Treatment of Skin Inflammation with Benzoxaborole Phosphodiesterase Inhibitors: Selectivity, Cellular Activity, and Effect on Cytokines Associated with Skin Inflammation and Skin Architecture Changes

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ABSTRACT
Psoriasis and atopic dermatitis are skin diseases affecting millions of patients. Here, we characterize benzoxaborole phosphodiesterase (PDE)-4 inhibitors, a new topical class that has demonstrated therapeutic benefit for psoriasis and atopic dermatitis in phase 2 or phase 3 studies. Crisaborole [AN2728, 4-{1-(hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl)oxy}benzonitrile], compd2 [2-ethoxy-6-{1-(hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl)oxy}nicotinonitrile], compd3 [6-{1-(hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl)oxy}-2-(2-isopropoxyethoxy)nicotinonitrile], and compd4 [5-chloro-6-{1-(hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl)oxy}-2-{[(4-oxopentyl)oxy]nicotinonitrile} are potent PDE4 inhibitors with similar affinity for PDE4 isoforms and equivalent inhibition on the catalytic domain and the full-length enzyme. These benzoxaboroles are less active on other PDE isoforms. Compd4 binds to the catalytic domain of PDE4B2 with the oxaborole group chelating the catalytic bimetal and overlapping with the phosphate in cAMP during substrate hydrolysis, and the interaction extends into the adenine pocket. In cell culture, benzoxaborole PDE4 inhibitors suppress the release of tumor necrosis factor-α, interleukin (IL)-23, IL-17, interferon-γ, IL-4, IL-5, IL-13, and IL-22, and these cytokines contribute to the pathologic changes in skin structure and barrier functions as well as immune dysregulation in atopic dermatitis and psoriasis. Treatment with compd3 or N6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate increases cAMP response element binding protein phosphorylation in human monocytes and decreases extracellular signal-regulated kinase phosphorylation in human T cells; these changes lead to reduced cytokine production and are among the mechanisms by which compd3 blocks cytokine release. Topical compd3 penetrates the skin and suppresses phorbol myristate acetate–induced IL-13, IL-22, IL-17F, and IL-23 transcription and calcipotriol–induced thymic stromal lymphopoietin expression in mouse skin. Skin thinning is a major dose-limiting side effect of glucocorticoids. By contrast, repeated application of compd3 did not thin mouse skin. These findings show the potential benefits and safety of benzoxaborole PDE4 inhibitors for the treatment of psoriasis and atopic dermatitis.
effects are particularly concerning in children, who comprise the majority of patients with AD (Kivelevitch et al., 2013; Schäkel et al., 2014). Calcineurin inhibitors have additional potential risks of infection and cancer and have boxed warnings required by the U.S. Food and Drug Administration (Guttman-Yassky et al., 2011b; Ring et al., 2012). For these reasons, there is a need for novel, safe, and effective topical therapeutic agents for patients with mild-to-moderate AD or psoriasis.

Psoriasis is associated with hyperactivity of Th1 and Th17 immune responses. Psoriatic skin lesions exhibit prominent infiltrates of Th1 and Th17 T cells and dendritic cells (Guttman-Yassky et al., 2011a), as well as elevated levels of interferon (IFN)-γ, TNF-α, IL-17, and IL-23 (Guttman-Yassky et al., 2011b; Lowes et al., 2013; Martin et al., 2013). AD is characterized as a disorder of Th2 immunopathology. Pruritus is a prominent feature of AD and significantly affects the sleep and quality of life of patients with AD. AD lesional skin contains pronounced infiltrates of Th2 cells, Th22 cells, and dendritic cells, as well as increased numbers of eosinophils and mast cells (Guttman-Yassky et al., 2011a; Gittler et al., 2012).

The levels of cytokines associated with the Th2 phenotype, such as IL-4, IL-5, IL-13, and IL-31, are increased in both acute and chronic AD lesions (Leung et al., 2004; Gittler et al., 2012). When AD lesions become chronic, IFN-γ expression increases and the levels of Th2 cytokines remain high or even become further elevated (Gittler et al., 2012). The levels of itch mediators, such as thymic stromal lymphopoietin (TSLP) and IL-31, are increased in AD skin lesions (Soumelis et al., 2002; Bilsborough et al., 2006; Sonkoly et al., 2006). In both psoriasis and AD, IL-22 is highly expressed, and this cytokine prevents terminal differentiation of keratinocytes and promotes epidermal hyperplasia (Fujita, 2013).

Cyclic nucleotide-specific phosphodiesterases (PDEs) control the intracellular level of cAMP or cGMP and thus play critical roles in many cellular functions. Among the 11 families of PDEs, PDE4 has received great attention as a target for treating inflammatory diseases. Leukocytes are highly sensitive to PDE4 inhibition in part because PDE4 is a major PDE isozyme in immune cells, including T cells, B cells, eosinophils, neutrophils, monocytes, and macrophages (Torphy, 1998). PDE4 inhibitors block cytokine synthesis by elevating the cAMP level and subsequently activating protein kinase A (PKA), which negatively modulates signaling pathways that lead to cytokine secretion (Torgersen et al., 2002; Maurice et al., 2014). Continuing efforts have been devoted to the development of safer and more efficacious PDE4 inhibitors (Kumar et al., 2013; Maurice et al., 2014; Schäkel et al., 2014). Crisaborole [AN2728, 4-[[1-hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl]oxy]benzonitrile] (Akama et al., 2009) has shown significant clinical benefit to patients with mild-to-moderate AD in phase 2 (Murrell et al., 2015; Stein Gold et al., 2015) and phase 3 (data on file, Anacor Pharmaceuticals, Inc., 2015) clinical trials.

Here, we characterize the anti-inflammatory properties of four benzoxaborole PDE4 inhibitors, including crisaborole. These molecules potently inhibited PDE4 enzymatic activity. Crystallography revealed that interaction of benzoxaboroles with the hydrophobic pocket in the PDE4 catalytic domain increased their affinity for PDE4. These benzoxaboroles strongly suppressed the secretion of cytokines associated with psoriasis and AD. We also explored the intracellular pathways modulated by Camp/PKA and PDE4 to further elucidate the mechanism by which benzoxaborole PDE4 inhibitors suppress cytokine secretion. Finally, we demonstrated that a benzoxaborole PDE4 inhibitor reduced skin inflammation and inflammatory cytokine production in mice, without causing skin thinning. These findings provide a better understanding of the potential therapeutic benefits and safety of benzoxaborole PDE4 inhibitors.

