Anti-Pruritic Effect of the Topical Phosphodiesterase 4 Inhibitor E6005 Ameliorates Skin Lesions in a Mouse Atopic Dermatitis Model

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Abstract

Phosphodiesterase (PDE) 4 inhibition is a well known anti-inflammatory mechanism, but the development of PDE4 inhibitors has been hampered by side effects such as nausea and emesis. Local delivery of a PDE4 inhibitor to the site of inflammation may overcome these issues. The purpose of this study was to assess the therapeutic potential of E6005, a novel PDE4 inhibitor developed as a topical agent for atopic dermatitis (AD). E6005 potently and selectively inhibited human PDE4 activity with an IC$_{50}$ of 2.8 nM, and suppressed the production of various cytokines from human lymphocytes and monocytes with IC$_{50}$ values ranging from 0.49 to 3.1 nM. In mice models, the topical application of E6005 produced an immediate anti-pruritic effect as well as an anti-inflammatory effect with reduced expression of cytokines/adhesion molecules. Based on these observed effects, topical E6005 ameliorated the appearance of atopic dermatitis-like skin lesions in two types of AD models, hapten- and mite-elicited models, exhibiting inhibitory effects comparable to that of tacrolimus. The use of $^{14}$C-labeled E6005 showed rapid clearance from the blood and low distribution to the brain, contributing to the low emetic potential of this compound. These results suggest that E6005 may be a promising novel therapeutic agent with anti-pruritic activity for the treatment of AD.
Introduction

Atopic dermatitis (AD) is a major pruritic inflammatory skin disease. Although its pathogenesis remains unclear, both genetic and environmental factors are thought to play critical roles in the onset of AD (Leung et al., 2004). The resulting atopic inflammation causes severe itching of the affected skin, which in turn amplifies the inflammation by eliciting scratching behavior. Within the inflamed skin, distinct subsets of leukocytes and keratinocytes are activated by immunologic and non-immunologic stimulation to release inflammatory mediators that orchestrate atopic skin inflammation (Novak and Bieber, 2003; Homey et al., 2006). The generation of a vicious itching/scratching cycle eventually leads to chronic skin lesions (Wahlgren, 1999).

Itching is not only a major complaint but is also an important pathogenic factor for AD. Management of the itching response, therefore, is as important as managing inflammation when treating AD. The itching associated with AD does not respond well to therapy with antihistamines. Additionally, topical corticosteroids do not have an immediate antipruritic effect and their use is associated with a high frequency of side effects such as skin atrophy (Draelos et al., 2008). Therefore, there is an existing need to develop safe alternative agents with immediate antipruritic effects for AD therapy.
Phosphodiesterases (PDEs) are cyclic nucleotide-degrading enzymes, and 11 families of PDEs have been identified in mammals. The PDE4 isozyme is expressed in a variety of inflammatory cells including lymphoid and myeloid cells, and it catalyzes the conversion of cyclic AMP to 5′-AMP. PDE4 plays a critical role in the pathogenesis of inflammatory disorders (Manning et al., 1999; Abrahamsen et al., 2004; Jin et al., 2005), and its activity increases during abnormal immune reactions, such as increased cytokine production (Grewe et al., 1982; Sawai et al., 1995). AD appears to be associated with the dysregulation of PDE activity in inflammatory cells (Hanifin et al., 1996).

Several PDE4 inhibitors have been developed for the treatment of chronic inflammatory disorders, but most have been discontinued due to systemic side effects such as nausea and emesis. In order to minimize systemic exposure to these inhibitors, topical delivery methods such as inhalation and dermal applications have been explored (Pagès et al., 2009). Although topical application of the PDE4 inhibitor atizoram has demonstrated efficacy in its clinical evaluation as an AD treatment (Hanifin et al., 1996), there has been no further investigation into these findings. Because systemic exposure of absorbed drugs from the application site could cause undesirable effects, a PDE4 inhibitor compound with low transdermal bioavailability is preferable.
In our efforts to achieve a higher therapeutic index, we have identified a potent, topically active PDE4 inhibitor that appears to produce only minimal systemic side effects. In this report, we describe and characterize a novel PDE4 inhibitor, E6005, which was developed as a potent, selective, and less emetic compound for the topical treatment of AD. E6005 exhibited a broad spectrum of cytokine inhibition, and inhibited the aggravation of AD-like skin lesions through both antipruritic and anti-inflammatory mechanisms. The immediate antipruritic effect of E6005 may represent a novel activity of PDE4 inhibitors, and its rapid elimination from the body and low distribution to the brain could also reduce negative side effects. In summary, these results suggest that E6005 has significant potential as a topical agent for the treatment of AD.
Materials and Methods

Reagents

E6005, cilomilast, and roflumilast were synthesized at Eisai Laboratories. The chemical structure of E6005 is shown in Figure 1. FK506 (tacrolimus) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Forskolin, betamethasone, and oxazolone were purchased from Sigma-Aldrich (St. Louis, MO, USA), and *Dermatophagoides farina* body (Dfb) ointment (Biostir-AD) was purchased from Biostir Inc. (Kobe, Japan). Vaseline–based E6005 ointment was prepared in our laboratory.

