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Pharmacological activity of PF-904 in guinea pig in vivo, and on human bronchus and neutrophils in vitro

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Abstract

The effects of PF-904 (4-amino-1-ethyl-6-methylpyrazino[2,3-c][1,2,6]thiadiazine 2,2-dioxide), a pyrazinothiadiazine derivative, were examined in guinea-pig airways in vivo, in human isolated bronchus and human polymorphonuclear leukocytes. PF-904 (12.5–200 mg/kg, intraduodenal) reduced bronchoconstriction in response to histamine, arachidonic acid, platelet-activating factor (PAF) and methacholine. PF-904 (50–200 mg/kg) prevented PAF-induced airways hyperreactivity and inhibited antigen-induced bronchoconstriction, airway microvascular leakage and eosinophil lung accumulation, but antigen-induced airways hyperresponsiveness was not reduced. PF-904 (1 μM–1 mM) produced complete inhibition of spontaneous ($-\log EC_{50} = 3.57 \pm 0.04$; $n = 10$) and histamine-stimulated tone ($-\log EC_{50} = 3.66 \pm 0.07$; $n = 10$) of human isolated bronchus. Glibenclamide (10 μM) or precontraction with KCl (80 mM) did not impede PF-904-induced bronchial relaxation. PF-904 inhibited cyclic AMP ($-\log IC_{50} = 2.83 \pm 0.25$; $n = 8$) and cyclic GMP ($-\log IC_{50} = 2.90 \pm 0.21$; $n = 8$) phosphodiesterase activity in human bronchus. The activity of type IV phosphodiesterase was inhibited by PF-904 ($-\log IC_{50} = 3.43 \pm 0.11$; $n = 3$). PF-904 also inhibited superoxide release by *N*-formylmethionyl–leucyl–phenylalanine-stimulated human polymorphonuclear leukocytes, but the maximal effect was approx. 50% of that produced by rolipram (10 μM). This profile of activities of PF-904 suggests that this compound has potential therapeutic value as an anti-asthma drug. © 1997 Elsevier Science B.V.

Keywords: PF-904; Airway; Airway, hyperreactivity; Eosinophil infiltration; Microvascular leakage; Bronchus, human, isolated; Phosphodiesterase activity; Neutrophils, human; Superoxide anion generation; (Guinea pig)

1. Introduction

Asthma is a multifaceted condition that has manifestations including bronchospasm, non-specific bronchial hyperreactivity, inflammatory cell (especially eosinophils) infiltration and microvascular leakage in the airways (Barnes et al., 1988). Although asthma is currently treated with effective bronchodilator and anti-inflammatory medication, the search for new anti-asthma drugs continues (Molimard and Advenier, 1993). PF-904 (4-amino-1-ethyl-6-methylpyrazino[2,3-c][1,2,6]thiadiazine 2,2-dioxide) is a novel chemical entity which was selected from a series of pyrazynothiadiazine derivatives (Goya et al., 1992) because it showed bronchodilator and antibronchospastic effects in a pharmacological screening program. These findings suggested that PF-904 may have therapeutic value in asthma.

The present study evaluated the pharmacological activity of PF-904 on in vivo guinea-pig models of acute bronchoconstriction in response to different spasmogens, on platelet-activating factor PAF-induced airway hyperreactivity, and antigen-induced airway hyperreactivity, microvascular leakage and eosinophil infiltration. Additional experiments were carried out to assess the in vitro activity of PF-904 as relaxant of human bronchus, as inhibitor of cyclic nucleotide phosphodiesterase activity in human bronchus, and as inhibitor of superoxide release by human neutrophils. These experiments aimed to explore potential bronchodilator and anti-inflammatory activity of this compound of possible relevance to therapy of asthma. To evaluate the therapeutic potential of this new drug, the

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effects of PF-904 were compared to those of reference drugs.

