Bioactive Sphingolipids for DNA protection

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Abstract

Background: Sunlight triggers deleterious oxidative stress in the skin. The generation of reactive oxygen species (ROS) is a common process occurring during various cellular reactions. An overproduction or inadequate processing of ROS within the skin manifests itself on a biomolecular level by lipid peroxidation, protein degradation, enzyme dysfunction and even DNA mutations/breakage. Sphingolipids are well known for their contribution and relevance for a proper skin barrier function. Therefore, innovative bioactive Hydroxy-Ceramides were developed and screened for their biological activity.

Methods: The efficacy of Sphingolipid derivatives was analyzed using SimDerma® screening methods. For further investigation UV-irradiated human epidermal keratinocytes (NHEK) and reconstructed epidermal skin models were used and ROS-formation as well as DNA damage was analyzed. For determination of cosmetic benefits an *in vivo* study on sun-stressed skin was performed, showing improvement of skin tone, texture parameters and skin density by application of Hydroxybutyroyl-Phytosphingosine.

Results: Screening of various Sphingolipid derivatives showed anti-oxidation and DNA repair effects. Hydroxybutyroyl-Phytosphingosine showed most promising efficacy and was further investigated. A significant reduction in ROS formation and DNA damage after UV irradiation was shown for this molecule. This efficacy finally results in improving sun-stressed skin conditions like reduction of skin roughness and increase in skin density.

Conclusion: Hydroxybutyroyl-Phytosphingosine showed promising anti-oxidative benefits with a special efficacy in DNA protection. A protection from sun-induced premature aging could be shown *in vivo* which marks Hydroxybutyroyl-Phytosphingosine as multifunctional product for holistic skin protection.

Keywords: Sphingolipids; sun-stressed skin; anti-oxidation; DNA protection.

Introduction. The skin in its function as a biological barrier of the human body protects against multiple environmental influences. In particular, sunlight triggers deleterious oxidative stress in the skin. Adverse effects result in erythema, wrinkling, dryness, inflammation, autoimmune reactions and pigment abnormalities.

Sunlight consists of electromagnetic radiation of different wavelengths which lead to the generation of reactive oxygen species (ROS) in skin cells. The role of especially UVA and UVB radiation on the different skin layers (epidermis, dermis and subcutis) is well studied.

The generation of ROS is a common process occurring during various cellular reactions. Nevertheless, an overproduction or inadequate processing of ROS within the skin manifests itself on a biomolecular level by lipid peroxidation, protein degradation, enzyme dysfunction and even DNA mutations/breakage [1]. Accumulation of these effects over time were associated with premature aging and photoaging. It could be shown that regular exposure to ultraviolett (UV) radiation causes inflammation of the skin, weakens the skin‘s immunity and impedes cell function, leading to hyperpigmentation, skin barrier disruption and premature aging [1].

The skin aging process is equally driven by acute stress reactions, such as the up-regulation of matrix degrading enzymes and pro-inflammatory cytokines, as well as chronic damage responses caused by the accumulation of macromolecular damage in non-proliferating skin cells, such as alterations in cellular DNA [2]. Solar UV radiation is one of the most significant environmental DNA-damaging agents to which humans are exposed. UV induces DNA damage in skin both directly, via the absorption of UVB, or indirectly by photosensitization mediated through UVA [3]. The solar UV photons are directly absorbed by DNA or by other endogenous photosensitizers, which leads to DNA damage and oxidation of biomolecules (proteins, lipids). This stimulates numerous cellular consequences, which lead to photoaging [4].

Sphingolipids are well known for their contribution and relevance for a proper skin barrier function. Besides that, special Sphingolipids can also have biochemical functions in regulation of cell growth, differentiation, and pathways in skin aging processes [5] [6]. Therefore, bioactive Hydroxy-Ceramides were developed and screened for their biological

activity. One of the most interesting molecules is Hydroxybutyroyl-Phytosphingosine (HB-PS). This Sphingolipid is based on skin-identical Phytosphingosine acylated with an omega hydroxy fatty acid with a short chain length. Such structures fit into the category of omega-hydroxy Ceramides (Ceramide OP).

Although, Sphingolipids are broadly used in cosmetic products, the application field is still focused to the improvement of skin barrier function. The benefits of Sphingolipids on the consequences of UV-irradiation are not investigated as intensive as effects in skin barrier formation. Especially the Sphingolipid Hydroxybutyrol-Phytosphingosine seems to be an interesting molecule to be explored especially on the effects of UV-irradiation. This will be described in this work.

