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Ability of non-animal methods for skin sensitisation to detect pre- and pro-haptens

Report and Recommendations of an EURL ECVAM Expert Meeting

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Abstract

Significant progress has been made in the development, validation and regulatory acceptance of *in chemico* and *in vitro* test methods for skin sensitisation. Although these methods have been shown to perform relatively well (about 80% accuracy in predicting Local Lymph Node Assay (LLNA) classifications) a concern was raised on the regulatory acceptability of negative results since it was questioned whether these methods are able to predict chemicals that need to be activated to act as sensitisers.

In order to inform ongoing discussions at the regulatory level in the EU, EURL ECVAM held an expert meeting on 10-11 November 2015 to analyse the extent to which *in chemical* and *in vitro* methods are able to correctly identify chemicals that need to be activated either through abiotic activation (pre-haptens) and/or through biotic (enzyme-mediated) mechanisms (pro-haptens) to acquire skin sensitisation potential.

The expert group analysed a list of 127 chemicals, with available LLNA and *in vitro* data, 22% of which were considered to be pre- and/or pro-haptens. The pre-haptens, constituting the vast majority of chemicals requiring activation, were mostly correctly identified by both the *in chemico* and *in vitro* assays whereas the pro-haptens which represent a small subset of sensitising chemicals, were identified correctly by at least one of the cell-based assays.

As a result, the expert group recommended that negative *in vitro* data should be accepted unless there is a compelling scientific argument that a substance is likely to be an exclusively metabolically activated pro-hapten.

1. Introduction

In the last few decades it has become more obvious that a substantial minority of substances causing contact allergy are not sensitisers themselves, but need to be activated to become sensitising. We name them pre-haptens and pro-haptens (1).

Pre-haptens are activated abiotically outside the skin mainly by autoxidation while pro-haptens are activated in the skin. Pro-haptens are most often judged to be activated biotically by metabolic mechanisms, although abiotic activation (*e.g.* oxidation) can also take place in the skin. In addition, many pre-haptens can act as pro-haptens and this potential should be considered as and when a pre-hapten has been identified. The same haptens can be formed from both activation pathways even though the mechanisms may be different. Activation of pre-haptens most often occurs via a radical pathway with the formation of highly sensitising hydroperoxides as the primary oxidation products. Some of these hydroperoxides are unstable; often they cannot be detected and are only identified as a result of secondary oxidation products being formed (2; 3). The secondary oxidation products can also be sensitisers and have for some substances (*e.g.* geraniol (4; 5) and alpha-terpinene (6; 7) been shown to be identical with those formed via the metabolic pathway. However, it is important to remember that not all oxidation processes lead to sensitisation.

Although the picture of activation via abiotic and biotic ways may appear quite complex, *in silico* tools such as TIMES-SS (http://oasis-lmc.org/products/models/human-health-endpoints/skin-sensitization.aspx) (8; 9; 10) or the OECD Toolbox (http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm) (11), which are underpinned by adequate experimental data and valid (Q)SARs, may be helpful in detecting and discriminating between alternative activation routes. With regard to the *in vitro* methods, the complexity of the problem must be first considered so that the skin sensitisation potential of a new substance will be correctly identified.

Besides the *in silico* approaches ((Q)SARs, expert systems and read-across, (12) currently available for skin sensitisation, significant developments have been made in the development and validation of *in chemico* and *in vitro* test methods (13; 14; 15). Two of these methods, the direct peptide reactivity assay (DPRA) and the KeratinoSens™, have been adopted by the OECD in 2015 as TG 442C (16) and TG442D (17) respectively. A third *in vitro* method, namely the human Cell Line Activation Test (h-CLAT), is in the final stages of the OECD adoption process at the time of writing of this report.

Although these methods have been shown to perform relatively well in predicting LLNA results (accuracy about 80%) they are proposed to be used in combination with other information for assessing the skin sensitisation potential of chemicals. One explanation put forward for this is that the validated methods are only addressing parts of the complex biological mechanisms that lead to the acquisition of skin sensitisation. One of the concerns commonly raised on the suitability of the results generated with these non-animal test methods is related to their ability to predict chemicals that need to be activated to act as sensitisers. The DPRA for example does not have a metabolic system and the two *in vitro* methods make use of cells that are not fully representing the *in vivo* metabolic situation.

