

Comparison of human skin irritation and photo-irritation patch test data with cellular in vitro assays and animal in vivo data

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Abstract

Replacement of the rabbit Draize skin irritation test or the animal photo-irritation test is in course in Europe under the REACH chemical strategy and the Cosmetics Directive. Various in vitro protocols, including 3D skin models, have been assessed. One key difficulty in determining the validity of alternative in vitro methods is that the in vivo animal data is both scarce and often of limited utility for prediction of effects in man. Consequently, we have examined in human 4h patch tests a number of chemicals of EU borderline classification. In addition, in a specific group of cosmetic ingredients we assessed the potential of photo-irritation using results obtained in 3D skin models and in human photopatch tests. Several chemicals reported to be irritant in the rabbit were found to be without effect in humans. 3D skin model assays and human patch tests provided concordant results particularly in case of non-irritating and non-phototoxic substances. In our view, skin model tests seem to be a useful tool for the prediction of human skin irritation or phototoxicity hazard, particularly for consideration of initial concentration for confirmatory human patch tests to prove substance and product safety.

Keywords: skin irritation, photo-irritation, human patch test, human epidermal model, rabbit Draize test

Introduction

The in vivo skin irritation/corrosion test in rabbits was introduced by Draize in the 1940s to predict hazardous effects of substances and formulations coming into contact with human skin (Draize et al., 1944). Several decades later, it was recognized that the design of the test and visual grading of the effects is highly subjective and that the test performed has low predictivity and relevance towards humans (Nixon et al., 1975, Basketter et al., 2004). Reproducibility of the assay has also been questioned (Weil and Scala, 1971). These studies concluded that results of the animal test should not be relied upon exclusively as 1) the test over predicts irritating effects of chemicals in relation to human skin; 2) although accepted by regulators, the test has never been scientifically validated. The replacement of the in vivo rabbit skin test is therefore not only matter of scientific effort but recently also a legal requirement of the European Cosmetics Directive (EC, 2003).

During the past two decades, a number of

reconstructed human epidermal (RHE) models became available, that can be used for studies of hazardous effects of topically applied substances. In the area of skin irritation, two assays using RHE models (EPISKINTM, EpiDermTM) were recently evaluated in the ECVAM Skin Irritation Validation study (Spielmann et al., 2007). For EPISKIN assay, satisfactory levels of specificity and sensitivity were obtained when compared to existing rabbit data. The EpiDerm assay revealed high specificity but relatively low sensitivity, and further optimization of the protocol was recommended to reach concordance with the regulatory accepted in vivo test. However, knowing the overpredictive nature of the in vivo rabbit assay, it was further recommended to search and analyze relevant human skin irritation data and to look at the reasons for discordant classifications. In consequence, we decided to perform a limited number of human patch tests with some substances from the ECVAM skin irritation validation study and several other commercially available chemicals and

to compare the obtained results with the classification based on in vivo rabbit test and two EpiDerm in vitro skin irritation assays.

In the area of phototoxicity, the in vivo photo-irritation assays have also never been validated. While the regulatory accepted in vitro 3T3 NRU Phototoxicity Test (PT) reliably predicts phototoxic hazard, the test is not able to estimate the phototoxic photopotency of tested substances when applied in real-life concentrations. Therefore, the aim of the second study was to demonstrate the usefulness of a tiered strategy for risk assessment of photopotency. The applied tiered testing strategy included spectral and chemical analyses of selected substances (4 different bergamot oils), in vitro 3T3 NRU Phototoxicity Test (Liebsch et al., 1998) and 3D Human Skin Model Phototoxicity assay (Liebsch et al., 1999), followed by skin photo-patch test in a group of volunteers (Neumann et al., 2000).

Materials and methods

Chemicals

Chemicals used in the skin irritation studies were purchased from Sigma - Aldrich, SAFC and Fluka, commercially non-available chemicals were kindly provided by ECVAM. Majority of EU classifications were derived from in vivo rabbit data published in the ECETOC Database No. 66 (ECETOC, 1995). Selected chemicals were carefully considered with regard to their general toxicological profile; chemicals exhibiting toxicologically unacceptable hazards were excluded from the studies.

