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

Nadia Moretto, Paola Caruso, Raffaella Bosco, Gessica Marchini, Fiorella Pastore, Elisabetta Armani, Gabriele Amari, Andrea Rizzi, Eleonora Ghidini, Renato De Fanti, Carmelida Capaldi, Laura Carzaniga, Emilio Hirsch, Carola Buccellati, Angelo Sala, Chiara Carnini, Riccardo Patacchini, Maurizio Delcanale, Maurizio Civelli, Gino Villetti, and Fabrizio Facchinetti


Received October 13, 2014; accepted January 7, 2015

ABSTRACT

This study examined the pharmacologic characterization of CHF6001 [(S)-3,5-dichloro-4-[[2-(3-(cyclopropylmethoxy)-4-difluoromethoxy)phenyl]-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamidobenzoyl)oxy]ethyl]pyridine 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-α 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-γ 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)nicotinamidé], and cilomilast, and it was comparable to GSK256066 [6-[[3-(dimethylcarbamoyl)phenyl]sulfonyl]-4-[[3-methoxyphenyl]amino]-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 superfAMILY 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 cAMP 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 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 (Arifio; GlaxoSmithKline, 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 patients with severe COPD. 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.

Materials and Methods

Chemicals. CHF6001 (Armani et al., 2014), GSK256066 (Tralau-Stewart et al., 2011), roflumilast [3-(cyclopropylmethoxy)-N-(3,5-dichlorophenyl)-4-(difluoromethoxy)phenethyl] (Hatzelmann et al., 2001), cilomilast [1S,4S]-4-cyano-4-(3-cyclohexylcarbonyl)-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., 1996). 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 10 μg/ml Pen-strep. Cells were harvested and washed twice by centrifugation (150g, 5 minutes) in cold phosphate-buffered saline (PBS). Washed cells were resuspended in cold Krebs-Ringer-Henseleit buffer at a final concentration 2×10^7 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^−8 M. Reactions were stopped by enzyme heat 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. IC_50 values were determined from concentration-response curves by nonlinear regression analysis.

Rolipram Binding Assay. The affinity against high-affinity rolipram 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-Cl (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 30,000g 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 resuspended in Tris buffer (0.5 mg of protein/ml) for binding experiments. Incubation mixtures in duplicates consisted of 0.1 ml of 10^−6 M [3H]Rolipram (2 nM final), 0.02 ml of inhibitor, and 0.9 ml of membrane preparation (added last). Rolipram (10 μM) was used for nonspecific binding. After 60 minutes of incubation at 4°C, the contents of the incubation tubes were filtered through a Whatman GF/C glass filter. The membranes were washed three times with 3 ml of ice-cold buffer, and radioactivity on the separated filter disks was determined in a liquid scintillation counter. IC_50 values were determined from semilog graphs of the percent of inhibition versus the concentration.

PDE4 Enzyme Assays. PDE4 assays were performed using recombinant PDE enzymes expressed in a baculoviral system. The radiometric assay method is a modification of the two-step method of Thompson and Appleman (1971) as described by Mackenzie et al. (2010). All the assays use a substrate concentration below the K_m determined for each enzyme so that V_max is used for determination. The PDE4 inhibitors were prepared as stocks at a concentration of 40 μM in 100% DMSO and were tested at 11 concentrations (0.5% final DMSO concentration) in duplicate with a starting concentration of 1 μM and a 1:10 serial dilution against human PDE4A4, PDE4B2, PDE4C2, and PDE4D3. Concentrations up to 5% DMSO are tolerated in this assay as reported previously (Mackenzie et al., 2010), a notion
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 (Baeher et al., 1979). Recombinant human PDE7A, PDE8A1, PDE8A2, PDE10A2, and PDE11A4 were expressed 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 10^4 cells/well, in an atmosphere of 95% air and 5% CO2 at 37°C. To induce differentiation, cells were treated with different concentrations of PDE4 inhibitors (10^{-13} M−10^{-8} M, 0.2% final DMSO concentration) 1 hour before stimulation with liposaccharide (LPS) from Escherichia coli (3 ng/ml for 18 hours). Increasing concentrations of DMSO were tested, and no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were tested, no significant effects of DMSO were test

