The Identification of a Novel PDE4 Inhibitor, EPPA-1, with Improved Therapeutic Index using Pica Feeding in Rats as a Measure of Emetogenicity


Running Title: **EPPA-1: A PDE4 Inhibitor with an Improved Therapeutic Index**

Corresponding author: Dr. Patricia L. Podolin, GlaxoSmithKline, Mail Code UW2532, 709 Swedland Road, King of Prussia, PA 19406

Phone: (610) 270-5846; Fax: (610) 270-5381; E-mail address: patty_podolin@gsk.com

Number of text pages: 42

Number of tables: 6

Number of figures: 4

Number of references: 27

Number of words in Abstract: 250

Number of words in Introduction: 693

Number of words in Discussion: 1591

Nonstandard abbreviations: PDE4, phosphodiesterase 4; EPPA-1, 1-ethyl-5-\{5-[(4-methyl-1-piperazinyl)methyl]-1,3,4-oxadiazol-2-yl]-N-(tetrahydro-2H-pyran-4-yl)-1H-pyrazolo[3,4-b]pyridin-4-amine; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; PBMC, peripheral blood mononuclear cell; COPD, chronic obstructive pulmonary disease; SPA, scintillation proximity assays; FP, fluorescence polarization; BAL, bronchoalveolar lavage; TI, therapeutic index; HARBS, high affinity rolipram binding site; LARBS, low affinity rolipram binding site; CNS, central nervous system

Recommended section assignment – Inflammation, Immunopharmacology, and Asthma
Clinical utility of phosphodiesterase 4 (PDE4) inhibitors as anti-inflammatory agents has, to date, been limited by adverse effects including nausea and emesis, making accurate assessment of emetic vs. anti-inflammatory potencies critical to the development of inhibitors with improved therapeutic indices. In the present study we determined the *in vitro* and *in vivo* anti-inflammatory potencies of the first-generation PDE4 inhibitor, rolipram, the second-generation inhibitors, roflumilast and cilomilast, and a novel third generation inhibitor, 1-ethyl-5-{5-[(4-methyl-1-piperazinyl)methyl]-1,3,4-oxadiazol-2-yl}-N-(tetrahydro-2H-pyran-4-yl)-1H-pyrazolo[3,4-b]pyridin-4-amine (EPPA-1). The rank-order potency against LPS-induced TNF-α production by human PBMCs was roflumilast (IC50=5 nM) > EPPA-1 (38) > rolipram (269) > cilomilast (389), and against LPS-induced pulmonary neutrophilia in the rat was EPPA-1 (D50=0.042 mg/kg) > roflumilast (0.24) > rolipram (3.34) > cilomilast (4.54). Pica, the consumption of non-nutritive substances in response to gastrointestinal stress, was used as a surrogate measure for emesis, giving a rank-order potency of rolipram (D50=0.495 mg/kg) > roflumilast (1.6) > cilomilast (6.4) > EPPA-1 (24.3). The low and high emetogenic activities of EPPA-1 and rolipram, respectively, detected in the pica model were confirmed in a second surrogate model of emesis, reversal of α2-adrenoceptor-mediated anesthesia in the mouse. The rank-order of therapeutic indices derived in the rat ((pica D50)/(neutrophilia D50)) was EPPA-1 (578) > roflumilast (6.4) > cilomilast (1.4) > rolipram (0.15), consistent with the rank-order derived in the ferret ((emesis D50)/(neutrophilia D50)). These data validate rat pica feeding as a surrogate for PDE4 inhibitor-induced emesis in higher species, and identify EPPA-1 as a novel PDE4 inhibitor with an improved therapeutic index.
Introduction

Phosphodiesterases are a superfamily of enzymes which hydrolyze cAMP and/or cGMP to their inactive nucleotides. PDE4 is selective for cAMP, and consists of the four subtypes A, B, C, and D. PDE4 inhibitors have shown efficacy in various in vitro and in vivo inflammatory models by increasing the intracellular levels of cAMP in many immune cells (T lymphocytes, monocytes, neutrophils and eosinophils). As such, PDE4 inhibitors have been pursued as therapeutics for pulmonary diseases with an inflammatory component, including chronic obstructive pulmonary disease (COPD) and asthma (Spina, 2004; Vignola, 2004). However, the clinical utility of PDE4 inhibitors has been dose-limited by a side effect profile including nausea and emesis (Lipworth, 2005). Second generation PDE4 inhibitors, such as cilomilast and roflumilast, have demonstrated moderate efficacy in Phase III clinical trials in COPD patients (Compton et al., 2001; Rabe et al., 2005), with reduced side effects compared to first generation inhibitors such as rolipram. Currently, third generation inhibitors are being pursued, with the goal of maximizing therapeutic efficacy, and further decreasing adverse effects.

The use of rodent systems to evaluate the anti-inflammatory effects of PDE4 inhibitors is widespread due to the abundance of well characterized models, the availability of commercial reagents to study underlying mechanisms, and the advent of PDE4B- and D-gene deficient mice (Jin et al., 1999; Jin and Conti, 2002). However, due to the lack of a vomit reflex in rodents (Borison et al., 1981) the evaluation of the emetic potential of PDE4 inhibitors has been limited to those higher-order species capable of an emetic response. This has typically involved the use of ferrets, dogs and non-human primates (King, 1988). Several issues contribute to the impracticality of using higher species to evaluate therapeutic index, including variability of
results in out-bred animals, limited availability of models and reagents to evaluate anti-inflammatory effects, increased costs associated with husbandry and housing, and ethical concerns and regulatory constraints in causing distress in these species. The use of rodent surrogate models of emesis would reduce these issues, and allow for assessment of the anti-inflammatory and adverse effects of PDE4 inhibitors in the same species. However, the use of non-emetic species, such as rodents, for the determination of therapeutic index requires confirmation that such models are representative of established models in higher order species.

A rodent behavior demonstrated to be analogous to vomiting in higher species is pica. Pica is the consumption of non-nutritive substances, which in rodents has been identified as a behavioral response to gastrointestinal distress (Mitchell et al., 1976). Support for the use of pica in rodents as a surrogate for emesis is based on the observations that pica in the rat is mediated by the same mechanisms as vomiting in higher species. Both pica in rats and vomiting in higher species can be induced through dopamine D2 receptors in the chemoreceptor trigger zone of the central nervous system, as well as through peripheral pathways such as serotonin 5HT3 receptors in the gastrointestinal tract (Takeda et al., 1993; Takeda et al., 1995a; Takeda et al., 1995b).

Based on the sensitivity of the pica model to both central and peripheral stimulation, we chose to utilize the pica model to assess the emetic potential of PDE4 inhibitors in the rat.