Animals

NC/Nga mice (female, more than 6 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan) or Charles River Japan (Kanagawa, Japan). BALB/c (male, 6–8 weeks old), ICR mice (female, 6–8 weeks old), and Sprague-Dawley rats (male, 8 weeks old) were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). All animals were housed under controlled temperature (23 ± 3 °C), humidity (55% ± 15%), and lighting (lights on from 7:00 to 19:00) conditions, and were given a commercially available diet and water *ad libitum*. All procedures were performed at the animal facility accredited by the Center for Accreditation of Laboratory Animal Care and Use, Japan Health Sciences.
Foundation. All protocols were approved by the Institutional Animal Care and Use Committee and carried out according to Eisai Animal Experimentation Regulations.

**Phosphodiesterase assay**

Phosphodiesterase (PDE) isoenzyme activities were quantified by measuring the formation of [³H] 5′-AMP or [³H] 5′-GMP from [³H] cAMP or [³H] cGMP, using an enzyme isolated from bovine brain (for PDE1), differentiated U-937 cells (for PDE2), human platelets (for PDE3 and PDE5), and U-937 cells (for PDE4), as described by Weishaar et al. (1986) and Torphy et al. (1992). The test compounds, reference compounds, or vehicle (water) was added to a buffer containing 40 mM Tris/HCl (pH 7.8), 3 mM MgCl₂, 1 mM DTT, 0.01% BSA, 200 mM NH₄Cl, 1 μM cAMP or cGMP, and 0.1 μCi [³H] cAMP or [³H] cGMP. The reaction was initialized by addition of the enzyme, and the mixture was incubated for 60 min at 22 °C. Following incubation, the reaction was stopped by heating the plate to 60 °C for 3 min, after which time SPA beads were added. After 20 min of shaking at 22 °C, the amount of [³H] 5′-AMP or [³H] 5′-GMP was quantified with a scintillation counter (Topcount, Packard). The results are expressed as percent inhibition of the control enzyme activity.

**Cytokine assay in human PBMCs**
PBMCs were isolated from the heparinized blood of healthy volunteers by means of density centrifugation using Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden). Cells were stimulated with Dynabeads® CD3/CD28 T-cell Expander (Dynal Biotech, Lake Success, NY) or PHA-P (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 3 days, or LPS for 1 day. Test compounds were dissolved in DMSO, diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μL/mL streptomycin, and 50 μM 2-mercaptoethanol, and then added to wells at a range of indicated concentrations, 30 min prior to stimulation. Cytokine production was determined by a Bio-Plex Human cytokine Th1/Th2 assay (Bio-Rad Laboratories, Hercules, CA, USA). The assay was performed in triplicate using PBMCs from 3 different donors.

**Skin inflammation model**

BALB/c mice were sensitized by painting 0.3% oxazolone solution dissolved in acetone onto both ears on day 0. On days 5, 8, and 12 the right ear of each animal was challenged with 0.1% oxazolone solution to elicit dermatitis. Test compounds were topically applied daily to the ear (i.e., 5 times per week) from days 5 to 15, except for the challenge day, when animals were treated twice at 4 h before and after the challenge. Twenty-four hours after each challenge, the ear thickness was measured with a dial thickness gauge (Ozaki
Mfg. Co., Kanagawa, Japan) as an indicator of swelling and expressed as $\Delta$ thickness (oxazolone challenged right ear – vehicle challenged left ear). In addition, mRNA expression in homogenates from inflamed skin was measured using mRNA expression analysis.

**mRNA expression analysis**

Total RNA was extracted using the RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer’s protocol. Complementary DNA was synthesized using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Hammonton, NJ), and polymerase chain reaction products were detected in real time using the ABI Prism 7900 Sequence Detector (Applied Biosystems) with the following probes: 18S ribosomal RNA (cat no. 4333760F), IL-1$\beta$ (cat no. Mm00434228), IL-4 (cat no. Mm00445260), VCAM-1 (cat no. Mm00449197), and ICAM-1 (cat no. Mm00516024). Relative expression levels of target genes were normalized to that of 18S ribosomal RNA and then determined using the $2^{-\Delta\Delta CT}$ method. Data were expressed as fold increase over normal skin.

**Pruritus model**

NC/Nga mice were sensitized by painting 0.5% oxazolone (dissolved in acetone) onto both ears on day 0. On days 4 and 7, the left ear was challenged with 0.3% oxazolone (dissolved
in acetone). On day 10, 10 μL of the test compound was applied to the left ear of each animal, which was followed by a re-challenge with either 0.3% oxazolone solution or acetone vehicle. Scratching behavior was measured for the next 2 h using the MicroAct system (Neuroscience Inc., Tokyo, Japan) (Inagaki et al., 2002).

