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The Presence of Essential and Non-Essential *Stratum Corneum* Proteases: The Vital Need for Protease Inhibitors

Rainer Voegeli[1], Peter Wikstroem[1], Remo Campiche[1], Stephan Doppler[1], Pierre Seroul[2], Nidhin Raj[3], Eileen Jackson[1], Torsten Steinmetzer[4], Marco Massironi[5], Majella E. Lane[3], Beverley Summers[6], Anthony V. Rawlings[3,7]

[1] DSM Nutritional Products Ltd., Basel, Switzerland – [2] Newtone Technologies, Lyon, France

[3] UCL School of Pharmacy, London, United Kingdom – [4] Philipps-University Marburg, Germany

[5] Cutech Srl, Padova, Italy – [6] Sefako Makgatho Health Sciences University, Pretoria, South Africa

[7] AVR Consulting Ltd., Northwich, United Kingdom

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Abstract

Dry skin is one of the most important concerns of consumers worldwide. Despite huge efforts over several decades, the personal care industry still does not offer complete solutions that satisfy the unmet needs of consumers for moisturizing treatments. The paucity of data for the underlying biochemical problems in and the effects of moisturizers on facial skin biology and physiology may partly explain this. Our recent color mapping studies based on bio-instrumental evaluations of skin capacitance and transepidermal water loss have revealed the complexity of facial skin. However, the biomolecular reasons for these subtle differences in the different zones of the face are unknown so far. As the maturation of the stratum corneum is vital for skin moisturization and optimal barrier function, we believe that the protease / protease-inhibitor balance particularly of the plasminogen system may be key in these processes. Thus, our aim was to develop a specific dual plasmin and urokinase inhibitor for topical application to barrier-impaired skin and demonstrate its efficacy.

INTRODUCTION

Facial skin dryness

To this day, dry skin is the number one unmet skincare consumer need globally. Thus, our aim is to understand in more depth the moisturization of the skin but also the needs of the differently pigmented skin types. Although various controversial data exist, it seems that the barrier of darkly pigmented skin has some advantages compared with that of lightly-pigmented skin. Most studies have been conducted on volar forearms, but our interest is facial skin.

Facial skin is unique in that it receives far more exposure to the external environment than skin on other areas of the body. Paradoxically, the *stratum corneum* of the face is particularly sensitive and thinner than elsewhere in the body [1] with the eye region having a *stratum corneum* as thin as 6 µm [2]. Environmental influences contribute to drying of facial skin but also damage to the *stratum corneum* [3]. There are fewer cell layers and smaller corneocytes than on most other body sites [4]. Whereas on the forearm there are approximately 15 - 20 cell layers, facial *stratum corneum* has only approximately 7 - 10 cell layers [5]. Compared to other body sites, facial skin shows elevated transepidermal water loss (TEWL), increased *stratum corneum* cohesion and higher proteolytic activity resulting in premature corneodesmosomal degradation and *stratum corneum* thinning.

Our recently developed color mapping analysis of bio-instrumental evaluations [6] enabled a continuous visualization of facial skin moisturization ***(Figure 1)***. We have concluded that a) remarkable gradients of skin hydration and TEWL exist within short distances and b) the gradients are distinctive in different ethnic groups. A four-week study using a cream with high levels of bench mark moisturizers led to a general improvement in hydration. However, relative to other facial sites, the moisturization of the nasolabial fold and parts of the cheek remained inadequate [7]. This study highlighted the complexity of facial skin and properties among different ethnic groups, which might be the reason why dry facial skin

