Supplemental material to this article can be found at:
http://jpet.aspetjournals.org/content/suppl/2014/04/30/jpet.114.214155.DC1

GSK356278, a Potent, Selective, Brain-Penetrant Phosphodiesterase 4 Inhibitor That Demonstrates Anxiolytic and Cognition-Enhancing Effects without Inducing Side Effects in Preclinical Species

A. Richard Rutter, Alessandro Poffe, Palmina Cavallini, T. Gregg Davis, Jessica Schneck, Michele Negri, Elena Vicentini, Dino Montanari, Roberto Arban, Frank A. Gray, Ceri H. Davies, and Paul B. Wren

GlaxoSmithKline, Discovery Medicine, Stevenage, Herts, United Kingdom (F.A.G., P.B.W.); GlaxoSmithKline, Neural Pathways Discovery Performance Unit, Biopolis, Singapore (A.R.R., C.H.D.); GlaxoSmithKline, Neurosciences Centre of Excellence for Drug Discovery, Verona, Italy (A.P., P.C., M.N., E.V., D.M., R.A.); GlaxoSmithKline, Molecular Discovery Research, Collegeville, Pennsylvania (J.S.); and GlaxoSmithKline, Respiratory Centre of Excellence for Drug Discovery, King of Prussia, Pennsylvania (T.G.D.)

Received February 20, 2014; accepted April 29, 2014

ABSTRACT

Small molecule phosphodiesterase (PDE) 4 inhibitors have long been known to show therapeutic benefit in various preclinical models of psychiatric and neurologic diseases because of their ability to elevate cAMP in various cell types of the central nervous system. Despite the registration of the first PDE4 inhibitor, rolipram, for the treatment of chronic obstructive pulmonary disease, the therapeutic potential of PDE4 inhibitors in neurologic diseases has never been fulfilled in the clinic due to severe dose-limiting side effects such as nausea and vomiting. In this study, we describe the detailed pharmacological characterization of GSK356278 [5-(5-(2,4-dimethylthiazol-5-yl)methyl)-1,3,4-oxadiazol-2-yl)-1-ethyl-N-(tetrahydro-2H-pyran-4-yl)-1H-pyrazolo[3,4-b]pyridin-4-amine], a potent, selective, and brain-penetrant PDE4 inhibitor that shows a superior therapeutic index to both rolipram and roflumilast in various preclinical species and has potential for further development in the clinic for the treatment of psychiatric and neurologic diseases. GSK356278 inhibited PDE4B enzyme activity with a pIC50 of 8.8 and bound to the high-affinity rolipram binding site with a pIC50 of 8.6. In preclinical models, the therapeutic index as defined in a rodent lung inflammation model versus rat pica feeding was >150 compared with 0.5 and 6.4 for rolipram and roflumilast, respectively. In a model of anxiety in common marmosets, the therapeutic index for GSK356278 was >10 versus <1 for rolipram. We also demonstrate that GSK356278 enhances performance in a model of executive function in cynomolgus macaques with no adverse effects, a therapeutic profile that supports further evaluation of GSK356278 in a clinical setting.

Introduction

Phosphodiesterase (PDE) 4 enzymes are a subclass of the phosphodiesterase enzyme superfamily that hydrolyze cAMP and/or cGMP to their inactive nucleotides. The four PDE4 subtypes, PDE4A, PDE4B, PDE4C, and PDE4D, are selective for cAMP. Inhibition of PDE4 with pharmacological agents has been shown to elevate cAMP in numerous cell types, including T lymphocytes, monocytes, and neurons, resulting in modulation of key signaling pathways in these cells. Based on the reported beneficial effects of PDE4 inhibitors in various in vivo models of behavior and inflammation, development of safe and well tolerated inhibitors for the treatment of chronic inflammatory diseases and psychiatric diseases has been pursued for many years (Press and Banner, 2009; Page and Spina, 2011). The anti-inflammatory properties of PDE4 inhibitors have been particularly well documented, demonstrating reduced immune cell activity, reduced airway smooth muscle contraction, and reduced antigen-induced bronchoconstriction (Torphy et al., 1993; Underwood et al., 1993). These properties fueled the development of inhibitors for the treatment of chronic obstructive pulmonary disease. However, it is the high expression of PDE4 enzymes in the brain that underlies many of the proposed therapeutic benefits of centrally penetrant PDE4 inhibitors (Bolger et al.,...
1994; Blokland et al., 2006; Ghavami et al., 2006; Richter et al., 2013). Regulation of cAMP levels in brain by PDE4 modulates one of the fundamental pathways underlying synaptic plasticity, long-lasting late-phase long-term potentiation, via activation of protein kinase A and cAMP response element-binding protein (Slack and Pockett, 1991; Frey et al., 1993; Matthies and Reymann, 1993). Consistent with this molecular mechanism, the brain-penetrant PDE4 inhibitor rolipram shows memory-enhancing properties in preclinical and clinical models (Fleischhacker et al., 1992; Rutten et al., 2009). In addition, rolipram has beneficial effects in various neuronal systems, for example, increased expression of brain-derived neurotrophic factor (Fujimaki et al., 2000) and increased proliferation of neural progenitor cells in the hippocampus (Li et al., 2009), two key effects thought to underlie the antidepressant effects of rolipram. Lastly, the elevation of cAMP in the brain is thought to have beneficial effects in models of tissue injury or neurodegeneration. Rolipram enhances axonal growth and neuronal survival in a rodent spinal cord injury model (Nikulina et al., 2004) and delays the onset and progression of atrophy and disease phenotypes in the R6/2 mouse model of Huntington’s disease (DeMarch et al., 2008).

Despite the validation of PDE4 as a therapeutic drug target, success in the clinical development of inhibitors has been hindered by a number of dose-limiting side effects, most notably nausea, emesis, and gastric acid secretion, which have been observed with both the first-generation inhibitors such as rolipram but also with second-generation inhibitors such as cilomilast. Various strategies have been employed to develop compounds with a reduced side effect profile (Press et al., 2004) and delays the onset and progression of atrophy and disease phenotypes in the R6/2 mouse model of Huntington’s disease (DeMarch et al., 2008).

Materials and Methods

Reagents. Rolipram and Ro20-1724 [4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one] were purchased from Sigma-Aldrich (St. Louis, MO). [3H]Rolipram (specific activity, 60–80 Ci/mmol; concentration, 200 mCi/ml) was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). GSK356278 was synthesized by GlaxoSmithKline (Stevenage, Hertfordshire, UK). Purified PDE 1–7 enzymes were generated by GlaxoSmithKline. PDE1, PDE2, and PDE3 were purified from bovine aorta. Recombinant human PDE4A (1–686), PDE4B2 (1–564), PDE4D3A (1–673), and PDE5A1 (1–874) were expressed in Saccharomyces cerevisiae and purified. PDE6 was purified from bovine retina. Recombinant human PDE7A1 (1–482) was expressed in a baculovirus/insect cell system. Recombinant full-length human PDE8A1, PDE9A1, PDE10A1, and PDE11A1 enzymes were expressed in a baculovirus/insect cell system and generated at Scottish Biomedical (Glasgow, UK).

