The effects of benzylsulfonyl-D-Ser-homoPhe-(4-amidino-benzylamide), a dual plasmin and urokinase inhibitor, on facial skin barrier function in subjects with sensitive skin

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Abstract

OBJECTIVE: The aim of this study was to optimize the synthesis of the plasmin and urokinase (uPA) inhibitor benzylsulfonyl-D-Ser-homoPhe-(4-amidino-benzylamide) (BSFAB), to characterize its activity and mechanism of action and to assess its use to improve stratum corneum (SC) barrier function.

METHODS: Peptide coupling methods were used to synthesize BSFAB, and high-performance liquid chromatography–mass spectrometry (HPLC-MS) together with 1H- and 13C-nuclear magnetic resonance spectroscopy (NMR) were applied to clarify its structure and determine its purity. Its binding mode was determined by docking studies to the catalytic domains of plasmin and uPA. Inhibition constants (Ki) were determined by enzyme kinetic studies, and the effect of BSFAB on plasmin, uPA and transglutaminase 1 expression was evaluated in non-cytokine and cytokine-stimulated keratinocytes. A vehicle-controlled clinical study on SC barrier function was conducted on facial skin of subjects with self-perceived sensitive skin.

RESULTS: BSFAB was synthesized with high purity (97.3%). In silico studies indicated that the amidine moiety of BSFAB was anchored in the S1 pocket of both enzymes by binding to Asp189, Ser190 and Gly219, whereas the backbone of the D-Ser residue makes an anti-parallel β-sheet interaction with Gly216. BSFAB was shown to be an effective inhibitor of plasmin and uPA with Ki values of 29 and 25 nM, respectively. BSFAB also inhibited keratinocyte-secreted protease activities in basal (plasmin inhibition 37.7%, P < 0.05 and uPA inhibition 96.6%, P < 0.01) and cytokine-induced conditions (plasmin inhibition 41.1%, P < 0.05 and uPA inhibition 97.0%, P < 0.001) and stimulated the gene expression of transglutaminase 1 in cytokine-stimulated keratinocytes (approximately 4.5 times increased expression, P < 0.01). Clinically, BSFAB was shown to improve SC barrier integrity (P < 0.02 on day 29) and subjective improvements in the perception of healthy skin (P < 0.05 on day 28).

CONCLUSION: BSFAB binds as a reversible competitive inhibitor to the active sites of plasmin and uPA. Additionally, BSFAB positively improved keratinocyte differentiation gene expression (transglutaminase 1). These effects were translated into improvements in SC barrier integrity clinically in subjects with dry and sensitive skin and improved their perception of having a healthy skin condition.

Résumé

OBJECTIF: Le but de cette étude était d’optimiser la synthèse de l’inhibiteur de plasmine et de l’urokinase (uPA) benzylsulfonyl-D-Ser-homoPhe- (4-amidino-benzylamide) (BSFAB), pour caractériser son activité et le mécanisme d’action et d’évaluer son utilisation pour améliorer la fonction de barrière du stratum corneum (SC).

MÉTHODES: Les méthodes de couplage peptidique ont été utilisées pour synthétiser BSFAB et la chromatographie liquide à haute performance-spectrométrie de masse (CLHP-SM) avec spectroscopie de résonance magnétique nucléaire 1H et 13C (RMN), qui servent à clarifier sa structure et déterminer sa pureté ont été employées. Son mode de liaison a été déterminé par l’étude d’amarrage aux domaines catalytiques de plasmine et d’uPA. Les constantes d’inhibition (Ki) ont été déterminées par des études de cinétique enzymatique et l’effet de BSFAB sur la plasmine, l’uPA et l’expression de la transglutaminase 1 a été déterminé chez les kératinocytes stimulés par les cytokines ou non. Une étude clinique contrôlée contre placebo sur la fonction barrière du SC a été réalisée sur la peau du visage des sujets à peau sensible auto-perçue.

RÉSULTATS: Le BSFAB a été synthétisé avec une pureté élevée (97.3%). Les études en silico ont indiqué que le groupement amidine de BSFAB a été ancré dans la poche S1 de deux enzymes par liaison à Asp189, Ser190 et Gly219, alors que le squelette du D-Ser résidu fait un anti-para-β-feuillet dinteraction avec Gly216 antiparallèle. BSFAB s’est montré être un inhibiteur efficace de la plasmine et de l’uPA avec des valeurs de Ki de 29 et 25 nM respectivement. BSFAB a également inhibé les activités des protéases kératinocytaires sécrétées dans les conditions de base (plasmine, l’inhibition de 37.7%, P < 0.05 et uPA inhibition de 96.6%, P < 0.01) et des conditions induites par la cytokine (plasmine inhibition de 41.1%, P < 0.05 et uPA inhibition de 97.0%, P < 0.001) et a stimulé l’expression du gène de la transglutaminase 1 dans les kératinocytes stimulés par des cytokines (une expression accrue d’environ 4.5 fois, P < 0.01). Cliniquement BSFAB a été permis d’améliorer l’intégrité de la barrière SC.