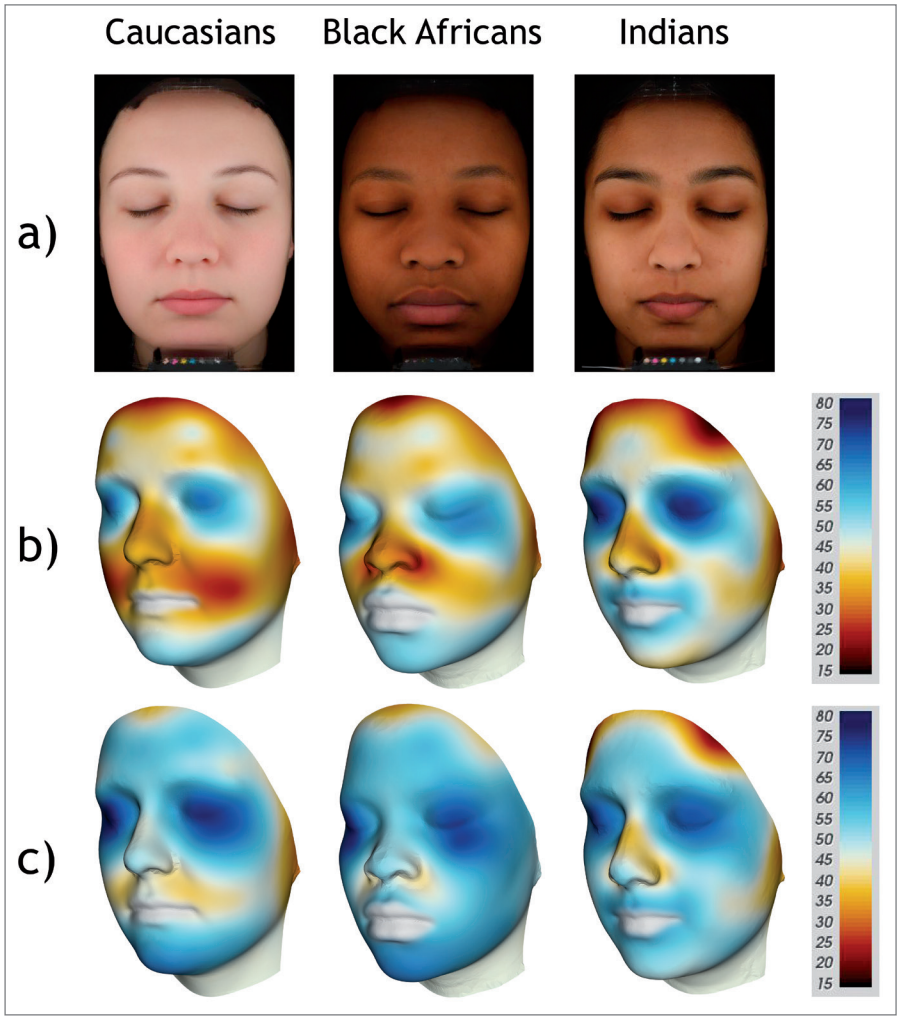

Figure 1 a) Composite facial images of Caucasian, Black African and Indian subjects (from left); below, continuous facial color mapping of skin capacitance b) baseline, and c) after a four-week moisturizer treatment. Color codes for corneometer values (AU) are shown on the scales; capacitance 15-80 AU, blue = normal skin condition, red = impaired skin condition.

is still a major unmet consumer need. The biomolecular causes for these differences in the different zones of the face are unknown to date. Obviously, a deeper molecular understanding of the *stratum corneum* is needed.

Surprisingly, it was only 29 years ago that Warner and colleagues demonstrated for the first time a water concentration profile within the epidermis [8]. They showed that there is a steep water gradient in the *stratum corneum* reaching a plateau level in the living epidermis. Based on these data it can be estimated that dry body skin needs only 3 ml of water to become well hydrated and dry facial skin as little as 100 µl, corresponding to 2 drops of water. So, why is it then that we still fail to moisturize dry skin reliably, effectively, and sustainably when such tiny amounts of water are required, especially if we consider the water movements through our skin which run in much larger dimensions (sweat and TEWL, more than 200 ml/day each)?

All these facts call for novel concepts for the treatment of dry skin. A promising approach can be found in Rawlings' moisturizing review in the IFSCC Magazine [9]. He concluded that the biology of skin moisturization, of which hydration is only one benefit, is highly complex and that the future of all new moisturizers lies in understanding the control of desquamation.

Stratum corneum proteases

Enzymes, in particular proteases, play important roles in lipid barrier homeostasis, tissue remodeling and inflammatory conditions but are also involved in the keratinization process [10]. Epidermal proteases control desquamation, corneocyte maturity and the number of cell layers in the *stratum corneum*. Changes in the proteolytic balance of the skin can result in inflammation, which leads to the typical somatosensory signs of redness, scaling, and itching. The activity of certain proteases is increased in skin disorders in which the epidermal barrier is impaired [11, 12], ranging from genetic diseases, such as psoriasis and atopic dermatitis, to subclinical barrier abnormalities induced, for example, by alkaline soaps or environmental influences [13]. This imbalance in protease activity causes structural changes in the *stratum corneum* and their excess can lead to activation of inflammatory pathways.

Desquamation of corneocytes can only occur once the extracellular part of corneodesmosomes, the cadherins, has been hydrolyzed, allowing a regulated shedding of corneocytes [11]. This is a very complex cascade in which mainly kallikreins (serine proteases), but also cathepsins (cysteine and aspartic proteases) and heparanase 1 (an endoglycosidase), are involved. Activities of desquamatory proteases are kept under control by several endogenous inhibitors, mainly lymphoepithelial Kazal-type inhibitors (LEKTI) [14, 15].

The proteases of the plasminogen system in the epidermis are activated by inflammatory conditions, such as UV light [16] [17], or by proinflammatory cytokines, such as IL-1β, IL-8 and TNFα [18, 19]. Plasminogen is activated to plasmin by plasminogen activators, such as urokinase ***(Figure 2)***. *Katsuta et al.* found that urokinase itself is immediately activated in the *stratum corneum* after barrier disruption [20]. Activated plasmin then leads to premature desquamation, impaired maturation of cornified envelopes and delayed barrier recovery. Moreover, plasmin activates proMMPs, which can lead to destruc-

