

**ESTABLISHMENT OF AN *IN VITRO* PHOTOALLERGY TEST USING NCTC2544 CELLS AND IL-18  
PRODUCTION**

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## Abstract

1 Differentiation between photoallergic and phototoxic reactions induced by low molecular weight  
2 compounds represents a current problem. The use of keratinocytes as a potential tool for the detection of  
3 photoallergens as opposed to photoirritants is considered an interesting strategy for developing *in vitro*  
4 methods. We have previously demonstrated the possibility to use the human keratinocyte cell line  
5 NCTC2455 and the production of interleukin-18 (IL-18) to screen low molecular weight sensitizers. The  
6 purpose of this work was to explore the possibility to use the NCTC2544 assay to identify photoallergens and  
7 discriminate from phototoxic chemicals.  
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16 First, we identified suitable condition of UV-irradiation ( $3.5 \text{ J/cm}^2$ ) by investigating the effect of UVA-  
17 irradiation on intracellular IL-18 on untreated or chlorpromazine (a representative phototoxic compound)-  
18 treated NCTC2544 cells. Then, the effect of UVA-irradiation over NCTC2544 cells treated with increasing  
19 concentrations of 15 compounds including photoallergens (benzophenone, 4-ter-butyl-4-methoxy-  
20 dibenzoylmethane, 2-ethylexyl-p-methoxycinnamate, ketoprofen, 6-methylcumarin); photoirritant and  
21 photoallergen (4-aminobenzoic acid, chlorpromazine, promethazine); photoirritants (acridine, ibuprofen, 8-  
22 methoxypsoralen, retinoic acid); and negative compounds (lactic acid, SDS and p-phenilendiamine) was  
23 investigated. Twenty-four hours after exposure, cytotoxicity was evaluated by the MTT assay or LDH  
24 leakage, while ELISA was used to measure the production of IL-18. At the maximal concentration assayed  
25 with non-cytotoxic effects (CV80 under irradiated condition), all tested photoallergens induced a significant  
26 and a dose-dependent increase of intracellular IL-18 following UVA irradiation, whereas photoirritants failed.  
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28 We suggest that this system may be useful for the *in vitro* evaluation of the photoallergic potential of  
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46 **Keywords:** photosensitization, UVA, NCTC2544, IL-18, *in vitro*, phototoxicity  
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## 1. Introduction

1 Safety evaluation of ingredients is an important part of the development of cosmetics and drugs.  
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3 Phototoxicity, photoirritation and photoallergy are health hazards arising from exposure of skin to normally  
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5 harmless levels of light in the presence of radiation-absorbing compounds. The growing use of cosmetics in  
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7 combination with relative high UV-light exposure potentiates this problem. Photoactivation and binding of  
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9 photoactive compounds to proteins is a known prerequisite for the formation of immunogenic photoantigens  
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11 and the induction of photoallergy. Ultraviolet A (UVA) is the action spectrum of this photoderivatization, as  
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13 protein and cells are photocoupled with photohaptenic compounds by irradiation with UVA but not UVB  
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15 (Tokura, 2000; Tokura, 2005). Photochemiotoxic reactions may be phototoxic or photoallergic in nature.  
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17 Acute phototoxic reactions are generally characterized by erythema and oedema followed by  
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19 hyperpigmentation and desquamation. Chronic repeated injury of this type may result in fragility, blistering  
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21 and milia formation or even actinic keratoses and skin cancers. The photochemical mechanisms involved  
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23 may differ. They include photoaddition of the chemical to biological targets such as DNA, the formation of  
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25 toxic products due to absorption of light by the phototoxic molecule, the generation of reactive oxygen  
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27 species or free radicals (Epstein and Wintroub, 1985). Phototoxic reactions are significantly more common  
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29 than photoallergic reactions and mostly resemble to exaggerated sunburns. Photoallergic reactions appear  
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31 only in a minority of individuals and resemble allergic contact dermatitis on sun-exposed areas, although  
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33 sometimes may extend into covered areas. Generally, the physical examination and a positive patient's  
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35 history of photosensitivity reactions on substances are of great importance for the diagnostics. Photocontact  
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37 allergic dermatitis is one of the undesirable adverse effects produced by chemicals and drugs in our  
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39 environment (Yamamoto and Tokura, 2003), it is a delayed type IV hypersensitivity reaction seen when an  
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41 exogenous agent comes into contact with the skin in the presence of UV. Various chemicals have been  
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43 reported to cause photocontact dermatitis. The physical manifestation of this increased photosensitivity will  
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45 depend on the specific photosensitizer; some will reduce the threshold to sunburn while others may induce a  
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47 photoallergy in susceptible individuals (Maverakis et al., 2010). Historically, the use of halogenated  
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49 salicylanilide and related compounds, especially 3,3',4',5-tetrachlorosalicylanilide and bithionol, resulted in a  
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51 large number of patients with this skin disease. More recent causative agents include cosmetic or sunscreen  
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53 products such as 6-methylcoumarin, musk ambrette, benzophenone. Likewise, there have been various  
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55 culprit drugs to evoke photosensitivity, including chlorpromazine, promethazine, quinolones, such as  
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57 sparfloxacin and enoxacin, piroxicum, afloqualone, and non-steroidal anti-inflammatory drugs (Kurita et al.,  
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59 2007).  
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1 Assessments of phototoxic properties of ingredients have generally been performed using animal tests  
2 including Guinea pigs, rabbits, rats and mice (Maurer et al., 1980; Jordan, 1982; Gerberick and Ryan, 1990;  
3 Ulrich et al., 1998). However, the development of alternative in vitro assays is now required following the 7<sup>th</sup>  
4 amendment of the European Cosmetics Directive (Directive 76/768/EEC), which aims for the complete  
5 replacement of animal testing by 2013. For phototoxicity testing, the in vitro 3T3 NRU phototoxicity test has  
6 been adopted as an alternative to in vivo phototoxicity test (OECD n°432). In vitro replacements for the in  
7 vivo photoallergy test have also been sought (Tokura, 2000; Lovell and Jones, 2000; Barratt et al., 2000;  
8 Neumann et al., 2005; Onoue and Tsuda, 2005; Kurita et al., 2007, Hoya et al., 2009; Karschk et al., 2010),  
9 but to date no accepted alternative is available to identify the photoallergenic potential of new chemicals.

