The EPISKIN Phototoxicity Assay (EPA): Development of an in vitro tiered strategy to predict phototoxic potential

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Abstract
The aim of the study was to investigate the ability of human reconstructed epidermis EpiSkin to assess the phototoxic potential of chemicals following topical or systemic treatment. Three categories of chemicals according to their available human phototoxic potential were evaluated: systemic phototoxic, topical phototoxic and non phototoxic chemicals. Products were applied topically or added to the underlying culture medium (systemic-like administration) at non cytotoxic concentrations. Following treatment, tissues were exposed to a non cytotoxic dose of UV A (50J.cm⁻²). Cell viability and pro-inflammatory mediators (IL-1α) were investigated 22h after UV A exposure. Our results showed that the phototoxic potential of chemicals can be assessed using cell viability endpoints combined with pro-inflammatory mediator measurements (cytokine IL-1α) in a 3-D epidermis model. The EPA was able to discriminate efficiently between phototoxic and non phototoxic products. Furthermore, the EPA is sensitive to the administration route in the prediction of the phototoxic potential. Differences observed between the 2 routes of administration (topical or systemic–like) may be linked in part to chemicals bioavailability which depends on specific penetration potential, epidermis barrier function and also on keratinocytes absorption/metabolization processes. The performances of the EPA showed that the EpiSkin model is an efficient tool able to integrate decision-making processes to address the question of phototoxicity linked to the administration route.

Keywords: in vitro phototoxicity, EpiSkin, reconstructed epidermis, 3D model, inflammatory mediators

Introduction
Photo-irritation is an adverse acute, inflammatory and non-immunologically mediated skin reaction that occurs after a single contact with a photoreactive chemical. Therefore, the identification of chemicals (or ingredients) and formulations able to elicit a phototoxic reaction is a crucial step in risk assessment processes. In recent years, extensive efforts have been made to develop in vitro methods for predicting the phototoxicity potential of chemicals in order to replace animal testing. In this respect, the in vitro 3T3 NRU phototoxicity test was scientifically validated by EU/COLIPA in 1998 (Spießmann, 1998) and was accepted as Method 41 in Annex V by directive 67/548/EEC in 2000, and as the Test Guideline 432 by the OECD in 2002 (Liebsch, 2005). However, some limitations exist: 1. this monolayer culture of fibroblasts is a simple basic system as compared to the three-dimensional architecture of skin involving connections and interactions between different cells types and extra cellular matrix (Augustin, 1997) 2. Some non-hydro-soluble chemicals can be tested only at low concentrations due to their lack of aqueous solubility, and results obtained may be questionable. 3. many complex mixtures or formulations cannot be tested 4. it provides only information about the direct photocytotoxic potential thus mimicking a systemic-like administration of the chemical (Liebsch, 2005).

To overcome these limitations, the use of reconstructed skin models is an interesting alternative, and several studies with such models have shown their capacity to predict photoirritancy (Augustin, 1997; Bernard, 2000; Cohen, 1994; Edwards, 1994; Liebsch, 1995; Liebsch, 1997; Medina, 2001; Portes, 2002). In this study, we developed an in vitro phototoxicity (EPA) test by using the human reconstructed epidermis EpiSkin (Tinois, 1994) which has been previously used for corrosion, irritation.
and photoirritation studies (Cohen, 1994; Portes, 2002; Tinois, 1994; Cotovio, 2005; Roguet, 2004). Complementary to cell monolayer phototoxicity tests, this 3-D model allows the topical application of a large panel of chemicals with different physicochemical properties as water insoluble or extreme pH values chemicals, finished products or complex formulations. Furthermore, it takes into account the influence of the administration route (topical treatment versus chemical directly diluted in the underlying culture medium to mimic a systemic-like administration) in the prediction of the phototoxic potency.

