The Identification of a Novel Phosphodiesterase 4 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), with Improved Therapeutic Index using Pica Feeding in Rats as a Measure of Emotogenicity


ABSTRACT

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 versus 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 lipopolysaccharide (LPS)-induced tumor necrosis factor- \((\text{TNF-}\alpha)\) production by human peripheral blood mononuclear cells was roflumilast (269) > cilomilast (389) > rolipram (269) > EPPA-1 (38) > rolipram (269) > cilomilast (389), and against LPS-induced pulmonary neutrophilia in the rat was EPPA-1 (D\(_{50}\) = 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 (D\(_{50}\) = 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 \(\alpha_2\)-adrenoceptor-mediated anesthenemia in the mouse. The rank order of therapeutic indices derived in the rat [(pica D\(_{50}\)/(neutrophilia D\(_{50}\))] was EPPA-1 (578) > rolumilast (6.4) > cilomilast (1.4) > rolipram (0.15), consistent with the rank order derived in the ferret [(emesis D\(_{50}\)/(neutrophilia D\(_{50}\))]. 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.
nary disease (COPD) and asthma (Spina, 2004; Vignola, 2004). However, the clinical utility of PDE4 inhibitors has been dose-limited by a side-effect profile that includes nausea and emesis (Lipworth, 2005). Second-generation PDE4 inhibitors, such as cilomilast and rolflumilast, 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 with 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 because of 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, because of 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 nonemetic 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, and through peripheral pathways such as serotonin 5HT3 receptors in the gastrointestinal tract (Takeda et al., 1993, 1995a,b). Based on the sensitivity of the pica model to both central and peripheral stimulation, we chose to use the pica model to assess the emetic potential of PDE4 inhibitors in the rat.

This study aimed to validate the use of pica feeding as a surrogate model of emesis in rats, and to 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 > rolflumilast > 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 α2-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 toward a well tolerated, efficacious PDE4 inhibitor for the treatment of respiratory diseases containing a significant inflammatory component.

**Materials and Methods**

**PDE Enzyme Assays.** [3H]cAMP, [3H]cGMP, and phosphodiesterase scintillation proximity assay (SPA) beads were obtained from GE Healthcare, Little Chalfont, Buckinghamshire, UK. Fluorescein-cAMP and IMAP binding reagent were from Molecular Devices (Sunnyvale, CA), and EGTA and magnesium chloride solution were from Sigma-Aldrich (St. Louis, MO). PDE1, PDE2, and PDE3 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 dimethyl sulfoxide (DMSO). Low-volume additions (0.5–2 μl) to assays were performed with use of a liquid-handling robot (Biomek Fx; Beckman Coulter, Fullerton, CA). PDE activity was measured by use of 96-well SPAs and, in addition, for PDE3, PDE4A to PDE4D, PDE5, and PDE6 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 of PDE enzyme in 50 mM Tris-HCl, pH 7.5, 8.3 mM MgCl2, 1.7 mM EGTA, 0.05% (w/v) BSA was preincubated with 2 μl of inhibitor or vehicle (2 μl of DMSO) for 30 min at room temperature. For PDE1 the assay buffer contained, in addition, 4 μl of calf thymus DNA and 1 μM CaCl2, and did not contain EGTA. The assay was initiated by addition of 25 μl of [3H]cAMP (10 nM final concentration: PDE3, -4, and -7 assays) or [3H]cGMP (36 nM final concentration: PDE1, -2, -5, and -6 assays). After 1 h of incubation at room temperature, assays were terminated by addition of 50 μl of phosphodiesterase SPA beads suspended in water (~1 mg/well) and bound radioactive product measured by liquid scintillation counting. For FP assays, 10 μl of 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 preincubated with 0.5 μl of inhibitor or vehicle (0.5 μl of DMSO) for 30 min at room temperature. Assays were initiated by addition of 10 μl of 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 min of incubation at room temperature by addition of 60 μl of IMAP binding reagent (1 in 400 dilution of stock suspension in binding buffer). The FP ratio of parallel to perpendicular light was measured with use of an Analyst or Acquest plate reader (Molecular Devices, Sunnyvale, CA).