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(\(P < 0.02\) le jour 29) et apporter des améliorations subjectives dans la perception de la peau saine (\(P < 0.05\) au jour 28).

CONCLUSION: BSFAB se lie comme un inhibiteur compétitif réversible aux sites actifs de la plasmin et de l’uPA. En outre, BSFAB améliore positivement l’expression du gène de la différenciation des kératinocytes (transglutaminase 1). Ces effets ont été traduits en améliorations dans l’intégrité de la barrière SC cliniquement chez les sujets ayant une peau sèche et sensible et ont amélioré leur perception d’avoir une condition de la peau saine.

Introduction

Proteases play important roles in the homoeostasis and diseases of skin, particularly of the stratum corneum (SC) [1]. Besides kallikreins (KLKs), other serine proteases, and especially members of the trypsin family such as urokinase (uPA) and plasmin together with theirzymogens, have started to receive more attention [1]. Activation of pro-uPA, the zymogen of uPA, generates catalytically active high molecular weight (HMW)-uPA and a low molecular weight (LMW)-uPA [2]. A number of serine and cysteine proteases can activate pro-uPA such as plasmin, matriglpase, tryptase, and the various trypsin-like KLKs and the cathepsins B and L [3, 4]. Outside of the circulation, plasminogen, the zymogen of plasmin, is mainly activated by uPA [5].

Concerning the skin, Grayson et al. observed that uPA was evenly distributed between the cytoplasm and lamellar granules in the epidermis [6] while others have reported that plasminogen and its binding sites are in the basal layer of the epidermis [7, 8]. Kramer et al. [9] reported that the epidermis contains the normal components of the plasminogen system, and uPA is reportedly the predominant plasminogen activator but in disease states, such as psoriasis, the levels of tissue-type plasminogen activator (tPA) increase. Others also demonstrated that psoriatic lesions display increased activity and supra basal expression of plasminogen [10–14]. Moreover, Fraki et al. [15–17] reported on the presence of ‘plasminogen activator’ protease in psoriatic SC. The source of plasmin in skin is uncertain but in situ synthesis has not been excluded [8].

During their studies on the identification of potential SC desquamatory proteases, Suzuki et al. [18] demonstrated the presence of plasmin and uPA activity in tape strippings of abdominal skin and sunburn peelings. Later, Komatsu et al. [19] also determined the activities of plasmin in non-lesional and lesional SC tape strippings of psoriatic skin compared with healthy skin and found them to be increased by approximately 3.5-fold and 8.5-fold in lesional skin. Similarly, the same group identified an approximate two-fold increase in plasmin activity in atopic subjects [20]. We have compared the (patho)physiological changes in the activities and mass levels of plasmin and uPA in acute eczematous lesional skin on ventral forearm of atopic dermatitis (AD) patients with non-lesional AD skin and skin of healthy subjects [21, 22]. uPA mass levels were very low and close or even at detection limit. However, increased mass levels of plasmin (8-fold) were observed in lesional skin. Additionally, activities of plasmin were increased 69-fold and uPA eight-fold in lesional skin compared with healthy skin.

We have also shown SC serine protease activities to be increased in non-diseased barrier-compromised conditions of the face [23, 24]. Furthermore, the increase in plasmin activity appears to be related to photodamage as greater activities of plasmin are observed in cheek vs. post-auricular facial sites of Caucasian and Albino African subjects implying that ultraviolet light (UV)-induced inflammation contributes to its increased levels [25]. These findings are consistent with the earlier studies on sunburn peelings [18]. Moreover, Kitamura et al. [26] were the first, who observed increased epidermal levels of plasmin in dry skin conditions, whereas Kawai et al. and Katsuta et al. found increased activities of uPA after barrier disruption [27, 28].

The question remains, do these elevated proteolytic activities of plasmin and uPA impair functioning of the SC or the epidermis? In fact, we have reported that a significantly thinner SC occurs in lesional skin of patients with acute eczematous atopic skin compared with normal and/or non-lesional skin implying that elevation of SC proteases, and potentially plasmin or uPA or their role in activating other SC KLKs, might cause premature desquamation and reduce the skin barrier reserve [21]. Moreover, in addition to the effects of plasmin on dry skin, Kitamura et al. also demonstrated that skin conductance was lower and transepidermal water loss (TEWL) was higher in such conditions [26, 29]. In this respect, the epidermal plasminogen system is thought to be involved in delaying epidermal barrier recovery [28, 30, 31].

Denda et al. [30] demonstrated that trypsin inhibitors and especially trans-4-(aminomethyl)cyclohexaneacarbonyl acid (t-AMCHA; tranexamic acid) accelerated the recovery of SC barrier function after a variety of insults to the epidermis. Katsuta et al. [32] also focussed on the role of uPA and identified a methylamide derivative of t-AMCHA that was more effective in alleviating disturbances in barrier function. Also, as the role of tight junctions has unfolded as the second line of defence in epidermal barrier function, Yuan et al. [33] have shown t-AMCHA to upregulate occludin expression in an SLS disturbed barrier and accelerated barrier repair.

