GSK256066, an Exceptionally High-Affinity and Selective Inhibitor of Phosphodiesterase 4 Suitable for Administration by Inhalation: In Vitro, Kinetic, and In Vivo Characterization


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Abstract

Oral phosphodiesterase (PDE) 4 inhibitors such as roflumilast have established the potential of PDE4 inhibition for the treatment of respiratory diseases. However, PDE4 inhibitor efficacy is limited by mechanism-related side effects such as emesis and nausea. Delivering the inhibitor by the inhaled route may improve therapeutic index, and we describe 6-[(3-(dimethylamino)carbonyl)phenyl)sulfonyl]-8-methyl-4-[(3-methoxy)phenyl]amino]-3-quinolinecarboxamide (GSK256066), an exceptionally high-affinity inhibitor of PDE4 designed for inhaled administration. GSK256066 is a slow and tight binding inhibitor of PDE4B (apparent IC50 3.2 pM; steady-state IC50 <0.5 pM), which is more potent than any previously documented compound, for example, roflumilast (IC50 390 pM), tofimilast (IC50 390 pM), and cilomilast (IC50 74 nM). Consistent with this, GSK256066 inhibited tumor necrosis factor α production by lipopolysaccharide (LPS)-stimulated human peripheral blood monocytes with 0.01 nM IC50 (compared with IC50 values of 5, 22, and 389 nM for roflumilast, tofimilast, and cilomilast, respectively) and by LPS-stimulated whole blood with 126 pM IC50. GSK256066 was highly selective for PDE4 (380,000-fold versus PDE1, PDE2, PDE3, PDE5, and PDE6 and 2500-fold versus PDE7), inhibited PDE4 isoforms A-D with equal affinity, and had a substantial high-affinity rolipram binding site ratio (>17). When administered intratracheally to rats, GSK256066 inhibited LPS-induced pulmonary neutrophilia with ED50 values of 1.1 μg/kg (aqueous suspension) and 2.9 μg/kg (dry powder formulation) and was more potent than an aqueous suspension of the corticosteroid fluticasone propionate (ED50 9.3 μg/kg). Thus, GSK256066 has been demonstrated to have exceptional potency in vitro and in vivo and is being clinically investigated as a treatment for chronic obstructive pulmonary disease.

Introduction

The importance of the second messenger, cAMP, in the control of immune and inflammatory cell function has been appreciated for many years. The intracellular concentration of cAMP depends in part on the activity of phosphodiesterases (PDEs) that catalyze its breakdown. There are currently 11 families of PDEs of which five are cAMP-selective and one has dual cAMP/cGMP specificity (Beswells-Smith et al., 2006). However, the availability of first-generation PDE4 inhibitors such as rolipram in the 1980s allowed the predominant role of PDE4 in catalyzing cAMP hydrolysis in immune and inflammatory cells to be demonstrated (Torphy and Undem, 1991). The therapeutic potential of PDE4 inhibition in...
asthma was underlined with the demonstration that rolipram blocked immune and inflammatory cell activity, reduced human airway smooth muscle contraction (Torphy et al., 1993), and inhibited antigen-induced bronchoconstriction and inflammation in vivo (Underwood et al., 1993). The anti-inflammatory and antibronchoconstrictor effects of PDE4 inhibition have subsequently been confirmed in numerous reports using a diverse array of PDE4 inhibitors (Dyke and Montana, 1999).

Unfortunately, first-generation PDE4 inhibitors were unsuccessful clinically because of the production of PDE4-dependent gastrointestinal side effects (Horowski and Sastre-y-Hernandez, 1985; Hebenstreit et al., 1989). Second-generation PDE4 inhibitors that would show a more favorable therapeutic index were vigorously pursued, and many have reached clinical development (Torphy et al., 1999; Dyke and Montana, 2002; Dastidar et al., 2007). However, only cilomilast and roflumilast have so far reached preregistration. Although the second-generation PDE4 inhibitors did show improved therapeutic index compared with rolipram, as a class they are still hampered by dose-limiting nausea and emesis (Boswell-Smith et al., 2006), and there are reports from preclinical toxicology studies of dose-limiting mesenteric and other organ inflammatory vasculitis, particularly in the rat (Daguès et al., 2007). There is thus still a need for novel PDE4 inhibitors with improved therapeutic index.

Although the majority of PDE4 inhibitors currently under investigation are intended and prepared for oral therapy, inflammatory diseases of the lung such as COPD and asthma currently are frequently treated with inhaled anti-inflammatory corticosteroids and bronchodilator agents acting within the lung. Consequently, we sought to identify potent anti-inflammatory PDE4 inhibitors that would have minimal systemic impact when administered by the inhaled route, reasoning that such a compound could show an improved therapeutic index and so be dosed to higher levels of both PDE4 inhibition and anti-inflammatory efficacy than is possible with oral PDE4 inhibitors. In this article, we describe the in vitro pharmacology and preliminary in vivo characterization of a novel tight binding inhibitor of PDE4, 6-[[3-[(dimethylamino)carbonyl]phenyl]sulfonfonyl]-6-methyl-4-[[3-methoxy]phenyl]amin]-3-quinoxinecarboxamide (GSK256066), which was derived from this rational design-based medicinal chemistry effort (Woodrow et al., 2009) and has been undergoing clinical trials for asthma and COPD. Studies described include enzyme kinetics, in vitro potency and selectivity, cellular and whole blood activity, and in vivo anti-inflammatory activity in a rat acute lung inflammation model. Some of these data were presented in poster form at the 2009 American Thoracic Society conference (Knowles et al., 2009).

