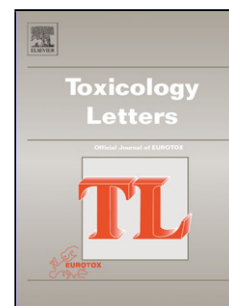


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**DEVELOPMENT OF AN *IN VITRO* METHOD TO ESTIMATE THE SENSITIZATION
INDUCTION LEVEL OF CONTACT ALLERGENS**

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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 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.

Keywords: reconstituted human epidermis, 3D model, *in vitro*, contact allergens, potency, contact hypersensitivity.

60 INTRODUCTION

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

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

77 Currently the evaluation of the contact sensitization potential of chemicals is done using the
 78 LLNA that, in addition to the hazard identification, has proven very useful in assessing the
 79 skin sensitizing potency of chemicals, based on the estimation of the concentration of
 80 chemical required to induce a stimulation index of three relative to concurrent vehicle-treated
 81 controls (EC3 value). Low EC3 values correlated well with sensitizers known to be potent in
 82 man, whereas high EC3 values were usually associated with weakly human sensitizers
 83 (Basketter et al., 1999 and 2014). Although the identification of potentially sensitizing
 84 chemicals is carried out using animal models, in Europe legislative changes impose and
 85 promote the use of non-animal methods (i.e. Cosmetic Directive, REACH). Skin sensitization
 86 is a very active research area and in the last 20 years significant progress has been made for
 87 the *in vitro* identification of contact sensitizers. Mechanistically based *in chemico* and *in vitro*
 88 test methods have been considered scientifically valid by the OECD for the evaluation of the
 89 skin sensitization hazard of chemicals. In particular, OECD test guidelines (TGs) on DPRA
 90 (TG 442C) and KeratinoSense™ (TG 442D) were published respectively in February and July
 91 2015, while h-CLAT and other methods will be soon available. Current available methods
 92 support the discrimination between skin sensitizers and non-sensitizers but they cannot be
 93 used to predict potency for safety assessment decision, which is necessary to fully replace
 94 animal testing. There is a need to define exactly what processes and events determine the
 95 potency with which contact allergens cause the acquisition of skin sensitization and to model
 96 this *in vitro* (Kimber et al., 2011; Corsini et al., 2016). This will require a better understanding
 97 of the molecular events, including pathway analysis and marker signature identification, that
 98 trigger cell activation, following exposure to contact allergens (Corsini et al., 2016). The
 99 identification of the mechanisms influencing the vigor of T cell responses, that can explain the

100 strength of contact hypersensitivity reactions to weak, moderate, strong, and extreme
 101 sensitizers is a challenge still to be solved. The idea being that most powerful contact
 102 allergens may cause stronger inflammatory reactions compared to less potent allergens,
 103 which may impact maturation, migration of skin dendritic cells that in turn may influence the
 104 quality of the elicited immune response.

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

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

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

140 and limitation of the RhE IL-18 potency test.

141

142

143 MATERIALS AND METHODS

144

145 *Chemicals*

146 A total of 10 contact sensitizers and 4 non-sensitizers were studied (Table 1). Chemicals
147 were selected from the ICCVAM database and the LLNA database of 341 chemicals
148 (Gerberick et al., 2005; Kern et al., 2010), and not previously tested in the RhE assay, namely
149 benzoquinone, chlorpromazine, chloramine T, benzyl salicylate, diethyl maleate,
150 dihydroeugenol, benzyl cinnamate, 2,4-dichloronitrobenzene, imidazolidinyl urea, and
151 limonene as contact sensitizers while benzyl alcohol, isopropanol, dimethyl isophthalate and
152 4-aminobenzoic acid as non-sensitizers in the LLNA. All reagents and chemicals were
153 purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA) at the highest purity available. Cell
154 culture medium (EPI-100-MM-60) was supplied by MatTek Corporation.

155

156 *Reconstituted Human Epidermis (RhE)*

157 The EpiDerm™ (MatTek In Vitro Life Science Laboratories, Bratislava Slovak Republic) was
158 used throughout the study. EpiDerm™ is produced from normal human keratinocytes
159 obtained from neonatal male skin donors. EpiDerm™ was maintained according to the
160 suppliers' instructions at 37 °C, 5% CO₂ and 95% relative humidity. After 24 h of equilibration
161 time, RhE were exposed to chemicals as described below.

162

163 *Chemical exposure*

164 The assay was performed in order to determine the EC50 value of the chemical (EC50 =
165 effective chemical concentration required to reduce RhE metabolic activity - corresponding to
166 cell viability - to 50% of the maximum value compared to vehicle exposed cultures) and the
167 release of IL-18 associated with its treatment. According to SOP (Gibbs et al., 2013; Teunis et
168 al., 2013, 2014), the maximum solubility of chemical was first identified by dissolving the
169 compound in different vehicles. Dilutions were made in the following order: 200, 100, 50, 25,
170 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.20 and 0.10 mg/ml until a clear solution was reached. The
171 vehicle with the highest dissolving capacity was chosen. Vehicles used are reported in the
172 last column of Table 1.

173 Chemicals were then tested in dose-response experiments starting with the highest soluble
174 concentration and decreasing with 2-fold serial dilutions for a total of five concentrations. Test
175 chemicals stock solutions were freshly prepared in acetone:olive oil (4:1) or in medium. For

176 exposure, Finn Chamber filter paper discs 8 mm (Epitest LTD Oy, Finland) were impregnated
 177 with chemicals or vehicle controls and applied topically to the RhE stratum corneum. Cultures
 178 were incubated with chemicals for 24 h. After chemical exposure, filter paper discs were
 179 removed and metabolic activity was determined immediately by the MTT assay, while culture
 180 supernatants were harvested and stored at -80°C for IL-18 quantification by ELISA as
 181 described below.

182 Chemicals were tested in single in two independent RhE batches. Control conditions
 183 (unexposed, vehicle(s) and positive control (DNCB 3 mg/ml in AOO)) were tested in double in
 184 each experiment.

185

186 *Viability*

187 The MTT assay measures mainly mitochondrial activity, which is representative of cell
 188 viability (Mosmann, 1983). After 24 h exposure, filter paper discs were removed from RhE.
 189 The MTT analysis was performed in 24-well plates. Thiazolyl blue tetrazolium bromide (MTT)
 190 (Sigma) solution in PBS (0.5 mL/well of a 5 mg/mL solution) was added to the 24-well plate,
 191 RhE cultures were placed on top and further incubated for 2 h under standard culture
 192 conditions. Cultures were then transferred to a new 24-well plate containing 0.3 mL
 193 isopropanol to dissolve the formazan crystals. Plates were incubated overnight, sealed with
 194 parafilm and protected from the light at room temperature. The absorbance was measured at
 195 595 nm and cell viability is expressed as a % relative to the absorbance value of vehicle
 196 control treated RhE. The RhE-EC50 values were obtained by linear regression analysis
 197 based on changes in metabolic activity (MTT).

