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# Development of an in vitro method to estimate the sensitization induction level of contact allergens

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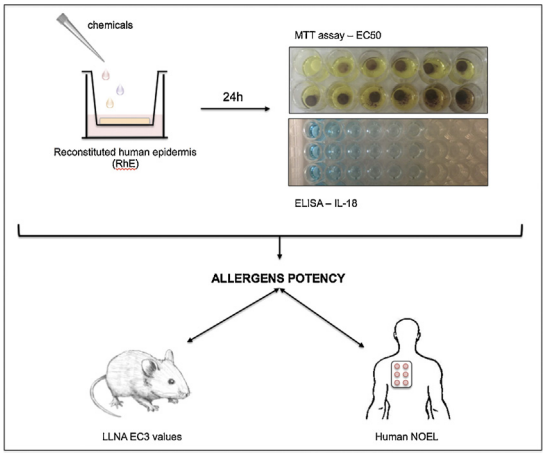
## HIGHLIGHTS

- The RhE IL-18 assay better predicts human skin sensitizers.
- A simple *in vitro* method to estimate *in vivo* potency was developed.
- In the current paper, 14 chemicals not previously tested were used.
- Good correlations between *in vitro* predicted LLNA EC3 and human NOEL values with *in vivo* available data were found.

## GRAPHICAL ABSTRACT

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## ABSTRACT

No standardized *in vitro* methods to assess potency of skin sensitizers are available. Recently, we standardized a procedure which combines the epidermal equivalent potency assay with assessment of IL-18 to provide a single test for identification and classification of skin sensitizers. This current study aimed to extend tested chemicals, and to provide a simple *in vitro* method for estimation of the expected sensitization induction level interpolating *in vitro* EC50 and IL-18 SI2 values to predict LLNA EC3 and/or human NOEL from standards curves generated using reference contact allergens. Reconstituted human epidermis was challenged with 14 chemicals not previously tested benzoquinone, chlorpromazine, chloramine T, benzyl salicylate, diethyl maleate, dihydroeugenol, 2,4-dichloronitrobenzene, benzyl cinnamate, imidazolidinyl urea, and limonene as contact sensitizers while benzyl alcohol, isopropanol, dimethyl isophthalate and 4-aminobenzoic acid as non-sensitizers in the LLNA. Where for benzyl salicylate and benzyl cinnamate no sensitization was observed in human predictive studies, positive responses to benzyl alcohol and dimethyl isophthalate were reported. The proposed method correlates better with human data, correctly predicting substances incorrectly classified by LLNA. With the

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exception of benzoquinone (interference with both MTT and IL-18 ELISA), and chloramine T (underestimated in the interpolation), a good estimation of LLNA EC3 and *in vivo* available human NOEL values was obtained.



## 1. Introduction

Contact allergy is caused by skin contact with low molecular weight chemicals, which may evolve to allergic contact dermatitis (ACD) if exposure exceeds the individual threshold (Rustemeyer et al., 2006). In industrialized countries, ACD is the most frequent manifestation of immunotoxicity (Thyssen et al., 2007). Over 4000 chemical substances are considered contact allergens and are linked to the induction of ACD in humans (De Groot, 1994). Chemical allergy is of considerable importance to the toxicologist and regulatory authorities worldwide require testing for ACD and appropriate hazard labeling to minimize exposures. ACD is a preventable disease that can be avoided by proper chemical and product testing to identify and label potential contact allergens; by characterization of potency; by understanding of human skin exposure; and by application of adequate risk assessment and management strategies (Corsini et al., 2014; Boeniger and Ahlers, 2003; Peiser et al., 2012).

Potency refers to the intrinsic property of a sensitizing chemical and is based on the concentration of chemical needed to induce a positive response (ICCVAM, 2011). Potency data can lead to improvements in hazard classification and also in risk management, and can facilitate improved risk assessment for skin sensitization. Risk assessment strategies serve to ensure that human exposure to skin sensitizers is managed in accordance with their potency.