10 We recently shown that exposure of the human keratinocyte cell lines NCTC2544, HaCaT or primary  
11 keratinocytes to contact allergens, but not to respiratory allergens or irritants, resulted in a dose-related  
12 induction of intracellular IL-18. The NCTC 2544 assay was developed within the SENS-IT-IV project  
13 sponsored by the European Union. The assay proved to be useful in the identification and discrimination of  
14 contact allergens from respiratory sensitizers and irritants (Corsini et al., 2009; Galbiati et al., 2011). IL-18  
15 was chosen as this cytokine, formerly known as IFN- $\gamma$ -inducing factor (IGIF), which belongs to the IL-1  
16 cytokine family, has been shown to play a key proximal role in the induction of allergic contact sensitization  
17 and to favour Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as  
18 TNF- $\alpha$ , IL-8 and IFN- $\gamma$ , (Okamura et al., 1995; Cumberbatch et al., 2001; Antonopoulos et al., 2008).

19 The purpose of this work was to explore the possibility to use the current NCTC 2544 IL-18 assay for the  
20 identification of photosensitizers and discriminate them from phototoxic chemicals, and to develop a new in  
21 vitro photosensitization test system.

## 2. Materials and methods

### 2.1. Chemicals

22 Phototoxic chemicals were selected based on compounds used in similar published studies (Hoya et al.,  
23 2009; Karschuk et al., 2010) and reported to cause allergic contact dermatitis (Ibbotson et al., 1997), and the  
24 commercially availability from credited sources. As photosensitizers the following chemicals were used:  
25 benzophenone, 2-ethylhexyl 4-methoxycinnamate (Parsol MCX), 4-tert-butyl-4'-methoxydibenzoylmethane  
26 (Avobenzone), 6-methylcoumarin, ketoprofen. As photosensitizers / photoirritants 4-aminobenzoic acid

(PABA), chlorpromazine, promethazine, and as photoirritants acridine, ibuprofen, 8-methoxy-psoralen, retinoic acid were used. As negative compounds lactic acid, sodium dodecyl sulphate (SDS) and *p*-phenylenediamine (PPD) were used. All reagents were purchased from Sigma (St Louis, MO, USA) at the highest purity available. All chemicals were dissolved in DMSO (final concentration of DMSO in culture medium 0.2%). DMSO (0.2% final concentration) was used as vehicle control. Solutions were freshly prepared for each experiment.

## 2.2. Cell culture, treatment and irradiation conditions

NCTC 2544 cells (Istituto Zooprofilattico di Brescia, Brescia, Italy) were cultured in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 10 µg/ml gentamycin supplemented with 10% heated-inactivated foetal calf serum (complete medium) and cultured at 37 °C in 5% CO<sub>2</sub>.

The irradiation of the cells was carried out in a photostability UV chamber equipped with four UVA lamps (lamp potency 0.84 mW/cm<sup>2</sup>; UVA EN-180L/F - Colaver, Vimodrone, Italia). Irradiance was routinely measured before cell exposure with a photoradiometer Delta OHM provided with a UVA probe (HD2302 - Italy) to determine UV dose using the following equation:

$$E (\text{J}/\text{cm}^2) = t(\text{s}) \times P (\text{W}/\text{cm}^2)$$

where E stands for UV energy, t represents the time expressed in seconds and, finally, P is the lamp potency. Cells were irradiated with a dosing of of 3.5-7 J/cm<sup>2</sup>.

Cells were seeded in 24-well plate or in 96-well plate at a cell density of 2.5 x 10<sup>5</sup>/ml (0.5 ml/well in 24-well plate and 0.1 ml/well in 96-well plate). After overnight adherence, cells were irradiated or not irradiated in the presence of increasing concentrations of the selected chemicals in PBS (200 µl/well in 24-well plate and 40 µl/well in 96-well plate). Each concentration was tested in quadruplicate (n= 4 wells). Non-irradiated cells were kept in the dark during irradiation. After irradiation, 300 µl or 60 µl of complete medium was added to each well. Cells were then incubated for 24 hours.

## 2.3. Cell viability

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Prior to study the effects of the selected chemicals on IL-18 production, cytotoxicity was assessed by the MTT test (Gerlier and Thomasset, 1986). Cells seeded in a 96-well plate were treated with increasing concentrations of the test chemical or DMSO as vehicle control, and exposed to UV light or kept in the dark. After irradiation, cells were incubated for 24 h, medium was then discarded and 100 µl/well of MTT solution 0.75 mg/ml in culture medium was added. Cells were incubated for 3 h at 37 °C, medium was discarded and cells lysed in 100 µl/well of a mixture of HCl 1 N and isopropanol (1:24). The absorbance of the resulting solutions was read at a wavelength of 595 nm in a microplate reader (Molecular Devices). 80 % cell viability (CV80) was calculated for each chemical by linear regression analysis of data in both irradiated and non-irradiated conditions, the average CV80 obtained from two independent experiments was used as the highest concentration.

Cell viability was also assessed by lactate dehydrogenase (LDH) leakage from damaged cells. LDH is a well-known indicator of cell membrane integrity and cell viability. LDH activity was determined in cell-free supernatants using a commercially available kit (Takara Bio Inc., Japan). Results are expressed as OD.

#### 2.4. Cytokine production

For intracellular IL-18 assessment, after incubation, culture medium was discarded, monolayers gently washed once with 1.0 ml of PBS and cells lysed in 0.25 ml of 0.5% Triton X- 100 in PBS. Cell lysates were stored at -80 °C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA commercially available (MBL, Nagoya, Japan). Limit of detection was 15.6 pg/ml. The protein content of the cell lysate was measured with the BCA assay. Results are expressed in pg/mg of total intracellular protein content, or as ratio between the stimulation index (SI) calculated in irradiated (I) conditions versus the SI in non-irradiated (NI) conditions (IL-18 SI I/NI), where the stimulation index is calculated dividing the intracellular IL-18 content in treated cells vs intracellular IL-18 in control cells (n= 4 wells).

