

The h-CLAT for Assessment of Dermal Sensitization Potency of Commercially Available Mixtures and the OECD Proficiency Chemicals

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ABSTRACT

In order to explore the applicability domain for more pure chemicals and mixtures (including commercial products), we performed a series of studies using the Human Cell Line Activation Test (h-CLAT) protocol under the latest OECD Test Guideline. THP-1 cell expression of CD86 and CD54 from an expanded set of validation chemicals and several complex mixtures were measured. A variety of products from the petroleum, agrochemical, food, beauty and chemical industries, were obtained via retail outlets and evaluated. Known positive (via safety data sheets) mixtures were assessed along with non-sensitizing mixtures. These mixtures included: non-PPD-containing hair dye, propolis extract, diesel fuel additive, a pesticide, and commercial acrylate-based sealants. In addition to mixtures, we evaluated the OECD proficiency test chemicals, which include DNCB, Phenylenediamine, Nickel Sulfate, 2-Mercaptobenzothiazole, R(+)-Limonene, Imidazolidinyl Urea, and the non-sensitizers Lactic Acid, Isopropanol, Glycerol, and 4-Aminobenzoic Acid. All chemicals were tested at a low or non-irritating concentration, yielding a CV₇₅ or higher viability, as determined by Flow Cytometry. Sensitizer potency was measured by the concentration of test chemical that induced a Relative Fluorescence Intensity (RFI) that was a threshold positive response (CD86 = 150%, CD54 = 200%) of control. Proficiency chemicals from draft and final OECD Test Guidelines (Jul 2014, Dec 2015 and Aug 2016) were tested for a total of 16 pure chemicals (six non-sensitizers and nine sensitizers). The h-CLAT correctly predicted nine of nine sensitizing and five of six non-sensitizing chemicals, for an overall Accuracy of 93%.

METHODS

THP-1 monocytic cell line from ATCC was cultured in RPMI-based media with 10% FBS, penicillin-streptomycin and 0.05 mM mercaptoethanol. Cells were seeded at 1×10^6 cells/ml in 24-well plates, and dosed with test chemicals in either media or 0.2% DMSO. After 24 hours of exposure, cells were harvested, rinsed with PBS containing 0.1% BSA and stained with antibodies to CD86 and CD54 for flow cytometry analysis. Relative Fluorescence Intensities (RFI) of >150% for CD86 and/or >200% for CD54 relative to isotype-corrected vehicle control values were considered to be a positive response. The concentrations that would yield a threshold-positive response, the EC150 and EC200, were calculated by a logarithmic regression of the data and interpolation. Chemicals were ranked according to their potency based on EC150 and EC200 values.

Test Chemicals: Chemicals from OECD Guideline 442E “*In Vitro* Skin Sensitisation: Human Cell Line Activation Test (h-CLAT)” (Drafts Jul 2014 and Dec 2015, Final Aug 2016) proficiency substances was tested and 14 of 15 chemicals were correctly predicted.

In addition to proficiency chemicals, 12 mixtures and six potential alternative vehicles were tested at two concentrations 0.2% and 1%. Finally, suitable (non-toxic) vehicles were selected for evaluation using the positive control agent DNCB.

RESULTS

Mixtures Validation Effort

Substance Name	CD86 EC150 (µg/ml) independent replicates	CD54 EC200 (µg/ml) independent replicates	h-CLAT Prediction
Thread Sealant	<232.4	<232.4	Sensitizer
Glue (All Purpose)	>5000	<1395	Sensitizer
Glue (School)	>5000	>5000	Non-sensitizer
Pesticide 1	644.5 >798.6 >798.6	>798.6	Non-sensitizer
Pesticide 2	59.8	78.4	Sensitizer
Fuel additive 1	>100.8	51.7	Sensitizer
Fuel additive 2	38.9	75.1	Sensitizer
Non-PPD hair dye	>159	83.8	Sensitizer
PPD hair dye	<254.5	477.1	Sensitizer
Hypoallergenic Shampoo 1	42.7 >47.3 >47.3	>47.3	Non-sensitizer
Hypoallergenic shampoo 2	>408.4	<114 >408.4 388.94	Sensitizer
Propolis	>1249.8	<348.9	Sensitizer

RESULTS (continued)

Summary of Validation Chemicals

Substance Name	CD86 EC150 (µg/ml)		CD54 EC200 (µg/ml)		Met OECD Reference Range	h-CLAT Prediction
	MB Research	OECD Reference Range	MB Research	OECD Reference Range		
Sensitizers:						
2,4-Dinitrochlorobenzene	2.2	0.5-10	2.2	0.5-15	Yes	Correct
4-Phenylenediamine	<12.68	<40	>1.5	>1.5	Yes	Correct
Nickel Sulfate	96.5	<100	32.3-67.0	10-100	Yes	Correct
2-Mercaptbenzothiazole	27.63	>10	28.93	10-140	Yes	Correct
R(+)-Limonene	45.87	>5	29.52	<250	Yes	Correct
Imidazoidinyl Urea	29.1-34.9	20-90	29.1-34.9	20-75	Yes	Correct
Chloramin T	250.8	NP	785.7	NP	N/A	Correct
Phenylacetaldehyde	20.5	NP	21.9	NP	N/A	Correct
Hydroxycitronellal	>1200	NP	771	NP	N/A	Correct
Non-Sensitizers:						
Isopropanol	>5000	>5000	>5000	>5000	Yes	Correct
Glycerol	>5000	>5000	>5000	>5000	Yes	Correct
Lactic Acid	>2411	>5000	>2000	>5000	Yes*	Incorrect [#]
4-Aminobenzoic Acid	>1200	>5000	>1200	>5000	Yes*	Correct
1-Butanol	>1000	NP	>1000	NP	N/A	Correct
Vanillin	>1000	NP	>1000	NP	N/A	Correct

