.1	>2000	0	0 of 2	>2000	0
Benzyl alcohol	100-51-6	>50	1.2	>2000	0	0 of 4	>2000	0
Fumaric acid *	110-17-8	>25	1.3	>2000	0	0 of 2	>2000	0
Glycerol	56-81-5	>100	1.2	>2000	0	0 of 4	>2000	0
1-Butanol	71-36-3	>20	1.1	>2000	0	0 of 2	>2000	0
Ethyl vanillin	121-32-4	>50	5.4	161.7	1	2 of 2	>2000	0
Sulfanilic acid	121-57-3	>25	1.3	>2000	0	0 of 2	>2000	0
Sodium lauryl sulfate	151-21-3	14	1.2	>2000	0	0 of 2	44.7	0
Octanoic acid	124-07-2	>50	1.1	>2000	0	0 of 2	>2000	0
Phenol	108-95-2	NC	1.3	>2000	0	0 of 2	>2000	0
2-hydroxypropyl methacrylate *	923-26-2	>50	1.95	1025	0	1 of 4	>2000	1

* chemicals marked with a star have not been tested previously and were not included in the Silver list

Table 1(continued). Results in the KeratinoSens assay for 114 chemicals selected based on a WoE assessment

Name	CAS-Number	LLNA EC3	KeratinoSens result					Adduct peptide reactivity
			I_{max}	EC 1.5	Pos / Neg	reps. Positive	IC50	
Sensitizers								
Methylisoeugenol *	93-16-3	Pos. [3)]	1.4	>2000	0	0 of 4	815.1	0
4-Methylcatechol *	452-86-8	Pos. [3)]	8.1	19.2	1	2 of 2	71.7	1
1-Bromododecane *	143-15-7	Pos. 3)	2.2	44.0	1	5 of 6	98.0	0
Cobalt chloride	7646-79-9	Pos. [3)]	23.3	298.6	1	2 of 2	1330.2	
Diphenylmethane-4,4'-diisocyanate *	101-68-8	Pos. [3)]	2.4	121.8	1	2 of 2	>2000	1
Dodecyl methanesulfonate *	51323-71-8	Pos. [3)]	2.03	12.14	at cytotox	3 of 4 at cytotox	19.3	0
4-Nitrobenzyl chloride *	100-14-1	Pos. [3)]	93.4	4.0	1	2 of 2	27.6	1
Chlorpromazine hydrochloride *	69-09-0	Pos. [3)]	1.1	>2000	0	0 of 9	10.1	0
Beryllium sulfate *	7787-56-6	Pos. [3)]	5.7	15.4	at cytotox	9 of 9 at cytotox	51.3	
Benzocaine	94-09-7	>50	3.0	18.2	1	2 of 2	>2000	0
Benzaldehyde	100-52-7	>25	2.3	443.1	1	2 of 2	>2000	0
Methyl methacrylate *	80-62-6	90	1.7	424.4	1	2 of 3	>2000	1
d-Limonene *	5989-27-5	52.7	1.2	>2000	0	0 of 2	82.3	0
Penicillin G *	61-33-6	30	10.7	1308.6	0	0 of 4	>2000	0
Butyl glycidyl ether	2426-08-6	28	107.1	59.4	1	3 of 3	729.2	1
Ethylene glycol dimethacrylate	97-90-5	28	188.4	57.4	1	2 of 2	1655.8	1
Methylhexanedione *	13706-86-0	25.8	23.4	49.8	1	2 of 2	1431.9	0
Imidazolidinyl urea	39236-46-9	24	2.9	45.4	1	3 of 4	90.4	1
Hydroxycitronellal	107-75-5	23	137.1	79.4	1	2 of 2	>2000	0
Cyclamen aldehyde *	103-95-7	22.3	3.1	111.9	1	3 of 4	190.8	0
Cinnamyl alcohol	104-54-1	21	1.7	123.6	1	4 of 4	774.6	0
Geraniol *	106-24-1	21.74	2.0	209.8	1	2 of 2	722.0	0
Butyl acrylate *	141-32-2	~20	6.9	37.7	1	2 of 2	200.9	1
Estragole *	140-67-0	20.2	1.3	>2000	0	0 of 2	419.0	0
2,4-Dichloronitrobenzene	611-06-3	20	2.9	68.3	1	4 of 4	816.0	1
Lilial *	80-54-6	18.7	1.1	>2000	0	0 of 2	94.5	0
Benzyl cinnamate	103-41-3	18.4	8.7	11.0	1	2 of 2	>2000	1
Phenyl benzoate	93-99-2	17.1	1.3	>2000	0	1 of 4	191.6	1
Lyral HMPCC	31906-04-4	17.1	16.1	79.6	1	2 of 2	355.4	0
Abietic acid	514-10-3	14.7	11.4	16.6	1	2 of 2	104.6	0
Eugenol	97-53-0	12.9	1.3	>2000	0	0 of 4	1505.7	1
alpha-Hexylcinnamaldehyde	101-86-0	12	2.7	17.3	1	2 of 2	26.3	0
Amylcinnamic aldehyde *	122-40-7	11.5	1.56	14.4	1	3 of 6	46.8	1
Bromohexane *	111-25-1	10	2.0	128.1	1	2 of 2	391.9	1
Citral	5392-40-5	9.2	96.4	23.2	1	2 of 2	182.8	1
Methylanisylidene acetone *	104-27-8	9.3	835.8	14.8	1	2 of 2	159.3	1
Dihydroeugenol	2785-87-7	6.8	1.54	462.0	1	2 of 2	759.2	0
Phenylpropionaldehyde *	93-53-8	6.3	9.1	64.8	1	2 of 2	195.1	0
Resorcinol	108-46-3	5.92	1.0	>2000	0	0 of 2	>2000	0
Creosol *	93-51-6	5.8	1.0	>2000	0	0 of 3	>2000	0
3,4-dihydrocoumarin *	119-84-6	5.6	1.0	>2000	0	0 of 2	>2000	1
Farnesol *	4602-84-0	5.5	1.6	13.0	1	2 of 2	23.3	0
trans-2-Hexenal *	6728-26-3	5.5	85.4	83.4	1	4 of 4	802.8	1
Tetramethylthiuram disulfide	137-26-8	5.2	6.8	0.8	1	2 of 2	39.1	1
Phenylacetaldehyde	122-78-1	3 / 4.7	11.3	28.5	1	2 of 2	116.2	0