The aims of the study described herein were to 1) validate the use of pica feeding as a surrogate model of emesis in rats, and 2) compare the therapeutic indices of first and second generation PDE4 inhibitors with that of a novel, orally active pyrazolo[3,4-b]pyridine inhibitor of PDE4, EPPA-1. Using the induction of pica feeding and inhibition of lipopolysaccharide (LPS)-induced pulmonary neutrophilia as measures of emetic potential and anti-inflammatory activity, respectively, we demonstrated the rank-order of therapeutic indices in the rat to be
EPPA-1 > roflumilast > cilomilast > rolipram, consistent with the rank-order observed in an emetic species, the ferret. In a second model of emesis, EPPA-1 did not shorten $\alpha_2$-adrenoceptor-mediated anesthesia induced by xylazine/ketamine in the C57BL/6 mouse, providing additional support for the low emetogenic potential of this inhibitor. The decreased emetogenicity of EPPA-1, combined with its increased anti-inflammatory potency, exemplifies an advance towards a well tolerated, efficacious PDE4 inhibitor for the treatment of respiratory diseases containing a significant inflammatory component.
Methods

PDE Enzyme Assays. \(^{3}\text{H}\) cAMP, \(^{3}\text{H}\) cGMP and phosphodiesterase scintillation proximity assays (SPA) beads were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). Fluorescein-cAMP and IMAP binding reagent was from Molecular Devices (Wokingham, UK), and EGTA and magnesium chloride solution were from Sigma-Aldrich (Gillingham, UK). PDE1, 2 and 3 were purified from bovine aorta. Recombinant human PDE4A 1-686, PDE4B2B 1-156, PDE4C 1-712, PDE4D3A 1-673 and PDE5 were expressed in Saccharomyces cerevisiae. PDE6 was purified from bovine retina. Recombinant human PDE7A1 1-482 was expressed in a baculovirus/insect cell system. Compound dilution series were prepared in DMSO. Low volume additions (0.5-2 µl) to assays were carried out using a liquid handling robot (Biomek Fx). PDE activity was measured using 96 well SPAs and additionally, for PDE3, 4A-D, 5 and 6 only, 384 well fluorescence polarization (FP) assays. In general, compound dilution series were tested from 10 or 30 µM with at least 10 3-fold dilutions per series. Enzymes were diluted to an appropriate concentration in the assay to give linear reaction progress curves over the duration of assay. For SPAs, 75 µl PDE enzyme in 50 mM Tris-HCl pH 7.5, 8.3 mM MgCl\(_2\), 1.7 mM EGTA, 0.05% (w/v) BSA was pre-incubated with 2 µl inhibitor or vehicle (2 µl DMSO) for 30 minutes at room temperature. For PDE1 the assay buffer contained additionally 4 µg/ml calmodulin and 1mM CaCl\(_2\), and did not contain EGTA. The assay was initiated by addition of 25 µl \(^{3}\text{H}\) cAMP (10 nM final concentration: PDE3, 4 and 7 assays) or \(^{3}\text{H}\) cGMP (36 nM final concentration: PDE1, 2, 5 and 6 assays). After 1 hour incubation at room temperature, assays were terminated by addition of 50 µl phosphodiesterase SPA beads suspended in water (~1mg per well) and bound radioactive product measured by
liquid scintillation counting. For FP assays, 10 µl PDE enzyme in 10 mM Tris-HCl buffer pH 7.2, 10 mM MgCl₂, 0.1% (w/v) BSA, 0.05% (w/v) NaN₃ was pre-incubated with 0.5 µl inhibitor or vehicle (0.5 µl DMSO) for 30 minutes at room temperature. Assays were initiated by addition of 10 µl fluorescein-cAMP (40 nM final concentration: PDE3 and 4 assays) or fluorescein-cGMP (40 nM final concentration for PDE5 and 6 assays) and were terminated after 40 minute incubation at room temperature by addition of 60 µl IMAP binding reagent (1 in 400 dilution of stock suspension in binding buffer). The FP ratio of parallel to perpendicular light was measured using an Analyst or Aquest™ plate reader.

**Human PBMC TNF-α Assay.** Periperal blood mononuclear cells (PBMCs) were prepared from heparinized human blood. Blood was centrifuged on histopaque at 1000g for 30 minutes and PBMCs collected from the interface, washed by centrifugation (1300g for 10 minutes) and resuspended in assay buffer (RPMI1640 containing 10% BSA, 1% L-glutamine and 1% penicillin/streptomycin) at 1x10⁶ cells/ml. Cells (5 x10⁴) were incubated with 0.5 or 1.0 µl inhibitor (10 uM – 1.5 nM in 3-fold dilutions) or vehicle (DMSO, 0.4 or 0.8% final concentration) and LPS (S. typhosa) (1ng/ml final) in a total assay volume of 125 µl for 20 hours at 37°C, 5% CO₂. Supernatants were removed and the concentrations of tumor necrosis factor- α (TNF-α) determined by electrochemiluminescence assay.

**Human Whole Blood TNF-α Assay.** Heparinized blood (100 µl) was incubated with 0.5 or 1.0 µl inhibitor (10 uM – 1.5 nM in 3-fold dilutions) or vehicle (DMSO, 0.4 or 0.8% final concentration) for 1 hour at 37°C, 5% CO₂. Samples were then stimulated with 50 ng/ml LPS (S. typhosa) in RPMI 1640 containing 1% L-glutamine and 1% penicillin/ streptomycin (25 µl). Following incubation for 20 hours at 37°C, 5% CO₂, 50 or 100 µl physiological saline (0.138%
NaCl) was added, and diluted plasma collected after centrifugation (1300g for 10 minutes) for TNF-α determination using an electrochemiluminescence assay.

**TNF-α Assay.** 50 µl supernatant from either whole blood or PBMC assays was incubated for 2 hours with 50 µl of streptavidin/biotinylated anti-TNF-α antibody mix, 25 µl ruthenium tagged anti-TNF-α monoclonal antibody (BioVeris, Gaithersburg, MD) and 100 µl PBS containing 0.1% BSA. Electrochemiluminescence was read on an IGEN instrument (BioVeris, Gaithersburg, MD) and TNF-α concentrations calculated from a standard curve of human recombinant TNF-α (R&D Systems, Abingdon, UK) included on each assay plate.

**Animals.** Male Lewis rats were obtained from Charles River (Portage, MI) and had access to 5001 Rodent Chow (PMI Nutrition International, Brentwood, MO) and water *ad libitum*. Male ferrets (Mustela Pulorius Furo) were obtained from Misay Consultancy (Hampshire, UK) and had access to SDS diet C pelleted food (Special Diet Services, Essex, UK) and water *ad libitum*, with supplemental Whiskers cat food (Mars Inc, UK) given 3 times per week. Male C57BL/6J mice were obtained from Charles River (Lyon, France) at 6 weeks of age and housed in specific pathogen free conditions, and given access to Altromin R diet (Rieper, Vandoies, Italy) and water *ad libitum*. All animals were housed on a 12-hour lights on, lights off cycle and acclimated to their housing facilities for a minimum of 1 week prior to use. All animal use protocols were performed in accordance with local laws and regulations governing animal research. Studies performed at GlaxoSmithKline, King of Prussia, Pennsylvania were performed with approval from GlaxoSmithKline Institutional Animal Care and Use Committee for the Valley Forge Area. Studies conducted at GlaxoSmithKline, Stevenage, UK were performed under Home Office License PPL 80/01537, procedure number 5. All experiments conducted in Verona were carried out in accordance with Italian regulation governing animal welfare and
protection (which acknowledges the European Directive 86/609/EEC) and according to internal GlaxoSmithKline Committee on Animal Research & Ethics (CARE) review.

**Drugs.** Rolipram and cisplatin were purchased from Sigma-Aldrich (St Louis, MO). Ketamine was purchased from Merial Animal Health Ltd. (Harlow, UK). Xylazine was purchased from Bayer Health Care (Leverkusen, Germany). Roflumilast, roflumilast-N-oxide and EPPA-1 (1-ethyl-5-{5-[(4-methyl-1-piperazinyl)methyl]-1,3,4-oxadiazol-2-yl}-N-(tetrahydro-2\(H\)-pyran-4-y1)-1\(H\)-pyrazolo[3,4-\(b\)]pyridin-4-amine) were synthesized by the Respiratory Department of Chemistry at GlaxoSmithKline (Stevenage, Hertfordshire, UK).