Animals. Male ferrets (Mustela putatorus fur), 0.860–1.180 kg in weight, were supplied by Marshall Europe (Lyon, France). Male CD rats (Sprague-Dawley–derived) were supplied by Charles River UK (Margate, Kent, UK). Male Lewis rats were obtained from Charles River Breeding Laboratories (Portage, MI). Common marmosets (Callithrix jacchus) over 2 years of age, weighing 300–500 g, were laboratory-bred animals (GlaxoSmithKline). Indonesian cynomolgus macaques (Macaca fascicularis) were held at Maccine Pte (Singapore). All studies conducted at Maccine Pte were done in accordance with the GlaxoSmithKline Policy on the Care, Welfare, and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee at Maccine Pte. Studies performed at GlaxoSmithKline were performed with approval from GlaxoSmithKline Institutional Animal Care and Use Committee for the Valley Forge Area. Studies conducted at GlaxoSmithKline 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. All experiments conducted in Verona were carried out in accordance with the Italian regulation governing animal welfare and protection (which acknowledges the European Directive 86/609/EEC) and according to internal GlaxoSmithKline Committee on Animal Research and Ethics Review.

HARBS Binding. Native tissue membranes were prepared by differential centrifugation from cortical brain tissue dissected from adult male animals (CD rats, 225–250 g; ferrets, ~1.5 kg; marmosets, ~350 g). For rats and ferrets, an identical protocol was followed: cortices were weighed, homogenized (Potter S homogenizer, 8 strokes, 800 rpm) in 10 volumes of ice-cold assay buffer (50 mM Tris buffer containing 2 mM MgCl2, pH 7.5), and centrifuged (15–20 minutes, 40,000g, 4°C). The supernatant was discarded, and the pellet was re-suspended in 30 volumes of ice-cold assay buffer, homogenized (Polytron Ultra-Turrax T8 homogenizer, 2 × 10 seconds, setting 5), and centrifuged, as before. This process was repeated twice. Upon final homogenization in 10 volumes of ice-cold assay buffer, the resultant membranes were subdivided into aliquots and stored at −80°C until required. In marmoset, prior to the first centrifugation, the initial homogenate was centrifuged at low speed (10 minutes, 1000g, 4°C); otherwise, the methodology was identical. Membrane protein concentrations were determined and were in concentration ranges of 1000–2000 μg/ml.

Binding assays to purified recombinant PDE4 enzymes were performed using membranes from yeast expressing recombinant human full-length PDE4 suspended in a Tris buffer (50 mM Tris, 0.2 M NaCl, 2 mM MgCl2, 5 mM dithiothreitol, 20% glycerol, pH 7.5). Binding data presented show that the preclinical therapeutic index is superior to both rolipram and roflumilast. We also show that GSK356278 has beneficial effects on anxiety and cognition in nonhuman primates at compound exposures that do not induce emesis or other dose-limiting side effects.
assays to native PDE4 enzymes were performed using membranes prepared from brain cortices of rats, mice, marmosets, and ferrets. Binding assays to both membrane preparations were performed in 96-well plates in duplicate or triplicate by incubating 5 μl of test compound prepared from weighed stock solutions in 100% dimethyl sulfoxide (DMSO), but not exceeding 1% final DMSO in the assay. over a seven-point concentration range with 100 μl of ([H]rolipram (final concentration 2 nM), 300 μl of assay buffer (50 mM Tris-HCl, 2 mM MgCl2, pH 7), and 100 μl of membranes at 30°C (final protein concentrations 40–50 and 100–150 μg/well for native tissue and recombinant membranes, respectively) until equilibrium had been reached (100 minutes). The reaction was stopped by rapid filtration through a GF/B filter, presoaked in 0.5% polyethyleneimine, using a 96-well harvester (Brandel, London, UK). Following this, six successive washes with 1 ml/well ice-cold 0.9% NaCl to separate free from bound radioligand. Bound radioactivity was counted by liquid scintillation counting on a TriCarb 2900TR (PerkinElmer Life and Analytical Sciences, Buckinghamshire, UK). Total binding was defined in the presence of 1% final DMSO. Nonspecific binding was determined in the presence of 100 μM rolipram, 100 μM Ro20-1724. IC50 values were derived from curves generated using a four-parameter logistic equation and were converted to inhibition constant values (Ki) following the Cheng-Prusoff equation (Cheng and Prusoff, 1973) using Ki values for ([H]rolipram binding determined in saturation-binding studies. The Ki values for human recombinant PDE4B, human recombinant PDE4D, and mouse, rat, ferret and marmoset native PDE4 were as follows: 16.5, 31.4, 5.0, 5.0, 5.0, 5.0, 5.0, and 5.0 μM, respectively.

The method to determine the kinetics of GS356278 in marmoset brain cortex membranes was adapted from established methods (Motulska and Mahan, 1984). In brief, inhibition of [H]rolipram binding by GS356278 or rolipram was determined using methods similar to those described above, except that membranes were added at different time points to follow the association of radioligand to the enzyme-ligand complex in the presence of a final competing concentration of 20 nM GS356278 (60%–60% enzyme occupancy). The specific binding values determined at each time point were plotted as dpm versus time-response curves and analyzed in GraphPad Prism using the kinetic of competitive binding equation to derive Kd, Km, and pKd values for the unlabeled compounds. Association constants, dissociation constants, and Bmax values were determined for [H]rolipram binding in previous experiments, and these values were constrained in the competitive binding equation.

Whole-Blood Assays. The concentrations of GS356278 in blood, plasma, or brain samples were determined in a 384-well luminescence cAMP detection assay (Cambrex Bio Science Wokingham, Berkshire, UK). PDE4A, PDE4B, and PDE4D purified enzymes in 2.5 μl of enzyme buffer: 40 mM Tris-HCl, 10 mM MgCl2, 1 mM 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid, 0.01% bovine serum albumin, pH 7.5 were first incubated with 50 nl of test compounds (in 100% DMSO) for 30 minutes at room temperature, and then enzyme reactions were initiated by the addition of an equal volume of cAMP (final concentration 1 μM in enzyme buffer). After 60 minutes, the reactions were first stopped by the addition of 1.25 μl of reconstituted PDELight stop reagent, and then cAMP concentrations were quantified by the addition of 2.5 μl of reconstituted PDELight AMP detection reagent. Luminescence was detected on a ViewLux plate reader (PerkinElmer Life and Analytical Sciences) using a 613±55-nm or 618±40-nm emission filter.

Enzyme mode of action studies were carried out in luminescence assays using the reagents and method, as described above, but with the following modifications. Compounds (400 nl) were dispensed into 384-well plates over a 0.03–30 nM range. cAMP solution was titrated from 0.03–30 μM added as a 5 μl of stock to the plate, followed by 5 μl of reconstituted PDELight AMP detection reagent and 5 μl of PDE4B enzyme (final concentration 15 pm). Luminescence was then detected immediately on a ViewLux plate reader, with readings taken every 1 minute for 30 minutes. Enzyme rates were calculated from the linear plots of relative light units versus time for each combination of cAMP and compound concentration. These were then plotted against cAMP concentration and analyzed in Grafit 5.0 for competitive and noncompetitive modes of interaction. The activity of test compounds at all other PDE isoforms was performed using purified bovine (PDE1, 2, 3, and 6) or recombinant human (PDE5A and 7A) PDE proteins in a 96-well [H]cAMP or [H]cGMP scintillation proximity assay exactly as previously described (Davis et al., 2009). Recombinant human PDE8A-11A enzyme assays were conducted at Scottish Biomedical in the same 96-well scintillation proximity assays.

