CHF6001 I: A Novel Highly Potent and Selective Phosphodiesterase 4 Inhibitor with Robust Anti-Inflammatory Activity and Suitable for Topical Pulmonary Administration

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ABSTRACT

This study examined the pharmacologic characterization of CHF6001 [((S)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl)pyridin-1-oxide], a novel phosphodiesterase (PDE) 4 inhibitor designed for treating pulmonary inflammatory diseases via inhaled administration. CHF6001 was 7- and 923-fold more potent than roflumilast and cilomilast, respectively, in inhibiting PDE4 enzymatic activity (IC50 = 0.026 ± 0.006 nM). CHF6001 inhibited PDE4 isoforms A-D with equal potency, showed an elevated ratio of high-affinity rolipram binding site versus low-affinity rolipram binding site (i.e., >40) and displayed >20,000-fold selectivity versus PDE4 compared with a panel of PDEs. CHF6001 effectively inhibited (subnanomolar IC50 values) the release of tumor necrosis factor-alpha from human peripheral blood mononuclear cells, human acute monocytic leukemia cell line macrophages (THP-1), and rodent macrophages (RAW264.7 and NR8383). Moreover, CHF6001 potently inhibited the activation of oxidative burst in neutrophils and eosinophils, neutrophil chemotaxis, and the release of interferon-gamma from CD4+ T cells. In all these functional assays, CHF6001 was more potent than previously described PDE4 inhibitors, including roflumilast, UK-500,001 [2-(3,4-difluorophenoxy)-5-fluoro-N-((1S,4S)-4-(2-hydroxy-5-methylbenzamido)cyclohexyl)nicotinamide], and cilomilast, and it was comparable to GSK256066 [6-[(3-(dimethylcarbamoyl)phenyl)sulfonyl]-4-[(3-methylphenoxy)phenyl]-8-methylquinoline-3-carboxamide]. When administered intratracheally to rats as a micronized dry powder, CHF6001 inhibited liposaccharide-induced pulmonary neutrophilia (ED50 = 0.205 μmol/kg) and leukocyte infiltration (ED50 = 0.188 μmol/kg) with an efficacy comparable to a high dose of budesonide (1 μmol/kg i.p.). In sum, CHF6001 has the potential to be an effective topical treatment of conditions associated with pulmonary inflammation, including asthma and chronic obstructive pulmonary disease.

Introduction

Phosphodiesterases (PDEs) form a superfamil of at least 11 intracellular isoenzymes that are involved in the modulation of signal transduction processes via the degradation of cyclic nucleotides (cAMP and/or cGMP) (Bender and Beavo, 2006). Type 4 cyclic nucleotide phosphodiesterases (PDE4s) are a family of cAMP-specific PDEs encoded by four genes (PDE4A, PDE4B, PDE4C, and PDE4D) sharing a highly conserved catalytic domain and abundantly expressed in leukocytes (Srivani et al., 2008). By increasing intracellular Ca2+ levels, PDE4 inhibitors show a broad spectrum of anti-inflammatory effects in almost all cells of the immune system. In particular, PDE4 is a major player in regulating proinflammatory cellular functions, such as proliferation and cytokine secretion, chemotaxis, degranulation, antibody IgE release and generation of lipid mediators (Spina, 2003). PDE4 regulates also the function of several structural cells that control lung functions such as airway smooth muscle, airway epithelium, vascular endothelium, and

ABBREVIATIONS: AWD 12-281, N-(3,5-dichloropyridin-4-yl)-2-(1-(4-fluorobenzyl)-5-hydroxy-1-indol-3-yl)-2-oxoacetamide; BALF, bronchoalveolar lavage fluid; CHF6001, (S)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl)pyridin-1-oxide; COPD, chronic obstructive pulmonary disease; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IMLP, N-formyl-L-leucyl-1-leucyl-phenylalanin; GSK256066, 6-[(3-(dimethylcarbamoyl)phenyl)sulfonyl]-4-[(3-methylphenoxy)phenyl]-8-methylquinoline-3-carboxamide; HARBS, high-affinity rolipram binding site; IFN-g, interferon-gamma; LARBS, low-affinity rolipram binding site; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PDE, phosphodiesterase; ROS, reactive oxygen species; TNF-alpha, tumor necrosis factor-alpha; UK-500,001, 2-(3,4-difluorophenoxy)-5-fluoro-N-((1S,4S)-4-(2-hydroxy-5-methylbenzamido)cyclohexyl)nicotinamide.
and airway sensory nerves (Fan Chung, 2006). This body of evidence, together with a large body of evidence supporting potent anti-inflammatory activity of PDE4 inhibitors in various experimental models, including models of pulmonary inflammation (Hatzelmann et al., 2010), has generated a considerable interest in targeting PDE4 in chronic inflammatory and obstructive airway diseases (Spina, 2003). Clinical investigations of second-generation oral PDE4 inhibitors, such as cilomilast (Arltö; GlaxoSmithKine, Brentford, UK) (Barnette et al., 1998) and roflumilast (Daxas; Takeda Pharmaceuticals, Zurich, Switzerland) (Hatzelmann and Schudt, 2001), have demonstrated efficacy in chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma (Press and Banner, 2009). However, the development of these compounds has been hampered by dose-limiting adverse events that are mechanism-related, in particular, gastrointestinal disturbances, such as nausea, diarrhea, abdominal pain, vomiting, and dyspepsia (Calverley et al., 2009). Roflumilast was recently approved in the European Union and the United States for once-daily treatment of severe COPD associated with chronic bronchitis and frequent exacerbations as add-on to bronchodilator treatment. However, target-related gastrointestinal side effects may limit roflumilast dosage and thus optimal clinical efficacy (Giembycz and Field, 2010; Page and Spina, 2011). In line with this consideration, the active doses of roflumilast determined in experimental models (Martorana et al., 2005; Hatzelmann et al., 2010) hardly support the daily dose of 0.5 mg approved in COPD patients.

