

The Pharmacology of Two Novel Long-Acting Phosphodiesterase 3/4 Inhibitors, RPL554 [9,10-Dimethoxy-2(2,4,6-trimethylphenylimino)-3-(*N*-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one] and RPL565 [6,7-Dihydro-2-(2,6-diisopropylphenoxy)-9,10-dimethoxy-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one]

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Received December 5, 2005; accepted May 4, 2006

ABSTRACT

The pharmacology of two novel, trequinsin-like PDE3/4 inhibitors, RPL554 [9,10-dimethoxy-2(2,4,6-trimethylphenylimino)-3-(*N*-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one] and RPL565 [6,7-dihydro-2-(2,6-diisopropylphenoxy)-9,10-dimethoxy-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one], has been investigated in a number of *in vitro* and *in vivo* assays. Electrical field stimulation-induced contraction of guinea pig superfused isolated tracheal preparations was significantly inhibited by RPL554 (10 μ M) and RPL565 (10 μ M) (percentage control; 93 ± 1.2 and 84.4 ± 2.7 , respectively). Contractile responses were suppressed for up to 12 h after termination of superfusion with RPL554 demonstrating a long duration of action. RPL554 and RPL565 inhibited, in a concentration-dependent manner, lipopolysaccharide-induced tumor necrosis factor α release from human monocytes [IC_{50} ; 0.52 μ M (0.38–0.69) and 0.25 μ M (0.18–0.35), respectively] and proliferation of human mono-

nuclear cells to phytohemagglutinin [IC_{50} ; 0.46 μ M (0.24–0.9) and 2.90 μ M (1.6–5.4), respectively]. The inhibitory effect of these drugs *in vitro* was translated into anti-inflammatory activity *in vivo*. RPL554 (10 mg/kg) and RPL565 (10 mg/kg) administered orally significantly inhibited eosinophil recruitment following antigen challenge in ovalbumin-sensitized guinea pigs. Likewise, inhalation of dry powder containing RPL554 by conscious guinea pigs (25% in micronized lactose) 1.5 h before antigen exposure significantly inhibited the recruitment of eosinophils to the airways. Exposure of conscious guinea pigs to inhalation of dry powder containing RPL554 (2.5%) and RPL565 (25%) in micronized lactose significantly inhibited histamine-induced plasma protein extravasation in the trachea and histamine-induced bronchoconstriction over a 5.5-h period. Thus, RPL554 and RPL565 are novel, long-acting PDE 3/4 inhibitors exhibiting a broad range of both bronchoprotective and anti-inflammatory activities.

It is now recognized that the second messenger cAMP plays a pivotal role in the regulation of cell function, and

cyclic nucleotide phosphodiesterases (PDEs) are a diverse family of enzymes (1–11) responsible for the degradation of cAMP and are therefore potential drug targets for modulating cell function (Essayan, 2001). In the context of lung diseases, it is of particular interest that inhibiting the PDE3 and PDE4 isoenzymes can modulate the function of a variety

This work was supported by Vernalis Plc.
Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.105.099192.

ABBREVIATIONS: PDE, phosphodiesterase; MKS492, 8-amino-3,7-dihydro-7-(2-methoxyethyl)-1,3-dimethyl-1*H*-purine-2,6-dione; RPL554, 9,10-dimethoxy-2(2,4,6-trimethylphenylimino)-3-(*N*-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one; RPL565, 6,7-dihydro-2-(2,6-diisopropylphenoxy)-9,10-dimethoxy-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one; K-H, Krebs-Henseleit solution; EFS, electrical field stimulation; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; EB, Evans blue; TNF, tumor necrosis factor; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; PHA, phytohemagglutinin; ANOVA, analysis of variance; CDP840, *R*-(+)-4-[2-(3-cyclopentylloxy-4-methoxyphenyl)-2-phenylethyl]pyridine; RP73401, 3-(cyclopentylloxy)-*N*-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide; AWD 12-281, *N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide; Cl-930, 3(2*H*)-pyridazinone-4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-5-methylmonohydrochloride; NVP-ABE171, 4-(8-benzo[1,2,5]oxadiazol-5-yl-[1,7]naphthyridin-6-yl)-benzoic acid; YM976, 4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-*d*]pyrimidin-2(1*H*)-one; Ro 20-1724, 4-3(Butoxy-4-methoxybenzyl)imidazolidin-2-one.

of cells in the lung including airway smooth muscle, mast cells, macrophages, eosinophils, neutrophils, and vascular endothelial cells (Spina, 2004). Indeed, a number of selective PDE4 inhibitors have entered clinical development for the treatment of lung diseases, including roflumilast and cilomilast (for review, see Lipworth, 2005), and although they seem to exhibit clinical efficacy in patients with asthma or chronic obstructive pulmonary disease, they have so far been reported to be poor bronchodilators (Bateman et al., 2003; Grootendorst et al., 2003). This is likely to be due to the importance of PDE3, rather than PDE4, in mediating airway smooth muscle relaxation, confirmed by the observation that PDE3 inhibitors cause bronchodilatation in subjects with asthma (Bardin et al., 1998). Thus, a combined PDE3/PDE4 inhibitor would have the ability to relax airway smooth muscle and suppress inflammation (Torphy, 1998; Essayan, 2001), and this has led to the development of combined PDE3/4 inhibitors for the treatment of airway disease.

To date, dose-dependent side effects, particularly gastrointestinal disturbance, have limited the use of PDE4 inhibitors (Lipworth, 2005), and there has for a long time been concerns about inhibiting PDE3 systemically (Packer et al., 1991). Traditionally, systemic side effects of treatments for lung disease have been reduced by administering drugs by inhalation, and it would seem sensible to consider this approach also for the development of novel PDE inhibitors. Indeed, administration of the PDE3 inhibitor olprinone (Myou et al., 1999) or MKS492 (Bardin et al., 1998) in asthmatics provided significant bronchodilator activity without systemic side effects, and experimentally, delivery of PDE4 directly to the lung is not associated with gastrointestinal disturbances (Kuss et al., 2003). Furthermore, successful chemical strategies have been developed to improve the duration of action of short-acting drugs administered by inhalation, e.g., the modification of the short-acting β_2 -adrenoceptor agonist salbutamol to give the longer acting salmeterol. The combined use of inhaled long-acting β_2 -adrenoceptor agonists with glucocorticosteroids provides asthmatic subjects with a treatment strategy that gives long-lasting symptomatic and anti-inflammatory treatment. However, recent concerns regarding the safety of regular treatment with long-acting β -agonists (Martinez, 2005) and continued concerns about the safety of glucocorticosteroids provide a rational basis for the development of novel single molecular entities with both bronchodilator and anti-inflammatory activity. Among known PDE inhibitors, we have reported that trequinsin demonstrated a significantly longer duration of action against contraction of airway smooth muscle than a number of PDE4-selective inhibitors (Spina et al., 1998). In the present study, we describe the pharmacology of two novel long-acting trequinsin analogs, RPL554 and RPL565, in a variety of in vitro and in vivo assays of relevance to airway disease.