198

199 *IL-18 ELISA*

200 The mission of the Alternatives Research & Development Foundation (ARDF) is to fund and
 201 promote the development, validation, and adoption of non-animal methods in biomedical
 202 research, product testing, and education. To do this, ARDF guidelines prohibit the use of
 203 monoclonal antibodies derived from mouse ascites fluid, therefore, we had developed an
 204 alternative IL-18 ELISA using antibodies not derived from ascites, as described below. ELISA
 205 plates were coated with capture antibody (Mouse anti-human IL-18-UNLB, Southern Biotech,
 206 AL, USA) 0.1 $\mu\text{g}/\text{ml}$ in PBS (dilution 1:5000 – 100 $\mu\text{l}/\text{well}$) and incubated o.n. at 4°C . After
 207 blocking for 1 h in 1% BSA in PBS, samples and standard curve (recombinant human IL-18,
 208 MBL International, MA, USA) were plated (100 $\mu\text{l}/\text{well}$) for 2h at RT. After washing, rabbit anti
 209 IL-18 (MyBioSource, CA, USA) was added (dilution 1:1000 in blocking buffer - 100 $\mu\text{l}/\text{well}$) for
 210 1 h at RT. After 1h, a goat anti rabbit IgG-horseradish peroxidase conjugate (BIO-RAD,
 211 Segrate, Italy) was added (dilution 1:15.000 in blocking buffer - 100 $\mu\text{l}/\text{well}$). After washing,
 212 the substrate solution (tetramethylbenzidine liquid substrate, Sigma Aldrich, Mo, USA) was
 213 added (100 $\mu\text{l}/\text{well}$). Plates were read at 595 nm. Results were calculated in pg/ml from a
 214 standard curve and then converted to a Stimulation Index (SI), calculated as:

$$IL18\ SI = \frac{(IL18\ pg/ml\ in\ chemical\ treated\ RhE)}{(IL18\ pg/ml\ in\ vehicle\ treated\ RhE)}$$

IL-18 SI2 values were obtained by linear regression analysis based on the chemical concentration resulting in a 2-fold increase in IL-18 release.

Determination of interference of chemical with MTT assay and IL-18 ELISA

If chemicals interfere with the readouts, they need to be excluded from the RhE IL-18 assay, following outside of the applicability domain of the assay. In order to determine whether a chemical interferes with the MTT assay, the highest soluble chemical concentration is incubated with the MTT solution in the absence of RhE. If a color change is observed, then the chemical must be excluded.

To determine if a chemical interferes with the ELISA, a known amount of recombinant human IL-18 (e.g. 250 pg/ml) is spiked directly into conditioned media of RhE treated with the chemical suspected for interference. If the calculated concentration differs from the nominal concentration of IL-18, the chemical must be excluded from the RhE potency assay as it interferes with the ELISA.

Data analysis

According to SOP (Gibbs et al., 2013; Teunis et al., 2013, 2014), all chemicals were tested in at least two independent experiments using different RhE batches. Data obtained in two independent experiments is presented. As results are incorporated into a prediction model, it is not feasible and not necessary to run stats on each independent experiment. The following readouts were used:

(1) For the assessment of allergenicity (YES/NO): if the fold increase in intracellular IL-18 is ≥ 2.0 in at least one of the concentrations tested the chemical is classified as contact sensitizer (H317). In case the two experiments gave inconsistent results (e.g. one positive and one negative), a third experiment was done, and the majority was used for classification (e.g. if two positives and one negative, the classification will be contact allergen).

(2) For potency assessment: the arithmetic means of the values obtained in the two experiments for both cytotoxicity (MTT assay) expressed as of EC50 and IL-18 SI2 (chemical concentration resulting in 2 fold increases in IL-18 release) were considered.

Statistical analysis was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

RESULTS

Effect of selected chemicals on IL-18 production

A total of 14 chemicals were tested (Table 1). Chemicals were selected from the ICCVAM database. Allergens of different potency (1 extreme, 2 strong, 3 moderate and 4 weak) were investigated together with chemicals misclassified at the LLNA, namely benzyl salicylate and benzyl cinnamate for which no sensitization was observed in human predictive studies, and benzyl alcohol for which positive responses were reported in humans. In Table 1, the LLNA EC3 and the human NOEL or LOEL values are reported together with the vehicles used for the *in vitro* study.

Experiments were run accordingly with the SOP developed for the EE IL-18 assay (Gibbs et al., 2013; Teunis et al., 2013, 2014), which foresees two independent runs for each chemical to be tested. RhE was exposed to the selected chemicals for 24 h as described in Materials and Methods section. Cell viability was assessed by the MTT assay and IL-18 release by ELISA. Of the selected chemicals, only benzoquinone had to be excluded due to its interference with both MTT assay and the ELISA (data not presented).

The results obtained in two independent experiments are shown in Figure 1. Five increasing concentrations were tested for all chemicals. Graphics on the left show cell viability (% vs vehicle treated RhE), while IL-18 release is shown on the right (Stimulation Index). Dotted lines were set at 50% viability and IL-18 SI-2. The contact allergens chlorpromazine, chloramine T, diethyl maleate, dihydroeugenol, 2,4-dichloronitrobenzene, imidazolidinyl urea and limonene were able to a different extend to induce a IL-18 stimulation index ≥ 2.0 , while the non-sensitizers isopropanol and 4-aminobenzoic acid (Figure 2) failed to induce IL-18 release. Comparing the two experiments, cytotoxicity data are less variable compared to the release of IL-18, which may reflect a different degree of KCs activation between RhE batches. This greater variability is also seen in the data obtained with the positive control DNCB 2 mg/ml: IL18 SI 15.4 ± 4.9 (Min 8.0, Max 24.4), viability 11.6 ± 3.6 % (Min 6.9, Max 16.2), data is mean $\pm$ SD, n=11 independent experiments. Despite this, the positive/negative classification is not affected as for the prediction model the fold increase in intracellular IL-18 is ≥ 2.0 in at least one of the concentrations tested the chemical in both independent experiments is used to classify chemical as contact sensitizer.