#### 2.5. Data analysis

For classification of chemicals, the following PREDICTION MODEL was used: if the IL-18 SI (I/NI) is  $\geq 1.3$  and the increase in IL-18 is statistically significant from vehicle treated cells (Dunnnett multiple comparisons test) the chemical is classified as potential photoallergen. For a given chemical, the same classification must be obtained in two out of three independent experiments. The 1.3 fold increase is meant for at least one of

1 the concentrations tested. The 1.3 induction must be observed at CV  $\geq$  80%. All experiments with the  
2 selected chemicals were therefore performed at least three times, and one of the three experiments is shown  
3 as representative. Data are expressed as mean  $\pm$  standard deviation (SD), n=4. A 20% variation among  
4 replicates is considered acceptable. Statistical analysis was performed using InStat software version 3.0a  
5 (GraphPad Software, La Jolla, CA, USA). Statistical differences were determined using ANOVA followed by  
6 a multiple comparison test as indicated in the legends. Effects were designated significant if p< 0.05. Data  
7 obtained from the different experiments are not pooled, as each experiment must be considered  
8 independently for the classification.  
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### 10 11 12 13 14 15 16 17 18 **3. Results**

#### 19 *3.1. Establishment of the irradiation conditions*

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21 The dose of UVA irradiation was assessed exposing cells to increasing UVA doses. Under our experimental  
22 conditions, as shown in Fig. 1 A, the dose of 3.5 J/cm<sup>2</sup> represents the optimal UVA dose. As at this dose, no  
23 effects of UVA alone was observed, while UVA dose of 7 J/cm<sup>2</sup> resulted in a loss of IL-18 production and  
24 LDH leakage (cytotoxicity), compared to not irradiated cells.  
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33 The photoallergen/photoirritant chlorpromazine was then used to establish the optimal irradiation conditions  
34 in the presence of potential phototoxic compound. Initially, as highest concentration the CV80 calculated in  
35 non-irradiated condition was used (Fig.1 B, C), hoping to use the same SOPs developed for the NCTC 2544  
36 IL-18 assay. In this condition, however, following UVA irradiation a dramatic cytotoxicity was observed as  
37 assessed by LDH leakage, which resulted in the impossibility to assess the effect of irradiated  
38 chlorpromazine on IL-18 production. Therefore, CV80 was assessed in irradiated condition. As shown in  
39 Fig.1 D, using the CV80 calculated in irradiated condition as the highest concentration, irradiated  
40 chlorpromazine induced a dose-related increase in intracellular IL-18. To appreciate the effect of the  
41 combination of chemical + UVA, the ratio of IL-18 SI in irradiated vs non-irradiated conditions is reported as  
42 dotted line (Fig. 1D).  
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55 Based on these experiments, the UVA dose of 3.5 J/cm<sup>2</sup> was selected, and as highest concentration the  
56 CV80 calculated in irradiated condition was used.  
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### 3.2. Determination of 80% cell viability

1 The concentration of chemical resulting in 80% of viability (CV80) respect to vehicle treated cells 24 h was  
2 calculated for all chemicals in both non-irradiated and irradiated conditions. Results are presented in Table 1.  
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4 For many chemicals, including photoallergens and photoirritants, a lower CV80s were observed in irradiated  
5 condition.  
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### 3.3. Selective induction of intracellular IL-18 by photoallergens

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14 NCTC2544 cells were treated for 24 h with the selected photoallergens (Fig.2), photoallergens/photoirritants  
15 (Fig. 3), photoirritants (Fig. 4), and non-phototoxic compounds (Fig.5). Following treatment and UVA  
16 irradiation, the intracellular IL-18 content was assessed as described in the Materials and Methods section.  
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18 Results are reported as the ratio of IL-18 SI in irradiated vs non-irradiated conditions (IL-18 SI (I/NI)).  
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26 As shown in Fig. 2 and Fig. 3, all irradiated photoallergens and photoallergens/photoirritants, induced a  
27 statistical significant increase in intracellular IL-18, with an IL-18 SI (I/NI) up to 7 for chlorpromazine. The  
28 lowest inductions were observed for avobenzone and benzophenone. In all figures, a dotted line was set at a  
29 SI of 1.3, as provisional criteria for identification of photoallergens and discriminate them from photoirritants.  
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35 We then tested the effect of the photoirritants ibuprofen, acridine, retinoic acid and 8-methoxypsoralene (Fig.  
36 5). Irradiated ibuprofen and acridine failed to induced IL-18 production, while a slight increase, below or  
37 close to IL-18 SI (I/NI) of 1.3, was observed at the highest concentration of 8-methoxypsoralene and retinoic  
38 acid.  
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44 Finally, we assessed the effects of not phototoxicants, namely PPD, lactic acid and SDS on intracellular IL-  
45 18 production. Following UVA irradiation, none of them was able to increase intracellular IL-18 (Fig. 5),  
46 confirming the selective up-regulation of IL-18 by irradiated photoallergens.  
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### 3.4 Reproducibility of data

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55 To appreciate the variability among independent experiments performed on different days over one year  
56 period, we reported in Fig. 6 the IL-18 SI (I/NI) obtained for chlorpromazine 1.25  $\mu$ M (15 experiments),  
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ketoprofen 2.5  $\mu\text{M}$  (4 experiments) and 6-methylcoumarin 10  $\mu\text{M}$  (5 experiments). The reason for which we tested these chemicals repeatedly was to identify a positive control to be used routinely in the assay. We initially thought to use chlorpromazine 1.25  $\mu\text{M}$ , but due to variability in the SI, we later decided to use ketoprofen or 6-methylcoumarin. IL-18 SI (I/NI) for chlorpromazine 1.25  $\mu\text{M}$  ranged from 1.25 to 7.7 with an average of 3.16; for ketoprofen 2.5  $\mu\text{M}$  from 1.28 to 1.42 with an average of 1.33, and for 6-methylcoumarin 10  $\mu\text{M}$  from 1.2 to 3.20 with an average of 1.76. As for the NCTC 2544 IL-18 assay (Corsini et al., 2009; Galbiati et al., 2011), the differences observed in the absolute value of IL-18 may be due to many factors including the number of cells seeded and adhering to plate wells, to the use of a different cell batch, the performance of IL-18 ELISA and protein assay as well as to differences in the chemical concentration (fresh solutions are used) and slight variation in the irradiation conditions. Despite the variability, the discriminatory capacities based on defined prediction model are, however, preserved.