Materials and Methods.

SimDerma® screening

SimDerma® is a screening system that includes 23 laboratory assays. This tool has been developed to identify novel biological activities for cosmetic and skincare products.

Hydroxybutyroyl-Phytosphingosine was screened using SimDerma® to offer a wide and fast overview of the ingredient's activity profile and potential skin care claims.

Protection from environmental influences on keratinocytes

For analysis of ROS production, keratinocytes were pre-incubated in 96-well plates for 48 hours at 37°C. The medium was then replaced by assay medium containing or not (irradiated control) the test compounds (Hydroxybutyroyl-Phytosphingosine at 3 and 8 µM which corresponds to 1 and 3 ppm) or the references (Vitamin E at 100 µM and EGCG at 10 µM) and the cells were incubated for 24 hours. Afterwards, the fluorescent probe, 2,7-dichlorodihydrofluorescein diacetate (2,7-DCDHF-DA at 10 µM in assay medium) was added and cells were incubated for another 30 minutes at 37°C. Cells were washed and irradiated with 100 mJ/cm² UVB + UVA (+ 0.7 J/cm²) without compounds or references.

The lamp used was a SOL500 Sun Simulator equipped with an H2 filter (Dr. Hönle, AG). After irradiation, cells were post-incubated for 30 minutes. A non-irradiated control and a condition with no probe (background noise) were performed in parallel.

The emitted fluorescence intensity (λex = 485 nm, λem = 538 nm) was measured. Fluorescence intensity of the metabolized probe (DCF) was proportional to the formation of ROS. The production of ROS was therefore expressed as relative fluorescence intensity.

For analysis of DNA damage, a classical comet assay was used. Keratinocytes were seeded in 6-well plates and pre-incubated for 48 hours at 37 °C. Afterwards, medium was replaced by culture medium containing or not (control) the test compounds (Hydroxybutyroyl-Phytosphingosine at 3 and 8 µM which corresponds to 1 and 3 ppm) or the reference (Tiron at 3 mM) followed another 24 hours incubation. Afterwards, cells were irradiated with 250 mJ/cm² UVB + UVA (+ 1.6 J/cm²) in absence of the compounds. The lamp used was a SOL500 Sun Simulator equipped with an H2 filter (Dr. Hönle, AG). A non-irradiated control condition was performed in parallel.

For the analysis of DNA damage a Comet assay was performed. Normal human epidermal keratinocytes were seeded in 6-well plates and incubated in culture medium for 48 hours with medium renewal after 24 hours. The medium was then replaced by culture medium containing or not (control) the test compound or the reference (tiron at 3 nM) and the cells were incubated for 24 hours. After pre-incubation. The culture medium was removed and replaced by assay medium and the cells were irradiated with 250 mJ/cm2 UVB + UVA (+ 1.6 J/cm2) in absence of the compound. The lamp used was a SOL500 Sun Simulator equipped with an H2 filter (Dr. Hönle AG). A non-irradiated control condition was performed in parallel. All experimental condition were performed in n=2.

At the end of the irradiation, the culture supernatants were discarded and the cells were washed in a phosphate buffered saline (PBS) solution before analyzing.

To perform an alkaline Comet assay, the cells were trypsinized and counted, and the supernatant was removed after centrifugation. Cells were then washed in PBS solution and were suspended in the same PBS solution to achieve a cell concentration of 1x 105 cells/ml. Cell suspension was mixed with molten (37 °C) 1% low-melting-point agarose gel and pipetted onto the Comet slides (analysis in n=2 for each condition). For cell lysis, the slides were immersed in freshly prepared alkaline solution (200 mM NaOH containing 1 mM EDTA, pH >13) on electrophoresis support. Gel electrophoresis was performed at 21 Volt for 30 minutes. The Comet slides were washed twice with ultrapure water for 5 minutes, then

with 70% ethanol for 5 minutes and air dried for 15 minutes at 37°C. After electrophoresis, each dried sample was stained with a fluorescent DNA intercalation dye (SYBR Green solution). Cells were then observed in epifluorescence using an automated microscope imager INCell AnalyzerTM 2200 (GE Healthcare) (Objective lens X10). When excited (λex = 494 nm, λem = 524 mm), the DNA-bound SYBR® Green emits green light. In healthy cells the fluorescence is confined to the nucleoid: undamaged DNA is supercoiled and does not migrate very far of the nucleoid under the influence of an electric current.

