TRPV1 antagonist can suppress the atopic dermatitis-like symptoms by accelerating skin barrier recovery

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ABSTRACT

Background: Transient receptor potential vanilloid type 1 (TRPV1) is a cation channel activated by diverse noxious stimuli like capsaicin, low pH or heat. Recently, it was revealed that TRPV1 might be deeply associated with skin permeability barrier function, suggesting that modulation of TRPV1 might be beneficial for the skin disorders with barrier damages.

Objective: We aimed to investigate whether the blockade of TRPV1 activation might accelerate skin barrier recovery and alleviate atopic dermatitis (AD)-like symptoms, employing a novel TRPV1 antagonist, PAC-14028.

Methods: TRPV1 antagonistic effects of PAC-14028 in human keratinocytes and skin were confirmed through capsaicin-evoked calcium influx assay and capsaicin-induced blood perfusion increase. Effects of PAC-14028 on skin barrier recovery were examined in vivo tape-stripping-induced barrier disruption in hairless mice. To determine the effects of PAC-14028 on AD, Dermatophagoides farina (DF)- and oxazolone (OXZ)-induced AD models were employed.

Results: PAC-14028 could inhibit capsaicin-evoked calcium influx in keratinocytes at sub-micromolar concentrations. This potent TRPV1 antagonistic activity in keratinocytes was manifested in vivo as the blockade of capsaicin-induced blood perfusion increase, and the accelerated barrier recovery from tape-stripping-induced barrier damages in hairless mice. PAC-14028 could also attenuate dermatitis-associated barrier damages in DF and OXZ models as determined by lower TEWL (trans-epidermal water loss), reformation of neutral lipid layer and reversion of changes in loricrin and filaggrin expression. Importantly, along with accelerated recovery of skin barrier function, PAC-14028 alleviated the general AD-like symptoms, including serum IgE increase, mast cell degranulation, scratching behavior and clinical severity of dermatitis.

Conclusions: These results reflect that the blockade of TRPV1 activation can suppress the atopic dermatitis-like symptoms by accelerating skin barrier recovery.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by pruritic and eczematous skin lesions with erythema, excoriation, erosions, lichenification and dryness, frequently accompanied by increased serum immunoglobulin E (IgE) levels [1,2]. AD is a disease arising from the complex interactions between multiple factors, like genetic background, immunologic abnormalities and exposure to environmental sensitizers or allergens. Among these, the dysfunction and breakdown of skin barrier is suggested as an important contributor to the development of AD [3,4]. The patients with AD generally exhibit xerotic skin resulting from impaired epidermal barrier [5] and the impaired skin barrier function allows the facile penetration of allergens and subsequently stronger sensitization responses [6–11], indicating that skin barrier dysfunction plays a critical role in the aggravation and the flares of AD [12–14].

While topical and oral corticosteroid therapies are being frequently used to control the exacerbation of AD [15], they do not restore the structure of the lamellar body and lipid bilayer in the lower stratum corneum, which constitute the epidermal barrier function. What is worse, corticosteroids frequently accompany immune-suppression [16] and consequently, prolonged use of high dose corticosteroids is often associated with a variety of side-effects including skin atrophy, increased infection
and paradoxical barrier damages [17]. For this reason, novel anti-AD therapies, which can prevent the development of AD without major side effects or barrier damages, are being continuously and actively sought for.

Transient receptor potential vanilloid type 1 (TRPV1) was highly expressed in epidermal keratinocyte as well as in the nerve fibers distributed in epidermis and dermis [18–21]. It is a non-selective cation channel activated by heat, acid (low pH), capsaicin and endogenous inflammatory mediators [22,23]. Several studies have suggested a potential therapeutic utility of TRPV1 antagonists in somatic pain, migraine, respiratory disease, bladder and gut related pain [24,25]. Recently, Denda et al. [18] reported that the application of a TRPV1 antagonist, capsazepine could accelerate the recovery from barrier damages, indicating that TRPV1 activation in epidermal keratinocytes might be closely linked with skin barrier disruption. However, the effect of TRPV1 antagonists on AD, a representative skin disorder with severe barrier disruption has never been examined to our best knowledge.

In this study, we aimed to investigate whether TRPV1 antagonists might be beneficial for AD especially in terms of barrier damages using Dermatophagoides farina (Df)- and oxazolone (OXZ)-induced AD models in vivo. We employed a novel, orally active and potent TRPV1 antagonist, PAC-14028 ((E)-N-[(R)-1-(3,5-Difluoro-4-methanesulfonylamino-phenyl)-ethyl]-3-(2-propyl-6-trifluoromethyl-pyridine-3-yl)-acrylamide) (Fig. 1a) instead of the classical TRPV1 antagonist, capsazepine which is a rather weak TRPV1 antagonist (IC50 > 500 nM) with diverse nonspecific effects and was known to be orally incompatible [26,27].