**Inhaled LPS-Induced Pulmonary Neutrophilia in the Lewis Rat.** Male rats (n=6-8) weighing approximately 320-400 g were pretreated orally with the appropriate dose of test compound or vehicle alone (0.5% methylcellulose (Sigma-Aldrich, St. Louis, MO)) at a dose volume of 10 ml/kg. Thirty minutes following pretreatment, the rats were exposed to aerosolized 0.1 mg/ml LPS solution from *E. coli*, serotype 055:B5 (Sigma-Aldrich, St. Louis, MO) at a rate of 4.5 L/min for 20 minutes. At 4 hours post LPS exposure the study rats and two naïve rats were euthanized by pentobarbital (Vortech, Dearborn, MI) overdose (390 mg, i.p.). Bronchoalveolar lavage (BAL) was performed through a 14 gauge blunt needle into the exposed trachea in five, 5 ml washes of PBS to collect a total of 20-23 ml of BAL fluid. 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 PBS. The red blood cells were lysed with hemolytic Gey’s solution (Mishell and Shiigi, 1980), centrifuged and resuspended as described above. Total cell counts were performed on a Beckman-Coulter Z1 particle counter. Leukocyte differentials were performed on BAL smears after staining by Criterion Three-Step Stain (Richard-Allen Scientific, Kalamazoo, MI), containing 1% Azure A, 1% methylene blue and 1% eosin Y.
Pica Feeding. On the first day of the experiment, treatment groups of 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 (Languna Clay Company, 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 acclimated to the new conditions for at least 72 hours prior to compound administration. After the acclimation period, the rats were transferred to clean cages and the weights of the clay to which the animals would have access over the next 24 hours were measured to the nearest 0.1 gram using a calibrated Sartorius 1203 MP top-loading balance. Immediately prior to the dark cycle, the rats were administered compound or vehicle. Rolipram, cilomilast, roflumilast and EPPA-1 were suspended in 0.5% methylcellulose (Sigma, St. Louis, MO) and administered orally at a dose volume of 2 ml/kg. Twenty four hours after compound or vehicle administration any clay which had fallen through the cage grate was separated from food and waste, and was dried and weighed in a previously tared container, if necessary. This clay weight was added to the uneaten clay remaining in the food cup in order to calculate the total clay consumed by each rat following administration of compound or vehicle. A drug-treated rat was defined as pica positive if the animal consumed greater than 0.3 grams of clay over the average clay consumed by the vehicle-treated rats. This threshold (0.3 grams) was established because it was the standard deviation in pica consumption exhibited by the vehicle-treated control animals across multiple pilot studies.

Anti-inflammatory Potency and Emetogenic Effects in the Conscious Ferret. Male ferrets weighing 1-2.5 kg were fasted overnight prior to the study, but allowed free access to
water throughout the study. Food was returned to the animals 60-90 minutes post-dosing. Thirty minutes prior to LPS challenge, compound was orally administered to conscious animals (n=3-8) in 20% Cremophor El (Sigma-Aldrich, Gillingham, UK), at a dose volume of 1ml/kg. After dosing, the animals were returned to holding cages, and were continuously observed for up to 2.5 hours for emesis. Ferret therapeutic index studies were conducted in a dose-escalating manner, and the escalation was halted if the incidence of vomiting was exhibited in greater than 50% of the animals at any given dose. Thirty minutes after oral administration of compound or vehicle, the ferrets were placed into sealed perspex containers and exposed to a nebulizer-generated aerosol of a 30 μg/ml LPS solution, (E. coli, serotype 0127:B8 (Sigma-Aldrich, Gillingham, UK) at a rate of 6L/min for 10 minutes. Following the LPS challenge, the animals were returned to their holding cages. Six hours after LPS exposure the animals were killed by overdose of sodium pentobarbitone (i.p.) (Vericore Ltd, Dundee, Scotland). BAL was performed through the exposed trachea with 2 x 20ml of heparinised (10 units.ml⁻¹) PBS. The BAL samples were centrifuged at 300g for 7 minutes at room temperature. The supernatant was removed and the resulting cell pellet resuspended in 1ml PBS. A slide of the resuspended cells was prepared by cytospin, and then treated with Leishmans stain (Sigma-Aldrich, Gillingham UK) for 20 minutes to allow differential cell counting. The total cells were counted with a Sysmex K1000 automated haematology analyzer. From these two counts, the total numbers of neutrophils in the BAL samples were determined.

α2-Adrenoceptor-Mediated Anesthesia in the C57BL/6J Mouse. Mice were anaesthetized with an intraperitoneal injection consisting of 80 mg/kg ketamine (Merial, UK) and 10 mg/kg xylazine (Bayer, Germany). Fifteen minutes later, 10 animals per group were subcutaneously treated with rolipram (3 mg/kg), EPPA-1 (3, 10 and 30 mg/kg), or vehicle (0.5%
methylcellulose) and placed in dorsal recumbency. The time to the recovery of righting reflex was used as an endpoint to measure the duration of anesthesia.

**Statistical Analysis.** For *in vitro* assays, percent inhibition values were generated relative to uninhibited controls. IC50 values were determined from concentration-response curves by non-linear least squares curve fitting using a 4-parameter logistic equation in Activity Base (IDBS, Guilford, Surrey, UK).

Initially, the raw neutrophil dose response data was fit using a 4-parameter logistic model. Following this, dose-response curves of the percent inhibition of neutrophils were generated by normalization, using the estimated control and baseline asymptotes of the 4-parameter logistic curve. D50 (defined as the dose that produces a 50% increase in mean response over vehicle) was back-interpolated from the percent inhibition curve. Dose-response curves of the incidence of pica positive rats and vomiting ferrets were generated using binary logistic regression analysis. Significance in the mouse anesthesia reversal study was determined by one-way ANOVA, with a Dunnett’s post-test. The data for this study are expressed as mean ± SEM with statistical significance achieved at p<0.05.

For the experiments discussed in this paper, we define the therapeutic index as the ratio of the D50 for the pica feeding or emesis endpoints divided by the D50 for the neutrophil inhibition response. For both the rat and ferret model experiments, we conducted tests of the (null) hypotheses of "no difference" among the therapeutic indices for the different compounds tested. Only if such an overall null hypothesis was rejected (at a 5% significance level) did we go on to make statistical comparisons between two compounds. If the null hypothesis of "no difference" among the therapeutic indices was rejected, pairwise tests to compare the therapeutic
index of EPPA-1 against the therapeutic indices of the other compounds in the respective studies were performed.

For each of the four compounds, confidence intervals for both the neutrophil and pica D50 values are reported, as well as a confidence interval for the therapeutic index. These confidence intervals were adjusted for multiplicity (by the Bonferroni criterion) across the four compounds. For improved accuracy, all confidence intervals for the D50 values and therapeutic indices were initially computed for their log values and then anti-logged. Furthermore, for each compound, coefficients of determination (i.e. $R^2$ values, see (Neter and Wasserman, 1974)) were reported for the neutrophil dose response curves. For the (quantal response) pica data, $R^2$ values were computed using the generalized coefficient of determination proposed by Nagelkerke (Nagelkerke, 1991).