Protection from sunburn cell and CPD formation in reconstructed human epidermis

To further investigate the effects of Hydroxybutyroyl-Phytosphingosine UV-irradiated reconstructed human epidermis (RHE) models were used. The effects of the product against UV-irradiation were evaluated on the formation of sunburn cells (SBC) using histological staining (hematoxylin-eosin staining) and image analysis and the formation of cyclobutane pyrimidine dimers (CPD) using in situ fluorescent immunolabeling and image analysis.

For the study, ten-days-old RHE were systemically treated or not (control) with 3 µM Hydroxybutyrol-Phytosphingosine or topically treated with the reference Sun cream SPF 30 (5 mg/cm²) and incubated for 24 hours. RHE were then irradiated (in absence of the test compounds) with UVB at 500 mJ/cm² (+ UVA at 3.2 J/cm²) using a SOL500 Sun Simulator equipped with an H2 filter (Dr. Hönle, AG). After irradiation, the treatments were renewed and RHE were incubated for further 24 hours. A non-irradiated control was performed and kept in the dark during the irradiation time.

At the end of the incubation, RHE were washed in a phosphate buffered saline (PBS) solution and embedded in paraffin.

The sunburn cells (SBC) were identified on reconstructed epidermis sections after classic hematoxylin-eosin (HE) staining according to the 2 following criteria: dark colored condensed and/or fragmented nucleus and eosinophilic cell (bright pink staining in cytoplasm). The SBC were counted and normalized to the epidermis area (number of SBC/mm2).

In situ immunohistochemical labeling was performed for analysis of CPD (cyclobutane pyrimidine dimers. CPDs are molecular lesions which are formed from thymine or cytosine

bases in DNA by sunlight exposure. CPD formation leads to severe damages in the DNA by destroying the normal base-pairing double-strand structure in that area of the DNA.
Fluorescent CPD-positive cells were counted (objective lens x40) and normalized to the epidermal living layer area identified by PI staining of nuclei (number of CPD positive nuclei/mm²).

Regeneration of sun-stressed skin

For the *in vivo* study 16 panelists per test formulation (men and women aged between 18 and 65 years) were recruited. The study was started in mid of September after summer period, so the panelists presented sun-stressed skin. They applied the test emulsions twice daily for overall 8 weeks on the forearm. The test formulation was an O/W emulsion containing either 0.1% (Hydroxybutyroyl-Phytosphingosine or no active ingredient (vehicle). Untreated skin served as control. The study was designed as a half-side test in an incomplete block-design.
Before the application started (baseline), after 2, 4 and 8 weeks various skin parameters were measured.
The skin tone was evaluated on the outer forearm measuring skin color parameters with a colorimeter (L* and ITA: the higher the value, the lighter the skin color). Skin texture was measured on the inner forearm using a Visioscan VC 98 camera. Various texture parameters were summarized to show an overall skin texture value (calculated as % improvement of the initial value).
Skin structure was evaluated by measuring skin roughness (Visioscan VC 98, summarized roughness values) and skin density (Ultrasound, Dermascan C, Cortex Technology, Denmark) on the outer forearm.

Results.

SimDerma® screening as first step in product development indicates the main activity profile for Hydroxybutyroyl-Phytosphingosine in “Skin Defense” with a very high activity in inhibition of reactive oxygen species (ROS) showing a strong anti-oxidative efficacy (Table 1).

Experimental models	Age-Defying			Sensitive Skin		Nourishing		Skin Evenness		Skin Defense				Barrier Fortify			Hair Care
ROS Inhibition (k)	+++	+++	·	·	+++	+++	·	+++	+++	+++	+++	+++	+++	+++	+++	·	+++
CB1 Antagonism	·	·	·	·	·	·	·	·	·	+++	·	·	·	·	·	·	+++
STAT3 Inhibition	·	·	·	·	·	·	·	·	·	+	·	+	·	·	·	+	+

+ low activity, ++ medium activity, +++ high activity, · No relation

(f) fibroblasts, (k) keratinocytes, (m) melanocytes, (mac) macrophages

Table 1: Overview of SimDerma® screening results.

In addition, Hydroxybutyroyl-Phytosphingosine seems to work as CB1 antagonist.