Sixteen chemicals belonging to "the list of chemicals with sufficient human data" published in the ECVAM Report 2 (Spielmann, 1994) plus an additional chemical (coumarin) were selected and tested in the EpiSkin<sup>LM</sup> EPA. Chemicals can be classified into three classes according to their available phototoxic potential human data. The first class is composed of 5 systemic phototoxic compounds (no case reports of phototoxic effects after topical applied to human skin exist), the second class includes 8 topical phototoxic chemicals (essentially evaluated by photopatch testing; known to be also systemic phototoxic compounds) and the third class contains 4 non phototoxic compounds.

This study had 2 main objectives. The first one was to show that phototoxic potential of the tested agents could be predicted by using a tiered strategy involving cytotoxicity and pro-inflammatory mediator release (cytokine IL-1α). The second objective was to show the influence of the administration route (topical treatment versus chemical directly diluted in the underlying culture medium to mimic a systemic-like administration) in the prediction of the phototoxic potency. The overall aim of the study was to demonstrate that this model combined with an adapted protocol can closely simulate in vivo conditions thus confirming previous studies in similar 3-D models (Bernard, 2000; Cohen, 1994; Edwards, 1994; Liebsch, 1995; Liebsch, 1997; Medina, 2001; Portes, 2002).

**Materials**

**In vitro Tissue culture**

The EpiSkin<sup>LM</sup> model (LM: large model; manufactured by EPISKIN S.N.C., Lyon, France) is a reconstructed organotypic culture of human adult keratinocytes reproducing a multilayered and differentiated human epidermis. Briefly, human adult keratinocytes were seeded on a dermal substitute consisting of a collagen I matrix coated with a layer of collagen IV fixed to the bottom of a plastic chamber. Epithelial differentiation was obtained by an air-exposed step leading to a 3-dimensional epidermis construct (1.07 cm<sup>2</sup> surface), with basal, spinous, granular layers (with specific markers) and a stratum corneum (Fig. 1). The kit system was presented as 12 unit packs with all necessary culture media, sterile additional plates and control certificate (IC50 of SDS, cell viability by MTT conversation test, histology).

EpiSkin<sup>LM</sup> units were delivered to the laboratory within 24 hours. Upon arrival, tissues were transferred to 12 well plates containing 37°C pre-warmed maintenance media (2 ml/well) and incubated overnight at 37°C, 5% CO<sub>2</sub> and 95% humidity.

**Test chemicals with human data:**

Chemicals used for this study were selected from "the list of chemicals with sufficient human data" published in the ECVAM Report (Spielmann, 1994) except for coumarin. Histidine, penicillin G, sodium lauryl sulfate (SDS) and coumarin were used as non-phototoxic reference compounds (e.g. NPT class). Two phenotiazine derivatives (chlorpromazine = CPZ and promethazine), 3 psoralen compounds (8-methoxypsoralen = 8-MOP, 5-methoxysporalen = 5-MOP and angelicin), bergamot oil and 2 non steroidal anti-inflammatory drugs (ketoprofen, tiaprofenic acid) were used as topical phototoxic (PT) compounds (e.g. topic PT class) but are also systemic phototoxic compounds. Three antibiotics (demeclocyclin, lomefloxacin, and ofloxacin),

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**Fig. 1:** Histology of the EpiSkin<sup>LM</sup> Model. Hematoxilin & eosin staining of a 13-days old culture. Human keratinocytes are seeded onto a Type I collagen matrix coated with Type IV collagen. Epithelial differentiation is obtained by an air-emersion step that leads to a fully-differentiated functional 3-dimensional epidermis with different layers.

**Fig. 2:** Spectral power distribution of the 1600W 6"x6" solar UV simulator Oriel equipped with WG335/3mm filter.
fenofibrate and furosemide were used as systemic PT reference compounds (e.g. systemic PT class; no case reports of phototoxic effects after topical applied to human skin exist). All compounds were provided by Sigma-Aldrich, Fluka or Pierre Fabre. 5-MOP, bergamot oil and coumarin were first dissolved in ethanol (final concentration 1% in water). 8-MOP was first dissolved in DMSO (final concentration 1% in water). Tiaprofenic acid was dissolved in corn oil. The other chemicals were dissolved in the main solvent used: water containing 1% of ethanol.