Materials and Methods

Animals. Female CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). All animal studies were performed with approval from the Anacor Pharmaceuticals Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

Drugs. Crisaborole, compd2 [2-ethoxy-6-(1-hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl)oxy]nicotinonitrile], compd3 [6-[[1-hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl]oxy]2-(2-isopropanoyloxy)nicotinonitrile], and compd4 [5-chloro-6-[[1-hydroxy-1,3-dihydrobenzo[c][1,2]oxaborol-5-yl]oxy]-2-(4-oxopentyl)nicotinonitrile] were synthesized by Anacor Pharmaceuticals, Inc. (Palo Alto, CA) and were > 95% pure. Roflumilast [3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide] and apremilast [[S,N-2-(1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl)-1,3-dioxoisindolin-4-yl]acetamide] were synthesized by Sundia (Shanghai, China).

Enzymes. The human recombinant PDE4B catalytic domain (amino acids 152–484) was purchased from Proteos (Regensburg, Germany). The full-length human recombinant PDE4 enzymes were purchased from BPS Bioscience (San Diego, CA).

PDE4 Assays. The PDE4-mediated cAMP enzymatic hydrolysis assay was performed as described by Saldou et al. (1998). The enzymatic activities of other PDEs were assessed by a fluorescent polarization assay by BPS Bioscience (San Diego, CA) using purified human recombinant full-length PDE enzymes. The IC50 value was calculated based on a four-parameter logistic equation, and the geometric mean of replicates is reported because the IC50 values are not normally distributed.

X-Ray Crystallography. The human recombinant PDE4B catalytic domain (amino acids 152–484) was used in the crystallization with compd4. Phase information, which was used to analyze the structure, was obtained by molecular replacement with a previously solved structure of PDE4B (Freund et al., 2012) as the search template. The model included residues Asn162 to Ile484 with a resolution of 1.86 Å. The electron density in the geometric mean of replicates is reported because the IC50 values are not normally distributed.

Cytokine Assays. Human peripheral blood mononuclear cells (PBMCs) were stimulated in vitro to produce TNF-α, IL-2, IFN-γ, IL-5, IL-4, IL-13, IL-17, and IL-22. Human PBMCs were stimulated with 1 μg/ml lipopolysaccharide (LPS) for 24 hours to produce TNF-α; stimulated with 20 μg/ml phytohemagglutinin (PHA)-L for 24 hours to induce IL-2 and IFN-γ, or for 48 hours to induce IL-5, IL-4, IL-13, and IL-17 were induced in human PBMCs with 20 μg/ml concanavalin A for 48 hours. To induce IL-22 production, PBMCs were stimulated for 24 hours with anti-CD2/CD3/CD28 beads (cell/bead = 1:1) (Miltenyi Biotec, Bergisch Gladbach, Germany). Human monocytes were isolated as described by Dong et al. (2013) with a purity of 85%–90% and were stimulated with 1 μg/ml LPS and 100 ng/ml IFN-γ for 24 hours to induce IL-23 production. The cell culture supernatants were collected...
for cytokine analysis. The levels of TNF-α, IL-2, IL-4, IL-5, IL-13, IL-17, IL-23, and IFN-γ were determined using Cisbio HTRF cytokine determination kits (Cisbio, Bedford, MA); the level of IL-22 was quantified by an enzyme-linked immunosorbent assay (eBioscience, San Diego, CA). The results are not normally distributed and are thus presented as the geometric mean of the IC₅₀ values from multiple independent experiments.

### Protein Phosphorylation and Western Blot Analysis

To evaluate the effect of the benzoxaborole PDE4 inhibitors on cAMP response element binding protein (CREB) phosphorylation, human monocytes were incubated at 37°C for 3 hours and then either not treated or treated with 1 μM compd3 or 100 μM N⁶,²'-O-dibutyryladenosine 3'⁵'-cyclic monophosphate (dBcAMP) (Sigma, St. Louis, MO), a cell-permeant cAMP analog, for 15 minutes prior to stimulation with 1 μg/ml LPS. The nuclear fraction was isolated using a nuclear extract kit (Active Motif, Carlsbad, CA), separated by SDS-PAGE, and transferred to a polyvinylidene fluoride membrane. Phospho-CREB was detected by an antibody specific to phospho-threonine (T202) and phospho-serine (S133) (Santa Cruz Biotechnology, Santa Cruz, CA). To address the effect of the benzoxaborole PDE4 inhibitors on extracellular signal-regulated kinase (ERK) phosphorylation, human T cells were treated or treated with compd3 or dBcAMP for 15 minutes prior to stimulation with human T-activator CD3/CD28 beads (Thermo Fisher Scientific, San Diego, CA). The results are not normally distributed and are thus quantified by an enzyme-linked immunosorbent assay (eBioscience, San Diego, CA).

### In Vivo Skin Thinning Assay

The effect of each compound on mouse skin thickness was evaluated using hairless SKH-1 mice (eight mice per group) generally following the protocol of Kirby and Munro (1976), with the addition of histopathology as reviewed by Schoepf et al. (2006). The baseline thickness of the dorsal skin was measured using calipers prior to drug treatment. Each mouse received 30 μl of a topical drug once a day for 16 consecutive days on a 1-cm² area of the dorsal skin between the shoulder blades near the neck. The compounds were dissolved in acetonitrile/ethanol (1:1(v/v)). The thickness of the dorsal skin was measured using calipers prior to drug treatment. Each mouse received 30 μl of a topical drug once a day for 16 consecutive days on a 1-cm² area of the dorsal skin between the shoulder blades near the neck. The compounds were dissolved in acetonitrile/ethanol (1:1(v/v)). The thickness of the dorsal skin was measured on days 1, 7, and 16. At the end of the treatment period, the dorsal skin was removed and fixed in neutral buffered formalin, followed by hematoxylin and eosin staining. The thicknesses of the epidermis and dermis was quantified by measuring three representative areas. Statistical significance was analyzed using one-way analysis of variance and Dunnett’s multiple comparison test.

