Session 5.4
Development and validation of alternatives for dermal toxicity testing

Poster

Phototoxicity testing: Relevance of the 3T3-NRU assay for UVB absorbers

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Many types of chemicals have been reported to induce phototoxic effects. They all absorb radiation in the wavelength range of 290-700 nm and are distributed in sun-exposed tissues. The *in vitro* 3T3 Neutral Red Uptake (NRU) phototoxicity test is based on a comparison of the cytotoxicity of a compound when tested in the presence and absence of exposure to UVA light. This assay was recently adopted by the European Agency for the Evaluation of Medicinal Products (EAMA/CPMP, 20021) and the Food and Drug Administration (FDA/CDER, 2003) for guidance on photosafety testing. The 3T3-NRU phototoxicity test was shown to be predictive of acute effects in animals and in human for UVA/visible absorbers (Spielmann et al. 1998). Positive chemicals in this test are highly likely to be phototoxic *in vivo* following systemic or topical applications (OECD, 2002). In order to determine the reliability and relevance of this assay for strict UVB absorbers, 8 chemicals that absorb in UVB range only were tested in which 6/8 have been reported to be photo-irritants *in vivo*. The correlation between *in viv*o published data and those obtained with the 3T3-NRU *in vitro* assay will be shown.

Lecture

The COLIPA strategy for the development of *in vitro* alternatives: Skin irritation

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Skin irritation is a multi-endpoint non-immune response of skin to insult, e.g. by substances or preparations. It covers both clinical (e.g. erythema, dryness) and sensory (e.g. burning, itching, stinging) reactions. In the past, the only accepted model for the predictive identification of agents which could cause skin irritation was the Draize rabbit skin test. More recently, *in vitro* methods allowing the identification of corrosive substances have been adopted, allowing the complete replacement of animals for the assessment of corrosivity, as well as a refinement and reduction in animal usage in the general strategy for skin irritation. Our present aim is to undertake the work necessary to ensure the final step, replacement of animal testing, can be achieved. To this end, the COLIPA Skin Tolerance Task Force (STTF) has funded work on genomic analysis of the early phase of the response of skin to insult with a range of irritant substances. In addition, it has supported the analysis of the response to irritants of 3D skin models at the protein level. This work has yielded valuable insights, but also demonstrates the complexity of the response to irritants at the molecular level. Related to this is the concern about how to interpret *in vitro* data in relation to skin irritation risk assessment. Ultimately, the ability of skin to tolerate a novel cosmetic formulation is assessed by carefully controlled human testing. The role of *in vitro* skin irritation alternatives remains to be fully characterised.

Lecture

The ECVAM skin irritation validation study

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In 1998, the European Centre for the Validation of Alternative Methods (ECVAM) commissioned a pre-validation study of five *in vitro* methods for identifying skin irritants. The predictive ability of two reconstituted human skin methods (EpiDerm and EPISKIN) and one animal skin model (the mouse skin integrity function test) was found to be inadequate in this study but, following refinements to the protocols or the methodology for statistical analysis, ECVAM concluded that all three methods could proceed to a full validation study. This was conducted in two phases; in phase I, 20 coded chemicals (9 irritant and 11 non-irritant, as defined by the EU classification system) were tested in a single (lead) laboratory. The overall predictive ability was 75% for EpiDerm, 80% for EPISKIN but only 45% for SIFT; for the human skin models, incorrect predictions were restricted to chemicals which were close to the classification borderline (mean *in vivo* erythema scores between 1.7 and 2.4). EpiDerm and EPISKIN progressed to phase II in which 60 coded chemicals (26 irritant and 34 non-irritant) were tested in three laboratories. Interleukin-1-alpha release was evaluated as a test endpoint for both models in addition to the usual MTT cell viability assay to determine whether this would improve the predictive ability for chemicals on the borderline of classification. ECVAM will review the data from phase II in July 2005 and the results will be presented at this meeting.

Poster

Effects of THz radiation on human keratinocytes *in vitro*

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Terahertz frequencies are beginning to be employed in medical imaging. It is a non-ionising energy capable of producing images of skin tumours and teeth, speculatively at no damage to the body. In this laboratory primary human keratinocytes exposed *in vitro* to between 0.1 and 3 THz are still able to differentiate and commence production of the skins natural barrier functions. The sensory innervation of the skin and the corneal epithelium are know to affect differentiation and therefore barrier functionality. A sensory nerve cell line, ND7/23, can be maintained in an undifferentiated state, and then driven to differentiate and develop dendrite-like projections. Our studies using human keratinocytes, gave no adverse cell reactions or affected their ability to differentiate and form cornified envelopes. Hence more extensive exposure periods of up to 24 hrs, for the NHK and ND7/23 cells, were applied.

Exposed to THz radiation for 10 to 60 minutes both cell types alone or in co-culture converted rezazurin, (a viability assay), and the fluorescein leakage assay (barrier function assay) gave no observed adverse effects, c.f. controls; 16 hours (ND7/23) and 24 hours (NHK), exposure in culture medium or HBSS, at room temperature or 37°C in a CO_2 cabinet, for up to 16 hours, also showed no statistical loss of function compared to unexposed cells. Hence there is no indication that this level of THz results in cell damage. Repeat experiments are underway along with exposure to a more power source under construction.

Funded from the THz-BRIDGE EU grant (QOL-2000-4.2).