Materials and Methods

Materials

GSK256066 (Fig. 1) as a free base (Woodrow et al., 2009) and in micromized form for in vivo studies, roflumilast, N-(3,5-dichloropyrid-4-yl)-(1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl)glyoxylic acid amide (AWD 12-281), tofimilast, N-(9-amino-4-oxo-1-phenyl-3,4,6,7-tetrahydro(1,4)-diazepane(6,7-h1)indol-3-yl)nicotinamide (CT1044), cilomilast, 3-(2-(3-cyclopentyloxy)-4-difluoromethoxy)phenyl]-2-(5-2-(1-hydroxy-1-trifluoromethyl)-2,2,2-trifluoroethyl)thiazolo[5,2-a]pyridine N-oxide (L689286), 3-(cyclopentyloxy)-N-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide (RP73401), 4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-d]pyrimidin-2(1H)-one (YM976), limilast, 4-[(3-(cyclopentyloxy)-4-methoxyphenyl)-2-phenylethyl]pyridine (CDP840), and fluticasone propionate (FP) were synthesized at the GlaxoSmithKline Respiratory-Centre of Excellence for Drug Discovery Chemistry, Stevenage, Hertfordshire, UK.

[3H]cAMP, [3H]cGMP, [3H]GSK256066, [methyl-3H]rolipram, and phosphodiesterase scintillation proximity assay (SPA) beads were obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Fluorescein-cAMP and IMAP binding reagent were from Molecular Devices Ltd. (Wokingham, Berkshire, UK). TNFα standard was from R&D Systems Europe Ltd. (Abingdon, Oxfordshire, UK), and streptavidin/biotinylated anti-TNFα antibody mix and ruthenium-tagged anti-TNFα monoclonal antibody were from IGEN International Inc. (Gaithersburg, MD). Lipopolysaccharide (LPS), cAMP, bovine serum albumin (BSA), EGTA, and magnesium chloride solution were from Sigma-Aldrich Company Ltd. (Gillingham, Dorset, UK). Slide-A-Lyser dialysis cassettes (3500 molecular weight cutoff) were obtained from Perbio Science U.K. Ltd. (Cramlington, Northumberland, UK). Other general laboratory chemicals and reagents were from various sources and were the highest grade available.

Human recombinant PDE4B, splice variant 2B, (HSPE4B2B: 1-564) was obtained from Dr. M. McLaughlin (Pathway Genomics, GlaxoSmithKline, Upper Merion, PA). The enzyme was expressed in PDE-deficient S. cerevisiae strain GL62, with induction by 150 μM CuSO4. Supernatant fractions from a 100,000g centrifugation of the yeast cell lysate were prepared as described by McLaughlin et al., (1993) and stored at -80°C in the presence of 20% (v/v) glycerol. Human recombinant PDE4B 152-503 truncate, which encodes almost the entire catalytic domain (Houslay, 2001), was expressed in human fibroblasts in cell culture and purified by Dr. Stuart Ballantine (Gene Expression and Protein Biochemistry, GlaxoSmithKline, Stevenage, UK). In outline, purification was by ion-exchange chromatography followed by gel-filtration chromatography. PDE1, PDE2, and PDE3 were purified from bovine aorta. Recombinant human PDE4A4 (1-886), PDE4C1 (1-712), PDE4D3 (1-673), and PDE5A1 (1-875) were expressed in S. cerevisiae, and recombinant human PDE7A1 (1-482) was expressed in a baculovirus/insect cell system. PDE6 was purified from bovine retina.

In Vivo Reagents. LPS (serotype 0127:B8) was used for all studies and dissolved in phosphate-buffered saline (PBS). Aqueous suspensions of test compounds were prepared immediately before dosing, by adding the required volume of 0.2% Tween 80 in normal saline to the preweighed compound. The suspension was sonicated for 10 min before use.

Fig. 1. Structure of GSK256066 (Woodrow et al., 2009).
Compound Preparation for In Vitro Assays. Dilution series for pIC50 determinations generally comprised eight successive 3-fold dilutions in DMSO. Low-volume additions (0.5–2.0 μl) to assays were carried out using a Biomek Fx liquid-handling robot (Beckman Coulter, Fullerton, CA).

PDE Enzyme Assays. For routine assay, PDE activity was measured at room temperature using 96-well SPA and additionally, for PDE4B and D only, 384-well fluorescence polarization assays. In both cases, enzymes were used at in-assay concentrations that gave linear reaction progress curves over the duration of the assay.

For SPA assays, 15 μl of PDE enzyme in 50 mM Tris-HCl, pH 7.5, containing 8.3 mM MgCl2, 1.7 mM EGTA, and 0.05% (w/v) BSA was preincubated with 2 μl of inhibitor solution or DMSO for 30 min. For PDE1 assays the assay buffer contained additionally 4 μg/ml calmodulin and 1 mM CaCl2 and did not contain any EGTA. Assays were initiated by the addition of 25 μl of [3H]cAMP (10 nM final concentration: PDE3, PDE4, and PDE7) or [3H]cGMP (36 nM: PDE1, PDE2, PDE5, and PDE6). After a 1-h incubation, assays were terminated by addition of 50 μl of aqueous suspension of SPA beads (approximately 1 mg per well) and, after an incubation of at least 30 min, bound radioactivity was measured by liquid scintillation counting. For fluorescence polarization assays, 10 μl of PDE enzyme in 10 mM Tris-HCl buffer, pH 7.2, containing 10 mM MgCl2, 0.1% (w/v) BSA, and 0.05% (w/v) Na2S2O3 was preincubated with 0.5 μl of inhibitor or DMSO for 30 min. Assays were initiated by the addition of 10 μl of fluorescein-cAMP (40 nM final concentration) and terminated after 40 min by the addition of 60 μl of IMAP binding reagent (1 in 400 dilution of stock suspension in binding buffer). The ratio of parallel to perpendicular light was measured using an Analyst (Molecular Devices, Sunnyvale, CA) or Aquest (Molecular Devices) plate reader.