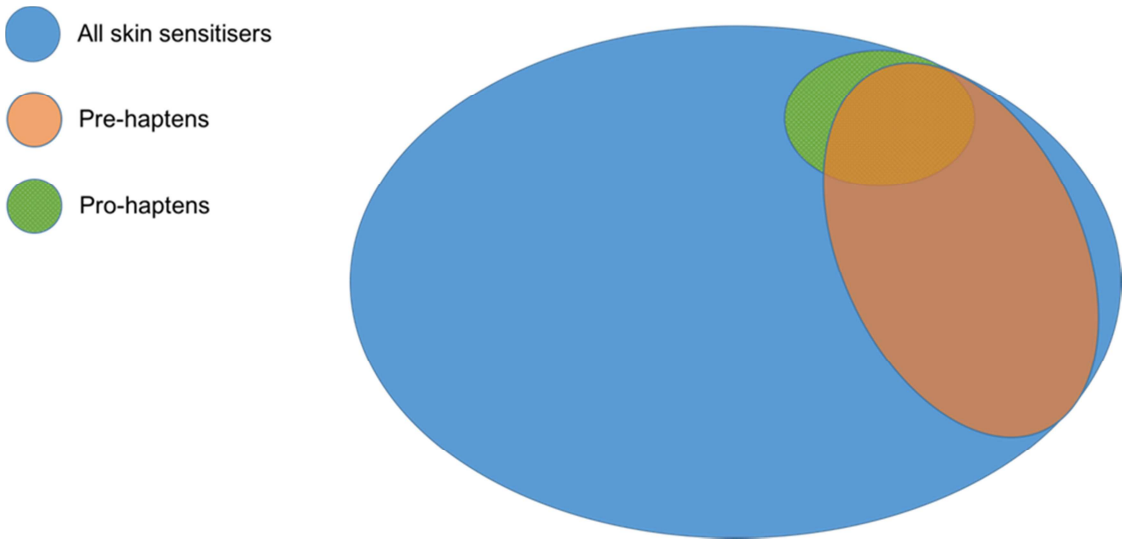
In order to inform ongoing discussion at the regulatory level in the EU and specifically the revision of the ECHA guidance on Information Requirements and Chemical Safety Assessment (Chapter R.7a: endpoint specific guidance) (12), the expert group considered the general topic of skin sensitisers which are known not to be directly acting substances, *i.e.* that require some type of "activation" before they can initiate key event 1 (KE1) in the skin sensitisation Adverse Outcome Pathway (AOP) (18; 19). The first key event in the published AOP for skin sensitisation is considered also to be the Molecular Initiating Event (MIE) and represents the covalent interaction with skin proteins.

Pre-haptens require abiotic activation (*e.g.* air oxidation, hydrolysis) to trigger KE1; pro-haptens require metabolic activation to trigger KE1, but abiotic activation (oxidation) also can take place in the skin. The group noted that for a proportion of non-direct acting haptens it remains uncertain whether they are pre- or pro- (or possibly pre- and pro-) haptens. It does appear that many of the substances previously suspected to be pro-haptens requiring metabolic activation are actually also pre-haptens (*e.g.* p-phenylenediamine (20; 21), geraniol (4), cinnamic alcohol (22; 23; 24)). Consequently, the possibility that a substance can act both as a pre-hapten and as pro-hapten should be considered.

2. Analysis of in vivo and in vitro data

An assessment of a published database of 319 LLNA results demonstrates that approximately 25% of sensitising chemicals were reported to be pre- or pro-haptens (25). A similar conclusion was reached by the expert group following review of an EURL ECVAM dataset of 127 substances for which LLNA and non-animal data, generated with DPRA, h-CLAT and Keratinosens™, was available (see table 1). Of the substances identified as sensitisers by the LLNA, 22% were considered to be pre- and/or pro-haptens. Unlike the published dataset from Kern et al. (25), a view was taken for this dataset on the distribution of pre- and pro-haptens (see Figure1).

Figure 1: Distribution of pre-haptens and/or pro-haptens among skin sensitisers



The majority of the group of non-direct acting haptens are pre-haptens, substances which generally were identified by the DPRA and the cell-based assays. However, the rate of oxidation varies and slow oxidisers may not be identified by these methods, just as they might fail to be detected by the *in vivo* assays depending on their purity at the time point for the experiment. It is therefore important that the possibility for a substance to be activated by oxidation is considered based on structure–activity relationship (SAR) analysis and if necessary autoxidation studies should be performed together with experimental sensitisation studies.