The hypothesis test of the equality of all four therapeutic indices was done using a likelihood ratio test (Huet et al., 1996). Pairwise comparisons of the three therapeutic indices against that for EPPA-1 were done as (one-sided) tests involving the maximum likelihood estimates and their standard errors. For each compound, a dose response curve was fit to a neutrophil data set and also to the corresponding pica (quantal response) data set. Each of these data sets produces a D50 estimate. Because the therapeutic index (TI) is a positive entity, we found a confidence interval for the logTI, and then anti-logged it to get a confidence interval for the TI, which must be positive. The confidence interval for the logTI was obtained using the following 
(approximate) formula,

$$
\hat{\theta}_P - \hat{\theta}_N \pm z_{\alpha/2} \sqrt{\hat{\sigma}^2(\hat{\theta}_P) + \hat{\sigma}^2(\hat{\theta}_N)}.
$$
which is valid for independent data samples. Here $\hat{\theta}_P$ is the maximum likelihood estimate of the logD50 for the pica assay and $\hat{\theta}_N$ is the maximum likelihood estimate of the logD50 for the neutrophil assay. Furthermore, $\sigma^2(\hat{\theta}_P)$ is an estimate of the variance of $\hat{\theta}_P$ and $\sigma^2(\hat{\theta}_N)$ is an estimate of the variance of $\hat{\theta}_N$. (Here, $z_{\alpha/2}$ is the $(1-\alpha/2)^{th}$ percentile of the standard normal distribution.) In a similar fashion, pairwise tests to compare the TIs were based upon maximum likelihood estimates of the difference of the logTI's and the estimated variances of the logTI estimates. These estimates were obtained from the NLMIXED Procedure using the SAS statistical package (SAS v9.1). The NLMIXED Procedure has a general maximum likelihood estimation capability which can be used to compute the needed logD50 estimates and their associated estimated variances. The Bonferroni adjustment to the significance level was used for multiple comparisons among the pairwise tests of the therapeutic indices.
Results

**EPPA-1 is a Potent and Selective Inhibitor of PDE4.** The structure of EPPA-1 can be seen in Figure 1. The potency of EPPA-1 was assessed against the enzyme activity of the four PDE4 isoforms PDE4A, PDE4B2B, PDE4C, PDE4D3A, and also assayed for selectivity against PDE1, 2, 3, 5, 6, 7. As seen in Table 1, EPPA-1 is a potent inhibitor of PDE4, with IC50 values against the four isoforms ranging from 35 to 142 nM. EPPA-1 was selective for PDE4 over PDE1, 2, 3, 5, 6, and 7, with IC50 values ranging from >11 to >30 μM. The potencies of EPPA-1 against PDE8, 9, 10 and 11 are unknown.

**EPPA-1 is a Potent Inhibitor of LPS-induced TNF-α in Human PBMCs and Whole Blood.** To assess anti-inflammatory activity *in vitro*, EPPA-1, rolipram (4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone), cilomilast (c-4-cyano-4-[3-(cyclopentyloxy)-4-methoxyphenyl]-r-1-cyclohexane carboxylic acid), roflumilast (3-cyclopropylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide) and roflumilast’s active N-oxide metabolite were assayed to determine their ability to inhibit TNF-α production by LPS-stimulated human PBMCs and human whole blood. The results can be seen in Table 2. EPPA-1 displayed an IC50 against LPS-induced TNF-α production by isolated human PBMCs of 38 nM. Roflumilast and its active N-oxide metabolite were more potent in PBMCs with IC50s of 5 nM and 2 nM, respectively. Cilomilast and rolipram were least potent in isolated PBMCs, exhibiting IC50s of 389 nM and 269 nM, respectively. There was a decrease in potency across all of the compounds tested in the human whole blood assay. EPPA-1, roflumilast, and rolipram displayed 2.5- to 4-fold decreases in IC50 between the isolated PBMC assay and the human whole blood assay, while roflumilast’s
active N-oxide and cilomilast showed greater decreases in potency, with differences between the PBMC and whole blood IC50s of 10-fold and >15-fold, respectively.

**EPPA-1 is a Potent Inhibitor of LPS-induced Pulmonary Neutrophilia in the Lewis Rat.** To assess the anti-inflammatory activity of each PDE4 inhibitor in a model of pulmonary inflammation in the rat, the compounds were tested for their ability to attenuate LPS-induced pulmonary neutrophilia. Each compound was administered orally 30 minutes prior to the aerosolized LPS challenge. Four hours following the LPS challenge BAL was performed on the animals, and differential cells analysis performed to quantify neutrophils. As shown in Figure 2, all of the compounds inhibited LPS-induced pulmonary neutrophilia in a dose-dependent manner (for ease of interpretation, the percent inhibition values relative to vehicle-treated controls have been plotted). The D50 values, generated by analyzing the raw neutrophil numbers and then fitting the data, are listed in Table 5. Cilomilast, the least potent compound, exhibited a D50 of 4.54 mg/kg, followed by rolipram and roflumilast with D50s of 3.34 and 0.24 mg/kg, respectively. EPPA-1 exhibited the most potent anti-inflammatory activity, with a D50 of 0.042 mg/kg. The somewhat small R^2 values obtained (Table 5) were due to substantial variation in response at each dose level. Nonetheless, statistically significant dose responses were observed for all four of the inhibitors tested. For the sake of completeness, the normalized data represented in Figure 2 were analyzed as well, and comparable D50 values obtained (4.34, 3.13, 0.24 and 0.035 mg/kg for cilomilast, rolipram, roflumilast and EPPA-1, respectively).

**Oral Administration of Rolipram (5 mg/kg) Induces an Increase in the Consumption of Clay by Lewis Rats.** In order to assess the emetic potential of PDE4 inhibitors in the same species used to conduct the anti-inflammatory studies, rat pica feeding was used as a surrogate for the emetic response that occurs in higher-order species. In this model, rats were
acclimated for 72 hours in cages with free access to food, dried clay pellets and water, but without bedding or additional environmental enrichment. Immediately prior to the first dark cycle following the 72 hour acclimation period, compound was administered orally to the rats, and clay consumption over the next 24 hours was measured. Initially we attempted to validate the pica model using cisplatin to induce pica under conditions identical to those previously reported (Takeda et al, 1993), with the exception that commercially available modelling clay was used instead of kaolin pellets. Under these conditions we were able to reproduce the results of Takeda, et al, by inducing pica in rats following a 10 mg/kg intraperitoneal dose of cisplatin (data not shown). Next, we assessed the ability of the PDE4 inhibitor rolipram at 5 mg/kg, p.o. to induce pica in rats. The clay consumption was measured every 24 hours over the 72 hour acclimation period and again 24 hours after rolipram administration. As seen in Table 3, over the first 24 hours of acclimation, the rats did consume a measurable amount of clay, but between 48 and 72 hours the rats consumed almost no clay, indicating that clay consumption from exploratory behaviour had ceased. Following the 72 hour acclimation period, the rats were orally dosed with 5 mg/kg of rolipram or vehicle, and after 24 hours the clay was measured again. The rolipram-treated animals consumed 1.2 ± 0.45 grams of clay, which was greater than the 0.1 ± 0.07 grams of clay consumed by the vehicle control group, indicating the induction of pica feeding by rolipram. This indication was substantiated by a statistically significant positive slope for dose dependency for rolipram as discussed below.