Table 1(continued). **Results in the KeratinoSens assay for 114 chemicals selected based on a WoE assessment**

Name	CAS-Number	LLNA EC3	KeratinoSens result					Adduct peptide reactivity
			I_{max}	EC 1.5	Pos / Neg	reps. Positive	IC50	
Sensitizers (continued)								
Benzylidene Acetone	122-57-6	3.7	503.9	9.7	1	2 of 2	174.5	1
Propylidene phthalide *	17369-59-4	3.7	1.1	>2000	0	0 of 2	717.4	1
Thioglycerol	96-27-5	3.55	1.36	>2000	0	1 of 6	>2000	1
Diethylenetriamine	111-40-0	3.28	1.49	>1000	0	2 of 6	>2000	0
3-Aminophenol *	591-27-5	3.2	1.4	>2000	0	1 of 6	>2000	0
Cinnamic aldehyde	104-55-2	3.1	16.2	16.1	1	4 of 4	194.4	1
Benzyl salicylate	118-58-1	2.9	5.5	8.4	1	2 of 2	111.0	0
Methyl-2-nonynoate	111-80-8	2.5	33.1	1.8	1	2 of 2	121.9	1
1,2-Benzisothiazolin-3-one	2634-33-5	2.3	24.0	3.2	1	2 of 2	50.9	1
Ethylenediammine	107-15-3	2.2	13.2	99.9	1	4 of 4	>2000	0
3-Dimethyl-amino-1-propylamine *	109-55-7	2.2	30.2	85.8	1	4 of 4	1337.9	0
Diethylmaleate *	141-05-9	2.1	60.7	9.4	1	4 of 4	361.1	1
Methylisothiazolinone *	2682-20-4	1.9	22.6	11.8	1	4 of 4	139.0	1
Isoeugenol	97-54-1	1.8	6.4	16.1	1	4 of 4	731.4	1
2-Mercaptobenzothiazol	149-30-4	1.7	8.8	48.1	1	4 of 4	1003.1	1
Trimellitic anhydride *	552-30-7	1.42	1.1	>2000	0	0 of 2	>2000	0
Glyoxal	107-22-2	1.4	28.2	89.1	1	4 of 4	677.9	1
2-Hydroxy-ethyl-acrylate	818-61-1	1.4	54.9	32.3	1	2 of 2	207.2	1
Methyldibromo glutaronitrile	35691-65-7	0.9	4.0	7.8	1	2 of 2	25.6	1
Metol	55-55-0	0.78	10.3	2.2	1	6 of 6	18.6	1
Formaldehyde	50-00-0	0.7	16.9	63.2	1	2 of 2	201.6	1
1,3-phenylenediamine *	108-45-2	0.49	2.5	82.5	1	2 of 2	>2000	0
N,N-dimethyl-4-nitrosoaniline *	138-89-6	0.48	8.2	0.5	1	2 of 2	15.1	1
Methyl 2-octynoate *	111-12-6	0.45	46.6	2.5	1	2 of 2	87.6	1
2-amino-phenol *	95-55-6	0.4	13.1	1.1	1	2 of 2	138.2	1
Chloramine T *	127-65-1	0.4	50.2	248.4	1	2 of 2	404.7	1
Propyl gallate *	121-79-9	0.32	8.2	199.8	1	2 of 2	650.3	1
Phthalic anhydride	85-44-9	0.16	1.3	>2000	0	0 of 2	>2000	1
1,4-Phenylenediamine	106-50-3	0.16	26.8	5.0	1	2 of 2	438.9	1
Toluene 2,4-diisocyanate *	584-84-9	0.11	4.6	135.0	1	4 of 4	359.0	1
1,4-Hydrochinone *	123-31-9	0.1	16.4	9.8	1	2 of 2	130.7	1
Glutaraldehyde	111-30-8	0.1	80.7	24.3	1	2 of 2	242.6	1
4-nitrobenzylbromide	100-11-8	0.05	6.9	1.3	1	2 of 2	9.1	1
2,4,6-Trinitrochlorobenzene *	88-88-0	0.05	1.6	121.3	1	2 of 2	616.8	1
2,4-Dinitrothiocyanatobenzene *	1594-56-5	0.047	7.2	2.1	1	2 of 2	6.4	1
1-Chloro-2,4-dinitrobenzene	97-00-7	0.04	14.8	2.5	1	2 of 2	8.2	1
Tetrachlorsalicylanilide *	1154-59-2	0.04	4.9	<0.98	1	4 of 4	9.15	0
p-Benzoquinone	106-51-4	0.01	15.2	6.5	1	4 of 4	104.5	1
5-chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.009	7.2	8.7	1	2 of 2	7.1	1
Benzoyl peroxide	94-36-0	0.004	1.4	>2000	0	0 of 2	567.6	1
Oxazolone	15646-46-5	0.003	2.4	175.5	1	4 of 4	1370.9	1