In the respiratory field, a logical way to improve the therapeutic index is to deliver drugs directly into the lung via the inhalation route, allowing high topical efficacy coupled with low oral availability and limited systemic exposure. The PDE4 inhibitor UK-500,001, developed for inhaled administration, was tested in a double-blind, placebo-controlled 6-week trial in 209 patients with moderate to severe COPD; results were disappointing, however, and the study was stopped after a planned interim analysis for futility (Vestbo et al., 2009). Lack of efficacy in phase 2 trials in asthma and COPD led to the planned interim analysis for futility (Vestbo et al., 2009). Lack of efficacy in phase 2 trials in asthma and COPD led to the planned interim analysis for futility (Vestbo et al., 2009). Lack of efficacy in phase 2 trials in asthma and COPD led to the planned interim analysis for futility (Vestbo et al., 2009).

**Materials and Methods**

**Chemicals.** CHF6001 (Armarni et al., 2014), GSK256066 (Tralau-Stewart et al., 2011), roflumilast [3-(cyclopentylmethoxy)-N-(3,5- dichloroaryl)-4-(difluoromethyl)benzamide] (Hatzelmann et al., 2001), cilomilast [15,45S]-4-epoxy-4,3-(cyclopropyl)-4-methoxyphenyl cyclohexane-1-carboxylic acid (Pagès et al., 2009), and UK-500,001 (Pagès et al., 2009) were synthesized at Chiesi Farmaceutici S.p.A. (Parma, Italy). Unless otherwise stated, all other chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

**PDE4 Enzymatic Assays from U937 Extracts.** PDE4 activity was determined in U937 human monocytic supernatant cell lysate (American Type Culture Collection, Manassas, VA). Cells were cultured and harvested, and supernatant fraction prepared essentially as previously described (Barnette et al., 1998). All the materials used for cell culture were from Gibco (Monza, Italy). U937 cells were grown at 37°C, 5% CO₂ in RPMI 1640 with GlutaMAX-I medium supplemented with 10% fetal bovine serum and 100 μg/ml Pen-strep. Cells were harvested and washed twice by centrifugation (150 g) in cold phosphate-buffered saline (PBS). Washed cells were resuspended in cold Krebs-Ringer-Henseleit buffer at a final concentration 2 × 10⁵ cells/ml and sonicated. After centrifugation at 15,000g for 20 minutes, the supernatants were pooled, divided in aliquots, and stored at −80°C. PDE4 activity was determined in cell supernatants by assaying cAMP disappearance from the incubation mixtures. The concentration of the test compounds ranged between 10⁻¹² M and 10⁻⁶ M. Reactions were stopped by enzymeheat inactivation (2.5 minutes at 100°C), and residual CAMP content was determined using the LANCE CAMP Detection kit from PerkinElmer (Milan, Italy) according to provider instructions. IC₅₀ values were determined from concentration-response curves by nonlinear regression analysis.

**Rolfipram Binding Assay.** The affinity against high-affinity rolfipram binding site (HARBS) was evaluated in a radioligand binding assay performed in rat brain membranes using [³H]rolipram as radioligand. Fresh rat brains were homogenized in 20 volumes of ice-cold 50 mM Tris-HCl (pH 8.0) buffer containing 1.2 mM MgCl₂ in a polytron PT-10 homogenizer (Brinkman Instruments, Westbury, NY). The resulting homogenate was centrifuged at 30,000 g for 20 minutes at 4°C. The pellet was washed by resuspension in 20 volumes of fresh buffer and recovered by centrifugation as before. The final pellet was suspended in Tris buffer (0.5 mg of protein/ml) for binding experiments.

Incubation mixtures in duplicates consisted of 0.1 ml of calf thymus PDE4 enzymes expressed in a baculoviral system. The resulting homogenate was centrifuged at 30,000 g for 20 minutes at 4°C. The pellet was washed by resuspension in 20 volumes of fresh buffer and recovered by centrifugation as before. The final pellet was suspended in Tris buffer (0.5 mg of protein/ml) for binding experiments. Incubation mixtures in duplicates consisted of 0.1 ml of calf thymus PDE4 enzymes expressed in a baculoviral system. The resulting homogenate was centrifuged at 30,000 g for 20 minutes at 4°C. The pellet was washed by resuspension in 20 volumes of fresh buffer and recovered by centrifugation as before. The final pellet was suspended in Tris buffer (0.5 mg of protein/ml) for binding experiments.

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confirmed also by a preliminary study aimed at evaluating tolerance to DMSO (estimated up to 5%).

**PDE Enzyme Assays.** PDE1 was prepared from bovine brains (Gietzen et al., 1982). PDE2, PDE3, and PDE5 were purified from human platelets (Schudt et al., 1991). PDE6 was purified from bovine retinas (Baehr et al., 1979). Recombinant human PDE7A, PDE8A1, PDE9A2, and PDE11A4 were prepared in a baculovirus/insect cell system. The radiometric assay method is a modification of the two-step method of Thompson and Appleman (1971). Briefly, the assay mixture contained 50 mM Tris (pH 7.4), 5 mM MgCl₂, 0.5 mM cAMP or cGMP, and [³H]cAMP or [³H]cGMP (1 μM/ml); the indicated concentration of the inhibitor and an aliquot of the enzyme solution. CHF6001 was prepared as stock at a concentration of 0.3 mM in 100% DMSO and tested at 5 concentrations (1% final DMSO concentration) in duplicate starting at 30 μM and a 1:10 serial dilution against human PDEs. PDE1 soenzyme was assayed in the presence of Ca²⁺ (2 mM) and calmodulin (100 U/ml) using cAMP as substrate. PDE2, PDE3, PDE7A, PDE8A1, PDE10A2, and PDE11A4 were assayed in the presence of cAMP as substrate. PDE5, PDE6, and PDE9A2 were assayed using cGMP as substrate.