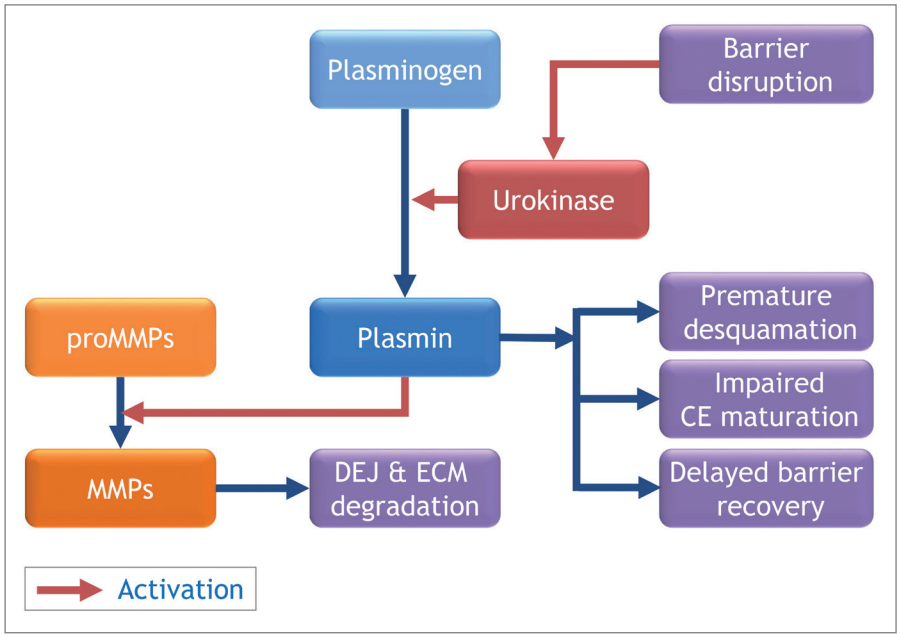

Figure 2 Activation cascade of the plasminogen system and its impact on desquamation, cornified envelope (CE) maturation, barrier recovery and components of the extracellular matrix (ECM) and the dermal-epidermal junction (DEJ).

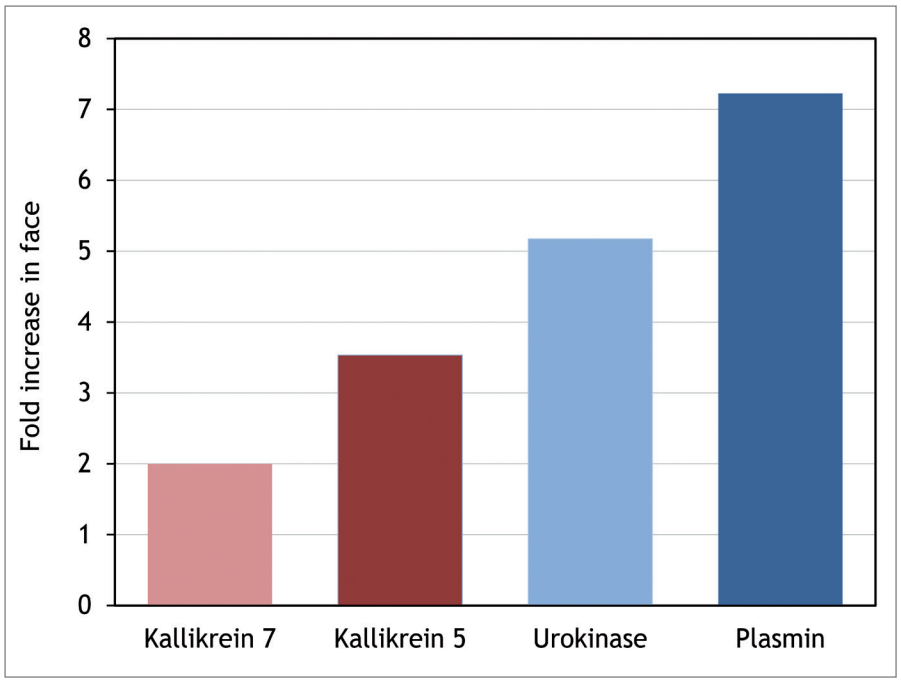

Figure 3 Fold increase (cheek vs. forearm) of protease activities in extracts of tape strippings taken from Caucasian subjects.

tion of components of the extracellular matrix (ECM) and the dermal-epidermal junction (DEJ) [21]. Thus, the activation of the plasminogen system is detrimental for epidermal homeostasis. It is proposed to be the major protease activity involved in the delay of barrier recovery [20, 22, 23, 24]. Obviously, the inhibition of the plasminogen system will lead to improved barrier function and reduced ECM and DEJ degradation.

To our knowledge, there is no evidence for corneodesmosomal degradation by urokinase or plasmin. However, there are indications that the plasminogen system can activate certain pro-kallikreins and vice versa, which may lead to abnormal desquamation and may play a role in inflammatory skin diseases [25].

A tape stripping based depth profiling of desquamatory kallikreins and proteases of the plasminogen system in the *stratum corneum* of healthy Caucasian subjects revealed significantly increased activities of all the tested proteases on the cheek *versus* forearm ***(Figure 3)*** [4]. Kallikrein 7 was elevated two and kallikrein 5 three and a half times *versus* forearm, and the inflammatory proteases urokinase five and plasmin seven times. Later, we found a highly significant correlation of plasmin activity with TEWL ***(Figure 4)*** [26].