### Results

#### Benzoxaborole PDE Inhibitors Potently Inhibit PDE4

Crisaborole, compd2, compd3, and compd4 (Supplemental Fig. 1) inhibited the activity of the catalytic domain of PDE4B2, with IC₅₀ values of 75, 1.8, 3.0, and 0.32 nM, respectively (Table 1). Although some types of PDE4 inhibitors interact with both the catalytic domain and the upstream conserved region (UCR) 2 region (Saldou et al., 1998; Burgin et al., 2010), the benzoxaborole PDE4 inhibitors described here displayed essentially equal affinity for the full-length form and the catalytic domain of PDE4 (Table 1). Crisaborole displayed IC₅₀ values of 61 nM and 75 nM against the full-length PDE4B1 and the PDE4B2 catalytic domain, respectively, and compd4 showed equivalent activities on the full-length and truncated forms of PDE4B2, with IC₅₀ values

#### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ Value (No. of Independent Experiments)</th>
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<tbody>
<tr>
<td></td>
<td>PDE4B2_cat</td>
</tr>
<tr>
<td>Crisaborole</td>
<td>75 (2)</td>
</tr>
<tr>
<td>Compd2</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Compd3</td>
<td>3.0 (2)</td>
</tr>
<tr>
<td>Compd4</td>
<td>0.32 (10)</td>
</tr>
<tr>
<td>Apremilast</td>
<td>39 (1)</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>0.47 (6)</td>
</tr>
</tbody>
</table>

PDE4B2_cat is the catalytic domain of human PDE4B2; the sequences of the catalytic domain of PDE4B1 and PDE4B2 are identical. The others are full-length human PDE4 enzymes. In the assays, 1.5 μM [³H]-cAMP was used as a substrate. NT, not tested.
of 0.22 nM and 0.32 nM, respectively. Similar to benzoxaborole inhibitors, apremilast and roflumilast displayed equal affinity for full-length PDE4 and its catalytic domain. Thus, crisaborole, compd2, compd3, and compd4 bound to the catalytic domain but did not interact with other regulatory regions of PDE4.

Four PDE4 genes and their alternatively spliced mRNAs encode more than 20 PDE4 proteins (Houssay et al., 2007). The effects of benzoxaboroles on some of these PDE4 isoforms were further evaluated. Crisaborole, compd2, compd3, and compd4 exhibited comparable activity for PDE4A1A, PDE4B1, PDE4B2, PDE4C1, and PDE4D7 (Table 1), which was mirrored by apremilast and roflumilast. Thus, the examined benzoxaborole PDE4 inhibitors, apremilast and roflumilast, were not selective among PDE4 isoforms.

In addition to PDE4, 10 other homologous gene families have been identified in mammals; these genes constitute the PDE superfamily, classified as PDE1–PDE11 (Maurice et al., 2014). These PDE family proteins exhibit selectivity for cAMP or cGMP, and have diverse regulatory mechanisms, subcellular localization, and tissue expression (Maurice et al., 2014). The effects of the tested benzoxaborole PDE4 inhibitors, as well as apremilast and roflumilast, on the 10 PDE families other than PDE4 were explored (Table 2). Apremilast and roflumilast were more selective for PDE4 and displayed no appreciable inhibitory activity on other PDE isoenzymes. Crisaborole, compd2, compd3, and compd4 showed moderate inhibitory activity on PDE enzymes outside the PDE4 family, with IC50 values ranging from 24 to 9400 nM, the majority of which were > 500 nM.

**X-Ray Crystal Structures of Compd4 and PDE4B.** Compd4 was co-crystallized with the human recombinant PDE4B catalytic domain. Similar to the crystallographic structure of compd1 (300J) (Freund et al., 2012), a close analog of crisaborole, the benzoxaborole moiety of compd4 interacted with the two metal ions at the catalytic site in a tetrahedral configuration. The substituted pyridine ring of the benzoxaborole core was tightly sandwiched between Phe446 and Phe414, whereas the nitrile substituent at C(5) of the pyridine ring was positioned to form a key H-bond with Gln443. The 4-(keto)pentyloxy side chain occupied an enclosed hydrophobic pocket formed by Ile410, Tyr233, Asn395, Tyr403, and Pro396. Collectively, these interactions likely contribute to the substantially increased affinity of compd4 (0.32 nM) relative to crisaborole (75 nM) (Fig. 1).

**Cytokine Inhibition.** Cytokines play key roles in initiating, promoting, and maintaining the pathology of psoriasis and AD (Brandt and Sivaprasad, 2011; Biedermann et al., 2004; Brandt and Sivaprasad, 2011; Guttmann-Yassky et al., 2011a; Gittler et al., 2012; Fujita, 2013; Lowes et al., 2014). The levels of TNF-α, IL-23, and IL-17 are elevated in psoriatic lesions, and these cytokines are central to the pathogenesis of this disorder. As shown in Table 3, crisaborole, compd2, compd3, and compd4 potently inhibited LPS-induced TNF-α secretion from human PBMCs, with IC50 values of 170, 24, 3.4, and 0.56 nM, respectively. Crisaborole also blocked TNF-α secretion from monocyte-derived dendritic cells, with an IC50 value of 890 nM; and compd3 reduced TNF-α secretion from human primary monocytes, with an IC50 of 8 nM (Supplemental Table 1A). Crisaborole, compd2, compd3, and compd4 were potent inhibitors of LPS/INF-γ-induced IL-23 secretion from human primary monocytes, with IC50 values of 2400, 32, 7.7, and 73 nM, respectively. In addition, these compounds inhibited IL-17 release from human PBMCs. Similarly, apremilast and roflumilast inhibited TNF-α, IL-23, and IL-17 production. The IC50 values of apremilast against TNF-α, IL-23, and IL-17 were 15, 22, and 1500 nM, respectively, and those of roflumilast were 0.49, 0.39, and 520 nM, respectively.

Th2 cytokines contribute to the pathogenesis of AD (Brandt and Sivaprasad, 2011; Guttmann-Yassky et al., 2011a; Gittler et al., 2012). Therefore, we determined the effects of the benzoxaboroles crisaborole, compd2, compd3, and compd4, as well as those of apremilast and roflumilast, on Th2 cytokine production. Crisaborole inhibited IL-4, IL-5, and IL-13 secretion, with IC50 values of 480, 2000, and 22,000 nM, respectively (Table 3). Compd3 and

<table>
<thead>
<tr>
<th>PDE Isozyme</th>
<th>IC50 Value</th>
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<tbody>
<tr>
<td></td>
<td>Crisaborole</td>
</tr>
<tr>
<td>PDE1A1</td>
<td>a</td>
</tr>
<tr>
<td>PDE1B</td>
<td>3700</td>
</tr>
<tr>
<td>PDE1C</td>
<td>b</td>
</tr>
<tr>
<td>PDE2A1</td>
<td>6700</td>
</tr>
<tr>
<td>PDE3A</td>
<td>5100</td>
</tr>
<tr>
<td>PDE3B</td>
<td>4500</td>
</tr>
<tr>
<td>PDE4A1</td>
<td>c</td>
</tr>
<tr>
<td>PDE5C</td>
<td>6700</td>
</tr>
<tr>
<td>PDE7A1</td>
<td>c</td>
</tr>
<tr>
<td>PDE7B</td>
<td>9400</td>
</tr>
<tr>
<td>PDE8A1</td>
<td>c</td>
</tr>
<tr>
<td>PDE9A2</td>
<td>c</td>
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<tr>
<td>PDE10A1</td>
<td>c</td>
</tr>
<tr>
<td>PDE10A2</td>
<td>c</td>
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</table>

A fluorescent polarization assay was performed using full-length human PDE enzymes and 0.1 μM FAM-cAMP or FAM-cGMP as a substrate. FAM, 5-(and-6)-carboxyfluorescein.