**Peripheral Blood Mononuclear Cells.** Cells were purchased from Lonza (Basel, Switzerland), washed, resuspended in RPMI 1640 medium (w/o Phenol red), supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin, and 100 μg/ml streptomycin and plated in 96-well tissue culture plates at a density of 10⁵ cells/well, in an atmosphere of 95% air and 5% CO₂ at 37°C. To induce differentiation into adherent macrophages, cells were plated in 48-well plates (2.5 × 10⁶ cells/well) and incubated for 4 days (THP-1) with 50 nM of phorbol 12-myristate 13-acetate as previously described (Diugnautel et al., 2010). Subsequently, cells were treated with different concentrations of PDE4 inhibitors (10⁻¹³ M–10⁻⁶ M, 0.2% final DMSO concentration) 1 hour before stimulation with lipopolysaccharide (LPS) from *Escherichia coli* (3 ng/ml for 18 hours). Increasing concentrations of DMSO were tested, and no significant effects of DMSO were tested above 0.4% on cell viability and tumor necrosis factor-α (TNF-α) release were noticed. Human TNF-α in the supernatant was assayed using a paired antibody quantitative enzyme-linked immunosorbent assay (ELISA) kit (Bender Medsystems, Vienna, Austria).

**Human Myeloid Leukemia THP-1 Cell Line.** Human myeloid leukemia cell line (THP-1) was obtained from Sigma-Aldrich and cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin, and 100 μg/ml streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C. To induce differentiation into adherent macrophages, cells were plated in 48-well plates (2.5 × 10⁵ cells/well) and incubated for 4 days (THP-1) with 50 nM of phorbol 12-myristate 13-acetate as previously described (Diugnautel et al., 2010). Subsequently, cells were treated with different concentrations of PDE4 inhibitors (10⁻¹³ M–10⁻⁶ M, 0.2% final DMSO concentration), stimulated with LPS (100 ng/ml final concentration), and incubated for 18 hours in RPMI (without Phenol red) supplemented with 10% FBS. Human TNF-α in the supernatant determined by quantitative ELISA (Bender Medsystem).

**Murine (RAW264.7) and Rat (NR8383) Macrophage Cell Lines.** RAW264.7 and NR8383 cells were cultured from ATCC and cultured in RPMI 1640 medium (w/o Phenol red) supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin, and 100 μg/ml streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C. RAW264.7 and NR8383 cells were seeded in RPMI (w/o Phenol red) containing 10% FBS in 48-well tissue culture plates at the density of 7.5 × 10⁵ cells/well and grown for 24 hours at 37°C with 5% CO₂. Subsequently, cells were treated with different concentration of PDE4 inhibitors (10⁻¹³ M–10⁻⁶ M, 0.2% final DMSO concentration), stimulated with LPS (100 ng/ml final concentration) and incubated for 18 hours in RPMI (without Phenol red) supplemented with 10% FBS. Murine and rat TNF-α in the supernatant determined by quantitative ELISA kit (Bender Medsystem). As an indirect index of nitric oxide, accumulation of nitrite in the medium production was measured by a colorimetric assay method based on the Griess reaction, as previously described (Faucinetti et al., 2004).

**CD4⁺ T Lymphocytes.** CD4⁺ T lymphocytes were purchased from Lonza (Basel, Switzerland). CD4⁺ T lymphocytes were cultured in lymphocyte growth medium supplemented with 10% FBS, albumin, insulin, transferrin, and gentamicin. Cells were stimulated via the T-cell receptor CD3 and CD28 coreceptor by using selective monoclonal antibodies (mAbs) (Hatzelmann and Schudt, 2001). For this purpose, 96-well microtiter plates were prepared on the day before cell plating: each well was incubated with 50 μl of PBS containing 6 μg/ml of anti-CD3 mAb (Orthoclone OKT-3; Janssen-Cilag, Neuss, Germany) for about 2.5 hours at 37°C; plates were then stored overnight at 4°C and washed three times with PBS (200 μl) before use. CD4⁺ T lymphocytes were plated at a density of 10⁵ cells/well in 96-well tissue culture plates, precoated with the anti-CD3 mAbs, and treated with different concentrations of PDE4 inhibitors (10⁻¹³ M–10⁻⁸ M, 0.2% final DMSO concentration). Subsequently, anti-CD28 (clone CD28.2; Coulter-Immunotech Diagnostics, Hamburg, Germany) was added to the final concentration of 3 μg/ml, and the plates were further incubated at 37°C and 5% CO₂ for 72 hours. Human interferon-γ in the supernatant was assayed using a paired-antibody quantitative ELISA kit (Life Technologies, Grand Island, NY).