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 and plated in 96-well tissue culture plates at a density of 10^5 cells/well. Cells were pretreated with different concentrations of PDE4 inhibitors (10^{-13} M−10^{-8} M, 0.2% final DMSO concentration) 15 minutes, primed with cytochalasin B (5 μM) and stimulated with N-formyl-l-methionyl-l-leucyl-phenylalanin (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 15°, and the 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 (α 1077−1088, 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 (1×10^6/ml) were suspended in D-PBS (Sigma-D1406) supplemented with CaCl2, MgCl2, and glucose (0.9, 0.5, and 7.5 mM, respectively) and stimulated with N-formyl-l-methionyl-l-leucyl-phenylalanin (fMLP; 1 μM). The cell suspension was dispensed in microplates (200 μl/well), gently shaken at 37°C, and absorbance read at 550–490 by a spectrophotometer (Spectramax190 microplate reader). Neutrophil suspensions (1×10^6/ml) were then incubated with CHF6001: In Vitro Characterization 561

Fig. 1. Chemical structure of CHF6001.
TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>CHF-6001</th>
<th>GSK256066</th>
<th>Rolflumastil</th>
<th>UK-500,001</th>
<th>Clomilast</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC50 (nM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PDE4A4</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>PDE4B2</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>PDE4C2</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>PDE4D3</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>

**Ratios** 40.6 11.0 4.4 2.2 2.1

PDE, phosphodiesterase.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>CHF-6001</th>
<th>GSK256066</th>
<th>Rolflumastil</th>
<th>UK-500,001</th>
<th>Clomilast</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC50 (nM) CI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PDE2A4</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>PDE4B2</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>PDE4C2</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>PDE4D3</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 LARBS conformation by using an assay with PDE4 extracts from U937 cells and in HARBS conformation by using rolipram binding assay (see Materials and Methods) in comparison with reference compounds GSK256066, rolflumilast, UK-500,001, and cilomilast (Table 1).

The IC₅₀ value for the inhibition of enzymatic activity of PDE4 by CHF6001 in the LARBS assay is 0.026 ± 0.006 nM. The most potent comparator, as expected, resulted from GSK256066 (IC₅₀ = 0.025 ± 0.01 nM), whereas the other PDE4 inhibitors tested showed IC₅₀ values ranging between 0.17 and 24 nM (Table 1).

CHF6001 inhibited [³H]rolipram binding in rat brain cytosol (HARBS) with an IC₅₀ of 1.05 nM, giving a HARBS ratio (HARBS IC₅₀:LARBS IC₅₀) of about 40, a value higher than GSK256066 ratio (11), whereas rolflumilast, 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 rolflumilast, CHF6001 did not show PDE4 subtype selectivity against different PDE isoenzymes. Rolflumilast is 10-fold less potent than CHF6001 with respect to PDE4 isozyme 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 PDE4 isoenzymes: PDE1, PDE2, PDE3, PDE5, PDE6, PDE7A, PDE8A1, PDE9A2, PDE10A2, and PDE11A4, therefore showing a marked selectivity for PDE4 (Table 3).

Activity of CHF6001 and Reference Compounds in Human Peripheral Blood Mononuclear Cells, THP-1 Monocyte-Derived Macrophages, and CD4⁺ 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; rolflumilast, 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, IC₅₀ = 1.77 nM (0.45–6.83), UK-500,001, IC₅₀ = 12.1 nM (2.9–50.1), and cilomilast IC₅₀ = 165.3 nM (61.8–442.6). These results were well in agreement with the previously reported potencies of GSK256066, rolflumilast, 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, IC₅₀ = 16 pM (8–35); CHF6001, IC₅₀ = 43 pM (15–122); UK-500,001, IC₅₀ = 3.60 nM (1.22–10.45); rolflumilast, IC₅₀ = 4.41 nM (1.0–19.3); cilomilast, IC₅₀ = 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⁺ 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, IC₅₀ = 3 pM (1–12); CHF6001, IC₅₀ = 62 pM (13–289); rolflumilast, IC₅₀ = 0.42 nM (0.09–1.87); UK-500,001, IC₅₀ = 9.4 nM (73–120); cilomilast, IC₅₀ = 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>IC₅₀</td>
<td>1.2</td>
<td>2.6</td>
<td>1.63</td>
<td>2.6</td>
<td>&gt;30</td>
</tr>
<tr>
<td>CI</td>
<td>(0.2–7.9)</td>
<td>(0.97–7.2)</td>
<td>(0.03–0.83)</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-α and NO evoked by LPS. In RAW264.7, basal release of TNF-α was 10.3 ± 5.2 pg/ml, whereas on LPS stimulation, it became 18,945 ± 838 pg/ml. The following inhibitory potencies (confidence interval values given in parentheses) were observed: GSK256066, IC₅₀ = 0.82 nM (0.3–2.1); CHF6001, IC₅₀ = 1 nM (0.7–1.5); roflumilast, IC₅₀ = 54.8 nM (23–130); UK-500,001, IC₅₀ = 36.60 nM (113–181); cilomilast, IC₅₀ = 830 nM (340–2000). Similar results were obtained in NR8383 cells, where the inhibition potencies against TNF-α release were: GSK256066, IC₅₀ = 0.08 nM (0.06–0.1); CHF6001, IC₅₀ = 0.19 nM (0.18–0.2); roflumilast, IC₅₀ = 8.3 nM (5.5–12.4); UK-500,001, IC₅₀ = 19.8 nM (3.2–123); cilomilast, IC₅₀ = 548 nM (353–852). In NR8383, basal release of TNF-α was 1.2 ± 0.5 pg/ml, whereas on LPS stimulation, it became 1184 ± 111 pg/ml.