Materials and Methods

Animals. All in vivo experiments were performed with male Dunkin-Hartley guinea pigs (250–500 g). All experiments were conducted in accordance with the United Kingdom Animal Scientific Procedures Act, 1986 and were approved by the ethics committee of Kings College, London. Guinea pigs were housed with free access to standard food and water before the experimental procedure.

Inhibition of PDE3 and PDE4. The inhibitory effects of RPL554 on isolated PDE3 from human platelets and on PDE4 obtained from

human neutrophils were determined by Pneumolabs Ltd. using previously described methods (Giembycz et al., 1996).

Superfusion of Isolated Guinea Pig Tracheal Muscle. Superfusion of guinea pig tracheal rings was performed according to a previously described method (Coleman et al., 1996). In brief, guinea pigs were sacrificed by cervical dislocation, and the trachea were excised and cut into rings. Each tracheal ring was opened by sectioning the ring opposite the smooth muscle, and the tissue was then suspended between two platinum electrodes under 1-g tension and superfused at a rate of 3 ml/min with Krebs-Henseleit solution (K-H) (37°C, 5% CO₂, 95% O₂) containing the cyclooxygenase inhibitor, indomethacin (Sigma Chemical, Poole, Dorset, UK) (5 μ M). The tracheal preparations were then equilibrated for 40 min before commencement of electrical field stimulation (EFS) delivered as a 10-s train of square wave pulses at 3 Hz, 0.1-ms duration, and 30 V, generated every 100 s by means of a physiological square wave stimulator.

Ten minutes thereafter, the trachea was superfused with K-H solution containing RPL554 or RPL565 (0.1, 1, or 10 μ M) at a rate of 0.26 ml/min until maximal inhibition of contraction was observed, at which point superfusion with drug was terminated. The tissues were then superfused with drug-free K-H solution, and tension was monitored for a further 5 to 24 h.

Lung Function Studies. Male Dunkin Hartley guinea pigs (200–400 g) were anesthetized with urethane (1.5 g/kg), and the trachea were cannulated. The cannula was attached to a pneumotachograph that was in turn connected to a Validyne pressure transducer (± 2 cm H₂O). Changes in airflow were measured using a Lung Function Recording system (version 3; Mumed Systems, London, UK) and displayed in real time on a personal computer. The flow signal was integrated to give a measure of tidal volume.

A cannula was inserted into the thoracic cavity between the third and fifth ribs and connected to a Validyne pressure transducer (± 20 cm H₂O). The positive side of the pressure transducer was connected to the side of the pneumotachograph proximal to the animal to obtain a measure of transpulmonary pressure (difference between mouth and thoracic pressure). The lung function parameter total airway resistance (R_L; centimeters of water per liter per second) was derived from each measure of flow, tidal volume, and transpulmonary pressure by the method of integration. The jugular vein and carotid artery were cannulated for i.v. administration of drug and measurement of blood pressure, respectively.

Conscious guinea pigs were placed in a "volumetric" chamber (800 ml) and exposed at regular intervals to inhalation of RPL554 or RPL565 (2.5 or 25% in micronized lactose) or micronized lactose alone. The micronized compounds were delivered into the chamber using a dry powder delivery device (Penn Century, Philadelphia, PA). Approximately 3 to 5 mg of drug-lactose blend or lactose was loaded into the device and delivered to the chamber by firing 3 ml of air through the delivery device. A fine mist of dust particles was achieved, which the conscious animal inhaled. A total of nine deliveries were made over a 3-min interval. Thereafter, assessment of airway responsiveness to histamine (1–8 μ g/kg i.v.), 1.5, 2.5, 3.5, 4.5, and 5.5 h after exposure to RPL554 and RPL565 was undertaken.

Inflammatory Studies. Male Dunkin Hartley guinea pigs (200–300 g) were immunized according to a previously described technique (Seeds et al., 1995). In brief, animals were injected i.p. with ovalbumin (grade V; 40 μ g/ml in aluminum hydroxide). Eighteen days later, animals were dosed with RPL554 and RPL565 (10 mg/kg; orally dissolved in PEG 200). One hour later, they were challenged with an aerosol of ovalbumin (100 μ g/ml) in an exposure chamber for 1 h at a rate of approximately 10 ml/h ovalbumin solution. In another series of experiments, conscious animals were required to inhale micronized RPL554 (25% in micronized lactose) 1.5 h before antigen challenge as described above.

Twenty four hours after antigen challenge, animals were killed by an overdose of anesthetic, and bronchoalveolar lavage (BAL) was performed. Cytospins of the lavage fluid were prepared using a

Shandon Cytospin 2 centrifuge. Slides were stained with Lendrum's stain, and differential cell counts were performed. In some experiments, the supernatant was collected for determination of eosinophil peroxidase (EPO) levels using a previously published technique (Banner et al., 1995).

Vascular Permeability Studies. Conscious guinea pigs (200–500 g) were placed in a volumatic chamber (800 ml) and exposed at regular intervals to inhalation of micronized RPL554 (2.5%, 3 puffs/min, 3 min) or RPL565 (25%, 3 puffs/min, 3 min) as described above. The animals were left for 1 h before being anesthetized with urethane, as described above, for the measurement of airway edema. Animals were assessed 1.5 h after dry powder inhalation of test drug by the extravasation of Evans blue (EB) dye, which binds to serum albumin. The jugular vein was cannulated, and EB dye (30 mg/kg) was administered i.v. After 2 min, saline or histamine (4 µg/kg) was administered i.v., and 5 min later, the thoracic cavity was opened, and the vena cava was clamped and then cut below the level of the clamp. Saline (5 ml/min) was infused with the aid of a roller pump via the jugular vein for a period of 7 min before the trachea and lungs were removed and placed in saline. The trachea was cleared of connective tissue, weighed and cut into 3-mm rings. EB dye was extracted by incubating the tissue in 2 ml of 100% formamide at 37°C for 16 to 18 h.

