Short Communication

Adaptation of the KeratinoSens™ Skin Sensitization Test to Animal-Product-Free Cell Culture

Nathalie Belot, Bushra Sim, Christopher Longmore, Lottie Roscoe and Carol Treasure

XCellR8 Ltd, The Innovation Centre, Sci-Tech Daresbury, Daresbury, Cheshire, UK

Summary

Skin sensitization is the process by which a substance induces an allergic reaction following skin contact. The process has been described as an adverse outcome pathway (AOP), including several key events, from skin penetration and covalent protein binding, to keratinocyte activation, dendritic cell activation and T-lymphocyte proliferation. The *in vitro* assay KeratinoSens™ measures the activation of keratinocytes. It is fully accepted at a regulatory level (OECD TG 442d) and complies with a range of legislation including the EU Cosmetics Regulation, REACH, and the CLP Regulation.

Currently, many *in vitro* methods use animal-derived components in their cell culture systems. Many stakeholders in the cosmetics industry have both scientific and ethical concerns relating to this issue and have stated a strong preference for fully human *in vitro* test systems. We have adapted the KeratinoSens™ method to animal product-free conditions, and carried out an in-house validation with 21 reference substances, including those listed in the Performance Standards associated with OECD TG442d. The modified method was shown to be equivalent to the Validated Reference Method (VRM), with comparable values for accuracy (85.7%), sensitivity (84.6%) and specificity (87.5%), and all acceptance criteria being met. In Europe, data generated by the adapted method may be used in REACH submissions, and we are now seeking approval to list the adaptation in OECD TG 442d, enabling formal compliance with a range of global regulations.

Keywords: skin sensitization, KeratinoSens, alternatives to animal testing, 3R, *in vitro*

1 Introduction

Skin sensitization is a multi-step process that has been described as an Adverse Outcome Pathway (AOP). Following skin penetration, chemicals may bind covalently to skin proteins (key event 1), followed by the activation of keratinocytes (key event 2) and activation of dendritic cells (key event 3). Dendritic cells migrate to the lymph nodes and induce the proliferation of T-lymphocytes (key event 4).

Under pressure from legislation, including the European Cosmetics Regulation 1223/2009 and REACH (Registration, Evaluation, Authorization and restriction of CHemicals), many groups have worked in recent years on identifying relevant alternatives to traditional animal methods to determine the skin sensitization potential of chemicals (Reisinger et al., 2015). Alternatives for key events 1-3 have recently been adopted as OECD guidelines: DPRA (OECD TG 442c), KeratinoSens™ (OECD TG 442d) and h-CLAT (OECD TG 442e).

The KeratinoSens™ method addresses the second key event of skin sensitization, i.e., the activation of keratinocytes. KeratinoSens™ cells are derived from the human keratinocyte cell line HaCat, containing a luciferase gene that is under the control of a constitutive promoter fused with the antioxidant response element (ARE) from a gene that is known to be up-regulated by contact sensitizers. The majority of skin sensitizers induce this pathway and, therefore, the luciferase signal reflects the activation by sensitizers of endogenous Nrf2 dependent genes. This method was fully validated and gained regulatory acceptance in 2015 (Natsch et al., 2010, 2011, 2013).

The KeratinoSens™ method represents a great advancement in the replacement of animal testing. However, the published protocol currently includes the use of animal-derived cell culture components (bovine serum and porcine trypsin). XCellR8's mission is the full replacement of animal testing for cosmetics and their ingredients globally, motivated by an industry requirement to maximize the human relevance of *in vitro* models, along with

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animal welfare considerations. This strategy includes eradication of the significant number of animal-derived components that are still widely used in cell culture methods (including serum, tissue extracts and antibodies). In this project, minor adaptations to an existing method enable the complete replacement of animals for testing key event 2 in the skin sensitization AOP. They enable the method described in OECD TG 442d to be conducted without the need for a number of animal components by replacing them with human equivalents. Key animal components replaced are fetal calf serum and porcine trypsin. The significant animal welfare concerns around the production of these components has been well documented (Jochems et al., 2002; van der Valk et al., 2017).

Here, we describe the internal validation of the modified method using 21 reference chemicals, including those described in the Performance Standards (OECD, 2015) for skin sensitization and in OECD TG 442d. The reference chemicals are comprised of a panel of different sensitizer categories (minimal to severe and non-sensitizers), as defined by the animal-based Local Lymph Node Assay (LLNA).

