Antipruritic 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 (methyl 4-[[3-[(6,7-dimethoxy-2-(methylamino)quinazolin-4-yl)]phenyl]amino)carbonyl]benzoate), a novel PDE4 inhibitor developed as a topical agent for treating AD. The use of 14C-labeled E6005 showed rapid clearance from the blood and low inhibitory effects comparable to that of tacrolimus. The topical application of E6005 produced an immediate antipruritic effect as well as an anti-inflammatory effect with reduced expression of cytokines/adhesion molecules. On the basis of 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. These results suggest that E6005 may be a promising novel therapeutic agent with antipruritic 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 nonimmunologic 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, 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 because of systemic side effects such as nausea and emesis. To minimize systemic exposure to these inhibitors, topical delivery methods such as inhalation and dermal applications have been explored (Pages 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, topical active PDE4 inhibitor that appears to produce only minimal systemic side effects. In this report, we describe and characterize a novel PDE4 inhibitor, E6005 (methyl 4-[(3-[6,7-dimethoxy-2-(methylamino)quinazolin-4-yl]phenyl)amino]carbonyl]benzoate), 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, cilemistat, and rolfumistat were synthesized at Eisai Laboratories. The chemical structure of E6005 is shown in Fig. 1. FK506 (tacrolimus) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Forskolin, betamethasone, and Dermatophagoides farinae (cat no. Mm00434228), IL-4 (cat no. Mm00445260), vascular cell adhesion protein (VCAM-1) (cat no. Mm00441917), and intercellular adhesion molecule-1 (cat no. Mm00516024) from 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. Mm00449197), and intercellular adhesion molecule-1 (cat no. Mm00516024). Relative expression levels of target genes were normalized to that of 18S ribosomal RNA and then determined using the 2−ΔΔCT method. Data were expressed as fold increase over normal skin.

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 hour 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 Δthickness (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), interleukin (IL)-1β (cat no. Mm00434228), IL-4 (cat no. Mm00445260), vascular cell adhesion protein (VCAM-1) (cat no. Mm00441917), and intercellular adhesion molecule-1 (cat no. Mm00516024). Relative expression levels of target genes were normalized to that of 18S ribosomal RNA and then determined using the 2−ΔΔCT 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 rechallenge with either 0.3% oxazolone solution or acetone vehicle. Scratching behavior was measured for the next 2 hours 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
Inhibition of PDE isozyme activities by E6005 and cilomilast
Data given as the mean maximum inhibition rate at 30 μM ± S.E.M. or mean IC₅₀ values (nM) calculated from concentration-inhibition curves from three separate experiments; 95% confidence intervals are shown in parentheses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PDE1</th>
<th>PDE2</th>
<th>PDE3</th>
<th>PDE4</th>
<th>PDE5</th>
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<tr>
<td>E6005</td>
<td>46.1 ± 6.2% at 30 μM</td>
<td>51.6 ± 4% at 30 μM</td>
<td>69.4 ± 5.4% at 30 μM</td>
<td>IC₅₀ = 2.8 nM (1.6–3.9 nM)</td>
<td>57.4 ± 7.2% at 30 μM</td>
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<td>Cilomilast</td>
<td>12.7 ± 6.2% at 30 μM</td>
<td>26.6 ± 1.9% at 30 μM</td>
<td>6.4 ± 1.8% at 30 μM</td>
<td>IC₅₀ = 24 nM (6.1–42 nM)</td>
<td>19.9 ± 1.8% at 30 μM</td>
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Shortening Effect on the Duration of Xylazine/Ketamine-Induced Anesthesia. ICR mice were anesthetized by a single intramuscular injection of xylazine/ketamine. After 15 minutes, E6005, cilomilast, or vehicle was subcutaneously (10 mg/kg) administered to the mice, which were placed in a dorsal recumbent position. The measurement of duration was initiated 15 minutes 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 minutes after administration of the test compounds or vehicle until return of the righting reflex was recorded as the duration of anesthesia.