Detailed enzyme studies were carried out at 25°C, and all reagents were pre-equilibrated to this temperature. GSK256066 (0.036 ml, 3–150 μM final concentration) and [3H]cAMP (0.45 μl) were mixed, and assays were initiated by the addition of 1.35 ml of PDE4B enzyme (10–20 μM final concentration) in 66.7 mM Tris-HCl, pH 7.5, containing 11.1 mM MgCl2, 2.27 mM EGTA, and 0.07% (w/v) BSA. Assays were made at three concentrations of [3H]cAMP (0.010, 8.0, and 20 μM); the specific activities were 51.8, and 12.0 Ci/mmol, respectively. Samples (100 μl, 16 from each assay) were taken at intervals over 90 min and added to 50 μl of PDE SPA bead suspension (approximately 1 mg per well). At least 30 min after the final sample was mixed with the beads bound radioactivity was measured by liquid scintillation counting.

Reversal of Inhibition by Dialysis. For dialysis studies, 2.7 nM PDE4B in dialysis buffer (PDE assay buffer lacking BSA) was incubated for 10 min at room temperature in the presence of 2.7 nM GSK256066 to establish >90% inhibition of enzyme activity. Samples of inhibited enzyme preparation (300 μl) were dialyzed for 4 h at room temperature in dialysis cassettes in which the membranes had previously been hydrated by immersion in dialysis buffer. Dialysate was replaced with fresh dialysis buffer at 2 h. At 4 h, 75–100 μl samples were removed from the cassettes, diluted 100-fold in PDE assay buffer, and assayed for enzyme activity with product being determined at 15-min intervals over 1 h after substrate addition.

Reversal of the Binding of [3H]GSK256066. These experiments were done using full-length PDE4B and PDE4B152-503 truncate. Equimolar concentrations (2.7 nM) of PDE4B and [3H]GSK256066 were mixed and incubated for 10 min at room temperature such that >90% of the enzyme activity was inhibited. Samples (0.4 ml) of the mixes were diluted with 3.6 ml of PDE assay buffer lacking BSA or 3.6 ml of 2.7 μM unlabeled GSK256066 in the same buffer. Samples of the dilutions (0.5 ml) were immediately processed through three rounds of ultra centrifugation using Millipore (Billericia, MA) Microcon YM-10 centrifugal filter units (10,000 molecular weight cutoff). The filter units were centrifuged at 10,000g for 45 or 50 min, and 0.4 ml of buffer was added to the reservoirs on each occasion. The amount of radioactivity in the combined filtrates from each filter was determined by liquid scintillation counting. Further samples were filtered at intervals between 5 and 48 h. For each enzyme construct, two separate experiments were done. In each experiment, to obtain a suitable spread of sampling times, two different PDE4B-[3H]GSK256066 mixes were made, but at different times. Control mixes that lacked enzyme were similarly treated.

Rolipram Binding Assay. The binding of rolipram to the high-affinity rolipram binding site (HARBS) in rat brain cytosol was carried out using a modification of the previously described method (Torphy et al., 1992). Cytosol from rat brain cerebral cortex was prepared by homogenization of the tissue in four volumes of ice-cold 50 mM Tris-HCl, pH 7.5, containing 5 mM MgCl2, 2 mM benzamidine, 100 μM N-tosyl-L-lysine chloromethyl ketone, 100 μg/ml bacitracin, 20 μg/ml trypsin inhibitor, 50 μM phenylmethylsulfonyl fluoride, and 100 μM leupeptin. The homogenate was centrifuged at 100,000g for 1 h at 4°C, and the supernatant was stored in aliquots at −80°C. Competition binding studies involved incubation of GSK256066 or DMSO (1% final concentration) and 2 nM [methyl-3H]rolipram in assay buffer (50 mM Tris HCl, pH 7.5 containing 5 mM MgCl2). The assay was initiated by addition of 700 μl of a 1:10 dilution in assay buffer of rat brain cytosol; total assay volume was 750 μl. After a 1-h incubation at 30°C the reaction mixture was cooled on ice for 10 min and then rapidly filtered under vacuum (Tomtec (Hamden, CT) harvester) through glass microfiber filters (Filtermat A, PerkinElmer Life and Analytical Sciences, Waltham, MA) that had been soaked in 0.3% polyethyleneimine. The filters were rapidly washed with ice-cold 20 mM Tris HCl, pH 7.5 + 2 mM MgCl2 and dried in a microwave oven before annealing to Meltleic scintillant sheets with a hot press (Wallac Microsealer) and counting for radioactivity.

Human Peripheral Blood Monocyte and Whole-Blood TNFα Assays. Whole blood and PBMCs were assayed as described previously (Davis et al., 2009). In brief, PBMCs were isolated from heparinized human blood by centrifugation on histopaque. Washed cells were then incubated (5 × 10^6 cells/well) with inhibitor solution or DMSO and LPS (1 ng/ml final concentration) in assay buffer (RPMI medium 1640 containing 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin) for 20 h at 37°C and 5% CO2. For whole-blood assays, heparinized blood was incubated with inhibitor solution or DMSO for 1 h at 37°C and 5% CO2 and then stimulated with LPS (50 ng/ml final) for 20 h at 37°C and 5% CO2.

TNFα concentrations in supernatants from both assays were measured by electrochemiluminescence assay as described previously (Davis et al., 2009).

In Vivo Studies. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act of 1986 and the GlaxoSmithKline Corporate Policy on the Care and Ethical Use of Animals in Scientific Research.

Animals. Male CD rats (Sprague-Dawley derived), weighing between 220 and 250 g, were supplied by Charles River U.K. Ltd. (Margate, Kent, UK). The animals were housed in plastic cages with absorbent bedding material and maintained on a 12-h daylight cycle. Food and water were provided ad libitum. The animals were acclimatized for a minimum of 7 days before commencing studies.