#### 4. Discussion

Given that there **is** no in vivo or in vitro methods developed which regulatory authorities to assess photosensitization induced by chemicals, in the present work, we describe the possibility to use the NCTC2544 IL-18 assay to identify in vitro photoallergens. We found that irradiated photoallergens induced a significant increase in intracellular IL-18 compared to photoirritants or not photoxic compounds. As provisional prediction model, based of the tested chemicals, an IL-18 SI (I/NI) > 1.3 was set as criteria for identification of photoallergens.

Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of keratinocytes and skin organotypic culture as a simplified in vitro model to evaluate the potential toxicity of chemicals destined for epicutaneous application is amply justified. In the present work, intracellular IL-18 was investigated after exposure of NCTC 2544 cells to several photoallergens, and photoirritants. We showed that UVA irradiation of NCTC 2544 cells treated with photoallergens results in a dose-related induction of intracellular IL-18, indicating the possibility to use IL-18 to specifically identify photoallergens and distinguish them from photoirritants.

As mentioned in the Introduction, IL-18 was chosen, as this cytokine is mainly associated with Th1-mediated immune responses. Interestingly, it has been proposed that UV damage can be considered a “danger” signal. Recent data linking IL-1 $\beta$  and IL-18 production to “inflammasomes” supports this notion. IL-1 $\beta$  and IL-

18 are normally expressed in human keratinocytes as an inactive precursor. Upon exposure to UV radiation, both precursors are cleaved by caspase-1 to yield active IL-1 $\beta$  and IL-18. Caspase-1 activation is dependent upon its recruitment to inflammasomes, which links UV-induced cytokine production to the innate immune system. Inflammasomes are composed of NOD-like receptor (NLR)-family proteins (Feldmeyer et al., 2007). These intracellular proteins contain a nucleotide-binding oligomerization domain called NACHT and several leucine rich repeat domains that may bind to microbial ligands similar to Toll-like receptor family proteins. The relationship between inflammasomes and UV-induced IL-1 $\beta$  and IL-18 production demonstrates that this pathway can also sense UV-induced tissue injury. Under our experimental condition, the UVA dose used alone didn't induce IL-18. Only the combination chemical (photoallergen) + UVA resulted in IL-18 induction. However, a similar mechanism of inflammasome activation is likely to be triggered by photoallergens. For allergens, we previously demonstrated a role for oxidative stress, NF- $\kappa$ B and p38 MAPK activation in PDD-induced IL-18 production (Galbiati et al., 2011), consistent with an inhibition of IL-18 transcription as in 5' upstream region of the human IL-18 gene AP-1, PU.1, NF- $\kappa$ B and SP-1 transcription binding sites have been identified (Takeuchi et al., 1999).

A critical initial point in our study was the choice of UVA dose and chemical exposure conditions. Suitable irradiation conditions for the test were determined using chlorpromazine as a representative photoallergen. This drug and several others related phenothiazines are known to cause both phototoxic and photoallergic reaction in the skin and eyes of patients. The original SOP developed for the NCTC2544 IL-18 assay had to be adapted to the UVA irradiation. A UVA dose of 3.5 J/cm<sup>2</sup> was chosen, and the CV80 calculated in irradiated condition was selected as the highest concentration.

We then investigated if commercially available photosensitizers could selectively up regulate IL-18 in human keratinocytes after UVA exposure. We could indeed demonstrate that compounds classified as photosensitizers or photoallergens/photoirritants have the ability to increase IL-18 intracellular levels in a dose related-manner, whereas photoirritants failed. A slight increase in IL-18 was observed with 8-methoxypsoralene and retinoic acid. It is important to mention that both chemicals are reported to possess photoallergenic effects. Recent literature and FDA-approved package for all-trans-retinoic acid products state explicitly that all-trans-retinoic acid is both a phototoxin and a photosensitizer (Moore, 2002; Fu et al., 2003; Tolleson et al., 2005; Stein and Scheinfeld, 2007). Even if photoallergy to furocoumarins has rarely been reported, Bonamonte et al. (2010) clearly demonstrated photoallergic contact dermatitis to 8-methoxypsoralen. In the establishment of our prediction model, both compounds were, however, considered as photoirritants. Therefore, based on the effect on IL-18 observed for these two chemicals, the cut off of IL-

18 SI (I/NI) > 1.3 was set to identify and discriminate photoallergens from photoirritants. Based on human classification, an overall accuracy of 100 % of the proposed assay was obtained.

In conclusion, we propose a new reliable *in vitro* photosensitization assay using the keratinocyte cell line NCTC2544 and IL-18 production. The induction of IL-18 following UVA irradiation may represent a promising *in vitro* model for the screening and evaluation of potential photoallergens, reducing the risk of photoallergic reactions. Although we need to further confirm the applicability of the test evaluating a wider battery of chemicals, we consider that this assay may become a useful cell-based *in vitro* test for evaluating the photosensitizing potential of chemicals.

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**Conflict of Interest.** For the past 3 years since the beginning the work, all Authors declare not having any financial, personal, or association with any of the individuals or organizations, that have could inappropriately influence the submitted work.

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**Table 1.** Concentration ( $\mu\text{M}$ ) of the tested chemicals that induced 80% viability ( $\text{CV}_{80}$ ) in non-irradiated and irradiated conditions ( $3.5 \text{ J/cm}^2$ ), and their classification.

