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A human keratinocyte tissue model can differentiate between phototoxicity and direct skin toxicity

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The issue

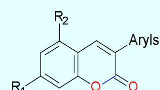
A heteroaromatic compound (with structural similarities regarding phototoxicity to coumarin and related analogs) investigated in early screens for toxicity displayed phototoxic effects in vitro based on its properties to absorb UVA. In rats, there was evidence for in vivo phototoxicity with UV irradiation. However, when given to monkeys, the test item produced skin toxicity even in the absence of UV. To judge on relevance for humans and to provide a model to investigate backup compounds, we investigated the effects of the test item in the human skin model epiCS® with and without irradiation with artificial sunlight.

Objectives

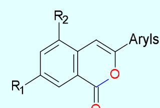
Objectives of using our in vitro model of human skin were

1) to reproduce the histopathologic phenotype of skin toxicity observed in monkeys and rats in an in vitro human skin equivalent model,
2) to investigate the influence of UV irradiation on the toxicity
3) to evaluate the model as a potential screening tool for back-up compounds.

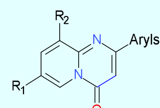
The test compound



Coumarin nucleus



Isocoumarin nucleus



The test compound, ROXXX

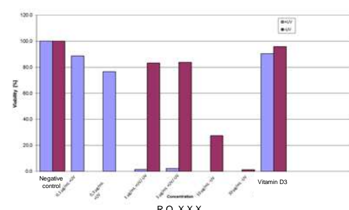
Animal findings

Distinct histopathologic skin findings were repeatedly observed in non-human primates with and without exposure to UV irradiation and ROXXX. Main histopathologic features comprised parakeratosis with variable epithelial hyperplasia and interspersed dyskeratotic keratinocytes in basal layers, in more severe cases, reduced keratinocyte layers with loss of stratum spinosum were observed. Epithelial lesions were variably accompanied by a sparse mixed inflammatory infiltrate. In Crl:LE (Long-Evans) pigmented rats, treated for two weeks, the test compound produced erythema, edema and flaking with increased incidence and severity on lightly and darkly pigmented skin. Histologically, epidermal to dermal necrosis, serocellular crusts, ulceration and deep dermal inflammatory infiltrate were observed. This is consistent with UVA damage and histological features induced by the positive control 8-methoxypsoralene but distinctly different from the histological presentation of the skin effects seen in the monkey.

Establishment of an in vitro human skin model

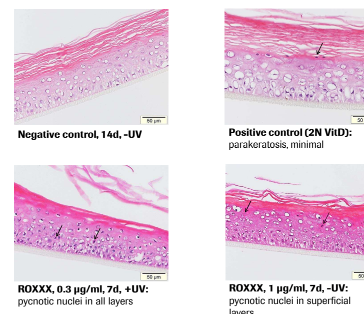
Human keratinocytes, reconstituted on a collagen matrix layer were supposed to be ideal means to differentiate between direct effects on keratinocyte differentiation and effects mediated by UV absorption and subsequent phototoxicity of the test item. treated for 7 and 14 days at ascending doses with and without UVA and ROXXX An MTT assay for cell viability, cytokine measurements and histopathology data support both a phototoxic effect and a direct toxic effect, as signals of cellular damage were observed in both irradiated and non-irradiated samples

Cell viability (MTT assay) after 7d



- **With UV:** dose-dependent ↓ cell viability at ≥ 0.1µg/ml, with significant drop at 1 µg/ml
- **Without UV:** ↓ cell viability at ≥ 1 µg/ml, significant drop at 10 µg/ml

Histopathology: phototoxicity vs. primary skin toxicity



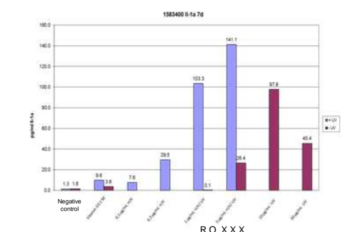
Negative control, 14d, -UV

Positive control (2N VitD): parakeratosis, minimal

ROXXX, 0.3 µg/ml, 7d, +UV: pycnotic nuclei in all layers

ROXXX, 1 µg/ml, 7d, -UV: pycnotic nuclei in superficial layers

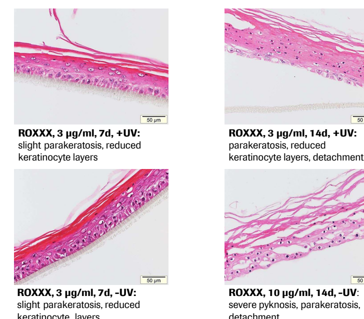
Cytokine detection: Il-1a after 7d



- **With UV:** dose-dependent ↑ at ≥ 0.1 µg/ml after 7d
- **Without UV:** dose-dependent ↑ at ≥ 3 µg/ml after 7d

General increase of cytokines (Il1a, Il-8, TNFa) detected, mostly higher doses affected in non-irradiated vs. irradiated samples with drop at very high doses most likely due to decreased cell viability.