2. Materials and methods

2.1. Activity in in vivo guinea-pig models

2.1.1. Assessment of the effects against spasmogen-induced bronchoconstriction in normal, anaesthetized guinea pigs

Male Dunkin–Hartley guinea pigs (Interfauna Iberica S.A., Barcelona, Spain) ranging in weight from 300 to 500 g were used. They were housed six per cage under standard conditions (22 ± 2°C, 40–60% humidity and a 12 h light (7:00–19:00)-dark cycle), and used after adaptation for at least one week with free access to food (Bantin and Kingman) and water. All animals used in the studies involving non-parenteral routes of administration were fasted overnight, but water was allowed prior to experiments. All protocols were in accordance with internationally accepted principles in the care and use of experimental animals (E.E.C. Council Directive 86/609, OJ L358/1-28; Dec. 12, 1986; ratification by Spain as Royal Decree in 1987). Guinea pigs were anaesthetized with urethane (2 g/kg, i.p.) and the trachea, jugular vein and duodenum were cannulated. Additional urethane was given as required to maintain anaesthesia. Body temperature was maintained at 37–38°C. Suxamethonium (5 mg/kg, i.m.) was administered to prevent spontaneous respiration. Animals were ventilated using a Harvard pump at 60 strokes/min with a stroke volume of 1 ml/100 g body weight. Pulmonary inflation pressure was recorded at the side arm of the tracheal cannula by means of a pressure transducer connected to a polygraph (Letica, Barcelona, Spain). After stabilization of the animals, changes in airway resistance were induced by i.v. injection of histamine (7.5 μg/kg), methacholine (17.5 μg/kg), arachidonic acid (1 mg/kg) or PAF (150 ng/kg). These doses were selected from preliminary experiments to produce a bronchoconstrictor response ranging from 40 to 64% of the maximum response obtained by tracheal occlusion. Ten minutes after two similar responses to the spasmogen had been obtained, a single dose of PF-904 or theophylline was given intraduodenally in a volume of 2 ml/kg. Bronchoconstrictor challenges were repeated at 15, 30, 60, 120 and 180 min after administration of the test compound except for PAF that was only injected at 30 min. Test compound activity was calculated by comparing the increase in pulmonary inflation pressure in response to each bronchoconstrictor before and after compound administration and expressing the difference as percentage change. Dose–response curves for PF-904 (12.5–200 mg/kg) and theophylline (6.25–100 mg/kg) were made and the 50% inhibitory dose (ID_{50}) with its 95% confidence limits was calculated by linear regression.

2.1.2. Assessment of the effects against PAF-induced airways hyperresponsiveness in normal, anaesthetized guinea pigs

Guinea pigs were anaesthetized as reported before and the trachea, carotid artery, jugular vein and duodenum were cannulated for measurement of pulmonary inflation pressure and systemic blood pressure for agonist challenge and drug administration, respectively. The animals were maintained anaesthetized with supplemental doses of urethane as required, they were paralyzed with suxamethonium (5 mg/kg, i.m.), ventilated with room air at 60 strokes/min with a stroke volume of 1 ml/100 g body weight, and pulmonary inflation pressure recorded as indicated above. The animals were challenged with histamine (4 μg/kg, i.v.) at 10 min intervals. Two stable responses to histamine were obtained 45 and 35 min before the start of a 60 min i.v. infusion of PAF (600 ng/kg over 1 h, infused at three increasing rates according to Robertson and Page, 1987). Five min after cessation of the infusion, the pulmonary inflation pressure values were similar to their pre-infusion values and the guinea pigs were re-challenged with histamine. PF-904 (50 or 100 mg/kg), theophylline (25 or 50 mg/kg) or their vehicle (carboxymethylcellulose, 0.5%) was given intraduodenally 30 min prior to the PAF infusion. The results are expressed as the difference (expressed in mmHg) between the responses to histamine obtained pre- and post-infusion of PAF or its vehicle.