The expert group agreed that the number of substances that were exclusively pro-haptens in the EURL ECVAM dataset represented a small subset of the overall category of classifiable skin sensitisers. This was also consistent with the assessment by Urbisch and colleagues (26).

The data analysis suggested that this pro-hapten subset (n=5) of which only one was identified by the DPRA, were all identified correctly by h-CLAT and two were correctly identified by Keratinosens™ (see Table 2). Thus, when using *in vitro* methods, an approach might be first to conduct an assessment of KE1 (*e.g.* DPRA) and if this proved negative, then in the absence of other data to support an argument that it is non-sensitising, there should be a follow up with a cell-based assay. If both tests were negative, the substance would not be regarded as sensitising.

The expert group agreed that all decisions relating to classification should be based on an assessment of the overall weight of evidence.

2.1 Table 1: List of chemicals with LLNA and non-animal data

#	Chemical name	SMILES	LLNA	Human[1]	DPRA	Keratino-Sens™	h-CLAT	Reaction Mechanistic Domain[2]
1	1,2,4-Benzenetricarboxylic anhydride (Trimellitic anhydride)	O=C(OC(=O)c1ccc(C(=O)O)c2)c12	1		1	0	1	Acyl[b]
2	1,2-Benzisothiazolin-3-one (Proxel active)	O=C(NSc1cccc2)c12	1	1	1	1	1	Special case SN2[b]
3	1,2-Dibromo-2,4-dicyanobutane (MDGN)	C(#N)C(Br)(CCC(#N))CBr	1	1	1	1	1	pre-MA, SN2[b]
4	1,4-Phenylenediamine	Nc(ccc(N)c1)c1	1	1	1	1	1	pre-MA[a,b]
5	1-Bromobutane	BrCCCC	0		1	0	1	SN2[a]
6	1-Bromohexane	CCCCCCBr	1		1	1	0	SN2[b]
7	1-Butanol	OCCCC	0	0	0	0	0	non-reactive

1 indicates a positive outcome, 0 indicates a negative outcome.

[1] Human data derived from Basketter et al. (27).

[2] Reaction mechanistic domains as defined by Aptula et al. (20): MA, Michael Acceptors; SN2, SN2 electrophiles; Acyl, acyl transfer agents; SNAr, SNAr electrophiles; SB, Schiff base formers. Substances that do not fall into one of these domains may be categorised as special cases to reflect other potential mechanisms such as SN1 or refinements to an existing reaction pathway e.g. MA via its keto-tautomer, N-nitroso derivatives which act as hard SN2 or pro-SN2 electrophiles. Assignment to a reaction domain does not automatically signify that a substance is a sensitiser, it may not be sufficiently reactive to be sensitising. Substances that exhibit no features indicative of reaction potential are denoted as non-reactive.
Reaction domain assignments are taken from references a = (20); b = (28); c = (29); d = (25).

8	1-Chloro-2,4-dinitrobenzene (Dinitrochlorobenzene, DNCB)	N(=O)(=O)c(ccc(c1N(=O)(=O))Cl)c1	1	1	1	1	1	SNAr[a]
9	1-Iodohexane	C(CCCCC)I	0		1	1	1	SN2[b]
10	1-Naphthol	Oc(c(c(ccc1)cc2)c1)c2	1		1	1	1	Special case pre-MA[b]
11	1-Phenyl-1,2-propanedione	O=C(c(cccc1)c1)C(=O)C	1		1	1	1	SB
12	2,3-Butanedione	O=C(C(=O)C)C	1		1	1	1	SB or MA[b]
13	2,4-Dichloronitrobenzene	C1=CC(=C(C=C1Cl)Cl)[N+](=O)[O-]	1		0	1	1	SNAr
14	2,4-Heptadienal	CCC=CC=CC=O	1		1	1	1	MA[b]
15	2,5-Diaminotoluene sulphate (PTD)	Nc1ccc(N)c(c1)C	1	1	1	1	1	pre-MA[b]
16	2-Acetyl-cyclohexanone	O=C(C(C(=O)CCC1)C1)C	0		1	1	1	SB but not reactive enough to sensitise[b]
17	2-Aminophenol	Oc(c(N)ccc1)c1	1	1	1	1	1	pre-MA[b]
18	2-Ethylhexyl acrylate	O=C(OCC(CCCC)CC)C=C	1		1	1	1	MA
19	2-Hydroxyethyl acrylate	O=C(OCCO)C=C	1	1	1	1	1	MA[b]
20	2-Hydroxypropyl methacrylate	O=C(OCC(O)C)C(=C)C	0		1	1	0	MA[a]
21	2-Mercaptobenzothiazole	N(c(c(S1)ccc2)c2)=C1S	1	1	1	1	1	Weakly reacting SN2, Acyl[b]