Additionally, the potential protective effects of HB-PS were evaluated in UVB (+UVA)-irradiated normal human epidermal keratinocytes (NHEK) on reactive oxygen species (ROS) production and DNA damage.

Figure 1 shows a strong induction of reactive oxygen species (ROS) production after UV irradiation (vertical stripes). The positive controls Vitamin E and Epigallocatechingallate (EGCG) show the expected reduction of ROS compared to the untreated/stressed control. It could be shown in this experiment, that HB-PS is able to reduce ROS production after UV irradiation at the higher concentration even better than the positive controls. An up to 40% protection effect from UV induced ROS production can be seen by application of Hydroxybutyroyl-Phytosphingosine.

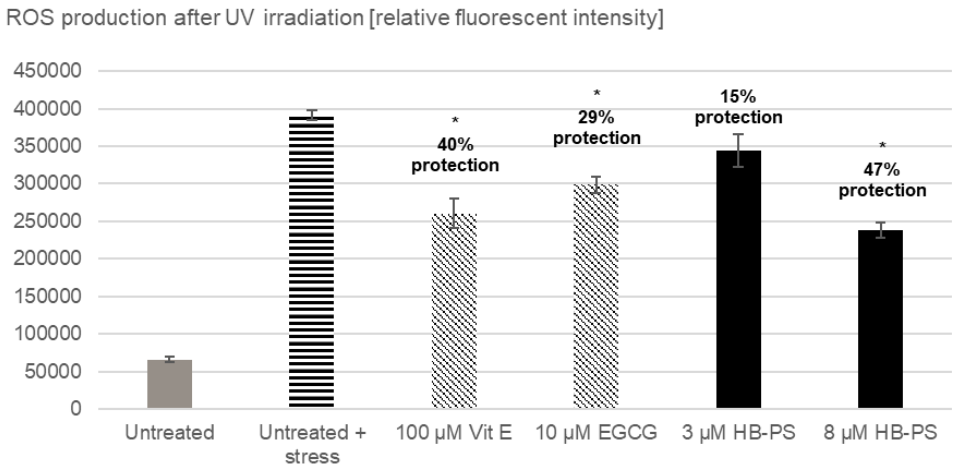

Figure 1: ROS production after UV irradiation measured as relative fluorescence signal. Statistical significance: * vs. stressed cells ($p<0.05$).

A simple method for measuring deoxyribonucleic acid (DNA) strand breaks is the comet assay. In healthy cells the fluorescence is confined to the nucleoid: undamaged DNA is supercoiled and thus does not migrate very far of the nucleoid under the influence of an electric current. In case of DNA damage, the alkaline treatment unwinds the DNA, releasing fragments that migrate from the cell when subjected to an electric field. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage. When using alkaline electrophoresis conditions, the distribution of DNA between the tail and the head of the comet can be used to evaluate the degree of DNA damage.

The effect of reduction of ROS production can be directly seen in reduced DNA damage. Figure 2 shows that UV irradiation leads to a strong increase in tail DNA in the comet assay (vertical stripes).

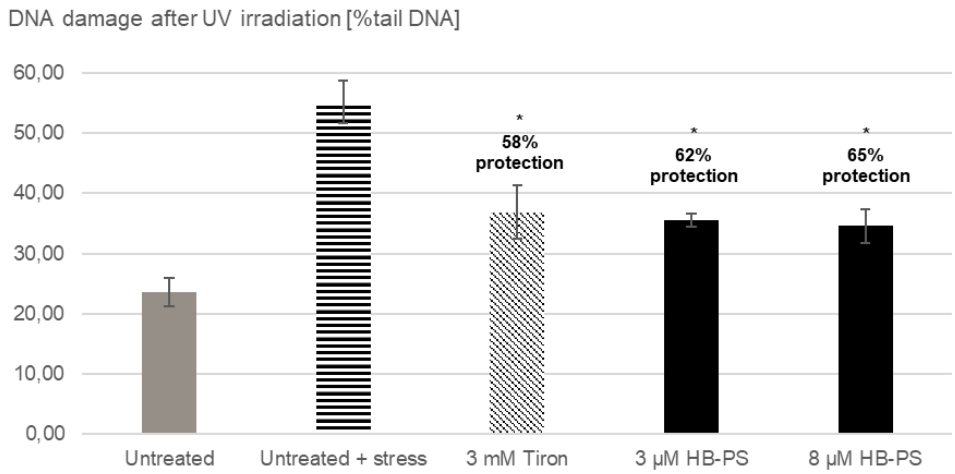

Figure 2: DNA damage after UV irradiation measured as DNA tail formation. Statistical significance: * vs. stressed cells ($p<0.05$).