2.1.3. Assessment of the effects against antigen-induced bronchoconstriction and airway microvascular leakage in sensitized guinea pigs

The guinea pigs were sensitized as previously reported (Ortiz et al., 1996). In brief, two i.p. injections of 0.5 ml of sterile saline containing 20 μg ovalbumin and 100 mg of $Al(OH)_3$ were given 24 h apart. Non-sensitized animals were treated similarly except that they did not receive the antigen. Experiments were carried out 20 to 28 days after sensitization. The animals were anaesthetized and prepared for pulmonary inflation pressure recording as described before. Ten minutes after connection to the ventilator, the animals were given an intraduodenal dose of PF-904 (100 mg/kg), theophylline (50 mg/kg), rolipram (1 mg/kg) or drug vehicle in a volume of 2 ml/kg. Drug or vehicle was followed 30 min later by the injection of Evans blue dye (20 mg/kg, i.v.). One minute later, antigen was administered (5 mg/ml, 30 breaths) and 5 min after antigen inhalation the animals were hyperinflated with twice the tidal volume by manually blocking the outflow of the ventilator and the animals were then killed by exsanguination. Aerosols were generated from a DeVilbiss ultrasonic nebulizer. Animals pretreated with drug vehicle and then receiving 30 breaths of the antigen vehicle (saline) were used as sham controls. The effect of drugs in animals receiving antigen vehicle was also tested. At the end of the

experiments, the lower portion of the trachea, main bronchi, proximal and the distal intrapulmonary airways, as well as samples from oesophagus and urinary bladder were collected and the Evans blue dye was extracted and quantified as previously described (Rogers et al., 1989). No antigen-induced extravasation was found in oesophagus and bladder (data not shown), demonstrating the absence of extravasation outside the airways.

2.1.4. Assessment of the effects against antigen-induced airway hyperresponsiveness and eosinophil infiltration in sensitized guinea pigs

Preparation of guinea pigs and experimental protocol were as outlined previously (Ortiz et al., 1996). Briefly, 20–28 days after sensitization, conscious animals were placed in a chamber (approx. 4 l) which was connected to the output (approx. 8–10 ml/h) of a DeVilbiss ultrasonic nebulizer. The nebulizer chamber was filled with an ovalbumin (0.1% in saline) or saline solution. The duration of the antigen challenge was 60 min. Twenty-four hours after exposure to the aerosol, the animals were anaesthetized and instrumented as noted above and airways reactivity was determined (histamine 2–25 μg/kg, i.v.). The animals were then killed with an overdose of urethane and their lungs were lavaged with 6 aliquots of 10 ml each of saline with heparin 10 IU/ml. Total fluid recovery exceeded 85%. All suspensions were concentrated by low speed centrifugation, and the cell pellet was resuspended. Total cell counts were made in a haemocytometer. Differential counts were made from cytospin preparations stained with May–Grünwald–Giemsa. PF-904 (200 mg/kg) and theophylline (100 mg/kg) were given orally 30 min before the antigen exposure. Methylprednisolone (37.5 mg/kg, i.p.) given at 24 and 2 h before the antigen was used as reference compound.

2.2. Activity in human isolated bronchus

2.2.1. Pharmacomechanical study

Lung tissue was obtained from patients who were undergoing surgery for lung carcinoma. None of the patients had a history of asthma. After the resection of one or more lung lobes, a piece of macroscopically normal tissue was cut free and submerged in physiological salt solution (PSS, composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.1) at 4°C for transport to the laboratory. In the laboratory, parts of the bronchus were dissected free from parenchymal lung tissue and preparations were cut (3–4 mm length × 3–4 mm internal diameter) as previously described (Cortijo et al., 1993). Preparations were stored in PSS, equilibrated with 5% CO_2 in O_2 at 4°C until used. Experiments were routinely completed within 24 h of storage. Bronchial rings were suspended on tissue hooks in 10 ml organ baths containing PSS, gassed with 5% CO_2 in O_2 at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Grass FTO3) and isometric tension changes were recorded by means of a data acquisition program for isolated tissues Proto5®. The preparations were equilibrated for 60–90 min with changes of bath PSS every 20 min before any pharmacological intervention occurred. A load of 2 g was maintained throughout the equilibration period and the resting tone was stable at the end of this period.

