GSK256066, An Exceptionally High Affinity and Selective Inhibitor of PDE4 Suitable for Administration by Inhalation: *In vitro, Kinetic and In vivo* Characterisation


Running title: GSK256066, an exceptionally high affinity inhibitor of PDE4
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Non-standard abbreviations:
GSK256066, 6-\{(3-\{(dimethylamino) carbonyl\}phenyl} sulfonyl\}-8-methyl-4-\{[3-methyloxy) phenyl]amino\}-3-quinolinecarboxamide
PDE, Phosphodiesterase
PBMC, Peripheral Blood Mononuclear cell
HARBS, High-Affinity Rolipram Binding Site
pIC_{50}, Negative log of concentration producing 50% of maximum possible inhibition
FP, Fluticasone Propionate
COPD, Chronic Obstructive Pulmonary Disease
hERG, (Human Ether-a-go-go Related Gene) a potassium ion channel responsible for the repolarizing I_{Kr} current in the cardiac action potential.
BSA, bovine serum albumin
LPS, lipopolysaccharide
PBS, phosphate-buffered saline
SPA, scintillation proximity assay
BAL, bronchoalveolar lavage

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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 herein we describe GSK256066 (6-((3-[(dimethylamino) carbonyl]phenyl) sulfonyl)-8-methyl-4-{[3-methyloxy) phenyl]amino}-3-quinolinecarboxamide), 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 < 0.5pM), more potent than any previously documented compound, for example roflumilast (IC₅₀ 390 pM), tofimilast (IC₅₀ 1.6 nM) and cilomilast (IC₅₀ 74 nM).
Consistent with this, GSK256066 inhibited TNFα production by lipopolysaccharide (LPS) -stimulated human peripheral blood monocytes with 0.01nM IC₅₀ (compared with IC₅₀s of 5, 22 and 389nM for roflumilast, tofimilast and cilomilast) and by LPS-stimulated whole blood with 126 pM IC₅₀. GSK256066 was highly selective for PDE4 (>380,000-fold vs PDEs 1, 2, 3, 5 and 6 and >2500-fold against PDE7), inhibited PDE4 isoforms A-D with equal affinity and had a high HARBS (High-Affinity Rolipram Binding Site) ratio (>17). When administered by the intratracheal route to rats, GSK256066 inhibited LPS-induced pulmonary neutrophilia with ED₅₀s 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 COPD.
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 is in part dependent on the activity of phosphodiesterases (PDE) which catalyse its breakdown. There are currently 11 families of PDE of which 5 are cAMP selective and a further one has dual cAMP/cGMP specificity (Boswell Smith et al., 2006). However, the availability of first generation PDE4 inhibitors such as rolipram in the 1980’s allowed the predominant role of PDE4 in catalysing 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 anti-bronchoconstrictor 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 due to 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 favourable 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 pre-registration. Although the second generation PDE4 inhibitors did show improved therapeutic index compared to rolipram, as a class they are still hampered by dose limiting nausea and emesis (Boswell-Smith et al., 2006) and there are reports from pre-clinical toxicology studies of dose limiting mesenteric and other
organ inflammatory vasculitis, particularly in the rat (Dagues et al., 2007). There is thus still a need for novel PDE4 inhibitors with improved therapeutic index.

Whilst the majority of PDE4 inhibitors currently under investigation are intended and designed for oral therapy, inflammatory diseases of the lung such as COPD and asthma are currently 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 manuscript, we describe the in vitro pharmacology and preliminary in vivo characterisation of a novel tight binding inhibitor of PDE4, GSK256066, which was derived from this rational design based medicinal chemistry effort (Woodrow et al., 2009) and which 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 this data was presented in poster form at the 2009 American Thoracic Society conference (Knowles et al., 2009).

Methods

Materials

GSK256066 (6-\{(3-[(dimethylamino) carbonyl]phenyl} sulfonyl)-8-methyl-4-\{[3-methyloxy) phenyl]amino\}-3-quinolinecarboxamide, Fig. 1) as a free base (Woodrow et al., 2009) and in micronised form for in vivo studies, Roflumilast, AWD 12,281, Tofimilast, CI1044, Cilomilast,
L869298, RP73401, YM976, Lirimilast, CDP840, and fluticasone propionate (FP) were synthesised in GSK Respiratory-CEDD Chemistry, Stevenage, Hertfordshire, U.K. 