2 Materials and methods

Test chemicals

All chemicals were purchased from Sigma-Aldrich (UK) and selected following description in the Performance Standards (OECD, 2015) for skin sensitization and in OECD TG 442d. Cinnamic aldehyde was used as the positive control in the assays (Sigma-Aldrich, UK CAS # 14371-10-9). Test chemicals were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 mM. They were further diluted in culture medium to a final concentration of 2000 µM in 1% DMSO and then serially diluted in culture medium with 1% DMSO (DB-ALM Protocol 155 (DB-ALM, 2013). The reference chemicals were: isopropanol (67-63-0); salicylic acid (69-72-7); lactic acid (50-21-5); glycerol (56-81-5); 4-methoxy-acetophenone (100-06-1) chlorobenzene (108-90-7); methyl salicylate (119-36-8); sulfanilamide (63-74-1); ethylene glycol dimethacrylate (97-90-5); phenyl benzoate (93-99-2); eugenol (97-53-0); 2-mercaptobenzothiazole (149-30-4); citral (5392-40-5); isoeugenol (97-54-1); methyldibromo glutaronitrile (35691-65-7); 4-methylaminophenol sulphate (55-55-0); para-phenylenediamine (106-50-3); 2,4-dinitrochlorobenzene (97-00-7); 4-nitrobenzylbromide (100-11-8); oxazolone (15646-46-5); cinnamyl alcohol (104-54-1).

Cell culture

The KeratinoSens™ cell line contains a stable insertion of a luciferase gene under the transcriptional control of a constitutive promoter fused with the ARE (Antioxidant Response Element) from the AKR1C2 gene (Lou et al., 2006). KeratinoSens™ cells were developed by Emter et al. (2010) and purchased from Givaudan. Upon first thawing from cryopreservation, cells were adapted to cell culture medium containing human serum. Cells were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) containing Glutamax (Life Technologies), 10% human serum (pooled human male AB plasma, 60-70 donors, Sigma-Aldrich UK) and Geneticin (500 µg/ml, Life Technologies) at 37°C, 5% CO_2 to a maximum passage number of 22. They were grown to a maximum of 80-90% confluence. Sub-culture was performed using TrypZean® (Sigma-Aldrich), a recombinant, animal-free equivalent of porcine trypsin.

KeratinoSens™ assay

The KeratinoSens™ assay was performed as previously described (Emter et al., 2010; DB-ALM, 2013) with the following adaptations and optimization under animal-product-free conditions.

Cells were seeded in 3 parallel replicate 96-well plates for luciferase activity testing and in one 96-well plate for MTT viability testing in medium containing 10% human serum. Plates were incubated at 37°C, 5% CO_2 for 24 h prior to treatment. At the dosing time point, culture medium was removed and replaced with fresh culture medium containing 1% human serum and 1% DMSO. Cells were then exposed to the reference chemicals and controls at 12 different concentrations (max. 2000 µM) and incubated for 48 h at 37°C, 5% CO_2. After the incubation period, luciferase activity was evaluated by luminescence measurement (Luciferase Assay System, Promega, UK) and cell viability was evaluated using the MTT viability assay (Sigma-Aldrich). The assay was repeated 3 times. For each chemical in each repetition and at each concentration, the luciferase gene induction was compared to the negative control (1% DMSO). A maximum of 3 outliers out of 9 values were removed to obtain a CV value below 20%. As per the VRM (Validated Reference Method), the following parameters were calculated: the I_{max} value (maximal average fold induction of luciferase activity observed at any concentration of the tested chemical and positive control); the $EC_{1.5}$ value, representing the lowest concentration for which induction of luciferase activity is above the 1.5 fold threshold (i.e., 50% enhanced luciferase activity); and the IC_{50} and IC_{30} concentration values for 50% and 30% reduction of cellular viability, respectively.

The positive control, cinnamic aldehyde, was used at a concentration range of 8 to 128 µM and required to meet the following acceptance criteria: average induction (I_{max}) in the three replicates for cinnamic aldehyde at 32 µM between 1.6 and 3; $EC_{1.5}$ value between 6 µM and 39 µM.