In all experiments, the human bronchi were first contracted with acetylcholine (1 mM) and relaxed with theophylline (3 mM) to obtain maximal contraction and relaxation values (Advenier et al., 1991). After washout and recovery of resting tension, the relaxant and anti-spasmogenic effects of PF-904 were investigated. In a first group of experiments, cumulative concentration–response curves for PF-904 (1 μM–1 mM) or theophylline (1 μM–1 mM) were obtained in preparations with either spontaneous tone or tone induced by histamine (0.1 mM). Only one curve was made with each preparation. Experiments were terminated by the addition of theophylline (3 mM). In a second group of experiments, two consecutive concentration–response curves for histamine (1 nM–1 mM) were obtained, with the second curve made in the absence (time-matched control) or presence of either PF-904 or theophylline (each at 0.1 mM). Additional experiments were carried out to compare the relaxant effect of PF-904 with that of cromakalim, a K_{ATP}-channel activator (Small et al., 1993). Curves were made for PF-904 (1 μM–1 mM) or cromakalim (0.1–30 μM) in the absence or presence of glibenclamide (10 μM), an antagonist of K_{ATP} channels (Small et al., 1993). In separate experiments, PF-904 (1 mM)- or cromakalim (10 μM) was used to relax KCl (10 mM)-contracted bronchus and glibenclamide (10 μM) was added to assess its capacity to reverse the relaxation. In other experiments, the ability of PF-904 (1 μM–1 mM) to relax bronchus contracted with either 10 mM or 80 mM of KCl was compared with that of cromakalim (0.1–30 μM). Another set of experiments was done to examine the ability of PF-904 (0.1 mM) to potentiate the concentration–response curves to isoprenaline (10 nM–3 μM) or sodium nitroprusside (0.1 μM–1 mM). Theophylline (0.1 mM), a non-selective inhibitor of phosphodiesterase activity (Raeburn and Advenier, 1995), was used for comparison. Changes in force were measured from isometric recordings and expressed in g. The molar concentration required to produce 50% (EC_{50}) of the maximal contraction or relaxation was calculated by linear regression from the individual concentration–response curves.

2.2.2. Biochemical study

The activity of PF-904 to inhibit cyclic nucleotide phosphodiesterase activity in human bronchi was assayed as previously outlined (Cortijo et al., 1993). In brief, individual human bronchi (1–2 g) were homogenized for 60 s with an Ultraturrax at 9000 rpm in 5 volumes of ice-cold buffer A (20 mM Bis Tris, pH 6.5, containing 50

mM sodium acetate, 5 mM β-mercaptoethanol and 50 mM phenylmethylsulphonylfluoride). The homogenate was centrifuged at 15 000 × *g* for 10 min and the clear supernatant was filtered through 0.22 μm Millex filters. Phosphodiesterase activity was assayed following the procedure of Thompson and Strada (1984). Inhibition assays for PF-904 (10 μM–3 mM) or theophylline (10 μM–3 mM) were run in duplicate at 30°C for 20 min at a substrate (cyclic AMP or cyclic GMP) concentration of 1 μM. The IC_{50} values were obtained by non-linear regression using GraphPad software.

Additional experiments aimed to determine the activity of PF-904 against the isoenzyme type IV from human bronchial tissue. Supernatants were injected into a Mono-Q HR 5/5 column (Pharmacia) attached to an FPLC chromatography system and phosphodiesterases were eluted against a linear sodium acetate gradient from 50 to 1000 mM. A major peak of phosphodiesterase activity eluting at 0.9 M sodium acetate was found. This peak was identified in previous studies as containing mainly phosphodiesterase IV activity (Cortijo et al., 1993) although phosphodiesterase III may co-elute (Cortijo et al., 1996). Therefore, phosphodiesterase IV activity was assessed in these fractions in the presence of 10 μM SKF94120 (5-(4-acetimidophenyl)pyrazin-(1H)-one) as previously reported (Cortijo et al., 1996). Inhibition assays for PF-904 and theophylline were run on these fractions as indicated above.