"54% inhibition at 10 μM.

"80% inhibition at 10 μM.

"Less than 50% inhibition at 10 μM. For apremilast and roflumilast, inhibition was < 50% at 10 μM for all PDE isozymes.
compd4 were more potent than crisaborole in reducing the levels of IL-4, IL-5, and IL-13 (Table 3). These inhibitory properties were not unique to benzoxaborole PDE4 inhibitors, because apremilast and roflumilast also suppressed IL-4 and IL-5 secretion (Table 3).

Psoriatic lesions have increased numbers of Th1 cells, which produce high amounts of IFN-γ (Austin et al., 1999; Biedermann et al., 2004). IFN-γ is also significantly upregulated in chronic AD lesions (Gittler et al., 2012). Crisaborole, compd2, compd3, and compd4, as well as apremilast and roflumilast, potently blocked PHA-induced IL-2 and IFN-γ release from human PBMCs, with IC₅₀ values ranging from 0.10 to 700 nM (Table 3). IL-22 is upregulated in both psoriasis and AD skin lesions, and this cytokine promotes keratinocyte hyperplasia and Th2 polarization in AD (Fujita, 2013; Lowes et al., 2014). Compd2, compd3, compd4, apremilast, and roflumilast inhibited anti-CD2/CD3/CD28-induced IL-22 secretion, with IC₅₀ values of 51, 14, 1.8, 86, and 5.9 nM, respectively (Table 3).

The relationship between inhibition of PDE4 and suppression of cytokine secretion was explored by correlating the IC₅₀ for PDE4 inhibition with the IC₅₀ for cytokine suppression by up to 183 benzoxaborole and nonbenzoxaborole PDE4 inhibitors. A strong relationship would be shown by a slope close to 1 when plotted with log (IC₅₀ cytokine) versus log (IC₅₀ PDE4).

Inhibition of PDE4 enzymatic activity was strongly correlated with the suppression of LPS-induced TNF-α and IL-23, as well as PHA-stimulated IFN-γ, IL-2, and IL-5, with slopes of 0.70–0.80 (Supplemental Table 2). These results indicate that PDE4 is critical in the LPS and PHA signaling pathways that lead to the synthesis of these cytokines. There was a weak relationship between inhibition of PDE4 and suppression of concanavalin A–induced IL-17, IL-4, and IL-13 secretion, with slopes of 0.043–0.33 (Supplemental Table 2).

**Effect of Compd3 on the Phosphorylation of CREB and ERK1/2 in Human Primary Leukocytes.** Inhibiting PDE4 increases the intracellular level of cAMP, which then activates PKA (Giembycz et al., 1996; Torphy, 1998). PKA activation subsequently leads to the phosphorylation of target proteins, some of which are involved in the control of cytokine production (Supplemental Fig. 4), such as CREB (Gonzalez and Montminy, 1989; Wen et al., 2010), nuclear factor-κB (NF-κB) (Hou et al., 2003; Kwak et al., 2005), nuclear factor of activated T cells (Chow and Davis, 2000), Rap1 (Stork and Dillon, 2005), and Csk (Moseniden and Taskén, 2011).

In HL-60 promyelocytic leukemia cells, increasing the cAMP level prevents cytokine production, and this effect is attributed to the suppression of NF-κB activity by increased phospho-CREB (Parry and Mackman, 1997). To explore whether benzoxaborole PDE4 inhibitors use the same mechanism to inhibit LPS-induced cytokine production, the effect of compd3 on CREB phosphorylation in human primary monocytes was assessed. As shown in Fig. 2A, compd3 increased the phosphorylation of CREB in cells compared with LPS stimulation alone, and the same effect was observed in cells treated with dBcAMP. These results indicate that suppression of LPS-induced cytokines by a PDE4 inhibitor is at least partially mediated by increasing the phospho-CREB level in human monocytes. However, compd3 had no effect on anti-CD3/CD28–induced CREB phosphorylation in human primary T cells (data not shown).

TABLE 3
Effect of crisaborole, compd2, compd3, compd4, apremilast, and roflumilast on cytokine secretion from human leukocytes in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ Value (No. of Independent Experiments)</th>
<th>nM</th>
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<tbody>
<tr>
<td></td>
<td>TNF-α¹</td>
<td>IL-23¹</td>
</tr>
<tr>
<td>Crisaborole</td>
<td>170 (14)</td>
<td>2400 (3)</td>
</tr>
<tr>
<td>Compd2</td>
<td>24 (3)</td>
<td>32 (2)</td>
</tr>
<tr>
<td>Compd3</td>
<td>3.4 (8)</td>
<td>7.7 (6)</td>
</tr>
<tr>
<td>Compd4</td>
<td>0.56 (13)</td>
<td>73 (2)</td>
</tr>
<tr>
<td>Apremilast</td>
<td>15 (8)</td>
<td>22 (3)</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>0.49 (7)</td>
<td>0.39 (2)</td>
</tr>
</tbody>
</table>

NT, not tested.

¹LPS stimulation for 24 hours.
²LPS/IFN-γ stimulation for 24 hours.
³Concanavalin A stimulation for 48 hours.
⁴PHA-L stimulation for 48 hours.
⁵Anti-CD2/CD3/CD28 stimulation for 24 hours.
the expression and stability of c-Fos, a subunit of the activator protein 1 (AP-1) complex, and inhibiting ERK activity results in a reduction in AP-1–mediated cytokine transcription (Schade and Levine, 2004). Because inhibition of PDE4 increases the cAMP level in T cells, we assessed the effect of compd3 on ERK phosphorylation in human primary T cells. As shown in Fig. 2B, ERK was phosphorylated in activated T cells, and treatment with compd3 or dBcAMP reduced anti-CD3/CD28–induced ERK phosphorylation. Thus, the suppression of ERK phosphorylation in T cells is at least partially responsible for the inhibition of T cell receptor–mediated cytokine production by compd3 (Supplemental Table 1B).