Test items were considered as positive for skin sensitization if the following conditions were met in 2 of 3 repetitions: The I_{max} was higher than 1.5 fold and statistically significantly different as compared to the solvent (negative) control (as determined by a two-tailed, unpaired Student's t-test); the cellular viability was higher than 70% at the lowest concentration with induction of luciferase activity above 1.5 fold (i.e., at the $EC_{1.5}$ determining concentration); the $EC_{1.5}$ value was less than 1000 µM; there was an apparent overall dose-response for luciferase induction.

3 Results

The KeratinoSens™ protocol adapted to animal-free conditions was performed to predict the skin sensitization potential of 21 reference chemicals, including those listed in the Performance Standards (OECD, 2015) and OECD TG 442d.

For each chemical, the I_{max} value and the $EC_{1.5}$ concentration were calculated (Tab. 1) (I_{max} maximal average fold induction of luciferase activity observed at any concentration of the tested chemical and positive control); the $EC_{1.5}$ value, representing the lowest concentration for which induction of luciferase activity is above the 1.5 fold threshold (i.e., 50% enhanced luciferase activity).

Among the 8 reference compounds classified as non-sensitizers *in vivo* (based on published LLNA results), one was misclassified (4-methoxy-acetophenone), in common with the KeratinoSens™ VRM.

Regarding the 13 chemicals classified as sensitizers *in vivo* (based on published LLNA results), 2 were misclassified (phenyl benzoate and eugenol), in common with the KeratinoSens™ VRM.

Results show that the prediction for the 21 reference chemicals was identical to the VRM (Emter et al., 2010; Natsch and Emter, 2008).

Both assays correctly predicted 18 chemicals of the total 21 chemicals and incorrectly predicted the same 3 chemicals, when compared with LLNA results: 4-methoxy-acetophenone was predicted as a skin sensitizer ("false positive") while phenyl benzoate and eugenol were predicted to be non-sensitizers ("false negatives"). In compliance with the acceptance criteria of the Performance Standards, no strong or extreme sensitizer was under-predicted with the adapted method.

Furthermore, in the case of eugenol, the result obtained in our laboratory was a "borderline" classification, as the 2 last concentrations were determined as positive but with a cell viability below the 70% threshold.

Tab. 1: Results obtained for the 21 reference chemicals (mean of 3 independent repetitions in triplicate)
These results are compared to VRM KeratinoSens™ assay results and to *in vivo* classification of the chemicals, based on EC3 values from literature results of the LLNA test method: > 10 weak, < 10 and > 1 moderate, < 1 and > 0.1 strong, < 0.1 extreme (OECD, 2015).
The 3 underlined chemicals were misclassified as compared to *in vivo* results. S, sensitizer; NS, non-sensitizer; I_{max}, maximal fold gene induction; $EC_{1.5}$, concentration in µM for 1.5-fold induction; n.i., no induction.

Reference substances	*In vivo* category (according to LLNA)	VRM			Animal-product-free method		
		I_{max} (fold)	$EC_{1.5}$ (µM)	Prediction	I_{max} (fold)	$EC_{1.5}$ (µM)	Prediction
Isopropanol	NS	1.2[a]	n.i.[a]	NS[a]	1.2	n.i.	NS
Salicylic acid	NS	1.1[a]	n.i.[a]	NS[a]	1.4	n.i.	NS
Lactic Acid	NS	1.3[a]	n.i.[a]	NS[a]	1.3	n.i.	NS
Glycerol	NS	1.2[a]	n.i.[a]	NS[a]	1.4	n.i.	NS
4-methoxy-acetophenone	NS	1.7[b]	449.3[b]	S[b]	2.1	620	S
Chlorobenzene	NS	1.2[a]	n.i.[a]	NS[a]	1.2	n.i.	NS
Methyl salicylate	NS	1.2[a]	n.i.[a]	NS[a]	1.2	n.i.	NS
Sulfanilamide	NS	1.4[a]	n.i.[a]	NS[a]	1.1	n.i.	NS
Cinnamyl alcohol	S (weak)	1.7[a]	123.6[a]	S[a]	4.2	20	S
Ethylene glycol dimethacrylate	S (weak)	188.4[a]	57.4[a]	S[a]	4.8	29	S
Phenyl benzoate	S (weak)	1.3[a]	n.i.[a]	NS[a]	1.1	n.i.	NS
Eugenol	S (weak)	1.3[a]	n.i.[a]	NS[a]	2.2	286	NS borderline
2-Mercaptobenzothiazole	S (moderate)	8.8[a]	48.1[a]	S[a]	6.9	57	S
Citral	S (moderate)	96.4[a]	23.2[a]	S[a]	3.8	18	S
Isoeugenol	S (moderate)	6.4[a]	16.1[a]	S[a]	3.4	20	S
Methyldibromo glutaronitrile	S (strong)	4[a]	7.8[a]	S[a]	2.7	8	S
4-Methylaminophenol sulphate	S (strong)	5.9[a]	9.4[a]	S[a]	36.1	4	S
Para-phenylenediamine	S (strong/extreme)	26.8[a]	5[a]	S[a]	28.2	6	S
2,4 Dinitro-chorobenzene	S (extreme)	14.8[a]	2.5[a]	S[a]	8.5	1	S
4-Nitrobenzylbromide	S (extreme)	6.9[a]	1.3[a]	S[a]	10.5	< 0.98	S
Oxazolone	S (extreme)	2.4[a]	175.5[a]	S[a]	5.4	129	S