Compound	$\text{CV}_{80}$ Non-Irradiated	$\text{CV}_{80}$ Irradiated	Category
2-ethylhexyl-4-methoxycinnamate	3400	9.8	Photoallergen
6-methylcoumarin	> 1000	10	Photoallergen
Avobenzone	90	90	Photoallergen
Benzophenone	> 1000	31.2	Photoallergen
Ketoprofen	3000	4.9	Photoallergen
4-aminobenzoic acid (PABA)	7300	7300	Photoallergen/photoirritant
Chlorpromazine	20	1.25	Photoallergen/photoirritant
Promethazine	125	1	Photoallergen/photoirritant
8-methoxypsoralen	700	2	Photoirritant
Acridine	2116	0.05	Photoirritant
Ibuprofen	> 1000	> 1000	Photoirritant
Retinoic acid	50	20	Photoirritant
p-phenylenediamine (PPD)	55	27.5	Allergen
Lactic acid	> 8300	> 8300	Irritant
Sodium dodecyl sulfate (SDS)	100	100	Irritant

## LEGENDS TO FIGURES

1 **Figure 1.** Effects of UVA irradiation on intracellular IL-18 and viability of NCTC2544 cells. (A) Cells were  
2 irradiated at the UVA doses of 3.5 and 7 J/cm<sup>2</sup>. Intracellular IL-18 and LDH leakage were assessed 24 h  
3 later. Results are expressed as mean ± SD, n= 4. Statistical analysis was performed with Dunnett's multiple  
4 comparison test, with \*\* p < 0.01 vs non-irradiated cells (NI). Dose response effects of chlorpromazine on  
5 cell viability (B) and cell-associated IL-18 (C). NCTC2544 cells were irradiated (I) at the UVA dose of 3.5  
6 J/cm<sup>2</sup> or non-irradiated (NI) in the presence of increasing concentrations of chlorpromazine, using the CV80  
7 calculated under non-irradiated condition as highest concentration. (D) Dose response effects of  
8 chlorpromazine on cell-associated IL-18, using the CV80 calculated under irradiation condition as highest  
9 concentrations. Results are expressed as mean ± SD, n= 4. Statistical analysis was performed with  
10 Dunnett's multiple comparison test, with \* p < 0.05 and \*\* p < 0.01 vs vehicle treated cells (0).  
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25 **Figure 2.** Effects of the selected photoallergens on cell-associated IL-18. NCTC2544 cells were irradiated (I)  
26 at the UVA dose of 3.5 J/cm<sup>2</sup> or non-irradiated (NI) in the presence of increasing concentrations of the  
27 selected photoallergens, using the CV80 calculated under irradiated condition as highest concentration.  
28 Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each data is mean ± SD, n= 4.  
29 Statistical analysis was performed with Dunnett's multiple comparison test, with \* p < 0.05 and \*\* p < 0.01 vs  
30 vehicle treated cells.  
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41 **Figure 3.** Effects of the selected photoallergens/phototoxicants on cell-associated IL-18. NCTC2544 cells  
42 were irradiated (I) at the UVA dose of 3.5 J/cm<sup>2</sup> or non-irradiated (NI) in the presence of increasing  
43 concentrations of the selected photoallergens/phototoxicants, using the CV80 calculated under irradiated  
44 condition as highest concentration. Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of  
45 1.3. Each data is mean ± SD, n= 4. Statistical analysis was performed with Dunnett's multiple comparison  
46 test, with \* p < 0.05 and \*\* p < 0.01 vs vehicle treated cells.  
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57 **Figure 4.** Effects of the selected phototoxicants on cell-associated IL-18. NCTC2544 cells were irradiated (I)  
58 at the UVA dose of 3.5 J/cm<sup>2</sup> or non-irradiated (NI) in the presence of increasing concentrations of the  
59 selected phototoxicants, using the CV80 calculated under irradiated condition as highest concentration.  
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Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each data is mean  $\pm$  SD, n= 4. Statistical analysis was performed with Dunnett's multiple comparison test, with \* p < 0.05 and \*\* p < 0.01 vs vehicle treated cells.

**Figure 5.** Effects of the selected non-phototoxic compounds on cell-associated IL-18. NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm<sup>2</sup> or non-irradiated (NI) in the presence of increasing concentrations of the selected non-phototoxic compounds, using the CV80 calculated under irradiated condition as highest concentration. Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each data is mean  $\pm$  SD, n= 4. Statistical analysis was performed with Dunnett's multiple comparison test.

**Figure 6.** Reproducibility of data. Results obtained in several independent experiments performed over one year period are reported. NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm<sup>2</sup> or non-irradiated (NI) in the presence of chlorpromazine 1.25  $\mu$ M (15 experiments), ketoprofen 2.5  $\mu$ M (4 experiments) and 6-methylcoumarin 10  $\mu$ M (5 experiments). Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each dot represents an independent experiment. Mean  $\pm$  SD is also reported.

Figure 1 - Galbiati et al.