Inhibitors of PDE4 Induce a Dose-dependent Increase in the Incidence of Pica Feeding in Rats. Next, we attempted to measure a dose-response to rolipram in the pica model. As seen in Table 4, oral administration of rolipram induced pica in rats in a dose-dependent manner as measured by mean clay consumption. Although the rats consumed clay in a dose-
dependent manner as measured by clay mass consumed, we observed that the rats either engaged in pica behaviour, thus consuming clay, or did not. The quantal nature of the response skewed the continuous clay mass data at intermediate doses. Because of the binary nature of the pica response, we analyzed our dose-response data using a count system by establishing a mean baseline of clay consumption using the vehicle control group and rating PDE4 inhibitor-treated animals as pica positive based on clay consumption of > 0.3 grams over the mean clay consumed by vehicle-treated animals. This threshold (0.3 grams) was chosen because it was the standard deviation in pica consumption exhibited by the vehicle-treated control animals across multiple pilot studies. Based on the fact that it was applied consistently across the inhibitors, conclusions regarding the rank-order potency of the inhibitors should not be affected by it. The results of this analysis indicated that rolipram-treated rats consumed clay in a dose-dependent manner, with the binary logistic regression dose-response model exhibiting a statistically significant slope (p=0.0054). Similar to rolipram, incidence data were used to generate dose-response curves for roflumilast, cilomilast and EPPA-1 using logistic regression analysis. Rolipram and roflumilast induced pica with D50s of 0.495 and 1.57 mg/kg, respectively, while cilomilast and EPPA-1 were less potent exhibiting D50s of 6.41 mg/kg and 24.26 mg/kg, respectively (Figure 2 and Table 5). Similar to the rat neutrophilia data, while the somewhat small R^2 values (Table 5) are indicative of substantial variation in response at each dose level, statistically significant dose responses were observed for each of the four inhibitors tested. For the (quantal response) pica data, R^2 values were computed using the generalized coefficient of determination proposed by Nagelkerke (Nagelkerke, 1991).

**EPPA-1 Displays a Therapeutic Index Higher than Roflumilast, Cilomilast and Rolipram in the Lewis Rat.** The therapeutic index of each PDE4 inhibitor was calculated by
dividing the D50 value generated in the pica model by the D50 value generated in the LPS-
induced neutrophilia model. Thus, a higher quotient results from an increased anti-inflammatory
potency and/or a decreased pica potency, and represents an improved therapeutic index. As
seen in Table 5, the first generation PDE4 inhibitor rolipram exhibited the lowest therapeutic
index (0.15), followed by the second generation PDE4 inhibitors cilomilast (1.4) and roflumilast
(6.42). The third generation PDE4 inhibitor, EPPA-1, exhibited the highest therapeutic index
(577.5). A likelihood ratio test of the hypothesis of "no differences" among the therapeutic
indices for these compounds was statistically significant (p=0.008). As such, further testing was
done to compare the therapeutic indices of rolipram, cilomilast and roflumilast against the
therapeutic index of EPPA-1. The resulting p-values for the rolipram, cilomilast and roflumilast
comparisons were 0.00013, 0.0191, and 0.0195, respectively. As there were three comparisons,
the Bonferroni adjustment for multiple comparisons required a significance level of
0.05/3=0.01667.

Calculation of the therapeutic index values using the normalized (percent change from
control mean) rat neutrophilia data gave the following: for rolipram (D50=3.13 mg/kg),
cilomilast (D50=4.34 mg/kg), roflumilast (D50=0.24 mg/kg) and EPPA-1 (D50=0.035 mg/kg),
the therapeutic index values were 0.16, 1.5, 6.4 and 697, respectively, with a likelihood ratio test
of the hypothesis of "no differences" among the therapeutic indices giving p=0.004. Comparison
of the therapeutic indices of rolipram, cilomilast and roflumilast against that of EPPA-1 resulted
in p-values of 0.0007, 0.001, and 0.019, respectively, with the Bonferroni adjustment for
multiple comparisons requiring a significance level of 0.05/3=0.01667.

Hence, the differences between the therapeutic indices of EPPA-1 and rolipram were
highly statistically significant, while the therapeutic index comparisons for EPPA-1 vs.
cilomilast and roflumilast attained at least borderline statistical significance after the Bonferroni adjustment for multiple comparisons.

EPPA-1 Displays a Therapeutic Index Higher than Roflumilast and Cilomilast in the Ferret. In order to compare the therapeutic indices generated in the rat using pica with those generated in an emetic species, the panel of PDE4 inhibitors was evaluated in the ferret. Compounds were administered orally 30 minutes prior to the aerosolized LPS challenge, and six hours later animals sacrificed and BAL fluid collected for differential cell analysis. The incidence of vomiting animals was observed and recorded for the first 2.5 hours following dosing. For ethical reasons, each dose-response study was performed in a dose-escalating manner. The dose escalation was terminated if the incidence of vomiting ferrets was greater than 50%.

As seen in Table 6, cilomilast was evaluated at 1, 3 and 10 mg/kg. Cilomilast had little or no effect with respect to either neutrophilia or emesis at 1 and 3 mg/kg. At 10 mg/kg, cilomilast inhibited neutrophilia by 51%, but induced vomiting in 100% of the animals. Hence the D50 for the cilomilast-induced emesis could not be estimated, as the maximum likelihood estimates do not exist in this case (Albert and Anderson, 1984). As such, the therapeutic index value was taken to be less than 1. For roflumilast, the neutrophilia D50 (calculated by analyzing raw neutrophil numbers) was found to be 0.41 mg/kg; however an emesis effect rate (between 0% and 100%) could only be observed at 1 mg/kg. Here 3 out of 6 (50%) animals exhibited emesis. In this case, the maximum likelihood estimates also do not exist. Therefore the therapeutic index was taken as 1/0.4=2.4.

Like cilomilast and roflumilast, EPPA-1 inhibited LPS-induced pulmonary neutrophilia and induced vomiting in ferrets in a dose-dependent manner. EPPA-1 inhibited the influx of
neutrophils with a D50 of 3.2 mg/kg, and induced vomiting with a D50 of 9.5 mg/kg, resulting in
a therapeutic index of 3.0. Analysis of the normalized neutrophil data gave the same D50 value
for roflumilast as that generated via analysis of the raw neutrophil numbers (0.41 mg/kg), and
D50 = 2.6 mg/kg for EPPA-1, resulting in therapeutic index values of 2.5 and 3.7, respectively.
Thus the rank order of therapeutic indices in the ferret (EPPA-1 > roflumilast > cilomilast)
remained the same, and was aligned with the rank order of therapeutic indices exhibited by the
rat. Graphic representation of the therapeutic indices for roflumilast and EPPA-1, presented as
percent inhibition relative to vehicle-treated controls, can be seen in Figure 3.