Intratracheal Dosing. GSK256066 (0.1–100 μg/kg) and FP (1–100 μg/kg) were administered intratracheally as an aqueous suspension or a dry powder 2 h before LPS challenge. In both cases, in preparation for dosing rats were anesthetized by exposure to a gas mixture of isoflurane (4.5%), nitrous oxide (3 l/min), and oxygen (1 l/min).

For aqueous suspension dosing, the animals were transorally intubated by inserting a blunt stainless-steel dosing needle into the trachea via the larynx, and 200 μl of vehicle, GSK256066, or FP formulation was administrated with a plastic syringe attached to the dosing needle.
For dry powder dosing, formulations of GSK256066 and FP blended with inhalation-grade lactose were used. A known quantity of drug/lactose blend, or lactose alone for the vehicle control, was contained in a preweighed, sterile, plastic three-way tap (Vycor, Auchenleck, UK) attached to a stainless-steel dosing needle and plastic 5-ml syringe. The needle was inserted into the trachea, and 4 ml of air was expelled through the tap to deliver the dry powder blend into the trachea and lung. After dosing, the tap was reweighed and the quantity of dry powder delivered to the animal was calculated.

After recovery from anesthesia, the animals were returned to the holding cages and given free access to food and water.

**Exposure to LPS.** Two hours after intratracheal dosing with vehicle, GSK256066, or FP, the rats were placed into sealed Perspex containers and exposed for 15 min to an aerosol of LPS, generated by a compressed-air driven nebulizer (DeVilbiss, Somerset, PA). The LPS concentration in the nebulizer was 150 μg/ml. The animals were then returned to the holding cages and allowed free access to food and water.

**Bronchoalveolar Lavage and Cell Counting.** Four hours after LPS exposure, the animals were killed by an intraperitoneal overdose of sodium pentobarbitone. The trachea was cannulated, and the lungs were lavaged three times with 5 ml of heparinized (25 units/ml) PBS. The bronchoalveolar lavage samples were centrifuged at 1300 rpm for 7 min. The cell pellets were resuspended in 1 ml of PBS, and 100 μl of cell suspension was added to cytospin holders, which were centrifuged at 5000 rpm for 5 min. The slides were air-dried and then stained with Leishmans stain for 20 min for differential cell counting. The total number of cells were counted using a Sysmex (Wym-bush, UK) mautomatic cell counter.

**Data Analysis**

**pIC<sub>50</sub> Determinations.** Percentages of inhibition values were generated relative to uninhibited controls. Values for pIC<sub>50</sub> were determined from concentration-response curves by nonlinear least-squares curve fitting, in general using a four-parameter logistic equation in Activity Base (IDBS, Guildford, Surrey, UK).

**Kinetic Studies.** All fits were made using Grafit, version 4 or version 5 (Erithacus Software Ltd., Horley, Surrey, UK). The Robust Weighting option within Grafit was used throughout to remove the effect of any outliers from fits.

**Fitting of Progress Plot Data for PDE4B.** Progress plots for uninhibited PDE4B assays were fitted by linear regression. Progress plot data showing time-dependent inhibition of PDE4B by GSK256066 were fitted by nonlinear regression using an integrated equation describing time-dependent, tight binding inhibition (Cha, 1976; Williams et al., 1979)

\[ P = v_o d + \frac{(v_o - v_s)(1 - d)}{dk} \ln \left( \frac{1 - d e^{-kt}}{1 - d} \right) \]  

where \( P \) is product formed at time \( t, v_o \) is initial rate, \( v_s \) is the final or steady-state rate, and \( k \) and \( d \) are functions of the various rate constants and the concentrations of substrate, inhibitor, and enzyme.

**Determination of Values for Inhibitor Constant (K<sub>i</sub>) for GSK256066.** Relationships between rate and GSK256066 concentration were fitted using an equation that describes tight binding inhibition (Morrison, 1969; Dharmasena et al., 2002):

\[ v = \left( \frac{v_o}{2E} \right) [E - I - K_i^*] + \sqrt{[E - I - K_i^*]^2 + 4EK_i^*} \]  

where \( v \) is the measured rate at the inhibitor concentration \( I, v_o \) is the control rate in the absence of inhibitor, \( E \) is the enzyme concentration, and \( K_i^* \) is the apparent inhibitor constant at the substrate concentration used. If an inhibitor is competitive with respect to the substrate, \( K_i' = K_i (1 + S/K_s) \), where \( K_i \) is the true inhibitor (or dissociation) constant and \( S \) is the substrate concentration.

**Determination of the Rate of Dissociation of [H]GSK256066 from PDE4B-[H]GSK256066 Complex.** The total counts that were recovered in the combined filtrates at each time point were plotted against the sampling time and the data were fitted using the first-order rate equation

\[ A_t = A_0 (1 - e^{-kt}) + A_0 \]  

where \( A_t \) represents the counts at time \( t, A_0 \) represents the final counts at infinite time, and \( A_0 \) represents the counts at time 0.

The values for \( \Delta \text{cpm} \) at each time were then expressed as a percentage of the extrapolated maximum counts displaced \( A_0 - A_0 \). In this way, data from four different mixes for each PDE4B construct were normalized and then combined. The combined data were then fitted using eq. 3 to determine the rate constant \( k \) for the dissociation of [H]GSK256066 from the PDE4B-[H]GSK256066 complex.

**In Vivo Studies.** Data were analyzed using Prism (GraphPad Software Inc., San Diego, CA). Parametric tests performed to determine statistical significance were an analysis of variance with Dunnnett’s post test, and nonparametric tests used were Kruskal-Wallis and Dunn’s post test.