**Human Primary Peripheral Eosinophils.** Human primary peripheral eosinophils were purchased from 3H Biomedical (Uppsala, Sweden). Human primary eosinophils were washed; resuspended in RPMI 1640 medium (w/o Phenol red); supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin, and 100 μg/ml streptomycin; and plated in 96-well white OptiPlate (PerkinElmer) at a density of 10⁵ cells/well. Cells were pretreated with different concentrations of PDE4 inhibitors (10⁻¹³ M–10⁻⁸ M, 0.2% final DMSO concentration) for 30 minutes, primed with cytochalasin B (5 μM) for 15 minutes, and stimulated with N-formyl-L-methionine-L-leucyl-phenylalanine (fMLP; 1 μM) in RPMI (without Phenol red) supplemented with 10% FBS. After the addition of fMLP, cells were incubated with the chemiluminescence probe L-012 (500 nM) (Wako Chemicals GmbH, Neuss, Germany), and reactive oxygen species (ROS) generation was recorded repeatedly over 1 hour (one reading every minute) using a luminescence reader (Centro LB 960; Berthold Technologies, Oak Ridge, TN). During luminescence measurement, the plate was rotated agitatively at 37°C. ROS generation was determined by calculating the area under the curve.

**Neutrophil Oxidative Burst.** Human neutrophils (PMNLs) were purified from diluted “buffy coat” (Blood Bank, University of Milan) centrifuged on a discontinuous Percoll gradient (1077–1098, 4–10°C), followed by red cells lysis (1 volume 0.2% NaCl, balanced with 1 volume 1.6% NaCl and 0.2% saccharose, 4–10°C). Handling in sterile buffer prevented spontaneous activation of PMNLs. Purified neutrophils (1 × 10⁶/ml) were suspended in D-PBS (Sigma-D1408) supplemented with CaCl₂, MgCl₂, and glucose (0.9, 0.5, and 7.5 mM, respectively) and antibiotics. The cell suspension was dispensed in microplates (200 μl/well), gently shaken at 37°C, and absorbance read at λ550–490 by a spectrophotometer (Spectramax190 microplate reader). Neutrophil suspensions (1 × 10⁶/ml) were then incubated with

![Chemical structure of CHF6001](Fig. 1)
the direct inhibitor of neutrophil NADPH-oxidase diphenyleneiodonium), the PDE4 inhibitor compounds, or its vehicle, DMSO, for 10 minutes at 37°C before challenge with FMLP (1 μM); ROS production was monitored by measuring the variation in absorbance of the reduced cytochrome c: duplicate samples containing superoxide dismutase (180 U/ml) were used as blanks. As the PMNL suspension obtained from different donors significantly differs in response to challenge, results were expressed as percentage of inhibition of ROS release. Each treatment had four replicates with cells from the same donor, and concentration-response curves were originated using the average values from at least three different donors.

**Neutrophil Chemotaxis.** Male C57BL/6J mice were bred in the animal house of the Center for Molecular Biotechnology (University of Turin, Italy) and maintained on a standard diet with tap water ad libitum and a 12-hour light/dark cycle. Mice were sacrificed by cervical dislocation. The femur and the tibia bones from both hind legs were dislocated. The femur and the tibia were cut into small parts and the bone marrow was flushed with PBS. Erythrocytes and epithelial cells were ignored. The number of each cell type was quantified by expressing the cell number as a percentage of the total count.

**Results**

**CHF6001 Selection.** The structure of CHF6001 is shown in Fig. 1. It was identified as a potent inhibitor of PDE4 activity (compound 32a) from a series of novel ester derivatives of 1-(S)-5-(cyclopentyloxy)methylphenoxy)-2-(3,5-dichloropyridin-4-yl) ethanol (Armani et al., 2014). In particular, esters of variously substituted benzoic acids were explored and structural modifications of the benzoic moiety were performed to maximize the inhibitory potency. Besides CHF6001, several other novel PDE4 inhibitors with potent anti-inflammatory activity in vitro were obtained (Armani et al., 2014).

**Activity of CHF6001 and Reference Compounds in PDE Enzyme Assays.** PDE4 isoforms exist in both low-affinity rolipram binding sites (LARBS) and HARBS conformations. CHF6001 enzymatic activity was tested both in

### TABLE 1

Inhibitory potencies (IC<sub>50</sub>, nM ± S.E.M.) of CHF6001 and other reference PDE4 inhibitors against the enzymatic activity of HARBS and LARBS conformers

<table>
<thead>
<tr>
<th></th>
<th>CHF-6001</th>
<th>GSK256066</th>
<th>Roflumilast</th>
<th>UK-500,001</th>
<th>Cilomilast</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HARBS</strong></td>
<td>0.265 ± 0.006 (n = 6)</td>
<td>0.025 ± 0.010 (n = 3)</td>
<td>0.176 ± 0.095 (n = 5)</td>
<td>1.28 ± 0.168 (n = 2)</td>
<td>24.17 ± 7.83 (n = 2)</td>
</tr>
<tr>
<td><strong>LARBS</strong></td>
<td>1.05 ± 0.195 (n = 6)</td>
<td>0.274 ± 0.115 (n = 2)</td>
<td>0.784 ± 0.191 (n = 3)</td>
<td>2.84 ± 0.413 (n = 2)</td>
<td>50.9 ± 10 (n = 2)</td>
</tr>
<tr>
<td><strong>RATIOS</strong></td>
<td>40.6</td>
<td>11.0</td>
<td>4.4</td>
<td>2.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

PDE, phosphodiesterase.

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the direct inhibitor of neutrophil NADPH-oxidase diphenyleneiodonium), the PDE4 inhibitor compounds, or its vehicle, DMSO, for 10 minutes at 37°C before challenge with FMLP (1 μM); ROS production was monitored by measuring the variation in absorbance of the reduced cytochrome c: duplicate samples containing superoxide dismutase (180 U/ml) were used as blanks. As the PMNL suspension obtained from different donors significantly differs in response to challenge, results were expressed as percentage of inhibition of ROS release. Each treatment had four replicates with cells from the same donor, and concentration-response curves were originated using the average values from at least three different donors.