Samples of bergamot oil (CAS No. 8007-75-8) for phototoxicity studies were supplied by AROMA s.r.o. (Czech Republic), BIOMEDICA s.r.o. (Czech Republic), SIGMA AG (Germany) and SCHUPP GmbH (Germany).

Skin irritation assays

4-hour human patch test

The selection of volunteers and the test methods were carried out in accordance with the ethical principles as set out in the Declaration of Helsinki and International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS, 2002). The patch test procedure (Basketter et al., 2004) involves application of 0.2 ml (0.2 g for moistened solids) on a 25 mm plain Hill Top Chamber containing a Webril pad (Hill Top Companies, Cincinnati, Ohio, USA) to the skin of the upper outer arm of 30 human volunteers for up to 4h. Exposure time progressively increases from 15 and 30 min through 1, 2, 3 and 4h, with each progressive application at a new skin site, until a positive irritation reaction is reported by the volunteer and/or recorded by a trained assessor. Treatment sites are assessed for the presence of irritation using a 4 point scale at 24, 48 and 72 h after

patch test removal. Sodium lauryl sulphate (SLS) at 20% aq is used as the positive control; water is used as the negative control. A volunteer exhibiting a weak but unequivocal erythema over most or all of the test site or greater reaction at any of the observation times is considered to have demonstrated a positive irritant reaction and further treatment with that substance does not proceed. The number of panellists who develop a positive irritant reaction after progressive exposure up to 4h is determined. The substance is classified as skin irritant (R38), when the incidence of positive irritation reactions to the undiluted test substance is significantly greater than or not significantly different (using Fisher's exact test) than the level of reaction in the same panel of volunteers to 20% SLS.

EpiDerm™ in vitro skin irritation assay

In vitro data were obtained using the EpiDerm Skin Irritation Test Protocol (Kandarova et al., 2005), as evaluated in ECVAM Skin Irritation Validation study (Spielmann et al., 2007). In this assay, the prediction model is based on determining the viability of 3 tissues exposed to a chemical (15 min initial exposure followed by rinse off and a 42h post-incubation) referenced to unexposed, negative control tissues. The modified protocol employed prolonged exposure (60 min). If the tissue viability (as determined using the MTT assay) of chemical-exposed tissues is reduced to 50% or less, the chemical is classified (in both protocols) as an irritant; if the tissue viability exceeds 50%, the material is classified as a non-irritant.

Photo-irritation assays

Spectral and chemical analysis

Absorbance in the UV/vis range was determined by means of Spectrophotometer Varian Cary 1E according to OECD Test Guideline 101 (OECD, 1981). Analyses of diluted oils were performed by capillary gas chromatography (Restek RTX-1 F&F: 30m x 0.32 mm i.d. x 0,5 µm d.f.) coupled to a mass spectrometer Fisons Trio 1000.

Source of irradiation

A doped mercury-metal halide lamp (SOL 500, Dr. Hönle, Germany), which simulates the spectral distribution of natural sunlight, was used as the UV light source in all of the in vitro and in vivo experiments.

3T3 neutral red uptake phototoxicity test

The test was performed according to INVITTOX Protocol No. 78 (Liebsch and Spielmann, 1998), using 3T3 Balb/c fibroblasts. The photoirritation factor (PIF) was calculated as the ratio of toxicity for each substance with and without UV light (EC,

2000). A second predictor of phototoxicity (MPE), the mean photo effect, was also calculated (Holzhütter, 1997). According to the OECD Test Guideline 432, a test substance with a PIF > 2 and < 5 or an MPE > 0.1 and < 0.15 is predicted as "probably phototoxic" (OECD, 2004).