EPPA-1 does not Attenuate $\alpha_2$-Adrenoceptor-Mediated Anesthesia in the C57BL/6J

Mouse. To confirm the lack of emetogenic activity of EPPA-1 in a second surrogate model for
emesis in the rodent, mice were anesthetized with a mixture of xylazine and ketamine. Fifteen
minutes after the administration of anesthesia, the mice were subcutaneously treated with
rolipram (3 mg/kg) or EPPA-1 (3, 10 and 30 mg/kg) and placed in dorsal recumbency. The
recovery of the righting reflex was used as an endpoint to measure the duration of anesthesia. As
seen in Figure 4, rolipram significantly decreased the duration of anesthesia by 53% (p<0.001).
EPPA-1 had no effect on duration of anesthesia with respect to the vehicle control group at any
dose tested.
Discussion

The broad range anti-inflammatory effects exhibited by PDE4 inhibitors have provided the rationale for the use of these compounds in the treatment of respiratory diseases with an inflammatory component, including COPD and asthma. The major obstacle in the development of PDE4 inhibitors for clinical use has been their association with adverse effects, primarily nausea and emesis. Thus, critical to the identification of PDE4 inhibitors with improved therapeutic indices is the application of models that facilitate accurate assessment of both the anti-inflammatory (therapeutic) and gastrointestinal (adverse) effects of these inhibitors, ideally in the same species. While therapeutic index screening in rodents offers practical benefit over the use of higher-order species, the absence of a vomit reflex in rodents necessitates identification of a surrogate measure for emesis. Herein we report on the use of rat pica feeding as a surrogate for the evaluation of the emetic potentials of PDE4 inhibitors. In conjunction with a rat model of inflammation, therapeutic index values were determined for the first-generation inhibitor, rolipram, the second-generation inhibitors, roflumilast and cilomilast, and a novel third generation inhibitor, EPPA-1. The rank-order of the therapeutic indices generated in the rat aligned with the rank-order generated in an emetic species, the ferret. The low and high emetogenic activities of EPPA-1 and rolipram, respectively, detected in the pica model were confirmed in a second surrogate model of emesis, reversal of xylazine/ketamine-induced anesthesia in the mouse. Collectively, these data identify the representative tool compound EPPA-1 as a novel PDE4 inhibitor with increased anti-inflammatory potency and reduced emetogenic potential, resulting in an improved therapeutic index.
In our determinations of the anti-inflammatory activities of the first and second generation PDE4 inhibitors (rolipram, roflumilast and cilomilast), the rank-order potency in both the \textit{in vitro} assays (LPS-induced TNF-\(\alpha\) production by human PBMCs and whole blood) and the \textit{in vivo} assay (LPS-induced pulmonary neutrophilia in the rat) was roflumilast > rolipram > cilomilast. This is consistent with the rank-order potency reported by other investigators against LPS-induced TNF-\(\alpha\) production by human monocytes and dendritic cells (Hatzelmann and Schudt, 2001), LPS-induced TNF-\(\alpha\) production by human whole blood (Draheim et al., 2004; Hatzelmann and Schudt, 2001), and ovalbumin-induced leukocyte and TNF-\(\alpha\) accumulation in rat BAL fluid (Bundschuh et al., 2001). EPPA-1 demonstrated a relatively high degree of anti-inflammatory activity in the assays, exhibiting potencies intermediate to those of roflumilast and rolipram in the PBMC and whole blood assays, and a potency greater than that of roflumilast in the pulmonary neutrophilia model.

To measure the emetogenicity of the PDE4 inhibitors in the same species in which anti-inflammatory potency was being determined, we utilized rat pica feeding. Pica feeding is defined as the consumption of non-nutritive substances as a result of gastrointestinal distress, and in rodents appears to be analogous to vomiting in that both responses mitigate the effects of ingested noxious agents (Mitchell et al., 1976) and are mediated via common physiological pathways (Takeda et al., 1993; Takeda et al., 1995a; Takeda et al., 1995b). The rank-order potency observed in the pica model was rolipram > roflumilast > cilomilast > EPPA-1, implicating rolipram as the most emetic, and EPPA-1 as the least emetic, of the PDE4 inhibitors tested. These results were confirmed in a second surrogate model of emesis in the rodent, xylazine/ketamine-induced anesthesia in the mouse, with rolipram at 3 mg/kg significantly reducing the duration of anesthesia, while EPPA-1 at up to 30 mg/kg had no effect. Using a
panel of PDE4 inhibitors, Robichaud et al. previously demonstrated that reduction of xylazine/ketamine-induced anesthesia in the mouse positively correlates with induction of vomiting in an emetic species, the ferret (Robichaud et al., 1999; Robichaud et al., 2002).

The therapeutic indices derived from the rat LPS-induced pulmonary neutrophilia and pica models gave a rank-order of EPPA-1 > roflumilast > cilomilast > rolipram, consistent with the rank-order of therapeutic indices derived from the LPS-induced pulmonary neutrophilia and emesis models in the ferret (EPPA-1 > roflumilast > cilomilast), thus validating rat pica feeding as a surrogate of PDE4 inhibitor-induced emesis in higher species. The first generation PDE4 inhibitor, rolipram, exhibited relatively low anti-inflammatory potency and was the most potent inducer of pica in the rat, leading to a low therapeutic index (0.15). Conversely, the third generation inhibitor, EPPA-1, exhibited the strongest anti-inflammatory activity and weakest induction of pica, resulting in the highest therapeutic index (578). The second generation inhibitors, roflumilast and cilomilast, demonstrated intermediate therapeutic indices of 6.4 and 1.4, respectively, with roflumilast exhibiting increased potencies in both the neutrophilia and pica models compared to cilomilast.

Various therapeutic index models have been used in order to pursue PDE4 inhibitors with increased anti-inflammatory activity and decreased emetogenicity. These models, several of which are described herein, are performed in different species, including mice, rats, ferrets, dogs and non-human primates. Models performed in lower-order species such as mice and rats offer the advantages of being higher throughput and having reagents available to measure a range of anti-inflammatory readouts. Alternatively, in species such as ferrets, dogs and non-human primates the anti-emetic effects of inhibitors can be tested directly, rather than indirectly on a surrogate of emesis, which may not fully represent the mechanisms underlying emesis. While
testing PDE4 inhibitors in emetic species is associated with the aforementioned advantage, these models often necessitate measuring, in addition to vomiting, behavioural correlates of emesis (ie, salivation, retching) which can be difficult to quantitate. As such, confirmation of an improved therapeutic index in multiple models, if possible, increases confidence in the improved efficacy/tolerability of the inhibitor. In this regard, validation of surrogates such as pica for PDE4 inhibitor-induced emesis provides a method to rapidly evaluate novel compounds, with those demonstrating an improved therapeutic index in rodents being selected for further evaluation in higher-order, emetic species in order to increase confidence in their enhanced efficacy/tolerability. EPPA-1 exemplifies such a compound, demonstrating a non-emetic phenotype in the $\alpha_2$-adrenoceptor-mediated anesthesia model in the mouse, and improved therapeutic indices in both the rat pica/neutrophilia (surrogate) model, and in an emetic species, the ferret.

Several hypotheses have been proposed regarding the molecular basis underlying the anti-inflammatory vs. emetic potential of PDE4 inhibitors. Evidence supports the existence of two non-interconvertible pharmacologically distinct conformers of PDE4, one of which binds rolipram with high affinity (High Affinity Rolipram Binding Site, HARBS) and the other which binds rolipram with low affinity (LARBS). It has been suggested that many of the beneficial anti-inflammatory effects of PDE4 inhibitors are associated with inhibition of LARBS, while adverse effects such as nausea and emesis are associated with inhibition of HARBS. Thus, an improved therapeutic index would be expected from a PDE4 inhibitor exhibiting decreased affinity for HARBS and/or increased affinity for LARBS. A second hypothesis proposes that the gastrointestinal side effects occur via the central nervous system (CNS), and thus that PDE4 inhibitors with reduced CNS penetrability will be less emetogenic (Burnouf and Pruniaux,
A third hypothesis is focused on selectivity of inhibitors for the different PDE4 subtypes, with inhibition of PDE4B, and possibly PDE4A, mediating anti-inflammatory efficacy, and inhibition of PDE4D mediating emetogenicity (Jin and Conti, 2002; Robichaud, Stamatiou, Jin, Lachance, MacDonald, Laliberté, Liu, Huang, Conti, and Chan, 2002).