Lecture

Optimisation of the EpiSkin protocol combining a tiered strategy in the framework of the ECVAM skin irritation validation

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In recent years there has been considerable interest in the development of *in vitro* model substitutes for animals in cutaneous pharmaco-toxicology. Particularly suited to test chemicals with wide and diverse physical properties, the reconstructed epidermis model EpiSkin allows current use in fundamental and screening studies. Able to mimic *in vivo* situation, 3D models were included in the ongoing ECVAM-funded validation for acute skin irritation. *In vitro* selected tests should be capable of discriminating between irritant chemicals (EU risk R38) and non-irritant chemicals (EU risk "no classification"). In order to meet this specific need, an optimisation of the already published (Portes et al., 2002) EpiSkin protocol, based on a specific extended post treatment incubation period (42 hours) was applied to a set of 48 chemicals. Sensitivity, specificity and accuracy of the MTT-based PM were, 85%, 78.6% and 81.3% (respectively) with low false negative rate (12%). Stronger performances of this optimised protocol were sustained by robustness properties and an efficient separation between I and NI classes. IL-1α, IL-8 and the adenylate kinase were also investigated. Combining selected end points in a simple tiered strategy (TSTS), MTT being the first sort followed by IL-1α determination, resulted in a clear improvement of predictive capacities (95% sensitivity, drop of false negatives (4.3%)).

The results demonstrated the usefulness of the TSTS as a decision-making tool able to strengthen the PM performances. Furthermore, the transferability of this final optimised protocol 15 min - 42 hours to other skin models (Kandarova et al., 2004) was a great advantage shared in the ongoing ECVAM skin irritation test validation.

Lecture

7-ethoxycoumarin metabolism in a viable pig ear skin model: New alternative model for absorption and metabolism studies

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The skin is the largest organ and most accessible drug metabolising organ. Skin expresses many cytochromes P450 that have critical roles in exogenous and endogenous substrates metabolism. The role of absorption rate and enzyme activity on cutaneous metabolism of topically applied xenobiotics was assessed by determining simultaneously percutaneous penetration/metabolism of 7-ethoxycoumarin (7-EC) in a newly developed skin organ culture model, coming from ear of domestic pigs.

Six doses of 7-EC (from 10 to 400 nmol/cm^2) in two vehicles (PBS/ethanol 2:1 solution and myritol/ethanol solutions 2:1) were applied on the viable pig ear skin model. Diffusion and metabolism of 7-EC was assessed by Radio-HPLC during a time period of 72 h. All the experiments were carried out in triplicate.

More than 65% and 40% of the applied dose of 7-EC with the hydrophilic vehicle and with the lipophilic vehicle respectively were recovered in the receptor medium. Metabolism occurred with both vehicles and remained active during 72 h. About 15% of the applied 7-EC dose were metabolised for the low doses (10-50 nmol/cm^2) and 5% for the highest doses (100-400 nmol/cm^2) suggesting a saturation of the enzymes. More than 95% of the metabolites was the glucuronide form and found in the receptor medium. The other 5% was hydroxy-7-EC, located in the skin.

This viable pig ear skin model exhibits cytochromes P450 dependant phase I and phase II activities. This model may provide a suitable, relevant and alternative model to animal and human studies for cutaneous uptake and detoxification metabolism.

Poster

In vitro dermal penetration studies with excised pig skin and reconstructed skin

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The OECD Test Guideline 428 "Skin Absorption: *in vitro* method" recommends the use of excised human or pig skin for the *in vitro* investigation of dermal absorption and penetration. Reconstructed skin models can be acceptable for this purpose as well, if their suitability is proven by appropriate correlation studies with both the respective skin model and excised skin.

Our studies with pig skin and reconstructed skin (Epiderm™, MatTek, USA) with cosmetic formulations (finite dose) confirmed the distinctly higher penetration rates through the reconstructed skin barrier as compared to pig skin for substances with lower octanol-water partition coefficients.

After topical application of e.g. Sodium-Dodecyl-Sulphate solutions (infinite dose) in a wide range of concentrations (0.05 mmol/l to 100 mmol/l including 14C-SDS as a radio-labelled tracer) we found an approximately 100 times higher permeability with Epiderm™ in comparison to split thickness pig skin (750+/-50 µm). Results for substances with higher octanol-water partition coefficients (log P_{OW}>8) were more comparable in both models.

Poster

ECVAM Key Area Topical Toxicity: Summary of ongoing activities

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The area of topical toxicity managed and co-ordinated at ECVAM includes eye and skin irritation/corrosion, phototoxicity and percutaneous absorption. Validated alternative tests for skin corrosion, photoxicity and percutaneous absorption have obtained regulatory acceptance at both the EU and OECD level. In contrast, alternative methods for eye and skin irritation are urgently needed, especially in light of the 7th Amendment to the Cosmetic Directive and REACH. Since topical application represents a main route of exposure to cosmetics, eye and skin irritation data are essential. As a base set requirement, around 30,000 substances produced or imported in quantities greater than 1 tonne/annum will require eye and skin irritation data under REACH.

An update on key activities to validate the most promising *in vitro* tests for skin and eye irritation will be provided. Highlights include: an update on the ECVAM validation study on the EPISKIN and EpiDerm assays for acute skin irritation, which is in its final phase and involves 6 laboratories. A review of ICCVAM/ECVAM joint activities to develop a tiered testing strategy for eye irritation are provided. Lastly, technical support to DG Enterprise and DG Health and Consumer Protection in relation to Directive 76/768/EEC and to the Scientific Committee on Consumer Products (SCCP) are highlighted.

Poster

Skin irritation: Prevalence, variability and regulatory classification of existing *in vivo* data from industrial chemicals

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In vivo rabbit data for skin irritation registered in the European New Chemicals Database (NCD) and an ECETOC Database were evaluated to characterise the distribution of irritation potential among chemicals and to assess the variability of the animal test. These databases could be used to determine experimental and rudimentarily within-laboratory variability, but not between-laboratory variability. Our evaluation suggests that experimental variability is small. Using two classification systems – the system currently used in Europe and the Globally Harmonised System (GHS) – the prevalence of skin irritation data obtained from NCD was analysed. This analysis revealed that out of 3121 chemicals tested, less than 10% showed an irritation potential in rabbits which would require an appropriate hazard label and 64% did not cause any irritation. Furthermore, it appears that in practical use the European classification system introduces bias towards over-classification. Based on these findings, we conclude, that the classification systems should be refined taking prevalence into account. Additionally, prevalence should be incorporated into the design and analysis of validation studies for *in vitro* test methods and in the definition of testing strategies.