The correlation between cytotoxicity and the strength of IL-18 release is not always straightforward. For some allergens, the decrease in cell viability is associated with the increase in IL-18 release. In other cases, increased IL-18 release can be observed at not cytotoxic concentrations, as in the case of limonene where a dose related release of IL-18 was observed in the absence of cytotoxicity.

An unexpected dose-related IL-18 release was observed with dimethyl isophthalate. While no EC50 values were obtained, IL-18 SI-2 could be calculated: 69.2 mg/ml in the 1° exp and 26.1 mg/ml in the 2° exp. This compound is classified as a not sensitizer in the LLNA. In spite of phthalates being widely used additives in plastics, there are few reports of skin problems

caused by them (Johansen et al., 2011), and two cases of contact allergy to dimethyl phthalate has been reported (Capon et al., 1996), which may support the positive result observed.

Benzyl cinnamate (Figure 3A, 3B) and benzyl salicylate (Figure 3C, 3D) are classified in the LLNA as contact sensitizers (moderate and weak, respectively), and in the RhE IL-18 assay as non-sensitizers since they failed to induce IL-18 release. Benzyl salicylate is classified as a not relevant human allergen (Lapczynski et al., 2007; Schnunch et al., 2007; Urbisch et al., 2013) and there are no human data for benzyl cinnamate that support its classification as contact sensitizer (Bathia et al., 2007; Ohkawara et al., 2010; Api et al., 2008). These two compounds are considered as false positives in the LLNA, both of which were properly classified by the RhE IL-18 assay. Otherwise, benzyl alcohol (Figure 3E, 3F), classified as a non-sensitizer based on the LLNA data, scored as a contact sensitizer with IL-18 RhE assay as it was able to reach an IL-18 stimulation index ≥ 2.0 . Literature research showed that benzyl alcohol is sensitizer in humans (Api et al., 2007; Belsito et al., 2012; Scognamiglio et al., 2012). Thus, these three chemicals were correctly classified in the IL-18 RhE assay. As it is emerging in many other *in vitro* studies, data obtained using human cells seem to correlate better with *in vivo* human data. However, additional weak allergens should be tested to define whether the RhE IL-18 assay can correctly identify weak allergens and false negative compounds at the LLNA.

RhE and potency estimation

As a good correlation between the *in vitro* EC50 and IL-18 SI2 values and *in vivo* LLNA EC3 and human DSA05 were observed in previous publication (Gibbs et al., 2013), we next investigated, the possibility to use a simple approach to estimate the *in vivo* induction sensitization level. Curves were created, using reference skin sensitizers of different potency, namely DNCB, isoeugenol, cinnamal and benzocaine. In Table 2, the *in vivo* and *in vitro* values for the reference contact allergens are reported. Linear regression curves were created by plotting *in vivo* LLNA EC3 or human NOEL values against *in vitro* EC50 or IL-18 SI2 arithmetic mean values (Figure 4). A $R^2 > 0.90$ with a $p < 0.05$ was obtained for all combinations (Figure 4 inserts). The predicted LLNA EC3 (%) and human NOEL ($\mu\text{g}/\text{cm}^2$) values for the tested chemicals were then calculated based on the arithmetic means of *in vitro* EC50 or IL-18 SI2 values obtained in two independent experiments. Despite different dose response profiles obtained for some chemicals in IL-18 release, comparing the *in vitro* predicted values using the arithmetic means of EC50 and IL-18 SI2 values with *in vivo* available data reported in Table 1, with the exception of chloramine T for which the LLNA EC3 was underestimated (and no human data is available), an excellent correspondence was observed. The predicted LLNA EC3 values were very close to the actual values, and each compound remained within the same LLNA class. The predicted human data are more difficult to interpret because of the limited human NOEL data available for the tested compounds (*de facto* available only for imidazolidinyl urea and limonene). For the latter two

compounds, however, the values were comparable, suggesting that the proposed approach can be indeed a valid strategy to estimate the *in vivo* LLNA EC3 and human NOEL starting from *in vitro* data.

DISCUSSION

While incredible progress has been made in the development of alternative *in vitro* tests to assess contact hypersensitivity, currently it is not possible to estimate sensitizing potency, necessary for quantitative risk assessment and full replacement of animals. The main aim of this study was to provide a simple, mechanistic based *in vitro* method to classify chemicals according to their sensitization potential and to estimate their induction sensitization level. Results obtained in this study support the possibility of using the RhE IL-18 assay for the identification and potency estimation of contact sensitizers.

The use of reconstituted epidermis overcomes the limitation of chemical solubility and stability in water solution, which are the major limitations of many *in vitro* methods based on the used of submerged cultures. Topical application in relevant vehicles is only possible in RhE models, which mimic *in vivo* chemical bio-availability more closely, and therefore may lead to improved assessment of sensitizer potency (Van der Veen et al., 2014; Corsini et al., 2016).

The predicted potency values we estimated were very close to the actual values, suggesting that the proposed approach can indeed be a valid strategy to estimate the *in vivo* LLNA EC3 and human NOEL starting from *in vitro* data. Overall, the arithmetic means of EC50 and IL-18 SI-2 values obtained in two independent experiments predicted similar *in vivo* values. The advantage of IL-18 SI-2 is evident in cases of non-cytotoxic compounds for which it is not possible to calculate an EC50, e.g. limonene.

Generally, a good relationship between LLNA EC3 values and Human Repeated Insult Patch Test (HRIPT) thresholds was historically shown (Griem et al., 2003; Schneider and Akkan, 2004; Basketter et al., 2005) but it is important to mention the limitations associated with the human data: a lot of data comes from older studies; Human Maximization Test (HMT) and HRIPT have differences in sensitivity; there is not a well standardized protocol; the intrinsic potency of chemical skin sensitizers in humans requires experimental studies of dubious ethics; and the intraspecies variability of human susceptibility to skin sensitization may confound the results (Gerberick et al., 2001; Griem et al., 2003; Schneider and Akkan, 2004; Basketter et al., 2005; ICCVAM 2011).

More potent contact allergens are expected to evoke at lower concentrations a stronger innate inflammatory reaction than less potent allergens, which will in turn influence the migration of skin dendritic cells, their degree of activation and, therefore, the quality of immune responses elicited (Kimber et al., 2012). Results obtained in this study confirmed the previous ones (Gibbs et al., 2013; Teunis et al., 2014) that showed how there is a general trend for IL-18 induction/cytotoxicity at lower concentrations for extreme/strong sensitizers, whereas higher concentrations are required in the case of weak sensitizers (Corsini et al., 2012).