2. Materials and methods

2.1. Animals and materials

Female hairless mice (HR-1, 7 weeks, Charles River, Yokohama, Japan) and male NC/Nga mice (8 weeks, SLC, Hamamatsu, Japan) were kept under controlled environmental conditions (23 ± 3 °C, 40–60% relative humidity, 12/12 h dark-light cycle) with ad libitum access to laboratory diets (Purina, Seoul, Korea) and tap water in the specific pathogen-free facility (Amorepacific R&D Center, Yongin, Korea). All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Amorepacific R&D Center. OXZ (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one), capsaicin and A23187 were purchased from Sigma–Aldrich (St. Louis, MO, USA). OXZ was dissolved in ethanol. Df extract (Biosir AD) was purchased from Biosir Inc. (Hiroshima, Japan). The TRPV1 antagonist, PAC-14028 was synthesized by the drug discovery team, AmorePacific Corporation R&D Center. PAC-14028 was dispersed in 1% methyl cellulose containing 0.5% Tween 80 for oral administration.

2.2. Measurement of calcium influx in primary culture of neonatal rat dorsal root ganglia (DRG) neurons and normal human epidermal keratinocytes (NHEK)

DRG neurons from neonatal Sprague–Dawley (SD) rats were cultured in their appropriate medium according to the method as previously described [28]. For the experiment, 10 μl aliquots of sample solution, the combinations of capsaicin and 10 μCi/ml 45Ca (Perkin Elmer, Boston, MA, USA) in the presence or absence of PAC-14028, were added to each well and then cells were incubated for 10 min at room temperature. After the cells were washed, 0.3% sodium dodecyl sulfate (SDS) (10 μl) was added to each well to extract the 45Ca and counted by liquid scintillation counter, Microbeta (Wallac, Turku, Finland).

NHEK (LONZA, Basel, Switzerland) were grown in keratinocyte growth medium (LONZA), supplemented with SingleQuots (LONZA) containing bovine pituitary extract, hEGF, insulin, hydrocortisone, gentamycin/amphotericin-B. We measured calcium influx in NHEK based on the method described by Li et al. [29]. In brief, NHEK were grown in the 8 well chambered coverglass (NUNC, Roskilde, Denmark) overnight and loaded with 4 μM Fluo-
4 AM (Molecular Probes, Eugene, OR, USA) at room temperature for 45 min. PAC-14028 (0.25 μM) and capsaicin (final 100 μM) were prepared in Tyrode’s buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM glucose and 10 mM HEPES, pH 7.2). NHEK were incubated with PAC-14028 at room temperature for 40 min, and then coverglass was mounted on Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) and controlled by SCAN Ware 5.10 software (Carl Zeiss). After baseline fluorescence ($F_0$) was measured for 20 s under confocal laser-scanning microscope, capsaicin was added, followed by measuring post-baseline fluorescence ($F_1$) for 3 min. The change in fluorescence ($F_1/F_0$) was used to evaluate calcium influx. Calcium ionophore A23187 (final 5 μM) was also investigated in place of capsaicin in the same way.

2.3. Capsaicin-enhanced microcirculation test in SD rat

To indirectly assess the capsaicin-induced erythema and vascular permeability, we obtained an image of the spatial skin blood perfusion after capsaicin treatment. After PAC-14028 (30 mg/kg) or vehicle was orally administered daily for 2 weeks, the hair on the dorsal region of SD rats anesthetized with pentobarbital sodium (Sigma–Aldrich) was shaved on a day before microcirculation test. On the day of measurement, 200 μl of 0.075% capsaicin cream (Hi-Tech Pharmacal Inc., Amityville, NY, USA) was applied on a 2 cm × 2 cm area on the dorsal region for 30 min and washed away. The cutaneous microcirculation of individual test sites was measured using a Laser Doppler Perfusion Imager (PIM3.0, Perimed AB, Stockholm, Sweden).