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**Statistical Analysis.** All values are expressed as means ± S.E.M. of the given number (n) of independent experiments. IC<sub>50</sub> values were calculated by the analysis of the sigmoidal dose-response curve (variable slope), elaborated by Graph Pad Prism 4 program (Graph-Pad Software, San Diego, CA). Statistical analysis was performed using one-way analysis of variance followed by Dunnett's post hoc test for multiprogroup comparisons.

### TABLE 2

Inhibitory potencies (IC<sub>50</sub>, nM, CI) of CHF6001 and other reference PDE4 inhibitors against a panel of four PDE4 isoforms

<table>
<thead>
<tr>
<th></th>
<th>CHF6001</th>
<th>GSK256066</th>
<th>Roflumilast</th>
<th>UK-500,001</th>
<th>Cilomilast</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PDEA4</strong></td>
<td>0.032 (0.02–0.05)</td>
<td>0.017 (0.008–0.039)</td>
<td>0.35 (0.27–0.46)</td>
<td>26.1 (12.9–52.2)</td>
<td>375 (118–1181)</td>
</tr>
<tr>
<td><strong>PDEB2</strong></td>
<td>0.025 (0.01–0.05)</td>
<td>0.015 (0.006–0.037)</td>
<td>0.18 (0.08–0.44)</td>
<td>22.8 (13.2–39.4)</td>
<td>440 (269–719)</td>
</tr>
<tr>
<td><strong>PDEC2</strong></td>
<td>0.061 (0.04–0.09)</td>
<td>0.061 (0.042–0.088)</td>
<td>2.07 (1.44–2.99)</td>
<td>21 (93.2–506)</td>
<td>1700 (928–3102)</td>
</tr>
<tr>
<td><strong>PDED3</strong></td>
<td>0.021 (0.01–0.04)</td>
<td>0.015 (0.010–0.020)</td>
<td>0.24 (0.15–0.38)</td>
<td>0.28 (0.13–0.61)</td>
<td>86.2 (54.4–136.6)</td>
</tr>
</tbody>
</table>

CI, confidence interval; PDE, phosphodiesterase.
a LARS conformation by using an assay with PDE4 extracts from U937 cells and in HARS conformation by using rolipram binding assay (see Materials and Methods) in comparison with reference compounds GSK256066, roflumilast, UK-500,001, and cilomilast (Table 1).

The IC<sub>50</sub> value for the inhibition of enzymatic activity of PDE4 by CHF6001 in the LARS assay is 0.026 ± 0.006 nM. The most potent comparator, as expected, resulted from GSK256066 (IC<sub>50</sub> = 0.025 ± 0.01 nM), whereas the other PDE4 inhibitors tested showed IC<sub>50</sub> values ranging between 0.17 and 24 nM (Table 1).

CHF6001 inhibited [3H]rolipram binding in rat brain cytosol (HARBS) with an IC<sub>50</sub> of 1.05 nM, giving a HARBS ratio (HARBS IC<sub>50</sub>:LARBS IC<sub>50</sub>) of about 40, a value higher than GSK256066 ratio (11), whereas roflumilast, UK-500,001 and cilomilast showed ratios between 2 and 4 (Table 1).

CHF6001 inhibited most of PDE4 splicing variants at subnanomolar concentrations (Table 2); similarly to GSK256066 and roflumilast, CHF6001 did not show PDE4 subtype selectivity against different PDE isoenzymes. Roflumilast is 10-fold less potent than CHF6001 with respect to PDE4 isoenzyme inhibition, apart from PDE4C, which is inhibited with a slightly lower potency. In contrast, UK-500,001 and cilomilast showed some subtype selectivity for PDE4D (Table 2).

CHF6001 is at least 25,000-fold less potent against the following panel of PDE isoenzymes: PDE1, PDE2, PDE3, PDE5, PDE6, PDE7A, PDE8A1, PDE9A2, PDE10A2, and PDE11A4, therefore showing a marked selectivity for PDE4 (Table 2).

**Activity of CHF6001 and Reference Compounds in Human Peripheral Blood Mononuclear Cells, THP-1 Monocyte-Derived Macrophages, and CD4<sup>+</sup> T Lymphocytes.** We selected two in vitro models of LPS-stimulated TNF-α to evaluate the anti-inflammatory potency of CHF6001 in comparison with other known PDE4 inhibitors, including GSK256066, which is the most potent PDE4 inhibitor so far described in the literature; UK-500,001; roflumilast, the only clinically approved PDE4 inhibitor; and cilomilast. All the compounds tested inhibited TNF-α release up to a maximum of about 90% in peripheral blood mononuclear cells (PBMCs), an outcome in line with the notion that PDE4 is the predominant isoform expressed in monocytes (Fig. 2, upper panel). In PBMCs, basal release of TNF-α (mean ± S.D.) was 98.6 ± 6.1 pg/ml, but on LPS stimulation, it became 40,146 ± 2227 pg/ml. The rank order of potency of the compounds (confidence interval values given in parentheses) is the following: GSK256066, IC<sub>50</sub> = 3 pM (2–4); CHF6001, IC<sub>50</sub> = 28 pM (21–40); roflumilast IC<sub>50</sub> = 1.77 nM (0.45–6.83), UK-500,001, IC<sub>50</sub> = 12.1 nM (2.9–50.1), and cilomilast IC<sub>50</sub> = 165.3 nM (61.8–442.6). These results were well in agreement with the previously reported potencies of GSK256066, roflumilast, and cilomilast against TNF-α release in PBMCs (Hatzelmann and Schudt, 2001; Tralau-Stewart et al., 2011). In human THP-1 monocyte-derived macrophages, basal release of TNF-α (mean ± S.D.) was of 10.3 ± 2.5 pg/ml but on LPS stimulation became 18,945 ± 838 pg/ml. All the compounds tested inhibited TNF-α release up to a maximum of 65%–70% (Fig. 2, lower panel) with the following potencies: GSK256066, IC<sub>50</sub> = 16 pM (8–35); CHF6001, IC<sub>50</sub> = 43 pM (15–122); UK-500,001, IC<sub>50</sub> = 3.60 nM (1.22–10.45); roflumilast, IC<sub>50</sub> = 4.41 nM (1.0–19.3); cilomilast, IC<sub>50</sub> = 264.0 nM (121.2–575.0). The observed potency ranking in THP-1 monocyte-derived macrophages is similar to that observed in PBMCs, with both CHF6001 and GSK256066 displaying a potency in the low picomolar range.