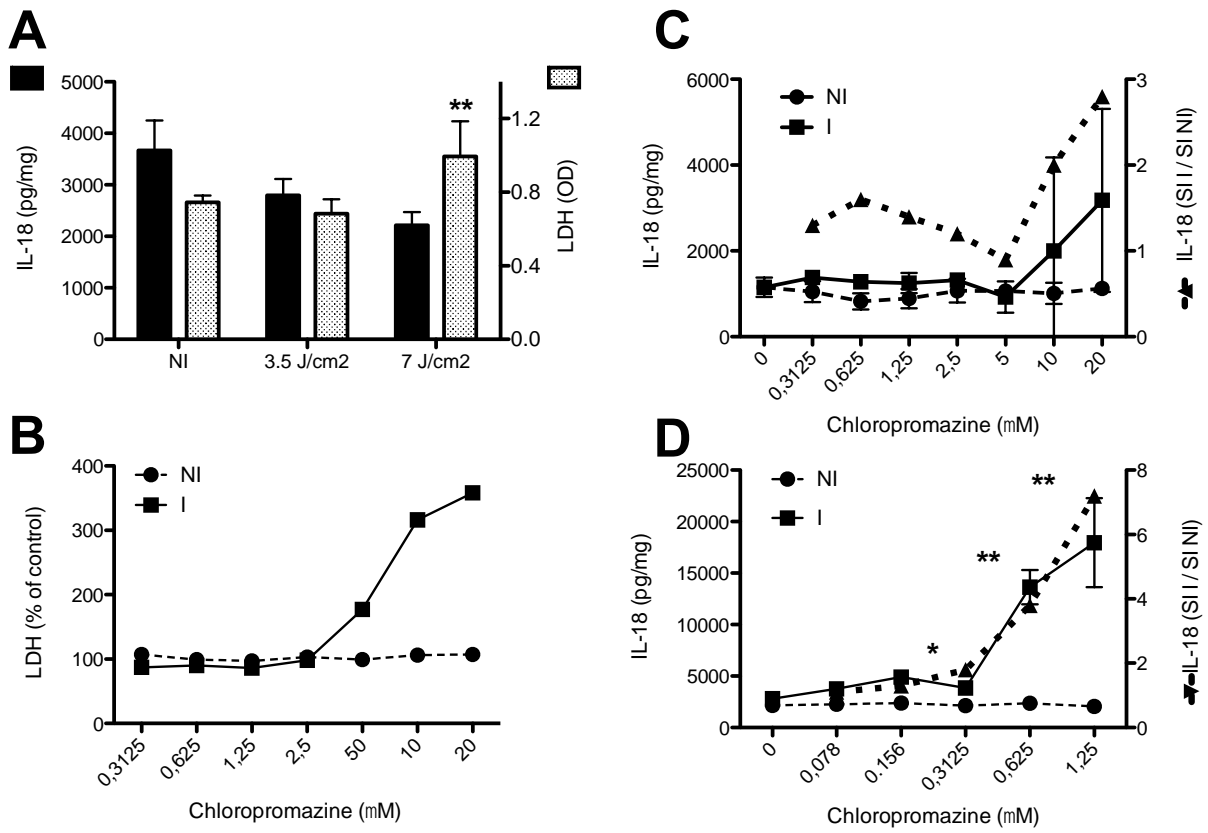


Figure 2 - Galbiati et al.

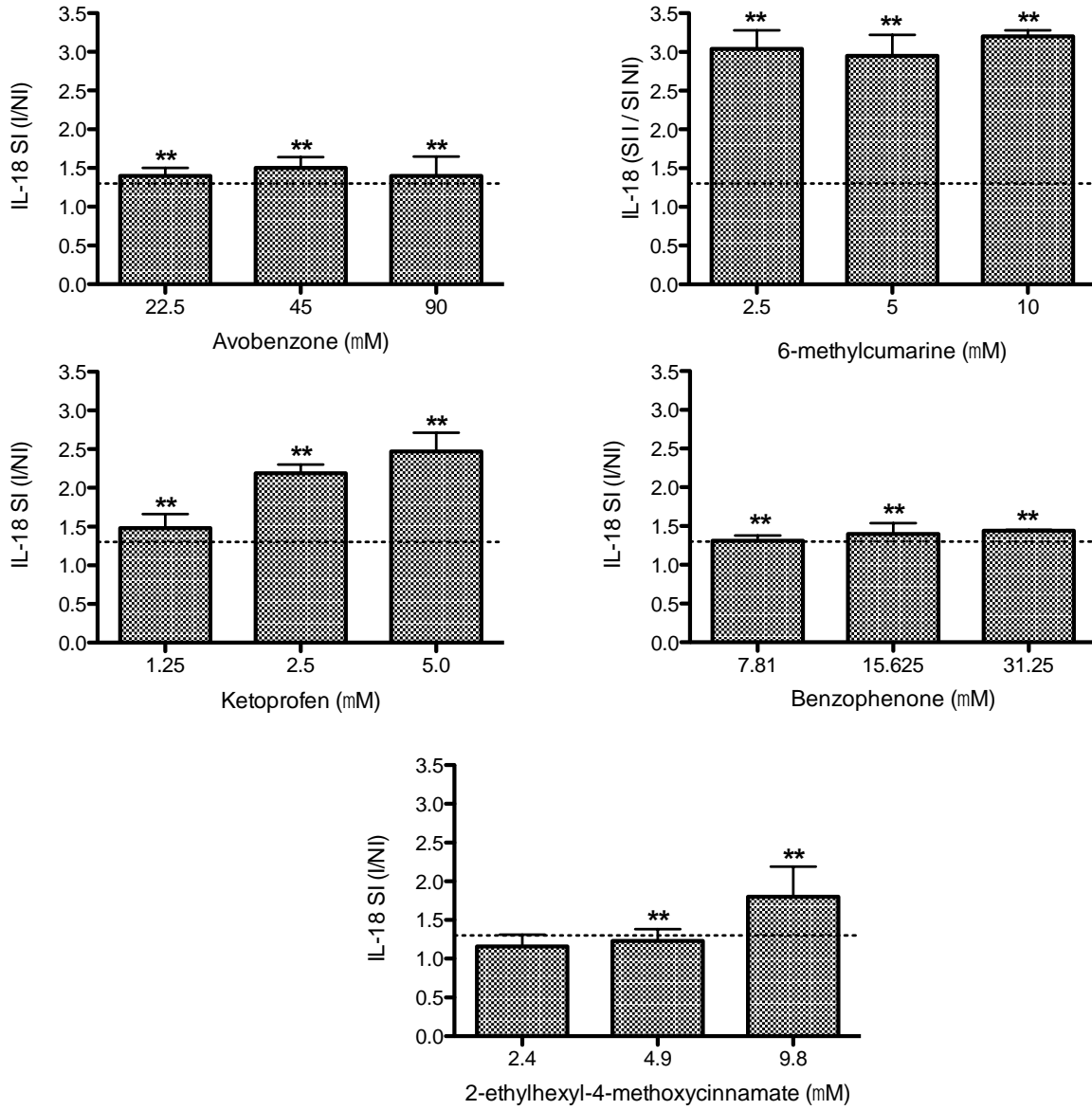


Figure 3 - Galbiati et al.