The positive control Tiron shows the expected reduction in DNA damage compared to the untreated/stressed control. Hydroxybutyroyl-Phytosphingosine shows a significant reduction in DNA tail production and therefore, protects the cells from UV induced DNA damage already at the lower concentration that was tested. The effects can also be seen in the comet assay images (figure 3).

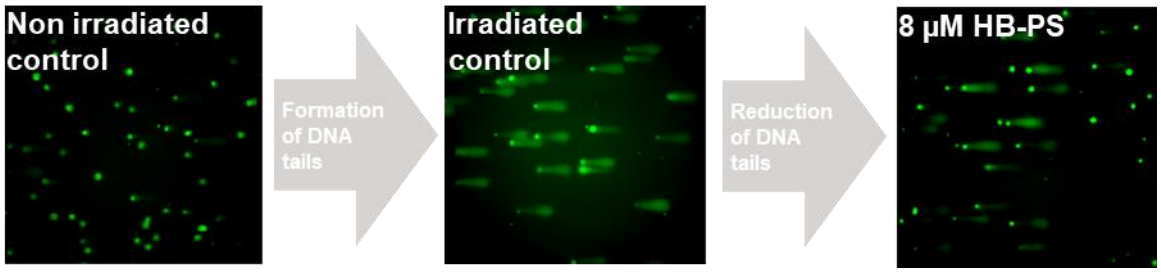

Figure 3: Formation of DNA damage after UV irradiation (Comet assay).

DNA damage as consequence of UV irradiation can lead to the formation of so-called sunburn cells. Sunburn cells are apoptotic keratinocytes, which show nuclear shrinkage and uniform cytoplasm and are eosinophilic and deeply stained. Sunburn cell formation is a defense mechanism for preventing malignant changes of epithelial cells.

The amount of sunburn cell formation can be used as sign of damages in the skin related to UV irradiation. Figure 4 shows the formation of sunburn cells after irradiation of epidermal skin models with UV light.

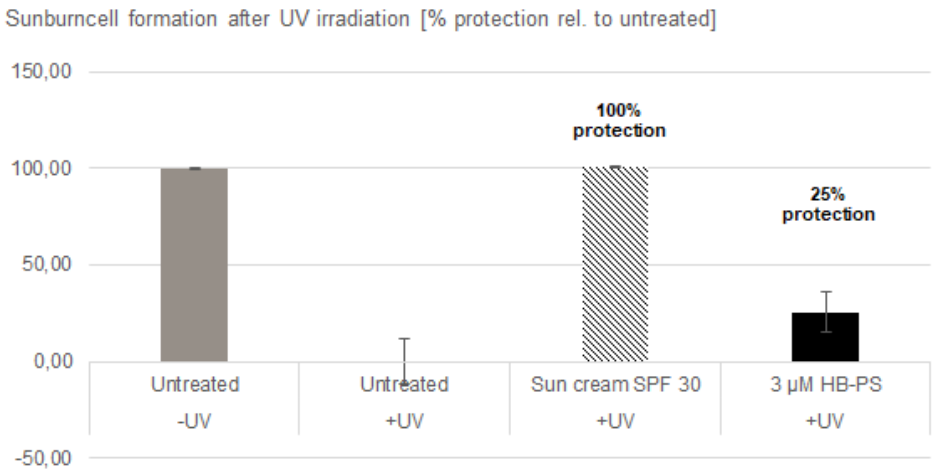

Figure 4: Sunburn cell formation after UV irradiation as % protection compared to untreated and non-stressed control.

Figure 5 shows the formation of CPD after UV irradiation as % protection compared to the untreated control.

UV irradiation leads to a strong induction in CPD formation and therefore, no protection effect. Application of a sun cream with SPF 30 shows a complete protection effect from UV induced CPD formation, because the UV rays are blocked by the cream on top of the skin models and cannot harm the DNA within the skin cells. Systematic application of Hydroxybutyroyl-Phytosphingosine leads to a protection effect of 25% compared to the untreated control.