The amount of EB dye extracted from tissue was measured spectrophotometrically using an Anthos Labtec HT3 96-well plate reader at 620 nm. The amount of dye extracted from the tissue was quantified by interpolation from a standard curve of EB dye concentrations (1.95–62.5 µg/ml). EB content of each tissue was expressed as nanograms per wet weight tissue.

Experiments with Isolated Human Mononuclear Cells and Neutrophils. Permission for these studies was obtained from the ethics committee of King's College London. Mononuclear cells and neutrophils were isolated from the peripheral blood of healthy volunteers by density dependent centrifugation of the blood with Histopaque-1077 according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). After aspiration of the plasma, the mononuclear cell fraction (250g, 5 min, 20°C) was washed twice with modified Hanks' balanced salt solution and used in TNF α and proliferation assays.

In the case of the neutrophils, cells were separated by gentle centrifugation (35g, 15 min), and the supernatant was centrifuged for a further 7 min (188g) in modified Hanks' balanced salt solution. The cell pellet was resuspended, red blood cells were lysed before centrifugation (1000g, 1 min), and the resulting cell pellet was used in cAMP assays.

cAMP Levels in Neutrophils. Neutrophils isolated from healthy subjects were resuspended into normal Hanks' solution (2.5×10^6 cells/ml) and transferred to 96-well plates in the absence or presence of RPL554 and RPL565 (0.1–10 µM) for 30 min in a final volume of 250 µl. Each treatment was performed in triplicate. Plates were then centrifuged (1000g, 1 min), the supernatants were discarded, and the remaining cells were lysed with 0.1 M HCl containing 0.1% Triton X-100. The supernatants were then collected following a further centrifugation step and stored at –70°C until assayed for cAMP according to the manufacturer's instruction (cAMP, Enzyme Immunoassay Kit; Assay Designs Inc., Ann Arbor, MI).

Inhibition of TNF α Release from Monocytes Stimulated by Lipopolysaccharide. The mononuclear cells were re-suspended at a concentration of 10^6 cells/ml in RPMI 1640 containing fetal calf serum (1%) and were seeded into 96-well plates (10^5 cells/well) and allowed to adhere (5% CO₂, 37°C, 2 h). Medium containing nonadherent cells was aspirated, and adherent cells were washed once with fresh medium. Adherent cells were then incubated (5% CO₂, 37°C) with fresh RPMI 1640 containing 1% fetal calf serum in the absence and presence of lipopolysaccharide (LPS; 10 ng/ml) with PDE inhibitors (RPL554, RPL565, or siguazodan, 0.001 nM–10 µM) or vehicle (0.1% DMSO). After 18 h, cell supernatants were removed by centrifugation and frozen until required. TNF α concentrations

were determined from the supernatants using a sandwich enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Genzyme, Cambridge, MA). Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium dye exclusion method.

Inhibition of Proliferation of Mononuclear Cells Stimulated by Phytohemagglutinin. Cells were resuspended at a concentration of 10^6 cells/ml in SMEM containing glutamine (2 mM), nonessential amino acids (1% v/v), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and HEPES (20 mM) and were seeded into 96-well plates (10^5 cells/well) and allowed to adhere (5% CO₂, 37°C, 2 h). Medium containing nonadherent cells was aspirated, and adherent cells were washed once with fresh medium. Adherent cells were incubated (5% CO₂, 37°C) with phytohemagglutinin (PHA) (2 µg/ml) in the presence of PDE inhibitors (RPL554 and RPL565; 0.001 nM–10 µM) or vehicle (0.1% DMSO). Twenty four hours later, [³H] thymidine (0.1 µCi) was added to each well and incubated with the cells for a further 24-h period. Cells were then harvested onto glass fiber filters using a cell harvester (ICN Flow, Buckinghamshire, UK) and counted in a scintillation counter.

Statistical Analysis. Data are expressed as mean \pm S.E.M. The time taken to cause 50% inhibition of the maximal inhibition of tracheal muscle was defined as the onset time (OT₅₀). Likewise, the time taken for the contractile response to recover by 50% of the predrug value was defined as the recovery time (RT₅₀). If the contractile response to EFS failed to reach 50% of the predrug response after 5.5-h superfusion, an RT₅₀ value of >240 min was recorded.

In some cases, data were analyzed using a Kruskal-Wallis test (nonparametric ANOVA), and differences between mean values were compared using Dunnett's test (Prism, version 2.01; GraphPad Software Inc., San Diego, CA) and considered significant for $P < 0.05$. In other cases, data were analyzed using ANOVA, and differences between mean values were compared using a Student's t test with a suitable multiple comparison test (e.g., Bonferroni correction; Prism, version 2.01) and considered significant for $P < 0.05$.

Drugs. K-H solution was composed of: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂·2H₂O, 1.2 mM MgSO₄·7H₂O, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11.1 mM D-glucose. All reagents were obtained from Sigma Chemical. CDP840 was a kind gift of Celltech UK (Berkshire, UK). RPL554 and RPL565 (Fig. 1) were synthesized by Tocris Cookson (Southampton, UK) under the guidance of Dr. Alec Oxford.

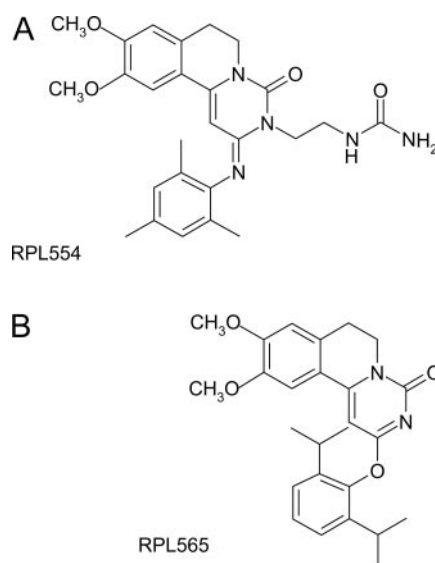


Fig. 1. Chemical structure of RPL554 (A) and RPL565 (B).

Results

The Inhibitory Actions of RPL554 and RPL565 on Isolated PDE3 and PDE4. The inhibitory activities of RPL554 and RPL565 on purified PDE3 (isolated from human platelets) and PDE4 (isolated from human neutrophils) are summarized in Table 1. In further experiments, we measured the levels of intracellular cAMP produced by human neutrophils. Basal intracellular levels of cAMP (picomoles per milliliter per 2.5×10^6 cells) were significantly ($P < 0.05$, ANOVA) increased following incubation of human neutrophils ($n = 3$ subjects) with increasing concentration of RPL554 or RPL565 (Fig. 2).