[a] Data from Emter et al. (2010)
[b] Data from Natsch and Emter (2008)

4 Discussion

Many advances have been made in terms of achieving full regulatory acceptance for scientifically advanced *in vitro* methods to replace traditional animal-based safety testing. Examples include: OECD TG431, OECD TG439 and OECD TG492 using reconstructed human tissue models to assess skin corrosion, skin irritation and eye irritation, respectively. Recently, three methods for the assessment of key events in the skin sensitization AOP have gained full acceptance and have been incorporated into the REACH Regulation in Europe as the methods of choice for this endpoint, i.e., OECD TG442c, OECD TG442d and OECD TG442e. The traditional animal-based method for the assessment of skin sensitization, OECD TG429, the Local Lymph Node Assay (LLNA), may now only be used as a last resort, representing a significant shift towards the adoption of *in vitro* technologies in the context of regulatory safety testing.

In spite of this substantial progress, it is important to recognize that many *in vitro* methods currently use animal-derived components such as bovine serum, mouse antibodies, rat tissue extracts, and a variety of animal-derived cell lines. As such, these methods still ultimately require the sacrifice of animals and cannot be considered completely animal-free. Such an approach presents scientific limitations by unnecessarily compromising the direct relevance of the test system to humans. The significant animal welfare concerns around the production of these components has also been well documented (Jochems et al., 2002; Even et al., 2006; van der Valk et al., 2017). Importantly, these scientific and ethical drawbacks give rise to concerns within the cosmetics and personal care industry. The European Cosmetics Regulation 1223/2009 includes an animal testing ban for cosmetic products and ingredients.

Globally, animal testing is a highly emotive issue in many countries, and there is increasing demand from consumers for "cruelty-free" cosmetic products, in which neither the formulation nor individual ingredients have been tested on animals. Many ethical cosmetic companies seek to go beyond the "bare minimum" expectation, and to satisfy consumer demand by avoiding the use of animal-derived components altogether. In response to this demand, new methods developed at XCellR8 are animal-product-free from the outset, and the organization openly encourages other laboratories to adopt the same approach, avoiding the need for adaptation *after* the formal process of validation and regulatory acceptance has been completed. In addition, we have been asked by leading cosmetic companies to adapt existing regulatory methods to animal-product-free conditions, and to seek regulatory acceptance for the updated protocols.

In this project, the widely used KeratinoSens™ test method for skin sensitization (OECD TG 442d) was adapted to animal-product-free conditions and further optimized by making the following minor adaptations to the established protocol: 1. The use of human serum in cell culture (OECD TG 442d states only "serum" but the Validated Reference Method (VRM) protocol states "foetal calf serum"); 2. non-animal recombinant trypsin (TrypZean®) used to harvest cells; 3. MTT method adapted to the widely-used protocol consisting of 3 h incubation, solubilization in isopropanol and absorbance measurement at 570 nm; 4. positive control (cinnamic aldehyde) concentration range optimized to 8-128 µM for the adapted test conditions.

The KeratinoSens™ cell line adapted well to routine culture in human serum (in place of bovine serum), showing healthy morphology and comparable growth rates. This enabled the rapid creation of an internal cell bank for long term use in the test. A maximum passage number of 22 was set to reflect the optimum passage window for assay performance using the cells cultured in human serum.

Since there have been no changes to the cell lines or key steps in the protocols, the changes constitute an adaptation to OECD TG442d, and not a "me-too" method.