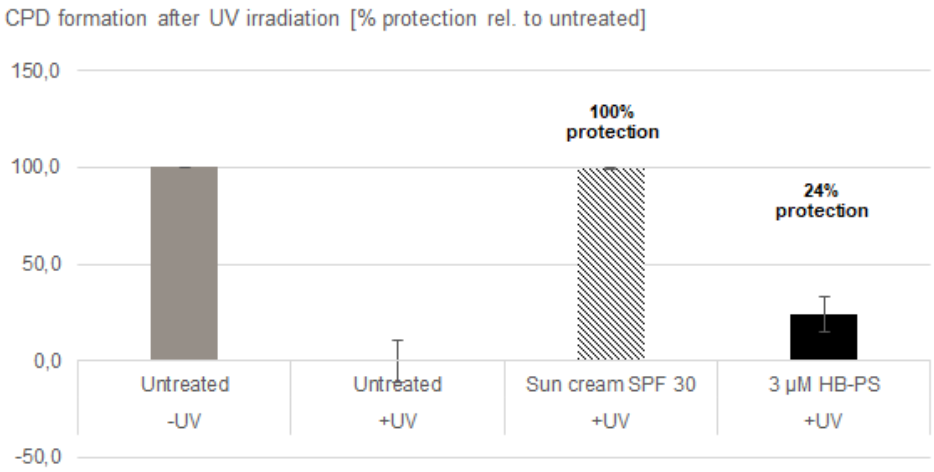

Figure 5: CPD formation after UV irradiation as % protection compared to untreated and non-stressed control.

This can also be visualized by fluorescent staining as shown in figure 6.

Figure 6: Visualization of CPD in human reconstructed epidermal models. Left: Untreated tissue sample after UV-irradiation; Right: Tissue sample treated before and after irradiation with Hydroxybutyroyl-Phytosphingosine.

An *in vivo* study was performed in order to investigate effects of Hydroxybutyroyl-Phytosphingosine on people with sun-stressed skin. It could be shown that the overall skin tone is getting lighter during the study period (figure 7+8).

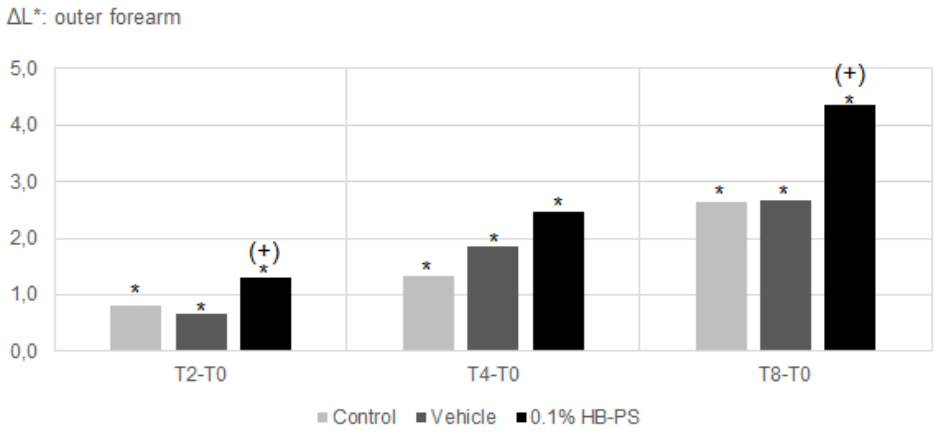

Figure 7: Skin tone parameter L* after 2, 4 and 8 weeks. Statistically significant ($p<0.05$): * vs. start, + vs. vehicle.

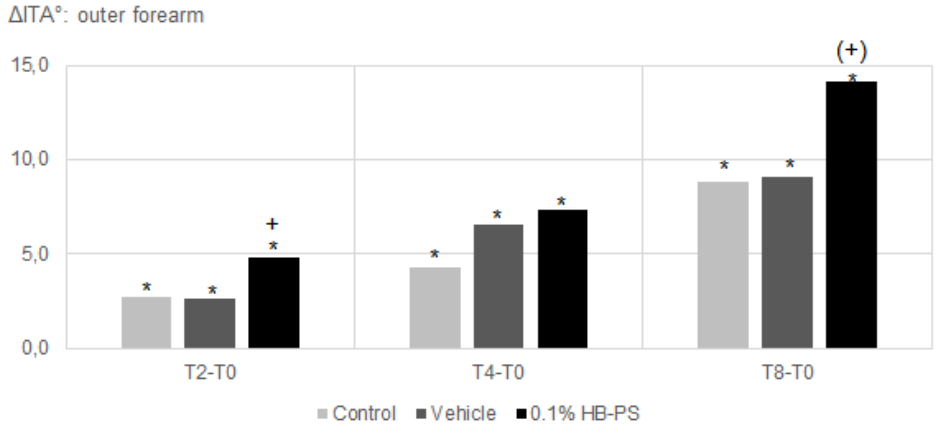

Figure 8: Skin tone parameter ITA after 2, 4 and 8 weeks. Statistically significant ($p<0.05$): * vs. start, + vs. vehicle.