The Inhibitory Actions of RPL554 and RPL565 on EFS-Induced Contractions of Isolated Guinea Pig Tracheal Smooth Muscle. Reproducible contractile responses induced by EFS were maintained up to 6 h (Fig. 3). RPL554 (0.1, 1, and 10 μM) inhibited in a concentration-dependent manner the contractile response to EFS, with the highest concentration abrogating the contractile response for the duration of the study. RPL565 also inhibited the contractile response to EFS in a time- and concentration-dependent manner, but it was approximately 10 times less active than RPL554, and its inhibitory effect at 10 μM was submaximal (Fig. 4). Their onset times and recovery times are shown in Table 2. In further experiments, we demonstrated that RPL554 (10 μM) suppressed EFS-induced contractile responses over at least a 12-h time period after termination of perfusion with this drug (Fig. 5).

The Bronchoprotective Effect of Inhaled RPL554 and RPL565. Histamine induced a dose-dependent bronchoconstriction that was reproducible over a 5.5-h time period in anesthetized guinea pigs (Fig. 6). Bronchoconstriction in response to histamine was significantly reduced in animals following inhalation of RPL554 (Fig. 6). This inhibition was maintained for 5.5 h with the 25 and 2.5% dry powder formulations of RPL554 (Figs. 6 and 7). RPL565 (25%) also caused a significant but lesser inhibition of histamine-induced bronchoconstriction (Fig. 7). In control animals, the change in resistance following i.v. administered histamine (4 $\mu\text{g}/\text{kg}$) over baseline R_L was 232 ± 18 cm $\text{H}_2\text{O}/\text{l/s}$ ($n = 21$).

TABLE 1

IC_{50} values (nanomolar) for RPL554 and RPL565 for purified human platelet PDE3 and purified human neutrophil (PDE4)

Compound	PDE3	PDE4	Ratio
RPL554	0.4	1479	3440
RPL565	107.2	1195	11

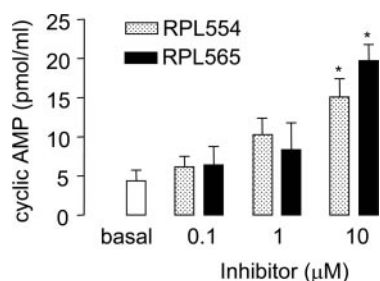


Fig. 2. Bar graph showing cAMP levels in neutrophils in response to increasing concentrations of RPL554 (light stippled bars) and RPL565 (closed bars). Bar, mean of cAMP levels from three subjects (performed in triplicate); vertical lines, S.E.M. *, $P < 0.05$ compared with basal levels.

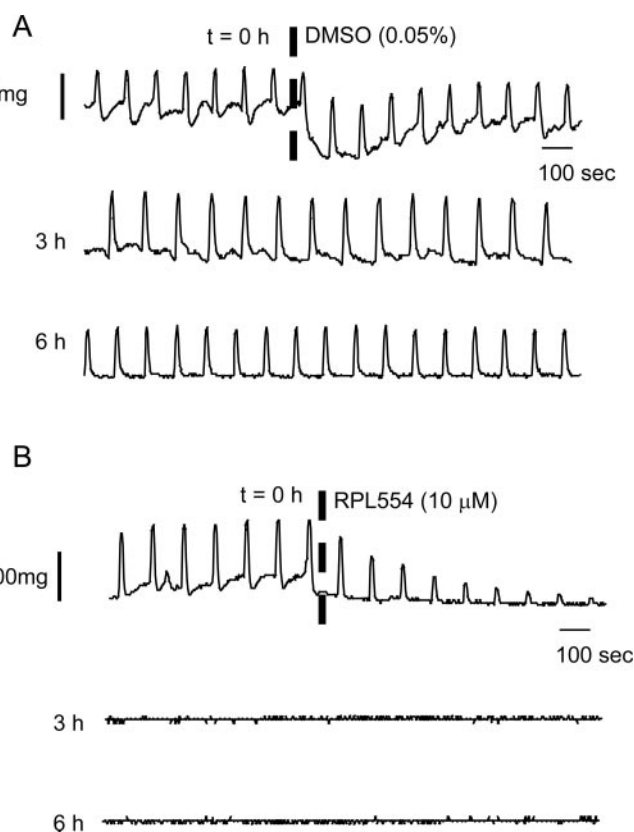


Fig. 3. Representative line drawing of contractile responses to electrical field stimulation (3 Hz) in guinea pig superfused isolated trachea over a 24-h period. Dotted line, commencement of superfusion with 0.05% DMSO for a period of 30 min, after which the superfusion with DMSO was terminated (A) and RPL554 (10 μM) (B), where the dotted line denotes commencement of superfusion with the drug for a period of 45 min, after which the superfusion with RPL554 was terminated and continued with drug-free K-H solution.

The Effect of Inhalation of RPL554 or RPL565 on Mean Arterial Blood Pressure. Mean arterial blood pressure was maintained over a 3-h period in anesthetized guinea pigs. Inhalation of RPL554 (25%) significantly reduced mean arterial blood pressure over a 4.5-h period by approximately 60% of control (Fig. 8). Lower concentrations of RPL554 (2.5%) also reduced the mean arterial blood pressure compared with lactose controls. RPL565 had no significant effect on mean arterial blood pressure compared with lactose controls (Fig. 8).

The Effect of RPL554 and RPL565 on Airway Edema. Histamine (4 $\mu\text{g}/\text{kg}$ i.v.) caused marked extravasation of Evans blue dye into tracheal mucosa and submucosa. Inhalation of RPL554 (2.5%) or RPL565 (25%) both significantly reduced extravasation of the dye (Fig. 9).