**Oxazolone-induced dermatitis model**

NC/Nga mice were sensitized by painting 0.3% oxazolone (dissolved in acetone) onto both ears or rostral back on day 0, and were challenged on the left ear with 0.3% oxazolone solution on days 5, 8, 12, and 15. Test compounds were administrated in single daily doses from day 12 to day 18. The severity of dermatitis was evaluated as the sum of individual skin symptom score graded as follows: 0 (none), 1 (slight), 2 (moderate), and 3 (severe). The detail skin symptoms are shown in figure legend. The dermatitis score was recorded from day 12 to day 18 before application of the test compound. The efficacy of the compound was evaluated on day 19 in a blinded manner, where the researcher was unaware of which animals received which type of treatment.

**Mite-induced dermatitis model**

Dermatitis was induced in NC/Nga mice as described (Gao XK., 2004). Briefly, on days 0, 3, 7, 10, 14, and 17, 50 mg of Dfb ointment was applied on the shaved rostral back skin, 1 h
after the barrier disruption, by applying 150 μL of 4% SDS solution. Treatment with 50 μL of vehicle or E6005 solution applied from day 14 to day 21. Each compound was dissolved in a 1:1 acetone/ethanol solution. The severity of dermatitis was evaluated as the sum of individual skin symptom score graded as follows: 0 (none), 1 (slight), 2 (moderate), and 3 (severe). The detail skin symptoms are shown in figure legend. The dermatitis scores were recorded on days 14, 17, and 21, prior to application of the drug solution. The efficacy of the compound was evaluated on day 21 in a blinded manner, where the researcher was unaware of which animals received which type of treatment.

**Histological evaluation**

At the end of the experiment, skin tissue was collected, fixed with 10% neutral formalin, embedded in paraffin, and sectioned into 40-μm slices. Sections were stained with hematoxylin-eosin. Epidermal thickness was determined by measuring 20 spots in each section.

**Shortening effect on the duration of xylazine/ketamine-induced anesthesia**

ICR mice were anesthetized by a single intramuscular injection of xylazine/ketamine. After 15 min, E6005, cilomilast, or vehicle was subcutaneously (10 μL/g) administered to the mice, which were placed in a dorsal recumbent position. The measurement of duration was
initiated 15 min after administration of the test compounds or vehicle. The restoration of the righting reflex was used as an endpoint to determine the duration of anesthesia. The time period from 15 min after administration of the test compounds or vehicle, until return of the righting reflex, was recorded as the duration of anesthesia.

**Animal study using $^{14}$C-E6005**

Rats were administered 14C-E6005 intravenously or transdermally. For transdermal administration, hair was removed from the dorsal regions. The damaged skin was prepared by stripping with twenty applications of mending tape. Doses for intravenous administration and dermal administration were 1 mg·2 mL$^{-1}$·kg$^{-1}$ (6.54 MBq/kg) and 60 mg of 0.2 w/w% of dermal formulation per 6 cm$^{2}$ area of each rat (0.785 MBq/body), respectively. After each administration, blood was withdrawn serially from the tail vein of the animals at the indicated times to measure the blood concentration of radioactivity. Metabolites were identified on the basis of the retention time of HPLC and the mass number corresponding to E6005 or each metabolite. In addition, the tissues were excised from euthanized animals at the indicated time points to analyze tissue distribution of radioactivity, with radioactivity measured by a liquid scintillation counter. Pharmacokinetic parameters of radioactivity in the blood were calculated using the non-compartment model.
Statistical analysis

IC$_{50}$ values were determined by non-linear regression analysis of the inhibition curves using the Hill Software (Cerep, Redmond, WA) or GraphPad Prism v4.00. The Student’s $t$-test was used to analyze differences between two groups, and the Dunnett test was used for multiple comparisons among treatment groups. $P$ values $<$0.05 were considered statistically significant.
Results

**E6005 inhibits cytokine production in lymphocytes and monocytes**

Chemical structure of E6005 is shown in Figure 1. E6005 inhibited human PDE4 enzymatic activity with a half-maximal inhibitory concentration (IC$_{50}$) of 2.8 nM, which was more potent than its inhibition of the isozymes PDE1, PDE2, PDE3, and PDE5. Cilomilast, a second-generation PDE4 inhibitor, also selectively inhibited PDE4 with an IC$_{50}$ of 24 nM (Table 1, Supplemental Figure 1). In a cell-based assay in which PDE4 activity plays an important role, E6005 strongly inhibited the production of various cytokines in activated human lymphocytes and monocytes in a dose-dependent manner (Table 2). Lymphocyte production of interleukin (IL)-2, IL-4, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α was inhibited, with IC$_{50}$ values in the range 0.78–3.1 nM, whereas lipopolysaccharide (LPS)-stimulated monocyte production of IL-12 and TNF-α was inhibited with IC$_{50}$ values of 0.49 nM and 0.79 nM, respectively. Cilomilast also inhibited cytokine production in this assay, but less strongly than E6005 did.

**E6005 reduces skin inflammation by suppressing anti-inflammatory cytokine expression**

To examine *in vivo* anti-inflammatory activities, we assessed the inhibitory effect of E6005
ointment on a chronic skin inflammation model that mimics human AD pathogenesis.