2.4. Skin barrier disruption by tape-stripping in hairless mice in vivo

PAC-14028 (10, 30 mg/kg) was orally administered daily from 2 weeks before tape-stripping. The flank region of mice was undergone tape-stripping using OPP tape (TOP-300, Tapex, Incheon, Korea). And, barrier function was evaluated by measuring transepidermal water loss (TEWL) using a vapometer SWL4102 (Delfin Technologies, Kuopio, Finland) at 1, 2, 3, 5, 24 and 30 h after tape-stripping. The percentage of barrier recovery was calculated using the following formula: [1 – (TEWL at indicated time – average TEWL of normal group at indicated time)/(TEWL immediately after tape-stripping – average TEWL of normal group at indicated time)] × 100. After sacrifice, dorsal cutaneous tissues were embedded in OCT compound and stained with Nile red (Sigma–Aldrich) and examined under fluorescence microscopy (Axiovert200, Carl Zeiss).

2.5. Df-induced AD model in NC/Nga mice

According to the previously described method [30] with minor modifications, AD-like symptoms were elicited with topical application of 100 mg Df extract on the shaved dorsal skin and both surfaces of each ear twice a week for 3 weeks. PAC-14028 (10, 30 mg/kg po) was administered daily during this period. The scratching behavior of NC/Nga mice was measured using an apparatus, MicroAct (Neuroscience, Tokyo, Japan). After mice were acclimatized for 30 min in an observation chamber (11 cm in diameter, 18 cm high), the number of scratching movements was measured for 4 h. After sacrifice, serum was collected for determination of the concentrations of total IgE using an apparatus, MicroAct (Neuroscience, Tokyo, Japan). After mice were acclimatized for 30 min in an observation chamber (11 cm in diameter, 18 cm high), the number of scratching movements was measured for 4 h. After sacrifice, serum was collected for determination of the concentrations of total IgE using an apparatus, MicroAct (Neuroscience, Tokyo, Japan). After mice were acclimatized for 30 min in an observation chamber (11 cm in diameter, 18 cm high), the number of scratching movements was measured for 4 h. After sacrifice, serum was collected for determination of the concentrations of total IgE using an apparatus, MicroAct (Neuroscience, Tokyo, Japan). After mice were acclimatized for 30 min in an observation chamber (11 cm in diameter, 18 cm high), the number of scratching movements was measured for 4 h.
Capsaicin-induced erythema and vascular permeability is a good surrogate and noninvasive marker for evaluating the effects of TRPV1 activation in the skin [33]. Therefore, we measured the skin blood perfusion increase from capsaicin-induced erythema using Laser Doppler Imager in rats after oral administration of PAC-14028 to determine the antagonistic activity of PAC-14028 against TRPV1 in vivo. Firstly, we confirmed that sufficient amount of PAC-14028 reaches to skin \( (1.4 \pm 0.2 \mu g/g \text{ tissue, data not shown}) \) after repeated oral administration for 2 weeks. As shown in Fig. 2, the blood flow increased by topical application of capsaicin cream \( (0.075\%) \) was significantly reversed in PAC-14028 treated group \( (30 \text{ mg/kg oral once daily for 2 weeks}) \) in comparison to vehicle group, indicating that PAC-14028 has a TRPV1 antagonistic activity in vivo.

3.2. Effects of TRPV1 antagonist, PAC-14028 on the recovery from tape-stripping-induced skin barrier disruption in hairless mice

Aberrant intracellular calcium increase in keratinocytes can delay the recovery from skin barrier damages by impairing lamellar body exocytosis and neutral lipid formation in the upper epidermis [34]. To examine if PAC-14028 could affect skin barrier function, skin barrier was disrupted by repetitive tape-stripping in hairless mice with or without oral administration of PAC-14028 and the recovery of barrier function was evaluated by examining neutral lipid formation and TEWL changes. As a result, while the loss of neutral lipids in the upper epidermis were not recovered in the skin of vehicle control group at 7 h after tape-stripping, apparent recovery of intercellular lipids in the upper epidermis was observed in PAC-14028 treated group \( (\text{Fig. 3a}) \). Consistently with this, while increased TEWL by tape-stripping returned to the basal level in all the groups, the speed of recovery was significantly higher in the mice treated orally with PAC-14028 \( (\text{Fig. 3b}) \).

3.3. Effects of TRPV1 antagonist, PAC-14028 on the skin barrier damages in murine AD models

AD is the representative skin disorder with severe skin barrier damages [32]. To investigate if the barrier recovering effects of PAC-14028 described above, might also attenuate the barrier damages associated with AD, we employed in vivo Df-induced AD

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Fig. 2. Effects of PAC-14028 on capsaicin-enhanced skin blood perfusion. Effects of oral administration of PAC-14028 \( (30 \text{ mg/kg, once daily for 2 weeks}) \) on skin blood perfusion (flare) evoked by topical application of capsaicin were measured with Laser Doppler Imager. The data are presented as means ± SE \( (n = 3) \). *, Significantly different from vehicle group \( (p < 0.05) \).