The Effect of RPL554 and RPL565 on Antigen-Induced Eosinophilia. In ovalbumin-immunized guinea pigs, there was no significant increase in total cell numbers in BAL fluid taken 24 h following challenge with ovalbumin (100 $\mu\text{g}/\text{ml}$). This concentration of antigen represents a mild antigen load, which does not cause significant apnea in conscious guinea pigs. There was however, a significant increase in the percentage of eosinophils in BAL fluid 24 h following antigen challenge compared with sham-immunized guinea pigs (Fig. 10A; $P < 0.05$). There was also a significant reduction in the percentage of monocytes in BAL fluid (data not

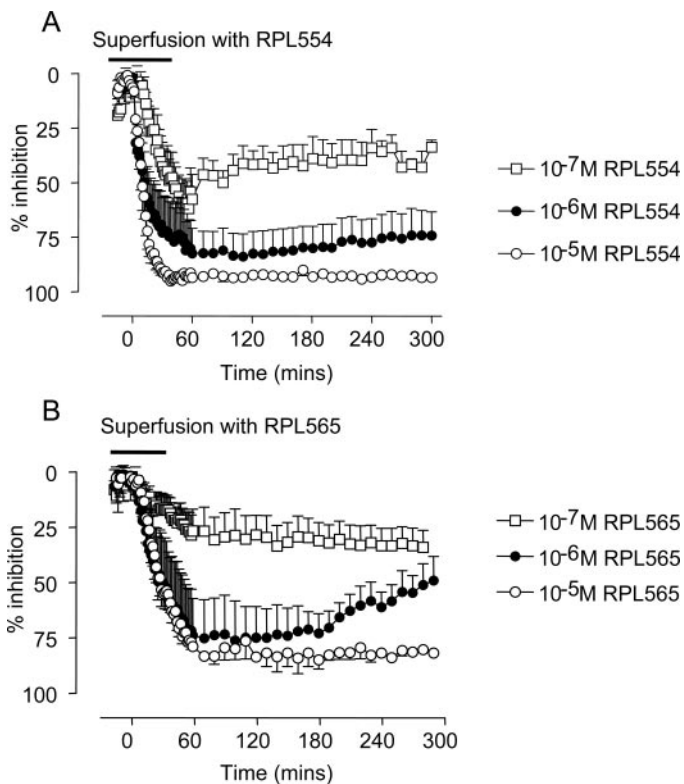


Fig. 4. Inhibition of contractile responses to electrical field stimulation (3 Hz) in superfused isolated guinea pig trachea to RPL554 (open circles, 10 μ M, $n = 5$; closed circles 1 μ M, $n = 6$; and open squares, 0.1 μ M, $n = 4$) (A) or RPL565 (open circles, 10 μ M, $n = 3$; closed circles 1 μ M, $n = 5$; and open squares, 0.1 μ M, $n = 4$) (B). Vertical bars, S.E.M. Closed bar, superfusion with inhibitor.

TABLE 2

Onset (OT_{50}) and recovery time (RT_{50}) values (minutes) for RPL554 and RPL565 (0.1–10 μ M) in isolated guinea pig perfused trachea

	Inhibition	OT_{50}	RT_{50}	n
	%	min		
RPL554				
0.1 μ M	55.8 \pm 12.1	28.4 \pm 5.5	N.D.	4
1.0 μ M	89.6 \pm 3.7	14.8 \pm 3.4	>240	6
10.0 μ M	93.0 \pm 1.2	16 \pm 0.4	>240	5
RPL565				
0.1 μ M	26.7 \pm 8.2	22.0 \pm 4.8	N.D.	4
1.0 μ M	66.3 \pm 15.1	32.0 \pm 7.4	>168 \pm 29.4	5
10.0 μ M	84.4 \pm 2.7	24.9 \pm 5.0	>240	3

Results expressed as mean \pm S.E.M. N.D., not determined.

shown, $P < 0.001$) but no change in the percentage of neutrophils (data not shown, $P > 0.05$). RPL554 (10 mg/kg) but not RPL565 (10 mg/kg) administered orally 1 h before antigen challenge caused a significant reduction of eosinophil infiltration into the lung (Fig. 10A; $P < 0.05$) following antigen challenge in ovalbumin-sensitized animals compared with sham-immunized guinea pigs.

Inhalation of RPL554 (25%) in conscious animals 1.5 h before antigen exposure also significantly attenuated the antigen-induced eosinophilia seen 24 h after antigen exposure (Fig. 10B). This was also associated with a significant inhibition of the increase in eosinophil peroxidase levels in BAL fluid following allergen challenge (Fig. 11).

The Inhibitory Effect of PDE Inhibitors on TNF α Release from Human Mononuclear Cells. RPL554 and

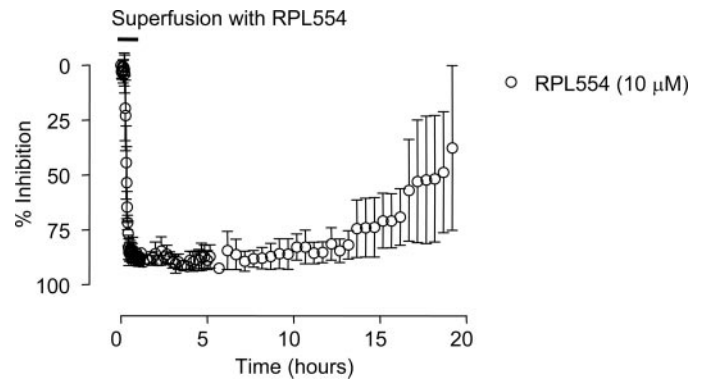


Fig. 5. Inhibition of contractile responses to electrical field stimulation (3 Hz) in superfused isolated guinea pig trachea to RPL554 (10 μ M, $n = 4$) over a 24-h time period. Vertical bars, S.E.M.

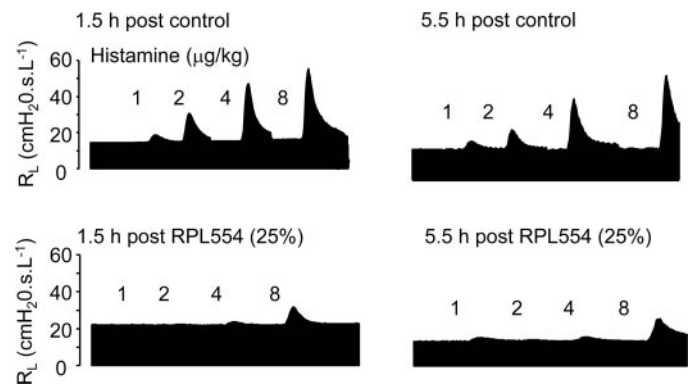


Fig. 6. Representative drawing of measurements of total lung resistance in anesthetized and ventilated guinea pigs at 1.5 and 5.5 h following inhalation of lactose or RPL554 (25%) by conscious animals. Increasing doses of histamine were administered at regular intervals (1, 2, 4, and 8 μ g/kg).