[1)], level not specified in ICCVAM List
[2)] NC, not calculated, maximal level not given
[3)] EC3 not specified, Positive ICCVAM validation paper
* chemicals marked with a star have not been tested previously and were not included in the Silver list

Table 2. Cooper statistics for the extended set of 114 chemicals

	KeratinoSens	**Adduct formation / direct peptide reactivity**	**WoE [1]: KeratinoSens and/or adduct forming positive**
Correct positives	66	52	73
False-positives	5	1	6
Correct-negatives	23	27	22
False-negatives	20	34	13
N	114	114	114
Sensitivity	76.7%	60.5%	84.9%
Specificity	82.1%	96.4%	78.6%
Accuracy	78.1%	69.3%	83.3%

[1] Chemicals are rated positive if they either are positive in the KeratinoSens assay or form an adduct

4. Discussion

4.1. Applicability domain of correctly classified substances

The list contains altogether 85 sensitizers, and the sensitivity of the KeratinoSens assay alone is 76.1% for these chemicals. This figure is somewhat below the number reported for the Silver list before, and this is mainly due to the inclusion of more pro-haptens and a relatively larger number of Acyl-transfer agents as decribed below. Nevertheless, this list covers chemicals in a broad range of different mechanistic applicability domains, which were correctly classified: Metals, S_N2 and S_NAr-reactive chemicals, Michael acceptors, aldehydes, diketones, thiols, epoxides, aromatic and aliphatic amines, catechols, (hydro)quinones and gallates, isocyanates and miscellaneous agents.

4.2. Correctly classified putative pre- and prohaptens

The list of chemicals classified correctly by the KeratinoSens assay also contains a number of chemicals which are listed as putative pro- and prehaptens in the publication of Kern et. al. (Kern et al.):

- Geraniol 106-24-1
- Dihydroeugenol 2785-87-7
- Ethylenediammine, 107-15-3
- 1,4-Hydrochinone, 123-31-9
- 3-Dimethyl-amino-1-propylamine, 109-55-7
- Abietic acid, 514-10-3
- 2-amino-phenol, 95-55-6
- 1,4-Phenylenediamine, 106-50-3
- Metol, 55-55-0
- Methyldibromo glutaronitrile, 35691-65-7
- 1,3-phenylenediamine, 108-45-2
- Cinnamyl alcohol, 104-54-1