PDE4s are critical regulators in T-cell receptor signaling, and their inhibition blunts T-cell cytokine production (Abrahamsen et al., 2004). To mimic the physiologic conditions of T-cell activation, we stimulated the CD4<sup>+</sup> T lymphocytes via the T-cell receptor (by plate-bound anti-CD3 antibody) and the CD28 coreceptor (by soluble anti-CD28 antibody). Basal release of interferon-γ (IFN-γ) (mean ± S.D.) was 54 ± 8.5 pg/ml but on LPS stimulation became 3036 ± 489 pg/ml. All the PDE4 inhibitors tested, including CHF6001, inhibited IFN-γ release up to a maximum of 50%–60% (Fig. 3) with the following potencies: GSK256066, IC<sub>50</sub> = 3 pM (1–12); CHF6001, IC<sub>50</sub> = 62 pM (13–289); roflumilast, IC<sub>50</sub> = 0.42 nM (0.09–1.87); UK-500,001, IC<sub>50</sub> = 9.4 nM (73–120); cilomilast, IC<sub>50</sub> = 386.7 nM (149.8–998.5).

**TABLE 3**

<table>
<thead>
<tr>
<th>CHF6001</th>
<th>PDE1</th>
<th>PDE3</th>
<th>PDE5</th>
<th>PDE7A</th>
<th>PDE2, -6, -8A1, -9A2, -10A2, -11A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.2</td>
<td>2.6</td>
<td>1.63</td>
<td>2.6</td>
<td>ND</td>
</tr>
<tr>
<td>CI</td>
<td>(0.2–7.9)</td>
<td>(0.97–7.2)</td>
<td>(0.03–83)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

CI, confidence interval; ND, not determined; PDE, phosphodiesterase.
The observed potency ranking in activated CD4\(^+\) T cells is similar to that observed in PBMCs and THP-1, with both CHF6001 and GSK256066 being active in the low picomolar range.

**Activity of CHF6001 and Reference Compounds in Rodent Macrophagic Cell Lines.** In two different rodent macrophagic cell lines, namely, RAW264.7 (mouse) and NR8383 (rat), all the PDE4 inhibitors tested inhibited the release of TNF-\(\alpha\) and NO evoked by LPS. In RAW264.7, basal release of TNF-\(\alpha\) (mean \(\pm\) S.D.) was 10.3 \(\pm\) 5.2 pg/ml, whereas on LPS stimulation, it became 18,945 \(\pm\) 838 pg/ml. The following inhibitory potencies (confidence interval values given in parenthesis) against TNF-\(\alpha\) (Fig. 4A) were observed: GSK256066, IC\(_{50}\) = 0.82 nM (0.3–2.1); CHF6001, IC\(_{50}\) = 1 nM (0.7–1.5); roflumilast, IC\(_{50}\) = 54.8 nM (23–130); UK-500,001, IC\(_{50}\) = 36.60 nM (11.3–181); cilomilast, IC\(_{50}\) = 830 nM (340–2000). Similar results were obtained in NR8383 cells, where the inhibition potencies against TNF-\(\alpha\) release (Fig. 4C) were the following: GSK256066, IC\(_{50}\) = 0.08 nM (0.06–0.1); CHF6001, IC\(_{50}\) = 0.19 nM (0.18–0.2); roflumilast, IC\(_{50}\) = 8.3 nM (5.5–12.4); UK-500,001, IC\(_{50}\) = 19.8 nM (3.2–123); cilomilast, IC\(_{50}\) = 548 nM (353–852). In NR8383, basal release of TNF-\(\alpha\) (mean \(\pm\) S.D.) was 1.2 \(\pm\) 0.5 pg/ml, whereas on LPS stimulation, it became 1184 \(\pm\) 111 pg/ml.

In both NR8383 and RAW264.7, the potencies of all the tested inhibitors showed slightly higher IC\(_{50}\) values against nitric oxide release (Fig. 4, B and D), but the ranking order observed in the previous assays was maintained.

**Activity of CHF6001 and Reference Compounds in Human Eosinophils and Neutrophils.** Since PDE4 inhibitors have been shown to negatively modulate fMLP-stimulated release of ROS from human eosinophils and neutrophils (Hatzelmann and Schudt, 2001; Press and Banner, 2009), we evaluated the ability of CHF6001 in inhibiting fMLP-induced ROS production. CHF6001 and GSK256066 fully inhibited fMLP-induced ROS generation in human eosinophils, at low picomolar concentrations (Fig. 5, upper panel), with IC\(_{50}\) values of 5 pM (0.002–0.013) and 5 pM (0.002–0.015), respectively. Similarly, in human neutrophils, CHF6001 and GSK256066 inhibited fMLP-induced ROS generation (Fig. 5, lower panel) with IC\(_{50}\) values of 0.55 nM (0.07–4.20) and 0.49 nM (0.17–1.43), respectively. Roflumilast, with an IC\(_{50}\) = 8.51 nM (1.51–47.88), was again less potent than CHF6001 and GSK256066.