368 The only compound apparently underestimated was chloramine T: an LLNA EC₃ value of
 369 27.14 % based on EC₅₀ or of 8.70 % based on IL-18 SI-2 were predicted versus a value of
 370 0.14%. Chloramine T is classified as a strong allergen, GHS class 1A in the animal studies. It
 371 is important to mention that in the LLNA positive responses are obtained by both contact and
 372 respiratory sensitizers, while the RhE IL-18 assay identify only contact allergens. It is thus
 373 possible that chloramine T would be mainly a type I sensitizer, and a weak contact sensitizer.
 374 This is likely to be the case in humans, where IgE-mediated allergic asthma caused by
 375 chloramine T is well know (Wass et al., 1989; Kramps et al., 1991), while skin symptoms,
 376 mainly allergic contact urticaria, have only seldom been reported (Dooms-Goossens et al.,
 377 1983; Lombardi et al., 1989; Kanerva et al., 1997), perfectly fitting our findings.
 378 Regarding the yes/no classification, based on animal data benzyl salicylate and benzyl
 379 cinnamate were incorrectly classified as not sensitizers, and 2 non sensitizers (benzyl
 380 alcohol, dimethyl isophthalate) were incorrectly classified as sensitizers. However, based on
 381 human data all chemicals, except dimethyl isophthalate, were correctly classified by the RhE
 382 IL-18 assay. Benzyl salicylate and benzyl cinnamate are fragrance ingredient compounds that
 383 can be found in cosmetics, shampoos, and toilets soaps as in household cleaners and
 384 detergents. It has emerged that both compounds, that share a similar chemical structure, are
 385 classified as not relevant human allergens (Lapczynski et al., 2007; Schnunch et al., 2007;
 386 Bathia et al., 2007; Ohkawara et al., 2010). Benzyl alcohol is a fragrance used like benzyl
 387 salicylate and benzyl cinnamate with a different chemical structure. Due to the presence of
 388 electronegative substituent and a side chain on the aromatic ring able to generate H bonds,
 389 benzyl alcohol acts as a sensitizer in human (Api et al., 2007; Belsito et al., 2012;
 390 Scognamiglio et al., 2012). Lastly, dimethyl isophthalate, considered as a not sensitizer in the
 391 LLNA, was classified as positive in the RhE IL-18 assay, inducing a dose-related release of
 392 IL-18. Only few cases of contact allergy to dimethyl phthalate has been reported (Capon et
 393 al., 1996), which may support our findings of dimethyl phthalate being a weak sensitizer.
 394 A comment should be made regarding 2,4-dichloronitrobenzene, mostly present in
 395 agrochemical products, which is often used as non-sensitizer structural analogue to 2,4-
 396 dinitrochlorobenzene (DNCB) in animal studies. Even if the weak sensitizing potential of
 397 DCNB (LLNA EC₃ of 20%) has been demonstrated in the LLNA by Basketter et al., 1996,
 398 there are evidences in literature that this compound is a human contact sensitizer, supporting
 399 our results (Basketter et al., 1996; Aleksic et al., 2007; Cruz et al., 2007; Roberts et al., 2014).
 400 To achieve a complete replacement of animals in skin sensitization assessment, dose-
 401 response information and evaluation of relative skin sensitizing potency to support effective
 402 risk assessment is necessary. Here, we propose a very simple and straightforward approach
 403 to estimate the LLNA EC₃ or human NOEL of a chemical sensitizer. In all biological
 404 responses, there are of course unavoidable variabilities, which also apply to in vitro and in
 405 vivo values we used in this paper. However, when it comes to risk assessment we still tend to
 406 use one value and apply a safety factor to take care of variability. This is also the case of
 407 quantitative risk assessment for skin sensitization where to calculate the acceptable exposure

408 level, the NESIL is divided by a Sensitization Assessment Factor, which takes care of inter-
409 individual variability, vehicle and matrix effects, and use considerations (Api et al., 2008).

410 In conclusion, even if further compounds must be tested to better define the applicability and
411 limitation of the RhE IL-18 potency test, using reference contact allergens, standard curves
412 can be created to estimate *in vivo* sensitization induction level, which can be useful in risk
413 assessment. A consortium of academic and industrial partners has now established a generic
414 SOP for the method applicable to several RhE models, and the validation of RhE IL-18
415 potency test is ongoing, results are expected by the end of 2017.

416
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419
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421 any of individuals or organizations that have could inappropriately influence the submitted
422 work. Susan Gibbs is co-founder of A-Skin BV, a VUmc tissue engineering spin off company.

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425 **Table 1** – Chemical tested, vehicle used and *in vivo* LLNA potency classification

CHEMICAL NAME	CAS #	LLNA EC3 (%) ^a	LLNA potency category	Human NOEL (µg/cm ²)	In vitro vehicle
Benzoquinone	106-51-4	0.01	Extreme	N.A.	AOO
Chlorpromazine	69-09-0	0.14	Strong	1,150*	Medium
Chloramine T	127-65-1	0.4	Strong	N.A.	Medium
Benzyl salicylate ^b	118-58-1	2.9	Moderate	17,717**	AOO
Diethyl maleate	141-05-9	2.1	Moderate	1600*	AOO
Dihydroeugenol	2785-87-7	6.8	Moderate	N.A.	AOO
Benzyl cinnamate ^b	103-41-3	18.4	Weak	4,720**	AOO
2,4-Dichloronitrobenzene	611-06-3	20	Weak	N.A.	AOO
Imidazolidinyl urea	39236-46-9	24	Weak	5,000	Medium
Limonene	5989-27-5	69	Weak	5,517	AOO
Benzyl alcohol ^c	100-51-6		N.C.	5,900	AOO
Isopropanol	67-63-0		N.C.	N.A.	AOO
Dimethyl isophthalate ^c	1459-93-4		N.C.	N.A.	AOO
4-Aminobenzoic acid	150-13-0		N.C.	N.A.	AOO

426 ^aLLNA EC3: concentration (%) of chemical required to induce a stimulation index of 3 relative
 427 to concurrent vehicle-treated controls obtained in the local lymph node assay (LLNA). ^bNo
 428 sensitization was observed in human predictive studies. ^cSensitizer in humans (weak). In vivo
 429 values were obtained from ICCVAM database (NIH Publication No. 11-7709), Research
 430 Institute for Fragrance Materials (RIFM) database, Griem et al. (2003), Api et al. (2008),
 431 Natsch et al. (2013), Basketter et al. (2014), Urbisch et al. (2015). In vitro EC50 and IL-18 SI2
 432 were calculated from RhE exposed to the selected compounds as described in the Materials
 433 and Methods section. *When the human NOEL is not available the human LOEL is reported,
 434 and when differing values have been reported the lower value has been adopted. **No
 435 sensitization was observed in human studies, dose reported reflect the highest concentration
 436 tested, not necessarily the highest achievable NOEL. N.A., no data available. N.C., no
 437 category. AOO, acetone:olive oil (4:1). Medium, EPI-100-MM-60.