**Human PBMC TNF-α Assay.** Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized human blood. Blood was centrifuged on histopaque at 1000 g for 30 min, and PBMCs were collected from the interface, washed by centrifugation (1300 g for 10 min), and resuspended in assay buffer (RPMI 1640 medium containing 10% BSA, 1% L-glutamine and 1% penicillin/streptomycin) at 1 × 106 cells/ml. Cells (5 × 105) were incubated with 0.5 or 1.0 μl of inhibitor (10 μM–1.5 mM in 3-fold dilutions) or vehicle (DMSO, 0.4 or 0.8% final concentration) and LPS (Salmonella typhosa) (1 ng/ml final) in a total assay volume of 125 μl for 20 h at 37°C, 5% CO2. Supernatants were removed and the concentrations of tumor necrosis factor-α (TNF-α) were determined by electrochemiluminescence assay.
Human Whole-Blood TNF-α Assay. Heparinized blood (100 μl) was incubated with 0.5 or 1.0 μl of inhibitor (10 μM−1.5 nM in 3-fold dilutions) or vehicle (DMSO, 0.4 or 0.8% final concentration) for 1 h at 37°C, 5% CO₂. Samples were then stimulated with 50 ng/ml LPS (S. typhosa) in RPMI 1640 medium containing 1% l-glutamine and 1% penicillin/streptomycin (25 μl). After incubation for 20 h at 37°C, 5% CO₂, 50 or 100 μl of physiological saline (0.138% NaCl) was added, and diluted plasma was collected after centrifugation (1300g for 10 min) for TNF-α determination with use of an electrochemiluminescence assay.

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

Animals. Male Lewis rats were obtained from Charles River Breeding Laboratories (Portage, MI) and had access to 5001 Rodent Chow (PMI Nutrition International, Brentwood, MO) and water ad libitum. Male ferrets (Mustela putorius 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., McClean, VA) given three times per week. Male C57BL/6J mice were obtained from Charles River Laboratories (Les Oncins, France) at 6 weeks of age and housed in specific pathogen-free conditions, and given access to Altromin Rat diet (Rieper, Vandoes, Italy) and water ad libitum. All animals were housed on a 12-h lights-on, lights-off cycle and acclimated to their housing facilities for a minimum of 1 week before 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 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 (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). Rolflumilast, rolflumilast-N-oxide, and EPPA-1 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 to 400 g were pretreated orally with the appropriate dose of test compound or vehicle alone (0.5% methylcellulose; Sigma-Aldrich) at a dose volume of 10 ml/kg. Thirty minutes after pretreatment, the rats were exposed to aerosolized 0.1 μg/ml LPS solution from Escherichia coli, serotype 055:B5 (Sigma-Aldrich) at a rate of 4.5 min/for 20 min. At 4 h after LPS exposure the study rats and two naive 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 to 23 ml of BAL fluid. BAL fluid was centrifuged at 500g for 10 min 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 (Thermo Fisher Scientific, Waltham, MA), 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 to 10 rats were placed in individual caged 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. Modelling clay (Languna Clay Company, City of Industry, CA) was cut into a size and shape similar to standard rat chew pellets, and dried at 60°C for 24 h. 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 h before 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 h 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 administered compound or vehicle. Rolipram, cilomilast, roflumilast, and EPPA-1 were suspended in 0.5% methylcellulose (Sigma-Aldrich) and administered orally 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 in a previously tared container, if necessary. This clay weight was added to the un eaten 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 greater than 0.3 g of clay over the average clay consumed by the vehicle-treated rats. This threshold (0.3 g) was established because it was the standard deviation in clay 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 to 2.5 kg were fasted overnight before the study, but allowed free access to water throughout the study. Food was returned to the animals 60 to 90 min after dosing. Thirty minutes before LPS challenge, compound was orally administered to conscious animals (n = 3–8) in 20% Cremophor EL (Sigma-Aldrich), at a dose volume of 1 ml/kg. After dosing, the animals were returned to holding cages, and were continuously observed for up to 2.5 h 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) at a rate of 6 ml/min for 10 min. After the LPS challenge, the animals were returned to their holding cages. Six hours after LPS exposure the animals were killed by overdose of sodium pentobarbital (intraperitoneally) (Vericore Ltd, Dundee, Scotland). BAL was performed through the exposed trachea with 2 × 20 ml of heparinized (10 units/ml) PBS. The BAL samples were centrifuged at 300g for 7 min at room temperature. The supernatant was removed and the resulting cell pellet resuspended in 1 ml of PBS. A slide of the resuspended cells was prepared by cytopsin, and then treated with Leishmans stain (Sigma-Aldrich) for 20 min to allow differential cell counting. The total cells were counted with a Sysmex K1000 automated hematology analyzer (GMI Inc., Ramsey, MN). From these two counts, the total numbers of neutrophils in the BAL samples were determined.