Repeated applications of oxazolone increased the thickness of ear skin over time, and it reached a plateau on day 15. Post-sensitization treatment with E6005 ointment significantly reduced the increase in ear thickness relative to vehicle alone in a dose-dependent manner (Figure 2a). Inflammatory cytokine messenger RNA (mRNA) levels were measured in inflamed ears that had been treated with oxazolone. IL-1β, IL-4, and vascular cell adhesion protein 1 (VCAM-1) mRNA levels were up-regulated in the inflamed ear, indicating that this up-regulation was suppressed by E6005 (Figure 2b). E6005 also tended to reduce expression of intercellular adhesion molecule (ICAM)-I mRNA in treated mice.

**E6005 shows antipruritic effect**

The antipruritic effect of E6005 ointment was investigated by monitoring scratching behavior in mice. The mean number of scratches made by mice in the vehicle-treated and oxazolone-challenged group (1164 ± 101) was significantly higher than that in the vehicle-treated and acetone-challenged group (430 ± 51). Pretreatment with E6005 ointment inhibited the number of scratching that was statistically significant at doses ≥0.003% (Figure 3a).

To study the relationships between PDE4 inhibition, cAMP elevation, and antipruritic
activity, the effects of several compounds on pruritus were compared. Topical application of a structurally different PDE4 inhibitor, roflumilast, and the cAMP-elevating reagent forskolin, also significantly inhibited scratching behavior at doses of 0.1% and 4%, respectively. In contrast, the potent anti-inflammatory drug betamethasone failed to inhibit scratching behavior at a dose of 0.12%, a dose that was previously shown to be clinically sufficient for it to act as an anti-inflammatory agent (Figure 3b).

**E6005 therapeutically inhibits oxazolone-induced dermatitis**

AD-like skin lesions were induced in the ears of sensitized mice by repeated exposure to oxazolone. The onset of symptoms included redness of the ears, and the symptoms became exacerbated over time with the appearance of erythema, excoriation, and oozing/crusting (Figure 4a). The application of E6005 ointment was started after the development of slight skin lesion therapeutically. The dermatitis score (mean ± SEM) of the vehicle-treated and oxazolone-challenged group at 7 days after the treatment (day 19) was 4.5 ± 0.4, compared to 4.3 ± 0.4, 3.4 ± 0.4, and 2.8 ± 0.4 for the groups treated with 0.003%, 0.01%, and 0.03% E6005 ointment, respectively. A significant difference was observed with the 0.03% dose (Figure 4b).
Comparison of E6005 with tacrolimus in the oxazolone-induced dermatitis model

The effect of E6005 was compared with that of tacrolimus in the oxazolone-induced dermatitis model in the therapeutic application as well in figure 4. The mean ± SEM dermatitis score at day 19 in the vehicle-treated and oxazolone-challenged group was 5.0 ± 0.8, compared to 3.2 ± 0.8 and 2.5 ± 0.7 for the 0.03% and 0.1% solutions of E6005, respectively, and 3.1 ± 0.9 for 0.1% tacrolimus solution. A significant difference was observed in the 0.1% E6005 solution. There was tendency of inhibition in the 0.1% tacrolimus solution (Figure 5a). In the histological evaluation, the mean ± SEM value of epidermal thickness at day 19 was 23.5 ± 1.2 µm for uninvolved skin of oxazolon-challenged mice, 58.9 ± 3.4 µm for the vehicle-treated and oxazolone-challenged mice, 48.6 ± 3.1 µm and 46.1 ± 1.8 µm for the 0.03% and 0.1% E6005, respectively, and 45.0 ± 3.8 µm for the 0.1% tacrolimus (Figure 5b). A significant difference was observed in case of 0.03% E6005, 0.1% E6005 and 0.1% tacrolimus.

E6005 inhibits mite-induced dermatitis

Therapeutic effects of E6005 on mite-induced dermatitis were examined. Repeated application of mite extract induced various symptoms of dermatitis including erythema,
edema, excoriation, and oozing, which reached a plateau level at dat14 after the
mite-challenge. The application of E6005 ointment was started at day14. The mean ± SEM
dermatitis score at day 21 in the vehicle-treated group was 4.6 ± 0.7, compared to 3.7 ± 0.7
and 2.3 ± 0.6 in the 0.03% and 0.1% E6005 groups, respectively (Figure 6a). The mean ±
SEM epidermal thickness at day 21 was 20.6 ± 1.2 μm for healthy mice, 25.7 ± 1.1 μm for
the uninvolved skin of mite-challenged mice, 66.3 ± 4.1 μm for the vehicle-treated and
mite-challenged mice, 64.1 ± 4.5 for 0.03% E6005-treated mice, and 48.2 ± 3.8 for the
0.1% E6005-treated mice (Figure 6b, Supplemental Figure 2). A significant difference in
the dermatitis score and epidermal thickness was observed with the 0.1% E6005.