**Activity of CHF6001 and Roflumilast on Neutrophilic Chemotaxis.** Neutrophil-dominant pulmonary inflammation is an important feature of COPD. Since PDE4 inhibitors have a functional role in mediating chemotaxis (Ariga et al., 2004), the ability of CHF6001 in inhibiting neutrophilic chemotaxis was tested in comparison with roflumilast in vitro in a Boyden two-chamber chemotactic assay. CHF6001 inhibited about 60% neutrophilic migration with a subnanomolar potency, IC\(_{50}\) = 0.093 nM (0.036–0.241). Roflumilast, by showing an IC\(_{50}\) = 0.937 nM (0.678–1.296), resulted in being less potent than CHF6001 (Fig. 6).

**CHF6001 Inhibits LPS-Induced Pulmonary Leukocytes Infiltration in Rats.** CHF6001 (0.01, 0.1, 0.3, 1 \(\mu\)mol/kg), GSK256066 (1 \(\mu\)mol/kg), or budesonide (1 \(\mu\)mol/kg) was...
administered intratracheally as micronized dry powder 1 hour before and 6 hours after LPS challenge. BALF was collected 24 hours after challenge and total, and differential cell counts performed.

When administered 1 hour before and 6 hours after the inflammatory stimulus, CHF6001 elicited a dose-dependent inhibition of pulmonary neutrophilia, which reached a statistical significance at 0.1 \( \mu \text{mol/kg} \) (\( P < 0.05 \)) and was maximal at 1 \( \mu \text{mol/kg} \) (77%) similarly to budesonide at 1 \( \mu \text{mol/kg} \) (77%). CHF6001 elicited also a dose-dependent inhibition of pulmonary white blood cell counts in BALF, which reached significance at 0.3 \( \mu \text{mol/kg} \) (\( P < 0.01 \)) and was maximal at 1 \( \mu \text{mol/kg} \) (61%), similarly to budesonide at 1 \( \mu \text{mol/kg} \) (62%). Calculated ED\(_{50}\) values for CHF6001 were 0.188 \( \mu \text{mol/kg} \) against total white blood cells and 0.205 \( \mu \text{mol/kg} \) against neutrophilia. Similarly to CHF6001, GSK256066, when tested at 1 \( \mu \text{mol/kg} \), showed an inhibitory effect on LPS-evoked pulmonary neutrophilia (70% inhibition) and white blood cell infiltration (60% inhibition) (Fig. 7).

**Discussion**

The recent approval of roflumilast as an oral drug for COPD treatment defines the standing of PDE4 as a therapeutic target in respiratory diseases. Nevertheless, this second-generation PDE4 inhibitor is still not without side effects that could compromise optimal dosing and affect patient compliance. Several strategies have been proposed to minimize PDE4 inhibition-related side effects, including designing inhibitors suitable for inhaled delivery that would limit systemic exposure while increasing the pulmonary anti-inflammatory activity achievable. In the present article, we report the pharmacologic characterization of a novel highly potent and selective PDE4 inhibitor (CHF6001) optimized for inhaled delivery through a rational drug-design and screening program (Armani et al., 2014). CHF6001 inhibitory potency was evaluated in enzymatic cell-free assays and subsequently in several cell-based assays taking advantage of different immunocompetent cells known to play key roles in asthma and COPD, including monocytes, macrophages, CD4\(^+\) T cells, neutrophils, and eosinophils. Finally, for testing how in vitro anti-inflammatory potency and efficacy translates in vivo, CHF6001 was delivered as micronized dry powder in a rat model of endotoxin-induced acute neutrophilia. A further preclinical in vivo safety and efficacy profile is presented in a companion paper.

CHF6001 was compared with two oral (roflumilast and cilomilast) and two inhaled (GSK256066 and UK-500,001) PDE4 inhibitors already described in detail in the scientific literature. Roflumilast was chosen because it is the only PDE4 inhibitor approved in the European Union and United States for once-daily treatment of severe COPD; cilomilast is the first oral PDE4 that reached preregistration, and the inhaled PDE4 inhibitors UK-500,001 and GSK256066 were both tested in controlled clinical trials of phase 2 in COPD and asthma, respectively. Head-to-head comparison with the four reference compounds showed that CHF6001 is highly potent in inhibiting all the four PDE4 (A–D) isoforms tested with a potency comparable to that of GSK256066, which is the most potent inhibitor described in the literature so far and 10- to 100-fold more potent than roflumilast. Indeed, the following ranking could be determined for inhibition of PDE4 isoforms: GSK256066 \( \geq \) CHF6001 > roflumilast > UK-500,001 > cilomilast. Such ranking was substantially maintained when CHF6001 inhibitory PDE4 activity was determined in U937 human monocytic cells lysate.

Two distinct PDE4 conformers can be distinguished based on their affinity to bind the prototypic inhibitor rolipram: the LARBS conformer, encompassing the catalytic domain of PDE4,
CHF6001 (CHF; 0.01, 0.1, 0.3, 1 μmol/kg), GSK256066 (GSK; 1 μmol/kg) or budesonide (Bude; 1 μmol/kg) were administered intratracheally as micronized dry powder (in lactose vehicle) 1 hour before and 6 hours after LPS challenge. BALF was collected 24 hours after challenge; total and differential cell counts were performed. Bars are the counts of neutrophils (left) and total white blood cell (right) in BALF. Treatment groups were micronized dry powder (in lactose vehicle) 1 hour before and 6 hours after LPS challenge. BALF was collected 24 hours after challenge; total and differential cell counts were performed. Bars are the counts of neutrophils (left) and total white blood cell (right) in BALF. Treatment groups were compared with LPS/vehicle-treated group by using analysis of variance followed by Dunnett's test.