4.3. Specificity

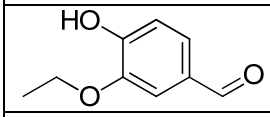
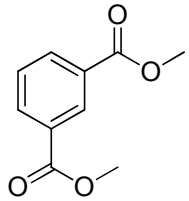
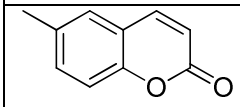
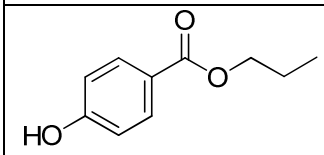
A clear limitation of the selection criteria applied to create the current list is the fact that relatively few non-sensitizers with congruent data from multiple sources are to be found in the published databases and reference lists. Therefore the list contains, as compared to the previous ‘Silver list’, only four

additional non-sensitizers. It should however be noted, that Attachment 4b contains data on a few additional non-sensitizers (streptomycin sulphate, Xylene and Clofibrate), and Attachment 12d contains an assessment of a number of surfactants considered non-sensitzers based on a WoE analysis.

4.4. False-positives in the KeratinoSens assay

Table 3 lists the chemicals which are false-positive along with their structure and a discussion for the individual chemicals.

Table 3. False-positives in the KeratinoSens assay

Structure	Name	Discussion
	Ethyl Vanillin	Clear false-positive, little evidence for sensitization by Ethyl by vanillin in the literature
Not defined	Tween 80	Clear false-positive, scattered evidence for sensitization by Tween 80 from patch testing and human tests.
	Dimethyliso-phtalate	Variable false-positive, positive in 5 of 8 repetitions, with an average EC1.5 of 778 µM, close to threshold of 1000 µM.
	6-methyl coumarin	Generally considered a non-sensitizer with photosensitization potential. However, the ICCVAM database reports a low sensitization threshold in a human maximisation test of 226 µg/cm^2 although the original reference could not be found
	Propylparaben	Nonsensitizer in LLNA and GP. ICCVAM validation paper lists a +/- for HMT and it is positive with low frequency in human patch tests. May be considered a very weak sensitizer. In the skin it is rapidly hydrolyzed to the non-sensitizing 4-HBA, and this detoxification process might be slower in the cell-culture.

4.5. False-negatives in the KeratinoSens assay

Tables 4a-c list all the false-negative chemicals, grouped according to structural features

Table 4a lists some of the chemicals which are positive in the direct binding assay (covalent adduct formation observed by LC-MS) and negative in KeratinoSens. These chemicals belong to the Acyl-transfer agents: They have an ester bond with a good-leaving group (in most cases a phenyl-group), and the transfer of the Acyl-group in most cases can be verified in the LC-MS peptide binding assay. This group of chemicals (with the exception of Benzoyl peroxide, which is unclear) have a selective reactivity with amine groups, and given the established molecular mechanism of Nrf2 activation by thiol-modifying compounds (Wakabayashi et al., 2004), it appears logical that they therefore lack the ability to activate Nrf2. Although this group is relatively small, it illustrates the need to perform peptide binding in parallel to KeratinoSens to maximize sensitivity as discussed before (Natsch, 2010). This group of chemicals is mainly responsible for improved accuracy of the combined tests in Table 2.

Table 4a: Acyl-transfer agents negative in the KeratinoSens assay, but positive in direct peptide binding assay

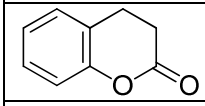
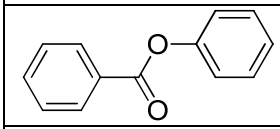
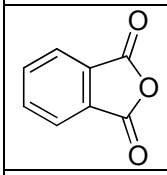
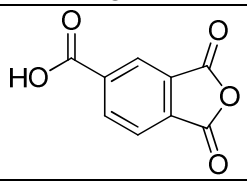
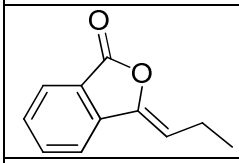
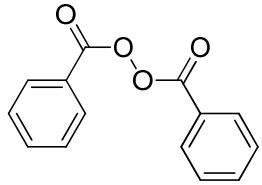
Structure	Name	Discussion
	3,4-dihydrocoumarin	Specific reaction with Lysine, adduct formation in LC-MS assay, also in (Aleksic et al., 2009)
	Phenyl benzoate	Reaction with Lysine, adduct formation in LC-MS assay, also in (Aleksic et al., 2009)
	Phtalic anhydride	Selective reaction with Lysine, adduct formation in LC-MS assay, specific reaction with Lysine also reported in DPRA (Gerberick et al., 2007)
	Trimellitic anhydride	Reaction with Lysine, but variable results in DPRA, considered labile to hydrolysis (Mitjans et al., 2008), and difficult to test in presence of water
	Propyliden-phthalide	Adduct formation in LC-MS assay specific with Lys, also considered to form part of Acyl-group transfer agents in (Roberts et al., 2007)
	Benzoyl peroxide	Adduct formation in LC-MS assay, reaction with Lysine and Cysteine reported in DPRA (Gerberick et al., 2007), specific reaction with Lys less clear in this case

Table 4 b lists a group of compounds, which generally are considered as prohaptens (Kern et al.). Whereas the KeratinoSens detects a number of putative prohaptens (see above), this group of chemicals overall is negative both in the KeratinoSens and in the LC-MS direct binding assay. One borderline case is Eugenol – it forms traces of covalent adducts in the LC-MS assay and thus is considered positive and it was positive in KeratinoSens in 2 of 5 labs in the ring-study.