22	2-Methoxy-4-methylphenol	O(c(c(O)ccc1C)c1)C	1		0	0	1	pro/pre-MA[b]
23	2-Methyl-2H-Isothiazol-3-one	S1N(C)C(=O)C=C1	1	1	1	1	1	SN2
24	2-Nitro-1,4-phenylendiamine	O=N(=O)c(c(N)ccc1N)c1	1	1	1	1	1	pro/pre-MA[b]
25	2-Phenylpropionaldehyde	O=CC(c(cccc1)c1)C	1		1	1	1	SB
26	3.4-Dihydrocoumarin	O=C(Oc(c(ccc1)C2)c1)C2	1		1	0	1	Acyl[a]
27	3-Aminophenol	Oc(cccc1N)c1	1		0	0	1	pro-MA[b]
28	3-Dimethylamino propylamine	N(CCCN)(C)C	1	1	0	1	1	pre-SB[b]
29	3-Phenoxypropiononitrile	C(Oc1ccccc1)CC(#N)	0		1	0	1	non-reactive
30	3-Propylidenephthalide	O=C(OC(c1cccc2)=CCC)c12	1		1	0	1	Acyl[a] or pre
31	4-(4-Hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde (Lyral)	O=CC(CCC(=C1)CCCC(O)(C)C)C1	1	1	1	1	1	SB[b]
32	4-(N-Ethyl-N-2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3)	Nc1c(C)cc(N(CC)CCNS(=O)(=O)C)cc1	1		1	1	1	pre/pro-MA[b]
33	4-Allylanisole	O(c(ccc(c1)CC=C)c1)C	1		1	0	1	pro-MA[b]
34	4-Aminobenzoic acid	C1=CC(=CC=C1C(=O)O)N	0	0	0	0	0	non-reactive

35	4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)	N1=C(c2ccccc2)OC(=O)C1=COCC	1		1	1	1	Acyl[b]
36	4-Hydroxybenzoic acid	O=C(O)c(ccc(O)c1)c1	0		0	0	0	non-reactive
37	4-Methoxyacetophenone	COc1ccc(cc1)C(C)=O	0		0	1	0	SB but not reactive enough to sensitise
38	4-Nitrobenzyl bromide	O=N(=O)c(ccc(c1)CBr)c1	1		1	1	1	SN2[b]
39	5-Methyl-2,3-hexanedione	O=C(C(=O)CC(C)C)C	1	1	1	1	1	SB[a]
40	6-Methylcoumarin	c1c(C)cc2C=CC(=O)Oc2c1	0		0	1	0	MA[a] but not reactive enough to sensitise
41	α-Amyl cinnamic aldehyde	O=CC(=Cc(cccc1)c1)CCCCC	1	1	0	1	1	MA[b]
42	α-methyl-trans-cinnamaldehyde	O=CC(=Cc(cccc1)c1)C	1		1	1	1	MA[b]
43	Abietic acid	O=C(O)C(C(C(C(C(=C1)C=C(C2)C(C)C)C2)(CC3)C)C1)(C3)C	1	1	1	1	0	pre[c] due to formation of hydroperoxides
44	Aniline	Nc(cccc1)c1	1	1	0	0	1	pre/pro-MA or pseudo SB
45	Bandrowski's Base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine)	Nc1ccc(cc1)/N=C3/C=C(/N)C(=Nc2ccc(N)cc2)C=C3N	1		1	1	1	pro/pre-MA[d]
46	Benzaldehyde	O=Cc(cccc1)c1	0	0	0	1	1	SB but not reactive enough to sensitise[b]

