Implementation of Three *in-vitro* Test Methods for Skin Sensitisation Safety Assessment

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Introduction

Until 2016, assessment of skin sensitising potential of chemicals required classical *in-vivo* tests. Skin sensitisation testing is expected to make up the largest proportion of tests required for the 2018 REACH deadline and the Annex VII update in 2016 now means non-animal tests are the default data requirement for skin sensitisation testing. Development of adverse outcome pathways (AOPs) for assay development and Integrated Testing Strategies (ITS) as part of Integrated Approaches to Testing and Assessment (IATA) have led to multiple OECD accepted *in-vitro/in-chemico* testing methods for hazard identification and thus classification and labelling of chemicals for this endpoint.

IATA requires multiple data sources *(in-silico, in-chemico, in-vitro)* to replace currently used *in-vivo* approaches as no single method provides a broad coverage of key sensitisation events in the AOP. This work describes implementation of three OECD accepted test methods, which when combined give comparable predictive power for skin sensitisation to the Local Lymph Node Assay (LLNA). Direct Peptide Reactivity Assay (DPRA),[1] KeratinoSens™ Assay (KSA)[2] (Givaudan Schweiz AG, Switzerland) and the human Cell Line Activation Test (h-CLAT)[3,4] have all been successfully implemented at Covance.

We have tested a total of 28 chemicals for their sensitising potential across the three assays and demonstrated the ability to successfully differentiate skin sensitisers from non-skin sensitisers using these three *in-vitro* assays (in accordance with OECD Test Guidelines).

Principle of the Tests

DPRA

The DPRA is an *in-chemico* method that quantifies the reactivity of a test chemical through its depletion of synthetic peptides containing cysteine- or lysine-, as measured by HPLC-UV. Percent depletion values are then calculated and the prediction model applied to discriminate between potential sensitisers and non-sensitisers.

KeratinoSens™ Test

The KeratinoSens™ test is an *in-vitro* method on human keratinocyte HaCaT (skin) cells containing a luciferase reporter gene (light producing), controlled by the mechanism in the cell that reacts to a potential sensitiser. Sensitisers therefore directly increase the luciferase readout. Cytotoxicity is measured in parallel.

h-CLAT Assay

The h-CLAT is an *in-vitro* method on human dendritic-like cells (part of the immune system) expressing surface proteins needed to trigger an immune response. Increases in these proteins are detected using fluorescent-labelled antibodies and a flow cytometer. Sensitisers increase the number of these proteins on the exposed cells compared to unexposed cells. Cytotoxicity is also measured.

Materials and Methods

DPRA (OECD Test Guideline 442C)

- Cysteine (Ac-RFAA**C**AA-COOH) and lysine (Ac-RFAA**K**AA-COOH) peptide stocks incubated for 24 hours with test chemical (1:10 cysteine; 1:50 lysine)
- Positive control – cinnamic aldehyde (CAS 104-55-2)
- Cysteine / lysine calibration standards and reference standards prepared
- Post incubation, analysis by HPLC-UV
- Considered positive if mean of cysteine and lysine depletion >6.38% OR cysteine depletion >13.89%
- Chemicals can be classified as negative, low, moderate or high reactivity

KeratinoSens™ Test (OECD Test Guideline 442D)

- $1x10^4$ cells seeded into wells of a 96-well plate on day prior to assay
- Positive control – 5 concentrations of *trans*-cinnamaldehyde (CAS 14371-10-9)
- 12-point dose series of test chemical
- 48-hour treatment period, then read luminescence and assess viability
- Considered positive with a >1.5-fold statistically significant luciferase induction

h-CLAT Assay (OECD Test Guideline 442E)

- $1x10^6$ cells seeded into 24-well plate on day of assay
- Positive control – 2,4-Dinitrochlorobenzene (CAS 97-00-7)
- Negative control 1% DMSO and/or medium
- After 24 h treatment period read CD54-FITC and CD86-FITC and check viability with propidium iodide
- A test chemical is positive if >1.5- (CD86) or >2.0- (CD54) fold fluorescence induction

Results

Table 1. Chemicals Predicted as Non-Skin Sensitisers in the *in-vitro* Assays (with LLNA EC3 as reference)