Fig. 3. Effects of PAC-14028 on the recovery of skin barrier function after tape-stripping. (a) Representative results of Nile Red staining of mouse dorsal skin \( (\text{bar} = 34 \mu \text{m}) \). The neutral lipids were shown in the upper epidermis (arrows). (b) TEWL. The data are presented as means ± SE of nine to ten animals. **, Significantly different from Tape-stripping group \( (p < 0.01) \). *, Significantly different from Tape-stripping group \( (p < 0.05) \). Naive, Negative control group; TS + Veh, tape-stripping + vehicle; TS + 14028, tape-stripping + PAC-14028.
model in NC/Nga mice and OXZ-induced AD model in hairless mice. As a result, the treatment of PAC-14028 indeed could significantly recover the loss of intercellular lipids in the upper epidermis of mice in Df-induced AD model (Fig. 4a). Consistently with this, PAC-14028 significantly and dose-dependently suppressed TEWL increase in OXZ model (Fig. 4b), indicating that PAC-14028 could alleviate the skin barrier damages indeed. These barrier protective effects of PAC-14028 in AD-like skin were further evidenced by the reversal of AD-associated expression changes in keratinocyte differentiation markers, loricrin and filaggrin. Loricrin was expressed as a thin band in stratum corneum in control group (Fig. 4c, upper panel). In contrast, in Df-challenged mice, the intensity of loricrin expression in stratum corneum was significantly reduced although the expressed region of loricrin extended to the whole layer of stratum granulosum in line with epidermal thickening. The treatment of PAC-14028 resulted in the retrogression of loricrin expression toward normal skin where it was contained in the upper layer of the stratum granulosum. Meanwhile, in normal skin, filaggrin was expressed in the junction between stratum corneum and stratum granulosum whereas it was extended to supraspinosum layer of epidermis in Df group (Fig. 4c, lower panel). In PAC-14028 treated group, immunoreactivity of filaggrin was retracted back to stratum granulosum of epidermis. The patterns of loricrin and filaggrin expression in OXZ-induced AD model were similar to those observed in Df-induced AD-like skin lesion. Loricrin extended to granular layer in OXZ-treated group, whereas the distribution of it was contained in the upper granular layer in PAC-14028 treated group. In addition, strong and condensed immunoreactivity of filaggrin was detected in the epidermis of PAC-14028 treated group compared with OXZ group.

3.4. Effects of TRPV1 antagonist, PAC-14028 on the representative symptoms and markers of AD

Skin barrier protection might attenuate the disease severity of AD by cutting off the vicious circle of barrier dysfunction, facile penetration of allergen or hapten, hyper-allergic reaction and barrier dysfunction again [6]. To investigate if PAC-14028 treatment could improve general AD-like symptoms, serum IgE increase, mast cell degranulation and pruritis, the hallmarks of AD, were assessed. As a result, serum IgE increase was significantly attenuated by PAC-14028 in a dose-dependent manner in both Df and OXZ models (Fig. 5a). The increased degranulation of mast cells in AD-like skin lesions was also significantly reduced by PAC-14028 treatment (Fig. 5b). Particularly, the treatment of PAC-14028 significantly suppressed the scratching behavior in Df model (Fig. 6a). Consistently with these results, severe erythema, edema, scaling and excoriation observed in the Df applied skin were significantly alleviated by the treatment of PAC-14028 (Fig. 6b and c). Histological examination also demonstrated that...
PAC-14028 significantly attenuated epidermal hyperplasia and dermal inflammation induced by Df challenges (Fig. 6d). In addition, PAC-14028 dose-dependently and significantly reduced the AD-like symptoms, including edema, scaling and excoriation in OXZ model (Fig. 7a and b). Histological observation also revealed that PAC-14028 improved epidermal hyperplasia and attenuated the infiltration of inflammatory cells induced by OXZ challenge. Furthermore, epidermal structure was recovered without crust formation and corneal layer were almost fully restored by PAC-14028 treatment (Fig. 7c).

4. Discussion

In the present study, we demonstrated that the blockade of TRPV1 activation by the TRPV1 antagonist, PAC-14028 could accelerate the recovery from skin barrier damages and suppress...
the development of AD-like symptoms such as serum IgE increase, mast cell degranulation, and skin inflammation, providing an important line of evidence for the role of TRPV1 activation in AD-associated skin barrier damages and for the potential therapeutic utility of TRPV1 antagonists in the management of AD.