47	Benzalkonium chloride	CCCCCCCCCCCCCC[N+](C)(C)c1ccccc1	0	0	0	0	0	non-reactive
48	Benzocaine	O=C(OCC)c(ccc(N)c1)c1	0	1	1	1	1	General aromatic amine pre/pro SB but free radical reactions are also possible
49	Benzoic acid	O=C(O)c(cccc1)c1	0		1	0	0	non-reactive
50	Benzoyl peroxide	O=C(OOC(=O)c(cccc1)c1)c(cccc2)c2	1	1	1	0	0	Special case – oxidising agent
51	Benzyl alcohol	C1=CC=C(C=C1)CO	0		0	0	1	non-reactive, pro-SN2 (but weak)
52	Benzyl benzoate	O=C(OCc(cccc1)c1)c(cccc2)c2	1	0	0	1	0	Acyl or SN2[b]
53	Benzyl bromide	BrCc(cccc1)c1	1		1	1	1	SN2[b]
54	Benzyl cinnamate	C1=CC=C(C=C1)COC(=O)/C=C/C2=CC=CC=C2	1		0	1	0	MA[d]
55	Benzyl salicylate	C1=CC=C(C=C1)COC(=O)C2=CC=CC=C2O	1	0	0	1	0	SN2[d]
56	Benzylidene acetone (4-phenyl-3-buten-2-one)	O=C(C=Cc(cccc1)c1)C	1		1	1	1	MA[a]
57	Bisphenol A-diglycidyl ether	O(C1COc(ccc(c2)C(c(ccc(OCC(O3)C3)c4)c4)(C)C)c2)C1	1	1	1	1	1	SN2[b]
58	Butyl acrylate	O=C(OCCCC)C=C	1		1	1	1	MA
59	Butyl glycidyl ether	CCCCOCC1CO1	1	1	1	1	0	SN2[b]
60	Chloramine T	CC1=CC=C(C=C1)S(=O)(=O)[N-]Cl.[Na+]	1		1	1	1	Acyl

61	Chlorobenzene	c(cccc1)(c1)Cl	0		0	0	1	non-reactive
62	Chlorpromazine hydrochloride	CN(C)CCCN1c2ccccc2Sc3c1cc(cc3)Cl.Cl	1	1	0	0	1	pro/pre-SB
63	Cinnamic aldehyde	O=CC=Cc(cccc1)c1	1	1	1	1	1	MA[a]
64	Cinnamic Alcohol	OCC=Cc(cccc1)c1	1	1	1	1	1	pre/pro-(MA or SN2)/pre
65	Citral	O=CC=C(CCC=C(C)C)C	1	1	1	1	1	MA, SB[b]
66	Clofibrate (Ethyl (2-(4-chlorophenoxy)-2-methylpropanoate)	CCOC(=O)C(C)(C)Oc1ccc(Cl)cc1	0		0	0	1	non-reactive
67	Coumarin	c1cc2OC(=O)C=Cc2cc1	0	1	0	1	0	MA but not reactive enough to sensitise[b]
68	Cyclamen aldehyde	O=CC(C)Cc(ccc(c1)C(C)C)c1	1		1	1	0	Weakly reacting SB[a]
69	Diethyl acetaldehyde	O=CC(CC)CC	1		1	1	1	SB[b]
70	Diethyl maleate	O=C(OCC)C=CC(=O)OCC	1	1	1	1	1	MA[b]
71	Diethyl phthalate	CCOC(=O)c1ccccc1C(=O)OCC	0	0	0	0	1	non-reactive
72	Dihydroeugenol (2-methoxy-4-propyl-phenol)	O(c(c(O)ccc1CCC)c1)C	1		0	1	1	pro-MA
73	Dimethyl isophthalate	COC(=O)C1=CC(=CC=C1)C(=O)OC	0		0	1	0	non-reactive
74	Diphenylcyclopropenone	c(ccc1C(C2=O)=C2c(ccc3)cc3)cc1	1	1	1	1	1	SB