[^3]H]-cAMP, [^3]H]-cGMP, [^3]H]-GSK256066, [methyl-[^3]H]-rolipram and phosphodiesterase SPA beads were obtained from GE Healthcare U.K. Ltd., Chalfont St. Giles, Buckinghamshire, U.K., Fluorescein-cAMP and IMAP binding reagent were from Molecular Devices Ltd., Wokingham, Berkshire, U.K., TNF\(\alpha\) standard was from R&D Systems Europe Ltd., Abingdon, Oxfordshire, U.K., and streptavidin/biotinylated anti-TNF\(\alpha\) antibody mix and ruthenium-tagged anti-TNF\(\alpha\) monoclonal antibody were from IGEN International Inc., Gaithersburg, MD., U.S.A. 

Lipopolysaccharide (LPS), cAMP, bovine serum albumin (BSA), EGTA, and magnesium chloride solution were from Sigma-Aldrich Company Ltd., Gillingham, Dorset, U.K. Slide-A-Lyser dialysis cassettes (3500 molecular weight cut off) were obtained from Perbio Science U.K. Ltd, Cramlington, Northumberland, U.K. Other general laboratory chemicals and reagents were from various sources and were the highest grade available.

Human recombinant PDE4B, splice variant 2B, (HSPDE4B2B: (1-564) was obtained from Dr. M. McLaughlin, Pathway Genomics, GSK, Upper Merion, PA., U.S.A. The enzyme was expressed in PDE-deficient Saccharomyces cerevisiae, strain GL62, with induction by 150\(\mu\)M CuSO\(_4\). Supernatant fractions from a 100,000g centrifugation of the yeast cell lysate were prepared as described by McLaughlin et al., (1993) and were 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 a baculovirus/insect cell system and purified by Dr. Stuart Ballantine, GEPB, GSK, Stevenage, U.K. In outline, purification was by ion-exchange chromatography followed by gel-filtration chromatography. PDE1, 2 and 3 were purified from bovine aorta. Recombinant human PDE4A4 (1-886), PDE4C1 (1-712), PDE4D3
(1-673) and PDE5A1 (1-875) were expressed in Saccharomyces cerevisiae, recombinant human PDE7A1 (1-482) was expressed in a baculovirus/insect cells system. PDE6 was purified from bovine retina.

**In vivo reagents.**

LPS (Serotype0127:B8 ) was used for all studies and was 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 pre-weighed compound. The suspension was sonicated for 10 minutes prior to use.

**Compound preparation for in vitro assays.**

Dilution series for pIC50 determinations generally comprised 8 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.

**PDE enzyme assays.**

For routine assay, PDE activity was measured at room temperature using 96-well scintillation proximity assays (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 assay.

For SPA assays, 75 µl of PDE enzyme in 50 mM Tris-HCl, pH7.5, containing 8.3 mM MgCl2, 1.7 mM EGTA, and 0.05% (w/v) BSA were pre-incubated with 2 µl of inhibitor solution or DMSO for 30 minutes. For PDE1 assays the assay buffer contained additionally 4µg/ml calmodulin and 1mM CaCl2 and did not contain any EGTA. Assays were initiated by addition of 25 µl of [3H]-cAMP (10 nM final concentration: PDE 3, 4 and 7) or [3H]-cGMP (36 nM: PDE1, 2, 5 and 6). After a 1 hour incubation, assays were terminated by addition of 50 µl of aqueous
suspension of SPA beads, (approximately 1mg per well) and, after an incubation of at least 30 minutes, bound radioactivity was measured by liquid scintillation counting. For fluorescence polarization assays, 10 µl of PDE enzyme in 10 mM Tris-HCl buffer, pH7.2, containing 10 mM MgCl₂, 0.1% (w/v) BSA, and 0.05% (w/v) NaN₃ were pre-incubated with 0.5 µl of inhibitor or DMSO for 30min. Assays were initiated by addition of 10µl of fluorescein-cAMP (40 nM final concentration) and were terminated after 40 minutes by 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 or Aquest™ 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 pM final concentration) and ³H-cAMP (0.45 ml) were mixed and assays were initiated by addition of 1.35 ml of PDE4B enzyme (10 to 20 pM final concentration) in 66.7 mM Tris-HCl, pH7.5, containing 11.1 mM MgCl₂, 2.27 mM EGTA and 0.07% (w/v) BSA. Assays were made at 3 concentrations of ³H-cAMP, 0.010, 8.0 and 20 µM; the specific activities were 51, 0.88 and 0.12 Ci/mmol, respectively. Samples (100 µl, 16 from each assay) were taken at intervals over 90 minutes and added to 50 µl of PDE SPA bead suspension (about 1mg per well). At least 30 minutes 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, 2.7nM PDE4B in dialysis buffer (PDE assay buffer lacking BSA) was incubated for 10 minutes at room temperature in the presence of 2.7nM GSK256066 to establish >90% inhibition of enzyme activity. Samples of inhibited enzyme preparation (300 µl) were dialysed for 4 hours 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 hours. At 4 hours, 75 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 minute intervals over a 1 hour period after substrate addition.