EpiDerm™ phototoxicity test

The test was conducted according to Liebsch et al. (1999). EpiDerm EPI-200 tissues were exposed to the test materials diluted in distilled water or sesame oil, applied for 24 h in a volume of 50 µl per tissue in water and 20 µl per tissue in oil. One set of tissues was irradiated with a non-toxic dose of 6 J/cm² (as measured in the UVA range). One day after the treatment and UVA exposure the cytotoxicity was detected as reduction of mitochondrial conversion of MTT to formazan. The results of mean tissue values in the presence and absence of UV light were

compared and a test substance was considered to be phototoxic, if one or more test concentration of the (+UVA) part of the experiment revealed a decrease in viability exceeding 30% when compared with identical concentrations of the (-UVA) part of the experiment.

Photopatch test in human volunteers

The assay was performed according to Neumann et al. (2000) in a limited group of 5 healthy females, aged 27-62. Test concentrations were selected according to the EpiDerm phototoxicity test results, i.e. for each bergamot oil the highest non-phototoxic/non-cytotoxic concentration of the samples diluted in water and sesame oil was employed. The test samples were applied in occlusion (Finn Chamber, USA), using saturated filter paper discs (diameter of 10 mm), on the lower back in two areas on both sides of the spine. After 1 h exposure and patch test removal

Table 1. Classification of 25 chemicals by rabbit Draize test, EpiDerm assay (15/60 min exposure) and human patch test

| | Chemical identification | CAS No. | Rabbit | EpiDerm 60' | EpiDerm 15' | HPT | Positive reactions | Positive reactions to SLS |
|----|---|---------------|---------|-------------|-------------|-----|--------------------|---------------------------|
| 1 | Nonanoic acid | 112-05-0 | R34/R38 | R38 | R38 | I | 19/29 | 26/29 |
| 2 | Heptaldehyde | 111-71-7 | R38 | R38 | R38 | I | 17/29 | 23/29 |
| 3 | Decanoic acid | 334-48-5 | R38 | R38 | R38 | I | 28/29 | 26/29 |
| 4 | 20% SLS | 334-48-5 | R38 | R38 | R38 | I | 94/118 | 94/118 |
| 5 | 1-Bromohexane | 111-25-1 | R38 | R38 | NI | I | 16/30 | 22/30 |
| 6 | 10-Undecenoic acid | 112-38-9 | R38 | R38 | R38 | NI | 1/29 | 23/29 |
| 7 | 1-Decanol | 112-30-1 | R38 | R38 | R38 | NI | 1/30 | 23/30 |
| 8 | bis[(1-Methylimidazol)-(2-ethyl-hexanoate)],zinc complex | not allocated | R38 | R38 | R38 | NI | 0/29 | 26/29 |
| 9 | Butyl methacrylate | 97-88-1 | R38 | R38 | NI | NI | 0/30 | 22/30 |
| 10 | Alpha terpineol | 98-55-5 | R38 | R38 | NI* | NI | 0/29 | 23/29 |
| 11 | 2-Isopropyl-2-isobutyl-1,3-dimethoxypropane | 129228-21-3 | R38 | NI | NI | NI | 6/29 | 26/29 |
| 12 | di-n-Propyl disulphide | 629-19-6 | R38 | NI | NI | NI | 6/30 | 22/30 |
| 13 | Linalyl acetate | 115-95-7 | R38 | NI* | NI | NI | 0/30 | 23/30 |
| 14 | Hexyl salicylate | 6259-76-3 | R38 | NI | NI | NI | 0/30 | 22/30 |
| 15 | Terpinyl acetate | 80-26-2 | R38 | NI* | NI | NI | 0/30 | 22/30 |
| 16 | Naphthalene acetic acid | 86-87-3 | NI | NI | NI | NI | 0/30 | 22/30 |
| 17 | Heptyl butyrate | 5870-93-9 | NI | NI | NI | NI | 0/30 | 22/30 |
| 18 | di-Propylene glycol | 25265-71-8 | NI | NI | NI | NI | 0/30 | 22/30 |
| 19 | Dodecanoic acid (lauric acid) | 143-07-7 | NI | NI | NI | NI | 0/29 | 23/29 |
| 20 | Hydroxycitronellal | 107-75-5 | NI | NI* | NI | NI | 0/30 | 23/30 |
| 21 | Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane, 1-(3-isopropylphenyl)-1-phenylethane, 1-(4isopropylphenyl)-1-phenylethane | 52783-21-8 | NI | NI | NI | NI | 0/29 | 26/29 |
| 22 | Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one,1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one | 224031-70-3 | NI | NI | NI | NI | 0/29 | 26/29 |
| 23 | 4-Methylthio-benzaldehyde | 3446-89-7 | NI | R38 | NI | NI | 0/30 | 22/30 |
| 24 | 1-Bromo-4-chlorobutane | 6940-78-9 | NI | R38 | R38 | NI | 0/30 | 22/30 |
| 25 | 3,4-Dimethyl-1-H-pyrazole | 2820-37-3 | NI | R38 | R38 | NI | 11/29 | 26/29 |