Irradiation source:
A 1.6kW LOT-Oriel solar simulator equipped with a Schott WG335/3mm filter emitting UVA and visible light but no UVB radiation was used for all irradiation experiments. Irradiance was measured using an IL1700 radiometer equipped with a UVA and a UVB probes (International Light Research Radiometer / Photometer, BFI Optilas International SA, Evry, France) before each UVA exposure (Fig. 2). These UVR source characteristics comply with the recommendations of the European Centre for the Validation of Alternative Methods (Spielmann, 1994).

Measurements of IL-1α:
IL-1α release in the culture medium was determined by a classic quantitative sandwich enzyme immunoassay technique (Quantikine, R&D Systems, UK). Protein levels of IL-1α were expressed as pg/ml.

Methods
UVA-induced effects on EpiSkin™ non treated tissues
After overnight incubation at 37°C, epidermis were transferred to 12-well plates containing 1.5ml of PBS (Ca²⁺ & Mg²⁺). 100µl of solvent used for phototoxicity evaluations were topically applied for 2 hours. Excess of solvent was eliminated and tissues were exposed to increasing doses of UVA (irradiance, 97W.m⁻²) from 0 to 70J.cm⁻². After exposure, tissues were incubated overnight at 37°C on fresh maintenance medium. Cell viability assessment by MTT conversion test (Mosmann, 1983) and histological studies were performed. Observations were made using a Leica Type LB–30T microscope equipped with a CCD camera Leica DC-300F (Leica Microsystems, Rueil-Malmaison, France). Aliquots of culture media were kept frozen (-20°C) for cIL-1α measurements.

In vitro EPA (EpiSkin™ Phototoxicity Assay)
Following overnight incubation at 37°C upon reception, epidermis were transferred to 12-well plates containing 1.5ml of PBS (Ca²⁺ & Mg²⁺). Tissues were treated in duplicate. 100µl of each test-chemical concentration was applied topically or added to the underlying culture medium (systemic-like administration). Appropriate control cultures were prepared with 100µl of the corresponding solvent. Duplicate plates were prepared for UVA and dark (controls) exposure purposes. After 2 hours of treatment, excess of product was eliminated and tissues were then exposed to UVA at a well defined non cytotoxic and physiologic radiation dose (50J. cm⁻² – irradiance, 97W.m⁻²). During irradiation, tissues were placed onto a refrigerating plate in order to avoid temperature increase. In parallel, the non-irradiated treated tissues were placed in the dark under the same conditions. After a 2 hours post-exposure period, tissues were incubated overnight at 37°C. 5% CO₂ and 95% humidity in fresh maintenance medium. Cytotoxicity was determined and culture media aliquots were kept frozen (-20°C) for pro inflamatory mediator measurements (IL-1α).

Calculations
Cell viability: Means from duplicate measurements of test-product concentrations for irradiated and non-irradiated tissues were expressed in % relative to the controls.
IL-1α: For the irradiated and non irradiated sets, mean IL-1α released from duplicate tissues for each test-product concentration was expressed as [IL-1α treated tissues - IL-1α control tissues]. Whenever the difference of IL-1α level between the test-product concentration and the control tissues was negative, the result was set to 0.
IL-1α levels and cell viabilities of exposed and non exposed conditions were compared for each test-product concentration.

EPA Prediction Model:
Basic phototoxic mechanisms can be described as an increased toxicity accompanied or initiated, in most cases, by releases of pro-inflammatory mediators by keratinocytes (Spielmann, 1998; Augustin, 1997; Cotovio, 2005; Barker, 1991b). Thus, an UVA-visible-absorbing chemical was predicted as phototoxic after systemic or topical treatment if it induces a significant toxicity increase (≥ 25% of C8V=25%) or if a significant difference of IL-1α releases ≥ 40 pg/ml (C8IL1=40pg) was set for one of the tested concentrations after UVA exposure as compared to the non exposed tissues. In contrast, a product was predicted as non phototoxic if neither C8V>25% nor C8IL1=40pg was detected. Similar PM based only on viability decrease (≥ 30%) have been previously established (Edwards, 1994; Liebsch, 1995) and used onto different skin reconstructed models (Bernard, 2000; Edwards, 1994; Liebsch, 1995; Liebsch, 1997).