E6005 shortens the duration of xylazine/ketamine-induced anesthesia

A reduction in the duration of xylazine/ketamine-induced anesthesia in mice is indicative of
the emetic potential of PDE4 inhibitors (Robichaud et al., 1999; 2001; 2002). We have used
this phenomenon to assess the emetic potential of E6005 and compare it with that of
cilomilast. The mean duration of anesthesia in the vehicle group was 91.6 ± 8.3 min, and it
was 57.0 ± 5.2 min and 56.6 ± 6.0 min for the 0.5 mg/kg and 1 mg/kg cilomilast doses,
respectively. In the E6005-treated group, anesthesia lasted for 78.0 ± 5.7 min and 66.9 ± 5.1
min at doses of 5 mg/kg and 10 mg/kg, respectively. The duration of anesthesia was
significantly reduced compared to treatment with vehicle alone or with the 10 mg/kg dose of E6005 (Figure 7).

14C-E6005 is present at higher levels in plasma than in the central nervous system and is rapidly eliminated from tissues

The tissue distribution of radioactivity was evaluated in rats following the systemic administration of 14C-E6005. The radioactivity levels in the plasma were much higher than the levels in the central nervous systems (CNS) (Table 3). The elimination of radioactivity from tissues was rapid. In addition, radioactive concentration in blood decreased to approximately 1/1200 after 24 h of a single intravenous administration.
Discussion

Atopic inflammation reactions are the result of complex interactions between genetic and environmental factors. Inflammatory T lymphocytes and monocytes play roles in the onset and persistence of atopic inflammation through cell-to-cell interactions via cytokines and adhesion molecules (Homey et al., 2006). T lymphocytes produce T-helper 1 (Th1)- and Th2-type cytokines that contribute to the pathogenesis of AD lesions. Monocyte-derived IL-12 is involved in switching from the Th2- to the Th1-dominant stage of the disease (Grewe et al., 1998), although T cells in some AD lesions display mixed Th1/Th2 cytokine profiles (Hijnen et al., 2008). Inhibition of the production of cytokines such as IFN-γ and IL-4 would be beneficial for the treatment of AD irrespective of the disease stage because these cytokines cooperatively play roles in the process of acanthosis and recruiting of inflammatory cells through the induction of adhesion molecules.

The suppression of cytokine production by a PDE4 inhibitor was previously observed in an immune cell-driven, chronic skin inflammation model induced by repeated oxazolone challenges in which IFN-γ and IL-4 dominated the initial and later phases, respectively (Webb et al., 1998). IL-4 drives skin inflammation in AD patients by facilitating the recruitment of inflammatory cells through the induction of adhesion molecules such as...
VCAM-1 (Chan et al., 2001; Chen et al., 2010), whereas IL-1β plays an important role in the development of dermatitis (Shimada et al., 2003; Nambu et al., 2006). Therefore, the suppressive effect of PDE4 inhibitors on skin inflammation may be partly ascribed to the inhibition of IL-4, IL-1β, and adhesion molecule expression, which may lead to the inhibition/activation of inflammatory cells such as mast cells and lymphocyte (Torphy, 1998).

Itching is a primary symptom of AD that not only impairs the social activities of patients but also aggravates skin inflammation by eliciting the scratch response (Wahlgren et al., 1999). The scratching behavior in our model immediately following the oxazolone challenge was responsive to the µ-opioid receptor antagonist naloxone, but was resistant to the anti-histamine terfenadine, and similar results have been seen in human AD (data not shown). The rapid and dose-dependent inhibition of scratching behavior after a single application of E6005 ointment indicated that the antipruritic effect could not be due to an improvement in skin lesions. Because roflumilast also inhibited scratching behavior, this study suggests that PDE4 inhibitors have an antipruritic effect.

Several of the effects of PDE4 inhibitors are mediated by cAMP-activated protein kinases (Jimenez et al., 2001). The antipruritic action of forskolin, a cAMP activator, suggests that
cAMP signaling plays a suppressive role in the process of itch generation (Takano et al., 2004). Therefore, the inhibition of pruritus by E6005 may be mediated by activation of cAMP signaling through PDE4 inhibition. However, the strong anti-inflammatory agent betamethasone failed to inhibit scratching immediately, despite the fact that long-term use of steroids is thought to suppress itching. Although PDE4 inhibitors share some pharmacological similarities with steroids, the antipruritic mechanism of E6005 appears to be distinct from these and remains to be elucidated. It may result from the inhibition of itch mediators such as neuropeptides and proteases released from the granules of mast cells, although PDE4 inhibitors have little effect on mast cell degranulation (Shichijo et al., 1999).

Recent studies have demonstrated that keratinocytes are involved in eliciting the itch response by releasing pruritogens (Andoh et al., 2007; 2009). These cells also express PDE4 (Chujor et al., 1998), and their potential as a target of PDE4 inhibitors warrants further investigation. The immediacy of its antipruritic effect suggests that E6005 directly inhibits the C-fiber nerve activation that is responsible for the perception of itching and conveying it to the CNS. It has previously been reported that a PDE4 inhibitor, but not other PDE isozyme inhibitors, directly suppressed C-fiber activation (Qian et al., 1994),
and we are currently using electrophysiological methods to investigate the possibility of the direct inhibition of C-fiber activation by E6005.