**Effect of Topical Benzoxaborole PDE4 Inhibitors on Acute Contact Dermatitis.** To extend our in vitro observations, we studied the in vivo effect of benzoxaborole PDE4 inhibitors on the production of cytokines associated with AD and psoriasis during skin inflammatory reactions to contact irritants. Contact irritation induced by PMA is a widely used acute contact dermatitis model. Topical application of PMA triggers prominent neutrophil migration and macrophage activation in the skin. TNF-α, IL-6, and IL-1β, along with proinflammatory lipids such as prostaglandin E2 and leukotriene B4, are induced by topical PMA (Raederstorff et al., 1996; Jang and Pezzuto 1998; Dong et al., 2013). We examined the effect of compd3 on the mRNA levels of IL-23, IL-17F, IL-13, and IL-22 in this model. Challenge with topical PMA upregulated the transcription of IL-23, IL-17F, IL-13, and IL-22 in mouse skin; however, the mRNA levels of IL-4 and IL-5 were not significantly increased by PMA. The mRNA levels of IL-23, IL-17F, IL-13, and IL-22 were low, with cycle threshold values of 32, 33, 34, and 32, respectively. The cycle threshold values of induced IL-6 and the housekeeping gene hypoxanthine phosphoribosyltransferase 1 were 28 and 25, respectively. The levels of PMA-induced IL-23, IL-17F, IL-13, and IL-22 mRNA were low but significant. In accordance with the low mRNA level, we were not able to detect IL-22 protein in PMA-treated ear punches. Compd3 dose-dependently inhibited PMA-induced IL-22 and IL-23 transcription in mouse skin (Supplemental Fig. 2), and 3 mg compd3 completely blocked the upregulation of IL-23, IL-17F, IL-13, and IL-22 mRNA in mouse ears (Fig. 3A). The topical benzoxaborole PDE4 inhibitors crisaborole, compd2, and compd3 significantly suppressed PMA-induced ear swelling in this model: 0.3 mg of these compounds reduced the thickness of

![Fig. 2. Compd3 increased CREB phosphorylation in human monocytes and decreased ERK1/2 phosphorylation in human T cells. (A) CREB phosphorylation in nuclear extract from LPS-stimulated human monocytes. (B) ERK1/2 phosphorylation in anti-CD3/CD28–stimulated human T cells. stim, stimulated; unstim, unstimulated.](image)

![Fig. 3. Effect of compd3 on cytokine production in mouse models. (A) Compd3 inhibited PMA-induced cytokine transcription in mouse skin. (B) Crisaborole, compd2, and compd3 decreased PMA-induced mouse ear swelling. (C and D) Compd3 reduced calcipotriol-induced TSLP expression in mouse skin. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Dex, dexamethasone.](image)
PMA-treated ears by 40%, 68%, and 46%, respectively, and 1 mg decreased ear swelling by 46%, 78%, and 72%, respectively (Fig. 3B).

Compd3 Inhibited Calcipotriol-Induced TSLP Expression. Increased expression of TSLP is observed in skin lesions of patients with AD (Soumelis et al., 2002; Sano et al., 2013). Mice overexpressing TSLP in keratinocytes develop AD-like symptoms (Li et al., 2005; Yoo et al., 2005). TSLP promotes Th2 polarization (Ziegler and Artis, 2010), and injection of TSLP into mice evokes scratching, indicating that TSLP is an itch mediator (Wilson et al., 2013). To address the effect of a PDE4 inhibitor on TSLP expression, we developed a calcipotriol-induced TSLP murine model based on the studies by Li et al. (2005, 2006) with a shorter calcipotriol treatment time. Topical calcipotriol induced TSLP expression in mouse ears in a dose- and time-dependent manner (Supplemental Fig. 3). Treatment with compd3 significantly inhibited TSLP production at both mRNA and protein levels in a dose-dependent manner: 0.03, 0.3, or 3 mg compd3 reduced TSLP protein levels by 66%, 96%, and 99% (Fig. 3C) and TSLP mRNA levels by 59%, 95%, and 99% (Fig. 3D), respectively.

Skin Thinning. Skin atrophy is a major adverse effect of topical glucocorticoids, which are mainstays of therapy for both AD and psoriasis. We thus investigated the ability of a benzoxaborole PDE4 inhibitor to cause skin thinning.

An in vitro competition binding assay was conducted to assess the ability of compd3 to interact with glucocorticoid receptors. Dexamethasone, progesterone, and clobetasol bound to the glucocorticoid receptor with IC50 values of 4.64, 240, and 1.35 nM, respectively. However, compd3 at up to 10 μM was unable to compete with dexamethasone for binding to the glucocorticoid receptor.

The effect of compd3 on mouse skin was further investigated using hairless SKH-1 mice. The mice were topically treated with 30 μl 1% compd3 for 16 consecutive days, and no significant change in dorsal skin thickness was observed at the end of treatment (Fig. 4A). By contrast, 30 μl 0.25% dexamethasone and 30 μl 0.05% clobetasol significantly decreased skin thickness by 41.5% and 19.3%, respectively, on day 16 (Fig. 4A). Histopathologic assessment of the dorsal skin collected on day 16 also indicated that treatment with compd3 did not significantly alter the thickness of the epidermis or dermis compared with vehicle treatment. By contrast, dexamethasone significantly reduced the thickness of the epidermis and dermis, and clobetasol moderately decreased epidermal and dermal thicknesses (Fig. 4, B and C).

Discussion

Several benzoxaborole PDE4 inhibitors, including crisaborole, were characterized for their PDE selectivity and effect on cytokines associated with skin inflammation and architecture. crisaborole has shown clinical benefit in phase 2 (Murrell et al., 2015; Stein Gold et al., 2015) and phase 3 (data on file, Anacor Pharmaceuticals, Inc., 2015) studies for patients with mild-to-moderate AD. Eleven PDE families have been identified in mammals. PDE4, PDE7, and PDE8 selectively hydrolyze cAMP; PDE5, PDE6, and PDE9 specifically hydrolyze cGMP; and PDE1, PDE2, PDE3, PDE10, and PDE11 cleave both cAMP and cGMP (Maurice et al., 2014). These PDEs differ in their regulatory mechanisms and tissue and cellular distributions. PDE4 is the major PDE family in primary leukocytes, rendering these cells susceptible to PDE4 inhibition (Torphy, 1998). Thus, PDE4 is an important therapeutic target for inflammatory and autoimmune diseases. Four benzoxaboroles (crisaborole, compd2, compd3, and compd4) were potent PDE4 inhibitors, and most of them displayed an IC50 value < 10 nM for PDE4 (Table 1). These compounds also displayed moderate activities against other PDE isozymes, with IC50 values generally in the micromolar range (Table 2). Roflumilast and apremilast were more selective for PDE4 than the examined benzoxaboroles and had no activity on other PDE isoforms. The additional activities of benzoxaboroles on PDEs aside from PDE4 may enhance their Fig. 4. Effect of compd3 on mouse skin thickness. (A) Thickness of mouse dorsal skin after repeated topical application of compd3, clobetasol, or dexamethasone. (B) Quantitation of epidermal and dermal thickness on day 16 after consecutive treatment with compd3, clobetasol, or dexamethasone. (C) Hematoxylin and eosin staining of mouse dorsal skin on day 16 after topical treatment with compd3, clobetasol, or dexamethasone. ***P < 0.001; ****P < 0.0001. n.s., not significant.
anti-inflammatory effect, since other PDEs (e.g., PDE1 and PDE7) are involved in regulating immune cell functions (Kanda and Watanabe, 2001; Smith et al., 2004).