TRPV1, an ionotropic receptor with high calcium ion permeability, is widely and abundantly expressed in epidermal keratinocytes [35–37]. Denda et al. [18] demonstrated that the activation of TRPV1 could delay recovery from barrier damages while the topical application of TRPV1 antagonist, capsazepine could accelerate it. This effect was suggested to be from the increased calcium influx in keratinocytes through TRPV1 activation and the subsequent perturbation of epidermal barrier maturation [18]. Influx of calcium ions into epidermal keratinocytes perturbs lamellar body exocytosis and consequently delays the recovery from barrier disruption [34]. In agreement with this, our results confirmed that TRPV1 antagonist, PAC-14028 could block calcium influx into keratinocytes (Fig. 2) and accelerate the formation of intercellular lipids in the upper epidermis indeed, which is known to be closely linked with the normalization of lamellar body exocytosis, providing an important clue for the mechanism underlying the skin barrier protective effect of TRPV1 antagonist.

Although previous studies have frequently employed the classical TRPV1 antagonist, capsazepine, it is a rather weak antagonist with micromolar IC50 values. In addition, capsazepine has non-selective effects on other endogenous targets such as voltage-activated calcium channel [26] or acetylcholine receptor [27], which could not give a clear picture for the role of TRPV1 activation in AD. On the contrary, PAC-14028 was determined to be highly selective to TRPV1 through the 68 binding assays where nonspecific activities stronger than 50% of inhibition were not observed at 10 μM (MDS Pharma Services, King of Prussia, PA, USA, data not shown).

Furthermore, PAC-14028 (10 μM) did not inhibit or activate other TRP channels such as hTRPV2, hTRPV3, hTRPM8 and hTRPA1 but potently inhibited hTRPV1 (data not shown), indicating that PAC-14028 could provide with a clearer picture for the role of TRPV1 activation in AD than capsazepine.

Skin barrier dysfunction was manifested as increased TEWL and disrupted neutral lipid layers in OXZ-induced AD-like lesions in hairless mice and Df-induced AD-like lesions in NC/Nga mice, respectively. Especially, Df and OXZ could inhibit and alter the expression of keratinocyte differentiation markers, loricrin and filaggrin, important contributors to epidermal barrier function [38,39], resulting in further exacerbation of barrier damages. Meanwhile, the treatment of PAC-14028 significantly inhibited or reversed these parameters of skin barrier damages. Since barrier damages, including defective barrier protein expression, allow facile penetration of allergens or haptons into the skin, barrier disruption is regarded not only as an important pathological feature but also as a key element in the etiology of AD [3,4,40,41]. Therefore, we believe that the protective effects of TRPV1 antagonist PAC-14028 on skin barrier function might have contributed to the improvement of AD-like symptoms.

In the two representative AD models, Df and OXZ models [42], AD-like symptoms such as epidermal hyperplasia and dermal infiltration of inflammatory cells were significantly attenuated by treatment of TRPV1 antagonist, PAC-14028. In addition, increases in serum IgE and the degranulation of mast cells were also suppressed (Fig. 5a and b). While protective effects of PAC-14028 against AD-like symptoms are thought to be from the skin barrier recovery and subsequent inhibition of interaction of antigens with the antigen-presenting cells, participation of other positive effects of TRPV1 blockade on AD through the mediation of TRPV1 expressed in other tissues like nerve fibers might not be ruled out. Suppression of TRPV1 activation in nerve fibers may attenuate neuropeptide release, contributing to the alleviation of dermal inflammation and
neurogenic activation. Interestingly, we could observe that the treatment of PAC-14028 significantly suppressed the scratching behavior in Df-induced AD-like skin model. Scratching can induce the mechanical skin barrier damages and further aggravate skin inflammation [43]. Incidentally, TRPV1 is being suggested as a major integrator of itch signal in sensory neurons [44,45], reflecting that anti-pruritic effect of PAC-14028 might have contributed to the improvement of AD-like symptoms although further studies are required to clarify it.

The barrier damages can increase the immune reactions and exacerbate AD symptoms further. Increased production of inflammatory mediators from dermalitis can impair barrier recovery [46]. This vicious circle could be stopped by the recovery of barrier damages as well as by the modulation of immune reaction. While the immune reaction has been a major focus of conventional AD therapies, barrier recovery has been rarely targeted therapeutically. In this regard, we believe that TRPV1 antagonists could be a novel anti-AD drug, satisfying the unmet medical needs that could not be fulfilled by topical steroids or calcineurin inhibitors.

Acknowledgement

This work was supported by a grant from the Ministry of Knowledge Economy (Bio-Star, 10031636).

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