It is clear that inhibitors against proteases of the plasminogen system can alleviate symptoms of dry skin and improve barrier function [26–33]. Nevertheless, t-AMCHA is a non-competitive inhibitor of the plasminogen enzyme system inhibiting plasminogen activation [34]. It does not bind to the active site of plasmin and cannot inhibit any already activated plasmin (and uPA). As we have discussed, increased activities of these enzymes are already present in the SC even in mildly photodamaged skin states and especially in facial SC [23, 24, 26, 27, 29]. Thus, the effects of t-AMCHA will be limited. As a result, we have focussed on identifying a competitive inhibitor to both plasmin and uPA that does not have the disadvantages of t-AMCHA. This report describes the development and efficacy of the dual uPA and plasmin inhibitor, benzylsulfonyl-D-Ser-homoPhe-(4-amidino-benzylamide) (BSFAB) (INCI name: benzylsulfonyl-D-Seryl-homophenylalanine amidino-benzylamide acetate) for topical applications on barrier impaired, photodamaged, microinflammatory skin, preferably on the face.

Materials and methods

Synthesis of benzylsulfonyl-D-Ser-homoPhe-(4-amidino-benzylamide) (BSFAB)

The amino acid building blocks, H-D-Ser(tBu)-OBzl hydrochloride and Boc-homoPhe-OH were obtained from Iris Biochemicals Ltd. The amino acid building blocks, H-D-Ser(tBu)-OBzl hydrochloride from abcr GmbH (Karlsruhe, DE). Building block I was prepared via N-sulfonylation of H-D-Ser alanine (BSFAB).

Building block 1 was prepared via N-sulfonilation of H-D-Ser (tBu)-OBzl hydrochloride using benzylsulfonyl chloride and N,N-dimethylformamide (DMF) in tetrahydrofuran at 4°C (step 1) (Scheme 1). The formed benzylster derivative was in turn...
hydrogenated (step 2) in isopropanol with palladium on activated charcoal (Pd/C) as catalyst and hydrogen gas at 2 bar and 50°C, to give the corresponding substituted amino acid, benzylsulfonyl-D-Ser (tBu)-OH (I). Building block II was prepared via amidation (step 3) of Boc-homoPhe-OH with 4-(aminomethyl)benzonitrile hydrochloride using o-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium-tetrafluoroborate (TBTU) and DIPEA at 5°C followed by deprotection (step 4) with HCl in dioxane at room temperature to give H-homoPhe-NHBzl (4CN) hydrochloride (II). The two building blocks were reacted using standard peptide coupling conditions with TBTU and DIPEA at 5°C (step 5). The formed dipeptide nitrile derivative was converted to the corresponding hydroxylamino derivative with hydroxylamine at reflux in methanol (building block III, step 6). Deprotection of the t-butyl ether on the serine side chain was accomplished in trifluoroacetic acid (TFA) in the presence of dodecanethiol as scavenger at 4–8°C (step 7). Finally, catalytic reduction of the oxamidino function with Raney nickel as catalyst and hydrogen gas in acetic acid/water (80/20) at 1.5 bar and 30°C gave the active amidino derivative, BSFAB (IV, step 8) [35]. Upon crystallization, the inhibitor was afforded in a purity of >97%.

**Determination of BSFAB structure and purity**

**High-performance liquid chromatography–mass spectrometry (HPLC-MS) methodology**

The purity and molecular mass of BSFAB were determined by HPLC using the benchtop quadrupole Agilent 1100 LC/MSD Model G1946D Mass Spectrometer (Agilent, Basel, CH) with a reverse-phase YMC Pro C18 column (150 × 3.0 mm, 3 μm, YMC Europe, Dinslaken, DE) and a linear water/acetonitrile/methanesulfonic acid gradient with a column temperature of 23°C, a flow rate of 0.5 mL min⁻¹ and detection at 230 nm. The mass spectrum was generated by electrospray ionization in positive mode.

**Nuclear magnetic resonance (NMR) spectroscopy**

NMR spectra were recorded on a Bruker Avance 300 spectrometer (Bruker, Fällanden, CH) equipped with 5 mm BBO BB-1H probe head operating at 300 MHz for ¹H and 75.5 MHz for ¹³C. Spectra were recorded in DMSO-d₆. Spectra were referenced to residual DMSO (2.50 ppm, ¹H; 39.5 ppm, ¹³C).