Poster

The influence of light source and cell line on *in vitro* phototoxicity tests

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There are various *in vitro* assay methods for evaluating the phototoxicity of chemicals. One of these techniques is *in vitro* 3T3 cell Neutral Red Uptake Phototoxicity (3T3 NRU PT) Test, which had been scientifically validated by ECVAM between 1992 and 1996. In this assay, Balb/c 3T3 mouse fibroblast is recommended and a doped mercury metal halide lamp (SOL 500; Dr Honle, Martinsried, Germany), which artificially simulates the spectrum distribution of natural sunlight, is used as the UV light source.

The purpose of this study is to investigate the flexibility of this assay. We used some different cell lines in addition to Balb/c 3T3 and two light sources, SOL 500 (UVA plus visible light) and xenon lump with a filter which extracts only UVA.

The assay was carried out according to the method described by EU/COLIPA (Spielmann et al., 1994, *ATLA*, 314-348). Fourteen substances (nine phototoxic chemicals, two photosensitisers and three non-phototoxic chemicals) are assayed in this study.

Our results indicate that the application of different cell lines to 3T3 NRU PT test does not largely influence the sensitivity of this assay, however, the spectrum of light sources or the condition of irradiation affect that. Furthermore, we reveal that the exposure dose of UVA influences the sensitivity of this assay more strongly than the intensity of irradiation.

Poster

In vitro assessment of ingredients and formulations using commercially available human epithelial tissue models

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Human tissue models represent the most promising developments in *in vitro* toxicology. The production of such models requires specialist expertise, not available in all laboratories, and therefore commercial availability is required. Our laboratory sources cultures from several suppliers. In this report, the usefulness of human epithelial cultures in the selection and ranking of ingredients or formulations using a benchmark approach is illustrated by examples of irritation testing using MatTek EpiDerm™ skin and EpiOcular™ corneal, and SkinEthic™ buccal mucosa models. All models were multilayered, 3D tissues prepared from human cell lines or primary human keratinocytes grown on filter inserts at the air/liquid interface. Protocols involved topical application of test materials, at concentrations and exposures as recommended by manufacturers and/or from previous experience. Cytotoxicity was measured using MTT reduction. The cytotoxicity rank order of test materials was compared including benchmarks of known or acceptable irritation potential. In general, the most irritant materials were the most cytotoxic and differences/similarities between test materials and benchmarks could be identified. The conclusions of these studies were that 3D human tissue models are useful for screening for irritation potential to a variety of tissues, to identify more irritant materials and prioritise development of those potentially less irritant. Other advantages of the use of this type of model are that they enable the testing of water-insoluble materials and of both liquids and powders. These models will provide a useful tool for further investigation of the most appropriate protocols and more specific predictive endpoints/markers than general cytotoxicity.

Lecture

Skin irritation *in vitro*: EpiDerm™ test protocol developed and optimised for an ECVAM validation study on skin irritation testing of chemicals

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Several validation studies have been conducted on *in vitro* methods, to discriminate between skin irritating and non-irritating chemicals. Most promising results were obtained until now with human reconstructed epidermal models EpiDerm™ and EPISKIN®. Based on experience of similar performance of the two skin models, it was suggested that a common test protocol and prediction model should be developed for the prediction of skin irritation potential with the two models (Fentem et al., 2002).

When the EPISKIN protocol (Portes et al., 2003) was applied to the EpiDerm model, an acceptable specificity was achieved, whereas sensitivity was low. In 2003, the EPISKIN protocol was further refined (Cotovio et al., 2005) by extending the post-incubation period after exposure to test chemicals. This refinement was as well evaluated on EpiDerm. With the new test design and additional technical refinements, high sensitivity (80%) and specificity (78%) were obtained (Kandarova et al., 2004).

Since all optimisation steps had been conducted always with the same test chemicals, it was decided to verify the protocol with a new set of chemicals. In the second study 26 additional chemicals (10 rabbit irritants and 16 non-irritants) were evaluated on EpiDerm. With this unbalanced testing set a specificity of 94%, and a sensitivity of 60% were obtained. Positive and negative predictivity and accuracy remained almost unchanged (around 80%).

Overall, 45 chemicals were tested in the final protocol. The resulting high positive (86%) and negative predictive values (79%) confirm the reliability of the improved test protocol (accuracy of 80).

Poster

ECVAM feasibility study: Can the pre-validated *in vitro* skin model phototoxicity assay be upgraded to quantify phototoxic potency of topical phototoxins?

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The determination of phototoxicity in the 3T3 NRU-PT according to OECD Test Guideline 432 is often the first step in the sequential phototoxicity testing strategy. If the chemical provides a negative result in the 3T3 NRU-PT, in most instances no further testing is required. However, if the result is positive, the chemical may be still applied topically at safe concentrations, depending on the absorption and accumulation of the chemical in the skin.

Thus, in addition to the information on inherent phototoxicity potential assessed by the 3T3NRU-PT, additional testing may be required to obtain combined information on the phototoxicity and bioavailability of the chemical in the skin.

Ideally, confirmatory tests should be performed *in vivo* on human volunteers, but for ethical reasons, this is not acceptable, if the 3T3 NRU-PT has provided a positive result. Thus, to avoid confirmatory testing *in vivo* in animals, reconstituted human 3D skin models are offering an attractive *in vitro* alternative for testing, since such models are characterised by both skin barrier function and viable primary skin cells.

In the current study, several substances (mostly cosmetic ingredients) which are known to be safely used in humans, and which provided positive results in the 3T3 NRU PT were evaluated on the reconstructed human skin model EpiDerm and, if the result was negative, tested in a limited group of human volunteers. First results we obtained show, that the human skin model phototoxicity test represents a useful step in the sequential strategy for phototoxicity testing.