Currently the evaluation of the contact sensitization potential of chemicals is done using the LLNA that, in addition to the hazard identification, has proven very useful in assessing the skin sensitizing potency of chemicals, based on the estimation of the concentration of chemical required to induce a stimulation index of three relative to concurrent vehicle-treated controls (EC3 value). Low EC3 values correlated well with sensitizers known to be potent in man, whereas high EC3 values were usually associated with weakly human sensitizers (Basketter et al., 1999, 2014). Although the identification of potentially sensitizing chemicals is carried out using animal models, in Europe legislative changes impose and promote the use of non-animal methods (*i.e.* Cosmetic Directive, REACH). Skin sensitization is a very active research area and in the last 20 years significant progress has been made for the *in vitro* identification of contact sensitizers. Mechanistically based *in chemico* and *in vitro* test methods have been considered scientifically valid by the OECD for the evaluation of the skin sensitization hazard of chemicals. In particular, OECD test guidelines (TGs) on DPRA (TG 442C) and KeratinoSense™ (TG 442D) were published respectively in February and July 2015, while h-CLAT and other methods will be soon available. Current available methods support the discrimination between skin sensitizers and non-sensitizers but they cannot be used to predict potency for safety assessment decision, which is necessary to fully replace animal testing. There is a need to define exactly what processes and events determine the potency with which contact allergens cause the acquisition of skin sensitization and to model this *in vitro* (Kimber et al., 2011; Corsini et al., 2016). This will require a better understanding of the molecular events, including pathway analysis and marker signature identification, that trigger cell activation, following exposure to contact allergens (Corsini et al., 2016). The identification of the mechanisms influencing the vigor of T cell responses, that can explain the strength of contact hypersensitivity reactions to weak, moderate, strong, and extreme sensitizers is a challenge still to be solved. The idea being that most powerful contact allergens may cause stronger inflammatory reactions compared to less potent allergens, which may impact maturation, migration of skin dendritic cells that in turn may influence the quality of the elicited immune response.

Keratinocytes (KCs) play a key role in the activation of all skin immune responses. The second key event identified in the adverse outcome pathway (AOP) for skin sensitization takes place in the KCs. It includes inflammatory responses as well as expression of genes associated with specific cell signaling pathways. Among the different factors produced by KCs, IL-18 has been shown to play a key proximal role in the induction of allergic contact sensitization (Antonopoulos et al., 2008; Okamura et al., 1995), and recent evidence provided from our group, has shown that IL-18 production in human KCs (NCTC 2544) can be used as a sensitive method to identify contact allergens, discriminating them from respiratory allergens and irritants, with a sensitivity of 87%, specificity of 95% and an accuracy of 90% (Corsini et al., 2009, 2013; Galbiati et al., 2011). Replacing NCTC 2544 cell line with a standardized commercially available reconstituted human epidermis (RhE), as described in our previous manuscript (Gibbs et al., 2013), made the assay extremely transferable and easy to perform (Teunis et al., 2014; Andres et al., 2016).

Currently, methods based on full thickness skin models are being explored to resolve sensitizing potency (Corsini et al., 2016). RhE models have several advantages over traditional cell cultures, and are expected to have a broader applicability domain. Topical application in relevant vehicles (*e.g.* solvents used in animal tests or cosmetic/dermatological formulations) is only possible in RhE models, which mimic *in vivo* bio-availability of a chemical more closely, and which may therefore lead to improved assessment of sensitizer potency (Van der Veen et al., 2014). We previously demonstrated the possibility of combining the epidermal equivalent potency assay, based on the irritation potential, with the assessment of IL-18 release (RhE IL-18 potency assay) to provide a single test for identification and classification of skin sensitizing chemicals, including chemicals of low water solubility or stability (Gibbs et al., 2013). Correlating the *in vitro* RhE sensitizer potency data (*i.e.* EC50 and IL-18 SI2) with human and animal data a good correlation with both human DSA05 and LLNA EC3 values ($p < 0.001$) were observed. This work has led to a standard procedure which is currently being implemented using different commercially available and in house RhE in different countries to determine the applicability domain of the assay.

As part of this extensive and ongoing study, the aim of this paper was to extend the list of tested chemicals in the RhE IL-18 potency assay, and to provide a simple method for the *in vitro* estimation of the expected sensitization induction level, which is required for full replacement of animals. Using reference contact allergens, namely DNCB, isoeugenol, cinnamal and benzocaine, standard curves were created to predict *in vivo* data based on *in vitro* EC50 and IL-18 SI2, which can be very useful in risk assessment. Results obtained are very encouraging, and further compounds should be tested to better define the applicability and limitation of the RhE IL-18 potency test.