**Scheme 1** Summary of synthesis route of benzylsulfonyl-D-Ser-homoPhe-(4-amidino-benzylamide) (BSFAB). 1) DIPEA in tetrahydrofuran, 2) Palladium on carbon, H₂-gas in isopropanol, 3) TBTU, DIPEA in tetrahydrofuran, 4) HCl in dioxane, 5) TBTU, DIPEA in THF, 6) aqueous NH₂OH in methanol, 7) 1-dodecanethiol in trifluoroacetic acid, 8) Raney nickel, H₂-gas in acetic acid/water. Abbreviations: DIPEA, N,N-diisopropylethylamine; TBTU, o-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium-tetrafluoroborate.
Molecular modelling of interaction of BSFAB with plasmin and uPA

The catalytic domain of plasmin and uPA was retrieved from the protein data bank of Research Collaboration for Structural Bioinformatics (RSCB PDB) (codes: 1BUI and 1VJ9) and prepared for modelling studies using a protein preparation wizard (MAESTRO version 10.4; Schrödinger, LLC, New York, NY, US 2015). In short, bond orders were set, hydrogens were added, disulphide bridge was created, all water molecules were removed and hydrogen bonds were optimized using the automated procedure. BSFAB was docked to the catalytic domains (GLIDE, version 6.9; Schrödinger, LLC), and the Standard Precision (SP) setting was used. For docking, multiple conformers of BSFAB were generated (CONFGEN, version 3.1; Schrödinger, LLC). Among the different binding poses generated the best pose, based on visual inspection, was refined using PRIME, version 4.2; Schrödinger, LLC. Fig. 1 was prepared using the PYMOL Molecular Graphics System, version 1.8.0.4; Schrödinger, LLC [36,37].

Figure 1. Modelled binding modes of BSFAB. Cartoon view of BSFAB bound into uPA (a) and plasmin (b) highlighting key hydrogen bond interactions that result both in a short anti-parallel β-sheet and tight binding into the S1 pocket. Surface view of BSFAB bound into uPA (c) and plasmin (d) highlighting the fit of BSFAB into the S1 pocket and the main contact residues.
Enzyme inhibitor studies and determination of $K_i$ values

Plasmin was purchased from Merck Millpore (#527621, Darmstadt, GE), uPA from Medac (Wedel, GE), tetrameric skin β1-trypsin from Promega (#G706A, Madison, US), neutrophil elastase from Merck Millpore (#3244681, Darmstadt, GE), and KLK5 and KLK 7 from R&D Systems (#1108-SE-010 and #2624-SE-010, Minneapolis, MN, US). The structures and sources of the fluorogenic protease substrates are listed in Table I.

The used concentrations of the proteases and their substrates in the assays are shown in Table II. The substrate solutions were prepared from a 100 mM stock solution in DMSO and further diluted in water. BSFAB was dissolved in DMSO to make a 100 mM stock solution and diluted with the test buffer (50 mM Tris-HCl containing 154 mM NaCl, pH 8.0, room temperature). The proteases were dissolved in 0.9% NaCl containing 0.1% BSA. For the assay, test buffer or inhibitor solution, respectively (100 μL), was mixed with substrate solution (20 μL) and the reactions were started by adding of enzyme solution (20 μL) (total assay volume 140 μL). Measurements were performed at room temperature for 10 min, the data were recorded every 15 s and the reciprocal velocities (1/v) as function of the inhibitor concentrations at the used substrate concentration, and the third dashed slopes of each time course, and the $K_i$-values were determined from Dixon plots (Fig. 2a and b) [38]. The straight lines in the Dixon plots were obtained by linear regression of the data for the reciprocal velocities (1/v) as function of the inhibitor concentrations at the used substrate concentration, and the third dashed line corresponds to the value of 1/V$_{max}$. The $x$-value at the point of intersection between the three lines provides the value for $K_i$ and is located in the second quadrant of the Dixon plots, which reveals that BSFAB is a competitive reversible inhibitor of plasmin and urokinase.

Effect of BSFAB on keratinocyte expressed plasmin, uPA and transglutaminase 1 in vitro

Cell culture

Normal human keratinocytes (CELLnTEC advanced cell systems AG, Berne, CH) were seeded at subconfluence and grown in 6-well plates for 24 h in Cnt-07 plasmin-free media (CELLnTEC advanced cell systems AG). The cytokines TNFα (10 ng mL$^{-1}$), IL-1β (10 ng mL$^{-1}$; both from Peprotech, London, U.K.) and BSFAB (100 μM: 0.006%) were added simultaneously as a stimulus. All assays were performed after 48 h of incubation.

uPA and plasmin activity assay

Protease activity was quantified using the methods previously described by Voegeli et al. [21, 23, 24]. Cell supernatant (200 μL) was mixed with enzyme substrate (1.25 μL: 31.25 μM: Table I) shaken and incubated for 2 h at 37°C. The reaction was stopped after 2 h using 1% acetic acid (250 μL). The aminomethylcoumarin (AMC) released was quantified by reverse-phase high-performance liquid chromatography (Symmetry C18, 3.5 μm, 4.6 mm × 75 mm; Waters, Milford, MA, US) with gradient elution (80% water/20% acetonitrile 0.07% trifluoroacetic acid (TFA) to 50% water/50% acetonitrile/0.07% TFA) at a flow rate of 1 mL min$^{-1}$. The injection volume was 5 μL and the retention time of AMC was 3.5 min. The wavelengths for excitation and emission were 354 nm and 442 nm.