**Pharmacokinetics.** The pharmacokinetics of GSK256066 was studied in the male CD rat after intravenous bolus and oral gavage administration both at doses of 1 mg/kg. The dose was formulated as a 0.5 mg/ml solution in DMSO/polyethylene glycol200/water (10:67:23, v/v/v). Dose volume for both routes was 2 ml/kg. Blood samples (200 μl) were taken at a range of time points over 12 h and centrifuged to yield plasma. GSK256066 was extracted from the plasma samples using protein precipitation, and the samples were analyzed by quantitative liquid chromatography/mass spectrometry. The pharmacokinetic parameters of GSK256066 were then calculated from the plasma concentrations using GlaxoSmithKline software package PKTools v2.

**Results**

**Enzyme Assays.** The derived IC<sub>50</sub> values (1–5 pM) for the inhibition of PDE4 by GSK256066 (Table 1; pIC<sub>50</sub> 11.3–11.9) were similar to the in-assay enzyme concentrations (nominally 10–20 pM). This apparent tight binding behavior was confirmed in the enzyme kinetic studies outlined below. The PDE4 pIC<sub>50</sub> values shown in Table 1 are considered minimum estimates. In spite of this, the pIC<sub>50</sub> values are unprecedented for PDE4 inhibitors. The next most potent comparator compound is roflumilast (pIC<sub>50</sub> 9.4), whereas other known inhibitors had pIC<sub>50</sub> values in the range of seven to nine in our PDE4B assays (Table 2).

Based on a minimum pIC<sub>50</sub> value for PDE4B of 11.5, selectivity over the other PDEs tested was extremely high, being at least 2500-fold against PDE7 and at least 350,000-fold against PDE1, PDE2, PDE3, PDE5, and PDE6 (Table 1). GSK256066 was found to be inactive (pIC<sub>50</sub> < 5) when tested against a wide range of protein kinases, the human ether-a-go-go-related gene channel and the Cerep panel of receptor screens (data not shown), representing at least 3,000,000-fold selectivity.

GSK256066 inhibited [H]rolipram binding in rat brain cytosol with a pIC<sub>50</sub> of 10.3, giving a HARBs ratio (HARBs IC<sub>50</sub>/PDE4B IC<sub>50</sub>) of at least 17, compared with ratios of 2 to 3 for AMD 12-281, cilomilast, and roflumilast (Table 1). The Hill slope for inhibition of rolipram binding by GSK256066 was 0.9, suggesting that enzyme concentration did not limit the measured compound potency in this assay.
TABLE 1
Potency and selectivity of GSK256066, AWD 12-281, and roliflumast for PDEs
PDE activity was measured at room temperature using SPA assays and additionally, for PDE4B and PDE4D only fluorescence polarization assays. The binding of GSK256066 to the HARBS in rat brain cytosol was measured using a competition binding assay with 2 nM [methyl-3H]cAMP as radioligand. When n = 1 the values are means of duplicate determinations in a single experiment, except for HARBS data for GSK256066 (mean of three determinations in a single study) and cilomilast, which was a single determination only, for the latter the data are consistent with the 100 nM Kᵢ published previously (Barnette et al., 1994).

<table>
<thead>
<tr>
<th>PDE4B</th>
<th>AWD 12–281</th>
<th>Cilomilast</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIC₅₀ (mean ± S.E.M. or range)</td>
<td>pIC₅₀</td>
<td>pIC₅₀</td>
</tr>
<tr>
<td>PDE4B</td>
<td>≥11.5 (n = 12)</td>
<td>7.63 ± 0.23 (n = 2)</td>
</tr>
<tr>
<td>PDE4A</td>
<td>≥11.3 (n = 4)</td>
<td>6.91 ± 0.04 (n = 66)</td>
</tr>
<tr>
<td>PDE4C</td>
<td>≥11.4 (n = 12)</td>
<td>7.52 ± 0.64 (n = 2)</td>
</tr>
<tr>
<td>PDE4D</td>
<td>≥11.9 (n = 12)</td>
<td>7.52 ± 0.64 (n = 2)</td>
</tr>
<tr>
<td>PDE1</td>
<td>5.73 ± 0.03 (n = 2)</td>
<td>≥600,000</td>
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<tr>
<td>PDE2</td>
<td>5.92 ± 0.05 (n = 2)</td>
<td>≥390,000</td>
</tr>
<tr>
<td>PDE3</td>
<td>5.93 ± 0.12 (n = 5)</td>
<td>≥380,000</td>
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<td>PDE5</td>
<td>5.49 ± 0.04 (n = 5)</td>
<td>≥1,050,000</td>
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<tr>
<td>PDE6</td>
<td>5.28 ± 0.03 (n = 5)</td>
<td>≥1,700,000</td>
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<tr>
<td>PDE7</td>
<td>8.11 ± 0.26 (n = 2)</td>
<td>≥251,000</td>
</tr>
<tr>
<td>HARBS</td>
<td>10.27 (n = 1)</td>
<td>≥17</td>
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N.D., not determined
* PDE4 potency is likely to be an underestimate because the derived IC₅₀ value is similar to the estimated in-assay enzyme concentrations.

TABLE 2
Potencies of GSK256066, competitor PDE4 inhibitors, and FP on PDE4B and LPS-driven TNFα production in human PBMCs and whole blood.