* - may become irritant, depending on age and purity of the test sample

Table 2. Concordance of classification

| Classification methods | sensitivity | specificity |
|------------------------|-------------|-------------|
| Rabbit ► HPT | 100% | 50% |
| Human PT ► Rabbit | 33.3% | 100% |
| EpiDerm 60 ► Rabbit | 66.7% | 70% |
| EpiDerm 15' ► Rabbit | 46.7% | 80% |
| EpiDerm 60' ► HPT | 100% | 60% |
| EpiDerm 15' ► HPT | 80% | 75% |

Table 3. Bergamot oils phototoxicity - 3T3 NRU PT

| Bergamot oil | Solvent | Run | PIF | MPE |
|--------------|------------------------|-----|---------------|----------------|
| AROMA | Ethanol (1% in PBS) | 1 | 1.218 | 0.027 |
| | | 2 | 1.067 | -0.004 |
| | DMSO (1% in PBS) | 1 | 1.006 | 0.005 |
| | | 2 | 1.185 | -0.038 |
| | PBS | 1 | 1.075 | 0.004 |
| | | 2 | 0.905 | 0.064 |
| BIOMEDICA | Ethanol (1% in PBS) | 1 | 1.377 | -0.004 |
| | | 2 | 1.178 | 0.016 |
| | DMSO (1% in PBS) | 1 | 1.051 | 0.003 |
| | | 2 | 0.904 | -0.017 |
| | PBS | 1 | 1.310 | 0.113* |
| | | 2 | 1.113 | 0.031 |
| SIGMA | Ethanol (1% in PBS) | 1 | 1.159 | 0.000 |
| | | 2 | 1.383 | 0.147* |
| | DMSO (1% in PBS) | 1 | 1.266 | 0.048 |
| | | 2 | 1.666 | 0.144* |
| | PBS | 1 | 1.648 | 0.017 |
| | | 2 | 2.380* | 0.232** |
| SCHUPP | Ethanol (1% in PBS) | 1 | 1.126 | 0.126* |
| | | 2 | 1.358 | 0.156** |
| | DMSO (1% in PBS) | 1 | 1.419 | 0.081 |
| | | 2 | 1.410 | 0.088 |
| | PBS | 1 | 2.581* | 0.289** |
| | | 2 | 3.415* | 0.348** |

* probably phototoxic, ** phototoxic
(according to OECD TG 432)

one test area was irradiated (5 J/cm², as measured in the UVA range). The other non-irradiated area served as a control. Test reactions were recorded 24 h, 48 h and 72 h after irradiation. Any reaction (erythema, oedema or pigmentation) was recorded. The number of panellists who developed a positive reaction was expressed as ratio of positivity.