Transglutaminase 1 gene expression analysis

RNA was harvested and purified using the RNasy Mini Kit (Qiagen, Venlo, NL) according to the manufacturer’s protocol. RNA was rewritten to cDNA with the Superscript First Strand Synthesis Kit (Invitrogen, Waltham, MA US), and real-time PCR was performed on a Taqman 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, MA US) with the following primers and probe:

| Gene ID  | Forward  | Reverse  | Probe             |
|---------|----------|----------|-------------------|
| NM_000359.2 | 5’-TGGGGAAGGACATACCTA-3’ | 5’-GTCGGCTGCTGCTGCTGC-3’ | 5’-CACCCAGAAAGGCTAGACGAG-3’ |

Skin barrier clinical study

Caucasian subjects ($n = 46$; 38.7 ± 6.5 years old; Fitzpatrick skin phototype II–III) with self-assessed sensitive facial skin participated in the study, which took place from mid-February to mid-April 2013 in Lyon, France. Subjects were recruited with self-perceived sensitive skin dry skin and with a TEWL >15 g m$^{-2}$ h$^{-1}$ on their cheeks. The study was a blinded, vehicle-controlled, full face, parallel-grouped trial. The study was authorized from the Agence Régionale de Santé Rhône-Alpes (ARS) and was conducted in accordance with the Declaration of Helsinki Principles. Written, informed consent was obtained from all participants before enrolment. Before the start of the study, volunteers were advised to refrain from any skin manipulations involving shaving and
concentration at the used substrate concentrations. (b) Dixon plot for the competitive reversible inhibition of uPA by BSFAB in the presence of the fluorogenic substrate MeO-Sac-Ala-Phe-Lys-AMC TFA (200 μM). The dashed line represents 1/Vmax. The data on the Y-axis indicate the reciprocal steady-state velocities of each measurement in ‘second/change in relative fluorescence units’, which were obtained as function of the inhibitor concentration at the used substrate concentrations. (b) Dixon plot for the competitive reversible inhibition of plasmin by BSFAB in the presence of the fluorogenic substrate MeO-Sac-Ala-Phe-Lys-AMC TFA (200 μM). The dashed line represents 1/Vmax. The data on the Y-axis indicate the reciprocal steady-state velocities of each measurement in the unit ‘second/change in relative fluorescence units’, which were obtained as function of the inhibitor concentration at the used substrate concentrations.

Table III Placebo and test formulation (hydrogel)

| Ingredients          | Placebo | Active |
|----------------------|---------|--------|
| Carboxol Ul崔re 21   | 0.50%   | 0.50%  |
| BSFAB                | –       | 10 ppm |
| Glycerine 99.5%      | 0.67%   | 0.67%  |
| Optiphen plus        | 1.00%   | 1.00%  |
| Sodium hydroxide 30% | 0.25%   | 0.25%  |
| Water                | 97.38%  | 97.38% |

Table IV Questionnaire with structured scales (ranging from 0 to 10) filled in by the subjects at the beginning and in the end of the 4-week treatment

| Skin condition                   | 0 | 10 |
|----------------------------------|---|----|
| I have great looking skin        | Not at all | Very much |
| My skin is smooth                | Not at all | Very much |
| My skin is rough                 | Not at all | Very much |
| My skin appears silky            | Not at all | Very much |
| I have a comfortable skin feel   | Not at all | Very much |
| My skin is attractive            | Not at all | Very much |

depilation or application of any cosmetics and drugs for 3 days before the start of the study. In the 28-day application phase, the subjects applied the placebo or the test formulation (Table III) twice daily, once in the morning and once in the evening, under normal conditions of use. In the 1-day regression phase, treatment was stopped. During the whole study, the subjects cleansed the face in the morning with tepid water and in the evening with a standardized, mild facial cleanser (Gel Mousse Adoucissant Tolérieane; La Roche-Posay, FR). After cleansing, the face was carefully patted dry and not rubbed with a soft towel. Before conducting the bioinstrumental measurements, the skin was cleaned by gentle swabbing with a cotton pad soaked with distilled water of ambient temperature and allowed to dry for 30 min. Subjects were acclimatized for 30 min before any measurements, and measurements were performed in a room at a temperature of 24 ± 2°C and 35 ± 10% relative humidity. The facial test sites (three cm below outer edge of the eyes) were marked with a surgical marker to ensure that the measurement probe and the tapes were consistently applied to the same area of the subjects faces [39], and the biophysical measurements were performed at the same time each day, to minimize variations induced by the circadian rhythm [40, 41]. TEWL was measured using an Aqualux AF200 (Biox Systems, London, UK) following the published guidelines [42, 43]. Six consecutive standard D-Squame® discs (CuDerm Corporation, Dallas, TX, US) with a diameter of 2.2 cm and an area of 3.8 cm² were placed onto the test sites under 225 g cm⁻² of pressure with a pressure device (CuDerm Corporation, Dallas, TX, US) for 5 s. To minimize variations, the same technician for all volunteers, throughout the study, conducted the procedure. The interval between the tape stripplings was 20 ± 5 s [44, 45]. Basal TEWL was measured at baseline and on day 28. Then, the tape stripplings were taken. To determine the barrier repair and barrier improvement, TEWL (SC integrity) was again measured 24 h after tape stripping (day 1 and 29) [46–48]. SC protein levels on the tape stripplings were estimated using infra-red densitometry as previously described [49].