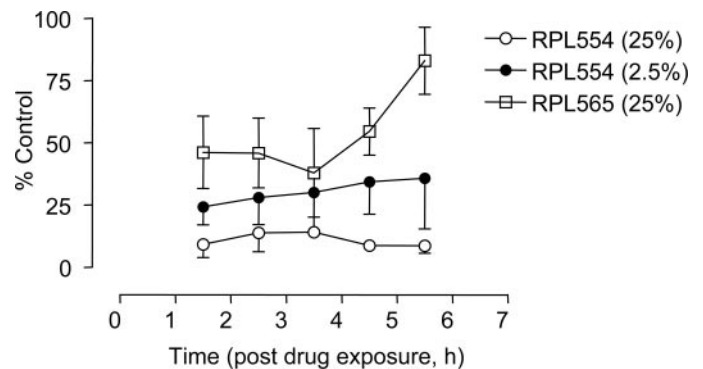


Fig. 7. Bronchoconstrictor response of anesthetized, ventilated guinea pigs to i.v. histamine (4 μ g/kg) expressed as percentage of control response at various times following inhalation of RPL554 by conscious guinea pigs (open circles, 25%, $n = 2-3$; closed circles, 2.5%, $n = 3-4$) or RPL565 by conscious guinea pigs (open squares, 25%, $n = 3-10$). Vertical lines, S.E.M.

RPL565 both caused concentration-dependent inhibition of TNF α production from human monocytes stimulated with LPS ($n = 6$; Fig. 12A), and IC_{50} values and 95% confidence limits for these compounds were calculated (Table 3). The effect of siguazodan, a PDE3-selective inhibitor, and CDP840, a PDE4-selective inhibitor, on TNF α release from human monocytes was also evaluated for comparison (Table 3). The PDE3-selective inhibitor, siguazodan, was relatively ineffective against TNF α release, but the PDE4 inhibitor,

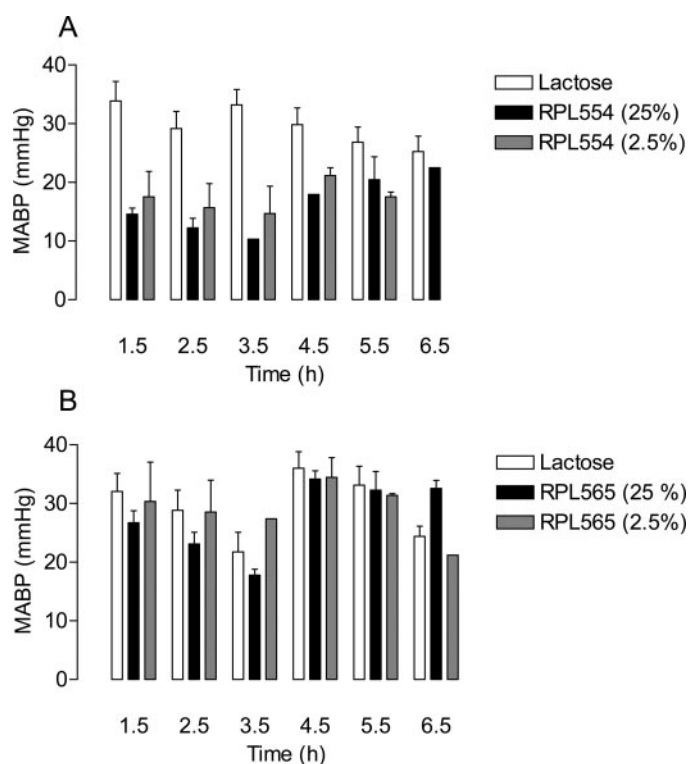


Fig. 8. Mean arterial blood pressure (millimeters of mercury) in anesthetized, ventilated guinea pigs following inhalation of lactose (open columns, $n = 6-11$) (A) or RPL554 (closed column, 25%, $n = 2-3$; stippled column, 2.5%, $n = 3-4$) or lactose (open columns, $n = 4-8$) or RPL565 (closed column, 25%, $n = 3-10$, stippled column, 2.5%, $n = 1-3$) (B). Vertical lines, S.E.M.

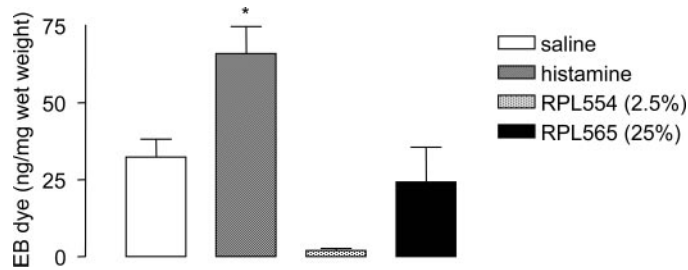


Fig. 9. Plasma albumin leakage in guinea pig trachea expressed as EB dye (nanograms per milligram wet weight) to i.v. administered saline (open column, $n = 6$) or histamine ($4 \mu\text{g}/\text{kg}$) in anesthetized guinea pigs following inhalation of lactose (heavy stippled column, $n = 6$), RPL554 (light stippled column, 2.5%, $n = 2$), or RPL565 (closed column 4, 25%, $n = 4$) in conscious guinea pigs. Vertical lines, S.E.M.

CDP840, potently inhibited this response (Fig. 12A). At all the concentrations examined, RPL554 and RPL565 did not reduce cell viability, as assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium dye exclusion technique (data not shown).

The Inhibitory Effect of PDE Inhibitors on PHA-Induced Proliferation of Mononuclear Cells. RPL554 and RPL565 both caused concentration-dependent inhibition of the proliferation of human mononuclear cells stimulated with PHA (Fig. 12B). The IC_{50} values and 95% confidence limits for these compounds are indicated in Table 3.

Discussion

We have demonstrated that by altering the two-substituent group in the pyrimido-isoquinoline nucleus of the mixed

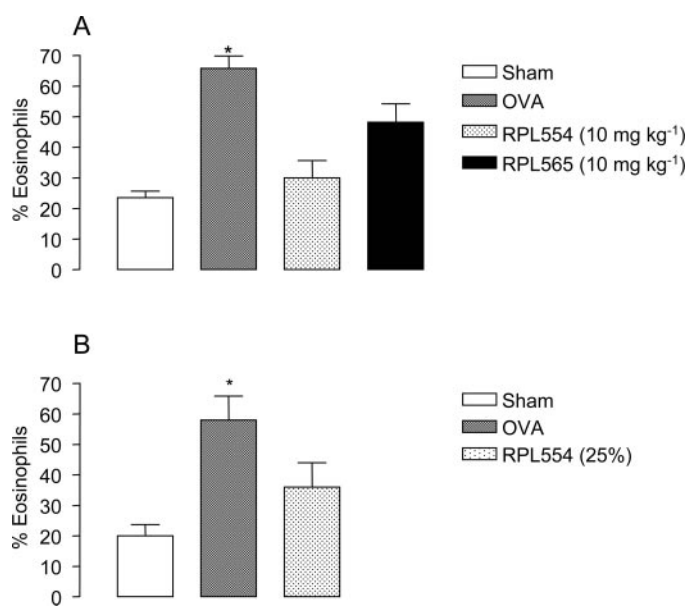


Fig. 10. Percent eosinophils recovered in bronchoalveolar lavage fluid, 24 h following antigen challenge of sham-immunized (open column), ovalbumin-immunized guinea pigs (heavy stippled column), and ovalbumin-immunized guinea pigs dosed with RPL554 (light stippled column; 10 mg/kg) or RPL565 (closed column, 10 mg/kg) (A). RPL554 (10 mg/kg) and RPL565 (10 mg/kg) were given by the oral route; and sham-immunized (open column), ovalbumin-immunized guinea pigs (heavy stippled column), and ovalbumin-immunized guinea pigs after inhalation of RPL554 (25%; light stippled column) (B). Vertical lines, S.E.M. of between four and five observations.