Whole-Blood Assays. Human whole blood was assayed in a modified version of a protocol described previously (Davis et al., 2009). Heparinized blood (100 μl) was incubated in a 96-well plate with 5 μl compounds prepared in assay buffer (RPMI 1640 medium containing 10% fetal calf serum, 1% l-glutamine, and 1% penicillin/streptomycin) for 1 hour at 37°C and 5% CO2. Blood was then stimulated with 20 μl of lipopolysaccharide (LPS; Salmonella typhosa; 50 ng/ml final concentration) in assay buffer overnight at 37°C and 5% CO2. The plate was then shaken vigorously for 10 minutes, and then saline (100 μl) was added to each well. Plasma was isolated by centrifugation at 1800g for 10 minutes, and tumor necrosis factor-α (TNF-α) concentrations were determined by electrochemiluminescence detection on an IGEN instrument (BioVeris, Gaithersburg, MD). Dose-response curves were analyzed using a four-parameter logistic equation to derive pIC50 values.

Pharmacokinetics. Pharmacokinetic parameters were determined in rats, marmosets, cynomolgous monkeys, and ferrets via oral administration using male CD rats were prepared with an cannula inserted via the femoral vein into the vena cava (for compound administration) and via the jugular vein (for blood sampling). The cannulae were exteriorized at the back of the neck, and the animals were placed in jackets with tethers and housed in plastic-bottom cages in facilities with a 12-hour dark/light cycle. Each animal had free access to food and water. After postoperative recovery, animals were dosed via intravenous infusion with GS356278 dissolved in 5% DMSO, 40% polyethylene glycol, and 35% saline to give a total dose of 1 mg/kg in a dose volume of 5 ml/kg. Blood samples (70 μl) were collected at various time points, mixed with 130 μl of water, and kept at 4°C. For oral dosing in male CD rats, GS356278 was administered in a hydroxypropylmethylcellulose (HPMC) vehicle (0.5% HPMC, 0.1% Tween 80 in 25 mM citrate, pH 3) at a dose volume of 5 ml/kg and a final dose of 1 mg/kg. In common marmosets, GS356278 was administered orally in the same HPMC vehicle at a dose volume of 1 ml/kg and a dose of 1 mg/kg. Blood samples (70 μl) were collected at various time points, mixed with 130 μl of water, and kept at 4°C. In male cynomolgus macaques, 2–6 kg in weight, GS356278 was administered orally as a suspension in 10% DMSO, 15% polyethylene glycol 400, and 75% saline at a dose of 0.2 mg/kg in a volume of 2 ml/kg. Intravenous dosing was performed using the same vehicle and dose but at a dose rate of 5 ml/kg per hour. Serial blood samples (500 μl) were collected into heparinized tubes by venipuncture, and plasma was purified by centrifugation and stored at −20°C. Cannulated M. putorius furo ferrets, weighing 0.8–1.6 kg, were administered GS356278 as an oral solution in 0.2% HPMC at a dose of 1 mg/kg in a volume of 2 ml/kg. Blood samples (200 μl) were collected at various time points, plasma purified by centrifugation, and stored at −20°C. Brain penetration of GS356278 was assessed in CD rats following a 1 mg/kg oral dose. Brain and blood samples were collected 2 hours postdose and frozen at −20°C. For all studies, samples of GS356278 were prepared to enable calibration curves to be derived during future sample analysis. A 10-μl sample of the working solution of GS356278 was added to 190 μl of fresh drug-free blood (diluted 1:2.71 with water) and stored at −20°C.

The concentrations of GS356278 in blood, plasma, or brain samples were determined by high-performance liquid chromatography–tandem mass spectrometry using a gradient chromatography system. Ionization of GS356278 took place in the positive mode using an electrospray source and multiple reaction monitoring to provide selectivity. Samples were prepared using protein precipitation with mixing/shaking, followed by centrifugation. Aliquots of the resultant supernatant were
injected directly onto the high-performance liquid chromatography–tandem mass spectrometry system. Calibration ranges were prepared to allow quantification of GSK356278 with suitable sensitivity and accuracy. Noncompartamental pharmacokinetic parameters were obtained from the blood concentration-time curves using WinNonlin Professional version 3.3 (Pharsight, Mountain View, CA). Oral bioavailability was calculated as the ratio of the area under the blood concentration versus time curve after oral and intravenous doses after normalizing for dose.

**Inhaled LPS-Induced Pulmonary Neutrophilia.** Quantification of LPS-induced pulmonary neutrophilia in the Lewis rat was performed, as previously described (Davis et al., 2009). Male rats weighing approximately 320–400 g were pretreated (30 minutes) orally with the appropriate dose of test compound or vehicle alone (0.5% methylcellulose in saline) at a dose volume of 10 ml/kg. Rats were then exposed to aerosolized 0.1 mg/ml LPS solution from *Escherichia coli* (Sigma-Aldrich) at a rate of 4.5 l/min for 20 minutes. At 4 hours post-LPS exposure, the study rats were killed by pentobarbital overdose (390 mg i.p.). Bronchoalveolar lavage (BAL) was performed through a 14-gauge blunt needle into the exposed trachea in five consecutive 5-ml washes of phosphate-buffered saline to collect a total of 20–23 ml of BAL fluid. This BAL fluid was centrifuged at 500g for 10 minutes at 4°C. The supernatant was discarded, and the cells were resuspended in 3 ml of phosphate-buffered saline. The red blood cells were lysed with hemolytic Gey’s solution, centrifuged, and resuspended, as described above. Total cell counts were performed on a Beckman Coulter Multisizer (Brea, CA) in particle counting mode. Cell counts of the animal were performed on BAL smears after staining by Criteron Three-Step Stain (Richard-Allen Scientific, Kalamazoo, MI), containing 1% azure A, 1% methylene blue, and 1% eosin Y.

**Pica Feeding.** The surrogate assay for the assessment of emetogenic potential in rodents, the pica-feeding assay in rats, was performed, as previously described (Davis et al., 2009). Treatment groups (n = 5–10 rats) were placed in individual cages suspended above the cage floor on a wire screen, with two stainless steel food cups, and without bedding or additional enrichment. Throughout the experiment, animals had access to food and water ad libitum. Modeling clay (Languana Clay, City of Industry, CA) was cut into a size and shape similar to standard rat Chow pellets and dried at 60°C for 24 hours. The clay pellets and food pellets were placed into individual stainless steel food cups. The rats were acclimatized to the new conditions for at least 72 hours before compound administration. After the acclimatization period, the rats were transferred to clean cages, and the weights of the clay to which the animals had access over the next 24 hours were measured to the nearest 0.1 g with a calibrated Sartorius 1203 MP top-loading balance (Sartorius AG, Goettingen, Germany). Immediately before the dark cycle, the rats were dosed orally with compound or vehicle (0.5% methylcellulose) at a dose volume of 2 ml/kg. Twenty-four hours after compound or vehicle administration, any clay that had fallen through the cage grate was separated from food and waste and was dried and weighed. This clay weight was added to the uneaten clay remaining in the food cup to calculate the total clay consumed by each rat after administration of compound or vehicle. A drug-treated rat was defined as pica-positive if the animal consumed 0.3 g more than the average amount of clay consumed by the vehicle-treated rats. This threshold (0.3 g) was established because it was the S.D. in pica consumption exhibited by the vehicle-treated control animals across multiple pilot studies.