Table 4b: Putative prohaptens negative in the KeratinoSens assay

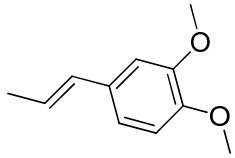
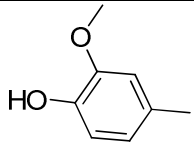
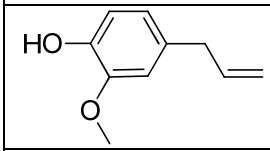
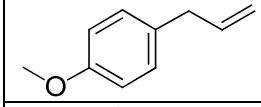
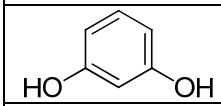
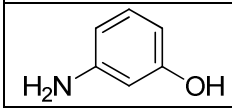
Structure	Name
	O-methyl-isoeugenol
	Creosol
	Eugenol
	Estragole
	Resorcinol
	3-Aminophenol

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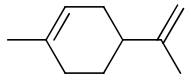
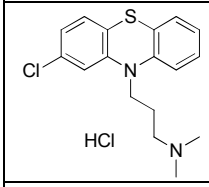
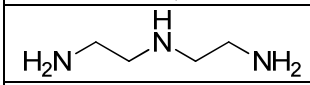
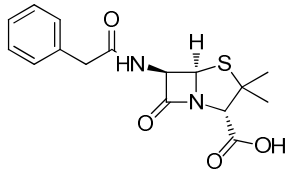
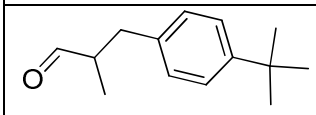
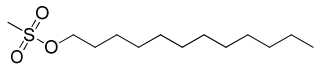
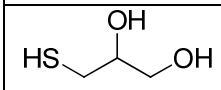
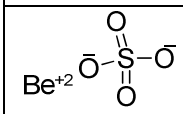
Table 4 c lists a miscellaneous group of false-negative compounds. Some of these compounds activate the luciferase gene, but only (i) after a photo- or oxidative- activation step or (ii) only at cytotoxic levels or (iii) beyond the thresholds set in the prediction model as discussed in detail in the Table.

Table 4c. Miscellaneous false-negatives

Structure	Name	Discussion
	Limonene	Probably sensitizer, but clearly *in vivo* sensitizer after air oxidation. As discussed in Attachment 4c, this chemically clearly becomes positive in the KeratinoSens assay after air oxidation, without forced oxidation the overall result is negative.
	Chlorpromazine hydrochloride	Sensitizer and esp. photosensitizer. As discussed in Attachment 4c, this chemical becomes positive in the KeratinoSens assay after a photoactivation step by UVA irradiation.
	Diethylene-triamine	Significant gene induction above the threshold of 1000 µM in few repetitions
	Penicillin G	Borderline case: Reproducible gene activation in 4/4 repetitions at 2000 µM only and at non-cytotoxic concentrations. Considered negative according to the prediction model which uses a threshold of 1000 µM
	Lilial	Many sensitizing aldehydes are positive in KeratinoSens, in the case of Lilial the result is clearly negative, could be due to relatively high cytotoxicity
	Lauryl methanesulfonate	Reproducible gene activation but at cytotoxic concentrations only. Very high cytotoxicity of this surfactant-like molecule could hamper detection. It is noteworthy, that the sensitizer Methyl-methanesulfonate acting by similar mechanism, but having low cytotoxicity, is positive.
	1-thioglycerol	Adduct forming by mixed disulfide formation, would be expected to be thiol-reactive, rapid metabolism in Keratinocytes might be an explanation.
	Beryllium sulfate	Positive at cytotoxic concentrations only. This is the only non-transition metal which is sensitizing, unusual case, and we do not understand mechanism. As discussed in attachment 4b it gives unusual cytotoxicity dose-response.

5. References

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6. Appendix

Below in Tables A – D the test chemicals and their *in vivo* data are listed.