In a further study, the proteolytic activity in the *stratum corneum* of eczema patients was evaluated [12]. All tested serine protease activities were significantly enhanced in lesional skin. Compared with healthy skin, kallikrein 7 activity was double and kallikrein 5 activity was increased five and urokinase activity eight times in lesional skin. Interestingly, these are approximately the same fold increases we found on the cheek of healthy subjects. The plasmin activity, however, was massively elevated in lesional skin: 30 times *versus* non-lesional and 70 times *versus* healthy skin ***(Figure 5)***. Moreover, there was a significant reduction of *stratum corneum* thickness in lesional skin.

The epidermal plasminogen system is activated in inflammatory conditions, even in subclinical, micro- or pre-inflammatory conditions, and in UV damaged skin. We believe that the plasminogen system is a key marker for underlying and sometimes non-observable barrier abnormalities but also an indicator of epidermal photodamage [27]. Thus, our aim was to develop a novel treatment for topical application to barrier-impaired facial skin based on a specific dual plasmin and urokinase inhibitor and show its efficacy *in silico*, *in vitro* and *in vivo*. The face represents a particular challenge. It has a low barrier reserve and needs special care to maintain its barrier function.

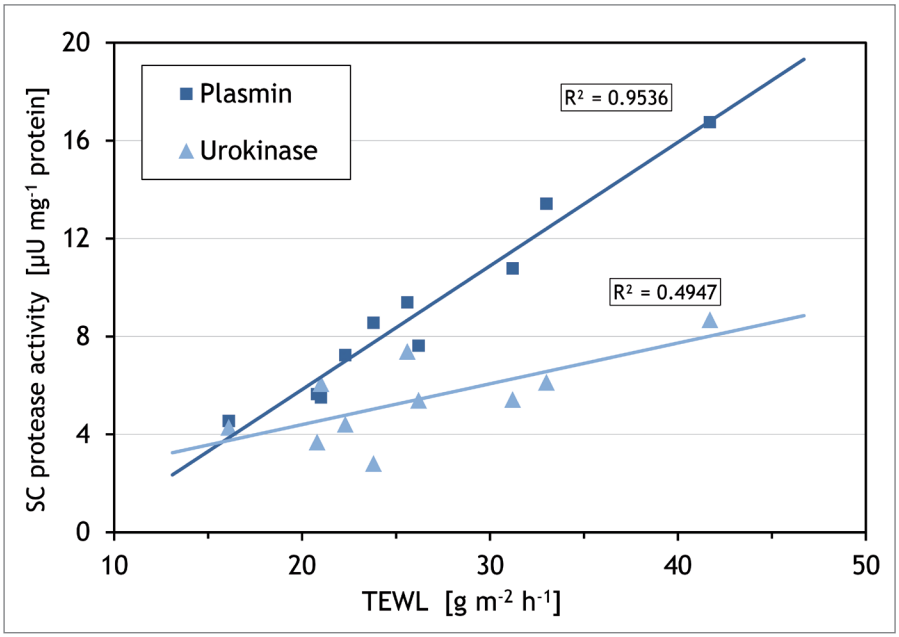

Figure 4 Correlation of plasmin and urokinase activities in extracts of tape strippings taken from Caucasian subjects and transepidermal water loss (TEWL), $p < 0.001$ for plasmin and $p < 0.05$ for urokinase.

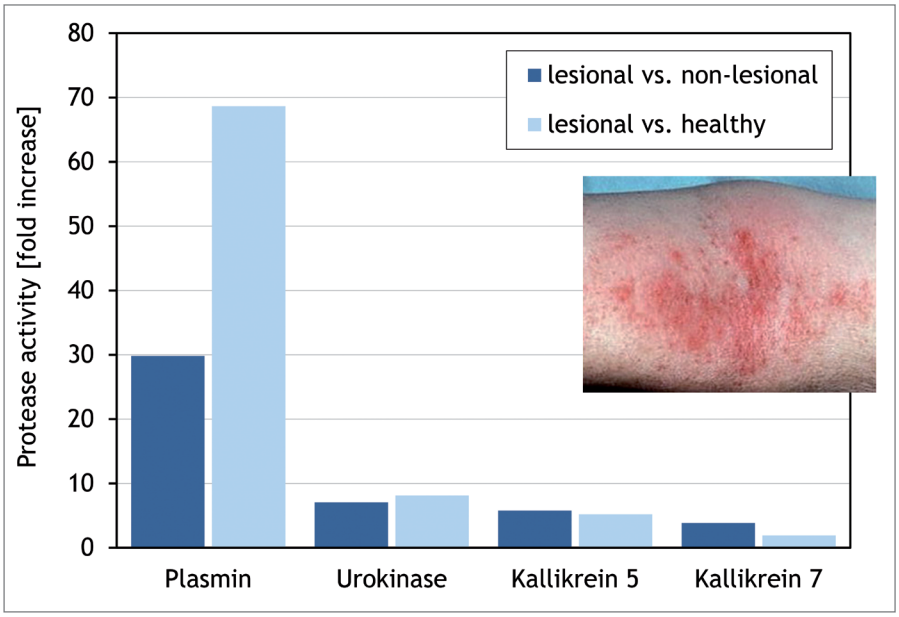

Figure 5 Fold increase of protease activities in extracts of tape strippings taken from atopic patients and healthy subjects (lesional vs. non-lesional atopic skin and lesional atopic skin vs. healthy skin, all $p < 0.05$). Inlay shows an example of lesional acute eczematous atopic skin.