Statistical analysis:
Cut-off values ("40pg/ml" for IL-1α and "25%" for viability) were graphically determined (ROC curve). Sensitivity (% prediction of PT compounds), specificity (% prediction of NPT compounds) and the accuracy (% of both PT and NPT chemicals correctly classified in vitro) were calculated. C8V>25% and
Results
Effects of UVA exposure on EpiSkin™

The sensitivity of the EpiSkin™ to UVA was first investigated. No significant cell viability decrease and no significant IL-1α release were observed in control tissues (Fig. 3) up to the highest UVA dose applied. No major modification of the epidermis histology was observed up to 50J.cm⁻² (Fig. 4.b,c,d,e). Some disorders in the basal layers became visible at 60J.cm⁻² (not shown) and were clearly evidenced at 70J.cm⁻² (Fig. 4.f). According to these results and to previous work published by Portes et al. (2002), the maximal non-cytotoxic UVA dose which did not provoke significant pro-inflammatory mediator release, morphological modifications or significant cell viability decrease was set to 50J.cm⁻². Similar experiments for negative control systemic-like conditions (final concentration of ethanol in medium was 0.06%) gave identical results (data not shown). The selected working dose of UVA was set to 50J. cm⁻² (1.6kW solar simulator equipped with an 320-400nm WG335/3mm filter ; irradiance, 97W.m⁻²) for the EPA study.

Phototoxic effects of 5-MOP (PT), promethazine (PT), CPZ (PT) and coumarin (NPT) after topical treatment on EpiSkin™

Results are illustrated in the Fig. 4. In unexposed treated tissues for all tested compounds, neither cell viability decrease nor IL-1α release was observed whatever the concentration (Fig. 4.A, 4.B, 4.C, 4.D). However, UVA irradiation considerably enhanced in a dose dependent manner, the cytotoxicity of 5-MOP, CPZ and promethazine, known as in vivo PT after topical contact. Indeed, in irradiated tissues, some disorders in the basal layers became visible at 60J.cm⁻² (not shown) and were clearly evidenced at 70J.cm⁻² (Fig. 4.f). According to these results and to previous work published by Portes et al. (2002), the maximal non-cytotoxic UVA dose which did not provoke significant pro-inflammatory mediator release, morphological modifications or significant cell viability decrease was set to 50J.cm⁻². Similar experiments for negative control systemic-like conditions (final concentration of ethanol in medium was 0.06%) gave identical results (data not shown). The selected working dose of UVA was set to 50J. cm⁻² (1.6kW solar simulator equipped with an 320-400nm WG335/3mm filter ; irradiance, 97W.m⁻²) for the EPA study.