Table A. Chemicals with congruent LLNA and Guinea pig data selected from the ICCVAM validation paper

			LLNA data		ICCVAM validation paper				ICCVAM database		
Name	**CAS-Number**	**WoE Sensiti-sation**	**LLNA EC3[4)]**	**LLNA class**	**LLNA**	**GP**	**HMT**	**HPT**	**Geometric Mean EC3 LLNA (%)**	**GPMT Overall Potency Category**	**BT Overall Potency Category**
Isoeugenol	97-54-1	+	1.8	Moderate	+	+		+	1.61	CAT 1	
Creosol	93-51-6	+	5.8	Moderate	+	+					
1,2-Benzisothiazolin-3-one	2634-33-5	+	2.3	Moderate	+	+		+	7.79		
o-methylisoeugenol	93-16-3	+			+	nonstd+					
3,4-dihydrocoumarin	119-84-6	+ [1)]	5.6	Moderate	+				4.27	CAT 2	
Cinnamic aldehyde	104-55-2	+	3.1	Moderate	+	+	+	+	1.28	CAT 1	CAT 1
Geraniol	106-24-1	+ [2)]	21.7	Weak	-	-	-	+	21.74	CAT 1	
2-hydroxypropyl methacrylate	923-26-2	-	>50	V.weak/none	-	-		+	NC	NOT CLASS	
Dihydroeugenol	2785-87-7	+	6.8	Moderate	+	+					
2,4-Dinitrothiocyanatobenzene	1594-56-5	+	0.047	Extreme	+	+					
Hydroxycitronellal	107-75-5	+	23	Weak	+	+	+	+	22.97	CAT 1	CAT 2
Propyl gallate	121-79-9	+	0.32	Strong	+	+		+	0.32	CAT 1	
4-hydroxybenzoic acid	99-96-7	-	>25	V.weak/none	-	-			NC	CAT 2	
Ethylenediammine	107-15-3	+	2.2	moderate	+	+		+	2.20	CAT 1	CAT 1
Lactic acid	50-21-5	-	>25	V.weak/none	-	-					
Phenyl benzoate	93-99-2	+	17.1	Weak	+	+		+	9.46		
Tween 80	9005-65-6	-		V.weak/none	-	-		+	NC	NOT CLASS	NOT CLASS
1,4-Hydrochinone	123-31-9	+	0.1	Strong	+	+		+	0.13	CAT 2	
4-Methylcatechol	452-86-8	+			+	+					
Glyoxal	107-22-2	+	1.4	moderate	+	+	+		0.75		
Salicylic acid	69-72-7	-	>25	V.weak/none	-	-	-		12.22	NOT CLASS	
Chlorobenzene	108-90-7	-	>25	V.weak/none	-	-					
3-Dimethyl-amino-1-propylamine	109-55-7	+	2.2	Moderate	+	+					
Phthalic anhydride	85-44-9	+	0.16	Strong	+	+			0.36	CAT 1	CAT 1
Butyl glycidyl ether	2426-08-6	+	31	weak	+	+	+		30.95	CAT 2	
Tartaric acid	87-69-4	-	NC (> 25%)	V.weak/none	-	nonstd-			NC		
Formaldehyde	50-00-0	+	0.7	Strong	+	+	+	+	0.84	CAT 1	CAT 1
Bromohexane	111-25-1	+			+	nonstd+					
2-Mercaptobenzothiazol	149-30-4	+	1.7	Moderate	+	+	+	+	3.71	CAT 1	CAT 2
1-Chloro-2,4-dinitrobenzene	97-00-7	+	0.04	Extreme	+	+			0.05	CAT 1	CAT 1
Sulphanilamide	63-74-1	-	NC	V.weak/none	-	-	+	+			
p-Benzoquinone	106-51-4	+	0.01	Extreme	+	+			0.01	CAT 1	
4-Aminobenzoic acid	150-13-0	-	>10	V.weak/none	-	-	-	+	NC	CAT 2	
Tetramethylthiuram disulfide	137-26-8	+	5.2	Moderate	+	nonstd+	+	+	3.07		
1-Bromododecane	143-15-7	+			+	nonstd+					
Abietic acid	514-10-3	+	14.7	Weak	+	+		+	11.64	CAT 2	NOT CLASS
Diethylenetriamine	111-40-0	+	3.28	moderate	+	+	+	+	3.28		
4-nitrobenzylbromide	100-11-8	+	0.05	strong	+	nonstd+					
Benzoyl peroxide	94-36-0	+	0.004	extreme	+	+		+	0.22		CAT 2

Table A (continued): Chemicals with congruent LLNA and Guinea pig data selected from the ICCVAM validation paper