METHODS

UV induction of plasmin *ex vivo*

For immunohistochemical evaluation of UV induction of plasmin, biopsies of excised abdominal human skin were radiated with a Bio-Sun solar simulator (Vilber-Lourmat, Eberhardzell, DE). The skin samples were irradiated with 3 Jcm^{-2} in total, composed as follows: 0.1 Jcm^{-2} UVB (312 nm) and 2.9 Jcm^{-2} UVA (365 nm). The biopsies were formalin fixed, paraffin embedded and cut into 5 µm thick sections. The sections were then deparaffinized in Kclear plus (#1276, Kaltek srl, Padova, IT) and graded alcohols, followed by specific antigen retrieval in EnVision™ FLEX Target Retrieval Solution, low pH (#K8005, DAKO, Glostrup, DK) in a steamer. After incubation with a polyclonal rabbit antibody for plasmin (#ab48350, Abcam, Cambridge, UK) the staining was detected with Dako Real Detection System (#K5005, DAKO, Glostrup, DK). The sections were counterstained with hematoxylin (#MHS80, Sigma-Aldrich, St. Louis, US) and mounted. Slides were photographed using an Olympus BX51 microscope equipped with an Olympus DP70 camera, at 200 × magnification. Twelve bright field images were acquired for each treatment. The amount of the antigen present in each slide was evaluated by estimating the intensity and the distribution of the red staining within the epidermis using ImageJ software (NIH, USA). The obtained data were normalized upon the dimension of the analyzed surface expressed in pixels, thus obtaining a semi-quantitative score for each image. Briefly, after background subtraction, a custom-modified color deconvolution matrix was applied to each picture in order to separate the color pictures according to the analysis needs. The obtained red channel pictures were selected and transformed into 8-bit images. The epidermis area was then selected in each image and the intensity and distribution of staining within the selected area was estimated and normalized on its dimension.

Stratum corneum protease profiling and corneocyte envelope phenotype studies from tape strippings

For protease profiling, tape strippings were taken from the sun-protected postauricular site and sun-exposed cheek of sixty healthy female volunteers living in Pretoria, South Africa [27]. There were three age-matched groups ($n = 20$ each) of Albino African (40 ± 3 years), Black African (38 ± 2 years) and Caucasian subjects (45 ± 3 years). Tape stripping extracts (0.1 M TRIS/HCl buffer, pH 8.0, containing 0.5 % Triton X-100) were assayed for the activity of plasmin by use of the aminomethylcoumarin-based fluorogenic peptide substrate (MeOSuc-Ala-Phe-Lys-AMC) and HPLC. Corneocyte maturation was evaluated by Nile red and involucrin antibody staining with a fluorescence microscope equipped with a Canon 70D camera. IMAGEJ™ image analysis software was used to analyze the red pixels

obtained from the Nile red-stained cells and the green pixels from the immunostained cells.

Synthesis of a plasmin and urokinase inhibitor and examination of its effects *in vitro*

Peptide coupling methods were used to synthesize the inhibitor BSFAB (INCI name: Benzylsulfonyl-D-Seryl-Homophenylalanine Amidino-Benzylamide Acetate) ***(Figure 6)***

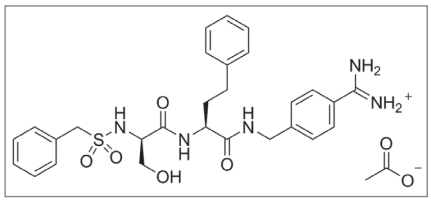

Figure 6 Chemical structure of BSFAB.

in high purity (>97 %) [28]. For the evaluation of Ki values of BSFAB, MeOSuc-Ala-Phe-Lys-AMC was used as the fluorogenic substrate for plasmin and Bz-β-Ala-Gly-Arg-AMC for urokinase.

For *in silico* docking studies, the catalytic domain of plasmin and urokinase were retrieved from the protein data bank of Research Collaboration for Structural Bioinformatics (RSCB), PDB codes: 1BUI and 1VJ9 and prepared for modeling studies using the protein preparation wizard (Maestro version 10.1, Schrödinger, LLC, New York, NY, USA) [28]. BSFAB was docked to the catalytic domains (Glide, version 6.9, Schrödinger, LLC, New York, NY, USA) and the Standard Precision (SP) setting was used. For docking, multiple conformers of BSFAB were generated (Confgen, version 3.1, Schrödinger, LLC, New York, NY, USA). Among the different binding poses generated the best pose, based on visual inspection, was refined using Prime, version 4.2 (Schrödinger, LLC, New York, NY, USA).