Histopathology: phototoxicity vs. primary skin toxicity



ROXXX, 3 µg/ml, 7d, +UV: slight parakeratosis, reduced keratinocyte layers

ROXXX, 3 µg/ml, 14d, +UV: parakeratosis, reduced keratinocyte layers, detachment

ROXXX, 3 µg/ml, 7d, -UV: slight parakeratosis, reduced keratinocyte layers

ROXXX, 10 µg/ml, 14d, -UV: severe pyknosis, parakeratosis, detachment

Results

Distinct cytotoxic effects of ROXXX were observed for the irradiated as well as for the non-irradiated cultures after 7 and 14 days repeated treatment in the MTT assay.

The cytotoxic effect was more severe in irradiated samples indicating phototoxicity.

Treatment with the compound triggered the release of IL1, IL-8 and TNFa (data on the the latter two not shown), which was generally more severe in irradiated as compared to non-irradiated samples confirming a phototoxic effect.

Histopathology assessment could distinguish a phototoxic effect from a direct toxic effect of the test item with presence of pycnotic nuclei being present only in irradiated samples and inter-cellular edema occurring at lower doses in irradiated versus non-irradiated samples.

Overall histopathologic toxicity scores correlated well with MTT results in non-irradiated samples after 7 and 14 days of treatment and irradiated samples after 14 days of treatment

Conclusions

- **This in vitro model of human skin was able to differentiate between the two types of toxicity of the test compound, its direct effects on keratinocytes as well as its effects upon UVA absorption**
- **The effect may be associated with post-transciptional control of gene expression**
- **Further studies are ongoing to investigate the effects of the test compound on a molecular level**

Materials and Methods

Study conduct

The study was conducted at Harlan Cytotest Cell Research GmbH (Harlan CCR); In den Leppsteinswiesen 19 64380 Rossdorf Germany

About epiCS

In this study cells of the "epiCS®-7 days airlift culture" model were used. This model was cultivated for only 7 days and was therefore not fully differentiated at the start of the treatment but full differentiation was obtained during the treatment phase of 7 and 14 days. The epiCS® kits were purchased from CellSystems® Biotechnologievertrieb GmbH (53842 Troisdorf, Germany). The epiCS® tissue consisted of normal, human-derived epidermal keratinocytes which were cultured to form a multilayered, highly differentiated model of the human epidermis. It consisted of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo.

Treatment and procedures

Treatment with the compound ROXXX was carried out on a daily basis for 7 and 14 days. After completion of the treatment phase the test item concentrations as well as the controls were rinsed off the skin equivalents. Each one set of treated skin tissues was irradiated ventilated with a fan while the other sets of treated tissues were kept in the dark. Irradiation was conducted for 60 minutes with 5.94 J/cm2

Both, the irradiated and non-irradiated skin equivalents were further incubated (18 to 24 hours) and subsequently one part of the skin equivalents was used for the MTT assay as well as for the determination of the cytokines whereas the second part of the skin equivalents was used for the histopathological evaluation.

Vitamin D3 (1 and 2 M solution) was used as possible positive control affecting skin differentiation. The positive control did not show effects in the MTT assay but it did show effects on the release of cytokines.

Read-outs

Cytotoxicity was assessed by MTT; cytokine release (IL-1 α, IL-6, IL-8, TNF-α) and histopathological evaluation completed the assessment after 7 and 14 days of culture/treatment

References

1) MatTek Corporation 1997, Phototoxicity: Protocol for use with EpiDermTM Model (EPI-200)
2) Medina, J., Elsaesser, C., Picarles, V., Grenet, O., Kolopp, M., Chibout, S., de Brugerolle de Fraissinette, A., 2001. Assessment of the Phototoxic Potential of Compounds and Finished Topical Products Using a Human Reconstructed Epidermis. In Vitro Mol Toxicol 14, 157-168
3) Rouget, R., Cohen, C., Rougier, A., 1994. A reconstructed human Epidermis to assess cutaneous irritation, photoirritation, and photoprotection in vitro. In: Alternative Methods in Toxicology , Vol. 10: In vitro Skin Toxicology - Irritation, Phototoxicity, Sensitization. Eds. Rougier, A., Goldberg, A., Maibach, H., Mary Ann Liebert Publ., New York, 141-149