OECD Proficiency substances	CASRN	DPRA			KeratinoSens™		hCLAT			Assay Predictions			
		Cysteine Depletion (%)	Lysine Depletion (%)	Mean Cys & Lys Depletion (%)	$EC_{1.5}$ (µM)	IC_{50} (µM)	CV75 µg/mL	h-CLAT CD86 (EC150) µg/mL	h-CLAT CD54 (EC200) µg/mL	DPRA	Keratino Sens™	hCLAT	LLNA EC3
isopropanol	67-63-0				> 2000	> 2000	>5000	>5000	>5000		Negative	Negative	NC
Glycerol	56-81-5				> 2000	> 2000	>5000	>5000	>5000		Negative	Negative	NC
Lactic acid	50-21-5	11.50	0.50	6.03	> 2000	> 2000	3035	>3644	>3644	Negative	Negative	Negative	NC
4-Aminobenzoic acid	150-13-0						>1000	>1000	>1000			Negative	NC
Salicylic acid	69-72-7				> 2000	> 2000					Negative		NC
1-Butanol	71-36-3	4.80	1.30	3.02						Negative			NC
6-Methylcoumarin	92-48-8	9.30	1.40	5.37	12.78	82.80				Negative	Positive		NC
4-Methoxyacetophenone	100-06-1	4.40	1.00	2.68	370.86	> 2000				Negative	Positive		NC
Potential solvents	**CASRN**	**DPRA**			**KeratinoSens™**		**hCLAT**			**Assay Predictions**			
		Cysteine Depletion (%)	Lysine Depletion (%)	Mean Cys & Lys Depletion (%)	$EC_{1.5}$ (µM)	IC_{50} (µM)	CV75 µg/mL	h-CLAT CD86 (EC150) µg/mL	h-CLAT CD54 (EC200) µg/mL	DPRA	Keratino Sens™	hCLAT	LLNA EC3
Acetone (@ 1%)	67-64-1				> tested	> tested					Negative		NC
Ethanol (@ 1%)	64-17-5				> tested	> tested					Negative		NC
Saline (@ 1%)	7647-14-5				> tested	> tested	> tested	> tested	> tested		Negative	Negative	NC
Saline (@ 10%)	7647-14-5				> tested	> tested					Negative		NC
DMSO (@ 1%)	67-68-5				> tested	> tested	> tested	> tested	> tested		Negative	Negative	NC
Dimethyl formamide (@ 1%)	68-12-2				> tested	> tested	> tested	> tested	> tested		Negative	Negative	NC

NC Not calculated because the substance was negative.
Conflicting data between assay results.

Table 2. Chemicals Predicted as Skin Sensitisers in the *in-vitro* Assays (with LLNA EC3 as reference)

OECD Proficiency substances	CASRN	DPRA			KeratinoSens™		hCLAT			Assay Predictions			
		Cysteine Depletion (%)	Lysine Depletion (%)	Mean Cys & Lys Depletion (%)	$EC_{1.5}$ (µM)	IC_{50} (µM)	CV75 µg/mL	h-CLAT CD86 (EC150) µg/mL	h-CLAT CD54 (EC200) µg/mL	DPRA	Keratino Sens™	hCLAT	LLNA EC3
2,4-Dinitrochlorobenzene	97-00-7	100	17.3	58.63	1.74	9.39	8.00	3.80	1.80	Positive	Positive	Positive	0.05
4-Phenylenediamine	106-50-3				18.26	420.87	61.60	1.90	>73.9 (N)		Positive	Positive	0.16
Nickel sulfate	10101-97-0				324.41	806.71	364.00	131.50	78.50		Negative	Positive	4.8
2-Mercaptobezothiazole	149-30-4				143.90	> 2000	166.00	116.20	64.50		Positive	Positive	1.7
R(+)-Limonene	5989-27-5				> 2000	112.22	>1000	>1000 (N)	973.20		Negative	Positive	30.0
Imidazolidinyl urea	39236-46-9				37.80	272.94	39.40	32.60	34.80		Positive	Positive	24.0
4-Methylaminophenol sulfate	55-55-0				1.62	26.20					Positive		0.8
Methyldibromo glutaronitrile	35691-65-7				6.17	31.20					Positive		0.9
Ethylene glycol dimethacrylate	97-90-5				66.10	1633					Positive		28.0
Cinnamic alcohol	104-54-1				58.70	1563					Positive		21.0
Oxazolone	15646-46-5	64.70	51.80	58.23	308.85	941.19				Positive	Positive		0.003
Formaldehyde	50-00-0	48.60	4.50	26.58	167.05	399.31				Positive	Positive		0.61
Benzylideneacetone	122-57-6	92.50	3.50	48.02	169.00	152.53				Positive	Positive		3.7
Farnesal	19317-11-4	40.00	7.20	23.60						Positive			12.0
2,3-Butanedione	431-03-8	86.10	34.80	60.45						Positive			11.0

(N) Indicates a negative result for that receptor.
Conflicting data between assay results.