**Ferret Emesis.** Assessment of emetogenic potential was performed in cannulated male *M. putorius furo* ferrets, 0.860–1.180 kg in weight. Sixty minutes before administration of the test substance, ferrets were placed in individual stainless steel cages (40 × 50 × 34 cm) with a grid floor. Animals were then administered (n = 5 per group) test compound in 0.2% HPMC in saline at a dose volume of 2 ml/kg and observed over a 4-hour period for retches and vomits. Retching was defined as a rhythmic respiratory movement against a closed glottis, whereas vomiting was defined as a forced expulsion of upper gastrointestinal contents. Blood samples (1 ml) were collected at various time points, plasma purified by centrifugation, and then frozen at −20°C.

**Human Threat Test in Marmosets.** The human threat test in the common marmoset (Costall et al., 1988) was used as a behavioral test to evaluate the anxiolytic activity of GSK356278 and rolipram. The studies used laboratory-bred male and female common marmosets (*C. jacchus*) over 2 years of age, weighing 300–500 g. The animals were held in couples, and both were involved in the test. Because of the natural variation in behavior between subjects, only responder animals were included in the study, as defined by a response of at least 10 postures exhibited in the 2 minutes of test period. For the behavioral test, animals were treated with either vehicle, GSK356278 or rolipram. GSK356278 was administered orally in HPMC vehicle (as for pharmacokinetic studies) at doses of 0.03, 0.1, 0.3, 1, and 3 mg/kg, 1 hour before the test. Rolipram was administered via subcutaneous injection in water at 0.001, 0.003, and 0.01 mg/kg also 1 hour before the test. Both treatment groups followed a Latin square treatment scheme. The number of postures and jumps in response to an observer standing in close proximity to the home cage was recorded over a 2-minute period. Postures were defined as any one of the following: 1) tail posture: the animal’s back is turned to the observer with elevation of the tail to expose the genital region; 2) scent marking: the animal scent marks the cage surfaces using circum-anal and circumgenital scent glands; 3) slit stare: the animal stares at the observer with flattened ear tufts and eyes reduced to slits; or 4) arch-piloerection: the animal moves around the cage with arched back and full-body piloerection, failing to make eye contact with the observer. The typical effect of anxiolytic compounds in the test is to decrease the number of postures exhibited by the responder animals, but without causing sedation, as determined by the number of jumps.

**Object Retrieval in Cynomolgus Macaques.** The animals used in this study were female cynomolgus macaques (*M. fascicularis*) with a weight range of 2.06–3.39 kg and an age range of approximately 3–5 years old. The animals were individually housed in an air-conditioned unit and were provided with cage toys for environmental enrichment. Animals were also closely observed and received treats such as raisins or peanuts and personal contact throughout the study with personnel familiar to them on a daily basis and after behavioral evaluations.

The object retrieval test was based on a previously described method (Rutten et al., 2008) and conducted at Maccine Pte. The test requires the subject to retrieve a food reward from a clear acrylic box (dimensions = 5 × 5 × 5 cm) with one open side that is positioned in front of the subject on a metal holder fixed to the outside of the home cage. The open side of the cube is presented to the subject with the open side facing left, right, or toward the monkey in a randomized set list of easy versus difficult trials. Food rewards (raisins) were placed on the outer edge, inner edge, line of sight (easy), or deep within (difficult) the box. Each test session consisted of 17 trials, with an initial easy phase of 3 easy trials, followed by a random phase of 9 trials (4 difficult and 5 easy, presented randomly for each session), followed by a difficult phase of 4 difficult trials and finally 1 easy trial. The final easy trial was for reward purposes, and, although the number of attempts was recorded, the data were not used for analysis. For successive test sessions, the subject was presented with the trials in the reverse order of the previous session (previous left-side exposure became right-side exposure and vice versa). Individual trials within one session were terminated if there were no reaches or successful retrieval of the reward within 2 minutes by the subject. The clear acrylic box was cleaned between trials to minimize cues that may influence subsequent task performance through easier identification of the cube entrance. Performance was classified as stable when each subject scored >75% correct on the easy-level trials and <40% on difficult trials.

Animals were habituated to oral dosing prior to compound testing to avoid confounding effects on behavior for a minimum of 1 week after a stable performance was obtained through training. During compound dosing, behavioral evaluations were conducted only once per week to discourage learning and thus mitigate the opportunity for a performance shift during the pharmacology study. The animals were fasted overnight prior to test article administration on each
Friday for the duration of the study. Animals were momentarily restrained in primate chairs to facilitate oral dosing. GSK356278 was administered by oral gavage at a dosing volume of 1 ml/kg body weight, 1.5 hours prior to testing. The compound solution was prepared as a suspension in dosing vehicle (0.5% HPMC, 0.1% Tween 80 in 25 mM citrate buffer, pH 3). The oral dose was washed down with approximately 5 ml purified water to ensure no residual test article remained in the gastric gavage tube. Normal chow was restricted until 1.5 hours postdose. During behavioral testing, the animals received 17 raisins but not normal primate chow. The study cohort received each dose level of GSK356278 (4 doses tested at 0.03, 0.1, 0.3, and 1.0 mg/kg) at escalating doses. The total number of reaches per object retrieval trial was recorded for each animal on each test day. The mean percentage of correct first reaches on the easy and difficult trials was analyzed to indicate any increase in performance following pharmacological treatment. Repeat dosing at the end of this dosing protocol was performed based on animal behavior throughout the study. Justification for repeating any dose level was based upon unusual strategy for that individual, unsettled behavior, or low easy-level performance.

**Results**

**GSK356278 Inhibits the Hydrolytic Enzyme Activity of Recombinant PDE4B with the Same Potency As It Binds to the HARBS.** The chemical structure of GSK356278 is shown in Fig. 1. The pharmacological activity of GSK356278 was determined in two primary assays, as follows: first in a luminescence-based cAMP detection assay using purified recombinant full-length human PDE4B2B enzyme that measures the potency of the compound to inhibit the cAMP hydrolytic activity of the enzyme; and second, in a competitive filtration-binding assay to the recombinant human PDE4B2B enzyme expressed in yeast membranes using [3H]rolipram, which measures binding affinity to the HARBS. As seen in Table 1, GSK356278 is a potent inhibitor of the cAMP hydrolytic activity of PDE4B, with a pIC50 of 8.8 ± 0.02 (n = 10). It competed with [3H]rolipram for the HARBS with a similar affinity, yielding a pKd of 8.6 ± 0.09 (n = 3). This contrasts with rolipram, which binds to the HARBS with high affinity but has a lower potency at inhibiting the hydrolytic activity of the enzyme. The pKd and pIC50 values for rolipram were 7.9 ± 0.01 (n = 11) and 6.8 ± 0.03 (n = 18), respectively. The PDE4-binding affinity shown for rolumilast was determined using marmoset brain membranes, and the resulting pKd of 9.4 ± 0.07 (n = 3) was similar to the pIC50 of 10.3 ± 0.03 (n = 50) derived for inhibition of the human recombinant PDE4B enzyme hydrolytic activity.