**Reversal of the binding of [³H]-GSK256066**

These experiments were done using full-length PDE4B and PDE4B 152 – 503 truncate. Equimolar concentrations (2.7 nM) of PDE4B and [³H]-GSK256066 were mixed and incubated for 10 minutes 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 unlabelled GSK256066 in the same buffer. Samples of the dilutions (0.5 ml) were immediately processed through 3 rounds of ultracentrifugation using Millipore Microcon YM-10 centrifugal filter units (10 000 molecular weight cut-off). The filter units were centrifuged at 10,000 g for 45 or 50 minutes and 0.4 ml of buffer were 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 hours. For each enzyme construct, two separate experiments were done. In each experiment, in order 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 4 volumes of ice cold 50mM Tris-HCl, pH 7.5, containing 5 mM MgCl₂, 2 mM benzamidine, 100 µM TLCK, 100 µg/ml bacitracin, 20 µg/ml trypsin inhibitor, 50 µM PMSF and 100 nM.
leupeptin. The homogenate was centrifuged at 100,000g for 1 hour at 4°C and the supernatant stored in aliquots at -80°C. Competition binding studies involved incubation of GSK256066 or DMSO (1% final concentration) and 2nM [methyl-\(^{3}\)H]-rolipram in assay buffer (50mM Tris HCl, pH 7.5 containing 5 mM MgCl\(_2\)). The assay was initiated by addition of 700µl of a 1:100 dilution in assay buffer of rat brain cytosol, total assay volume was 750 µl. After a 1 hour incubation at 30°C the reaction mixture was cooled on ice for 10 minutes and then rapidly filtered under vacuum (Tomtec harvester) through glass microfibre filtermats (Wallac Filtermat A) which had been soaked in 0.3% polyethyleneimine. The filtermats were rapidly washed with ice cold 20mM Tris HCl, pH 7.5 + 2mM MgCl\(_2\) and dried in a microwave oven before annealing to Meltilex scintillant sheets with a hot press (Wallac Microsealer) and counting for radioactivity.

**Human Peripheral Blood Monocyte (PBMC) and whole blood TNF\(\alpha\) Assays.**

Whole blood and PBMCs were assayed as previously described (Davis et al 2009). Briefly, PBMCs were isolated from heparinised human blood by centrifugation on histopaque. Washed cells were then incubated (5 x 10\(^4\) cells/well) with inhibitor solution or DMSO and LPS (1 ng/ml final concentration) in assay buffer (RPMI1640 containing 10% foetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin) for 20 hours at 37°C, 5% CO\(_2\). For whole blood assays, heparinised blood was incubated with inhibitor solution or DMSO for 1 hour at 37°C and 5% CO\(_2\) and then stimulated with LPS (50 ng/ml final) for 20 hours at 37°C and 5% CO\(_2\). TNF\(\alpha\) concentrations in supernatants from both assays were measured by electrochemiluminescence assay as described (Davis et al 2009).

**In vivo studies.**
All experiments were conducted in accordance with the Animals (Scientific Procedures) Act 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., Kent, U.K. The animals were housed in plastic cages with absorbent bedding material and were maintained on a 12 hour daylight cycle. Food and water were provided *ad libitum*. The animals were acclimatised for a minimum of 7 days prior to 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 hours prior to LPS challenge. In both cases, in preparation for dosing rats were anaesthetised by exposure to a gaseous mixture of isofluorane (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 administered 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 pre-weighed, sterile, plastic 3-way tap (Vycon, ref 876.00) attached to a stainless steel dosing needle and plastic 5 ml syringe. The needle was inserted into the trachea and 4 ml of air expelled through the tap to deliver the dry powder blend into the trachea.
and lung. After dosing, the tap was re-weighed and the quantity of dry powder delivered to the animal calculated.

Following recovery from anaesthesia, 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 minutes, to an aerosol of LPS, generated by a compressed air-driven nebuliser (DeVilbiss, Somerset, PA, USA). The LPS concentration in the nebuliser was 150 µg/ml. The animals were then returned to the holding cages and allowed free access to food and water.

**Bronchoalveolar lavage (BAL) 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 lavaged 3 times with 5 ml of heparinised (25 units/ml) PBS.