Poster

Skin corrosion *in vitro*: Assessment of the SkinEthic® reconstituted human epidermal (RHE) model for *in vitro* skin corrosion testing according to OECD TG 431

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In 2002 the National Co-ordinators of OECD Test Guideline Programme (WNT) endorsed a New Draft Test Guideline TG 431 (Human Skin Model) for *In Vitro* Skin Corrosion Testing which was finally adopted in April 2004. This guideline specifies general functional and performance criteria that have to be met, if a new skin or epidermal model is intended to be used for the skin corrosion testing of chemicals *in vitro*.

In 2003 ZEBET tested several chemicals with known corrosive potential on the SkinEthic reconstituted human epidermal (RHE) model using the validated EpiDerm test protocol and prediction model. After minor technical adaptations, classifications obtained were comparable to those obtained previously with the validated human skin models EPISKIN and EpiDerm.

From December 2003 to February 2004 ZEBET, SafePharm and BASF conducted an inter-laboratory trial with 12 coded reference chemicals proposed by the OECD TG 431 in order to confirm the performance of the SkinEthic skin corrosion assay.

In each laboratory, for each of the test chemicals, three independent tests were performed. Results obtained with the SkinEthic epidermal model were reproducible, both within and between laboratories, and over time. Concordance between the *in vitro* predictions of skin corrosivity potential obtained with the SkinEthic epidermal model and the predictions obtained with the accepted skin models was very good. The new test was able to correctly distinguish between corrosive and non-corrosive reference chemicals and can be regarded as valid method for the use in context with OECD TG 431.

Poster

Assessment of the SkinEthic® reconstituted human epidermal model for the prediction of the dermal irritation potential of chemicals

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During the past ten years, a lot of attention has been paid to the development of *in vitro* tests that could substitute irritation studies employing animals. The best results were until now obtained with 3D reconstructed human skin equivalents, for which several test protocols have been developed.

In areas of skin corrosion and phototoxicity, it has been proven that similarities between well developed 3D skin models allow to use common test protocols, obtaining similar results. Recently, efforts have been undertaken to develop a common protocol for the assessment of skin irritation of chemicals using skin models. This "common skin irritation protocol" for EPISKIN and EpiDerm™ reconstructed epidermal models is currently evaluated in an ECVAM validation study.

Concurrently with the ECVAM skin irritation validation study, ZEBET (Berlin, Germany) performed a small scale study applying the "common skin irritation protocol" on SkinEthic Reconstructed Human Epidermis (RHE) to verify whether this protocol can be successfully transferred to another epidermal model. Twenty substances from the ECVAM pre-validation study on skin irritation were tested on SkinEthic RHE. After minimal model specific adaptations, almost identical results (when compared to results of EpiDerm and EPISKIN models) were obtained.

Then the protocol's transferability was evaluated at ZEBET and Schering AG (Berlin, Germany) by testing six coded chemicals in three independent runs, obtaining very good results. In addition, an analysis of IL 1-α release was performed, with the aim to investigate if a second endpoint could add valuable information for the prediction of skin irritation potential of chemicals.

Poster

Wound healing response of the EpiDerm Full Thickness (EFT-200) *in vitro* human skin equivalent after solar UV irradiation: Comparison to excised human skin

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Normal human epidermal keratinocytes (EK) and dermal fibroblasts (FB) were cultured to produce full-thickness skin equivalent (EFT-200) consisting of FB-containing dermal and stratified epidermal components with a fully developed basement membrane at the dermal/epidermal junction. The wound healing response of EFT-200 and excised human skin after solar UV-irradiation were compared. H&E stained, EFT-200 histology sections displayed a dose-dependent increase in apoptotic sunburn cells 24 h post-irradiation. After 72 h, sunburn cells persisted in mid dose samples (40 J/cm^2, metal halide lamp), which were thinner than controls (indicating a major decrease in EK proliferation) but without major epidermal damage. However, after 48 and 72 h, high dose (61 J/cm^2) samples showed extensive epidermal and dermal damage. Nonetheless, viable basal cells remained in some areas, with signs of proliferation and epidermal regeneration. A 50% increase in MMP-1 released into the culture media was observed at 24 h and at 48 h, mid and high dose samples showed 100% and 150% increases, respectively. At 72 h, mid dose MMP-1 release was equivalent to control, but high dose release remained elevated at 125%. Similar experiments with excised human skin showed increased sunburn cell formation, tissue thinning at 40 J/cm^2 and extensive damage at 61 J/cm^2, and basal cell proliferation/epidermal regeneration at 72 h in high dose samples. These results show that EFT-200 behaves similarly to excised human skin in terms of UV induced damage and wound healing. The model will prove useful for additional applications in wound healing and other dermal/epidermal phenomena.

Poster

Dose-response evaluation of skin irritation using a 3-dimensional human skin model

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A 3-dimensional cultured human skin model has been developed and an alternative to skin irritation testing has been investigated. In the ECVAM validation protocol, hazard identification of chemicals has been evaluated using this model. However, we consider that this model is also useful for evaluating dose-response toxicity of chemicals. Therefore, we used the epidermal model (Labcyte™, Japan Tissue Engineering Co., Ltd.) and two skin models with a corneal layer (TESTSKIN™: Toyobo Co., Ltd. and Vitrolife-Skin™: Gunze Co., Ltd.), which are 3-dimensional human skin model made in Japan, and compared the cytotoxicity obtained by exposing of these models to 4 chemicals: Sodium lauryl sulfate, Benzethonium chloride, Polyoxyethylene oleyl ether(10) and Propylene glycol. As a result, the cytotoxicity of these chemicals was found to be stronger than the irritancy shown by human patch data, and there was a high level of false positivity compared to that shown in human patch data. Thus, we considered that these models were useful as an alternative to skin irritation testing for evaluating of strong irritancy.

Poster

How should we evaluate skin irritancy by patch test?