NP = Not Provided

N/A = Not Applicable

* = Concentrations approaching 5000 µg/ml limit induced >50% cell death

= Incorrect (mis-predicted) above 2000 µg/ml

Contingency Table for OECD Validation Chemicals

	Known +	Known -	
Tested +	9	1	10
Tested -	0	5	5
	9	6	15

Accuracy	93%	(14/15)
Sensitivity	100%	(9/9)
Specificity	83%	(5/6)
Positive Predictivity	90%	(9/10)
Negative Predictivity	100%	(5/5)

RESULTS (continued)

Alternative Vehicle Validation

Substance Name		Viability (%)	CD86 RFI ¹ multiple replicates	CD54 RFI ¹ multiple replicates	h-CLAT Prediction
Acetone	0.2%	96.4	97 107 17	132 77 59	Non-Sensitizer
	1%	96.9	130 133 3	184 62 38	Non-Sensitizer
Ethanol	0.2%	96.8	97 133 68	132 52 84	Non-Sensitizer
	1%	96.6	130 132 37	184 100 72	Non-Sensitizer
Dimethyl-formamide	0.2%	96.8	158 123 31	111 105 97	Non-Sensitizer
	1%	78.6*	306 228	173 175	Sensitizer
Dimethyl-acetamide	0.2%	81.4*	111 54 86	175 209 189	Viability <90%
	1%	13.5*	189 142 90	148 114 83	Viability <90%
Methyl Ethyl Ketone	0.2%	94.7	226 25 118	80 47 89	Non-Sensitizer
	1%	94.3	218 11 96	42 47 156	Non-Sensitizer
Propylene Glycol	0.2%	96.0	175 32 86	138 103 178	Non-Sensitizer
	1%	93.6	304 29 154	365 350 247	Sensitizer

★ = viability <90%; not suitable for h-CLAT

1 = all values compared to media control

Bold red indicates a positive response

RESULTS (continued)

DNCB in Alternative Vehicles

Substance Name	Viability (%)	CD86 RFI ^{1,2}	CD54 RFI ^{1,2}	Outcome
0.2% Acetone	96.5	91	90	Valid Alternative
4 µg/ml DNCB in 0.2% Acetone	59.7	324	517	
1% Acetone	96.2	100	81	Valid Alternative
4 µg/ml DNCB in 1% Acetone	66.0	372	778	
0.2% Ethanol	96.8	118	106	Valid Alternative
4 µg/ml DNCB in 0.2% Ethanol	61.2	247	848	
1% Ethanol	96.0	112	108	Valid Alternative
4 µg/ml DNCB in 1.0% Ethanol	55.9	276	377	
0.2% Dimethylformamide	96.5	114	134	Negative response in DNCB. Not valid.
4 µg/ml DNCB in 0.2% Dimethylformamide	58.2	187	186	
0.2% Methyl Ethyl Ketone	96.6	131	107	Valid Alternative
4 µg/ml DNCB in 0.2% Methyl Ethyl Ketone	60.9	223	629	
1% Methyl Ethyl Ketone	95.6	94	81	Valid Alternative
4 µg/ml DNCB in 1% Methyl Ethyl Ketone	54.4	271	635	
0.2% Propylene Glycol	97.3	129	135	Viability too low, negative response in DNCB. Not valid.
4 µg/ml DNCB in 0.2% Propylene Glycol	34.3	109	330	

1 = all values compared to vehicle or media controls

2 = average of three replicates

SUMMARY

- Chemicals from OECD Guideline 442E “*In Vitro* Skin Sensitisation: Human Cell Line Activation Test (h-CLAT)” proficiency substances were tested and 14 of 15 chemicals were correctly predicted.
- Known sensitizing and non-sensitizing mixtures were tested. Correctly predicted 10 of 11 materials.
- Additional vehicles were assessed for cytotoxicity and antigenicity. Acceptable vehicles were then used to solubilize DNCB for evaluation in the h-CLAT. Alternative vehicles were considered potentially useful if 1) Non-cytotoxic concentrations were identified while maintaining sufficient dissolving power; 2) Did not induce a positive CD86 or CD54 response in THP-1 cells; and 3) Allowed DNCB to produce positive responses in reactivity checks at the test guideline-prescribed concentration (4 µg/ml).
 - Acetone, Methyl Ethyl Ketone, and Ethanol were viable alternative vehicles.
- Contingency table analysis yields Cooper Statistics with an Accuracy of 93%, Sensitivity of 100%, and Specificity of 83% for the OECD validation chemicals.