**Fig. 7.** Inhibition of neutrophils and total leukocyte cell count in bronchoalveolar lavage fluid in endotoxin (LPS)-exposed rats treated with CHF6001. CHF6001 (CHF; 0.01, 0.1, 0.3, 1 μmol/kg), GSK256066 (GSK; 1 μmol/kg) or budesonide (Bude; 1 μmol/kg) were administered intratracheally as micronized dry powder (in lactose vehicle) 1 hour before and 6 hours after LPS challenge. BALF was collected 24 hours after challenge; total and differential cell counts were performed. Bars are the counts of neutrophils (left) and total white blood cell (right) in BALF. Treatment groups were compared with LPS/vehicle-treated group by using analysis of variance followed by Dunnett's t test. *P < 0.05 and **P < 0.01 for treatment groups compared with vehicle + LPS control group. Values are expressed as the mean ± S.E.M. values of each treatment group (n = 8).

and the HARBS conformer, which requires both amino terminal and the catalytic domain and is present in the brain but not in peripheral tissues (Barnette et al., 1996; Rocque et al., 1997). Targeting the conformer of PDE4 associated with the LARBS over the HARBS may retain anti-inflammatory activity and have a reduced capacity to cause nausea and emesis (Sonnens and Rao, 1997). Interestingly, CHF6001 results in a HARBS:LARBS ratio of 40, more than 10-fold higher than that of roflumilast, UK-500,001 and cilomilast, a characteristic suggestive of a potentially wider therapeutic window.

CHF6001 was tested in several functional cellular assays designed to evaluate its anti-inflammatory activities. CHF6001 showed subnanomolar potency in inhibiting the release of the clinically relevant proinflammatory cytokine TNF-α from PBMCs stimulated with LPS. Macrophage numbers are markedly increased in the lung and in alveolar space of patients with COPD and are preferentially localized to sites of alveolar destruction. Therefore, alveolar macrophages are critical players in the pathophysiology of COPD and a major target for future anti-inflammatory therapy. Indeed, CHF6001 was also tested in THP-1 monocytic-derived macrophages and two macrophagic cell lines, namely RAW264.7 which is derived from peritoneal mouse macrophages, and NR8383 which is derived from rat alveolar macrophages. Again, the anti-inflammatory effects of CHF6001 were evident in all macrophagic cell lines as TNF-α and nitric oxide release was inhibited by CHF6001 with potencies slightly inferior (2- to 10-fold potency difference) to those observed in PBMCs. This difference probably reflects different PDE4 levels of expression between macrophages and monocytes (Gantner et al., 1997).

CHF6001 was >6000-fold more potent than cilomilast in inhibiting release of IFN-γ in CD4+ T cells, which are known to play critical in the initiation and propagation of immune response in asthma (Kumar et al., 2006). CHF6001 was extremely potent (IC_{50} in the low picomolar range) in inhibiting fMLP-induced eosinophil activation, a finding consistent with the notion that PDE4 is prominently expressed in eosinophils.

Neutrophil-dominant pulmonary inflammation is an important feature of COPD (Watt et al., 2005; Quint and Wedzicha, 2007), and we found that CHF6001 was highly potent in inhibiting fMLP-evoked ROS production from human neutrophils (IC_{50} = 0.005 nM) and C5a-induced chemotaxis in mouse neutrophils (IC_{50} = 0.093 nM). Overall, these findings suggest that CHF6001 targets neutrophils both through direct inhibition of oxidative burst and chemotaxis, two biologic responses known to be scarcely sensitive to glucocorticoids (Kubo et al., 2012). This finding underlines a therapeutic potential for CHF6001, as well as PDE4 inhibitors, in treating COPD, which is often associated with lung neutrophilia.

The high potency of CHF6001 in all the in vitro assays used in this study translates well in anti-inflammatory efficacy in vivo when it is administered intratracheally as a micronized dry powder. By using an endotoxin rat model of pulmonary neutrophilia, we compared CHF6001 head-to-head with the corticosteroid budesonide and GSK256066 (same formulation and same delivery system). GSK256066 was chosen since it has been previously reported to be the most potent PDE4 inhibitor so far described in the literature and is effective in reducing allergen challenge responses in mild asthmatic patients (Singh et al., 2010). CHF6001 was highly potent and efficacious in inhibiting LPS-induced neutrophilia (full inhibitory effect equal to a maximal dose of budesonide or GSK256066). Potency is an important feature of an inhaled compound, and UK-500,001 failure in phase 2 clinical development may be, at least in part, a consequence of insufficient potency, as suggested by the head-to-head comparisons made in this study. Low systemic exposure is important in determining the therapeutic index of a PDE4 inhibitor being emesis and gastrointestinal disturbances directly associated with the mechanism of action. In a recent safety and tolerability study in healthy volunteers, CHF6001, administered as an inhaled dry powder formulation for 7 days, proved to be well tolerated up to 1.6 mg (Esposito et al., 2013). A complete preclinical in vivo safety and activity profiling of CHF6001 is presented in the companion article by Villetti et al. (2015). Given its excellent pharmacologic potency and efficacy as topical anti-inflammatory agent, CHF6001 holds promise as a novel inhaled PDE4 inhibitor for treating lung inflammatory diseases such as asthma and COPD. The testing of CHF6001 in clinical trials is currently ongoing (www.clinicaltrials.gov).
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