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440 **Table 2** - Reference contact sensitizers used to create the regression curves for the
441 estimation of the *in vivo* EC3 and human NOEL

CHEMICAL	CAS #	LLNA EC3 (%)	Human NOEL ($\mu\text{g}/\text{cm}^2$)	In vitro EC50 (% and $\mu\text{g}/\text{cm}^2$)		In vitro IL-18 SI-2 (% and $\mu\text{g}/\text{cm}^2$)	
DNCB	97-00-7	0.08	8.8	0.05	25	0.03	15
Isoeugenol	97-54-1	1.2	250	0.88	440	0.21	105
Cinnamal	104-55-2	3	591	1.1	550	0,5	250
Benzocaine	94-09-7	22	2,000	4.81	2,405	3.12	1,560

442 In vivo values were obtained from ICCVAM database (NIH Publication No. 11-7709),
443 Research Institute for Fragrance Materials (RIFM) database, Griem et al. (2003), Api et al.
444 (2008), Natsch et al. (2013), Basketter et al. (2014), Urbisch et al. (2015).
445 In vitro EC50 and IL-18 SI2 values are the arithmetic means obtained in two independent
446 experiments, and were calculated from RhE exposed to the selected compounds as
447 described in the Materials and Methods section.

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452 **Table 3** - Calculated *in vivo* LLNA EC3 and human NOEL for the tested chemicals based on453 *in vitro* EC50 and IL-18 SI-2 values

CHEMICAL	Predicted LLNA EC3 (%) based on EC50	Predicted LLNA EC3 (%) based on IL-18 SI2	Predicted Human NOEL ($\mu\text{g}/\text{cm}^2$) based on EC50	Predicted Human NOEL ($\mu\text{g}/\text{cm}^2$) based on IL-18 SI2	In vitro EC50 (% and $\mu\text{g}/\text{cm}^2$)		In vitro IL-18 SI-2 (% and $\mu\text{g}/\text{cm}^2$)	
Benzoquinone	-	-	-	-	-	-	-	-
Chlorpromazine	0.45	0.77	46	80	0.11	55	0.12	60
Chloramine T	27.14	8.70	2,797	911	6.65	3,325	1.36	680
Benzyl salicylate	-	-	-	-	-	-	-	-
Diethyl maleate	3.14	1.86	321	194	0.76	382	0.29	145
Dihydroeugenol	3.51	6.72	360	703	0.86	428	1.05	525
Benzyl cinnamate	-	-	-	-	-	-	-	-
2,4-dichloronitrobenzene	16.82	22.78	1,733	2,384	4.12	2,060	3.56	1,780
Imidazolidinyl urea	40.57	48.94	4,176	5,122	9.93	4,965	7.65	3,825
Limonene	-	61.55	-	6,442	-	-	9.62	4,810
Benzyl alcohol	28.00	23.03	2,952	2,410	6.86	3,430	3.60	1,800
Isopropanol	-	-	-	-	-	-	-	-
Dimethyl isophthalate	-	30.45	-	3,188	-	-	4.76	2,380
4-aminobenzoic acid	-	-	-	-	-	-	-	-

454 *In vivo* values were obtained from ICCAVAM database (NIH Publication No. 11-7709),

455 Research Institute for Fragrance Materials (RIFM) database, Basketter et al., (1996), Griem

456 et al. (2003), Api et al. (2008), Natsch et al. (2013), Basketter et al. (2014), Urbisch et al.

457 (2015).

458 *In vitro* EC50 and IL-18 SI2 were calculated by interpolation from linear regression analysis

459 of data reported in Figures 1-3 from RhE exposed to the selected compounds as described in

460 the Materials and Methods section. mg/ml values were converted to % and $\mu\text{g}/\text{cm}^2$ for an461 easier comparison with *in vivo* data. *In vitro* values are the mean of two independent

462 experiments. -, value not estimated.

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615

616 **FIGURE LEGENDS**

617

618 **Figure 1.** Dose-response effects of the selected contact sensitisers on cell viability and IL-18
 619 release. Data obtained from two independent experiments are represented (1° and 2° exp).
 620 RhE was exposed to increasing concentrations of the selected chemicals for 24h. Viability
 621 was assessed by MTT while IL-18 by ELISA. Viability (Figure 1A, 1C, 1E, 1G, 1I, 1M, 1O) is
 622 expressed as % vs vehicle treated RhE (100%), and IL-18 as stimulation index (SI) compared
 623 to vehicle treated RhE (Figure 1B, 1D, 1F, 1H, 1L, 1N, 1P). The dotted lines are set at 50%
 624 viability and IL-18 SI-2.

625

626 **Figure 2.** Dose-response effects of the selected non-sensitisers on cell viability and IL-18
 627 release. Data obtained from two independent experiments are represented (1° and 2° exp).
 628 RhE was exposed to increasing concentrations of the selected chemicals for 24h. Viability
 629 was assessed by MTT while IL-18 by ELISA. Viability (Figure 2A, 2C, 2E) is expressed as %
 630 vs vehicle treated RhE (100%), and IL-18 as stimulation index (SI) compared to vehicle
 631 treated RhE (Figure 2B, 2D, 2F). The dotted lines are set at 50% viability and IL-18 SI-2.

632

633 **Figure 3.** Effects on cell viability and IL-18 release of chemicals misclassified in the LLNA.
 634 RhE was treated for 24h with increasing concentrations benzyl salicylate (A, B), benzyl
 635 cinnamate (C, D), and benzyl alcohol (E, F). Viability was assessed by MTT while IL-18 by
 636 ELISA. Viability (Figure 3A, 3C, 3E) is expressed as % vs vehicle treated RhE (100%), and
 637 IL-18 as stimulation index (SI) compared to vehicle treated RhE (Figure 3B, 3D, 3F). The
 638 dotted lines are set at 50% viability and IL-18 SI-2.