The skin lesions in our experimental setting (Tomimori et al., 2005) developed in a manner similar to those of human AD with visible signs of pruritus, erythema, excoriation, and erosion. Scratching was an important pathogenic factor in our model, as it is in AD patients (Wahlgren et al., 1999), because NC/Nga mice exhibited diminished skin symptoms when their toe nails were clipped (data not shown), as reported previously (Hashimoto et al., 2004). E6005 ointment therapeutically inhibited the aggravation of AD-like symptoms at a dose of 0.03%. This dose exerted antipruritic and anti-inflammatory effects, both of which appeared to contribute to the improvement of skin lesions. The efficacy of E6005 was comparable to that of tacrolimus with respect to both the macroscopic and microscopic improvement of skin lesions observed in clinical practice. Furthermore, E6005 therapeutically improved both skin score and acanthosis in a mite-induced AD model in which Th2 cytokines such as IL-4 play important roles (Gao et al., 2004). This improvement may be, in part, due to the suppression of IL-4 by E6005 treatment.

A major challenge of PDE4 inhibitor use is to overcome side effects such as nausea and vomiting (Robichaud et al., 1999). The emetic potential is closely associated with the
α2-adrenoceptor antagonist-like activity of the PDE4 inhibitor, and can therefore be assessed by the ability to reverse xylazine/ketamine-induced anesthesia (Robichaud et al., 2001; 2002). In this model of emesis, the reversal of anesthesia by systemic treatment with E6005 was less potent than with cilomilast, despite the more potent PDE4 inhibition of E6005. This suggests that E6005 may have a more improved safety profile than cilomilast. Moreover, our results using 14C-E6005 demonstrated that it was rapidly eliminated from the plasma and showed limited distribution in the CNS. Such limited exposure results in reduced emetogenicity and contributes to a wide therapeutic index.

In conclusion, we identified a topical PDE4 inhibitor, E6005, with a wide therapeutic window that exhibited dual antipruritic and anti-inflammatory effects. Topical delivery of E6005 minimizes systemic exposure and allows for higher levels of PDE4 inhibition, which may provide prompt and strong relief from both itching and atopic inflammation.

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Authorship contributions

Participated in research design: Naoto Ishii, Shirato Manabu, Hishinuma Ieharu

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Wrote or contributed to the writing of the manuscript: Naoto Ishii, Shirato Manabu

References


Robichaud A, Stamiou PB, Jin SL, Lachance N, MacDonald D, Laliberté F, Liu S, Huang...


Conflicts of Interest

Eisai Co., Ltd. holds patents (US7939540, EP1992622) on the substance of E6005. Naoto Ishii, Manabu Shirato, Hisashi Wakita, Kazuki Miyazaki, Yasutaka Takase, Osamu Asano, and Kazutomi Kusano, are the inventor of the patent.
Figure Legends

Figure 1. Chemical structure of E6005

Figure 2. Topical effect of E6005 ointment on oxazolone-induced ear inflammation

(a) Ten µL of 0.01% E6005 ointment (■), 0.03% E6005 ointment (▲), 0.1% E6005 ointment (●), or vaseline-based vehicle ointment (♦) was applied 5 times per week from day 5 to day 15 in BALB/c mice that were sensitized and challenged with oxazolone (oxa) as described in Materials and Methods. Results are expressed as the mean ± SEM (n = 9 or 10). The degree of dermatitis was evaluated by calculating the increase in ear thickness from the basal value of day 0. (b) At the end of the experiment, skin tissues were collected for mRNA expression analysis. mRNA expression of IL-1β, IL-4, VCAM-1 and ICAM-1 in homogenates from inflamed skin was measured as described in Materials and Methods. The left ears in the vehicle (veh)-treated and acetone (ace)-challenged group were used as the normal skin. The results are expressed as mean ± SEM (n = 9). *P < 0.05 versus the vehicle-treated and oxazolone-challenged group.

Figure 3. Effect of compounds on oxazolone-induced scratching behavior in NC/Nga mice
(a) Ten µL of E6005 ointment (from 0.003 to 0.03%) or Vaseline-based vehicle ointment was applied on the ear of NC/Nga mice that were sensitized and challenged with oxazolone as described in Materials and Method. Scratching behavior was induced 4–5 h after the administration of each compound and then evaluated for 2 h. (b) Acetone/ethanol based-vehicle solution, 0.12% betamethasone solution (BETA), 4% forskolin solution (FORS), 0.1% roflumilast solution (ROFL), or 0.1% E6005 solution was administered to the ear of NC/Nga mice that were sensitized and challenged. Each compound was dissolved in a 1:1 acetone/ethanol solution. Scratching behavior was induced 1 h after the administration of each compound and then evaluated for 2 h. Results are expressed as the mean ± SEM (a, n = 24; b, n = 15). \(^* P < 0.05\) versus the vehicle-treated and acetone-challenged group. \(^* P < 0.05\) versus the vehicle-treated and oxazolone-challenged group.

Figure 4. Therapeutic effect of topically applied E6005 on oxazolone-induced dermatitis score in NC/Nga mice

(a) Representative macroscopic feature of skin lesion in NC/Nga mice with individual score.