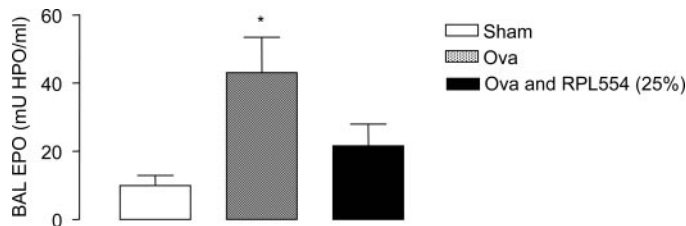


Fig. 11. Bar graph representing the levels of EPO in bronchoalveolar lavage fluid, 24 h following antigen challenge of sham-immunized (open column, $n = 4$), ovalbumin-immunized guinea pigs (heavy stippled column, $n = 5$), and ovalbumin-immunized guinea pigs after inhalation of RPL554 (25%; closed column, $n = 5$). Vertical lines, S.E.M.

PDE3/4 inhibitor, trequinsin, we could obtain potent, long-acting molecules assessed against cholinergic nerve-mediated contractile responses in superfused isolated guinea pig bronchus. In particular, two compounds, RPL554 and RPL565, were further investigated in a number of in vivo and in vitro pharmacological assays.

Biochemical investigations have documented PDE1-5 and PDE1-7 in human airway smooth muscle (Smith et al., 2003). Airway smooth muscle relaxation is observed following inhibition of PDE3 or PDE4 in canine (Torphy et al., 1988; Torphy and Undem, 1991), guinea pig trachea (Raeburn et al., 1993; Spina et al., 1995), and human airway preparations (de Boer et al., 1992; Rabe et al., 1995), which is primarily thought to be mediated by PDE3 (Cortijo et al., 1993). RPL554 and RPL565 both caused a concentration- and time-dependent inhibition of contractile responses elicited by EFS. Of major importance was the finding that these compounds had a considerably longer duration of action against EFS-induced contractile responses than other PDE4 inhibitors (Spina et al., 1998), more reminiscent of the long-acting β_2 -

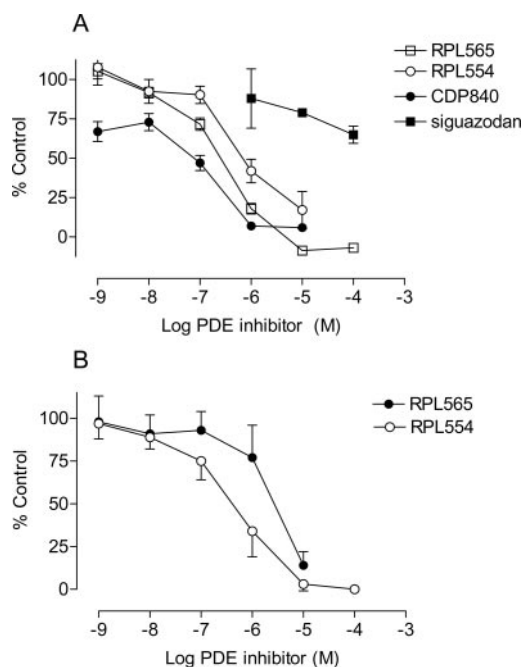


Fig. 12. Concentration-dependent inhibition of TNF α production in human monocytes stimulated with LPS following exposure to RPL554 (open circles, $n = 6$), CDP840 (closed circles, $n = 6$), RPL565 (open squares, $n = 6$), and siguazodan (closed squares, $n = 3$). A and B, concentration-dependent inhibition of proliferation of human mononuclear cells stimulated with PHA following exposure to RPL554 (open circles, $n = 6$) and RPL565 (open squares, $n = 6$). Each point represents S.E.M.

TABLE 3

IC₅₀ values for various RPL545 and RPL565 against TNF α production by human monocytes stimulated with LPS and proliferation of human mononuclear cells with PHA

Values are expressed as geometric mean together with 95% confidence interval of n subjects.

Compound	IC ₅₀	n
TNF α production		
RPL554	0.52 μ M (0.38–0.69)	5
RPL565	0.25 μ M (0.18–0.35)	6
Siguazodan ^a	>100 μ M	3
CDP840 ^b	92 nM (50–167)	6
Proliferation		
RPL554	0.46 μ M (0.239–0.897)	6
RPL565	2.90 μ M (1.56–5.41)	6

^a K_i values for siguazodan against PDE3 (3 μ M) and PDE4 (>100 μ M) (Nicholson and Shahid, 1994).

^b K_i values for CDP840 against PDE3 (>100 μ M) and PDE4 (12 nM) (Hughes et al., 1996).

selective agonist, salmeterol (Coleman et al., 1996). Very few studies have attempted to investigate the duration of action of PDE inhibitors, although we have shown previously that a wide range of PDE4 inhibitors generally had a short duration of action against spasmogen-induced contraction of guinea pig isolated bronchus, the exception being trequinsin (Spina et al., 1998). One other study has also reported on the relatively short duration of activity of the PDE4 inhibitors, rolipram and RP73401, on airway smooth muscle relaxation (Naline et al., 1996). It is of interest therefore that RPL554 (10 μ M) caused almost complete inhibition of the contractile response to electrical field stimulation that was maintained 12 h after superfusion with the drugs had ceased. Furthermore, both RPL554 and RPL565, when delivered by dry powder, significantly attenuated histamine-induced bronchoconstriction in the guinea pig, and in the case of RPL554, this

effect lasted for a period of at least 5 h. This is the first study to demonstrate a long duration of action of a dual PDE 3/4 inhibitor in vivo and is consistent with the long duration of action of these compounds in vitro.