639

640 **Figure 4.** Linear regression curves of RhE assay with LLNA EC3 and human NOEL. A, B)
 641 Regression analysis of RhE-EC50 data with LLNA EC3 (A) and human NOEL (B). B, D)
 642 Regression analysis of RhE-IL-18 SI2 data with LLNA EC3 (C) and human NOEL (D). Curves
 643 were created using data reported in Table 2. R^2 , p values, relative equations and slope errors
 644 are reported in each panel, and were calculated using GraphPad Prism (GraphPad Software,
 645 San Diego, CA, USA).

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, fourteen 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

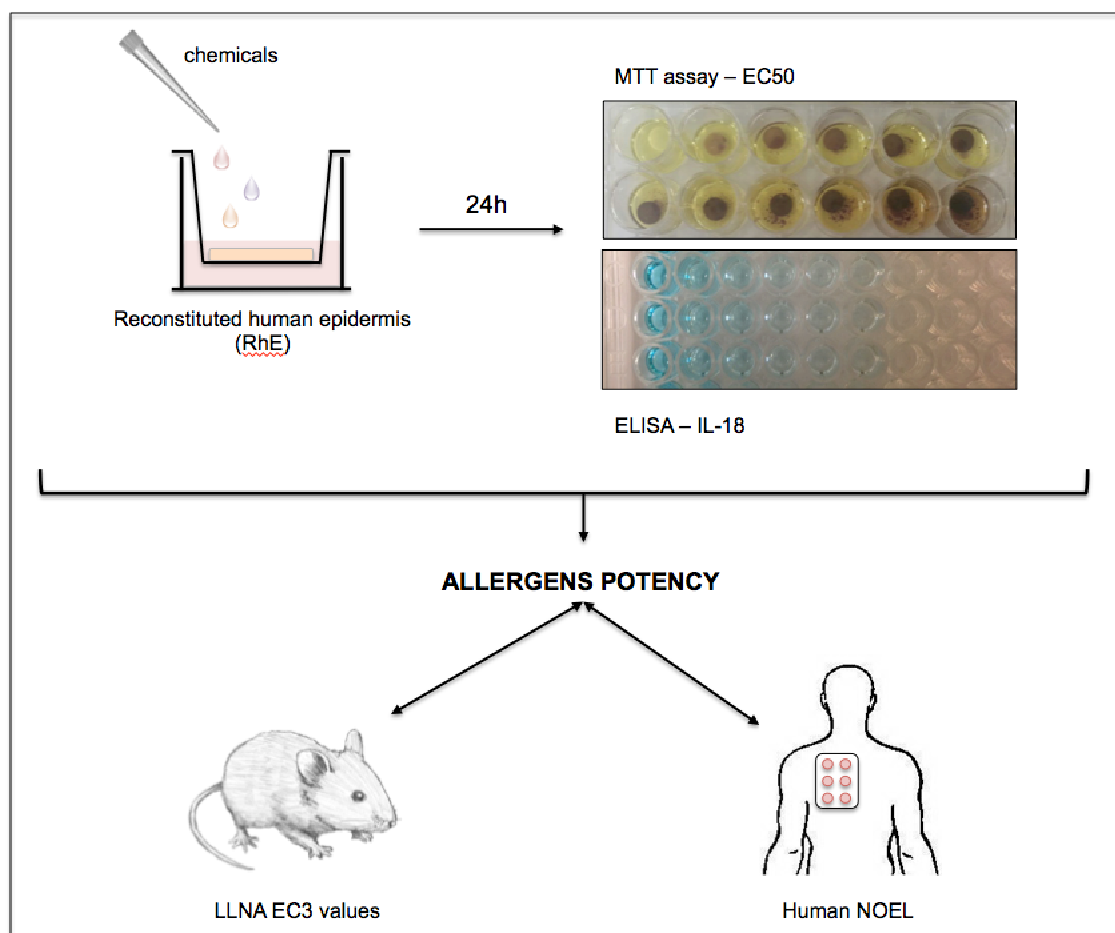


Figure 1a

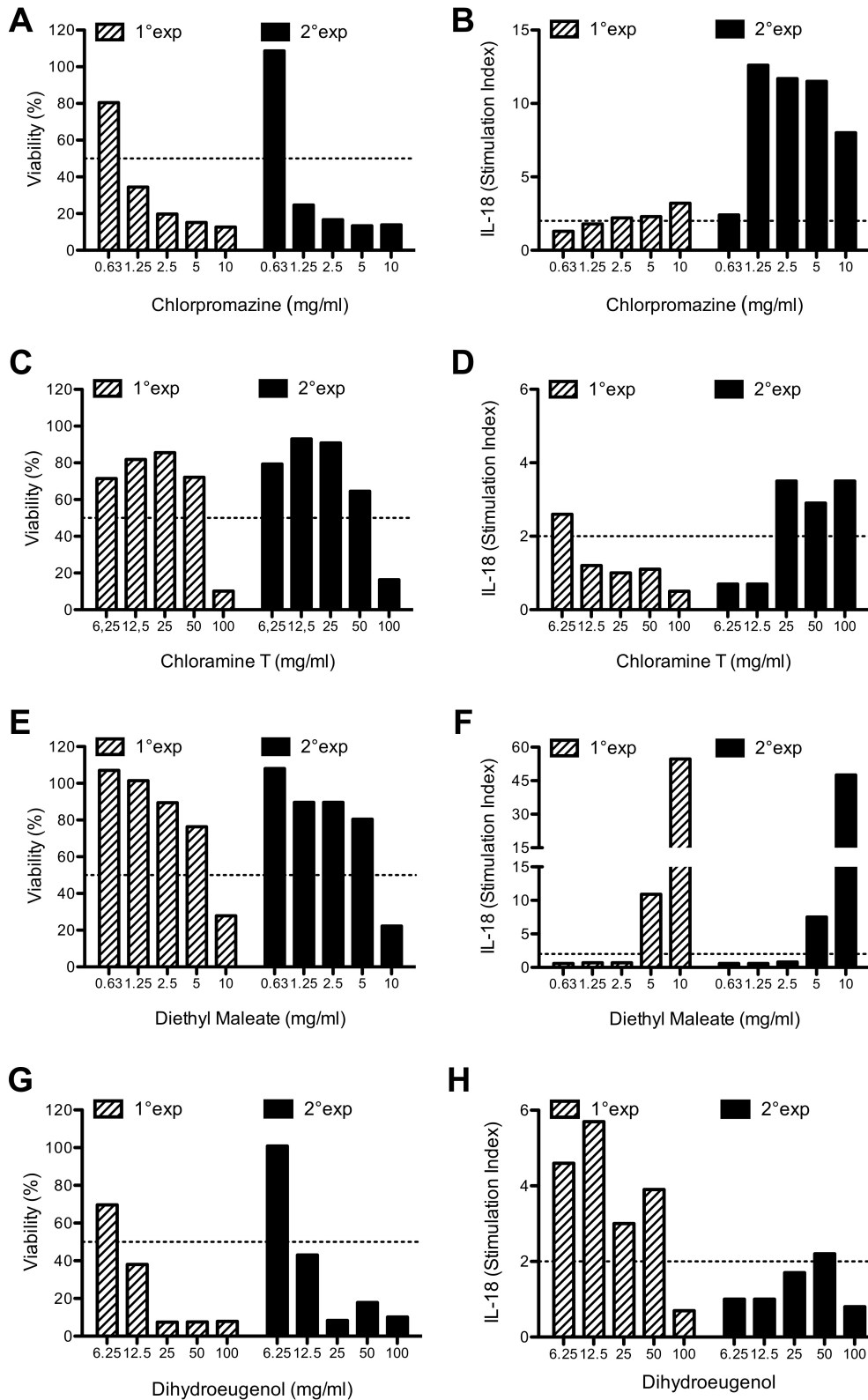


Figure 1b

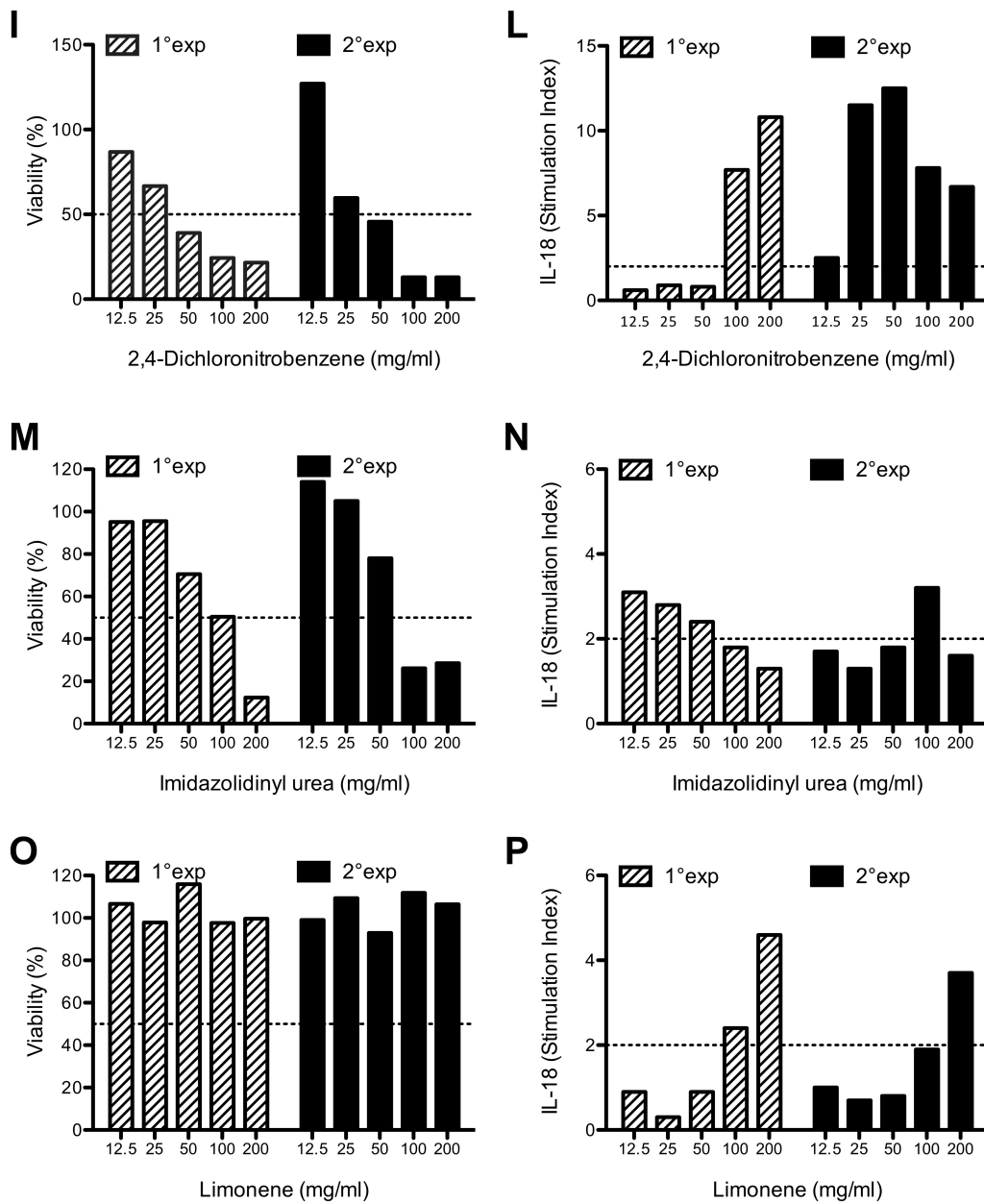


Figure 2

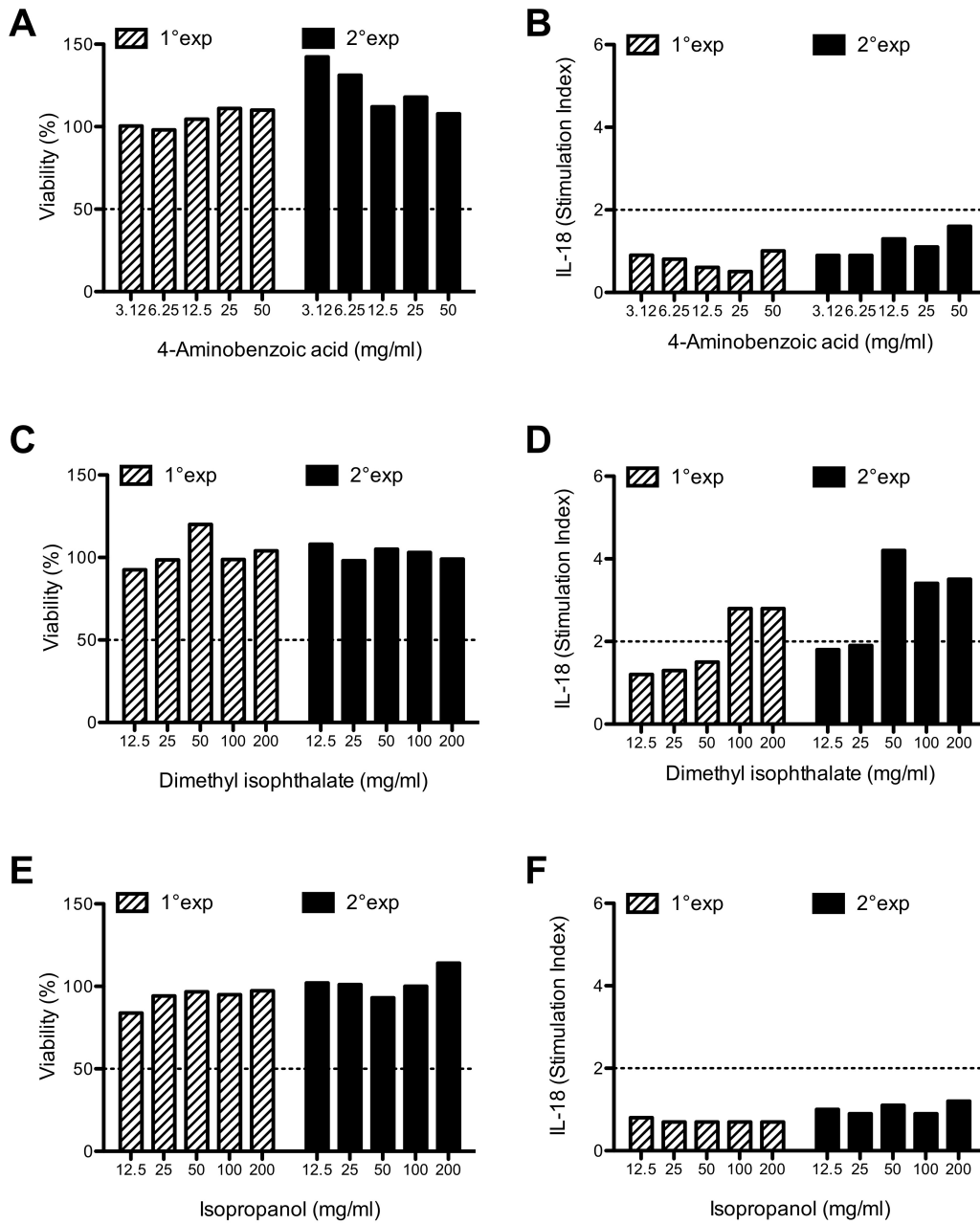


Figure 3

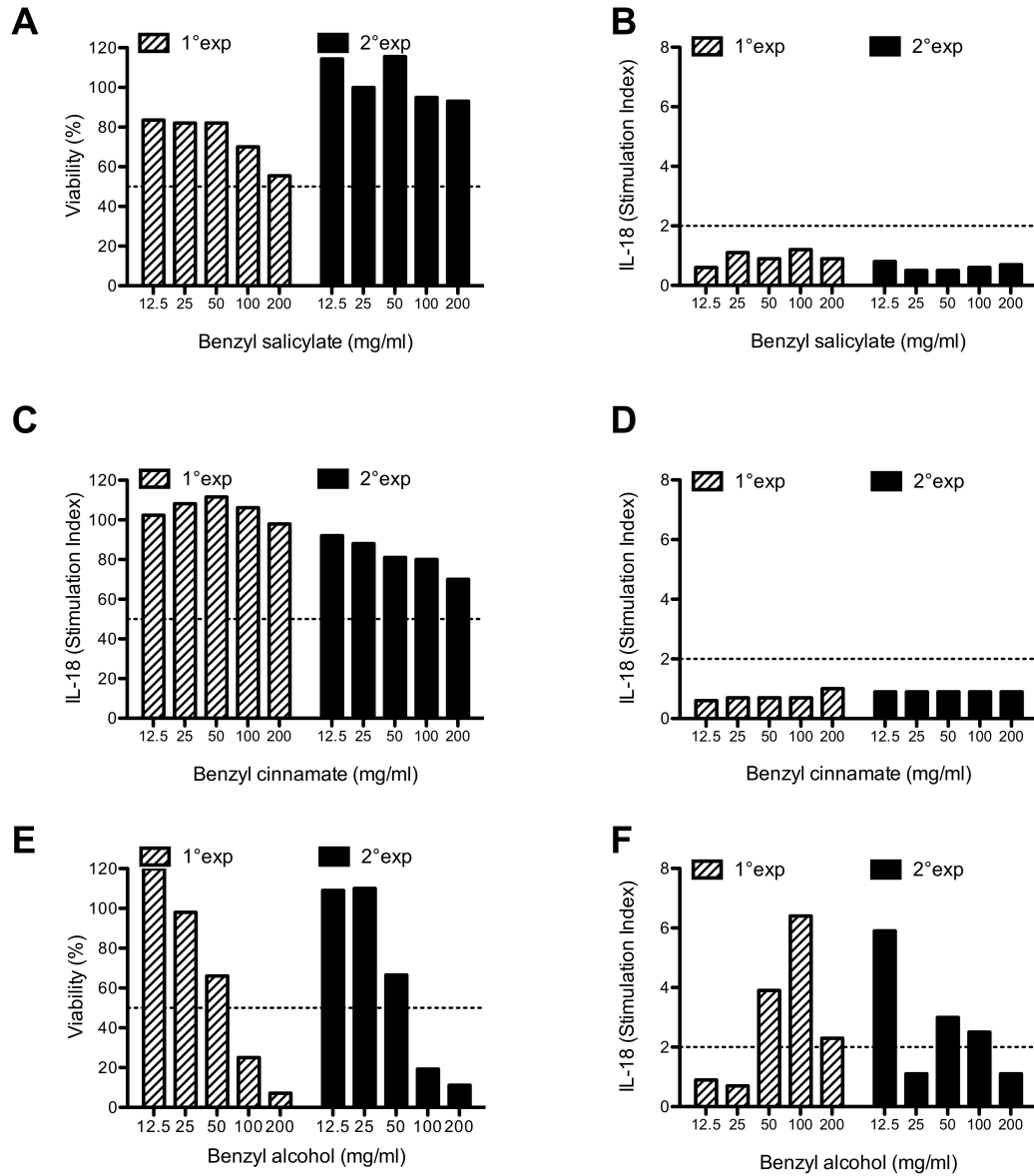


Figure 4

