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Acefylline activates filaggrin deimination by peptidylarginine deiminases in the upper epidermis

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ABSTRACT

Background: Peptidylarginine deiminases (PADs) catalyze deimination (or citrullination), a calcium-dependent post-translational modification involved in several physiological processes and human diseases, such as rheumatoid arthritis and cancer. Deimination of filaggrin (FLG) by PAD1 and PAD3 during the last steps of keratinocyte differentiation is a crucial event for the epidermis function and homeostasis. This allows the complete degradation of FLG, leading to the production of free amino acids and their derivatives that are essential for epidermal photoprotection and moisturizing of the *stratum corneum*.
Objective: To increase the flux of this catabolic pathway, we searched for activators of PADs.
Methods: A large chemical library was screened first *in silico* and then by using an automated assay based on an indirect colorimetric measurement of recombinant human PAD activity. Potential activators were then confirmed using a recombinant human FLG as a substrate, and secondly after topical application at the surface of three-dimensional reconstructed human epidermis.
Results: The data obtained after the library screening pointed to xanthine derivatives as potential PAD activators. Among seven xanthine derivatives tested at 50–300 μM, caffeine, theobromine and acefylline proved to be the most potent enhancers of *in vitro* deimination of FLG by PAD1 and PAD3. After topical application of a gel formulation containing 3% acefylline at the surface of reconstructed epidermis, immunoblotting analysis showed an increase in the total amount of deiminated proteins, and confocal microscopy showed an enhanced deimination in the *stratum corneum*. This demonstrated the activation of PADs in living cells.
Conclusion: As a PAD activator, acefylline will be useful to study the role of deimination and could be proposed to increase or correct the hydration of the cornified layers of the epidermis.

1. Introduction

Deimination (or citrullination) is a calcium-dependent post-translational modification of proteins involved in physiological processes, such as epidermal differentiation, inflammation, regulation of gene expression and also in severe human diseases such as rheumatoid arthritis, neurodegenerative diseases and cancer [1–4]. It corresponds to the transformation by a peptidylarginine deiminase (PAD) of a positively charged arginyl residue into a neutral citrullyl residue. This resulting loss of charge induces conformational changes of the substrates and therefore potentially modifies their interactions, structure and function. Several substrates, especially structural proteins, have already been identified (e.g. the α and β chains of fibrin, myelin basic protein, vimentin, histones H2A, H3 and H4). Five PAD isotypes (PAD1–4 and 6), encoded by five distinct genes clustered at 1p35–36 in the

Abbreviations: FLG, filaggrin; LUT, lookup table; PAD, peptidylarginine deiminase.

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human genome [5], have been characterized. Each displays a specific pattern of tissue expression [5].

In the epidermis, three isoforms of PAD are expressed, namely PAD1–3. Filaggin (FLG) is one of the substrates of PAD1 and PAD3 [6–8]. FLG is a protein essential to epidermal homeostasis. Nonsense mutations of its gene cause *Ichthyosis vulgaris* and are the main genetic risk factor for Atopic dermatitis (atopic eczema). These two frequent human diseases are associated with cutaneous dryness [9–11]. Silencing its expression in reconstructed human epidermis profoundly affects keratinocyte differentiation and the barrier function of the *stratum corneum* [12,13]. Absence of filaggrin in *Flg*$^{-/-}$ newborn mice induces dry scaly skin, and fragility and increased permeability of the*stratum corneum* [14]. At the molecular level, FLG interacts with and facilitates the aggregation of keratin intermediate filaments. It is partly cross-linked to a resistant pericellular structure called the cornified cell envelope. Finally, its complete proteolysis in the outer *stratum corneum* leads to the production of free amino-acids and two of their derivatives, i.e., pyrrolidone carboxylic and trans-urocanic acids, which are crucial for moisturizing the *stratum corneum* and for skin photo-protection, respectively [12,13,15–17]. Deimination of FLG improves its degradation, probably by promoting its detachment from the aggregated keratins and changes in its structure [18,19]. Therefore, in order to control *stratum corneum* moisturization, photo-protection of the skin and epidermal homeostasis through the FLG metabolism, it could be relevant to increase PAD1 and/or PAD3 activity in the epidermis.

In order to identify PAD1 and PAD3 activators, three successive assays were performed. Firstly, a large library of chemical compounds was screened*in silico*. Secondly, the selected candidates were tested using automated PAD activity assays *in vitro*. Thirdly, the best hits were confirmed using an *in vitro* FLG deimination assay. Finally, topical applications of gel formulations on three-dimensional reconstructed human epidermis (RHE) were used to demonstrate that acefylline increased deimination in the *stratum corneum*.