This effect is normal due to fading of brown pigments after summer. Compared to the untreated and vehicle control, HB-PS increases the fading of skin color significantly already after 2 weeks of application.

Looking at the skin structure, it can be clearly seen that HB-PS reduces skin roughness parameters significantly after 4 weeks application (figure 9).

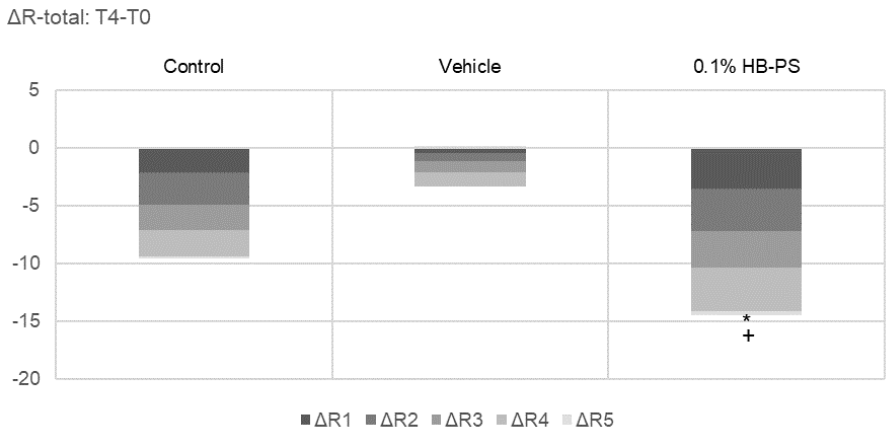

Figure 9: Skin roughness parameters after 4 weeks. Statistically significant ($p<0.05$): * vs. start, + vs. vehicle.

Ultrasound measurements can be used as non-invasive methods, to evaluate photoaged skin as a result of cumulative damage from UV radiation. Histological manifestations involve irregular epidermal thickness, nodular aggregations of elastotic material in the papillary dermis. The most obvious histological aspect is solar elastosis along with an increased amount of ground substance consisting of glycosaminoglycans and proteoglycans, and a decreased number of collagen fibers. This results for example in a reduction of skin density which can be measured.

In addition, figure 10 shows that also overall skin density can be improved over the study period of 8 weeks by Hydroxybutyroyl-Phytosphingosine.

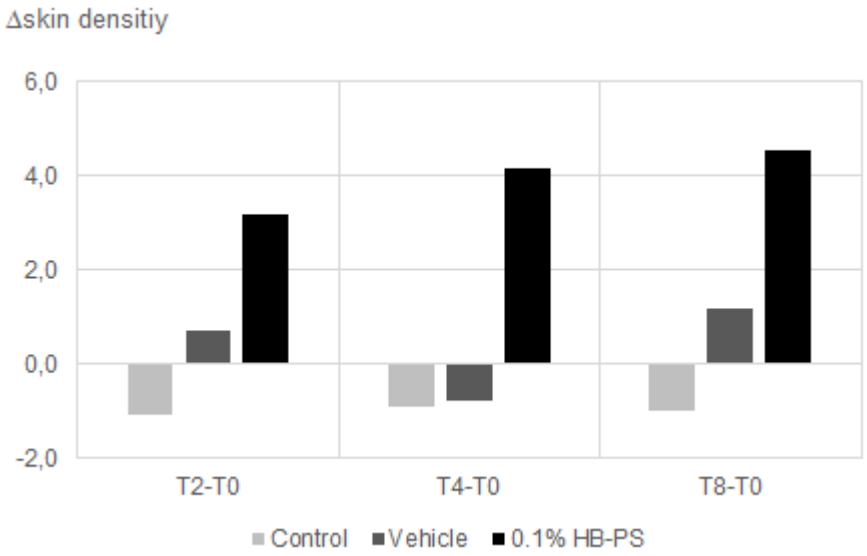

Figure 10: Skin density after 2, 4 and 8 weeks.