			LLNA data		ICCVAM validation paper				ICCVAM database		
Name	CAS-Number	WoE Sensiti-sation	LLNA EC3	LLNA class	LLNA	GP	HMT	HPT	Geometric Mean EC3 LLNA (%)	GPMT Overall Potency Category	BT Overall Potency Category
Cobalt chloride	7646-79-9	+			+	+	+	+			
Citral	5392-40-5	+	13 / 6.3 / 4.6-5.3	Moderate	+	+	+		9.16	CAT 2	
Diethyl phthalate	84-66-2	- [1)]	>100	V.weak/none	-				NC	NOT CLASS	
Estragole	140-67-0	+	20.2	Weak	+	+					
Eugenol	97-53-0	+	12.9	Weak	+	+		+	10.97	CAT 1	NOT CLASS
alpha-Hexylcinnamaldehyde	101-86-0	+	11.97 / 12	Weak	+	+			6.31	CAT 1	CAT 1
Isopropanol	67-63-0	-	>50	V.weak/none	-	-			NC	NOT CLASS	
Methyl salicylate	119-36-8	-	>20	V.weak/none	-	-	-		16.96	NOT CLASS	
Propylene glycol	57-55-6	-	>100	V.weak/none	-	-		+	NC	NOT CLASS	
6-methyl coumarin	92-48-8	-	>25	V.weak/none	-	-	-	+	NC		
Propylparaben	94-13-3	-	>50	V.weak/none	-	-	+/-	+	NC	NOT CLASS	
2-amino-phenol	95-55-6	+	0.4	Strong	+	nonstd+					
Oxazolone	15646-46-5	+	0.003	Extreme	+	+			0.003		CAT 1
5-chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	+	0.009	Extreme	+	+		+	0.010	CAT 1	CAT 1
1,4-Phenylenediamine	106-50-3	+	0.16	Strong	+	+	+		0.11	CAT 1	CAT 1
Metol	55-55-0	+	0.78	strong	+	+		+	0.80	CAT 1	
2-Hydroxy-ethyl-acrylate	818-61-1	+	1.4	Moderate	+	+		+	1.40	CAT 1	
3-Aminophenol	591-27-5	+	3.2	Moderate	+	nonstd+		+	0.88	CAT 1	
Methyldibromo glutaronitrile	35691-65-7	+	0.9	strong	+	+		+	1.92	NOT CLASS	NOT CLASS
Trimellitic anhydride	552-30-7	+		moderate	+	+			1.42	CAT 1	CAT 2
Penicillin G	61-33-6	+	30	weak	+	+	+		24.16	CAT 1	
Imidazolidinyl urea	39236-46-9	+	24	weak	+	+		+	25.17	CAT 2	
Benzalkonium chloride		-		non-sensitizer,	-	-		+	0.07	NOT CLASS	
Tetrachlorsalicylanilide	1154-59-2	+	0.04	extreme	+	+	+	+			
Thioglycerol	96-27-5	+	3.55	moderate	+	+	+		3.55	CAT 2	CAT 1
Dextran	9004-54-0	-		V.weak/none	-	-			NC	NOT CLASS	
Dimethyl isophthalate	1459-93-4	-		V.weak/none	-	-					
2,4-Dichloronitrobenzene	611-06-3	+ [3)]	20	weak	-	-					
N,N-dimethyl-4-nitrosoaniline	138-89-6	+	0.48	strong	+	+					
1,3-phenylenediamine	108-45-2	+			+	nonstd+		+			
2,4,6-Trinitrochlorobenzene	88-88-0	+	0.05	Extreme	+	+					
Chloramine T	127-65-1	+	0.4	strong	+	+		+			
Diphenylmethane-4,4'-diisocyanate	101-68-8	+			+	+		+			
Dodecyl methanesulfonate	51323-71-8	+			+	nonstd+					
4-Nitrobenzyl chloride	100-14-1	+			+	nonstd+					
Chlorpromazine hydrochloride	69-09-0	+			+	nonstd+	+				
Beryllium sulfate	7787-56-6	+			+	+	+				

[1)] GP data taken ICCVAM database, [2)] GP and LLNA data from newer ICCVAM database

[3)] Reported positive in LLNA and GPMT after ICCVAM validation, Basketter, Contact Dermatitis, 34(1),55-58,1996

[4)] LLNA result listed in our earlier publications and in the publication by Gerberick et. al., Dermatitis, 16 (4),2005, 157-202. The ICCVAM database for some chemicals lists several LLNA studies and reports the geometric mean of those ("Column Geometric Mean EC3 LLNA").