**Detailed In Vitro Pharmacological Profile of GSK356278.** The pharmacological profile of GSK356278 was further evaluated to determine the selectivity for recombinant PDE4 isoforms, selectivity versus other PDE enzymes, activity at native PDE4 enzyme across multiple species, and activity in cell-based assays. To determine PDE4 subtype selectivity, the potency of GSK356278 to inhibit the hydrolytic activity of full-length purified recombinant human PDE4A, PDE4B, and PDE4D enzymes was evaluated in the luminescence-based cAMP detection assays. As shown in Table 2, GSK356278 inhibited PDE4A, PDE4B, and PDE4D equally with pIC50 values of 8.6 ± 0.03 (n = 12), 8.8 ± 0.02 (n = 10), and 8.7 ± 0.02 (n = 13), respectively. To facilitate interpretation of in vivo experiments later performed across multiple species, the binding affinities of GSK356278 for the PDE4 enzyme expressed in rat, mouse, marmoset, and ferret brains were evaluated in a competitive [3H]rolipram-binding assay using purified membranes from brain cortices. As shown in Table 3, GSK356278 bound to the HARBS in rats, mice, marmosets, and ferrets with pKd values of 8.7 ± 0.13 (n = 11), 8.6 ± 0.13 (n = 13), and 8.5 ± 0.02 (n = 3), respectively. Neither rolipram nor rolumilast showed selectivity for binding to any particular recombinant PDE4 isoform or for native PDE4 in brain membranes from any particular species (Tables 2 and 3). GSK356278 also exhibited >100-fold selectivity for PDE4B over the other 10 known members of the phosphodiesterase enzyme family (Supplemental Table 1). GSK356278 (10 μM) was also tested at 154 diverse receptors, enzymes, and ion channels (Cerep, Poitiers, France). With the exception of PDE2 and PDE5 isoforms in this panel, less than 50% inhibition of specific radioligand binding was observed in each case with a concentration of 10 μM GSK356278, indicating >100-fold selectivity for PDE4B versus these targets (Supplemental Table 2). To confirm activity of GSK356278 in whole-cell systems, the potency of GSK356278 to inhibit LPS-induced release of TNF-α in human whole blood was determined, a well characterized assay commonly used to assess the potency of PDE4 inhibitors. GSK356278 inhibited TNF-α release with a pIC50 of 7.6 ± 0.08 (n = 12), similar to the potency of 7.7 ± 0.01 (n = 465) for rolumilast.

**GSK356278 Shows a Competitive Time-Independent Mode of Action at Recombinant PDE4B Enzyme.** To determine whether the different pharmacology of GSK356278 versus rolipram described above was driven by its mode of

---

TABLE 1

Summary of functional potencies and binding affinities of GSK356278, rolipram, and rolumilast for recombinant PDE4B.

<table>
<thead>
<tr>
<th></th>
<th>PDE4B pIC50</th>
<th>PDE4B HARBS pKd</th>
<th>Ratio IC50/Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK356278</td>
<td>8.8 ± 0.02 (10)</td>
<td>8.6 ± 0.09 (3)</td>
<td>0.63</td>
</tr>
<tr>
<td>Rolipram</td>
<td>6.8 ± 0.03 (18)</td>
<td>7.9 ± 0.01 (11)</td>
<td>13</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>10.3 ± 0.03 (50)</td>
<td>9.4 ± 0.07 (3)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

---

Fig. 1. Structure of GSK356278.
action at the enzyme, a number of detailed enzyme kinetic and binding kinetic experiments were carried out using the purified human recombinant PDE4B enzyme. First, the time dependency and cAMP dependency of inhibition were determined using standard methods in which the rate of cAMP hydrolysis was monitored over a 30-minute period in the luminescence cAMP detection assay in the presence of a range of concentrations of cAMP and GSK356278. As shown in Fig. 2A, the inhibition of cAMP hydrolysis by GSK356278 was concentration-dependent, but time-independent. As shown in Fig. 2B, increasing concentrations of GSK356278 resulted in a gradual increase in the apparent $K_{on}$ of the enzyme for cAMP, consistent with a competitive mode of action. The functional $pK_I$ value derived from these curves was $8.5 \pm 0.07 (n = 3)$, consistent with the functional $pIC_{50}$ value of $8.8 \pm 0.02 (n = 10)$ described above.

**GSK356278 Has a Faster Dissociation Rate from Marmoset Native PDE4 HARBS Versus Rolipram.** To identify whether there were any further differences between GSK356278 and rolipram in terms of its mode of binding to PDE4, association- and dissociation-binding kinetics were determined using brain cortex membranes from marmoset. $K_{on}$ and $K_{off}$ values were determined according to the method described by Motulsky and Mahan (1984). By measuring the association rate of $[^3H]$rolipram to membranes in the presence and absence of an $\sim IC_{70}$ concentration of competing ligand, GSK356278 or unlabeled rolipram, the $K_{on}$ and $K_{off}$ values for the competing ligands were derived. As shown in Table 4, the association rates of GSK356278 and rolipram were comparable, there was a difference ($P < 0.05$) in the dissociation rates, with GSK356278 dissociating faster ($k_{off} = 0.76 \pm 0.12$ minute$^{-1}$) compared with rolipram ($k_{off} = 0.16 \pm 0.02$ minute$^{-1}$).

**GSK356278 Is an Orally Bioavailable, Brain-Penetrant, and Low-Clearance PDE4 Inhibitor in Multiple Preclinical Species.** The pharmacokinetic properties of GSK356278 were determined in multiple species (rats, ferrets, cynomolgus macaques, and common marmosets) and are summarized in Table 5. In rats, following oral dosing at 1 mg/kg, GSK356278 was highly bioavailable (91%), achieving $C_{max}$ blood concentrations in the range of 141–264 nM at the 2-hour time point and an oral area under the curve (AUC$_{inf}$) in the range 314–448 ng h/ml. Following a 1-hour intravenous infusion to reach a target dose of 1 mg/kg, GSK356278 had a moderate blood clearance of 40 ml/min/kg (−47% liver blood flow) with a terminal half-life of 2.2 hours (range 1.7–2.7 hours). At steady state, the volume of distribution was 6.3 l/kg, indicating efficient distribution of GSK356278 into tissues. The pharmacokinetics of GSK356278 was similar in other species. Following oral dosing in cynomolgus macaques, common marmosets, and ferrets at 0.2, 1, and 1 mg/kg, respectively, similar exposure profiles were achieved as demonstrated by the oral AUC$_{inf}$ values of 51, 330, and 227 ng h/ml and blood $C_{max}$ values of 41, 146, and 193 nM, respectively, assuming a linear relationship between dose level and oral AUC$_{inf}$. In ferrets, exposure profiles generated at 3 and 10 mg/kg (data not shown) confirmed a linear relationship between exposure and dose in this species. The brain penetration of GSK356278 was evaluated in rat following oral dosing at 1, 3, and 10 mg/kg. Brain and blood samples were collected 2 hours after dosing and GSK356278 levels quantified.