The BAL samples were centrifuged at 1300 rpm for 7 minutes and the cell pellets resuspended in 1 ml of PBS. 100 µl of cell suspension were added to cytopsin holders which were centrifuged at 5000 rpm for 5 minutes. The slides were air dried and then stained with Leishmans stain for 20 minutes for differential cell counting. The total number of cells were counted using a Sysmex automatic cell counter.

**Data Analysis**

**pIC50 determinations.**
Percent inhibition values were generated relative to uninhibited controls. Values for pIC$_{50}$ were determined from concentration-response curves by non-linear least squares curve fitting, in general using a 4-parameter logistic equation in Activity Base (IDBS, Guildford, Surrey, U.K.).

**Kinetic studies.**

All fits were made using Grafit, version 4 or version 5 (Erithacus Software Ltd., Horley, Surrey, U.K.). 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 non-linear regression using an integrated equation describing time-dependent, tight-binding inhibition (Cha, 1976; Williams et al., 1979)

\[
P = v_0 t + \frac{(v_0 - v_s)(1 - d)}{d k} \ln \left( \frac{1 - d e^{kt}}{1 - d} \right) \quad \text{(equation 1),}
\]

where $P$ is product formed at time $t$, $v_0$ 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 ($K_i$) for GSK256066.**

Relationships between rate and GSK256066 concentration were fitted using an equation that describes tight-binding inhibition (Morrison, 1969; Dharmasena et al., 2002)

\[
v = \left( \frac{v_0}{2 E} \right) \left[ \left( E - I - K_i \right)^2 + \sqrt{\left( E - I - K_i \right)^2 + 4 E K_i} \right] \quad \text{(equation 2),}
\]
where $v$ is the measured rate at the inhibitor concentration $I$, $v_0$ 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, $K_i' = K_i (1 + (S / K_m))$, where $K_i$ is the true inhibitor (or dissociation) constant and $S$ is the substrate concentration.


The total counts that were recovered in the combined filtrates at each time point were plotted against the sampling time and the data fitted using the first-order rate equation

$$A_t = A_x (1 - e^{-kt}) + A_0 \quad \text{(equation 3)}$$

where $A_t$ represents the counts at time $t$, $A_x$ represents the final counts at infinite time, and $A_0$ represents the counts at time 0.

The values for $\Delta \text{cpm} (A_t - A_0)$ at each time were then expressed as a percentage of the extrapolated maximum counts displaced ($A_x - A_0$). In this way, data from 4 different mixes for each PDE4B construct were normalised and then combined. The combined data were then fitted using equation 3 to determine the rate constant ($k$) for the dissociation of $[^3]H$-GSK256066 from the PDE4B $[^3]H$-GSK256066 complex.

**In vivo studies.**

Data were analysed using Graph Pad Prism™ (GraphPad Software Inc., San Diego, CA, U.S.A.). Parametric tests performed to determine statistical significance were an analysis of variance (ANOVA) with Dunnett’s post test, and non-parametric tests used were Kruskal-Wallis and Dunn’s post test.

**Pharmacokinetics**
The pharmacokinetics of GSK256066 was studied in the male CD rat following intravenous bolus and oral gavage administration both at doses of 1mg/kg. The dose was formulated as a 0.5mg/mL solution in DMSO: PEG200: water 10: 67: 23: (v/v/v), dose volume for both routes was 2mL/kg. Blood samples (200µL) were taken at a range of time-points over a 12hr period and centrifuged to yield plasma. GSK256066 was extracted from the plasma samples using protein precipitation and the samples analyzed by quantitative LC-MS/MS. The pharmacokinetic parameters of GSK256066 were then calculated from the plasma concentrations using a GSK software package PKTools v2.
Results

Enzyme assays.

The derived IC₅₀ values (1-5pM) for inhibition of PDE4 by GSK256066 (Table 1: pIC₅₀ 11.3-11.9) were similar to the in-assay enzyme concentrations (nominally 10-20 pM). This apparent tight binding behaviour was confirmed in the enzyme kinetic studies outlined below and the PDE4 pIC₅₀ values shown in Table 1 are considered minimum estimates. In spite of this, the pIC₅₀ values are unprecedented for PDE4 inhibitors. The next most potent comparator compound is roflumilast (pIC₅₀ 9.4), whilst other known inhibitors had pIC₅₀ values in the range 7-9 in our PDE4B assays (Table 2).