(b) Fifteen microliters of 0.003% E6005 ointment (■), 0.01% E6005 ointment (▲), 0.03%
E6005 ointment (●), or Vaseline based-vehicle ointment (♦) was administrated once a day from day 12 to day 18 onto the ears of NC/Nga mice that were sensitized and challenged with oxazolone as described in Materials and Methods. Skin lesion was scored according to the following symptoms: erythema/hemorrhage, excoriation, and oozing/crusting, and evaluated as described in Materials and Methods. Results are expressed as the mean ± SEM (n = 19 or 20). *P < 0.05 versus the vehicle-treated group.

Figure 5. Comparison of therapeutic effect of E6005 with tacrolimus in the oxazolone-induced dermatitis model

Fifty microliters of 0.03% E6005 solution (▲), 0.1% E6005 solution (■), 0.1% tacrolimus solution (●), or acetone/ethanol based-vehicle solution (♦) was administered in single daily doses from day 12 to day 18 onto the rostral back of NC/Nga mice that were sensitized and challenged as described in Materials and Methods. Each compound was dissolved in a 1:1 acetone/ethanol solution. (a) Back skin lesion was scored according to the following symptoms: erythema/hemorrhage, excoriation, edema, and induration, and evaluated as described in Materials and Methods. (b) At the end of the experiment, skin tissues were collected from the rostral back for H&E staining. Epidermal thickness was determined by
measuring 20 spots on the epidermis on the H&E-stained slides. Non-challenged area in vehicle treated control group was used as un-involved control. Results are expressed as the mean ± SEM (n = 14 or 15). *p < 0.05 versus the vehicle-treated group.

**Figure 6. Therapeutic effect of topically applied E6005 on mite-induced dermatitis**

Fifty microliter of 0.03% E6005 solution (▲), 0.1% E6005 solution (■) or acetone/ethanol based-vehicle solution (●) was applied once daily from day 14 to day 21 to the rostral back of NC/Nga mice that were sensitized and challenged with mite antigen as described in Materials and Methods. Each compound was dissolved in a 1:1 acetone/ethanol solution. (a) Back skin lesion was scored according to the following symptoms: erythema/hemorrhage, excoriation, popular/induration, and edema, and evaluated as described in Materials and Methods. (b) At the end of the experiment, dorsal skin tissues were collected for H&E staining. Epidermal thickness of the rostral back of mice was determined by measuring 20 spots in the epidermis on H&E-stained slides. Results are expressed as the mean ± SEM (n = 18 or 19). *p < 0.05 versus the vehicle-treated group.

**Figure 7. Effect of E6005 and Cilomilast on the duration of xylazine/ketamine-induced**
anesthesia in mice

At 15 min following the induction of anesthesia, E6005 (5 or 10 mg/kg) or Cilomilast (0.5 or 1 mg/kg) was injected subcutaneously (10 μL/g). The duration of anesthesia was evaluated by the return of the righting reflex. Results are expressed as the mean ± SEM (n = 7 or 8). *P < 0.05 versus the vehicle-treated group.
Tables

Table 1. Inhibition of PDE isozyme activities by E6005 and Cilomilast

<table>
<thead>
<tr>
<th>Compound</th>
<th>PDE subtype</th>
<th>PDE1</th>
<th>PDE2</th>
<th>PDE3</th>
<th>PDE4</th>
<th>PDE5</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6005</td>
<td></td>
<td>46.1 ± 6.2%</td>
<td>51.6 ± 4%</td>
<td>69.4% ± 5.4%</td>
<td>IC$_{50}$ = 2.8 nM</td>
<td>57.4% ± 7.2%</td>
</tr>
<tr>
<td></td>
<td>at 30 µM</td>
<td>at 30 µM</td>
<td>at 30 µM</td>
<td>(1.6–3.9 nM)</td>
<td>at 30 µM</td>
<td></td>
</tr>
<tr>
<td>Cilomilast</td>
<td></td>
<td>12.7 ± 6.2%</td>
<td>26.6 ± 1.9%</td>
<td>6.4 ± 1.8%</td>
<td>IC$_{50}$ = 24 nM</td>
<td>19.9 ± 1.6%</td>
</tr>
<tr>
<td></td>
<td>at 30 µM</td>
<td>at 30 µM</td>
<td>at 30 µM</td>
<td>(6.1–42 nM)</td>
<td>at 30 µM</td>
<td></td>
</tr>
</tbody>
</table>

Data given as the mean maximum inhibition rate at 30µM ± SEM or mean IC$_{50}$ values (nM) calculated from concentration-inhibition curves from three separate experiments. 95% confidence intervals are shown in parentheses.
Table 2. Inhibition of cytokine release from PBMCs by E6005 and Cilomilast

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lymphocyte</th>
<th>Monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>IL-4&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>E6005</td>
<td>3.1</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>(2.0–4.9)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(0.90–8.3)</td>
</tr>
<tr>
<td>Cilomilast</td>
<td>59</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>(36–95)</td>
<td>(49–160)</td>
</tr>
</tbody>
</table>

Data given as mean IC<sub>50</sub> values (nM) calculated from concentration-inhibition curves from three different experiments. 95% confidence intervals are shown in parentheses.