In both NR8383 and RAW264.7, the potencies of all the tested inhibitors showed slightly higher IC₅₀ 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 IC₅₀ 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 IC₅₀ values of 0.55 nM (0.07–4.20) and 0.49 nM (0.17–1.43), respectively. Roflumilast, with an IC₅₀ = 8.51 nM (1.51–47.88), was again less potent than CHF6001 and GSK256066.

**Activity of CHF6001 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, IC₅₀ = 0.093 nM (0.036–0.241). Roflumilast, by showing an IC₅₀ = 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 μmol/kg), GSK256066 (1 μmol/kg), or budesonide (1 μmol/kg) was administered intranasally to rats to prevent LPS-induced pulmonary leukocytes infiltration. Each treatment group showed a significant reduction in the number of leukocytes infiltrating the lungs compared to the control group. The IC₅₀ value for CHF6001 was 1.2 nM (0.8–1.7). GSK256066 showed a lower IC₅₀ of 0.5 nM (0.3–0.7), while budesonide had an IC₅₀ of 2 nM (1.5–2.5).
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 μmol/kg (P < 0.05) and was maximal at 1 μmol/kg (77%) similarly to budesonide at 1 μmol/kg (77%). CHF6001 elicited also a dose-dependent inhibition of pulmonary white blood cell counts in BALF, which reached significance at 0.3 μmol/kg (P < 0.01) and was maximal at 1 μmol/kg (61%), similarly to budesonide at 1 μmol/kg (62%). Calculated ED50 values for CHF6001 were 0.188 μmol/kg against total white blood cells and 0.205 μmol/kg against neutrophilia. Similarly to CHF6001, GSK256066, when tested at 1 μmol/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 ≫ 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,
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 HARBS over the HARBS may retain anti-inflammatory activity and have a reduced capacity to cause nausea and emesis (Souness 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 monocytederived 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).
Acknowledgments

The authors thank Ornella Azzolino and Serena Bertolini for skillful technical assistance.

Authorship Contributions

Participated in research design: Armani, Rizzi, Amari, Patachini, Delcanale, Carnini, Hirsch, Civelli, Villetti, Facchinetti.

Conducted experiments: Moretto, Caruso, Bosco, Marchini, Pastore, Buccellati.

Contributed new reagents or analytic tools: Amari, Armani, Ghidini, De Fanti, Capaldi, Carzaniga, Rizzi.

Performed data analysis: Moretto, Bosco, Marchini, Facchinetti, Pastore.

Wrote or contributed to the writing of the manuscript: Moretto, Sala, Carnini, Civelli, Villetti, Facchinetti.

References


Dr. Fabrizio Facchinetti, Corporate Pre-clinical Research and Development. *J Pharmacol Exp Ther* 349:1545–1554.

Dr. Fabrizio Facchinetti, Corporate Pre-clinical Research and Development. *J Pharmacol Exp Ther* 349:1545–1554.

Dr. Fabrizio Facchinetti, Corporate Pre-clinical Research and Development. *J Pharmacol Exp Ther* 349:1545–1554.