Discussion.

A broad screening approach on relevant cosmetic targets and skin pathways (SimDerma®) has shown that the Sphingolipid Hydroxybutyroyl-Phytosphingosine has the main activity profile in "Skin Defense" with a very high activity in inhibition of reactive oxygen species (ROS). In addition, the molecule works as CB1 antagonist. Endocannabinoids and cannabinoids exert their actions by activating cannabinoid receptors which are involved in the regulation of physiological, immunological and metabolic functions. The use of cannabinoid receptor 1 (CB1R) antagonists could prevent against UV damage. Overall, the screening data already demonstrates a strong anti-oxidative efficacy of the Sphingolipid. In addition, results in normal human epidermal keratinocytes show that Hydroxybutyroyl-Phytosphingosine reduces oxidative cell stress and protects cellular DNA after UV irradiation. Taken together, *in vitro* data indicates that Hydroxybutyroyl-Phytosphingosine has anti-oxidative properties with a special efficacy in DNA protection. To further investigate the working mechanism of Hydroxybutyroyl-Phytosphingosine, a study on reconstructed human epidermal models was performed. Skin tissue was irradiated with a combination of UVB and UVA light and treated before and after irradiation with HB-PS. It could be shown that treatment with the Sphingolipid leads to a reduced formation of apoptotic cells (sunburncells) and a reduced formation of cyclobutene pyrimidine dimers. This confirms that

HB-PS is able to reduce oxidative cell stress and protects cellular DNA or even repairs DNA damages after UV-irradiation. The exact working mechanism of DNA protection efficacy needs to be investigated further. The molecule itself has no strong anti-oxidative potential so it is very likely, that Hydroxybutyroyl-Phytosphingosine works on a molecular cell level. Which markers in oxidative stress response and DNA repair pathways are involved needs to be part of future investigations.

In addition to the *in vitro* investigation, also positive effects on sun-stressed skin could be shown *in vivo*. The study was performed after summer in Europe, so panelists suffered from sun-stressed skin conditions. The daily application of Hydroxybutyroyl-Phytosphingosine over the study period of 8 weeks lead to an acceleration in skin regeneration which could be shown by increased skin lightening compared to the untreated and vehicle treated control. This could be explained by reduced DNA damages due to the Sphingolipid treatment which might lead to faster regeneration of the skin. In addition, various parameters linked to photoaging could be improved by application of Hydroxybutyroyl-Phytosphingosine. Various texture parameters were increased which indicates a more balanced skin tone (data not shown). Skin roughness parameters were decreased by Sphingolipid treatment which display a reduction of fine lines and wrinkles. Ultrasound measurements of the skin showed an increase of skin density and a strong reduction of age-bands between epidermis and dermis (data not shown). This part of the human skin is very prone to UV irradiation and oxidative stress leads to reduction of macromolecules like collagen and elastin. This can be visualized in ultrasound pictures by strongly reduced skin density profiles. Hydroxybutyroyl-Phytosphingosine can reduce the width of these age-bands leading to an increase in overall skin density (data not shown). Overall, it could be clearly demonstrated that Hydroxybutyroyl-Phytosphingosine can protect the human skin from sun-induced premature aging.

In summary, Hydroxybutyroyl-Phytosphingosine is a suitable active ingredient to fight environmental aggressions, protect skin from UV induced DNA damage and thereby reduce signs of sun-induced premature aging.

Conclusion. Hydroxybutyroyl-Phytosphingosine showed promising anti-oxidative benefits in a broad screening approach (SimDerma®) with a special efficacy in DNA protection. Positive effects on sun-stressed skin could be shown *in vivo*. A re-balanced skin tone after summer stress might be due to an accelerated skin regeneration based on DNA protection. Overall, a protection from sun-induced premature aging could be shown which marks Hydroxybutyroyl-Phytosphingosine as multifunctional product for holistic skin protection. Since DNA protection efficacy is not yet described for Sphingolipids, further studies are needed to investigate the exact working mechanism.

Acknowledgments. This research would not have been possible without support by the teams of Evonik's Active Ingredients product line for cosmetics, RD&I and innoHealth group.

Conflict of Interest Statement. All authors of this paper are directly or indirectly employees of Evonik Industries AG, based in Essen Germany. The SimDerma® technology referenced in this paper are commercially available services offered by Evonik.

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