2.3. Activity in human polymorphonuclear leukocytes

Human polymorphonuclear leukocytes were isolated from the blood of healthy donors obtained with informed consent as previously described (Cortijo et al., 1996). Human polymorphonuclear leukocytes were obtained by dextran-sedimentation and subsequent centrifugation on Ficoll-plaque. The purity of human polymorphonuclear leukocytes was about 95% and viability was greater than 97%. Cells were stored at 4°C in Krebs–Hepes buffer. Chemiluminescence of 10^5 human polymorphonuclear leukocytes in an assay volume of 0.5 ml was monitored using the luminol reaction, and measured in a Perkins Elmer LS-50 with *N*-formylmethionyl–leucyl–phenylalanine used as stimulant of superoxide release (Schudt et al., 1991).

2.4. Analysis of data

Data are presented as mean ± S.E.M. except for ID_{50} values in vivo that are given with 95% confidence limits. Differences between groups were assessed by analysis of variance (ANOVA) and when overall significance was found, a further analysis was done with the Bonferroni *t*-test. Statistical significance was assumed when $P < 0.05$.

2.5. Drugs and chemicals

PF-904 (4-amino-1-ethyl-6-methylpyrazino[2,3-c][1,2,6]thiadiazine 2,2-dioxide) was synthesized at Lab. Prodesfarma (Barcelona). Acetylcholine, arachidonic acid, bovine serum albumin (fraction 5), cyclic AMP, cyclic GMP, Evans blue dye, formamide, *N*-formylmethionyl–leucyl–phenylalanine, heparin, histamine dihydrochloride, methylcholine chloride, methylprednisolone, ovalbumin (Grade V, fatty acid-free), platelet-activating factor (PAF), theophylline and urethane were all purchased from Sigma-Aldrich Química (Madrid, Spain) or Merck S.A. (Barcelona, Spain). Rolipram and SKF94120 were gifts from Lab. Almirall (Barcelona, spain), and cromakalim from Smith-Kline Beecham (Guildford, UK). Suxamethonium chloride was obtained from Wellcome (Madrid, Spain). [8-^{3}H]-adenosine 3′:5′-cyclic monophosphate and [8-^{3}H]-guanosine 3′:5′-cyclic monophosphate were from Amersham (Amersham, UK). Drugs for oral or intraduodenal administration were prepared in carboxymethylcellulose (0.5% in distilled water). Drugs given intravenously or used for in vitro experiments were dissolved in physiological salt solutions or in dimethylsulphoxide (DMSO) as appropriate. Corresponding time-matched control experiments were routinely carried out with drug vehicle to ensure that drug effects were assessed without the influence of their vehicles. Evans blue dye was dissolved in 0.9% saline (20 mg/ml) and filtered through a 5 μm Millipore membrane. PAF was dissolved in absolute ethanol and diluted to the appropriate concentration with 0.25% bovine serum albumin in distilled water (w/v).

Table 1
Inhibitory effects (expressed as inhibitory dose 50%, ID_{50}) of PF-904 and theophylline on bronchoconstriction produced by different spasmogens in normal, anaesthetized ventilated guinea pigs, and measured at 30 min post-administration

Drug	ID_{50} (mg/kg, intraduodenal)			
	Histamine	PAF	Arachidonic acid	Methacholine
PF-904	36.27 (22.15–59.39)	36.79 (25.59–52.90)	13.77 (6.68–28.37)	103.32 (28.97–128.67)
Theophylline	16.91 (8.47–33.74)	26.63 (17.74–28.59)	7.47 (1.32–22.38)	22.38 (13.38–37.45)

Data are expressed as mean values from 9–10 animals, with the 95% confidence limits in parentheses.