Before the beginning of the study and after 4 weeks of treatment, the subjects completed a questionnaire with structured scales (ranging from 0 to 10) containing the description of skin conditions shown in Table IV. The results of the questions about skin smoothness, roughness, appearance, feel and attractiveness were summed as a global score for healthy skin.

One subject dropped out from the study on day 7 (vehicle group) due to itching and burning sensations.
Table V  Kᵢ values of BSFAB for serine proteases: urokinase, plasmin, tryp-tase, neutrophil elastase, kallikrein 5 and kallikrein 7

| Protease          | Kᵢ (nM) |
|-------------------|---------|
| Plasmin           | 29      |
| Urokinase         | 25      |
| Trypsinase        | 2010    |
| Neutrophil elastase| No inhibition |
| Kallikrein 5      | 21 000  |
| Kallikrein 7      | 25 000  |

Statistics
All data were collected in Microsoft Excel 2010 and checked for normality using the D'Agostino and Pearson omnibus normality test. In case of normal distribution, the data were analysed with a paired t-test and in case of non-normality with the Wilcoxon paired signed-ranks test.

Results

Synthesis and structural characterization

An 8-step synthetic route was devised to generate crystalline intermediates and a crystalline final product devoid of any impurities which might jeopardize its use in personal care products (Scheme 1). Thus, an efficient and cost-effective process was developed that delivered BSFAB in high purity (97.3%) and high overall yield (39%) without any need for ion-exchange chromatography.

The mass spectrum generated by electrospray ionization in positive mode revealed the molecular ion peak [M+H]+ (free peptide) at m/z = 552.2 (theoretical = 552.2) and a mass at 398.2, which corresponds to the fragment H-D-Ser-homoPhe-(4-amidino-benzyal-mide) lacking the N-terminal benzylsulfonyl group. The mass at 276.7 corresponds to [M-2H]2+/2.

The chemical shifts of 29 protons and 30 carbons detected are in agreement with the proposed structure of BSFAB (Scheme 1). The assignments are corroborated by a set of two-dimensional nuclear magnetic resonance spectroscopy (2D NMR) experiments including correlation spectroscopy (COSY), heteronuclear multiple-bond correlation spectroscopy (HMBC) and heteronuclear single-quantum correlation spectroscopy (HSQC).

Molecular modelling of interaction of BSFAB with plasmin and uPA

BSFAB was docked into the catalytic domains of plasmin (RCSB: 3UIR) and uPA (RCSB: 1V9). The catalytic domains of both enzymes are highly conserved, and in both models, BSFAB binds in a similar way with minor differences that can be accounted by the slight differences in their active sites (Fig. 1).

In both cases, BSFAB adopts a turn-like conformation with its peptide backbone forming a short anti-parallel β-sheet with resi-dues Ser214 and Gly216. The amidine moiety of BSFAB is tightly anchored into the S1 pocket by a salt bridge with the sidechain of Asp189 as well as an H-bond interaction with the carbonyl group of Gly219 and the hydroxyl sidechain of Ser190. Another common interaction in both complexes is the H-bond between the NH of Gly219 and the hydroxyl sidechain of BSFAB sulphonamide group.

The uPA catalytic domain has a specific insertion loop that is absent in plasmin. The D-Ser residue of BSFAB is located next to this loop, and its sidechain hydroxyl forms extra H-bond interactions with the carbonyl oxygen of Leu97B and imidazole side chain of His99. Another small difference is that plasmin forms an H-bond interaction with the sidechain of Asn192 that is rotated away in the uPA structure and thus not observed. This asparagine residue is known to be highly flexible adopting different orientations in different complexes.

BSFAB and enzyme kinetics

BSFAB was found to be an effective and reversible inhibitor of both uPA and plasmin with similar Kᵢ values of 25 nM and 29 nM, respectively (Fig. 2) (Table V). BSFAB possesses a significantly reduced potency against other key serine proteases found in SC. Its Kᵢ for trypptase was approximately 70- to 80-fold reduced and the potency against KLK5 and KLK7 approximately 700- to 1000-fold weaker, and it did not inhibit neutrophil elastase.