To demonstrate inhibition of cell-secreted proteases, normal human keratinocytes were seeded at sub-confluence and grown for 24 hours in Cnt-07 plasmin-free media (CELLnTEC, Bern, CH). The cells were stimulated with inflammatory cytokines (IL-1β and TNFα, 10 ng/ml each), followed by addition of BSFAB (100 µM). The proteolytic activities of urokinase and plasmin were quantified using previously described methods [4, 12] after 48 hours of incubation.

Table I Composition of Vehicle and Active (Hydrogels)

Ingredients	Vehicle	Active
Acrylates/C10-30 alkyl acrylate crosspolymer	0.50%	0.50%
Aqua	97.38%	97.38%
Glycerin 99.5%	0.87%	0.87%
Sodium hydroxide 30%	0.25%	0.25%
BSFAB	---	10 ppm
Phenoxyethanol, caprylyl glycol, sorbic acid	1.00%	1.00%

Clinical effects of BSFAB on barrier function *in vivo*

Forty-six Caucasian subjects (n=23 per aged matched group, 39±6 years) with self-assessed sensitive facial skin participated in the clinical study. On the 28 days of the application phase the subjects applied a hydrogel, either the vehicle or the test formulation (10 ppm BSFAB), twice daily to the face ***(Table I)*** [28]. Basal TEWL was measured and six consecutive standard D-Squame® disks (CuDerm Corporation, Dallas, USA) were taken from the cheek in order to mechanically challenge the barrier. TEWL was measured at baseline and day 28 and, in order to determine the barrier repair and barrier improvement, 24 hours after the tape strippings on day 1 and day 29. Before the beginning of the study and after four weeks of treatment, the subjects completed a questionnaire with structured scales containing the description of skin conditions.

RESULTS AND DISCUSSION

Changes in *stratum corneum* plasmin levels

For the first time, we demonstrated immunohistochemically that plasmin is significantly upregulated *ex vivo* in the *stratum corneum* and the *stratum granulosum* after exposure to UVA/B radiation (+102 %±33 %, p<0.05) ***(Figure 7)***.

Plasmin activity was also shown to be increased *in vivo* on sun-exposed cheek *stratum corneum* in all three subject groups, which was statistically significant (p<0.001) in Albino Africans (+107 %) and Caucasians (+109 %) ***(Figure 8)***. On both sun-exposed and sun-protected facial sites, the rank order of plasmin activity was Albino Africans > Caucasians > Black Africans. All interethnic differences were highly significant (p<0.001). Both photodamage and skin pigmentation seem to be related to plasmin activity. Moreover, elevated plasmin activity was associated with reduced corneocyte maturation (data not shown).

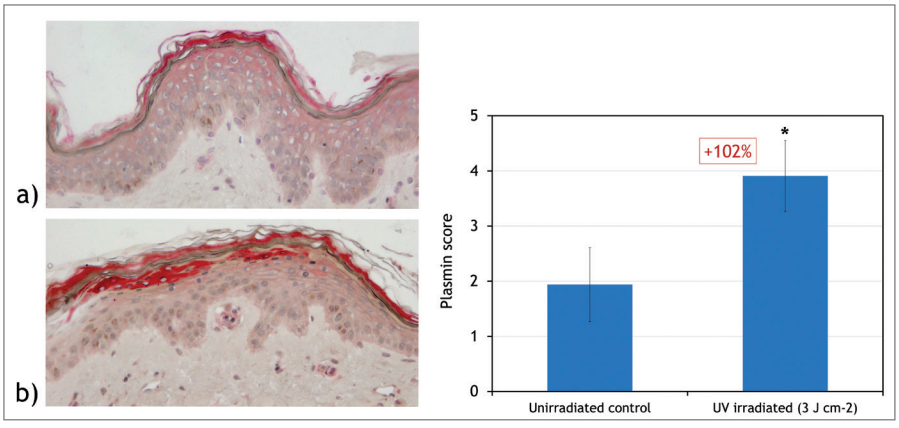

Figure 7 UV-induced expression of plasmin in a) unirradiated (control) and b) UVA/B-irradiated (3 J cm^{-2}) excised human skin. Plasmin was immunostained and is indicated by red color, n=12, data are the mean ± SEM, * p<0.05.