<table>
<thead>
<tr>
<th>GSK256066</th>
<th>AWD 12–281</th>
<th>Roliflumast</th>
<th>Cilomilast</th>
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</thead>
<tbody>
<tr>
<td>Mean pIC₅₀</td>
<td>S.E.M. or range (n)</td>
<td>Mean pIC₅₀</td>
<td>S.E.M. or range (n)</td>
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<tr>
<td>PDE4B</td>
<td>&gt;11.5 (12)</td>
<td>11.01 ± 0.04 (8)</td>
<td>9.90 ± 0.11 (5)</td>
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<td>CI1044</td>
<td>6.97 ± 0.02 (4)</td>
<td>7.22 ± 0.03 (15)</td>
<td>6.55 ± 0.11 (4)</td>
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<td>Cilomilast</td>
<td>7.13 ± 0.08 (4)</td>
<td>6.41 ± 0.14 (2)</td>
<td>5.24 ± 0.24 (2)</td>
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<td>L689298</td>
<td>8.99 ± 0.02 (2)</td>
<td>8.45 ± 0.04 (2)</td>
<td>8.02 ± 0.12 (4)</td>
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<td>Roliflumast</td>
<td>9.41 ± 0.04 (129)</td>
<td>8.30 ± 0.02 (40)</td>
<td>7.71 ± 0.02 (172)</td>
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<td>RP73401</td>
<td>9.18 (1)</td>
<td>8.62 ± 0.05 (4)</td>
<td>7.44 ± 0.07 (6)</td>
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<td>Tofimilast</td>
<td>8.80 ± 0.23 (2)</td>
<td>7.85 ± 0.09 (2)</td>
<td>6.44 ± 0.16 (2)</td>
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<td>YM976</td>
<td>8.69 ± 0.08 (3)</td>
<td>7.64 ± 0.06 (2)</td>
<td>6.42 (n = 1)</td>
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<tr>
<td>FP</td>
<td>9.71 ± 0.11 (11)</td>
<td>8.63 ± 0.18 (6)</td>
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Analysis of Progress Plot Data for the Inhibition of PDE4B by GSK256066. Under conditions where control progress plots for assays of full-length PDE4B were essentially linear for approximately 50 min with 0.010 μM cAMP and at least 80 min with 8.0 and 20 μM cAMP, progress plots made in the presence of GSK256066 were nonlinear. They showed a time-dependent decrease in rate that depended on the inhibitor concentration.

It was clear by inspection that steady-state rates had declined to zero at inhibitor concentrations that were similar to the nominal enzyme concentration. Thus, as well as showing time-dependent behavior, GSK256066 was binding very tightly to PDE4B. It was not possible to remove the need for tight-binding considerations in the data analysis by using much higher substrate concentrations because, at cAMP concentrations above 20 μM, the background readings became unacceptably high. We needed, therefore, to analyze the progress plot data using eq. 1 (see Data Analysis), which describes slow and tight binding behavior. Representative progress plots, with fits obtained using eq. 1, are shown in Fig. 2.

The fits enabled values for initial and steady-state rates to be obtained, and these are shown plotted against the concentration of GSK256066 in Fig. 3. At all concentrations of cAMP, values for both initial and steady-state rates decrease with increasing GSK256066 concentration. This behavior is consistent with a mechanism (Morrison and Walsh, 1988) in which an enzyme-inhibitor complex (E.I.), which is formed rapidly, undergoes a slow isomerization to a second complex (E⁻I⁻). Very similar behavior was observed for assays made at 0.010 μM cAMP using PDE4B 152-503 truncate (data not shown), showing that truncate 152-503 and full-length PDE4B have similar kinetic properties.

For a tight-binding inhibitor whose kinetic mechanism of inhibition is described by the proposed scheme, initial rate data against inhibitor concentration data can be fitted using eq. 2 to obtain a value for Kᵢ, the dissociation constant for the E⁻I⁻ dissociating to E + I. Steady-state rate against inhibitor concentration data can be fitted using eq. 2 to obtain a value for Kᵢ, the overall dissociation constant for the E⁻I⁻ dissociating to E + I.

Initial Rate Data. Inspection of Fig. 3 shows that, although the data obtained at the higher concentrations of cAMP seem suitable for fitting using eq. 2, data obtained at very low cAMP concentration result in an unusual relationship between initial rate and inhibitor concentration. The reason for this unusual behavior is not apparent. Although it may arise because of errors in the determination of values for
The values obtained from fits using eq. 2 are below the potency at which real changes can be discriminated. The limit of discrimination was estimated to be approximately 0.5 pM, based on simulated plots (not shown) of rate against inhibitor concentration that obey eq. 2. It is clear, however, that GSK256066 is a very potent inhibitor of PDE4B, and the value for the overall dissociation constant is \(<0.5 \text{ pM} (\text{pK}_d >12.3).\)

Reversibility of the Inhibition of PDE4B by GSK256066. Attempts to demonstrate reversibility of the enzyme inhibition using dialysis were unsuccessful. No activity was recovered after the E.I. had been processed through three rounds of dilution/ultracentrifugation using Millipore Microcon YM-10 centrifugal filter units. Recoveries of control enzyme in these experiments were in the range of 40 to 50%.

Reversibility of the Binding of \[^3H\]GSK256066 to PDE4B and PDE4B 152-503 Truncate. When PDE4B-\[^3H\]GSK256066 complexes were diluted into an excess of unlabeled GSK256066 and samples were subjected to several cycles of ultrafiltration and dilution, there were time-dependent increases in the number of counts recovered in the combined filtrates, which were shown to follow first-order kinetics (eq. 3).

For the truncate, the total counts recovered in the filtrates were equal to the counts recovered when compound alone was filtered, and the first-order rate constant for dissociation of the enzyme-\[^3H\]GSK256066 complex was 0.021 h\(^{-1}\) (t\(_{1/2}\) 6.3 h). The data for the full-length enzyme were more scattered. However, the differences between the data points and the fitted line were apparently randomly distributed, indicating that the fit made was an appropriate one. The rate of dissociation of \[^3H\]GSK256066 from full-length enzyme is clearly slower than from the truncate, and even after 48 h of incubation approximately 30% of the labeled compound had not appeared in the filtrate. The estimated rate constant for the dissociation of the full-length PDE4B-\[^3H\]GSK256066 complex was 0.021 h\(^{-1}\), enabling a value for t\(_{1/2}\) of 33 h to be derived.