Effect of BSFAB on cytokine- and non-cytokine-stimulated keratinocytes

The effect of BSFAB on plasmin and uPA activity in keratinocytes is shown in Fig. 3. Clearly, a significant inhibition of both enzymes was observed in both the non-cytokine (plasmin inhibition 37.7%, P < 0.05, uPA inhibition 96.6%, P < 0.01) and cytokine-induced conditions (plasmin inhibition 41.1%, P < 0.05, uPA inhibition 97.0%, P < 0.001). Plasmin is doubtlessly expressed by keratinocytes as they are grown in plasmin-free media. Additionally, transglutaminase 1 gene expression (approximately 4.5 times expression; P < 0.01) was induced by BSFAB in the cytokine-stimulated keratinocytes (Fig. 4).

Effects of BSFAB on skin barrier function

Clinically, BSFAB improved SC barrier function. Basal TEWL decreased numerically but non-significantly for both vehicle and BSFAB-treated sites at day 28 compared with day 0, and the BSFAB-treated site was numerically superior to the vehicle on day 28. However, there was a significant difference in TEWL between the two products on day 29 (24 h after the tape stripping) with the BSFAB treatment being more resistant to the mechanical challenge induced by tape stripping and allowed a faster epidermal barrier recovery (P < 0.02). 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 (Figs 5 and 6). There were no differences in the amounts of protein removed between the treatments on either challenges indicating subjects also perceived improvements in their skin condition which when summed as a global score of healthy skin (composite of results of the questions about skin smoothness, roughness, appearance, feel and attractiveness) was significantly different for the BSFAB-treated subjects (P < 0.05) (Fig. 7).

Discussion

The role of the plasminogen cascade enzymes in the epidermis has been discussed for 35 years [1–17]. However, it was not until recently that their relevance in the SC was established [18–33]. Elevated activities of plasmin and uPA in tape stripings of SC have been shown in disease states and in dry skin [19–22, 26–28]. Their levels have been profiled particularly on facial skin and have been shown to correlate with TEWL [23, 24]. Increased activities have been associated with a thinner SC [21].
In addition to the potential role of plasmin and uPA influencing SC thickness, the use of inhibitors to these enzymes has also shown their value in reducing the appearance of SLS-induced dry skin, basal TEWL, and skin conductance while normalizing skin turnover rates [26, 29]. Histologically, the distribution of plasminogen has been shown to be present in the basal layer in untreated skin but its distribution was enhanced in all cell layers in dry skin [26, 29].

Kawai et al. [27] performed fibrin autography and demonstrated that a 50 kDa plasminogen activator-like activity was detected in SC of experimental dry skin (2002) suggesting it was uPA (55 kDa) and not t-PA (70 kDa). Measuring uPA activity on tape stripplings of healthy and dry facial SC, they found uPA only in the dry samples. Moreover, these subjects had an increased TEWL.

Katsuta et al. [28] also observed that this activity was abolished by the addition of anti-uPA antibody.

Denda et al. [30] demonstrated that trypsin inhibitors (PMSF, leupeptin, TLCK and t-AMCHA) and especially t-AMCHA accelerated the recovery of epidermal barrier function after a variety of insults to human and murine epidermis (tape stripping, acetone lipid extraction or SLS damage). Yuan et al. [33] observed similar results in SLS-treated skin. Moreover, analogues of t-AMCHA such as aminocaproic acid had no effect and aminobutyric acid actually delayed barrier recovery in tape-stripped murine skin [26, 29]. Additionally, chymotrypsin inhibitors had no effect (chymostatin, TPCK). Furthermore, inhibitors of metalloproteases (EDTA), aspartate proteases (pepstatin) and cysteine proteases (E64) had no

Figure 3  Activity of uPA (a) and plasmin (b) secreted by keratinocytes under non-cytokine-stimulated and cytokine (IL-1β and TNF-α)-stimulated conditions and treatment with BSFAB for 48 h. Pro-inflammatory conditions increased activity of plasmin (a) and urokinase (b). Treatment with BSFAB significantly reduced activities of plasmin and urokinase, data are mean ± SEM, *P < 0.05; **P < 0.01; ***P < 0.001.
effect. Similarly, Egberts et al. [50] reported that cathepsin D and transglutaminase inhibitors delayed barrier recovery (pepstatin and monodansyl cadaverin).

The mechanism behind the reduced epidermal barrier recovery rates from elevated serine proteases includes degradation of the extracellular lipid processing enzymes required for the formation of mature lamellar membranes, reduced lamellar body secretion and activation of the G-protein-coupled protease activated receptor-2 (PAR-2) [31, 51]. More efficacious t-AMCHA derivatives have been identified by Katsuta et al. who focussed on the role of uPA demonstrating that its interaction with the SC led to its activation. Pro-uPA was activated by an insoluble component of SC homogenate (50 mM glycine pH 3.0), and its 33 kDa catalytic domain was shown to be formed. Furthermore, they identified that a methylamide derivative of t-AMCHA prevented that association and was more effective in alleviating disturbances in barrier function [28]. This inhibitory activity was greater for t-AMCHA methylamide than t-AMCHA, and its enhanced effectiveness was demonstrated by accelerated barrier recovery *in vivo*.