75	d-Limonene	CC1=CC[C@@H](CC1)C(=C)C	1	0	1	0	1	pre due to the formation of hydroperoxides
76	Ethyl benzoylacetate	O=C(OCC)CC(=O)c(cccc1)c1	0		0	1	0	SB but not reactive enough to sensitise
77	Ethyl vanillin	O=Cc(ccc(O)c1OCC)c1	0		0	1	0	non-reactive
78	Ethylene glycol dimethacrylate	O=C(OCCOC(=O)C(=C)C)C(=C)C	1	1	1	1	1	MA
79	Ethylenediamine (free base)	NCCN	1	1	0	1	1	pro-SB[b]
80	Eugenol	O(c(c(O)ccc1CC=C)c1)C	1	1	1	0	1	pro/pre-MA[b,c]
81	Formaldehyde	C=O	1	1	1	1	1	SB or cross linking[a]
82	Furil	C1=COC(=C1)C(=O)C(=O)C2=CC=CO2	0		1	1	0	SB[b]
83	Geraniol	OCC=C(CCC=C(C)C)C	1	1	0	1	1	pre/pro-(MA or SN2)/pre also due to the formation of hydroperoxides
84	Glutaraldehyde	O=CCCCC=O	1	1	1	1	1	SB or cross linking[a]
85	Glycerol	OCC(O)CO	0	0	0	0	0	non-reactive
86	Glyoxal	O=CC=O	1	1	1	1	1	SB[b]
87	Hexyl cinnamic aldehyde	O=CC(=Cc(cccc1)c1)CCCCCC	1	0	0	1	0	MA[a]
88	Hydroquinone	Oc(ccc(O)c1)c1	1	1	1	1	1	pre-MA[a]

89	Hydroxycitronellal	O=CCC(CCCC(O)(C)C)C	1	1	1	1	1	SB[a]
90	Imidazolidinyl urea	O=C(NCNC(=O)NC(NC(=O)N1CO)C1(=O))NC(NC(=O)N2CO)C2(=O)	1	1	1	1	1	Acyl[b]
91	Isoeugenol	O(c(c(O)ccc1C=CC)c1)C	1	1	1	1	0	pre-MA[a]
92	Isopropanol	OC(C)C	0	0	0	0	0	non-reactive
93	Lactic acid	O=C(O)C(O)C	0	0	0	0	0	non-reactive
94	Lauryl gallate	O=C(OCCCCCCCCCCCC)c(cc(O)c(O)c1O)c1	1	1	1	1	1	pre-MA[a,b]
95	Linalool	OC(C=C)(CCC=C(C)C)C	1	1	0	0	1	pre due to formation of hydroperoxides
96	Maleic anhydride	O=C1OC(=O)C=C1	1		1	0	1	MA or Acyl
97	Methyl 2-nonynoate	O=C(OC)C#CCCCCCC	1		1	1	1	MA[b]
98	Methyl methanesulphonate	O=S(=O)(OC)C	1		1	1	0	SN2[b]
99	Methyl salicylate	O=C(OC)c(c(O)ccc1)c1	0	0	0	0	0	non-reactive
100	Methylmethacrylate	CC(=C)C(=O)OC	1	1	1	1	0	MA
101	N,N-diethyl-3-methylbenzamide	CCN(CC)C(=O)C1=CC=CC(=C1)C	0	0	0	0	0	non-reactive
102	Nonanoic acid	O=C(O)CCCCCCCC	1		0	0	1	non-reactive False positive in the LLNA

103	Octanoic acid (Caprylic acid)	O=C(O)CCCCCCC	0	0	0	0	1	non-reactive
104	Oxalic acid anhydrous	O=C(O)C(=O)O	1		0	1	1	Non-reactive false positive in the LLNA or impurity
105	p-Benzoquinone	C1(=O)C=CC(=O)C=C1	1		1	1	1	MA[a,b]
106	Penicillin G	O=C(NC(C(=O)N1C(C(=O)O)C(S2)(C)C)C12)Cc(cccc3)c3	1	1	1	0	1	Acyl[b]
107	Pentachlorophenol	Oc(c(c(c(c1Cl)Cl)Cl)Cl)c1Cl	1	0	1	0	1	SNAr[b]
108	Perillaldehyde	C(=C)(C)C1CC=C(C=O)CC1	1		1	1	1	MA[b]
109	Phenylacetaldehyde	O=CCc(cccc1)c1	1		1	1	1	SB[b]
110	Phthalic anhydride	O=C(OC(=O)c1cccc2)c12	1		1	0	0	Acyl
111	Propyl gallate	O=C(OCCC)c(cc(O)c(O)c1O)c1	1	1	1	1	1	pre-MA[c]
112	Propyl paraben	O=C(OCCC)c(ccc(O)c1)c1	0	0	0	1	1	non-reactive
113	Propylene glycol	OCC(O)C	0	0	0	0	0	non-reactive
114	p-tert-Butyl-a-ethyl hydrocinnamal (Lilial)	O=CC(C)Cc(ccc(c1)C(C)(C)C)c1	1	1	1	0	1	SB[b]
115	Resorcinol	Oc(cccc1O)c1	1	1	0	0	1	pro-MA[b]
116	Saccharin	O=C(NS(=O)(=O)c1cccc2)c12	0		0	0	0	non-reactive
117	Salicylic acid	O=C(O)c(c(O)ccc1)c1	0	0	1	0	1	non-reactive