One approach to gain insight into the potential mechanism(s) contributing to PDE4 inhibitor therapeutic index is to determine whether a statistically significant correlation exists between a group of pharmacologically diverse inhibitors (ie, exhibiting a range of anti-inflammatory or emetic activities) and the mechanism of interest (ie, HARBS binding, PDE4 subtype selectivity, CNS penetration) (Barnette et al., 1995). In the rat pica feeding model, the rank-order potency of inhibitors was rolipram (D50 = 0.495 mg/kg) > roflumilast (1.6) > cilomilast (6.4) > EPPA-1 (24.3). In our hands, the two most potent inducers of pica / emetogenic compounds, rolipram and roflumilast, exhibited the highest affinity for HARBS (IC50 = 2, 1 and 3 nM for rolipram, roflumilast and roflumilast N-oxide, respectively), while the compounds with lower emetic potential, cilomilast and EPPA-1, exhibited decreased HARBS affinity (IC50 = 178 and 120 nM, respectively). In contrast, against PDE4D rolipram and roflumilast (the strongest pica inducers) exhibited the widest range of potencies amongst the inhibitors (IC50 = 65 and 0.3 nM, respectively) while EPPA-1 and cilomilast exhibited intermediate potencies (IC50 = 35 and 15 nM, respectively). Following an i.v. bolus of inhibitor, the proportion of compound present in the brain relative to the plasma was found to be similar for EPPA-1 and roflumilast (0.89 and 0.9, respectively), and lower for cilomilast (0.07). This indicates that EPPA-1 is CNS-penetrant, with nearly equal proportions of compound localizing in the brain and plasma. Thus, the data for this set of inhibitors is most consistent with the
HARBS hypothesis, although it should be noted that a more systematic analysis across a larger number of pharmacologically diverse PDE4 inhibitors is required to demonstrate statistically significant correlations, and mechanistic studies are required to demonstrate a cause-and-effect relationship. Given the complexity of PDE4 biology, it is possible that any one of these hypotheses alone is too simplistic to explain the differing therapeutic indices across every set of structurally related inhibitors, and that multiple mechanisms may contribute.

In conclusion, we validated a rodent system for the determination of therapeutic indices of PDE4 inhibitors. Using rat inhaled LPS-induced pulmonary neutrophilia to test anti-inflammatory activity, and induction of rat pica feeding as a measure of emesis, a compound with improved anti-inflammatory potency and tolerability, EPPA-1, was identified. The identification of PDE4 inhibitors with increased anti-inflammatory activity and decreased adverse effects offers a potential path forward for the development and clinical use of these compounds for inflammation-driven respiratory diseases including COPD and asthma.
Acknowledgments

We thank Dr. Nicola Aston, Dr. Kristen Belmonte, Dr. Ruth Mayer, Dr. Alison Redgrave and Dr. Peter Ward for program leadership.
References


Footnotes

Address reprint requests to: Dr. Patricia L. Podolin, GlaxoSmithKline, Mail Code UW2532, 709 Swedland Road, King of Prussia, PA 19406. E-mail: patty_podolin@gsk.com.
Legends for Figures

Figure 1. The chemical structure of EPPA-1, 1-ethyl-5-\{-5-\{(4-methyl-1-piperazinyl)methyl\}-1,3,4-oxadiazol-2-yl\}\{-N\)-(tetrahydro-2\textit{H}-pyran-4-yl\}-1\textit{H}-pyrazolo[3,4-b]pyridin-4-amine.

Figure 2. Inhibitors of PDE4 attenuate LPS-induced pulmonary neutrophilia, and induce pica feeding, in the rat. For the LPS-induced pulmonary neutrophilia studies, rats (n=6-8/group) were pretreated with PDE4 inhibitors or vehicle thirty minutes prior to LPS challenge. Four hours following the LPS challenge, the animals were sacrificed and BAL was performed. Cells in the BAL samples were counted, and leukocyte differentials conducted to determine the percent inhibition of neutrophil influx for each dose relative to the vehicle control group. For the pica feeding studies, rats (n=5-10/group) were individually housed in wire bottom cages with access to food, water and modeling clay. After a 72 hour acclimation period and prior to the 12 hour dark phase, the clay baseline weight was measured and the animals dosed with PDE4 inhibitors or vehicle. Twenty four hours following administration of compound or vehicle, the clay was re-weighed. Rats consuming >0.3 grams of clay more than the average clay consumed by the vehicle control group were deemed pica positive. A dose-response curve was generated by logistic regression using the incidence of pica feeding at each dose.

Figure 3. The therapeutic index of roflumilast and EPPA-1 in the ferret. Ferrets (n=3-8/group) were treated with PDE4 inhibitor or vehicle 30 minutes prior to LPS exposure. Animals were observed for vomiting for the first 2.5 hours after dose administration. Six hours following the LPS challenge, the animals were sacrificed and BAL was performed. Cells in the
BAL samples were counted, and leukocyte differentials conducted to determine for each dose the percent inhibition of neutrophil influx relative to the vehicle control group.

Figure 4. EPPA-1 does not inhibit α2-adrenoceptor-mediated anesthesia in mice.

C57BL/6J mice (n=10/group) were anesthetized with 10 mg/kg xylazine and 80 mg/kg ketamine. Fifteen minutes later, the mice were treated with rolipram, EPPA-1 or vehicle, after which time they were placed in dorsal recumbency. The time to the recovery of righting reflex was used to measure the duration of anesthesia. Data are expressed as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001 relative to the vehicle control group.
TABLE 1

Potencies of EPPA-1 against isoforms of PDE4

<table>
<thead>
<tr>
<th>EPPA-1 IC50</th>
<th>Mean (95% confidence limits)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1</td>
<td>&gt; 30.2 μM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>PDE2</td>
<td>&gt; 30.2 μM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>PDE3</td>
<td>29.0 (26.2, 32.0) μM</td>
<td>6</td>
</tr>
<tr>
<td>PDE4A</td>
<td>93.0 (51.3, 168.2) nM</td>
<td>4</td>
</tr>
<tr>
<td>PDE4B</td>
<td>45.7 (33.2, 62.9) nM</td>
<td>12</td>
</tr>
<tr>
<td>PDE4C</td>
<td>142.2 (61.2, 330.3) nM</td>
<td>4</td>
</tr>
<tr>
<td>PDE4D</td>
<td>35.1 (19.5, 63.2) nM</td>
<td>8</td>
</tr>
<tr>
<td>PDE5</td>
<td>&gt; 10.7 μM</td>
<td>3</td>
</tr>
<tr>
<td>PDE6</td>
<td>16.6 (9.1, 30.4) μM</td>
<td>6</td>
</tr>
<tr>
<td>PDE7</td>
<td>&gt; 30.2 μM</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>replication within a single experiment
TABLE 2

Potencies of PDE4 inhibitors in attenuation of TNF-α production by human PBMCs and whole blood

PBMCs were isolated from heparinized human blood, and following incubation of cells (5 x 10⁴) with LPS (1 ng/ml) and PDE4 inhibitor or vehicle for 20 hours, culture supernatants were assayed for TNF-α. Heparinized whole blood was incubated with PDE4 inhibitor or vehicle for 1 hour prior to the addition of LPS (50 ng/ml) for 20 hours; plasma was then collected and assayed for TNF-α.