Discussion Points

- EURL-ECVAM developed the skin sensitisation Adverse Outcome Pathway (AOP) (Figure 1) to determine the Key Events that lead to the Organism Response
- Several *in-vitro* assays were developed using this AOP with the DPRA, KeratinoSens™ and hCLAT passing EURL-ECVAM validation criteria first, leading to OECD Test Guidelines (OECD TG 442C, D and E)
- REACH 2018 deadline requires *in-vitro* testing where applicable over *in-vivo*
- IATA guidance (ENV/JM/MONO(2016)29) published by OECD to aid data interpretation depending on assay applicability domains to meet this need
- 2 compounds (6-Methylcoumarin and 4-Methoxyacetophenone) Positive in the KeratinoSens™ assay (as reported by Natsch et. al)[6] but Negative in the DPRA (not tested in h-CLAT yet)
- 2 compounds (Nickel sulfate and R(+)-Limonene) both Negative in the KeratinoSens™ assay but Positive in the h-CLAT (not tested in DPRA yet)
- Correct prediction of skin sensitisation potential of substances requires integration of information from multiple sources
  - Physio-chemical / *in-silico* / *in-vitro* etc.
  - IATA yields a weight-of-evidence prediction
  - Depending on chemical properties, a "2 out of 3" approach **MAY** be applicable (as suggested in IATA Guidance) (Figure 2)
- Each assay contains data that may inform about potency

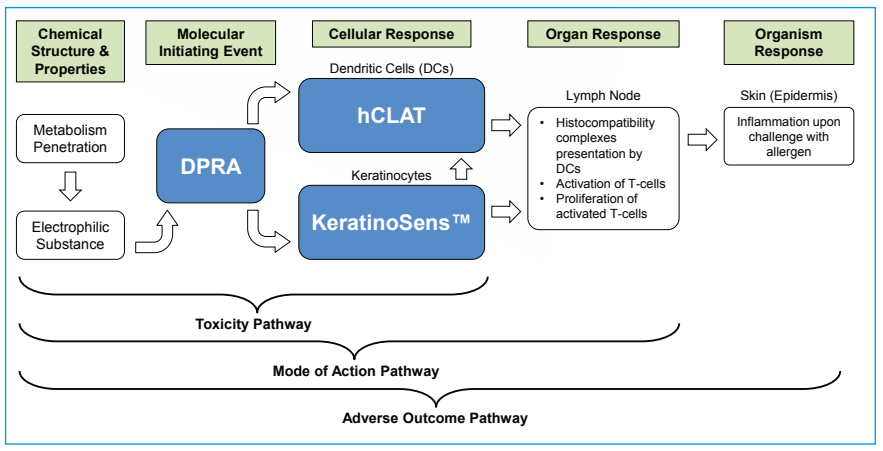

Figure 1. Adverse outcome pathway. Modified from ENV/JM/MONO(2012)10/PART1. Multiple non-animal tests are required to replace *in-vivo* skin sensitisation tests.

Pathway	Assay		Weight of Evidence	Prediction
Molecular	DPRA	→	2/3 Positive	Sensitiser
Cellular	KeratinoSens™			
	hCLAT	→	2/3 Negative	Non-Sensitiser

Figure 2. Skin sensitisation weight-of-evidence decision tree. The skin sensitisation safety assessment requires a 2 of 3 weight of evidence approach.[5] If an unequivocal 2-from-2 result – either 2 negative or 2 positive results – is obtained from the first two assays, there is no requirement for a third assay to make a decision.

Conclusions

- A total of 28 proficiency chemicals were tested according to the OECD guidelines (ANNEX II) across three skin sensitisation assays: DPRA, KeratinoSens™ and h-CLAT
- The correct skin sensitisation potential of all the proficiency substances was correctly predicted for all test chemicals
- Covance Laboratories has demonstrated proficiency in *in-vitro* / *in-chemico* skin sensitisation safety testing
- Using IATA, a weight of evidence approach will allow non-animal testing for skin sensitisation potential of substances registered for REACH registration 2018
- Completion of the additional data for the OECD listed chemicals is required before the relevance of any differences between assay predictions per chemical can be made

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Abstract

Until 2016, assessment of the skin sensitizing potential of chemicals required *in-vivo* tests. The Adverse Outcome Pathway (AOP) for skin sensitisation revealed key events that could be assayed and the OECD has recently published Test Guidelines for three *in-vitro* methods for skin sensitisation prediction. Further efforts from the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) led to additional OECD publications on Integrated Approaches to Testing and Assessment (IATA) and in 2016, the REACH Directive was amended in Annex VII so that *in-vitro* data became the default data source for this endpoint.

The first key event in the AOP is protein haptenation (required for chemical transfer through the skin) which is detected using The Direct Peptide Reactivity Assay (DPRA) - an *in-chemico* assay. The KeratinoSens™ Assay (Givaudan Schwiez AG, Switzerland) is a cell-based assay that detects basal epidermal response – the second key event – and the human Cell Line Activation Test (hCLAT) is also cell-based and detects dendritic cell activation (and hence the immune system) – the third key event.

Results from >24 chemicals tested (of varying potencies) across the three assays demonstrate that, within an IATA, the *in-vitro/in-chemico* assays better predict human skin sensitisation when compared to the existing *in-vivo* test data (local lymph node assay – LLNA). Although a measure of potency is not yet accepted from *in-vitro* assays at a regulatory level, each of these three assays yield useful information regarding this additional endpoint.

Key words: *in-vitro*, Skin sensitisation, Alternatives, REACH, Regulatory