Based on a minimum pIC₅₀ value for PDE4B of 11.5, selectivity over the other PDEs tested is extremely high, being at least 2500-fold against PDE7 and at least 350,000-fold against PDEs 1, 2, 3, 5 and 6 (Table 1). GSK256066 was found to be inactive (pIC₅₀ < 5) when tested against a wide range of protein kinases, the hERG channel and the CEREP panel of receptor screens (data not shown), representing at least 3,000,000-fold selectivity.

GSK256066 inhibited [³H]-rolipram binding in rat brain cytosol with a pIC₅₀ of 10.3, giving a HARBs ratio (HARBs IC₅₀/PDE4B IC₅₀) of at least 17, compared to ratios of 2-3 for AWD 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.

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 minutes with 0.010 µM cAMP and at least 80 minutes with 8.0 and
20 µM cAMP, progress plots made in the presence of GSK256066 were non-linear. They showed a time-dependent decrease in rate that was dependent 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 behaviour, 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 analyse the progress plot data using equation 1 (see Data Analysis) which describes slow- and tight-binding behaviour. Representative progress plots, with fits obtained using equation 1, are shown in Fig. 2.

The fits enabled values for initial rates 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 rate and steady-state rate decrease with increasing GSK256066 concentration. This behaviour is consistent with a mechanism (mechanism B in the terminology of Morrison and Walsh, 1988) in which an enzyme-inhibitor complex (E.I), which is formed rapidly, undergoes a slow isomerisation 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 against inhibitor concentration data can be fitted using equation 2 to obtain a value for $K_{EI}$, the dissociation constant for the E.I complex dissociating to E + I. Steady-state rate against inhibitor concentration data can be fitted using equation 2 to obtain a value for $K^*$, the overall dissociation constant for the E’.I complex dissociating to E + I.
Initial-rate data.

Inspection of Fig. 3 shows that, while the data obtained at the higher concentrations of cAMP seem suitable for fitting using equation 2, data obtained at very low cAMP concentration result in an unusual relationship between initial rate and inhibitor concentration. The reason for this unusual behaviour is not apparent. Whilst it may arise because of errors in the determination of values for initial rate, the same behaviour was seen with both PDE4B constructs used.

Fitting of the data obtained at 8.0 and 20 µM cAMP using equation 2 enabled apparent values for $K_{EI}$ of 89 and 178pM to be determined. An increase in the apparent value for $K_{EI}$ with increasing cAMP concentration is consistent with the inhibition of PDE4B by GSK256066 being competitive with respect to cAMP, although mixed inhibition cannot be ruled out. Assuming competitive inhibition, estimates for the true $K_{EI}$ value of 18 and 30 pM were obtained, from the relationship apparent $K_{EI} = true K_{EI} (1 + (S / K_m))$. A value for $K_m$ for cAMP of 4.0 µM was used (McLaughlin et al., 1993 and confirmed by us).

Steady-state rate data.

Steady-state rate against GSK256066 concentration data (Fig. 3) were also fitted using equation 2, allowing estimates for $K^*$ (the overall dissociation constant for the E'I complex) to be obtained. The values obtained were 0.03 and 0.06 pM for full-length PDE4B at 0.01 and 8.0 µM cAMP, respectively, and 0.03 pM for PDE4B 152 – 503 truncate at 0.01 µM cAMP. Somewhat surprisingly, it was not possible to obtain a fit using equation 2 for the data at 20 µM cAMP; the binding appears to be very tight and the rate decreases almost linearly with GSK256066 concentration.

The values that were obtained from fits using equation 2 are below the potency at which real changes can be discriminated. The limit of discrimination was estimated to be about 0.5 pM,
Based on simulated plots (not shown) of rate against inhibitor concentration that obey equation 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 pM (pKₐ > 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 from enzyme-inhibitor complex dialysed for 4 hours. GSK256066 does not, therefore, rapidly dissociate from PDE4B leading to recovery of enzyme activity. Significantly longer dialysis times could not usefully be used because control activity was poorly stable; the half-life for activity in the dialysed, control samples was about 3.5 hours.

No activity was recovered after enzyme-inhibitor complex had been processed through 3 rounds of dilution/ultracentrifugation using Millipore Microcon YM-10 centrifugal filter units. Recoveries of control enzyme in these experiments were in the range 40 – 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 unlabelled GSK256066 and samples 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 (equation 3). As the number of counts that did not bind to the enzyme, and the total number of counts recovered when [³H]-GSK256066 alone was diluted in buffer and concentrated by centrifugal filtration, varied from mix to mix, data from different mixes were normalized and combined as described in the Data Analysis section of Methods. The normalized data are shown in Fig. 4 together with the fits using equation 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. $[^3]H$-GSK256066 complex was 0.11 hour$^{-1}$ ($t_{1/2}$ 6.3 hours). 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 $[^3]H$-GSK256066 from full-length enzyme is clearly slower than from the truncate, and even after 48 hours of incubation approximately 30% of the labelled compound had not appeared in the filtrate. The estimated rate constant for the dissociation of the full-length PDE4B.$[^3]H$-GSK256066 complex was 0.021 hour$^{-1}$, enabling a value for $t_{1/2}$ of 33 hours to be derived.