α1-Adrenoceptor-Mediated Anesthesia in the C57BL/6J Mouse. Mice were anesthetized with an intraperitoneal injection consisting of 80 mg/kg ketamine (Merial Animal Health Ltd.) and 10 mg/kg xylazine (Bayer AG, Wuppertal, Germany). Fifteen minutes later, 10 animals per group were treated with rolipram (3 mg/kg s.c.), EPPA-1 (3, 10, and 30 mg/kg s.c.), or vehicle (0.5% methylcellulose) and placed in dorsal recumbency. The time to the recovery of the
righting reflex was used as an endpoint to measure the duration of anesthesia.

Statistical Analysis. For in vitro assays, percentage of inhibition values were generated relative to uninhibited controls. IC_{50} values were determined from concentration-response curves by nonlinear least-squares curve fitting with use of a four-parameter logistic equation in Activity Base (IDBS, Guilford, Surrey, UK).

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

For the experiments discussed in this article, we define the therapeutic index as the ratio of the D_{50} for the pica feeding or emesis endpoints divided by the D_{50} for the neutrophil inhibition response. For both the rat and ferret model experiments, we conducted tests of the (null) hypotheses of “no differences” 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 differences 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 D_{50} values are reported, and 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 D_{50} values and therapeutic indices were initially computed for their log values and then antilogged. 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 with use of the generalized coefficient of determination proposed by Nagelkerke (1991).

The hypothesis test of the equality of all four therapeutic indices was done with use of 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 produced a D_{50} estimate. Because the therapeutic index (TI) is a positive entity, we found a confidence interval for the log TI, and then antilogged it to get a confidence interval for the TI, which must be positive. The confidence interval for the log TI was obtained by the following (approximate) formula:

\[
(\hat{\theta}_N - \hat{\theta}_P) \pm z_{\alpha/2} \sqrt{\hat{\sigma}^2(\hat{\theta}_P) + \hat{\sigma}^2(\hat{\theta}_N)},
\]