Phototoxicity prediction of the 17 test chemicals in the in vitro EPA according to the prediction model indicators (CδV>25% and CδIL1>40pg) in comparison to the available in vivo human data.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Application</th>
<th>Phototoxic concentrations</th>
<th>Phototoxic Prediction</th>
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<tr>
<td></td>
<td></td>
<td>C&lt;sub&gt;δV&lt;/sub&gt;25%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;δIL1&lt;/sub&gt;40pg&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Topic</td>
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<td>NS</td>
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<td>SDS</td>
<td>Topic / Systemic</td>
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<td>NS</td>
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<td>S (0.133)</td>
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<td>NS</td>
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<td>S (1)</td>
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<td>Topic</td>
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<td>S (1)</td>
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<td>S (0.25)</td>
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<td>5-MOP</td>
<td>Topic</td>
<td>S (0.01)</td>
<td>S (0.01)</td>
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a: cell viability decrease >25% (comparison between treated irradiated and treated non irradiated tissues)  
b: IL-1α release >40pg/ml (comparison between treated irradiated and treated non irradiated tissues)  
S: Significantly different from non-irradiated epidermis (ANOVA, P<0.05). In this case, in parentheses, only the first phototoxic concentration was reported.  
NS: No significantly different from non-irradiated epidermis  
+ : topical and systemic phototoxic compound  
++ : strong topical and systemic phototoxic compound  
− : non-phototoxic chemical  
+*: systemic phototoxic chemical  
(*+*) = weak systemic phototoxic chemical
all concentrations of 5-MOP and CPZ induced a significant cell viability decrease exceeding 25% as compared to unexposed treated tissues (Fig. 4.A and 4.B). Promethazine showed photocytotoxic effects only at the highest concentrations (Fig. 4.C). Moreover, significant dose-dependent IL-1α releases, connected to cell death, were observed for these compounds. Results for the NPT chemical coumarin, showed no cytotoxic effects and no IL-1α release in irradiated tissues (Fig. 4.D). According to the PM (CδN>25% and CδIL1>40pg), the known in vivo topic PT and NPT compounds were correctly identified. Results for coumarin confirmed those previously published on Skin² tissues with photoallergenic products (Liebsch, 2005 ; Liebsch, 1995 ; Api, 1997)

**Phototoxic effects of Ofloxacin (weak systemic PT in vivo) and SDS (NPT) on Episkin™ after topical and systemic-like treatments.**

Results are presented in Fig. 5. After topical application in the non-exposed and exposed tissues, ofloxacin and SDS induced neither cell viability decrease nor significant IL-1α whatever
In contrast, ofloxacin (no case reports of phototoxic effects after topical applied to human skin exist). Although the number of NPT compounds was reduced as compared to PT compounds, the performance of the EPA (by taking into account the influence of the administration route) in the prediction of the phototoxic potency showed a very good sensitivity (92.3%) and an excellent specificity (100%) with an overall accuracy of 94.1%.

**Discussion**

The first step in risk assessment processes to assess the acute phototoxicity of UV-absorbing ingredients is to perform the *in vitro* 3T3 NRU phototoxicity test which was previously validated (Spielmann, 1998). However, one of the main disadvantages of this monolayer cell culture system is that water insoluble chemicals and cosmetic formulations can hardly be tested (Bernard, 2000; Liebsch, 1995). Moreover, this monolayer cell culture model test is over simplified in comparison with the complex 3-D skin structure and rather mimics a systemic-like administration route of the chemical. Reconstructed skin or epidermis tissues can be used as interesting models to evaluate *in vitro* phototoxicity. Their organic structure (multilayered and differentiated epidermis), the presence of barrier function (*stratum corneum*) simulate more closely the in vivo situation and allow topical applications of a large panel of chemicals with different physiochemical properties.

The aim of this study was to develop an *in vitro* assay able to identify the phototoxic potency of chemicals after topical or systemic-like administration using a 3-D reconstructed human epidermis model. The EpiSkin™ has been characterized physiologically and morphologically (Tinois, 1994). Moreover, recent (pre)validation studies have shown the capacity of the model to assess acute skin irritancy, skin corrosivity and phototoxicity of chemicals and cosmetics (Cohen, 1994; Portes, 2002; Cotovio, 2005; Roguet, 2004).