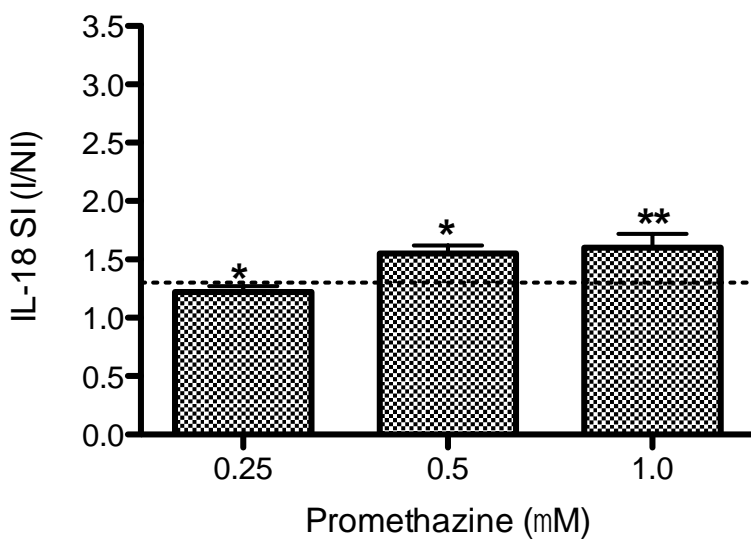
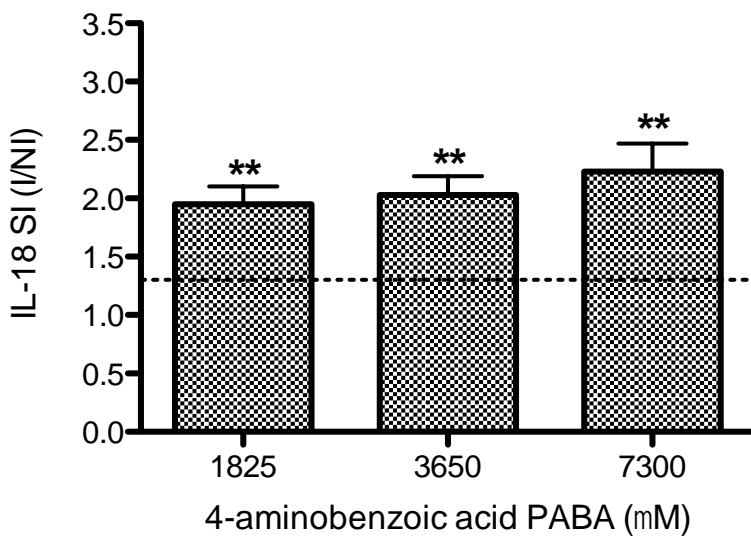
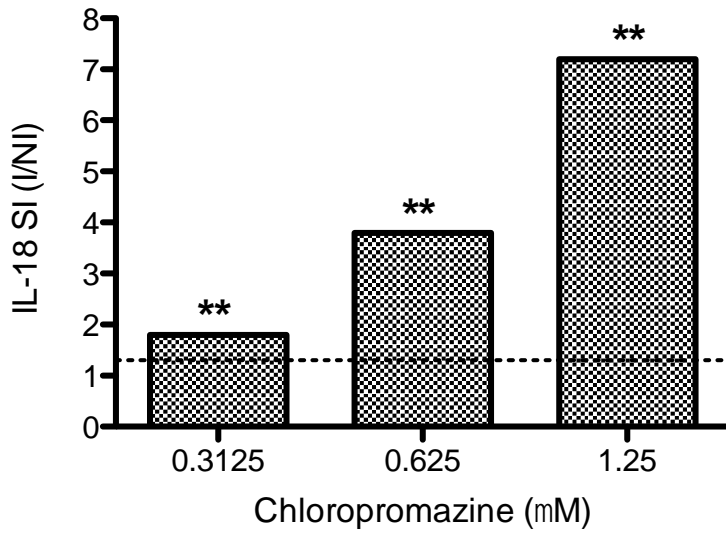


Figure 4 - Galbiati et al.

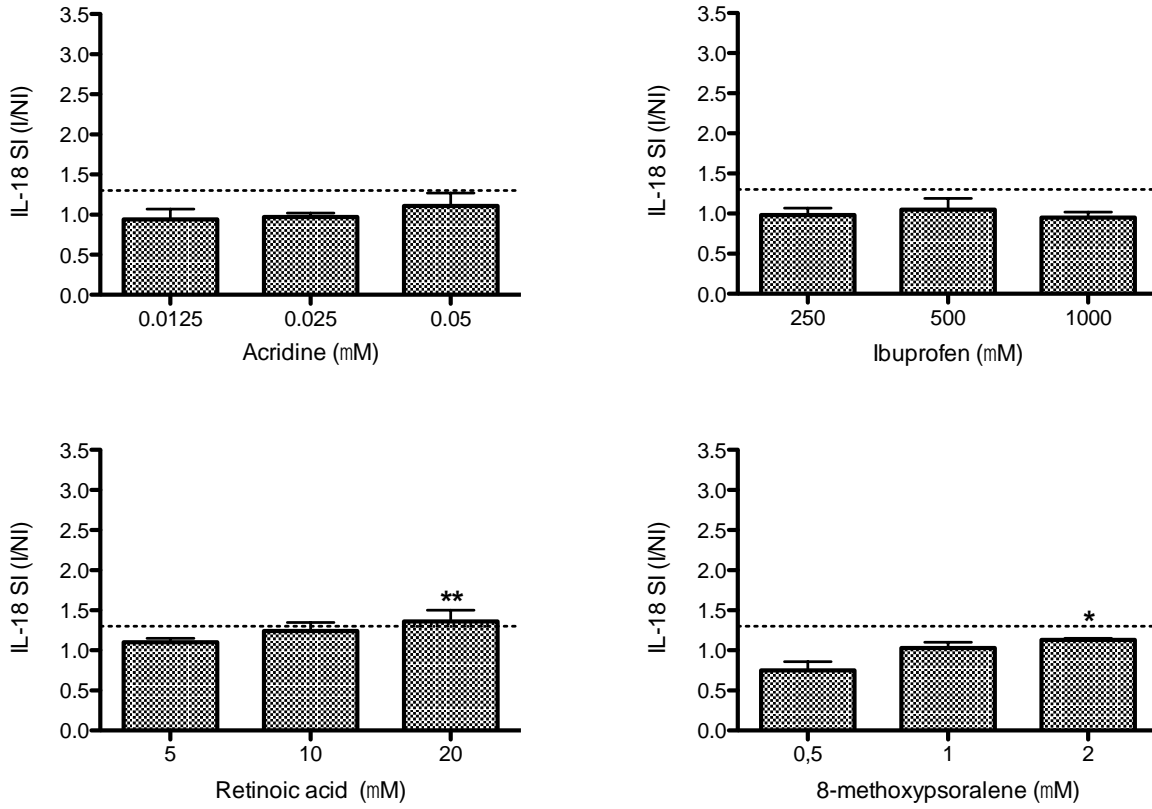


Figure 5 - Galbiati et al.