which is valid for independent data samples. Here, \(\hat{\theta}_N\) is the maximum likelihood estimate of the log D_{50} for the pica assay and \(\hat{\theta}_P\) is the maximum likelihood estimate of the log D_{50} for the neutrophil assay. Furthermore, \(\hat{\sigma}^2(\hat{\theta}_P)\) is an estimate of the variance of \(\theta_P\) and \(\hat{\sigma}^2(\hat{\theta}_N)\) is an estimate of the variance of \(\theta_N\). (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 on maximum likelihood estimates of the difference of the log TIs and the estimated variances of the log TI estimates. These estimates were obtained from the NLMIXED Procedure using the SAS statistical package (SAS v9.1, SAS Institute, Cary, NC). The NLMIXED Procedure has a general maximum likelihood estimation capability that can be used to compute the needed log D_{50} 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 Fig. 1. The potency of EPPA-1 was assessed against the enzyme activity of the four PDE4 isoforms, PDE4A, PDE4B2B, PDE4C, and PDE4D3A, and also assayed for selectivity against PDE1, -2, -3, -5, -6, and -7. As seen in Table 1, EPPA-1 is a potent inhibitor of PDE4, with IC_{50} 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 IC_{50} 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-cyclopyr-tyl-4-methoxyphenyl)-2-pyrridilone), cilomilast (c-4-cyano-4-[3-(cyclopyr-tyl)-4-methoxyphenyl]-r-1-cyclohexene carboxylic acid), roflumilast (3-cyclopropylmethoxy-4-difluoro-methoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide), and roflumi- last’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 IC_{50} against LPS-induced TNF-α production isolated human PBMCs of 38 nM. Roflumilast and its active N-oxide metabolite were more potent in PBMCs with IC_{50's} of 5 nM and 2 nM, respectively. Cilomilast and rolipram were least potent in isolated PBMCs, exhibiting IC_{50's} of 389 nM and 269 N M, 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 IC_{50} between the isolated PBMC assay and the human whole-blood assay, whereas roflumilast’s active N-oxide and cilomilast showed greater decreases in potency, with differences between the PBMC and whole blood IC_{50's} 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
min before the aerosolized LPS challenge. Four hours after
the LPS challenge BAL was performed on the animals, and
differential cell analysis was performed to quantify neutro-
phils. As shown in Fig. 2, all of the compounds inhibited
LPS-induced pulmonary neutrophilia in a dose-dependent
manner (for ease of interpretation, the percentage 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 R2 values obtained (Table 5) were due to
substantial variation in response at each dose level. None-
theless, statistically significant dose responses were ob-
served for all four of the inhibitors tested. For the sake of
completeness, the normalized data represented in Fig. 2 were
analyzed as well, and comparable D50 values obtained (4.34,
3.13, 0.24, and 0.035 mg/kg for cilomilast, rolipram, roflumi-
last, and EPPA-1, respectively).

**Oral Administration of Rolipram (5 mg/kg) Induces
an Increase in the Consumption of Clay by Lewis Rats.**
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 h in cages with free access to food, dried
clay pellets, and water, but without bedding or additional
environmental enrichment. Immediately before the first dark
cycle after the 72-h acclimation period, compound was ad-
ministered orally to the rats, and clay consumption over the
next 24 h was measured. We initially attempted to validate
the pica model using cisplatin to induce pica under conditions
identical to those reported previously (Takeda et al., 1993),
with the exception that commercially available modeling clay
was used instead of kaolin pellets. Under these conditions we
were able to reproduce the results of Takeda et al., by induc-
ing pica in rats after a 10 mg/kg i.p. 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 h during the 72-h accli-
maion period and again 24 h after rolipram administration.
As seen in Table 3, over the first 24 h of acclimation, the rats
did consume a measurable amount of clay, but between 48
and 72 h the rats consumed almost no clay, indicating that
clay consumption from exploratory behavior had ceased.
After the 72-h acclimation period, the rats were orally dosed
with 5 mg/kg rolipram or vehicle, and after 24 h the clay was
measured again. The rolipram-treated animals consumed
1.2 ± 0.45 g of clay, which was greater than the 0.1 ± 0.07 g
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
dependence 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 rolip-
ram induced pica in rats in a dose-dependent manner as
measured by mean clay consumption. Although the rats con-
sumed clay in a dose-dependent manner as measured by clay
mass consumed, we observed that the rats either engaged in
pica behavior, 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 with use of
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 g over the mean clay consumed by
vehicle-treated animals. This threshold (0.3 g) was chosen
because it was the standard deviation in pica consumption
exhibited by the vehicle-treated control animals across mul-
tiple pilot studies. Because 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

**Table 1** Potencies of EPPA-1 against isoforms of PDE4

<table>
<thead>
<tr>
<th>EPPA-1</th>
<th>IC50 Mean (95% Confidence Limits)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1</td>
<td>&gt;30.2 μM*</td>
<td>2</td>
</tr>
<tr>
<td>PDE2</td>
<td>&gt;30.2 μM*</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, 162.2) 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>

*Replication within a single experiment.