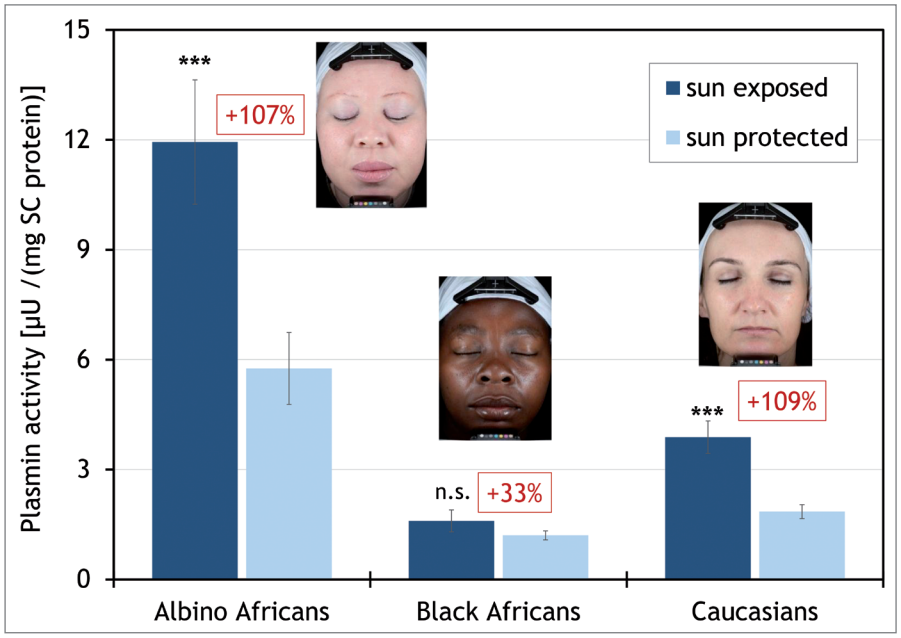

Figure 8 *Stratum corneum* plasmin activity on the sun-exposed cheek and sun-protected post-auricular site of differently pigmented ethnicities (Albino Africans, Black Africans, Caucasians). Data are the mean ± SEM, *** $p<0.001$, n.s., not significant.

Synthesis, *in silico* modeling and efficacy of BSFAB

An efficient and cost-effective process for the synthesis of BSFAB in high purity (97.3 %) and high overall yield (39 %) without any need for ion-exchange chromatography was developed. As the catalytic domains of urokinase and plasmin are highly conserved, BSFAB inhibits these enzymes by a very similar binding mode ***(Figure 9)***. In both modeled complexes the amidine moiety of BSFAB is tightly anchored in the S1 pocket by binding to the side chains of Asp189 and Ser190 and to the carbonyl oxygen of Gly219. The backbone of the P3 D-Ser residue binds to the NH and carbonyl oxygen of Gly216 and one oxygen of the sulfonamide group interacts with the NH of Gly219. Moreover, the urokinase complex is further stabilized by two additional hydrogen bonds between the P3 D-Ser hydroxyl group and the carbonyl of Leu97B and the side chain of His99.

BSFAB was shown to be an effective and reversible inhibitor of both proteases with Ki values of 25 nM for urokinase and 29 nM for plasmin. Moreover, we could demonstrate that BSFAB is a selective inhibitor and showed only negligible or no inhibitory potency against other key proteases occurring in the *stratum corneum*, such as tryptase or kallikrein 4 and kallikrein 7, and did not inhibit neutrophil elastase in the test conditions used. The Ki's for tryptase were approximately 45 - 80 and for kallikrein 5 and kallikrein 7 approximately 500 - 1000 fold weaker. As the kallikreins are vital for desquamation, we do not want to influence the desquamatory process and induce a rough or dry skin condition.

Inflammatory cytokines (IL-1β, TNFα, IL-8) are known to increase the activities of components of the plasminogen cascade [18, 19]. Therefore, we used IL-1β and TNFα to stimulate normal human keratinocytes and evaluated the inhibitory effect of BSFAB. We established that there was a significant reduction, 97 % and 40 %, in the activities of urokinase and plasmin, respectively, in both unstimulated and stimulated cells using this inhibitor (100 µM).

Clinically, BSFAB improved facial *stratum corneum* barrier function. Compared with day 0, basal TEWL of the BSFAB-treated subjects was numerically but nonsignificantly superior to the vehicle on day 28 ***(Figure 10)***. However, there was a significant difference between the two formulations on day 29 with the BSFAB treatment being more resistant to a repeated