Results

The EU classification of chemicals based on the available rabbit data, data obtained with the EpiDerm model applying 15 and 60 min exposure times and with the 4h human patch test (HPT), is shown in Table

Table 4. Bergamot oils phototoxicity – human photopatch test

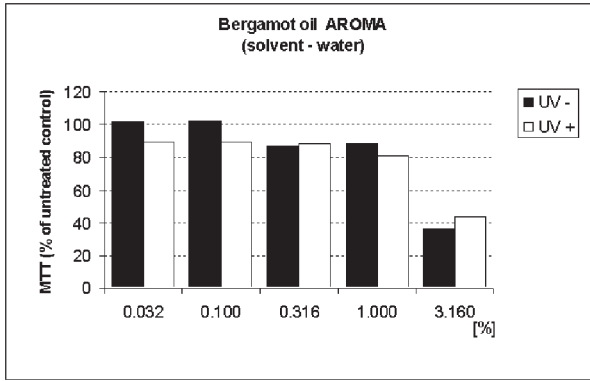
| Bergamot oil | ratio of positivity at different time intervals after irradiation | | |
|---------------------------|---|------------|------------|
| | 24 h | 48 h | 72 h |
| AROMA (1% in water) | 0/5 | 0/5 | 0/5 |
| BIOMEDICA (1% in water) | 0/5 | 0/5 | 0/5 |
| SIGMA (0.1% in water) | 5/5 | 5/5 | 5/5 |
| SCHUPP (0.0316% in water) | 5/5 | 5/5 | 5/5 |
| AROMA (1% in oil) | 0/5 | 0/5 | 0/5 |
| BIOMEDICA (10% in oil) | 0/5 | 0/5 | 0/5 |
| SIGMA (0.316% in oil) | 0/5 | 0/5 | 0/5 |
| SCHUPP (0.1% in oil) | 0/5 | 0/5 | 0/5 |

1. Amongst the 15 chemicals reported to be irritant in rabbits only 5 were found to be irritating to human skin. Table 2 shows the concordance of classification using the rabbit skin irritation test, EpiDerm 15 and 60 min in vitro tests and classification using 4h HPT is demonstrated.

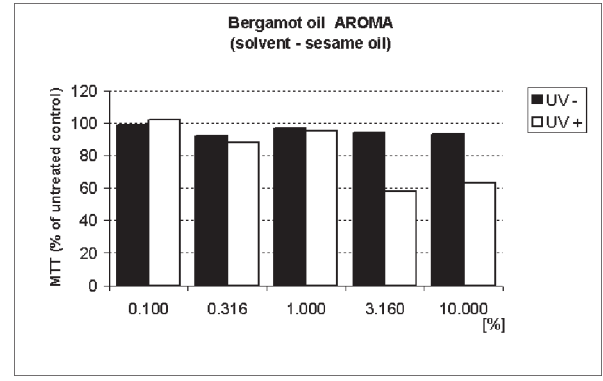
When compared to the 4h HPT results, the rabbit in vivo test provided 100% sensitivity (5/5), but only 50% specificity (10/20). The EpiDerm protocol with 15 min exposure corresponded better to the response seen in man – sensitivity 80% (4 of 5 irritants classified correctly), while the optimized EpiDerm protocol with 60 min exposure time reached higher concordance with the rabbit test.

In the phototoxicity study, spectrophotometric analysis in UV/VIS range revealed higher absorbance (in the range of 300 – 360 nm) in case of BO SCHUPP and BO SIGMA, compared to BO AROMA and BO BIOMEDICA. Chemical analysis identified a higher content of photoactive compounds present in BO SIGMA and BO SCHUPP. Besides bergapten, other potentially phototoxic components, as citropten, bergamoten, geranial and neral, were detected. In the 3T3 NRU PT, only BO SIGMA and BO SCHUPP were classified phototoxic, however, only on the basis of borderline phototoxicity results. Moreover, the test results were highly dependent on the solvent used for the test samples dilution (Table 3). The EpiDerm Phototoxicity Test identified as phototoxic BO AROMA in oil, BO SIGMA and BO SCHUPP