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Skin irritancy of a cosmetic product or topical medicament has been evaluated by a visual assessment of 24 or 48 hours human patch testing in Japan. We consider that patch testing is useful and achieving high accuracy is important to develop alternative to skin irritation testing. Since patch testing involves visual assessment, the results of such visual approaches can be subjective, and therefore variable between observers and institutions.

1. Visual assessment of human skin irritation might not be reliable and reproducible: According to visual assessment based on the Japanese patch-test reading-criteria (JPTRC: Kawamura et al., 1970), there was a significant variance in evaluating erythema among the readers. It is difficult to evaluate erythema from weak to moderate. The experience of the readers was not always associated with reliable and reproducible evaluation.

2. Newly proposed criteria to evaluate skin irritation on patch testing: Education and improvement of evaluation criteria seem important for standardising the evaluation of skin irritation. We have tried to develop new criteria for judging and colour atlas to education in the use of patch test to provide reliable and reproducible results. Seven grades of erythema were established according to the intensity, distribution of erythema, and presence or absence of edema or surface change.

3. Establishment of an education system: Using the newly proposed criteria, we provided seminars and lectures on judging skin irritation. Before and after lecture, all participants performed examination. As a result, reliability and reproducibility of responses from all members were increased.

Poster

Validation study for TESTSKIN™, a three-dimensional cultured human skin model as an alternative to skin irritation testing applied to forty cosmetic substances

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As a validation trial for an alternative to skin irritation testing in Japan, we performed a validation study of TESTSKIN™, a 3-dimensional cultured skin model. In this validation study, 7 laboratories participated, including the kit suppliers (TOYOBO Co., Ltd.). Participants performed the pre-test and main trial and obtained ET_{50} values (time to 50% reduction in MTT compared to the untreated control value) over a five month period from May to October 2002. Forty cosmetic ingredients in addition to 1% sodium lauryl sulphate solution (positive control), distilled water and olive oil (solvent) were selected, coded and supplied to the laboratories.

As a result, most chemicals did not show marked great differences in ET_{50} scores on tests repeated at each laboratory, using the estimation method as shown in the second paper. The feasibility of TESTSKIN™ was suggested through the experiment, although inter-laboratory variation was significant for some chemicals.

Furthermore, we compared the validation testing data and *in vivo* data in this study. *In vivo* primary skin irritation testing was performed using rabbits and humans. Comparing animal and human data, the consistency rate in humans was 66% of that in rabbits. The false positive rates were 32% and false negative rates were 40% in rabbits.

We compared human data with 200 data obtained from several laboratories in this validation study. The consistency rate of those data was 69%, the false positive rate was 38% and the false negative rate was 6%. These data show that the reliability of this method was similar to that of animal testing.

Poster

Validation study for Vitrolife-Skin™, a three-dimensional cultured human skin model as an alternative to skin irritation testing using ET_{50} protocol

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As a validation trial for an alternative to skin irritation testing in Japan, we performed a validation study of Vitrolife-Skin™, a 3-dimensional cultured skin model. In this validation study, 9 laboratories participated, excluding the kit suppliers (Gunze Co., Ltd.). Participants performed the pre-test and main trial and obtained ET_{50} values (time to 50% reduction in MTT compared to the untreated control value): ET_{50} protocol over a four month period from June to September 2004. Fourteen cosmetic ingredients, distilled water and olive oil (solvent) were selected, coded and supplied to the laboratories.

As a result, most chemicals did not show marked differences in ET_{50} scores on tests repeated at each laboratory, using the estimation method. The feasibility of Vitrolife-Skin™ using the ET_{50} protocol was suggested through the experiment, although inter-laboratory variation was significant for some chemicals.

Furthermore, we compared the validation testing data and *in vivo* data in this study. *In vivo* primary skin irritation testing was performed using humans. Compared to *in vivo* human data, the consistency rate of those data was 68.9%, the false positive rate was 54.5 % and the false negative rate was 3.6%. These data show the reliability of this method was similar to that obtained by animal testing such as using rabbits.

Poster

Validation study for Vitrolife-Skin™, a three-dimensional cultured human skin model as an alternative to skin irritation testing using Post-Incubation (PI) protocol

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As a validation trial for an alternative to skin irritation testing in Japan, we performed a validation study of Vitrolife-Skin™, a 3-dimensional cultured skin model. In this validation study, 9 laboratories participated, excluding the kit suppliers (Gunze Co., Ltd.). Participants performed the pre-test and main trial and obtained cytotoxicity using Post-Incubation (PI) protocol: treatment for 10 min after washing, incubated for a further 18 hrs (percent MTT viability) over a four month period from June to September 2004. Fourteen cosmetic ingredients, distilled water and olive oil (solvent) were selected, coded and supplied to the laboratories.

As a result, most chemicals showed marked differences in cytotoxicity on tests repeated at each laboratory. The low feasibility of the PI protocol was suggested through the experiment and inter-laboratory variation was significant for many chemicals.

Furthermore, we compared the validation testing data and *in vivo* data in this study. *In vivo* primary skin irritation testing was performed using humans. Compared to *in vivo* human data, the consistency rate of those data was 44.4%, the false positive rate was 41.2% and the false negative rate was 72.4%. These data show the reliability of PI protocol was lower than that obtained by animal testing such as using rabbits.