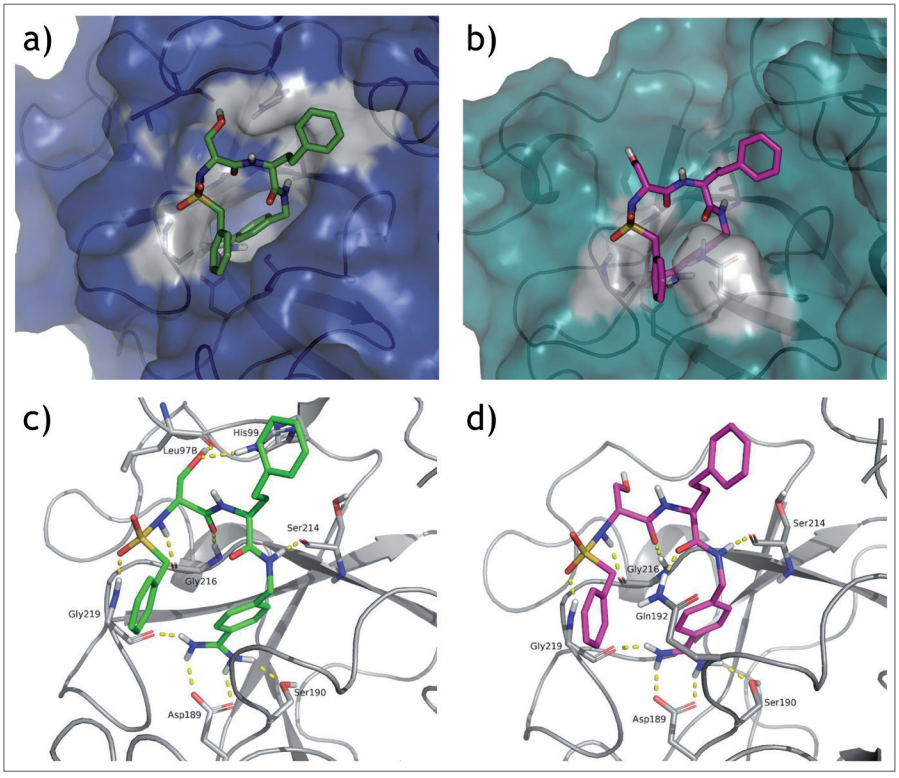

Figure 9 Modeled binding modes of BSFAB. Surface view of BSFAB bound to a) urokinase and b) plasmin, highlighting the fit of BSFAB into the S1 pocket and the main contact residues. Cartoon view of BSFAB bound to the active sites of c) urokinase and d) plasmin, highlighting key polar contacts.

tape stripping challenge, indicating an improvement in the biomolecular architecture of the *stratum corneum* and that BSFAB allowed a faster epidermal barrier recovery. In the subject group treated with BSFAB the TEWL of challenged skin on day 29 reached baseline TEWL value of unchallenged skin on day 0. Moreover, the BSFAB-treated subjects reported subjective perception of a more healthy and moisturized skin using this technology.

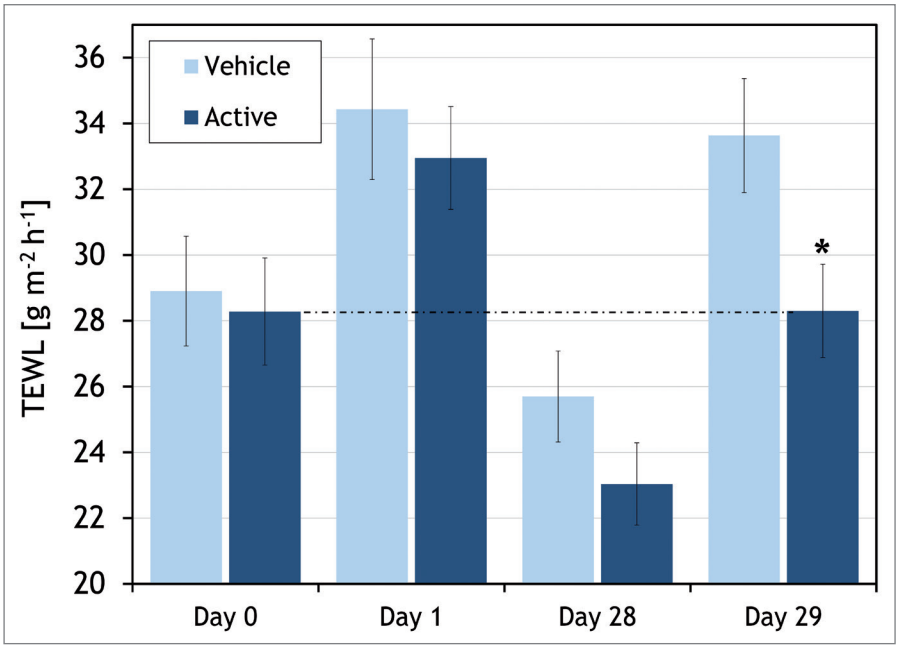

Figure 10 Basal TEWL data before and after a 28-day treatment with vehicle (light blue bars) and BSFAB (dark blue bars) and 24 hours after tape stripping on day 1 and on day 29. In the subject group treated with BSFAB, on day 29 challenged skin reached the baseline TEWL of unchallenged skin on day 0 (dotted line). Data are the mean ± SEM, * p = 0.02.

CONCLUSION

The enhanced activities of proteases of the plasminogen system in the *stratum corneum* are associated with premature desquamation and abnormal corneocyte maturation [12, 27]. There is evidence that the presence of elevated levels of these inflammatory serine proteases in photodamaged facial *stratum corneum* results in a thinner tissue compared with other body sites. Based on these data we developed a dual reversible competitive inhibitor of plasmin and urokinase for the treatment of compromised barrier skin condition. The efficacy of BSFAB was shown *in silico* and *in vitro* as well as in *in vivo* by a vehicle-controlled clinical study. The latter demonstrated that topical facial application of BSFAB led to an improvement in *stratum corneum* barrier function. As plasmin and urokinase activities are increased with increasing TEWL, inflammation and photodamage, we believe that BSFAB will be effective in any compromised barrier skin condition and our new approach will meet the so far unmet needs of facial skin globally.

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Corresponding Author
Rainer Voegeli
DSM Nutritional Products Ltd.
Basel
Switzerland
Tel. +41 61 815 82 98
rainer.voegeli@dsm.com