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 2016 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-vivo chemical 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 can give comparable predictive power for skin sensitisation to the Local Lymph Node Assay (LLNA). Direct Peptide Reactivity Assay (DPRA), KeratinoSens™ Assay (KSA) (Givaudan Schwedel AG, Switzerland) and the human Cell Line Activation Test (h-CLAT)1 have all been successfully implemented at Covance.

We have tested a total of 24 chemicals for their sensitising potential across the three assays and demonstrated the ability to successfully differentiate skin sensitisers from non-sensitisers using these three in-vitro assays (in accordance with OECD Test Guidelines).

Discussion
- 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.
- EU adoption of methods means UK facilities have to use in-vitro tests before considering in-vivo testing.
- It is an in-vivo 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 these numbers; these proteins on the exposed cells compared to unexposed cells. Cytotoxicity is also measured.

Materials and Methods
DPRA (OECD Test Guideline 442C)
- Cysteine (Ac-RFAAADD-COCH) and lysine (Ac-RFAAADD-COCH) 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 >638% or cysteine depletion >13.89%.
- Chemicals can be classified as negative, low, moderate or high reactivity.

KeratinoSens™ Test (OECD Test Guideline 442D)
- 1x10³ cells seeded into wells of a 96well plate on day prior to assay.
- Positive control – 5 concentrations of trans-cinnamaldehyde (CAS 14371-13-9).
- 13point 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.

hCLAT Assay (OECD Test Guideline 442E)
- 1x10³ cells seeded on 24-well plate on day of assay.
- Positive control – 2,4-Dinitrochlorobenzene (CAS 97-00-7).
- Negative control 1% DMSO and/or medium.
- Background 24 hour treatment period cd54-FITC and cd86-FITC and chelex back with propidium iodide.
- A test chemical is positive if >1.5-(CDS6) or >2.0 (CDS4) fold fluorescence induction.

Results

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

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

Table 3. Chemicals Predicted as In-vitro Non-Sensitisers in the in-vitro Assays (with LLNA EC3 as reference)

Conclusions
- A total of 24 proficiency chemicals were tested according to the OECD guidelines (ANNEX II) across three skin sensitisation assays: DPRA, KeratinoSens™ and hCLAT.
- 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 substances of registered for REACH registration (2016).
- European and national legislation will make in-vitro testing the standard data source for skin sensitisation regardless of industry.

References

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.

Figure 2. Skin sensitisation weight-of-evidence decision tree. The skin sensitisation safety assessment requires a 2 of 3 weight of evidence approach. If an unequivocal 2-from-3 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.

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Abstract

Until 2016, assessment of skin sensitising potential of chemicals required classical *in vivo* tests. The Adverse Outcome Pathway (AOP) for skin sensitisation revealed key events that could be assayed, thus enabling *in vitro / in chemico* testing to replace *in vivo* testing in many cases. In 2015/6, the OECD published Test Guidelines for 3 *in vitro* methods to predict skin sensitisation when used in conjunction with each other. Further efforts from the European Union Reference Laboratory – European Centre for Validation of Alternative Methods (EURL-ECVAM) led to 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 skin sensitisation.

The Direct Peptide Reactivity Assay (DPRA) detects protein haptenation (chemical transfer through the skin) - the first key event in the AOP. KeratinoSens™ Assay (Givaudan Schweiss, Switzerland) detects the cellular response in basal epidermal cells - the second key event - via a Nrf2 reporter and the human Cell Line Activation Test (hCLAT) detects dendritic cell (and hence the immune system) activation - the third key event. By combining these assays, it is now possible to detect skin sensitisation using *in vitro* and *in chemico* assays.