118	Sodium lauryl sulphate	CCCCCCCCCCCCOS(=O)(=O)[O-].[Na+]	1	0	0	0	0	False positive in the LLNA
119	Streptomycin sulfate	C[C@@H]1[C@]([C@@H]([C@H](O1)O[C@@H]2[C@H]([C@@H]([C@H]([C@@H]([C@H]2O)O)NC(=N)N)O)NC(=N)N)O[C@@H]3[C@@H]([C@H]([C@@H]([C@H](O3)CO)O)O)NC)(C=O)O.OS(=O)(=O)O	0		0	0	0	non-reactive
120	Sulphanilamide	O=S(=O)(N)c(ccc(N)c1)c1	0		0	0	0	non-reactive
121	Sulphanilic acid	O=S(=O)(O)c(ccc(N)c1)c1	0		0	0	0	non-reactive
122	Tetrachloro-salicylanilide	O=C(Nc(ccc(c1Cl)Cl)c1)c(c(O)c(cc2Cl)Cl)c2	1	1	1	1	1	Special case[c]
123	Tetramethylthiuram disulfide	N(C(=S)SSC(N(C)C)=S)(C)C	1	1	1	1	1	Special case SN2[b]
124	Thioglycerol	C(C(CS)O)O	1	1	1	0	1	SN2
125	trans-2-hexenal	O=CC=CCCC	1		1	1	1	MA[b]
126	Vanillin	O=Cc(ccc(O)c1OC)c1	0	0	0	0	0	non-reactive
127	Xylene	CC1=CC=CC=C1C	1	0	0	0	0	False positive in the LLNA

2.2 Table 2: List of chemicals briefly presented in Table 1 and assigned by the experts as being pre- and/or pro-haptens

#	Name	LLNA	Human	DPRA	Keratino -Sens™	h-CLAT	Chemical-based reason	Additional comments
1	1,2-Dibromo-2,4-dicyanobutane (MDGN)	1	1	1	1	1	pre-MA, SN2	
2	1,4-Phenylenediamine	1	1	1	1	1	pre-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alerts[1]
3	1-Naphthol	1		1	1	1	pre-MA	Pre-MA via keto tautomer
4	2,5-Diaminotoluene sulphate (PTD)	1	1	1	1	1	pre-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alerts
5	2-Aminophenol	1	1	1	1	1	pre-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alerts
6	2-Methoxy-4-methylphenol	1		0	0	1	pro/pre-MA	Quinone methide precursor and/or ortho-quinone via OMe alerts
7	2-Nitro-1,4-phenylendiamine	1	1	1	1	1	pro/pre-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alerts
8	3-Aminophenol	1		0	0	1	pro-MA	Aromatic meta: diamines, aminophenols, di-phenols, and aromatic monoamines
9	3-Dimethylamino propylamine	1	1	0	1	1	pre-SB	Aliphatic amines alert

[1] Alert, as referenced in this table, refers to a structural feature that is associated with reaction chemistry that could lead to sensitisation although the mechanism is unclear.