**TABLE 2** pIC$_{50}$ values for the inhibition of recombinant human PDE4 isoforms by GSK356278, rolipram, and roflumilast

Inhibitory pIC$_{50}$ values were determined against full-length recombinant human PDE4A, B, and D isoforms via luminescence detection of cAMP hydrolysis. Values are the mean ± S.E.M. of multiple independent replicates, with the $n$ number shown in brackets.

<table>
<thead>
<tr>
<th>Species</th>
<th>GSK356278</th>
<th>Rolipram</th>
<th>Roflumilast</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPDE4A</td>
<td>8.6 ± 0.03</td>
<td>10.1 ± 0.08</td>
<td>8.7 ± 0.02</td>
</tr>
<tr>
<td>hPDE4B</td>
<td>8.8 ± 0.02</td>
<td>10.3 ± 0.03</td>
<td>8.7 ± 0.02</td>
</tr>
<tr>
<td>hPDE4D</td>
<td>8.7 ± 0.02</td>
<td>10.3 ± 0.06</td>
<td>8.7 ± 0.02</td>
</tr>
</tbody>
</table>

**TABLE 3** Comparison of the binding affinities of GSK356278, rolipram, and roflumilast to native PDE4 enzyme in different species

Binding affinities to native PDE4 enzyme in brain cortex tissues were determined in competition filtration-binding assays, using 2 nM $[^3H]$rolipram as the radioligand. Values ($pK_I$) are the mean ± SEM. The $n$ number of independent determinations is shown in brackets.

<table>
<thead>
<tr>
<th>Species</th>
<th>GSK356278</th>
<th>Rolipram</th>
<th>Roflumilast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>7.8 ± 0.13</td>
<td>9.1 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Marmosets</td>
<td>8.4 ± 0.07</td>
<td>9.4 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Ferrets</td>
<td>8.5 ± 0.02</td>
<td>8.4 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

nd, not determined.
The mean \((n = 3 \text{ per group})\) brain/blood ratio of GSK356278 was in the range 0.5–0.6 at all doses tested.

**GSK356278 Shows Anti-Inflammatory Activity in Rodents at Exposures That Did Not Induce Pica Feeding.** The therapeutic index for GSK356278 was determined in rats using the LPS-induced rat lung neutrophilia model as a measure of in vivo potency (in peripheral tissues) versus activity in the rat pica-feeding model, a surrogate measure of emetogenic potential. In the rat lung neutrophilia model, GSK356278 was dosed orally and the incidence of pica feeding was quantified above. In the rat pica-feeding assay, GSK356278 was again extrapolation from the pharmacokinetic studies described above. Apart from one rat in the 0.03 mg/kg dose group (total \(n = 5\)), pica feeding was observed at 10 mg/kg and above. Because the threshold is defined as the limit of the S.D. of clay consumption in vehicle-treated animals, it is not surprising that, within a group of five animals, some animals may be classified as pica-positive by chance, and therefore, the occurrence in the one rat in the 0.03 mg/kg dose group is considered as nonsignificant within the variability of the assay. Because the response to GSK356278 was essentially all or nothing between 10 and 20 mg/kg in this assay, a true ED_{50} could not be calculated, but for comparative purposes an approximate ED_{50} of 15 mg/kg was assigned to GSK356278. The therapeutic index as defined by the ratio of the ED_{50} in pica feeding:ED_{50} in neutrophilia was 166. In comparison with rolipram and roflumilast, GSK356278 exhibited a superior therapeutic index. The equivalent therapeutic indices for rolipram and roflumilast were 0.15 and 6.4, respectively (Table 6), and have been published previously (Davis et al., 2009).

**GSK356278 Demonstrates Efficacy in a Nonhuman Primate Model of Anxiety at Exposures That Do Not Induce Emesis.** Given the apparent improved therapeutic index versus the prototypical brain-penetrant PDE4 inhibitor, rolipram, a number of studies were conducted to determine whether this improved therapeutic index was also observed when measuring behavioral responses to GSK356278, effects driven by inhibition of the PDE4 enzyme in the brain. The behavioral test chosen to explore the biologic effects of GSK356278 in the central nervous system (CNS) was the human threat test in the common marmoset, a measure of anxiety in which the physiologic and behavioral responses to a human threat are thought to bear similarities to the responses of humans to anxiety-inducing situations. Whereas emetogenic liability could also be evaluated in parallel in the marmoset, studies were also carried out in ferrets, a species in which the correlation with humans in terms of drug-induced emesis has been validated to a greater level. GSK356278 and rolipram were evaluated in the marmoset human threat test in dose response via oral and subcutaneous routes of administration, respectively. The number of postures and jumps was evaluated 1 hour after dosing, as described under Materials and Methods. GSK356278 significantly reduced the number of postures at 0.1, 0.3, and 1 mg/kg p.o. (Fig. 4A) without any

**TABLE 4**

Association- and dissociation-binding kinetics of GSK356278 versus rolipram

The binding kinetics of GSK356278 and rolipram was evaluated via inhibition of time-dependent [3H]rolipram binding to marmoset brain cortex membranes. GSK356278 and rolipram were used at 20 and 13 nM, respectively, to inhibit [3H]rolipram binding by \(-70\%.\) The resulting association-binding curves were analyzed according to a method described by Motulsky and Mahan (1984). Derived \(pK_a\) values and association- and dissociation-binding constants for GSK356278 and rolipram are summarized in the table and are mean values \(\pm\) S.E.M. from at least \(n = 3\) independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>% PDE4 Occupancy</th>
<th>(K_{a} (M^{-1} \text{ min}^{-1}))</th>
<th>(K_{d} (M^{-1}))</th>
<th>Derived (pK_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK356278B</td>
<td>62 \pm 3</td>
<td>3.21 \times 10^{8} \pm 0.48 \times 10^{8}</td>
<td>0.76 \pm 0.12</td>
<td>8.6 \pm 0.03</td>
</tr>
<tr>
<td>Rolipram</td>
<td>64 \pm 4</td>
<td>1.12 \times 10^{8} \pm 1.93 \times 10^{7}</td>
<td>0.16 \pm 0.02</td>
<td>8.9 \pm 0.05</td>
</tr>
</tbody>
</table>

