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

D. Kidd, C. Rothwell, J. Inns, D. Dreher and D. Henderson
Covance Laboratories Ltd., Harrogate, UK

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 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) has led to multiple OECD accepted in-vitro/in-chemo testing methods for hazard identification and thus classification and labelling of chemicals for this endpoint. IATA requires multiple data sources (in-silico, in-chemo, 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), KeratinoSens™ Assay (KSA) (Grauvand Salgany AD, Switzerland) and the human Cell Line Activation Test (h-CLAT) [1] have all been successfully implemented at Covance. We have tested a total of 28 chemicals for their sensitising potential across the in-silico and in-vitro domains and demonstrated the ability to successfully differentiate skin sensitizers from non-sensitizers using these three in-vitro assays (in accordance with OECD Test Guidelines).

Principle of the Tests

DPRA

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

KeratinoSens™ Test

The KeratinoSens™ test is an in-vitro method on human keratinoyte HaCaT (skin) cells containing a luciferase reporter gene (light emission) which when combined give comparable predictive power for skin sensitisation to the Local Lymph Node Assay (LLNA). Direct Peptide Reactivity Assay (DPRA), KeratinoSens™ Assay (KSA) (Grauvand Salgany AD, Switzerland) and the human Cell Line Activation Test (h-CLAT) [1] have all been successfully implemented at Covance.

Discussion Points

> **EUR-L-ECAM** 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 h-CLAT passing EUR-L-ECAM validation criteria first, leading to OECD Test Guidelines (OECD TG 442C, D and E) and 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 (4-Hydroxy-3-Methoxyacetophenone and 4-Methoxyacetophenone) Positive in the KeratinoSens™ assay but Negative in the h-CLAT (not tested in h-CLAT) yield correct prediction of skin sensitisation potential of substances requires integration of information from multiple sources – Physico-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

Materials and Methods

DPRA (OECD Test Guideline 442C)

- Keratinocytes (Ac-RFAA-COOH) and lysine (Ac-RFAA-COOH) peptide stocks incubated for 24 hours with test chemical (1:10 cysteine; 1:50 lysine)
- Positive control – cinnamaldehyde (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)

- 1x10⁶ 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 ≥5-fold statistically significant luciferase induction

h-CLAT Assay (OECD Test Guideline 442E)

- 1x10⁶ cells seeded in 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 induction

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

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CASRN</th>
<th>DPRA IC50 (%)</th>
<th>KeratinoSens™ IC50 (%)</th>
<th>h-CLAT IC50 (%)</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ioxetal</td>
<td>14371-10-9</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Negative</td>
</tr>
<tr>
<td>1,3-Dihydroxy-2-Propanone</td>
<td>110-62-7</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Negative</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>123-39-9</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Negative</td>
</tr>
<tr>
<td>Acetaminophenol</td>
<td>62-19-4</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Negative</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CASRN</th>
<th>DPRA IC50 (%)</th>
<th>KeratinoSens™ IC50 (%)</th>
<th>h-CLAT IC50 (%)</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Dinitrochlorobenzene</td>
<td>97-00-7</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Nickel sulphate</td>
<td>10017-87-2</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Nutmeg oleoresin</td>
<td>9888-27-1</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Indoxyl/indigo blue</td>
<td>2036-46-8</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Methylbutynoic acid-glucoside</td>
<td>9351-90-7</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Lysine</td>
<td>67-21-1</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Cinnamyl alcohol</td>
<td>106-46-0</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>3-Aminophenol</td>
<td>55-55-0</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>5-Aminosalicylic acid</td>
<td>97-65-1</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Acetaminophenoxide</td>
<td>15646-46-5</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>104-54-1</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>4-Methoxyacetophenone</td>
<td>105-05-1</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>4-Methoxyacetophenone</td>
<td>105-05-1</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
<tr>
<td>2-Methacrylamide</td>
<td>407-02-4</td>
<td>&gt; 1400</td>
<td>&gt; 2500</td>
<td>&gt; 1400</td>
<td>Positive</td>
</tr>
</tbody>
</table>

References


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-chemo 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
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