PDE4 family proteins contain a C-terminal catalytic domain and N-terminal regulatory regions termed UCR1 and UCR2. Regulatory proteins bind to or modify these UCR domains to modulate PDE4 catalytic activity (Torphy, 1998; Maurice et al., 2014). Some PDE4 inhibitors, such as rolipram, RS25344 [1-(3-nitrophenyl)-3-(4-pyridymethyl)pyridine(2,3-dipyrimidin-2,3(1H,3H)-dione), and PMNQP [6-(4-pyridymethyl)-8-(3-nitrophenyl)quinoline], bind to both the catalytic domain and the UCR2 and exert their actions by not only regulating catalytic activity but also modifying the functions of other domains (Saldou et al., 1998; Burgin et al., 2010). Crisaborole, compd2, compd3, and compd4 displayed similar activity for full-length PDE4 and its catalytic domain (Table 1), suggesting that these benzoxaboroles bind only to the catalytic domain and unlikely interact with the UCR domains of PDE4. Therefore, the effects of these compounds were mediated by inhibiting catalytic activity and elevating the cAMP levels, not by regulating the functions of other PDE4 regions.

The crystallography of PDE4 complexed with compd4 (Fig. 1) was determined in part to understand the mechanism underlying the increased affinity of compd4 to PDE4 compared with that of crisaborole (Table 1). Similar to crisaborole (unpublished observations) and its analog compd1 (Freund et al., 2012), compd4 interacted with the catalytic bimetal of PDE4 via the oxaborole group. The side chain of compd4 occupied a hydrophobic pocket in the catalytic domain, which increases the affinity of compd4 to PDE4 (Supplemental Fig. 1). The catechol PDE4 inhibitor roflumilast displayed equal affinity for PDE4 as compd4 (Table 1), and the crystal structure of roflumilast (1XOQ) (Card et al., 2004) shows that the difluoromethyl moiety fills the same hydrophobic pocket as the compd4 side chain. Thus, this interaction is important for the high affinities of roflumilast and compd4 for PDE4. In contrast with benzoxaborole PDE4 inhibitors, roflumilast does not directly interact with the bimetal center of PDE4. Because all PDEs have the bimetal center, the interaction of boron with the bimetal center contributes to the weak inhibition of benzoxaboroles to other PDEs.

Several cytokines are associated with skin inflammatory diseases. IL-23, IL-17, and TNF-α drive the pathology of psoriasis (Lowes et al., 2014), and elevation of Th2 cytokines is important in acute AD (Guttman-Yassky et al., 2011b; Martin et al., 2013). PDE4 inhibitors also blocked IL-22 secretion from human leukocytes (Table 3). These findings suggest that blockade of PDE4 would reduce IL-22-mediated epidermal hyperplasia in psoriasis and AD.

Itch is a major symptom of AD (Guttman-Yassky et al., 2011a). TSLP evokes itch and is increased in AD skin lesions (Sowemil et al., 2002; Sano et al., 2013). Injection of TSLP into mice triggers acute itch by directly activating sensory neurons (Wilson et al., 2013). TSLP also promotes Th2 polarization and initiates the Th2 phenotype, which is associated with allergic inflammation (Sowemil et al., 2002; Ziegler and Artis, 2010). Topical compd3 dose-dependently suppressed calcipotriol-induced TSLP expression in mouse skin (Fig. 3, C and D), suggesting that PDE4 inhibitors may inhibit TSLP expression in patients with AD and providing a potential explanation for pruritus suppression observed upon treatment with crisaborole (Murrell et al., 2015).

Benzoxaborole PDE4 inhibitors blocked Toll-like receptor-mediated TNF-α secretion from human PBMCs, monocytes, and monocyte-derived dendritic cells (Table 3; Supplemental Table 1). NF-κB is a transcription activator that modulates TNF-α synthesis. Optimal activity of NF-κB requires the association of its p65 subunit with CBP/p300 at the KIX region, to which phospho-CREB also binds (Fraser et al., 2007). In HL-60 promyelocytic leukemia cells, CAMP increases the phosphorylation of CREB, and phospho-CREB competes with NF-κB for a limited amount of CREB binding protein (CBP)/p300, thus inhibiting TNF-α synthesis (Parry and Mackman, 1997). Compd3 or dBCAMP increased CREB phosphorylation in LPS-stimulated human monocytes (Fig. 2A), and this effect likely reduces the association of p65 with CBP/p300 and blocks NF-κB-mediated TNF-α transcription.

Dysregulation of T cell–mediated immune responses drives the pathogenesis of psoriasis and AD (Leung et al., 2004; Guttman-Yassky et al., 2011b; Lowes et al., 2013). Optimal T-cell activation requires the CD28 costimulatory signal, which recruits the protein kinase B/β-arrestin/PDE4 complex to the proximity of plasma membrane and allows PDE4 to hydrolyze cAMP to overcome the inhibitory signal elicited by cAMP (Bjørgo et al., 2010). Increase of cAMP and activation of PKA as results of PDE4 inhibition directly or indirectly modulate the activities of NF-κB, AP-1, and nuclear factor of activated T cells, which regulate transcription of cytokines in T cells (Supplemental Fig. 4) (Torgersen et al., 2002). PKA phosphorylates Rap1 (Takahashi et al., 2013), and phospho-Rap1 suppresses the association of Ras with Raf-1 and inhibits the activation of ERK (Dumaz and Marais, 2005; Stork and Dillon, 2005; Kortum et al., 2013). ERK phosphorylates several proteins, including Elk-1, a transcription factor involved in c-Fos transcription (Karim, 1995). ERK also regulates the stability of c-Fos in T cells (Schade and Levine, 2004). Inhibition of PDE4 by compd3 or the addition of dBCAMP reduced the phosphorylation of ERK in activated T cells (Fig. 2B), and decreasing ERK activity results in reduced c-Fos expression and inhibition of AP-1–mediated cytokine production. Thus, a decrease in ERK phosphorylation at least
partially contributes to the suppression of cytokine expression by PDE4 inhibitors in human activated T cells.

Topical glucocorticoids are the first-line treatment of mild-to-moderate psoriasis and AD (Menter, et al., 2009; Ring, et al., 2012). Skin atrophy is one side effect preventing the long-term topical application of glucocorticoids, especially to sensitive skin regions (Schoepf et al., 2006; Ring, et al., 2012). A hairless mouse model is considered to be predictive of human skin responses and is used to evaluate the effect on skin atrophy (Schoepf et al., 2006). Topical dexamethasone or clobetasol resulted in substantial skin thinning, but repeated application of compd3 did not significantly affect skin thickness (Fig. 4A). These effects were confirmed by histopathologic evaluation of skin biopsies, in which dexamethasone and clobetasol, but not compd3, reduced epidermal and dermal thicknesses (Fig. 4B and C). These results indicate that long-term topical application of benzoxaborole PDE4 inhibitors has low potential to cause skin atrophy.