Nevertheless, t-AMCHA is an indirect inhibitor of the plasminogen activation system. It does not bind to the active site of plasmin, rather it binds to the kringle domains on plasmin(ogen) and thereby prevents tPA/uPA-induced activation of plasminogen to plasmin [34]. Thus, t-AMCHA cannot inhibit any already activated

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**Figure 4** Effect of BSFAB on stimulation of transglutaminase 1 gene expression in non-cytokine-stimulated and cytokine (IL-1β and TNF-α)-stimulated keratinocytes, data are mean ± SEM. **P < 0.01.

**Figure 5** Basal TEWL data before and after a 28-day treatment with vehicle (grey bars) and BSFAB (black bars) and 24 h after tape stripping challenge. Data are mean ± SEM, *P = 0.02. In the subject group treated with BSFAB, TEWL reached the same value on day 29 as baseline (dotted line).

**Figure 6** ATEWL values between BSFAB- and vehicle-treated group for the four time points. Barrier improvement of basal TEWL induced by the BSFAB corresponds to difference of basal TEWL between day 0 and day 28 (grey arrow). The barrier repair effect induced by the active is considered as difference in TEWL between day 1 and day 28 (black arrow), and the barrier improvement to challenge as differences in TEWL between day 0 and day 1 for the 1-day treatment and between day 28 and day 29 for the 4 weeks treatment (white arrows).
plasmin (and uPA) in the SC. As a result, we have focussed on identifying an efficient competitive inhibitor to both plasmin and urokinase that does not have the disadvantages of t-AMCHA and its derivatives.

The previously published 10-step synthetic route of BSFAB [36] was optimized and improved in order to reach a product of high purity and to reduce the cost for its industrial scale production. The number of synthetic steps was reduced to eight including elimination of the cost-pushing final chromatographic reversed-phase purification.

The binding mode of BSFAB was determined by docking experiments based on previously described X-ray structures of uPA and plasmin. As the catalytic domains are similar, the interaction with BSFAB was also similar but with some subtle differences. Nevertheless, in both complexes, the peptide backbone of BSFAB formed a β-sheet and its amidine moiety anchored into the S1 binding pocket, which result in an efficient enzyme inhibition of both enzymes.

BSFAB was found to be a competitive and reversible inhibitor of plasmin and uPA with $K_i$ values of 29 and 25 nM, respectively, which is similar to those published previously [36]. Moreover, we could demonstrate that BSFAB is a selective inhibitor as it was not an effective one of other key proteases found in the SC such as tryptase or KLK5 and KLK7 and did not inhibit neutrophil elastase in the test conditions used. As the KLKs 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 [52–54]. As a result, we chose to use IL-1β and TNFα to stimulate keratinocytes and evaluate the effect of BSFAB. We established that there was a 97% and 40% reduction in the activities of uPA and plasmin in both unstimulated and stimulated cells using this inhibitor (100 μM). Moreover, this inhibitor reduced the gene and protein expression of IL-8, MMP9 and CXCL5 (not shown). Conversely, t-AMCHA had no effect on diminishing the levels of these biomarkers when used at the same concentration in vitro (data not shown). An important finding from these experiments is that plasmin was secreted by keratinocytes. Others have only speculated on this fact [18].

Decreased transglutaminase activity and increased levels of immature corneocyte envelopes have recently been reported in the SC of subjects with sensitive skin [55]. Our in vitro findings of increased transglutaminase 1 expression with BSFAB may contribute to increases in barrier functionality in vivo by increasing epidermal differentiation as well as through the mechanisms previously discussed for other serine protease inhibitors.

Trypsin-like enzyme inhibitors and especially t-AMCHA have been used to accelerate epidermal barrier recovery following SLS and tape stripping challenges [26–33]. As a result, we chose to test BSFAB for its effects on barrier recovery in subjects with sensitive skin following a tape stripping challenge compared to its vehicle. As was shown after 4 weeks use of BSFAB, the SC was more resistant to tape stripping-induced TEWL compared with the vehicle. We found no differences in SC cohesion following treatment with the two products. In addition, subjects perceived themselves to have generally a better skin condition and when these scores were summed, a healthier skin condition.

In conclusion, we have optimized the synthesis of BSFAB and demonstrated it to be a reversible competitive inhibitor of plasmin and uPA as well as a transglutaminase 1 gene enhancer. Clinically, we have also shown BSFAB to be effective at improving the epidermal barrier recovery and the subjective perception of healthy skin. We believe that BSFAB will be effective in any compromised barrier skin condition as plasmin and uPA activities increase with increasing TEWL, inflammation and photodamage.

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Conflict of interests and disclosures

RV, PW, RC, EJ, DI and MG are employees of DSM. AVR is a consultant to DSM and TS reports no conflict of interests.

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