Animal Study Using ¹⁴C-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 20 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 cm² 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 high-performance liquid chromatography 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 of WinNonlin Professional Version 5.01 (Pharsight Corp., Sunnyvale, CA).

Statistical Analysis. IC₅₀ values were determined by nonlinear regression analysis of the inhibition curves using the Hill Software (Cerep, Redmond, WA) or GraphPad Prism v4.00 (GraphPad Software, La Jolla, CA). 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 Fig. 1. E6005 inhibited human PDE4 enzymatic activity with a half-maximal inhibitory concentration (IC₅₀) 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 IC₅₀ of 24 nM (Supplemental Fig. 1; Table 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 IL-2, IL-4, interferon (IFN)-γ, and tumor necrosis factor-α was inhibited, with IC₅₀ values in the range 0.78–3.1 nM, whereas...
lipopolysaccharide-stimulated monocyte production of IL-12 and tumor necrosis factor-α was inhibited with IC₅₀ values of 0.49 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. Postsensitization treatment with E6005 ointment significantly reduced the increase in ear thickness relative to vehicle alone in a dose-dependent manner (Fig. 2A). Inflammatory cytokine messenger RNA (mRNA) levels were measured in inflamed ears that had been treated with oxazolone. IL-1β, IL-4, and VCAM-1 mRNA levels were upregulated in the inflamed ear, indicating that this upregulation was suppressed by E6005 (Fig. 2B). E6005 also tended to reduce expression of intercellular adhesion molecule-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% (Fig. 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

**Fig. 2.** Topical effect of E6005 ointment on oxazolone-induced ear inflammation. (A) 10 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 ± S.E.M. (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 intercellular adhesion molecule (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 ± S.E.M. (n = 9). *P < 0.05 versus the vehicle-treated and oxazolone-challenged group.
that was previously shown to be clinically sufficient for it to act as an anti-inflammatory agent (Fig. 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 (Fig. 4A). The application of E6005 ointment was started after the development of slight skin lesion therapeutically. The dermatitis score (mean ± S.E.M.) of the vehicle-treated and oxazolone-challenged group at 7 days after the treatment (day 19) was 4.5 ± 0.4 compared with 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 (Fig. 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 Fig. 4. The mean ± S.E.M. dermatitis score at day 19 in the vehicle-treated and oxazolone-challenged group was 5.0 ± 0.8 compared with 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 (Fig. 5A). In the histologic evaluation, the mean ± S.E.M. value of epidermal thickness at day 19 was 23.5 ± 1.2 μm for uninvolved skin of oxazolone-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 (Fig. 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, that reached a plateau level at day 14 after the mite challenge. The application of E6005 ointment was started at day 14. The mean ± S.E.M. dermatitis score at day 19 in the vehicle-treated and oxazolone-challenged group was 5.0 ± 0.8 compared with 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 (Fig. 6A).
thickness at day 21 was $20.6 \pm 1.2 \mu m$ for healthy mice, $25.7 \pm 1.1 \mu m$ for the uninvolved skin of mite-challenged mice, $66.3 \pm 4.1 \mu m$ for the vehicle-treated and mite-challenged mice, $64.1 \pm 4.5$ for 0.03% E6005-treated mice, and $48.2 \pm 3.8$ for the 0.1% E6005-treated mice (Fig. 6B; Supplemental Fig. 2). A significant difference in the dermatitis score and epidermal thickness was observed with the 0.1% E6005.

**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 Th1- and Th2-type cytokines that contribute to the pathogenesis of AD lesions. Monocyte-derived IL-12 is involved in switching from
the mean evaluated by the return of the righting reflex. Results are expressed as injected subcutaneously (10 μg). The duration of anesthesia was evaluated by the return of the righting reflex. Results are expressed as the mean ± S.E.M. (n = 7 or 8). *P < 0.05 versus the vehicle-treated group.

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, 1999), because NC/Nga mice exhibited diminished skin symptoms when their toe nails were clipped (unpublished data), as reported previously (Hashimoto et al., 2004). E6005 ointment indicated that the antipruritic effect could not be attributable 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 AMP 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 was previously 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.

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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, attributable 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 wider 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.