3. Results

3.1. Effects against spasmogen-induced bronchoconstriction

Intraduodenal administration of PF-904 (12.5–200 mg/kg) and theophylline (6.25–100 mg/kg) produced a dose-related inhibition of bronchoconstrictor responses to intravenous histamine (7.5 μg/kg), arachidonic acid (1 mg/kg) and PAF (150 ng/kg). The potency (assessed from ID_{50} values) of PF-904 as inhibitor of these spasmogens was about half of that shown by theophylline (Table 1). The response to methacholine (17.5 μg/kg, i.v.) was about four times less sensitive to inhibition by PF-904 than was that to theophylline (Table 1). The duration of the inhibitory effects of equieffective doses of PF-904 and theophylline on histamine-induced bronchoconstriction is shown in Fig. 1. PF-904 exhibited a level of activity similar to that of the reference compound up to 30 min post-administration but PF-904 had significant inhibitory effects for 120 min (Fig. 1). Similar time course profiles were obtained against arachidonic acid and methacholine (data not shown).

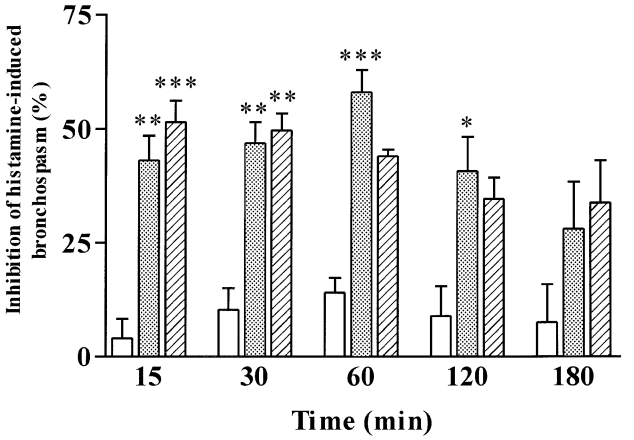

Fig. 1. Time-course of the inhibitory effect of PF-904 and theophylline on histamine-induced bronchospasm in normal anaesthetized ventilated guinea pigs. 10 min after a stable bronchoconstrictor response to histamine (7.5 μg/kg, i.v.) was established, vehicle (control; open columns), PF-904 (100 mg/kg, dotted columns) or theophylline (50 mg/kg, hatched columns) was administered intraduodenally and the histamine challenge was repeated at the times indicated on the abscissa. Data are means ± S.E.M. for 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control.

3.2. Effects against PAF-induced bronchial hyperresponsiveness to histamine

A repeated intravenous bolus of histamine (4 μg/kg) elicited an increase in pulmonary inflation pressure (mmHg) which was not modified by the infusion of 0.25% bovine serum albumin (4.27 ± 2.88 and 5.76 ± 2.73 before or after bovine serum albumin, respectively; $n = 6$ for each group). The histamine response was significantly enhanced by the infusion of PAF (600 ng/kg over 1 h), indicating the development of hyperresponsiveness (values of increase in pulmonary inflation pressure are 2.95 ± 0.79 and 11.36 before and after PAF, respectively; $P < 0.05$; $n = 6$ for each group). Drug vehicle (carboxymethylcellulose, 0.5%) did not modify the hyperresponsiveness but PF-904 (50 and 100 mg/kg) and theophylline (25 and 50 mg/kg) significantly reduced the hyperresponsiveness to histamine observed in PAF-treated animals (Table 2).

3.3. Effects against antigen-induced bronchoconstriction and airway microvascular leakage

Inhaled antigen (ovalbumin, 5 mg/ml, 30 breaths) produced bronchoconstriction that was markedly inhibited by PF-904, theophylline, and rolipram (Fig. 2). Animals treated with PF-904, theophylline or rolipram, but not receiving extravasation stimuli, had an Evans blue dye content of airways similar to that of control animals (data not shown). Sensitized animals challenged with aerosol antigen showed a significant extravasation of Evans blue dye at all airway levels (Fig. 3). PF-904 (100 mg/kg), theophylline (50 mg/kg), and rolipram (1 mg/kg) all inhibited antigen-induced extravasation in trachea and main bronchi (except theophylline in main bronchi). PF-904 and rolipram showed a tendency to reduce extravasation in intrapulmonary airways but the attenuation did not reach statistical significance.