10	3-Pr[4]opylidenephthalide	1		1	0	1	Acyl or pre	Pre - Possible autoxidation to a hydroperoxide
11	4-(N-Ethyl-N-2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3)	1		1	1	1	pre/pro-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alerts
12	4-Allylanisole	1		1	0	1	pro-MA	Quinone methide precursor and/or ortho-quinone via OMe alerts
13	Abietic acid	1	1	1	1	0	pre	Autoxidation to hydroperoxides
14	Aniline	1	1	0	0	1	pre/pro-MA or pseudo SB	Aromatic meta: diamines, aminophenols, di-phenols, and aromatic monoamines. Could be pro- or pre- (ring oxidation to pre/pro quinone imine, or oxidation of NH2 to NO - pseudo SB) or reacting via free radical
15	Bandrowski's Base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine)	1		1	1	1	pro/pre-MA	
16	Chlorpromazine hydrochloride	1	1	0	0	1	pro/pre-SB	Aliphatic amines alert. Pro- or pre- SB. -NCH2CH2CH2N- unit is an alert for malondialdehyde, CHOCH2CHO, highly reactive
17	Cinnamyl Alcohol	1	1	1	1	1	pro/pre-(MA or SN2)/pre	Pro-MA has been assumed to dominate but pro-SN2 via sulphate is also a possibility Pre also due to hydroperoxides
18	Dihydroeugenol (2-methoxy-4-propyl-phenol)	1		0	1	1	pro-MA	Quinone methide precursor and/or ortho-quinone via OMe alerts

19	d-Limonene	1	0	1	0	1	pre	Autoxidation to hydroperoxides
20	Ethylenediamine (free base)	1	1	0	1	1	pro-SB	Aliphatic amines alert
21	Eugenol	1	1	1	0	1	pro/pre-MA	Quinone methide precursor and/or ortho-quinone via OMe alerts
22	Geraniol	1	1	0	1	1	pro/pre-(MA or SN2)/pre	Autoxidation to hydroperoxide, aldehydesand epoxides. Maybe pro-SN2 also via sulphation
23	Hydroquinone	1	1	1	1	1	pre-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alerts
24	Isoeugenol	1	1	1	1	0	pre-MA	Quinone methide precursor and/or ortho-quinone via OMe alerts
25	Lauryl gallate	1	1	1	1	1	pre-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alerts
26	Linalool	1	1	0	0	1	pre	Autoxidation to hydroperoxides
27	Propyl gallate	1	1	1	1	1	pre-MA	Aromatic o- and p-diamine, aminophenol or di-phenol alert
28	Resorcinol	1	1	0	0	1	pro-MA	Aromatic meta: diamines, aminophenols, di-phenols, and aromatic monoamines alerts

Chemicals which autoxidise to hydroperoxides are generally weak sensitisers due to the limited amount of oxidation that can occur during exposure/testing. Test results are highly dependent on the sample, therefore these chemicals, as shown in Table 2, are not always correctly identified by the *in chemico* and *in vitro* assays in the same way as they would not be in the animal models.
Chemicals containing aromatic ortho (o) and para (p) diamines, aminophenols or di-phenol alerts are correctly predicted by the *in chemico* and *in vitro* assays as they are with the animal models.

Conclusion

It has long been recognised that *in vivo* predictive tests for skin sensitisation are not perfect, and do not deliver 100% accuracy in terms of the prediction of human hazard (30; 31). Nevertheless, the analysis of the Kern et al. database (25) indicated that the LLNA successfully identified all but 2 of the list of pre- and pro-haptens, a success rate of approximately 97%.

The expert group analysis of the EURL ECVAM dataset (n=127) similarly demonstrated that a large proportion of the pre- and pro-haptens were correctly identified by the non-animal methods.

As a result, it is recommended that negative *in vitro* data are accepted unless there is a compelling scientific argument that a substance is likely to be an exclusively metabolically activated pro-hapten. It is possible that *in silico* tools such as TIMES-SS (8; 9; 10) or the OECD Toolbox (11) would be able to inform such an argument.

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List of abbreviations and definitions

AOP	Adverse Outcome Pathway; The sequential progression of events from the molecular initiating event (MIE) to the in vivo outcome of interest
KE	Key Event; A change in biological state that is both measurable and essential to the progression of a defined biological perturbation leading to a specific adverse outcome
MIE	Molecular Initiating Event; A specialised type of key event that represents the initial point of chemical interaction on molecular level within the organism that results in a perturbation that starts the AOP
Pre-hapten	Chemical that is not protein reactive but is converted abiotically to protein-reactive derivatives
Pro-hapten	Chemical that is not protein reactive but is converted metabolically to protein-reactive derivatives
	(Q)SAR; (Quantitative) Structure Activity Relationship
TIMES-SS	TImes MEtabolism Simulator platform used for predicting Skin Sensitization

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