The maximal non-cytotoxic UVA dose which did not provoke significant pro-inflammatory mediator release, morphological modifications or significant cell viability decrease used in the assay was set to 50J.cm⁻² (Fig. 3.A and 3.B). These results are in agreement with those of Portes et al. (2002) and show that the EpiSkin™ model can be exposed to physiologic UVA doses. Basic phototoxic reaction induced by photoreactive chemicals usually leads to increased toxicity (Spielmann, 1998), initiated in most cases, by inflammatory reactions (Augustin, 1997; Barker, 1991b). Protocols based on viability were previously established for Skin² and were applied to different skin and epidermis reconstructed models.
Phototoxic classification of test-chemicals was evaluated by using a tiered strategy involving a similar cytotoxicity cut-off ($C_{\delta,V}>25\%$) and a pro-inflammatory IL-1-$\alpha$ release cut-off ($C_{\delta,IL1}>40\text{pg}$). An UVA-absorbing chemicals were classified as phototoxic after topical or systemic-like treatment if they are significantly above these limits. Using this optimized EPA, three classes of products according to available human data (5 systemic PT compounds chemicals, 8 topical PT chemicals and 4 NPT compounds) were evaluated (Table 1). All NPT chemicals were correctly identified in the EPA. Interestingly, coumarin, as in vivo and in vitro studies (Liebsch, 2005; Liebsch, 1995; Api, 1997) was correctly classified as negative. All in vivo topical PT chemicals (ketoprofen, tiaprofenic acid, 8-MOP, 5-MOP, bergamot oil, promethazine and CPZ) were correctly identified in the assay excepted for angelicin. These results showed the ability of the EPA to discriminate between NPT and topical PT compounds. Angelicin, reported to exhibit only a weak phototoxicity potential after topical application in few cases (Api, 1997) was not correctly identified in the assay. Reasons for this result could be due to a lack of penetration. The 5 in vivo systemic PT compounds evaluated (lomefloxacin, ofloxacin, demeclocyclin, furosemide and fenofibrate) were correctly identified: predicted as phototoxic only when using a systemic-like administration. These results show the ability of the model to discriminate topic PT from systemic PT chemicals and evidenced the influence of the administration route as an important issue in the prediction of chemicals phototoxic potency.

Many in vitro cytotoxic tests are based on the MTT conversion assay. This colorimetric method is linked to the mitochondrial dehydrogenases activity of viable cells. However, many chemicals are likely to interfere with the MTT assay. Therefore, identification of alternatives endpoints for skin phototoxicity evaluation (such as morphology or expression and release of cytokines), based on in vivo skin phototoxicity mechanisms needs to be performed. In the present study, phototoxicity

(*) Significantly different from non-irradiated epidermis (ANOVA, $P<0.05$), grey bars and black line.

Fig. 5: Phototoxic effects of Ofloxacin (weak systemic PT in vivo) and SDS (NPT) on EpiSkin™ after topical and systemic-like treatments and UVA exposure. Results are the mean of 3 and 2 independent experiments ± SEM for topical application and systemic-like administration respectively.
potency of chemicals can be determined using cell viability endpoint and pro-inflammatory mediator measurement. IL-1α produced by keratinocytes is part of the first events in inflammatory processes. As previously published (Augustin, 1997; Cohen, 1994), these results show that phototoxic reactions in vitro induce direct lethal effects on cells as well as indirect effects as cytokine release as possible messenger for keratinocytes. Results also show that IL-1α is an alternative endpoint to the cell viability test to assess the phototoxicity potency of chemical interfering with the MTT. Although the number of NPT compounds was reduced as compared to PT compounds, the EPA performances of the EPA by taking into account the influence of the administration route showed a very good sensitivity (92.3%) and an excellent specificity (100%) with an overall accuracy of 94.1%.

Conclusions

1. The phototoxicity of chemicals can be assessed by using a tiered strategy involving cytokotoxicity and pro-inflammatory mediator release (cytokine IL-1α) in a 3-D model.

2. Furthermore, the EPA takes into account the influence of the administration route (topical treatment versus a systemic-like administration) in the prediction of the phototoxic potency. Differences observed between the 2 routes of administration may be linked in part to chemicals bioavailability which depends on specific penetration potential, epidermis barrier function and also on keratinocytes absorption/metabolization processes.

3. The promising performances of the EPA showed that the EpiSkin model is an interesting tool able to integrate decision-making processes to address the question of phototoxicity linked to the administration route. Additional experiences need to be performed with a larger number of compounds and cosmetic formulations to confirm the robustness of our PM.

References