Table 2
Effects of intraduodenal PF-904 and theophylline on PAF(600 ng/kg, i.v. over 1 h)-induced airways hyperreactivity to histamine

Drug	Dose (mg/kg)	Pulmonary inflation pressure increase (mmHg)		Difference
		pre-PAF	post-PAF	
Vehicle		2.61 ± 0.79	10.50 ± 0.96	7.89 ± 0.87
PF-904	50	3.50 ± 0.88	6.45 ± 0.94	2.95 ± 0.84 [a]
	100	3.80 ± 0.98	5.15 ± 0.98	1.35 ± 0.94 [a]
Theophylline	25	2.11 ± 0.54	5.33 ± 0.71	3.22 ± 0.51 [a]
	50	3.61 ± 0.89	4.28 ± 1.23	0.67 ± 1.17 [a]

Results are expressed as means ± S.E.M. for 9–10 animals.
[a] $P < 0.05$ vs. vehicle group.

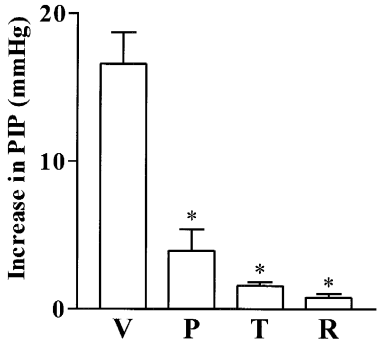

Fig. 2. Response to inhaled ovalbumin (5 mg/ml, 30 breaths) by sensitized animals pretreated intraduodenally with vehicle (V), PF-904 (P; 100 mg/kg), theophylline (T; 50 mg/kg) or rolipram (R; 1 mg/kg). Control animals were exposed to inhaled saline after treatment with vehicle and showed no response ($n = 4$; data not shown in the figure). Bronchoconstriction is expressed as increases in pulmonary inflation pressure (mmHg). Columns are means ± S.E.M. from 6 experiments. * $P < 0.05$ compared to V. No significant difference was observed among treated groups.

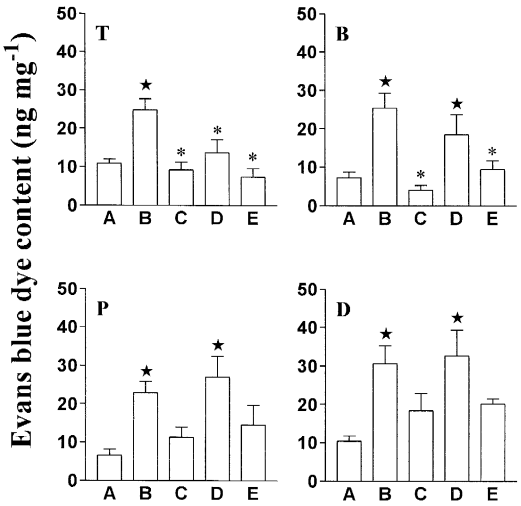

Fig. 3. Extravasation of Evans blue dye in guinea-pig trachea (T), main bronchi (B), proximal (P) and distal (D) intrapulmonary airways. Animals pretreated intraduodenally, with vehicle (A, B), PF-904 (100 mg/kg; C), theophylline (50 mg/kg; D) or rolipram (1 mg/kg; E), then exposed to inhaled saline (A) or antigen (25 mg/kg, 30 breaths; B to E). ★ $P < 0.05$ versus A. * $P < 0.05$ versus B. PF-904 effectively inhibited antigen-induced trachea and main bronchi extravasation.