Poster

Use of the cytosensor microphysiometer to predict results of a 21-day cumulative irritation patch test in humans

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The human 21-day Cumulative Irritation Patch Test (21-CIPT) (Lanman et al., 1968) is a standard test of new products intended for repeated dermal contact. However, the duration and cost limit its use to screen multiple formulas in early phase of the product development. Thus, there is a need for a rapid, relatively inexpensive test that predicts performance on the 21-CIPT. The Cytosensor Microphysiometer (μφ) (Molecular Devices Corp., Menlo Park, CA) was investigated as a screening tool. It measures metabolic changes in L929 cells as a function of test article dose in a cycle of exposure/wash/metabolism measurement. The dose producing 50% reduction in metabolic rate (MRD_{50}), relative to the baseline, is used as a measure of toxicity. It is quick and effective in predicting potential irritation of surfactants. The acute toxicity of the μφ assay can be compared to the chronic toxicity of the 21-CIPT, which is based largely on the exposure of wetting agents (surfactants) to the epidermal cells. Twenty wet wipe formulas were tested via the μφ and 21-CIPT. One material was a product with over five years of successful market experience. Samples with MRD_{50} greater than 50 mg/ml provided 21-CIPT scores consistent with a product that performs satisfactorily in the market. When the MRD_{50} was greater than 78 mg/ml, the 21-CIPT score was usually zero. The μφ assay showed greater sensitivity than the 21-CIPT for ranking materials with minimal irritancy. The μφ assay is useful as a screen for predicting the performance of wet wipe formulas on the 21-CIPT.

Poster

In vitro phototoxic potency assessment of chemicals using the human reconstructed epidermis EpiSkin

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The aim of the study was to evaluate the phototoxic potency of chemicals using the human reconstructed epidermis model EpiSkin. 8 phototoxic (ofloxacin, lomefloxacin, promethazine, chlorpromazine, menadione, demeclocycline, bergamot oil, 5MOP and 8MOP) and 3 non phototoxic (coumarin, histidine, penicillin G salt) compounds were evaluated. Chemicals were applied topically or systemically at non cytotoxic concentrations for 2 h. Following treatment, tissues were exposed to non cytotoxic dose of UVA. Viability and pro-inflammatory mediators (IL-1α, IL-8 and PGE2) were investigated 22 h after exposure. 2 criteria were used to evaluate phototoxicity: PIF>1 (Cmax-UV/CI.50+UV) or a significant decrease in viability (25%) on exposed tissues. The results showed that, excepting lomefloxacin and oxfloxacin, all known phototoxic chemicals induced a significant decrease in tissue viability (>25%) and a significant release of IL-1α after UVA exposure. Lomefloxacin and ofloxacin, described as systemic phototoxic chemicals *in vivo*, were found phototoxic after systemic treatment. Furthermore, viability decrease and IL-1α released levels were closely related. A significant release of PGE2 and IL-8 was detected with 8-MOP and 5-MOP after UVA exposure. These effects could be linked to specific furocoumarin phototoxic mechanisms. All tested non phototoxic chemicals, topically or systemically applied, were correctly identified.

Our results suggest that:

1. The phototoxic potential of chemicals can be determined using viability endpoints combined with inflammatory mediator measurements in a 3D epidermis model.
2. Episkin can be a relevant tool to predict the phototoxic potency of topically or systemically applied chemicals, bioavailability of the tested chemical depending on the administration route.

Poster

In vitro skin irritation screening of cosmetic products on reconstituted epidermis

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The *in vitro* skin irritation study is still under development to establish a standardised protocol to evaluate the irritation potential of developed cosmetic products. By using the human reconstituted epidermis model Skinethic, various cosmetic products such as surfactant and alcohol containing formulations, body and face creams or lotion, were tested by applying 20 µl of the test item on the epidermis of 0.5 cm^2. ELISA assays of IL-1 and IL-8 inflammation cytokines were performed on supernatants after 6, 24, and 72 hours. The cellular viability was assessed with a MTT coloration and a histological study was conducted at the same time. A positive control was systematically performed with a SDS 0.2% treatment, and compared with an untreated epidermis. An increase of IL-1 and IL-8 synthesis combined with a decrease in cellular viability was observed with the SDS treatment, whereas no cytokine was synthesised and cellular viability was high without treatment. Cytokine fluctuations induced by cosmetic products were analysed with histology features and the cytotoxicity by an analysis of variance (multifactor Anova). Human patch tests are currently being carried out with some of cosmetic products to correlate *in vitro* data with human skin reactivity. The results of this study should contribute to the determination of relevant endpoints for screening the *in vitro* skin irritation potential of different types of cosmetic products.

Poster

Use of the yeast *Saccharomyces cerevisiae* as a pre-screening approach for assessment of chemical-induced phototoxicity

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Photoreactive chemicals can induce dermatological reactions when present in the skin exposed to sunlight. Thus, new chemicals absorbing above 290 nm should have their potential phototoxicity tested. In order to screen a large number of molecules with various physico-chemical properties, a microbiological method is helpful. To this end, the yeast *Saccharomyces cerevisiae* was evaluated for its ability to detect phototoxic compounds. Twelve products known to be phototoxic *in vivo* and previously used as standards for validating the regulatory test 3T3 NRU were used in this work. Eleven of them could be detected in the yeast assay and, among them, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), angelicin and, to a lower extend, tiaprofenic acid induced genetic alterations. Interestingly, a pre-incubation with yeast cells in the dark before exposure decreased the phototoxicity of 5-MOP and 8-MOP but had no effect on this of chlorpromazine and ketoprofen. *Saccharomyces cerevisiae* and *Salmonella thyphimurium* (strains TA100 and TA102) were compared for the evaluation of 5-MOP and 8-MOP photogenotoxicity; only the yeast assay allowed performing experiments in exposure conditions close to those encountered in environmental situations. Finally, an application of this experimental approach to the detection of traces of furocoumarins in fragrance materials was developed.

Lecture

Validation of human skin models for skin corrosivity tests in Japan

Yasuo Ohno [1], Hajime Kojima [2], Tomoko Ando [3], Katsuhiro Inagaki [4], Mahito Ohhira [5], Tadashi Kosaka [6], Yosuke Nakamura [7], Hisashi Torishima [8], Noriyuki Morikawa [9], Takashi Omori [10], Jun Kanno [3], Mami Kuboki [5], Michiru Genno [8], Masaru Nokata [4], Takanori Harada [6], Takashi Morimoto [7] and Isao Yoshimura [11]

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As a validation trial of alternative for skin corrosivity testing in Japan, we performed a validation study of EPI-200 (EpiDerm™), a 3-dimensional cultured epidermal model and Vitrolife-Skin™, a 3-dimensional cultured skin model. In this validation study, 6 laboratories took part excluding the kit suppliers (Kurabo Industries Ltd. and Gunze Co, Ltd.). Participants performed the pre-test and main trial and obtained cut-off percentage cell viability values (viability after 3 minutes or 1 hour exposure) over a three month period from February to April 2004. Twelve chemicals were selected and coded, then 10 chemicals were supplied to each laboratory.