These studies demonstrated that benzoxaborole PDE4 inhibitors crisaborole, compd2, compd3, and compd4 preferentially inhibited PDE4. As exemplified by compd4, benzoxaborole PDE4 inhibitors directly interact with the bimetal center using the boron atom as a phosphomimetic, and compd4 gains additional affinity by binding to the adenine pocket of PDE4. These benzoxaborole PDE4 inhibitors suppressed TNF-α, IL-23, IL-17, IFN-γ, IL-2, IL-4, IL-5, IL-13, and IL-22 secretion from human leukocytes. This inhibitory effect is partially attributed to the altered phosphorylation of CREB and ERK and the subsequent reduction in the transcriptional activities of NF-κB and AP-1. Compd3 penetrated mouse skin, diminished skin inflammation, and decreased PMA-induced IL-13, IL-17F, IL-22, and IL-23 transcription and calcipotriol-induced TSLP expression. Repeated topical application of compd3 did not induce skin atrophy, in contrast with the significant effect of glucocorticoids. Benzoxaborole PDE4 inhibitors such as crisaborole could serve as a non-steroidal topical treatment of skin inflammatory diseases, including AD and psoriasis.

Authorship Contributions

Participated in research design: Dong, Rock, Jarnagin.

Conducted experiments: Dong, Virtucio, Zem ska, Baltazar, Baia, Jones-Iatauro, Sexton, Martin, Dee, Mak, Meewan.

Contributed new reagents or analytical tools: Akama.

Performed data analysis: Dong, Virtucio, Zem ska, Baltazar, Zhou, Baia, Jones-Iatauro, Dee, Mak, Meewan, Jarnagin.

Wrote or contributed to the writing of the manuscript: Dong, Zem ska, Jarnagin.

References


Jones-Iatauro, Sexton, Martin, Dee, Mak, Meewan.


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Treatment of skin inflammation with benzoxaborole PDE inhibitors: selectivity, cellular activity, and effect on cytokines associated with skin inflammation and skin architecture changes

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Figure S1. Structures of benzoxaborole and catechol PDE4 inhibitors and their IC50s for PDE4B2 catalytic domain.

X-ray crystallography. The human recombinant PDE4B catalytic domain (amino acids 152–484) was used in the crystallization process as described previously (Freund et al., 2012). The protein was 95% pure as evaluated by SDS-PAGE with Coomassie blue staining. Crystals of compd4 complexed with PDE4 were obtained from the protein-ligand solution (14 mg/mL PDE4, 1 mM MgCl2, and 2 mM compd4). Crystals were cryoprotected with 100% well solution before flash freezing. The phase information used to analyze the structure was obtained by molecular replacement with a previously solved structure of PDE4B (Freund et al., 2012) as the search model. The crystals belong to space group P 43 21 2. The model included residues Asn162 to Ile484 and was 99% complete with a resolution of 1.86 Å. The final structure showed r.m.s. deviation from ideal geometry of 0.007 Å for bond length, 0.97° for bond angle, an Rcryst of 20.5%, and an Rfree of 23.5%. The Ramachandran plot of the final model showed 93.5% of all residues in the most favored region, 6.5% in the additionally allowed region, and no residues in the disallowed region. Notably, based on refined temperature factors, 80% occupancy of the ligand was chosen. The electron density in the boron-containing heterocyclic ring was weaker than that in the rest of the ligand. This structure is available in the Protein Data Bank under code 5K6J.
Inhibition of cytokines from human monocytes, monocyte-derived dendritic cells and CD4+ T cells. The inhibitory effect of benzoxaborole PDE4 inhibitors on LPS-induced TNF-α secretion was further examined using purified human monocytes and monocyte-derived dendritic cells (MDDCs) for comparison to the effects of these compounds on TNF-α secretion from human PBMCs. Crisaborole showed similar activity on LPS/IFN-γ-induced TNF-α secretion from human MDDCs, and compd3 displayed a similar IC50 against LPS-induced TNF-α release from human primary monocytes (Table S1A). Purified human CD4+ T cells stimulated with anti-CD3/CD28 produced significant amounts of IL-2, IFN-γ, IL-4 and IL-5, and compd3 and compd4 inhibited the secretion of these cytokines (Table S1B), indicating that PDE4 plays an important role in TCR-mediated cytokine production in human T cells.

Table S1. Inhibition of cytokine production from human monocytes, MDDCs and CD4+ T cells.

A. Inhibition of TNF-α secretion from different types of cells in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nM)</th>
<th>PBMCa</th>
<th>monocyteb</th>
<th>MDDCc</th>
</tr>
</thead>
<tbody>
<tr>
<td>crisaborole</td>
<td>170</td>
<td>N</td>
<td>14</td>
<td>NT</td>
</tr>
<tr>
<td>compd3</td>
<td>3.4</td>
<td>8</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

B. Inhibition of IL-2, IFN-γ, IL-4 and IL-5 secretion from anti-CD3/CD28-stimulated human CD4+ T cellsd

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nM)</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>compd3</td>
<td>90</td>
<td>3</td>
<td>320</td>
<td>2</td>
<td>3,000</td>
</tr>
<tr>
<td>compd4</td>
<td>&lt;0.1</td>
<td>4</td>
<td>19</td>
<td>3</td>
<td>280</td>
</tr>
</tbody>
</table>

A: human PBMCs were stimulated with LPS for 24 h; b: purified human monocytes were stimulated with LPS and IFN-γ for 24 h; c: human monocytes were differentiated with GM-CSF and IL-4 for 6 days, and the generated MDDCs were stimulated with LPS and IFN-γ for 24 h; d: human CD4+ T cells were stimulated with anti-CD3/CD28 for 48 h.