**TABLE 5**

Summary of pharmacokinetics of GSK356278 in multiple species

The pharmacokinetics of GSK356278 in rats, ferrets, monkeys, and marmosets are summarized. All parameters were calculated from blood concentration-time data and are reported as mean (and range for \(n \geq 3\) or minimum and maximum values for \(n = 2\)). For \(T_{\text{max}}\), the median (and range) is given. Plasma protein-binding values are shown for reference and are mean \(\pm\) S.E.M. (number).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rats</th>
<th>Monkeys</th>
<th>Marmosets</th>
<th>Ferrets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain, sex</td>
<td>Sprague-Dawley, male</td>
<td>Cynomolgus, male</td>
<td>Common male, female</td>
<td>Male</td>
</tr>
<tr>
<td>(N)</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Doses (free base equivalents)</td>
<td>1 mg/kg i.v.</td>
<td>0.2 mg/kg i.v.</td>
<td>1 mg/kg p.o.</td>
<td>1 mg/kg p.o.</td>
</tr>
<tr>
<td>Blood clearance (ml/min/kg)</td>
<td>40 [28–53]</td>
<td>16 [16, 16]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Blood clearance (% liver blood flow) (^a)</td>
<td>47%</td>
<td>36%</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(V_s) (l/kg)</td>
<td>6.3 [5.1–7.0]</td>
<td>2.1 [2.0, 2.1]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>2.2 [1.7–2.7]</td>
<td>1.5 [1.4, 1.6]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Oral bioavailability (%)</td>
<td>91 [70–104]</td>
<td>23 [21, 24]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Oral (T_{\text{max}}) (h)</td>
<td>2.0 [2.0–2.0]</td>
<td>1.5 [1.0, 2.0]</td>
<td>2.0 [1.0–4.0]</td>
<td>0.5 [0.5–4.0]</td>
</tr>
<tr>
<td>Plasma protein binding (%)</td>
<td>16 ± 0.7 (7)</td>
<td>ND</td>
<td>15 (1)</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(\text{ND, not done.}\)

\(^a\)Calculated using the following liver blood flows (ml/min/kg): rats, 85; dogs, 31; monkeys, 44.

\(^b\)AUC_{inf}.
The emetogenic potential of GSK356278 was also evaluated in the ferrets at 1, 3, and 10 mg/kg dosed via oral administration. GSK356278 dosed at 1 mg/kg did not induce emesis in any one of five animals over a 4-hour observation period, 30 minutes after dosing. At 3 and 10 mg/kg, both retches and vomits were observed in one of five and two of five animals, respectively. On the basis of these data, a therapeutic index was defined as the ratio of the compound AUC (0–4 hours) or $C_{\text{max}}$ at the minimum effective dose in the marmoset human threat test to the compound AUC (0–4 hours) or $C_{\text{max}}$ at the maximum tolerated (nonemetic) dose in the ferret. In both cases, the compound AUC (0–4 hours) and $C_{\text{max}}$ values were extrapolated from the detailed pharmacokinetic profiles generated previously. The compound AUC (0–4 hours) values for the minimum effective dose in marmosets and maximum tolerated dose in ferrets were 18 and 226 ng h/ml, respectively, yielding a therapeutic index of 13-fold. The $C_{\text{max}}$ values were 15 and 193 nM at the respective doses, also yielding a therapeutic index of 13-fold.

**GSK356278 Enhances Performance in a Test of Executive Function.** The improved therapeutic index of GSK356278 in nonhuman primates versus rolipram facilitated further exploration of the effects of GSK356278 in behavioral and functional tests at doses in which the test outcomes would not be confounded by the sedating and other side effects commonly observed with rolipram.

GSK356278 was first evaluated in a behavioral test similar to one described by Rutten et al. (2008), the object retrieval test. The test, which involves the retrieval of a food object from a one-sided Perspex box, as described in detail under Materials and Methods, is considered to require the integration of multiple visual and sensory inputs for the animal to respond and successfully retrieve the food reward. As such, the test has been referred to as a measure of executive function in nonhuman primates. Single escalating doses of GSK356278 were evaluated in the object retrieval test in cynomolgus macaques over a 6-week testing period, one dose per animal per week, such that each animal received a single vehicle dose during week 1; a single acute dose of 0.03, 0.1, 0.3, and 1 mg/kg GSK356278 in weeks 2, 3, 4, and 5, respectively; and a single vehicle dose in week 6. As shown in Fig. 5, the performance of the animals in the easy tasks was stable over the 6-week testing period, with the percentage of correct first reaches remaining greater than 75%. Of note, the lower success rate in the difficult task also remained stable over the testing period, as demonstrated by the comparison in performance between the week 6 vehicle group and the week 1 vehicle group. During week 1, vehicle-treated animals responded with a 34 ± 6% (mean ± S.E.M.; $n = 10$) success rate versus 35 ± 4% (mean ± SEM; $n = 10$) success rate in the difficult task during week 6.

**TABLE 6**
Summary of therapeutic index of GSK356278 over emesis in various preclinical models

<table>
<thead>
<tr>
<th>Assay</th>
<th>Rolipram</th>
<th>Roflumilast</th>
<th>GSK356278</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS neutrophilia $ED_{50}$ mg/kg</td>
<td>3.2$^a$</td>
<td>0.24$^a$</td>
<td>0.09</td>
</tr>
<tr>
<td>Pica-feeding $ED_{50}$ mg/kg</td>
<td>0.5$^a$</td>
<td>1.8$^a$</td>
<td>15</td>
</tr>
<tr>
<td>Rat ($ED_{50}$ pica/$ED_{50}$ LPS neutrophilia)</td>
<td>0.15$^a$</td>
<td>6.4$^a$</td>
<td>166</td>
</tr>
</tbody>
</table>

$^a$The $ED_{50}$ values for rolipram and roflumilast are also shown for comparison and were derived using the same assays as for GSK356278 and have been previously described (Davis et al., 2009).
Therefore, there was no increase in baseline performance over the 6-week testing period. Administration of GSK356278 improved the performance in the difficult task, reaching significance at the 1 mg/kg dose, equivalent to a blood concentration of 93 $\pm$ 6 nM (mean $\pm$ S.E.M.; $n = 11$) in which animals responded with a success rate of 60 $\pm$ 6% (mean $\pm$ S.E.M.; $n = 10$) versus 34 $\pm$ 6% and 35 $\pm$ 4% (mean $\pm$ S.E.M.; $n = 10$) in vehicle-treated animals at weeks 1 and 6, respectively. GSK356278 did not affect the performance in the easy task at any dose tested, and no adverse effects were observed at any dose.

**Discussion**

The objective of the studies performed in this work was to determine whether the brain-penetrant PDE4 inhibitor GSK356278 displayed an improved therapeutic index in preclinical models compared with rolipram, the prototypical brain-penetrant PDE4 inhibitor. The medicinal chemistry campaign that led to the identification of GSK356278 initially targeted an in vitro profile in which the potency to inhibit cAMP hydrolysis was at least equal to its affinity for the HARBS. As shown in Table 1, the $pK_I$ value for binding to the HARBS of PDE4B is almost identical to the functional $pIC_{50}$ for enzyme inhibition. This contrasts with rolipram for which the binding affinity is approximately 10 times greater than the functional potency. Despite these differences, the pharmacology of GSK356278 was otherwise very similar to rolipram and other second-generation inhibitors such as roflumilast. In this respect, GSK356278 has a competitive, time-independent, mode of action at the cAMP binding site. It is nonselective for the PDE4 isoforms, A, B, and D, and there are no major differences in the binding potency between species. As such, GSK356278 may be considered similar to other advanced second-generation PDE4 inhibitors except that it is also brain penetrant.