**Cellular and whole blood potencies of GSK256066.**

GSK256066 was an extremely potent inhibitor of LPS-stimulated TNF$\alpha$ production in PBMC (pIC$_{50}$ 11.0, IC$_{50}$ 10pM) and human whole blood cultures (pIC$_{50}$ 9.90, IC$_{50}$ 126pM: Fig 5 and Table 2). The maximal inhibition achieved was 83 ± 3% in PBMC and 80± 4% for whole blood (mean +/- SEM). The compound was markedly more potent in both PBMC (at least 240-fold) and whole blood (at least 75-fold) than any of the comparator compounds tested (Table 2). GSK256066 was also approximately 20-fold more potent than the corticosteroid, FP, in PBMC and whole blood.

**Pharmacokinetics of GSK256066 in the Male CD Rat.**

GSK256066 showed a moderate plasma clearance (39mL/min/kg), a moderate volume of distribution (0.8L/kg) and a relatively short half-life (1.1hr) in the male CD rat. Bioavailability was low following oral administration as a solution (<1%). Blood samples were also taken from the hepatic portal vein following 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 behavioural changes.

When administered 2 hours prior to 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 which was significant (p<0.05) at 10 μg/kg and was 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 \textit{in vitro} and \textit{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). It is also a substantially more potent (>300-fold) inhibitor of PDE4B than the inhaled drug candidate UK 500001 (Vestbo et al 2009). GSK256066 is highly selective for PDE4 over other PDEs tested (>2500-fold against PDE7 & >380,000 against PDEs 1, 2, 3, 5 & 6) 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 following a mechanism in which an enzyme.inhibitor complex (E.I), which is formed rapidly, slowly isomerises 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 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 hypothesised above. As this very low concentration of cAMP is much lower than the value (4 µM) for \( K_m \) for cAMP for this enzyme, the unusual behaviour 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 complex 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\textsubscript{50} ≥ 11.5 shown in Table 1.

Due to the extremely tight binding of GSK256066 to PDE4B, reversal of inhibition could not be demonstrated by dilution of enzyme-inhibitor complex into assays, or by dialysis or ultrafiltration. The binding of [\textsuperscript{3}H]-GSK256066 to PDE4B was slowly reversible, however, with the half-life for dissociation being 6 hours for the truncated enzyme and 33 hours 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 was used as a crude extract whilst the truncate was a highly purified preparation from a baculovirus/insect cell system. Although the results show that the binding of [\textsuperscript{3}H]-GSK256066 to PDE4B is reversible, as the labelled compound was replaced on the enzyme by unlabelled inhibitor, it is not possible to say if, 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 as it is likely to provide prolonged duration of activity in the lung.

In this work, we have carried out detailed enzymatic studies both with full-length PDE4B and with PDE4B 152 - 503 truncate, which encodes almost the entire catalytic domain. The results were similar with both constructs. As well as providing confidence that the results obtained are real, the similarity of results means that the truncate may be a suitable species for trial crystallographic studies.
GSK256066 is an extremely potent and effective inhibitor of the production of the clinically relevant pro-inflammatory cytokine TNFα. The compound shows exceptional potency in both whole blood and PBMCs when compared to a wide range of competitor compounds (Table 2). For example, in PBMC it is >2000 fold more potent than both tofimilast and AWD 12-281, about 350-fold more potent than Merck Frosst’s L-869,298 and about 500-fold more potent than roflumilast. The decline in potency in the whole blood assay relative to the potency in PBMCs (about 13-fold) is likely to be due to plasma protein binding (98.4% bound at 1 µg/ml in human plasma: unpublished data) or possibly to red blood cell-binding of the compound.