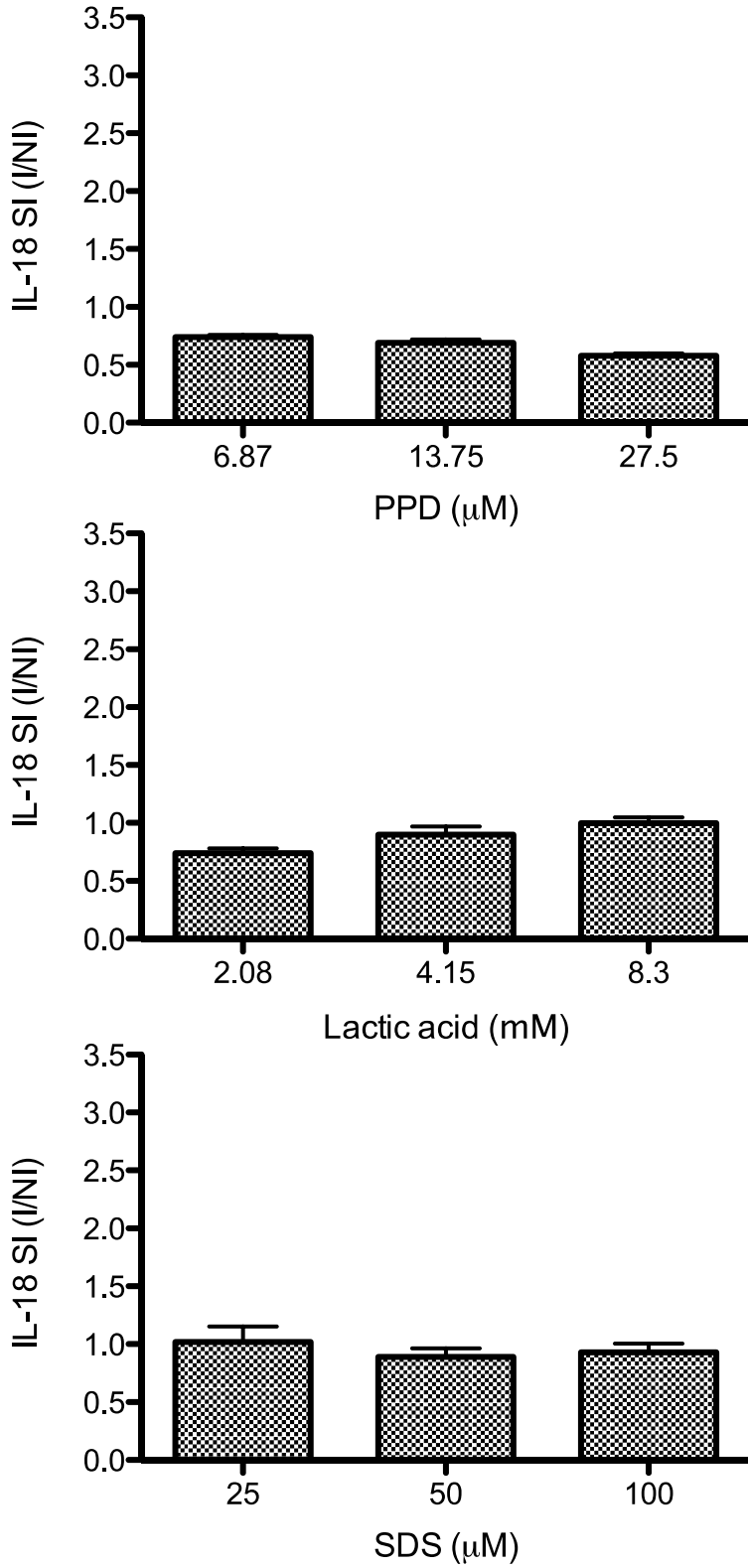
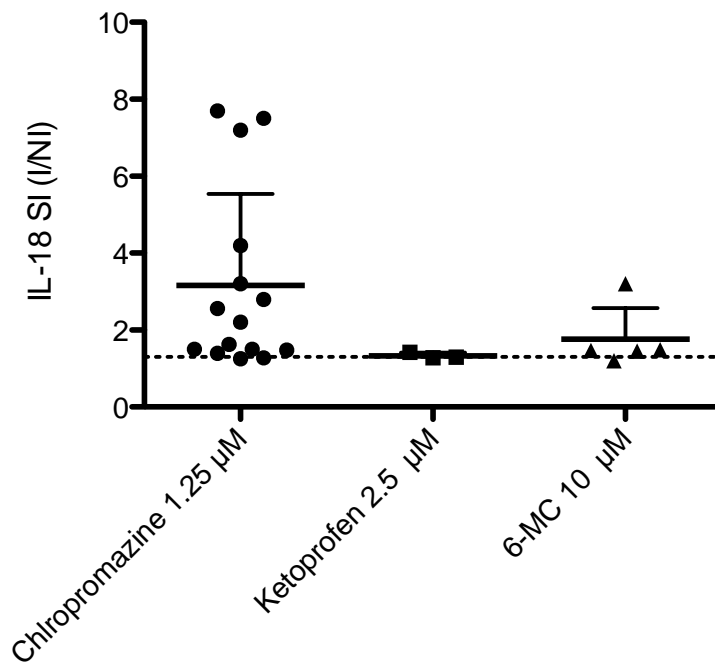


Figure 6 - Galbiati et al.



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**Highlights**

- Cell-based in vitro test for screening the photosensitizing potential of chemicals
- Development of a new in vitro method based on IL-18 production in keratinocytes
- Overall accuracy 100% was achieved