To evaluate the therapeutic index in preclinical species, we undertook two strategies. The first was to compare the therapeutic index with rolipram and the marketed compound, roflumilast, in rats. In this strategy, we measured the effect in the LPS-induced lung inflammation model that has been used previously to characterize a number of PDE4 inhibitors (Davis et al., 2009). As shown in Table 1, the $pK_I$ value for binding to the HARBS of PDE4B is almost identical to the functional $pIC_{50}$ for enzyme inhibition. This contrasts with rolipram for which the binding affinity is approximately 10 times greater than the functional potency. Despite these differences, the pharmacology of GSK356278 was otherwise very similar to rolipram and other second-generation inhibitors such as roflumilast. In this respect, GSK356278 has a competitive, time-independent, mode of action at the cAMP binding site. It is nonselective for the PDE4 isoforms, A, B, and D, and there are no major differences in the binding potency between species. As such, GSK356278 may be considered similar to other advanced second-generation PDE4 inhibitors except that it is also brain penetrant.
relationship of GSK356278 in a marmoset model of anxiety, the human threat test, whereas emetogenic effects and other side effects were determined through observation. The marmoset was chosen to explore the central effects of GSK356278 due to the greater similarity to humans in terms of anxiety behavior and centrally mediated side effects. In the rodent, GSK356278 demonstrated a 20- to 30-fold greater therapeutic index compared with rolipram and >1000-fold greater window versus rolipram. This method of calculating a therapeutic index enabled us to demonstrate that, at least in terms of peripheral biology, the pharmacology of GSK356278 at the PDE4 enzyme translated into a more desirable in vivo profile. However, our main interest in this compound was the therapeutic benefit in CNS disorders, and, therefore, the therapeutic index of >10 observed in the marmoset is significant and compares with a therapeutic index of <1 for rolipram. It is interesting that in the marmoset it was not emesis that was the first apparent behavioral side effect to occur following acute dosing of GSK356278 or rolipram. Whether these behavioral effects are compound-specific or directly related to inhibition of PDE4 enzyme is not clear, nor is it clear whether the dose-effect relationship for these observations is similar to the emetogenic effects of these compounds. However, the data demonstrate that the therapeutic index for GSK356278 is superior to that of rolipram. To further validate this apparent therapeutic index in higher species, we calculated the compound exposure required to elicit the minimum significant effect in cynomolgus macaque human threat test, and the maximum exposure that was nonemetic in ferrets. In this scenario, the therapeutic index was 13-fold, again demonstrating that efficacy in a behavioral model could be achieved at exposures that did not induce emesis or other acute dose-limiting side effects.

The reason for the improved therapeutic index of GSK356278 is not fully apparent in these studies. The original hypothesis that equivalent binding activity at the HARBS versus inhibition of the hydrolytic activity confers an improved therapeutic profile, perhaps, is a contributing factor in determining the profile of GSK356278 and goes some way to explain why both rolipram and GSK356278 have superior therapeutic indices versus rolipram. CNS penetration surprisingly does not appear to play a role in driving the emetic liability because the therapeutic index of GSK356278 is equivalent, if not superior, to other similar nonbrain-penetrant compounds like clomilast. Selectivity at PDE isoforms does not seem to account for this profile either, given that all three compounds in this work show a similar nonselective profile against PDE4A, B, and D. Differences in the mode of action at the enzyme can also be ruled out, given that both rolipram and GSK356278 have a competitive mode of inhibition. We did, however, identify some subtle differences in the kinetics of binding to the HARBS, in that GSK356278 dissociated faster than rolipram. It is thought that binding to the HARBS is the molecular mechanism that drives emesis (Hirose et al., 2007) as opposed to inhibition of cAMP hydrolysis, and, whereas it is not known why binding to the HARBS is responsible for inducing emesis or other behavioral side effects, it is possible that the kinetics of this binding may contribute to side effects for a given level of occupancy at the enzyme for a particular inhibitor, and perhaps the faster rate of dissociation for GSK356278 contributes to the improved therapeutic index over rolipram. We have not yet carried out a systematic comparison of other inhibitors to prove or disprove this concept, and this will be the focus for future experiments. Of course, other complex factors may also come into play in governing the therapeutic index for a particular PDE4 inhibitor. Subtle differences in the kinetics of compound exposure, differences in tissue distribution, and cellular penetration may also contribute to the final outcome in these assays. It is important to note that where cross species comparisons were made, we did not find any major differences in the pharmacology or pharmacokinetics of GSK356278 between species. For example, the blood exposures required to elicit the minimum effects in vivo were similar in the rat LPS neutrophilia test (8.9 nM) versus the marmoset human threat test (9.6 nM). In addition, GSK356278 was equipotent at binding to the HARBS in marmosets and ferrets, and therefore, the higher compound AUC required to induce emesis in ferrets compared with the compound AUC required for efficacy in the marmoset model was not due to differences in pharmacology between species.

Given the promising properties of GSK356278 in the marmoset anxiety test, we further evaluated the dose effect relationship of GSK356278 in another behavioral paradigm, again in nonhuman primates. The effect of GSK356278 to improve performance in the object retrieval test in cynomolgus macaques is consistent with the reported effects of rolipram in various tests of cognition (Rutten et al., 2009). Although in this experiment, a significant effect was only achieved at the 1 mg/kg dose compared with 0.1 mg/kg in the marmoset human threat test, we did observe a trend for improvement at the lower doses. While we cannot rule out that a higher level of enzyme inhibition is required to enhance performance in the cognition versus the anxiety test, the power to detect effects in the object retrieval test is less than that in the human threat test, and therefore, an apparent shift in the dose-response curve is not entirely unexpected.

In summary, we have shown that GSK356278 is a potent, selective, brain-penetrant PDE4 inhibitor that demonstrates pharmacological effects in rodent and nonhuman primate preclinical models of inflammation, anxiety, and cognition. These effects are elicited by GSK356278 at exposures that do not induce the dose-limiting side effects typically observed with PDE4 inhibitors, in particular emesis. As such, GSK356278 shows promise as a therapeutic for the treatment of psychiatric and neurodegenerative disorders.

Acknowledgments
The authors thank Federica Bianchi, Raffaella Ricci, and Sonia Delle Fratte for contributions to generating experimental data, and Charles H. Large, Paolo Cavanni, and Ruth Mayer for program leadership.

Authorship Contributions
Participated in research design: Montanari, Arban, Wren, Rutter, Gray.
Conducted experiments: Davis, Poffe, Schneck, Cavallini, Negri, Vicentini.
Performed data analysis: Davis, Poffe, Schneck, Cavallini, Negri, Vicentini, Rutter.
Wrote or contributed to the writing of the manuscript: Rutter, Davies, Wren, Gray.

References

Cheng Y and Prussow WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.


Davis TG, Peterson JJ, Kou JP, Capper-Spudich EA, Ball D, Nials AT, Wiseman J, Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.


Address correspondence to: Dr. A. Richard Rutter, Neural Pathways Discovery Performance Unit, GlaxoSmithKline R&D, 11 Biopolis Way, Biopolis, Singapore, 138667. E-mail: Richard.a.rutter@gsk.com