Table B. Chemicals with congruent LLNA and Guinea pig data, retrieved from the ICCVAM database, which were not included in the ICCVAM validation paper

Name	CAS-Number	WoE Sensiti-sation	ICCVAM database		
			Geometric Mean EC3 LLNA (%)	GPMT Overall Potency Category	BT Overall Potency Category
Amylcinnamic aldehyde	122-40-7	+	11.5	NOT CLASS	CAT 2
Benzoic acid	65-85-0	-	NC	NOT CLASS	NOT CLASS
Benzyl alcohol	100-51-6	-	NC	NOT CLASS	
Benzyl cinnamate	103-41-3	+	18.4	CAT 2	
Butyl acrylate	141-32-2	+	16.4	CAT 2	
Cinnamyl alcohol	104-54-1	+	20.8	NOT CLASS	CAT 2
Fumaric acid	110-17-8	-	NC	NOT CLASS	
Glutaraldehyde	111-30-8	+	0.12		CAT 1
Glycerol	56-81-5	-	NC	NOT CLASS	NOT CLASS
d-Limonene	5989-27-5	+	52.7	CAT 2	
Methyl dodecanesulfonate	2374-65-4	+	0.4	CAT 1	
Methyl methacrylate	80-62-6	+	73.5	CAT 2	CAT 2
Toluene 2,4-diisocyanate	584-84-9	+	0.1	CAT 1	CAT 1

Table C. Chemicals with congruent LLNA and human maximisation test results, retrieved from the ICCVAM database, which were not included in the ICCVAM validation paper

Name	CAS-Number	WoE Sensiti-sation	ICCVAM database			
			Geometric Mean EC3 LLNA (%)	Geometric Mean HMT Threshold (ug/cm^2)	Geometric Mean HRIPT Threshold (ug/cm^2)	Geometric Mean Threshold data (ug/cm^2)
Benzylidene Acetone	122-57-6	+	2.2	21	131	63
Cyclamen aldehyde	103-95-7	+	22.1		472	472
Diethylmaleate	141-05-9	+	3.1	15	88	57
Farnesol	4602-84-0	+	4.8	690	2755	1378
trans-2-Hexenal	6728-26-3	+	5.5	24	24	23.8
Lilial	80-54-6	+	15.0	2953	1244	1916
Lyral HMPCC	31906-04-4	+	17.1		1818	1818
Methylanisylidene acetone	104-27-8	+	8.5		41.2	41.2
Methylhexanedione	13706-86-0	+	26.0	3448		3448
Methylisothiazolinone	2682-20-4	+	1.2		1.5	1.5
Methyl-2-nonynoate	111-80-8	+	2.5	12	2	5
Methyl 2-octynoate	111-12-6	+	NC	19.4	118	35
Phenylacetaldehyde	122-78-1	+	4.5	118	58	67
Phenylpropionaldehyde	93-53-8	+	6.3		69.2	69
Propylidene phthalide	17369-59-4	+	3.1		115	115

Table D. Chemicals which had been previously included in the 'Silver list' and which are not in the Tables A–C

			LLNA data		ICCVAM database	
Name	**CAS-Number**	**WoE Sensiti-sation**	**LLNA EC3 (%)**	**LLNA class**	**GPMT Overall Potency Category**	**BT Overall Potency Category**
Benzyl salicylate	118-58-1	+	2.9	Moderate	NOT CLASS	
Resorcinol	108-46-3	+	5.92	moderate		
Benzocaine	94-09-7	+	>50	V.weak/none	CAT 2	CAT 2
1-Butanol	71-36-3	-	>20	V.weak/none		
Ethyl vanillin	121-32-4	-	NC (> 50%)	V.weak/none		
Sulfanilic acid	121-57-3	- [1)]	>25	V.weak/none	CAT 1	
Sodium lauryl sulfate	151-21-3	- [2)]	14	non-sens.	NOT CLASS	NOT CLASS
Octanoic acid	124-07-2	-	>50	V.weak/none		
Benzaldehyde	100-52-7	+ [3)]	>25	V.weak/none		
Phenol	108-95-2	- [4)]		V.weak/none		
Ethylene glycol dimethacrylate	97-90-5	+ [5)]	28	weak	NOT CLASS	

[1)] Considered False-positive in GPMT, Basketter and Kimber, Contact Dermatitis 56,1-4, 2007
[2)] False-positive in LLNA, WoE negative, Basketter and Kimber, Contact Dermatitis 56,1-4, 2007
[3)] Negative in LLNA, clearly sensitizing based on recent HRIPT test (Research Institute for Fragrance materials, RIFM)
[4)] Negative reference according D. Basketter, 1999, Food Chem. Toxicol. 37, 1167-1174
[5)] Included as optional substance in ICCVAM performance standards

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