The high potency of GSK256066 in 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 hours prior to inhaled LPS challenge. Acute pulmonary neutrophilia 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 6-7 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), roflumilast and cilomilast (Underwood 1998, Bundschuh
et al., 2001). Potency estimates ranged from about 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 only observed 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 (ED$_{50}$ < 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 (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 which 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., manuscript submitted for publication jointly with the current one). In addition, in the current studies GSK256066 was found to be well tolerated when administered by the intratracheal route to conscious rats, producing no overt behavioural 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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Contributed new reagents or analytic tools: Ward
Performed data analysis: Hart, Dawson, Wiseman, Williamson, Solanke, Angell, Gascoigne, Ranshaw and Lucas
Wrote or contributed to the writing of the manuscript: Williamson, Hart, Tralau-Stewart, Knowles, Nials and Ranshaw
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Legends for figures

Fig. 1. Structure of GSK256066 (as published in Woodrow et al., 2009)

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 µM. The lines drawn are fits of the data by an equation describing slow- and tight-binding inhibition (equation 1). The graphs shown are representative of a wider dataset, others being generated at 0.01 and 20µM cAMP.

Fig. 3. The variation of initial rate (a) and steady-state rate (b) with GSK256066 concentration for full-length PDE4B at cAMP concentrations of i, 0.010 µM; ii, 8.0 µM; iii, 20 µM. The lines drawn are fits to the data of an equation describing tight-binding inhibition (equation 2).

Fig. 4. GSK256006 slowly dissociates from PDE4B. Equimolar concentrations (2.7 nM) of enzyme and [3H]-GSK256066 were diluted 10-fold into an excess (2.7 µM) of unlabelled 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 4 different mixes which were normalised as described in the Methods section. The lines drawn are fits of the normalised data using equation 3, which describes first-order kinetics. Results for PDE4B 152–503 truncate are shown in a and for full length PDE4B in b.

Fig. 5. Dose-response curves for the inhibition of LPS-stimulated TNFα by GSK256066 and AWD 12-281 in PBMC and whole blood. Curve fits were generated using a 4-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 hours prior to LPS challenge. * p<0.05, *** p<0.01 vs control response to LPS.

**Fig. 7.** LPS–induced pulmonary neutrophilia in rats: effect of GSK256066 after administration as a dry powder 2 hours prior to LPS challenge. * p<0.05, ** p<0.01 vs control response to LPS.
Tables

**TABLE 1: Potency and selectivity of GSK256066, AWD 12-281 and Roflumilast for PDEs**

PDE activity was measured at room temperature using SPA assays and additionally, for PDE4B and D only fluorescence polarization assays. The binding of GSK256066 to the high affinity rolipram binding site (HARBS) in rat brain cytosol was measured using a competition binding assay with 2nM [methyl-3H]-rolipram as radioligand.

<table>
<thead>
<tr>
<th>Activity</th>
<th>GSK256066</th>
<th>AWD 12-281</th>
<th>Roflumilast</th>
<th>Cilomilast</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIC50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean ± SEM or range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDE4B</td>
<td>≥11.5 (n=12)</td>
<td>1.0</td>
<td>7.63 ± 0.23 (n=2)</td>
<td>9.41 ± 0.04 (n=129)</td>
</tr>
<tr>
<td>PDE4A</td>
<td>≥11.31 (n=4)</td>
<td>1.6</td>
<td>ND</td>
<td>9.36 ± 0.08 (n=56)</td>
</tr>
<tr>
<td>PDE4C</td>
<td>≥11.42 (n=4)</td>
<td>1.2</td>
<td>ND</td>
<td>8.78 ± 0.06 (n=30)</td>
</tr>
<tr>
<td>PDE4D</td>
<td>≥11.94 (n=12)</td>
<td>0.4</td>
<td>7.52 ± 0.64 (n=2)</td>
<td>9.52 ± 0.05 (n=112)</td>
</tr>
<tr>
<td>PDE1</td>
<td>5.73 ± 0.03 (n=2)</td>
<td>≥ 600,000</td>
<td>&lt;4.52 (n=1)</td>
<td>&lt;4.52 (n=1)</td>
</tr>
</tbody>
</table>
PDE4 potency is likely to be an underestimate as the derived IC₅₀ value is similar to the estimated in-assay enzyme concentrations.