Subsequently, the adapted method was validated in-house using the 10 proficiency chemicals listed in OECD TG 442d, and the 11 additional chemicals listed in the associated Performance Standards (of the 20 chemicals listed in the Performance Standards, 9 were already present in the Proficiency Chemicals listed in OECD TG 442d). These chemicals spanned the full range of levels of skin sensitization observed in the LLNA, from extreme to non-sensitizers. The adapted method showed full concordance of all 21 chemicals with the VRM, i.e., the KeratinoSens™ published protocol. In common with the VRM, the adapted method identified two "false negatives" (phenyl benzoate and eugenol) and one "false positive" (4-methoxyacetophenone) when compared with the LLNA. It is worth noting that the classification of chemicals as "false positive" or "false negative" by *in vitro* tests makes an assumption that the classification generated by the animal *in vivo* test (in this case the LLNA) is predictive of the human response. This is a common practice in the development of *in vitro* methods that is worthy of review.

The Performance Standards stipulate that "*The accuracy, sensitivity and specificity of the proposed similar or modified test method should be comparable or better to that of the VRM. The accuracy, sensitivity and specificity obtained with the 20 reference substances should all be equal or higher (≥) than 80.0% (actual for KeratinoSens™ based on the 20 reference substances and using a weighted calculation: 87.0% accuracy, 86.7% sensitivity and 87.5% specificity).*"

All acceptance criteria of the Performance Standards were met as follows: Results obtained showed that the accuracy of the adapted method was 85.7%, the sensitivity was 84.6%, and the specificity was 87.5%. The "false positive" and "false negative" results were the same as those obtained with the VRM and no strong or extreme sensitizers were under-predicted. The cell line was the one used in the VRM and the endpoint was the same (luciferase activity measurement).

Our animal-product-free adaptation of the KeratinoSens™ test has proven to be robust and reproducible in our Good Laboratory Practice (GLP) accredited laboratory for over two years, and is performed in full compliance with GLP requirements.

The adapted method provides added value from both scientific and ethical perspectives. From a scientific perspective, the eradication of animal-derived components from *in vitro* test systems, and their replacement with human equivalents, directly increases the relevance of the test system to humans and would be expected to at least equal, if not enhance, the predictivity of the tests across a wide range of chemical categories. The adaptations also provide

cosmetic companies with *in vitro* systems with enhanced ethical value, in response to consumer demands to avoid the use of animals and animal-derived components altogether in cosmetics testing. Human equivalents to animal-derived cell culture components are freely available commercially, and should be sourced from a reputable supplier with full donor consent and safety screening. In our experience, increases in the cost of such products over the animal-derived equivalents are small and do not have an impact on the overall cost of performing the test. We recommend the use of human serum from pooled donors. As with any type of serum, when a new batch is used, an internal validation of the batch including cell morphology, growth rates and I_{max} / $EC_{1.5}$ values with the positive control and representative reference chemicals should be conducted, with subsequent reservation of successfully performing batches for long term use. Over the past 2.5 years, more than 15 batches of human serum have been used in our laboratory to perform the test, each batch consisting of pooled serum from 60-70 donors. Over this time, the mean induction value of the positive control, cinnamic aldehyde, showed good reproducibility (1.84 ± 0.322 at a concentration of 32 µM).

The adaptation to the KeratinoSens™ test method, described here, will contribute to further international harmonization of hazard and risk assessment. Regulatory acceptance would enable incorporation into the skin sensitization IATA (Integrated Approach to Testing and Assessment) as a completely animal-product-free *in vitro* test option. We recently obtained clarification from the European Chemicals Agency (ECHA) that data using the adapted method may be used in REACH submissions, provided that the Performance Standards data, demonstrating equivalence with the VRM, is included in the dossier. The path to inclusion in OECD TG442d is already underway. Formal listing in the TG would mean that the proposed adaptation to the existing method will become globally available for use by test laboratories, for compliance with a range of chemical safety regulations around the world. Subsequent adoption by other laboratories will collectively avoid the unnecessary use of a large volume of animal-derived serum in the build-up to the 2018 REACH deadline and beyond, while maximizing the relevance of the test to human safety.

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Conflict of interest

None of the authors have potential conflicts of interest.

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Correspondence to

Carol Treasure, PhD
The Innovation Centre
Sci-Tech Daresbury
Keckwick Lane, Daresbury
Cheshire WA4 4FS, UK
Phone: +44 1925 607 134
e-mail: carol.treasure@x-cellr8.com