**Table 2** Potencies of PDE4 inhibitors in attenuation of TNF-α production by human PBMCs and whole blood

<table>
<thead>
<tr>
<th>EPPA-1</th>
<th>IC50 Mean (95% Confidence Limits)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38.0 (34.1, 42.4)</td>
<td>5.0 (4.6, 5.5)</td>
<td>4</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td></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>N</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>
clay in a dose-dependent manner, with the binary logistic regression dose-response model exhibiting a statistically significant slope ($p = 0.0054$). Similarly to rolipram, incidence data were used to generate dose-response curves for roflumilast, cilomilast, and EPPA-1 by use of logistic regression analysis. Rolipram and roflumilast induced pica with $D_{50}$s of 0.495 and 1.57 mg/kg, respectively, whereas cilomilast and EPPA-1 were less potent, exhibiting $D_{50}$s of 6.41 mg/kg and 24.26 mg/kg, respectively (Fig. 2 and Table 5). Similar to the rat neutrophilia data, although 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 with use of the generalized coefficient of determination proposed by 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 $D_{50}$ value generated in the pica model by the $D_{50}$ 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 ther-

### TABLE 3
Clay consumed by rats after administration of rolipram

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Treatment</th>
<th>Clay Consumption</th>
<th>Mean</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>1</td>
<td>None</td>
<td>1.2</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>1</td>
<td>None</td>
<td>0.4</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>1</td>
<td>None</td>
<td>0.1</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>96 h</td>
<td>1</td>
<td>Vehicle</td>
<td>0.2</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Rolipram, 5 mg/kg</td>
<td>1.2</td>
<td>0.45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 4
Rolipram induces pica in a dose-dependent manner

<table>
<thead>
<tr>
<th>Clay Consumed</th>
<th>Mean</th>
<th>S.E.M.</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.08</td>
<td>0.037</td>
<td>0/5</td>
</tr>
<tr>
<td>Rolipram</td>
<td>0.016 mg/kg</td>
<td>0.10</td>
<td>0.470</td>
</tr>
<tr>
<td></td>
<td>0.08 mg/kg</td>
<td>0.20</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>0.4 mg/kg</td>
<td>0.40</td>
<td>0.176</td>
</tr>
<tr>
<td></td>
<td>2 mg/kg</td>
<td>0.52</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>1.30</td>
<td>0.470</td>
</tr>
</tbody>
</table>

Fig. 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 30 min before LPS challenge. Four hours after the LPS challenge, the animals were sacrificed and BAL was performed. Cells in the BAL samples were counted, and leukocyte differentials were conducted to determine the percentage 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-h acclimation period and before the 12-h dark phase, the clay baseline weight was measured and the animals dosed with PDE4 inhibitors or vehicle. Twenty-four hours after administration of compound or vehicle, the clay was reweighed. Rats consuming >0.3 g 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.
The therapeutic indices of rolipram, roflumilast, cilomilast, and EPPA-1 in the rat

| Neutrophilia | Pica | Therapeutic Index
|-------------|-----|-----------------
| mg/kg       |     |                 
| Rolipram    | 3.34 (1.5, 7.6) $R^2 = 54.5\%$ | 0.495 (0.08, 3.2) $R^2 = 51.9\%$ | 0.15 (0.02, 1.2) |
| Roflumilast | 0.24 (0.10, 0.58) $R^2 = 81.5\%$ | 1.57 (0.56, 4.4) $R^2 = 60.1\%$ | 6.42 (1.7, 24.5) |
| Cilomilast  | 4.54 (0.05, 408.7) $R^2 = 24.5\%$ | 6.41 (0.67, 61.8) $R^2 = 32.6\%$ | 1.4 (0.01, 217.6) |
| EPPA-1      | 0.042 (0.001, 1.2) $R^2 = 54.1\%$ | 24.26 (0.37, 1594.9) $R^2 = 18.5\%$ | 577.5 (2.7, 122653.7) |

$^a$ Therapeutic index = (pica D$_{50}$/neutrophilia D$_{50}$)