2. Materials and methods

2.1. Material and chemicals

Purified recombinant human PAD1 and PAD3 (active forms without any tag), and purified recombinant human FLG (FLG-His) were produced and purified as previously described [6,19,20]. Dimethyl sulfoxide (DMSO), streptomycin, STI, trypsin and its synthetic substrate BAPNA were purchased from Sigma–Aldrich (L'Isle-d'Abeau Chesnes, France). Trypsin was used in a 100 mM Hepes buffer pH 7.5 (Sigma–Aldrich). The irreversible inhibitor of serine proteases, 4-(2-aminoethyl)-benzene-sulfonyl-fluoride-hydrochloride (AEBSF) was from Fisher Scientific (Illkirch, France). Deimination reactions were performed in conical bottomed 96-well microplates (ABgene, Thermo Fisher Scientific) and final optical densities were measured in clear flat bottomed 96-well microplates (Nunc, Thermo Fisher Scientific). The EpMotion® 5075 TMX automated pipetting system (Eppendorf, France) was used under the control of the Ep Blue ID software (Eppendorf). Caffeine, theobromine, acefylline and its TEA- and Na-salts were obtained from the Pierre Fabre Medicament Library of pure compounds. Acefylline TEA- and Na-salts were synthetized as previously described [21].

2.2. Antibodies

AHF11, a specific murine monoclonal antibody directed to human filaggrin was used as previously described [8]. The anti-modified citrulline (AMC) antibodies were used at 0.08 μg/mL for Western blotting and 0.2 μg/mL for immunohistology, as previously described [22,23]. Negative controls were performed with 3-h incubations at 37 °C with ultra-pure water instead of the chemical modification buffer normally used before AMC detection. The anti-tetra-His (Qiagen, Hilden, Germany) antibody was used at 0.10 μg/mL in Western blot experiments. An anti-actin monoclonal antibody (clone C4) was from Merck Millipore (Darmstadt, Germany). Horseradish peroxidase-labelled goat anti-mouse IgG (H + L) from Bethyl Laboratories Inc. (Montgomery, USA) were used at 1/10,000 for Western blotting analysis with the ECL detection kit from GE Healthcare Europe (Velizy-Villacoublay, France). Horseradish peroxidase-labelled goat anti-rabbit IgG (H + L) from Southern Biotech (Birmingham, USA) was used at 1/10,000 for histochemistry with 3,3′-diaminobenzidine (Dako France, Les Ullis, France) as the chromogenic substrate. The Alexa Fluor® 555 goat anti-rabbit IgG (H + L) from Invitrogen Life Technologies (Saint Aubin, France) was diluted to 1/1000 to perform confocal microscopy.

2.3. Automated indirect screening of PAD modulators

An indirect PAD activity assay (named indirect STI-trypsin assay) was derived from previously published reports [7,8,24,25], miniaturized using conical bottomed 96-well microplates and set up on the EpMotion® 5075 TMX automated pipetting system (Supplementary information Fig. S1). It was based on a two steps procedure. Firstly, PAD1 or PAD3 (40 mU for each test) were incubated for 30 min at 37 °C in 20 μL of deimination buffer (100 mM Hepes buffer pH 7.5, 10 mM $CaCl_2$ and 5 mM DTT) containing the Kunitz soybean trypsin inhibitor (STI) as the substrate and either a selected compound in 1% DMSO or 1% DMSO alone (as the control). The deimination reaction was stopped by the addition of EDTA at 10 mM. Secondly, 2.5 μg of trypsin and 400 μM of its synthetic substrate, Nα-benzoyl-arginine-*p*-nitro-anilide (BAPNA), were added. A second incubation was performed for 30 min at 37 °C. Cleavage of BAPNA produced yellow colored *p*-nitro-aniline. Trypsin activity was stopped by the addition of AEBSF to reach a final concentration of 2 mM. Finally, the amount of produced *p*-nitro-aniline was quantified at 405 nm. Difference in absorbance was related to the effect of the tested compound on PAD activity: increased deimination of STI reduced its inhibitory action on trypsin and therefore induced an enhancement of the absorbance whereas decreased deimination of STI induced a higher trypsin inhibition and therefore a decrease of the absorbance. Each assay was performed in triplicate.

2.4. Screening of PAD modulators with a direct FLG-deimination assay

Modulation of PAD1 and PAD3 activities was measured using purified human recombinant FLG-His as a substrate, as previously described [8], in the presence of 50 and 200 μM of the various compounds, especially xanthine derivatives (Supplementary information Table S1), or with the corresponding vehicle (1% DMSO or ultra-pure water) as a control. Each potent modulator was directly added into the reaction mixture containing 100 ng of Fil-His and 40 mU of PAD1 or PAD3. After incubation at 37 °C, samples were separated by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and immunodetected, as previously described [8], with AHF11 (0.40 μg/mL), AMC and anti-tetra-His antibodies. The intensity of the immunodetection signals was quantified using ImageJ 1.48c (National Institutes of Health).

2.5. Analysis of the effect of a topical application on RHEs of formulations containing xanthine derivatives

RHEs (0.33 cm^2) were produced as previously described [26]. At day 14, they were topically treated for 24 h with 5 mg/cm^2 of