<table>
<thead>
<tr>
<th></th>
<th>IC50 (nM)</th>
<th>Mean (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EPPA-1</td>
<td>Roflumilast</td>
</tr>
<tr>
<td>PBMC</td>
<td>38.0 (34.1, 42.4)</td>
<td>5.0 (4.6, 5.5)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=140</td>
</tr>
<tr>
<td>Whole blood</td>
<td>91.2 (53.2, 156.5)</td>
<td>19.5 (17.8, 21.3)</td>
</tr>
<tr>
<td></td>
<td>n=16</td>
<td>n=172</td>
</tr>
</tbody>
</table>
TABLE 3
Clay consumed by rats following administration of rolipram

Rats were separated into individual cages with access to food and clay pellets, and acclimated to
the new conditions for 72 hours. Following the acclimation period, rolipram (5 mg/kg) or
vehicle was administered. Clay consumption was measured every 24 hours during the 72 hour
acclimation period, and 24 hours after rolipram/vehicle administration.

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Mean (g)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>Group 1</td>
<td>None</td>
<td>10</td>
<td>1.2</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>None</td>
<td>10</td>
<td>0.4</td>
<td>0.17</td>
</tr>
<tr>
<td>48 hours</td>
<td>Group 1</td>
<td>None</td>
<td>10</td>
<td>0.1</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>None</td>
<td>10</td>
<td>0.1</td>
<td>0.14</td>
</tr>
<tr>
<td>72 hours</td>
<td>Group 1</td>
<td>None</td>
<td>10</td>
<td>0.1</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>None</td>
<td>10</td>
<td>0.2</td>
<td>0.15</td>
</tr>
<tr>
<td>96 hours</td>
<td>Group 1</td>
<td>Vehicle</td>
<td>10</td>
<td>0.1</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>Rolipram, 5 mg/kg</td>
<td>10</td>
<td>1.2</td>
<td>0.45</td>
</tr>
</tbody>
</table>
TABLE 4
Rolipram induces pica in a dose-dependent manner.
Rats were separated into individual cages with access to food and clay pellets, and acclimated to the new conditions for 72 hours. After the acclimation period, the clay weight was measured. Rolipram or vehicle was administered and the clay reweighed 24 hours later.

<table>
<thead>
<tr>
<th>Mean Clay Consumed (g)</th>
<th>SEM</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.08</td>
<td>0.037</td>
</tr>
<tr>
<td>Rolipram, 0.016 mg/kg</td>
<td>0.10</td>
<td>0.470</td>
</tr>
<tr>
<td>Rolipram, 0.08 mg/kg</td>
<td>0.20</td>
<td>0.169</td>
</tr>
<tr>
<td>Rolipram, 0.4 mg/kg</td>
<td>0.40</td>
<td>0.176</td>
</tr>
<tr>
<td>Rolipram, 2 mg/kg</td>
<td>0.52</td>
<td>0.089</td>
</tr>
<tr>
<td>Rolipram, 10 mg/kg</td>
<td>1.30</td>
<td>0.470</td>
</tr>
</tbody>
</table>
TABLE 5
The therapeutic indices of rolipram, roflumilast, cilomilast and EPPA-1 in the rat.

<table>
<thead>
<tr>
<th></th>
<th>Neutrophilia</th>
<th>Pica</th>
<th>Therapeutic Index&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(95% confidence limits)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rolipram</td>
<td>3.34 (1.5, 7.6)</td>
<td>0.495 (0.08, 3.2)</td>
<td>0.15 (0.02, 1.2)</td>
</tr>
<tr>
<td></td>
<td>[R^2 = 54.5%]</td>
<td>[R^2 = 51.9%]</td>
<td></td>
</tr>
<tr>
<td>Roflumilast</td>
<td>0.24 (0.10, 0.58)</td>
<td>1.57 (0.56, 4.4)</td>
<td>6.42 (1.7, 24.5)</td>
</tr>
<tr>
<td></td>
<td>[R^2 = 81.5%]</td>
<td>[R^2 = 60.1%]</td>
<td></td>
</tr>
<tr>
<td>Cilomilast</td>
<td>4.54 (0.05, 408.7)</td>
<td>6.41 (0.67, 61.8)</td>
<td>1.4 (0.01, 217.6)</td>
</tr>
<tr>
<td></td>
<td>[R^2 = 24.5%]</td>
<td>[R^2 = 32.6%]</td>
<td></td>
</tr>
<tr>
<td>EPPA-1</td>
<td>0.042 (0.001, 1.2)</td>
<td>24.26 (0.37, 1594.9)</td>
<td>577.5 (2.7, 122653.7)</td>
</tr>
<tr>
<td></td>
<td>[R^2 = 54.1%]</td>
<td>[R^2 = 18.5%]</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Therapeutic index = (pica D<sub>50</sub>)/(neutrophilia D<sub>50</sub>)
TABLE 6

Therapeutic indices of cilomilast, roflumilast and EPPA-1 inhibitors in the ferret.

Ferrets were administered PDE4 inhibitor or vehicle 30 minutes prior to LPS challenge. The animals were observed through the first 2.5 hours following dosing and the incidence of vomiting animals determined. Six hours following LPS challenge, the animals were sacrificed and neutrophil influx quantified.

Where statistically possible to compute, 95% confidence limits are given in parentheses.

<table>
<thead>
<tr>
<th>Neutrophilia</th>
<th>Emesis</th>
<th>Therapeutic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence (% or ratio) or D50 (mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cilomilast, 1 mg/kg</td>
<td>8%</td>
<td>0/3</td>
</tr>
<tr>
<td>Cilomilast, 3 mg/kg</td>
<td>13%</td>
<td>0/5</td>
</tr>
<tr>
<td>Cilomilast, 10 mg/kg</td>
<td>51%</td>
<td>8/8</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>0.41 mg/kg (0.16, 1.0)</td>
<td>50% emesis at 1 mg/kg</td>
</tr>
<tr>
<td></td>
<td>(R^2 = 78.6%)</td>
<td></td>
</tr>
<tr>
<td>EPPA-1</td>
<td>3.2 mg/kg (0.03, 301.8)</td>
<td>9.5 mg/kg (3.3, 27.8)</td>
</tr>
<tr>
<td></td>
<td>(R^2 = 20.2%)</td>
<td>(R^2 = 49.3%)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The therapeutic index of cilomilast could not be directly calculated because there were too few data points for both non-linear regression of neutrophil data and logistic regression of incidence of vomiting animals; however with inhibition of neutrophilia reaching 51% at 10 mg/kg and a 50% incidence rate of vomiting animals below 10 mg/kg, the therapeutic index is below 1.
Figure 2

Graphs showing the effect of different drugs on neutrophil influx and incidence of pica. The graphs compare Pica and Neutrophilia treatments with varying concentrations of Rolipram, Cilomilast, Roflumilast, and EPPA-1.
Figure 3

The figures show the relationship between the percentage inhibition of neutrophil influx and the incidence of vomits for different doses of roflumilast and EPPA-1. The graphs indicate that increasing the dose of roflumilast or EPPA-1 leads to a higher percentage inhibition of neutrophil influx and a higher incidence of vomits.

- **Roflumilast (mg/kg)**: The left graph shows a dose-response curve with vomits (○) and neutrophils (■) plotted against different doses of roflumilast. The graph suggests that at higher doses of roflumilast, the incidence of vomits increases.

- **EPPA-1 (mg/kg)**: The right graph shows a similar trend with vomits (○) and neutrophils (■) plotted against different doses of EPPA-1. The graph indicates that as the dose of EPPA-1 increases, both the percentage inhibition of neutrophil influx and the incidence of vomits also increase.