Cellular and Whole-Blood Potencies of GSK256066. GSK256066 was an extremely potent inhibitor of LPS-stimulated TNF\(\alpha\) production in PBMCs (pIC\(_{50}\) 11.0, IC\(_{50}\) 10 pM) and human whole-blood cultures (pIC\(_{50}\) 9.90, IC\(_{50}\) 126 pM) (Fig. 5 and Table 2). The maximal inhibition achieved was 83 ± 3% in PBMCs and 80 ± 4% for whole blood (mean ± S.E.M.). The compound was markedly more potent than any of the comparator compounds tested (Table 2). GSK256066 was also approximately 20-fold more potent than the corticosteroid FP in PBMCs and whole blood.

Pharmacokinetics of GSK256066 in the Male CD Rat. GSK256066 showed a moderate plasma clearance (39 ml/min/kg), a moderate volume of distribution (0.8 l/kg), and a relatively short half-life (1.1 h) in the male CD rat. Bioavail-

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**Fig. 2.** Progress plots showing the time-dependent inhibition of full-length PDE4B by the indicated concentrations of GSK256066 at a cAMP concentration of 8.0 \(\mu\)M. The lines drawn are fits of the data by an equation describing slow and tight binding inhibition (eq. 1). The graphs shown are representative of a wider dataset, others being generated at 0.01 and 20 \(\mu\)M cAMP.
ability was low after oral administration as a solution (<1%). Blood samples were also taken from the hepatic portal vein after oral administration, and the absorption of GSK256066 was found to be low (3.8%).

Rat LPS-Induced Pulmonary Neutrophilia: Inhibitory Potency of GSK256066 and FP. Aqueous suspension and lactose blended dry powder formulations of GSK256066 were well tolerated when administered via the intratracheal route to rats at doses up to 100 μg/kg and were not associated with any overt behavioral changes.

When administered 2 h before the inflammatory stimulus, GSK256066 caused a dose-dependent inhibition of the LPS-induced pulmonary neutrophilia, which reached statistical significance (p < 0.05) at 3 μg/kg and was maximal (72%) at 30 μg/kg (Fig. 6). In the same experiments, FP also caused a dose-related inhibition of the neutrophilia that was significant (p < 0.05) at 10 μg/kg and maximal (83%) at 100 μg/kg. The dose-response curves were used to derive ED50 values of 1.1 and 9.3 μg/kg for GSK256066 and FP, respectively.

When given in the dry powder formulation, GSK256066 inhibited the LPS-induced pulmonary neutrophilia with an ED50 of 2.9 μg/kg, achieving maximal inhibition of 62% (Fig. 7). Inhibition was significant (p < 0.001) at all doses tested.

Discussion

The aim of this series of experiments was to examine the in vitro and in vivo properties of the PDE4 inhibitor GSK256066. The data presented demonstrate that GSK256066 is an exceptionally high-affinity inhibitor of PDE4B, substantially more so than the other known PDE4 inhibitors tested (Table 2), including the most potent of the oral inhibitors roflumilast (Hatzelmann and Schudt, 2001) and the inhaled inhibitors AWD 12-281 (Kuss et al., 2003), tofimilast (Duplantier et al., 2007), and piclamilast (Hirose et al., 2008). GSK256066 is highly selective for PDE4 over other PDEs tested (>2500-fold against PDE7 and >380,000 against PDE1, PDE2, PDE3, PDE5, and PDE6) and also has a high HARBS/PDE4B ratio (>17).

Detailed enzyme kinetic studies showed that GSK256066 is a slow and tight binding inhibitor of PDE4B. Generally, the data are consistent with the inhibition being competitive with respect to cAMP (although mixed inhibition cannot be ruled out) and via a mechanism in which an E.I., which is formed rapidly, slowly isomerizes to a second complex (E·I). However, the data obtained at very low cAMP concentration (0.010 μM) suggest that, under these conditions, there may be an unusual relationship between initial rate and inhibitor concentration. The reason for this is not apparent, and the kinetic mechanism of inhibition may follow a more complicated scheme than that hypothesized above. Because this very low concentration of cAMP is much lower than the value (4 μM) for $K_m$ for cAMP for this enzyme, the unusual behavior may not be physiologically relevant, even if it is real.

There is no doubt that GSK256066 binds very tightly to PDE4B. The overall dissociation constant for the E·I. dissociating to E + I is too low (<0.5 pM) to allow a precise value.
to be obtained but is entirely consistent with the pIC$_{50}$ ≈ 11.5 shown in Table 1.

Because of the extremely tight binding of GSK256066 to PDE4B, reversal of inhibition could not be demonstrated by dilution of the E.I. into assays or by dialysis or ultrafiltration. The binding of $[^{3}H]$GSK256066 to PDE4B was slowly reversible, however, with the half-life for dissociation being 6 h for the truncated enzyme and 33 h for full-length PDE4B. The reason for the somewhat slower dissociation from the full-length enzyme is unclear. Construct length may be a factor, but differences in expression and extraction methods, which could, for example, influence phosphorylation status of the enzyme, cannot be ruled out. The full-length enzyme was expressed in yeast and used as a crude extract, whereas the truncate was a highly purified preparation from a baculovirus/insect cell system. Although the results show that the binding of $[^{3}H]$GSK256066 to PDE4B is reversible, because the labeled compound was replaced on the enzyme by unlabeled inhibitor, it is not possible to say whether, in a cell, dissociation of GSK256066 from PDE4B would be accompanied by recovery of enzyme activity. However, slow reversal of binding is considered a beneficial property because it is likely to provide prolonged duration of activity in the lung.