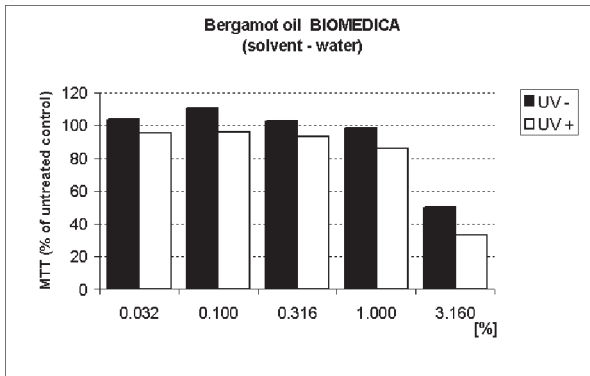
a) BO AROMA in water



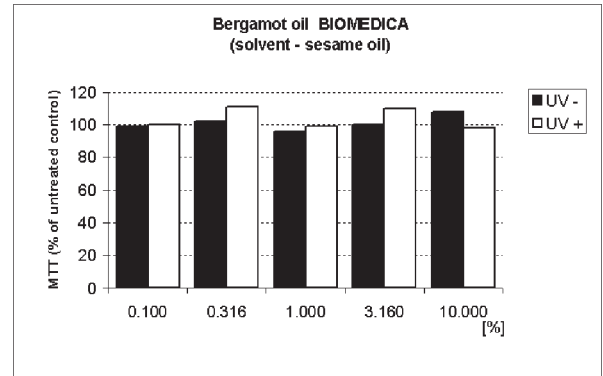
b) BO AROMA in sesame oil



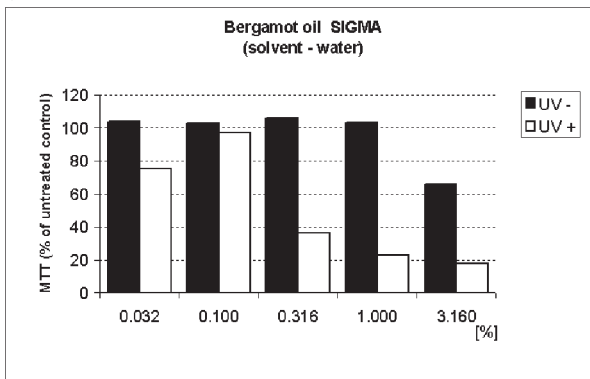
c) BO BIOMEDICA in water



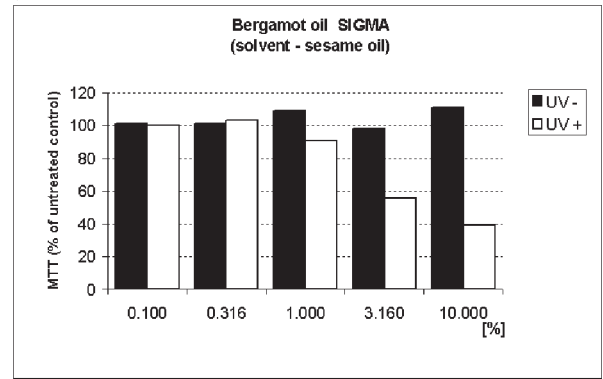
d) BO BIOMEDICA in sesame oil



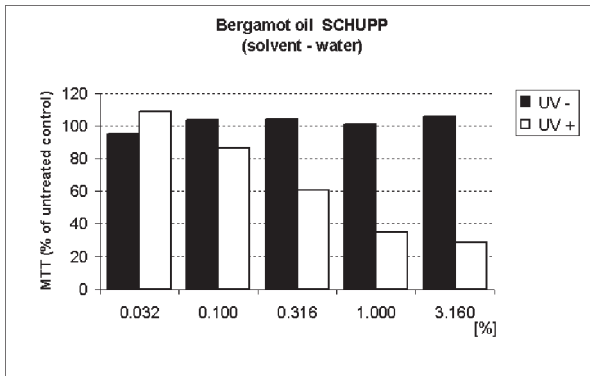
e) BO SIGMA in water



f) BO SIGMA in sesame oil



g) BO SCHUPP in water



h) BO SCHUPP in sesame oil

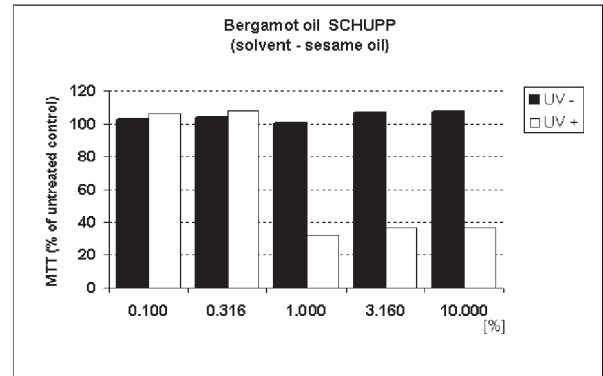


Fig. 1. Bergamot oils phototoxicity – EpiDerm Skin Phototoxicity Test. Each column represents the mean viability of EpiDerm tissues (n=2) in the presence (□) and absence (■) of UV light.

in both water and sesame oil (Figure 1a - h). No phototoxicity was proved for BO AROMA in water and BO BIOMEDICA in both water and sesame oil. Subsequent human photopatch tests employed the highest non-phototoxic/non-cytotoxic concentrations obtained in the EpiDerm Skin Phototoxicity Test. From all the tested samples, the potential of phototoxicity was identified in vivo only for BO SIGMA and BO SCHUPP diluted in water (Table 4).

Discussion

In the validation studies for replacement of in vivo tests, high quality in vivo data are rather scarce. Information on inter-laboratory reproducibility is almost non-existent and reproducibility was not systematically evaluated (Hoffman and Hartung, 2006). Human data from controlled human patch tests, that could be used to confirm the classifications, are usually not available or not existing. That is why we decided to perform a limited number of human studies using human patch tests in the field of skin irritation and phototoxicity.

From the 15 tested chemicals reported to be irritant in the rabbit only 5 (33.3%) were found irritant for humans. This result supports an extensive study published previously by David Basketter (2004), where about 40% of irritants classified by rabbit test did not trigger skin irritation when tested in 4h HPT. In our study, the rabbit test exhibited 100% sensitivity, but only 50% specificity, meaning that the rabbit test identifies irritants reliably, however 50% non-irritants are over predicted (wrongly labelled as irritants). Consequently, we compared the prediction of two in vitro EpiDerm assays with both rabbit and human data. The 15 min test