A variety of selective PDE4 inhibitors, including AWD 12-281 (Kuss et al., 2003), cilomilast (Underwood et al., 1998), roflumilast (Bundschuh et al., 2001), and the mixed PDE3/4 inhibitor zardaverine (Underwood et al., 1994), have been reported to significantly attenuate acute bronchospasm induced by antigen in sensitized guinea pigs. Likewise, CDP840 (Gozzard et al., 1996), but not rolipram, attenuated allergen-induced bronchoconstriction in the rabbit. Furthermore, the PDE3 inhibitor CI-930 (Howell, 1993) and to a lesser extent, siguazodan (Underwood et al., 1994), inhibited the allergen-induced bronchoconstrictor response in the guinea pig, whereas the PDE5 inhibitor, zaprinast, was without effect (Howell, 1993). However, the effect of PDE4 inhibitors on bronchospasm induced by allergen in these animal models is most likely due to inhibition of IgE/IgG-dependent mediator release from inflammatory cells, rather than functional antagonism of airway smooth muscle shortening. Thus, it is likely that the airway smooth muscle actions of RPL554 and RPL565 are dependent on the ability of these molecules to inhibit PDE3 rather than PDE4.

A similar long duration of action of RPL554 and RPL565 was also noted in a number of in vivo assays of airways inflammation. Airway wall edema and plasma protein extravasation can be induced by a variety of inflammatory mediators including leukotrienes, histamine, bradykinin, sensory neuropeptides, and platelet-activating factor administered either i.v. or applied topically to the mucosal surface (Evans et al., 1989). Both RPL554 and RPL565 when delivered as a dry powder significantly attenuated plasma protein extravasation induced by histamine in the guinea pig. This is in agreement with previous studies demonstrating that the PDE4-selective inhibitors NVP-ABE171 and YM976 inhibited antigen-induced edema in the airways (Aoki et al., 2001; Tigani et al., 2003). Together, these studies demonstrated the effectiveness of this class of drug on edema formation in the airways. In contrast, the PDE3 inhibitors milrinone (Ortiz et al., 1996) and siguazodan (Raeburn et al., 1993) have been shown to be ineffective against airway vascular leakage induced by PAF and antigen, suggesting this anti-inflammatory effect of RPL554 and RPL565 is probably via inhibition of PDE4, at the level of vascular endothelial cells. The relative lack of effect of RPL565 on systemic blood pressure when administered via the inhaled route, coupled with its demonstrable anti-inflammatory effect in vivo and in vitro, suggests that it is possible to synthesize novel molecules that demonstrate both bronchodilator and anti-inflammatory activity coupled with long effect duration, while lacking cardiovascular side effects. It is of interest, therefore, that the PDE3 inhibitor olprinone administered by the inhaled route (Myou et al., 1999) or MKS492 administered orally (Bardin et al., 1998) provided significant bronchodilator activity without systemic side effects in asthmatic subjects.

We have also shown that RPL554 and RPL565 can significantly inhibit TNF α release from LPS-stimulated human monocytes. For comparison, the PDE4 inhibitor CDP840, but not the PDE3 inhibitor, siguazodan, inhibited TNF α release from these cells. Furthermore, PDE4 inhibitors and to a lesser extent, PDE3 inhibitors, attenuated endotoxin or LPS-

induced TNF α production in monocytes (Barnette et al., 1998; Hatzelmann and Schudt, 2001). This is consistent with the view that PDE4 and not PDE3 is responsible for modulating cytokine release from monocytes (Essayan, 2001).

Both RPL554 and RPL565 inhibited mononuclear cell proliferation in a concentration-dependent manner. cAMP PDE activity in the soluble and particulate fraction of enriched T lymphocytes was inhibited by the PDE4 inhibitor Ro 20-1724 and the PDE3 inhibitor, CI-930 (Robicsek et al., 1991), and both PDE3 and PDE4 have been confirmed in membrane and cytosolic compartments of human CD4⁺ and CD8⁺ T lymphocytes (Giembycz et al., 1996). On closer inspection, PDE4A, PDE4B, PDE4D, and PDE7 were described in CD4⁺ and CD8⁺ human T lymphocytes (Giembycz et al., 1996). Functional studies have shown that PDE4 and to a lesser extent PDE3 inhibitors suppress proliferation of human T-lymphocytes in response to mitogen, anti-CD3, or allergen (Robicsek et al., 1991; Giembycz et al., 1996), cytokine generation (Crocker et al., 1996; Giembycz et al., 1996; Hatzelmann and Schudt, 2001), and mononuclear cell proliferation (Banner et al., 1995). The PHA- or anti-CD3-induced proliferation of CD4⁺ and CD8⁺ T-lymphocytes was inhibited in a concentration-dependent manner by rolipram, roflumilast, and cilomilast, consistent with the ability of PDE4 inhibitors to elevate intracellular cAMP in these cells (Giembycz et al., 1996; Hatzelmann and Schudt, 2001). We also confirmed in our in vitro experiments that both RPL554 and RPL565 elevated intracellular levels of cAMP in human neutrophils. Although both PDE3 and PDE4 can contribute toward the metabolism of this second messenger in cells, it seems that the regulation of intracellular cAMP levels within the vicinity of PDE4 is more critical in suppressing neutrophil degranulation (Jones et al., 2005).

RPL554 and RPL565 also significantly attenuated eosinophil recruitment 24 h after antigen challenge following oral administration and dry powder inhalation. This extends previous work showing that the PDE4 inhibitors rolipram, roflumilast, cilomilast, and AWD 12-281 all attenuated allergen-induced pulmonary eosinophilia in the guinea pig (Banner et al., 1995; Underwood et al., 1998; Bundschuh et al., 2001; Kuss et al., 2003). In addition, we have demonstrated that RPL554 reduced the activation of eosinophils as assessed by the retention of EPO with eosinophils recovered in BAL. Our data are consistent with previous studies showing that rolipram (Banner et al., 1995; Hughes et al., 1996) also attenuated the activation of eosinophils recruited to the lung following allergen exposure, as assessed by measurements of EPO contained in and/or secreted by the eosinophil. In conclusion, we have reported that RPL554 and RPL565 are novel, potent, long-acting mixed PDE3/4 inhibitors that exhibit both bronchodilator and anti-inflammatory activity as assessed by a variety of in vitro and in vivo assays.

Acknowledgments

RPL554 and RPL565 were synthesized by Tocris Cookson under the guidance of Alec Oxford based on an invention of David Jack (Oxford and Jack, 2004). We are grateful to Pneumolabs and M. Giembycz for the work on the isolated PDEs.

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