<sup>1</sup>anti-CD3/CD28 antibody-stimulated

<sup>2</sup>PHA-P-stimulated

<sup>3</sup>LPS-stimulated

<sup>4</sup>IC<sub>50</sub> (95% confidence interval) in nM
Table 3. Tissue distribution of radioactivity after a single intravenous administration of $^{14}$C-E6005 in rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Radioactive concentration (ng equivalent of E6005/g or mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5206 ± 108.7</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3331 ± 63.2</td>
</tr>
<tr>
<td>Cerebrum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>182.9 ± 4.3</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>175.8 ± 13</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1754 ± 170.5</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td></td>
</tr>
<tr>
<td></td>
<td>162.7 ±9.5</td>
</tr>
<tr>
<td>Spinal cord</td>
<td></td>
</tr>
<tr>
<td></td>
<td>126.3 ± 5.5</td>
</tr>
</tbody>
</table>

Data are shown as the mean values ± SEM (n = 4). ND, not detected; -, not determined.
Figure 2

(a) Relative mRNA expression of IL-1β and IL-4 across different treatments and time points.

(b) Relative mRNA expression of VCAM-1 and ICAM-1 across different treatments and time points.

Legend:
- **veh**: Vehicle
- **ace**: Acephen
- **oxa**: Oxaprene
- * indicates statistical significance

Days after sensitization:
- 0
- 3
- 6
- 12
- 15

ΔEar thickness (μm)
- 0
- 100
- 200
- 300
- 400

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Figure 3

(a) Scratching (counts/2h) of vehicle, acetone, 0.003% E6005, 0.01% E6005, and 0.03% E6005 oxazolone.

(b) Scratching (counts/2h) of vehicle, vehicle, 0.12% BETA, and 4% FORS.
Figure 4

a

Score = 0  Score = 1  Score = 2
Score = 3  Score = 4  Score = 5
Score = 6  Score = 7  Score = 8

b

Dermatitis score

Days after sensitization

*
Figure 5

a

Dermatitis score

Days after sensitization

b

Epidermal thickness (mm)

uninvolved vehicle 0.03% E6005 0.1% E6005 0.1% tacrolimus
Figure 6

(a) Dermatitis score over days after sensitization for uninvolved vehicle 0.03% E6005 and 0.1% E6005.

(b) Epidermal thickness (μm) measured in healthy and mite-infected skin with different treatments.
Figure 7

Duration time (min)

Vehicle  5 mg/kg  10 mg/kg  0.5 mg/kg  1 mg/kg
E6005     Cilomilast

* * *
Supplemental Data

Anti-Pruritic Effect of the Topical Phosphodiesterase 4 Inhibitor E6005 Ameliorates Skin Lesions in a Mouse Atopic Dermatitis Model

Naoto Ishii, Manabu Shirato, Hisashi Wakita, Kazuki Miyazaki, Yasutaka Takase, Osamu Asano, Kazutomi Kusano, Eiichi Yamamoto, Chiharu Inoue, and Ieharu Hishinuma

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The Journal of Pharmacology and Experimental Therapeutics
**Phosphodiesterase assay**

Phosphodiesterase (PDE) isoenzyme activities were quantified by measuring the formation of \[^{3}H\] 5′-AMP or \[^{3}H\] 5′-GMP from \[^{3}H\] cAMP or \[^{3}H\] cGMP, using an enzyme isolated from bovine brain (for PDE1), differentiated U-937 cells (for PDE2), human platelets (for PDE3 and PDE5), and U-937 cells (for PDE4), as described by Weishaar et al. (1986) and Torphy et al. (1992). The test compounds, reference compounds, or vehicle (water) was added to a buffer containing 40 mM Tris/HCl (pH 7.8), 3 mM MgCl₂, 1 mM DTT, 0.01% BSA, 200 mM NH₄Cl, 1 μM cAMP or cGMP, and 0.1 μCi \[^{3}H\] cAMP or \[^{3}H\] cGMP. The reaction was initialized by addition of the enzyme, and the mixture was incubated for 60 min at 22 °C. Following incubation, the reaction was stopped by heating the plate to 60 °C for 3 min, after which time SPA beads were added. After 20 min of shaking at 22 °C, the amount of \[^{3}H\] 5′-AMP or \[^{3}H\] 5′-GMP was quantified with a scintillation counter (Topcount, Packard). The results are expressed as percent inhibition of the control enzyme activity.
Supplemental Figure 1. Inhibition of PDE isozyme activities by E6005 and Cilomilast.
Supplemental Data

Anti-Pruritic Effect of the Topical Phosphodiesterase 4 Inhibitor E6005 Ameliorates Skin Lesions in a Mouse Atopic Dermatitis Model

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The Journal of Pharmacology and Experimental Therapeutics
Histological evaluation

At the end of the experiment, skin tissue was collected, fixed with 10% neutral formalin, embedded in paraffin, and sectioned into 40-μm slices. Sections were stained with hematoxylin-eosin.

Supplemental Figure 2. Histological features of mite-induced skin lesion treated with E6005