under predicted one of the human irritant (1-Bromo-hexane) and classified two substances as false positive. It is of note, that 1-Bromo-hexane was classified as irritant in the human patch test on a basis of almost borderline data; 16 out of 30 test persons responded positively in fully occlusive conditions. The 60 min protocol on the other hand did not miss any of the human irritants, however, provided couple of false positive results.

In summary, the data obtained in 4h HPT should represent a gold standard to detect acute skin irritation potential when approaching validation of alternative methods. Unfortunately, testing protocols for many endpoints must be optimized to achieve higher concordance with historical data obtained in animals, rather than with reliable and existing human data.

The phototoxicity study revealed, that phototoxicity of bergamot oils is dependent on furocoumarins content and the solvent (vehicle) used. Using aqueous dilutions, a clear phototoxic classification was obtained in both in vitro systems and in vivo human photopatch test. The aqueous solutions of

BOs revealed in HPPT even higher phototoxicity than initially expected, while concentrations of all bergamot oils diluted in sesame oil correlated well with findings determined by the RHE model. Consequently, even substances as essential oils of limited water solubility should be tested in aqueous dilutions if such applications are foreseen in practice. Data obtained with the 3D human skin model EpiDerm proved able to distinguish between non-phototoxic and phototoxic samples (Jírová et al., 2005, Jones et al., 2003) and to get a starting point for the human phototoxicity patch test. Nevertheless, the extrapolation of in vitro test results to the situation in man should be performed only to a limited extent and strictly respecting all ethical principles.

If the protocols for 3D RHE models are optimized, they might be more useful than the animal tests for the prediction of the human health hazard. When approaching validation of alternative methods, not only the animal data but also relevant human data should be taken into consideration, as only those provide a final judgment about the prediction ability of a new alternative method.

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