The therapeutic indices of rolipram, roflumilast, cilomilast, and EPPA-1 were calculated using the normalized percentage change from control mean rat neutrophilia data. The resulting p values for the rolipram, cilomilast, and roflumilast comparisons were 0.00013, 0.0191, and 0.0195, respectively. Because 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 percentage change from control mean rat neutrophilia data gave the following: for rolipram ($D_{50}$ = 3.13 mg/kg), cilomilast ($D_{50}$ = 4.34 mg/kg), roflumilast ($D_{50}$ = 0.24 mg/kg), and EPPA-1 ($D_{50}$ = 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, whereas the therapeutic index comparison for EPPA-1 versus 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. 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 min before the aerosolized LPS challenge, and 6 h later, animals were sacrificed and BAL fluid was collected for differential cell analysis. The incidence of vomiting animals was observed and recorded for the first 2.5 h after 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 $D_{50}$ 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 $D_{50}$ (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, three of six (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 $D_{50}$ of 3.2 mg/kg, and induced vomiting with a $D_{50}$ of 9.5 mg/kg, resulting in a therapeutic index of 3.0. Analysis of the normalized neutrophil data gave the same $D_{50}$ value for roflumilast as that generated via analysis of the raw neutrophil numbers (0.41 mg/kg), and $D_{50} = 2.6$ 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 percentage of inhibition relative to vehicle-treated controls, can be seen in Fig. 3.

**TABLE 6**
Therapeutic indices of cilomilast, roflumilast, and EPPA-1 in the ferret

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

$^a$ The therapeutic index of cilomilast could not be directly calculated because there were too few data points for both nonlinear 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 at less than 10 mg/kg, the therapeutic index is below 1.
Fig. 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 min before LPS exposure. Animals were observed for vomiting for the first 2.5 h after dose administration. Six hours after the LPS challenge, the animals were sacrificed and BAL was performed. Cells in the BAL samples were counted, and leukocyte differentials were conducted to determine for each dose the percentage inhibition of neutrophil influx relative to the vehicle control group.

EPPA-1 Does not Attenuate α2-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 treated with rolipram (3 mg/kg s.c.) or EPPA-1 (3, 10, and 30 mg/kg s.c.) 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 Fig. 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. Although 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 in vitro assays (LPS-induced TNF-α production by human PBMCs and whole blood) and the 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-α production by human monocytes and dendritic cells (Hatzelmann and Schudt, 2001), LPS-induced TNF-α production by human whole blood (Hatzelmann and Schudt, 2001; Draheim et al., 2004), and ovalbumin-induced leukocyte and TNF-α 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.

Fig. 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 ± S.E.M. * p < 0.05; **, p < 0.01; ***, p < 0.001 relative to the vehicle control group.
To measure the emetogenicity of the PDE4 inhibitors in the same species in which anti-inflammatory potency was being determined, we used rat pica feeding. Pica feeding is defined as the consumption of non-nutritive substances as a result of gastrointestinal distress, and in rodents seems 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, 1995a,b). 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, whereas EPPA-1 at up to 30 mg/kg had no effect. Using a panel of PDE4 inhibitors, Robichaud et al. (1999, 2002) demonstrated previously that reduction of xylazine/ketamine-induced anesthesia in the mouse positively correlates with induction of vomiting in an emetic species, the ferret.

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 a 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 with cilomilast.

Various therapeutic index models have been used 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 antiemetic effects of inhibitors can be tested directly, rather than indirectly, on a surrogate of emesis, which may not fully represent the mechanisms underlying emesis. Although testing PDE4 inhibitors in emetic species is associated with the aforementioned advantage, these models often necessitate measuring, in addition to vomiting, behavioral correlates of emesis (i.e., salivation, retching) that 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 to increase confidence in their enhanced efficacy/tolerability. EPPA-1 exemplifies such a compound, demonstrating a nonemetic phenotype in the α2-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 versus emetic potential of PDE4 inhibitors. Evidence supports the existence of two noninterconvertible 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, whereas 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 (Souness and Rao, 1997; Torphy, 1998; Burnouf and Pruniaux, 2002).

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 et al., 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 (i.e., exhibiting a range of anti-inflammatory or emetic activities) and the mechanism of interest (i.e., 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), whereas 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 among the inhibitors (IC50 = 65 and 0.3 nM, respectively) whereas EPPA-1 and cilomilast exhibited intermediate potencies (IC50 = 35 and 15 nM, respectively). After an intravenous 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. With the use of 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.

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References


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