As a result, most chemicals did not show any great differences in scores on tests repeated at each laboratory. Inter-laboratory variation was significant in sulfuric acid alone, and the feasibility of using EPI-200 and Vitrolife-Skin™ was suggested through the experiment.

Furthermore, we compared the validation testing data and *in vivo* database in ECVAM. Comparing corrosivity data, the consistency rate of tests using EPI-200 was 81.7%. The chemicals showing false positives were 5% potassium hydroxide and lactic acid but sulfuric acid alone showed a false negative response.

On comparison of the consistency rate of tests using Vitrolife-Skin™ showed 83.3%, the chemicals showing false positives were 5% potassium hydroxide and lactic acid whereas none of the chemical showed a false negative response. These data showed that the reliability of these two models was similar to the results obtained on the ECVAM validation.

Poster

Screening of skin irritation potential of surfactants with the Red Blood Cell Test. Comparison to EpiDerm™ skin model and human patch test data

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The assessment of the skin irritation potential of cosmetic formulations is normally carried out *in vivo* with the human epicutaneous patch test. However, test materials containing ingredients with an uncertain toxicity profile should be tested *in vitro* before testing on humans. Furthermore, time and cost reduction could be arguments for the use of *in vitro* tests. Since several years 3D skin models (e.g. EpiDerm™, MatTek) are routinely used for this purpose.

The Red Blood Cell Test (INVITTOX protocol no. 37) was originally developed to screen the mucous membrane irritation potential of surfactants and surfactant-based formulations. Twenty years of experience have demonstrated that the RBC test is a valuable tool for the assessment of eye irritation potential. Endpoints are haemolysis (damage of erythrocyte membranes) and haemoglobin denaturation. Over the years we have found that the haemoglobin denaturation correlates well also with the skin irritation potential of diluted surfactants and surfactant-based formulations.

In 2004 the DGK (Deutsche Gesellschaft für Wissenschaftliche und Angewandte Kosmetik) initiated a ring study for the validation of the human patch test. Eight diluted surfactants and mixtures of surfactants were tested in several laboratories. At Beiersdorf these test samples were additionally tested with the RBC test and EpiDerm™. A high correlation was found between the haemoglobin denaturation index of the RBC test compared to the results of EpiDerm™ and the patch test. Therefore, the RBC test is a valuable tool for the assessment of both eye and skin irritation potentials.

Poster

Report from an *in vitro* dermal absorption assay workshop

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The Institute for In Vitro Sciences (IIVS), Gaithersburg, Maryland, USA, hosted a workshop on *in vitro* percutaneous absorption (PA) methods for a small group of international stakeholders in July 2005. The purpose of the workshop was to provide a forum where stakeholders and method experts could come together to discuss the various OECD approved guidance on *in vitro* PA methods (OECD Test Guideline 428, April 2004 and Guidance Document for the Conduct of Skin Absorption Studies, March 2004) and how this guidance may be practically applied to the protocols in current use. The workshop participants compared and contrasted specific components of different *in vitro* protocols, and made recommendations on protocol components that are essential for obtaining useful toxicological data from the *in vitro* PA methods. A major goal of this workshop was to provide industry, contract research laboratories and the regulatory community with practical information to facilitate successful, wider, and earlier use of *in vitro* PA data in regulatory submissions. Detailed conclusions and recommendations from the workshop will be presented.

Poster

In vitro modelling of chromium and zinc interactions in human dermal fibroblasts

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One of the main goals of toxicology of heavy metals is to understand all adverse effects induced by individual members of this group of toxins. The studies of these effects are complicated by many factors, for example by ambivalent nature of many metals (toxicity *vs*. essentiality) and by the fact that in real environment heavy metals do not occur individually but in mixtures. Therefore, to investigate interactions of hexavalent chromium whose occupational exposure has been linked to various skin pathologies including skin ulcerations and allergic dermatitis and zinc (a bioelement implicated in many important cellular processes, with recognised cytoprotective effects in skin cells) in human dermal fibroblasts, a series of *in vitro* tests have been used during 48 hours. The followed markers included cell viability (WST assay), cell motility, cytoskeletal organisation, oxidative stress as well as measurement of cell death. Exposure to potassium chromate produced concentration and time dependent loss of cell viability, reorganisation of cytoskeleton and cessation of motile activity in fibroblasts. Cytoskeletal perturbation was accompanied by increased oxidative stress and activation of cell death. These changes were further enhanced in zinc-deprived fibroblasts. On the other hand, administration of zinc prevented chromium induced toxicity and, also, was able to reduce significantly cell death. These results demonstrate the important role of zinc in protection of skin homeostasis.

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Poster

A statistical method for estimating ET_{50} under the condition of small volume of data

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The median effective time (ET_{50}) is usually used as an index of skin irritancy of chemicals in a three dimensional human skin model such as Vitrolife-Skin or EpiDerm. Due to restrictions on time setting, cost and labor, at most 5 time points were available for each chemical in the validation study in Japan using Vitrolife-Skin. Assuming such a restricted experimental condition, we investigated to improve the estimating method for confidence intervals of ET_{50} (CI).

The least squares method is standard methods for estimating ET_{50} to fit a logistic curve or a linear regression line with time on horizontal axis and viability on vertical axis. However, simple application of these methods sometimes fails to get reasonable CI. We, consequently, propose the combining use of the non-linear least squares method assuming a logistic regression curve, with the least squares method assuming a linear curve with log(time) as independent variable.