Where n=1 the values are means of duplicate determinations in a single experiment, except for HARBS data for GSK256066 (mean of 3 determinations in a single study) and cilomilast which was a single determination only, for the latter the data is consistent with the 100nM Ki published previously (Barnette et al., 1994). ND = not determined

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC₅₀ ± SE (n)</th>
<th>Kᵢ ≥ (n)</th>
<th>IC₅₀ &lt;4.5 (n)</th>
<th>IC₅₀ &lt;4.5 (n)</th>
<th>IC₅₀ ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE2</td>
<td>5.92 ± 0.05</td>
<td>≥ 390,000</td>
<td>&lt;4.52 (n=1)</td>
<td>&lt;4.52 (n=1)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
<td></td>
<td></td>
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<tr>
<td>PDE3</td>
<td>5.93 ± 0.12</td>
<td>≥ 380,000</td>
<td>&lt;4.52 (n=1)</td>
<td>&lt;5 (n=3)</td>
<td>&lt;4.5 (n=1)</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDE5</td>
<td>5.49 ± 0.04</td>
<td>≥1,050,000</td>
<td>&lt;4.65 (n=3)</td>
<td>&lt;4.55 (n=3)</td>
<td>&lt;4.5 (n=1)</td>
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<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDE6</td>
<td>5.28 ± 0.03</td>
<td>≥ 1,700,000</td>
<td>&lt;4.52 (n=1)</td>
<td>&lt;5 (n=2)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PDE7</td>
<td>8.11 ± 0.26</td>
<td>≥2,510</td>
<td>&lt;4.52 (n=1)</td>
<td>&lt;6.6 (n=2)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
<td></td>
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<tr>
<td>HARBS</td>
<td>10.27 (n=1)</td>
<td>≥ 17</td>
<td>7.40 ± 0.37 (n=3)</td>
<td>8.98 ± 0.08 (n=11)</td>
<td>6.75 (n=1)</td>
</tr>
</tbody>
</table>

* PDE4 potency is likely to be an underestimate as 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 on LPS-driven TNFα production in human PBMC and whole blood.

PDE activity was measured at room temperature using SPA assays and additionally, for PDE4B and D only, fluorescence polarization assays. For cell and whole blood assays, PBMC or whole blood from healthy volunteers were incubated with LPS (1 and 50 ng.ml⁻¹ respectively) in the presence or absence of an inhibitor dilution series. After 20 hours of incubation, supernatants were removed and TNFα concentrations determined. Data are mean ± SEM or range (n=2). n= number of donors (each donor sample was generally assayed at least in duplicate within an assay).

<table>
<thead>
<tr>
<th></th>
<th>Mean pIC50 ± SEM or range (n)</th>
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<tbody>
<tr>
<td></td>
<td>PDE4B</td>
</tr>
<tr>
<td>GSK256066</td>
<td>&gt;11.5 (12)</td>
</tr>
<tr>
<td>AWD 12-281</td>
<td>7.63 (2)</td>
</tr>
<tr>
<td>CI1044</td>
<td>6.97 ± 0.02 (4)</td>
</tr>
<tr>
<td>Cilomilast</td>
<td>7.13 ± 0.08 (4)</td>
</tr>
<tr>
<td>L869298</td>
<td>8.99 ± 0.02 (2)</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>9.41 ± 0.04 (129)</td>
</tr>
<tr>
<td>RP73401</td>
<td>9.18 (1)</td>
</tr>
<tr>
<td>Tofimilast</td>
<td>8.80 ± 0.23 (2)</td>
</tr>
</tbody>
</table>
Other PDE4 inhibitors tested on isolated PDE4B were Bayer’s Lirimilast (pIC50 7.15) Celltech’s CDP840 (pIC50 6.91) and Mundipharm’s V11294 (pIC50 6.88). The corticosteroid, fluticasone propionate, was not tested on PDE4B.

<table>
<thead>
<tr>
<th></th>
<th>YM976</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.69 ± 0.08 (3)</td>
<td>7.64 ± 0.26</td>
<td>6.42 (n=1)</td>
</tr>
<tr>
<td>FP</td>
<td></td>
<td>9.71 ± 0.1 (11)</td>
<td>8.63 ± 0.18 (6)</td>
</tr>
</tbody>
</table>
Figure 2

Graphs showing CPM vs. Time (min) for different concentrations of substances (0pM, 15pM, 75pM, 6pM, 30pM, 150pM, 9pM, 50pM, 6pM, 20pM, 100pM).
Figure 5

The graph shows the percentage inhibition of an enzyme activity as a function of the concentration of the compound. The x-axis represents the concentration of the compound in nM, ranging from $10^{-4}$ to $10^4$. The y-axis represents the percentage inhibition, ranging from 0% to 100%. Different symbols represent different conditions or treatments, indicating distinct inhibition profiles at various concentrations.
Figure 6

The graph shows a dose-response relationship with % inhibition on the y-axis and Dose (µg/kg) on the x-axis. Two sigmoidal curves are depicted, with one curve having a higher % inhibition at higher doses. Significant differences are indicated by asterisks: '***' for p < 0.001 and '*' for p < 0.05.