Relationship between PDE4 inhibition and suppression of cytokine secretion. Up to 183 PDE4 inhibitors, including benzoxaboroles and non-benzoxaboroles such as rolipram, apremilast and roflumilast, were analyzed to determine the correlation of their affinities for PDE4 with their effects on cytokine production from human PBMCs. A plot of the Log(IC50 cytokine) and Log(IC50 PDE4) was well fit by a linear model. The strength of this relationship was measured by calculating the slope of the linear function, and the statistical merit of the linear model was judged based on the correlation coefficient. A slope close to 1 indicates a very tight association of PDE4 inhibition with cytokine suppression. As presented in Table S2, inhibition of PDE4 correlated well with the suppression of LPS-induced TNF-α and IL-23 release and PHA-induced IFN-γ, IL-2, IL-5 secretion, as indicated by the slopes of 0.70-0.80 and R² of 0.52-0.70. In contrast, inhibition of PDE4 showed weak connection with the ConA-induced IL-4, IL-13 and IL-17 production, with slopes of 0.043-0.33 and R² of 0.011-0.10.
Table S2. Correlation between suppression of cytokine secretion and PDE4 inhibition.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell</th>
<th>Stimulation</th>
<th>Slope (Log(ICI50(cytokine))/ Log(ICI50(PDE4))</th>
<th>Correlation (R²)</th>
<th>Number of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>PBMC</td>
<td>LPS, 24 h</td>
<td>0.76</td>
<td>0.70</td>
<td>183</td>
</tr>
<tr>
<td>IL-2</td>
<td>PBMC</td>
<td>PHA, 24 h</td>
<td>0.70</td>
<td>0.65</td>
<td>86</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>PBMC</td>
<td>PHA, 24 h</td>
<td>0.77</td>
<td>0.63</td>
<td>85</td>
</tr>
<tr>
<td>IL-5</td>
<td>PBMC</td>
<td>PHA, 48 h</td>
<td>0.80</td>
<td>0.52</td>
<td>68</td>
</tr>
<tr>
<td>IL-23</td>
<td>Monocyte</td>
<td>LPS/ IFN-γ, 24h</td>
<td>0.77</td>
<td>0.40</td>
<td>28</td>
</tr>
<tr>
<td>IL-17</td>
<td>PBMC</td>
<td>ConA, 48 h</td>
<td>0.33</td>
<td>0.10</td>
<td>32</td>
</tr>
<tr>
<td>IL-4</td>
<td>PBMC</td>
<td>ConA, 48 h</td>
<td>0.12</td>
<td>0.045</td>
<td>39</td>
</tr>
<tr>
<td>IL-13</td>
<td>PBMC</td>
<td>ConA, 48 h</td>
<td>0.043</td>
<td>0.011</td>
<td>36</td>
</tr>
</tbody>
</table>

Compd3 dose-dependently inhibited PMA-induced IL-22 and IL-23 transcription in mouse skin. Twenty microliters of 0.25 mg/mL PMA dissolved in 95% ethanol were topically applied to mouse ears. Compd3 dissolved in glycofurol:acetone:ethanol (1:2:2) was applied to the same areas 30 min before and 15 min after PMA challenge. After 6 h, the mouse ears were collected, and the mRNA levels of IL-22 and IL-23 in the ear samples was quantified by RT-PCR. Six mice were included in the PMA-only group, and 13 mice were included in each of the compd3 treated groups. Statistical significance of the results was analyzed based on comparison to the untreated controls using the Kruskal-Wallis non-parametric rank test followed by Dunn’s multiple comparison test to calculate multiplicity-adjusted p-values.

Figure S2. Compd3 dose-dependently inhibited PMA-induced IL-22 and IL-23 transcription in mouse skin. *P < 0.05; ***P < 0.001; ****P < 0.0001.

Time- and dose-dependent induction of TSLP by calcipotriol in mouse ear skin. This experiment was modified from the protocol described by Li et al. (2006, 2007), with only 1-2 days of treatment with calcipotriol. The ears of CD-1 mice, 6 per group, were treated with 0.4 nmol or 4 nmol of calcipotriol in ethanol once a day for either 1 or 2 days. At 24 h after the final
calcipotriol application, the ears were collected, and the TSLP mRNA and protein levels were measured by RT-PCR and ELISA, respectively. In comparison to the untreated mice, the mice treated with 0.4 nmol of calcipotriol for 1 day displayed a 9.2-fold increase in TSLP mRNA expression and a 4.3-fold increase in TSLP protein expression; the mice that received 4 nmol of calcipotriol for 1 day displayed a 63-fold increase in TSLP mRNA expression and a 210-fold increase in TSLP protein expression. Treatment with 0.4 nmol or 4 nmol of calcipotriol for 2 days resulted in even higher induction of TSLP: 17-fold and 540-fold increases in mRNA expression, respectively, and 51-fold and 1600-fold increases in protein expression, respectively. Based on these results, a single application of 4 nmol of calcipotriol was selected for subsequent experiments evaluating the effect of compd3 on TSLP expression. Statistical significance of the results was analyzed based on comparison to the untreated controls using the Kruskal-Wallis non-parametric rank test followed by Dunn’s multiple comparison test to determine multiplicity-adjusted p-values.

**Figure S3.** Time- and dose-dependent induction of TSLP by calcipotriol in mouse ear skin. TSLP mRNA (A) and protein (B) expression in mouse ear skin. *P < 0.05; **P <0.01; ***P < 0.001; ****P < 0.0001.

**Effect of PDE4 inhibitors on cAMP/PKA-mediated intracellular signaling pathways that lead to cytokine production in human monocytes and T cells.** Inhibition of PDE4 increases the intracellular levels of cAMP, which further activates PKA (Giembycz, et al., 1996; Torphy, 1998). PKA activation leads to the phosphorylation of target proteins such as CREB (Gonzalez and Montminy, 1989) and NFAT (Chow and Davis, 2000). Phospho-CREB reduces the activity of NF-κB by competing with NF-κB for CBP/p300 (Parry and Mackman, 1997; Wen et al., 2010). NF-κB is a transcriptional regulator of TNF-α, IL-23 and other inflammatory cytokines, and inhibition of NF-κB results in the reduction of LPS-induced TNF-α and IL-23 secretion from human monocytes.

Ras/Raf-1 activates the downstream effector MEK, which then phosphorylates ERK (Stork and Dillon, 2005). Active ERK phosphorylates several proteins, including Elk-1, a transcription factor involved in c-Fos transcription (Karin, 1995; Schade and Levine, 2004). c-Fos is a component of the transcription factor complex AP-1, which regulates the TCR-mediated transcription of cytokines such as IL-2 and IFN-γ. Increasing the level of cAMP (Stork and Dillon, 2005; Grader-Beck et al., 2003) via PDE4 inhibition activates PKA, and PKA further phosphorylates Rap-1 and subsequently inhibits the association of Ras with Raf-1 and reduces
MEK/ERK activation. Decreasing ERK activity reduces AP-1-mediated transcription of cytokines such as IL-2 and IFN-γ in human T cells.

PKA can also directly phosphorylate NFAT, which promotes the translocation of NFAT from the nucleus to the cytosol (Chow and Davis, 2000), therefore, blocks NFAT-mediated cytokine production in T cells.

Figure S4. Scheme of the signaling pathways by which a PDE4 inhibitor (PDE4i) suppresses cytokine production in monocytes and T cells.