We examined the performance of the proposed method using a Monte-Carlo method assuming logistic or linear model. According to the result of simulation, more reasonable estimate of ET_{50} was obtained in the proposed method compared with the simple use of respective method. However, the coverage probabilities of CI were less than the nominal confidence level 95% by 10% or more. The improvement of the proposed method is considered to be required in future studies.

Poster

Statistical considerations for positioning time points in ET_{50} estimation using three dimensional human skin model

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Appropriate experimental design is essential to precisely estimate effective time 50 (ET_{50}), which is an index of skin irritancy of chemicals in a three dimensional human skin model such as Vitrolife-Skin or EpiDerm. One of the most important issues in the design of experiment to obtain estimates of ET_{50} using as small number of kits as possible is appropriate positioning of time points within 24-hour interval, keeping the time points in a certain range, which is desirable for saving office hours of experimenters. It is also useful to reduce troublesome cases where reasonable confidence interval cannot be obtained, which frequently appeared in the validation study in Japan.

We conducted a Monte-Carlo simulation study using virtual data produced by a simulation model with a logistic curve on the time-response to examine the optimality of time positioning. In the simulation study, we used a logistic regression method to obtain estimates and confidence intervals of ET_{50}. We compared several cases of positioning of time points under the assumption that a preliminary experiment was performed in advance and a rough estimate of ET_{50} was given, keeping the number of time points at 3, 4, or 5. Based on the result of simulation, we concluded that the choice of time point within 2 hours around the preliminary estimate of ET_{50} was effective for obtaining reasonable estimates of ET_{50} and that at least five time points was necessary when preliminary estimate was not reliable, whereas four time points was sufficient when it was.

Poster

Occupational safety assessment of skin corrosion/ irritation using human reconstituted epidermis models

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The assessment of skin corrosion/irritation is an essential part of the safety evaluation of chemicals and is therefore demanded by international regulatory requirements. Because of ethical considerations and sometimes limited availability of compound for *in vivo* tests, alternative methods, including human reconstituted epidermis (hREP) models, gain more and more importance.

In an in house validation study with hREP models we used known corrosive or irritating chemicals and determined cell viability (MTT-assay) as the basis for classification of the respective hazard. The results of viability testing provided a good prediction for a wide spectrum of chemicals (e.g. organic acids and bases, anorganics, phenols). In addition, hematoxylin and eosin stained sections of the epidermis at the end of the study and the release of IL-1α in the assay medium were evaluated in order to investigate different endpoints for classification. In a comparability study for skin irritation performed together with ZEBET a good intra- and interlaboratory reproducibility of MTT-results could be shown over time and with separate lots of tissues. Therefore, *in vitro* skin corrosion and irritation assays using hREP models have now been implemented in the sequential testing strategy according to OECD test guideline 404 in our laboratory along with the evaluation of structure-activity-relationship (SAR) data and measurement of pH-value.

In conclusion, we have successfully established hREP models for the prediction of skin corrosion and irritation using cell viability as primary endpoint. These tests are integrated in a sequential test strategy for occupational safety assessment and replace the use of laboratory animals.

Poster

Validation study on the battery system for prediction of phototoxicity in Japan: The overview of the results

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The interlaboratory reliability of the battery system with the yeast growth inhibition phototoxicity assay (Yeast assay) and the red blood cell photohemolysis assay (RBC assay) developed in Shiseido Co. Ltd. was examined under the initiative of the Validation Committee of Japanese Society of Alternatives to Animal Experimentation. Six laboratories participated in the validation study for conducting experiments to evaluate the irritancy of nine chemicals, i.e. anthracene, amiodarone, chlorhexidine, chlorpromazine, bithionol, SLS, acridine, 6-methylcoumarin, and 4-t-butyl-4-methoxydibenzoylmethane. Different set of six chemicals out of nine was allocated to each laboratory and tested according to the SOP provided by Shiseido Co. Ltd. during the period from January to April, 2004. The irritancy of each chemical was evaluated whether it was positive, negative, or equivocal. All laboratories excluding one laboratory yielded consistent results, in the sense that both positive and negative judgements appeared in the same chemical. The combination of two assays yielded different results from single use of each assay. *In vivo* judgements could not always be reproduced by this battery, probably because sufficient data could not be obtained on the toxicity in *in vivo* assays. The consistency of judgement among laboratories was better when absorbance in 525 nm was used than when 540 nm was used.

Lecture

Validation study on the battery evaluation system for prediction of phototoxicity in Japan: The overview of the results

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The interlaboratory validation study of the battery evaluation system consisting of the yeast growth inhibition phototoxicity assay (Yeast assay) and the Red Blood Cell photohemolysis assay (RBC assay) was conducted under the charge of the Validation Committee of Japanese Society of Alternatives to Animal Experimentation. Six laboratories participated in the validation study for conducting experiments to evaluate phototoxic potential of nine chemicals, i.e. anthracene, amiodarone, chlorhexidine, chlorpromazine, bithionol, sodium lauryl sulfate, acridine, 6-methylcoumarin, and 4-t-butyl-4-methoxydibenzoylmethane. Each laboratory received six out of these nine chemicals under blinding so that each of the chemicals was evaluated in four laboratories. The experiments were finished by April with the start of January, 2004. The common SOP and work sheets were used in order to efficiently manage the experimental records. The phototoxicity of each chemical was classified into positive, negative, or equivocal by the battery system on the basis of both results of the Yeast assay and RBC assay. The intralaboratory reproducibility was good except for one laboratory. The difference in the results among laboratories was small, except for the data of amiodarone and sodium lauryl sulfate obtained from one laboratory. The results correlated with *in vivo* data when equivocal data were treated as positive although chlorhexidine and bithionol showed false positive. These results suggest that this battery evaluation system is effective in the prediction of the phototoxic potential of chemicals.