In this work, we have carried out detailed enzymatic stud-

Fig. 4. GSK256066 slowly dissociates from PDE4B. Equimolar concentrations (2.7 nM) of enzyme and $[^{3}H]$GSK256066 were diluted 10-fold into an excess (2.7 µM) of unlabeled GSK256066. Samples were filtered at intervals using centrifugal filters. The data points represent the percentage of the extrapolated maximum counts released into the filtrate plotted against the time of sampling for samples from four different mixes, which were normalized as described under Materials and Methods. The lines drawn are fits of the normalized data using eq. 3, which describes first-order kinetics. Results are shown for PDE4B 152-503 truncate (a) and for full-length PDE4B (b).

Fig. 5. Dose-response curves for the inhibition of LPS-stimulated TNFα by GSK256066 and AWD 12-281 in PBMCs and whole blood. Curve fits were generated using a four-parameter logistic equation and are representative of the larger data set. Data are shown for GSK256066 with PBMC (○) and whole blood (○) and AWD 12-281 with PBMC (▲) and whole blood (△).

Fig. 6. Inhibition of LPS-induced pulmonary neutrophilia in the rat: dose-related inhibition by GSK256066 (○) and FP (▲). Compounds were administered as an aqueous suspension 2 h before LPS challenge. *, p < 0.05 and ***, p < 0.01 versus control response to LPS.
GSK256066 is an extremely potent and effective inhibitor of the production of the clinically relevant proinflammatory cytokine TNFα. The compound shows exceptional potency in both whole blood and PBMCs compared with a wide range of competitor compounds (Table 2). For example, in PBMCs it is >2000-fold more potent than both tofimilast and AWD 12-281, approximately 350-fold more potent than Merck Frosst’s L869298 and approximately 500-fold more potent than roflumilast. The decline in potency in the whole-blood assay relative to the potency in PBMCs (approximately 13-fold) is probably caused by plasma protein binding (98.4% bound at 1 μg/ml in human plasma; L. Ranshaw, unpublished data) or possibly to red blood cell binding of the compound.

The high potency of GSK256066 in vitro assays translated into potent anti-inflammatory efficacy in vivo when the compound was administered intratracheally to rats as either an aqueous suspension or a dry powder formulation 2 h before inhaled LPS challenge. Acute pulmonary neutrophilia of this molecule is a characteristic of the response to inhaled LPS (Pauwels et al., 1990), and it was inhibited by an aqueous suspension of GSK256066 with approximately 7-fold greater potency (ED₅₀ 1.1 μg/kg) than was demonstrated by the corticosteroid FP, which is a current gold-standard inhaled treatment for asthma. Maximal inhibition of neutrophilia by GSK256066 was not significantly different from that of FP. As a dry powder, GSK256066 was marginally less potent (ED₅₀ 2.9 μg/kg) than as an aqueous suspension. Dry powder formulations of FP were not evaluated in this study, but reference to our unpublished data indicates the ED₅₀ is approximately 20 μg/kg, again suggesting that GSK256066 is 6- to 7-fold more potent.

Other workers have demonstrated efficacy with both systemic and inhaled PDE4 inhibitors in animal models of acute pulmonary inflammation, but, in general, not over the effective dose range seen with GSK256066. Efficacy has been demonstrated with orally administered PDE4 inhibitors rolipram (Evans et al., 1995) and roflumilast and cilomilast (Underwood et al., 1998, Bundschuh et al., 2001). Potency estimates ranged from approximately 0.1 mg/kg (roflumilast) to 30 mg/kg (cilomilast). There are fewer published studies with inhaled PDE4 inhibitors. Efficacy with inhaled piclamilast (RP73401) has been reported by Raeburn et al. (1994). In a rat model of antigen-induced pulmonary eosinophilia, significant anti-inflammatory effect was observed only at a dose of 400 μg/kg. In contrast, studies with a dry powder formulation of the inhaled PDE4 inhibitor AWD 12-281 (Kuss et al., 2003) demonstrated potent anti-inflammatory effect in a rat LPS model (ED₅₀ < 1 μg/kg). However, our own studies with this molecule were unable to reproduce this potent effect, and we did not see any anti-inflammatory effect at all with aqueous suspension formulations of this molecule (M. Gascoigne, unpublished observations). It is unclear why AWD 12-281 is more active when administered by dry powder than aqueous suspension.

Oral PDE 4 inhibitors such as roflumilast (Boswell-Smith et al., 2006) and cilomilast (Giembycz, 2001; Brown, 2005) have demonstrated some efficacy in COPD clinical studies. However, oral therapies by their nature require systemic exposure, and for PDE4 inhibitors this is associated with dose-limiting emesis and nausea (Boswell-Smith et al., 2006), which in turn limits the level of anti-inflammatory activity that is achievable. A highly potent, topically active PDE4 inhibitor delivered by the inhaled route, with limited systemic exposure, would be predicted to deliver an improved therapeutic index, allowing higher levels of inhibition and efficacy to be reached. Further studies with intratracheal delivery of dry powder GSK256066 in the ferret model of emesis support this hypothesis (Nials et al., 2010). In addition, in the current studies GSK256066 was found to be well tolerated when administered intratracheally to conscious rats, producing no overt behavioral effects at maximally inhibitory concentrations. GSK256066 thus holds promise as an inhaled anti-inflammatory therapy for COPD and asthma. Such a potent anti-inflammatory compound would also offer the potential for different combination therapies, for example, with B agonists or anticholinergics specifically targeting inflammation and bronchodilation. This would be similar to steroid/bronchodilator combination therapies such as Seretide (FP plus salmeterol) but with a novel anti-inflammatory mechanism.

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References


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