3.4. Effects against antigen-induced airways hyperresponsiveness and eosinophil accumulation

Twenty-four hours after antigen challenge, sensitized guinea pigs exhibited augmented airways responses to histamine compared with those of non-sensitized animals exposed to saline (Table 3). Oral treatment with methylprednisolone (75 mg/kg), theophylline (100 mg/kg) or PF-904 (200 mg/kg) failed to prevent the heightened airway responses to histamine induced by antigen exposure.

Sensitized guinea pigs showed increased total cell counts and an increased number of eosinophils and macrophages in bronchoalveolar lavage fluid 24 h after antigen challenge. Oral treatment with methylprednisolone (75 mg/kg), theophylline (100 mg/kg) or PF-904 (200 mg/kg) effectively reduced total cell and eosinophil counts (Table 3).

3.5. Relaxant and anti-spasmogenic activity in human isolated bronchus

PF-904 and theophylline caused concentration-dependent inhibition of spontaneous and histamine (0.1 mM)-induced tone of human bronchi as shown in Fig. 4. The two drugs produced full relaxation (i.e. the maximal relaxation was not significantly different from that obtained with theophylline 3 mM) and were equipotent, with EC_{50} values around 0.1 to 0.3 mM (Table 4). To assess anti-spasmogenic activity, response curves for histamine were obtained in the presence of PF-904 or theophylline (each at 0.1 mM, i.e., approx. EC_{50}). Theophylline showed no anti-spasmogenic effect but PF-904 depressed the contractile responses to histamine (Fig. 5).

Table 3
Airways reactivity to histamine and total and differential cell counts in bronchoalveolar lavage fluid recovered from guinea-pig lungs 24 h after exposure to an aerosol of antigen (sensitized animals) or saline (non-sensitized animals)

Group	Airway response (mmHg) to histamine (μg/kg, i.v.)				Total and differential cell count				
	n	2	10	25	Total cells	Neutrophil	Eosinophil	Macrophage	Lymphocyte
Non-sensitized	8	0.15 ± 0.10	3.16 ± 0.98	16.41 ± 4.89	5.17 ± 0.47	0.13 ± 0.06	0.46 ± 0.13	4.05 ± 0.38	0.51 ± 0.20
Sensitized untreated	6	1.30 ± 0.53	17.60 ± 3.85 [a]	35.60 ± 5.83 [a]	29.11 ± 4.26 [a]	0.77 ± 0.03	6.22 ± 1.07 [a]	15.42 ± 0.60 [a]	2.46 ± 1.00
Methylprednisolone	5	0.25 ± 0.19	7.39 ± 1.80	27.57 ± 5.36	16.28 ± 1.90 [a,b]	0.44 ± 0.12	2.16 ± 0.40 [b]	14.43 ± 2.55 [a]	2.11 ± 0.69
Theophylline	7	0.41 ± 0.32	11.0 ± 3.04	24.90 ± 3.86	12.97 ± 1.40 [b]	0.31 ± 0.18	2.51 ± 0.73 [b]	9.31 ± 0.67	0.78 ± 0.31
PF-904	5	1.35 ± 0.95	15.42 ± 2.84 [a]	35.14 ± 3.41 [a]	13.36 ± 1.83 [b]	0.22 ± 0.07	1.94 ± 0.50 [b]	9.74 ± 1.36	1.44 ± 0.40

Five groups of animals were tested: control group, untreated, non-sensitized animals, exposed to saline; untreated sensitized animals exposed to antigen; and sensitized animals treated with PF-904 (200 mg/kg, oral, 30 min before antigen), theophylline (100 mg/kg, oral, 30 min before antigen) or methylprednisolone (37.5 mg/kg i.p., at 24 and 2 h before antigen; $n = 7$). Results are expressed as means ± S.E.M. of cells × 10^6 for groups of $n = 5$–8 animals as indicated.

[a] $P < 0.05$ vs. non-sensitized animals.

[b] $P < 0.05$ vs. sensitized untreated.

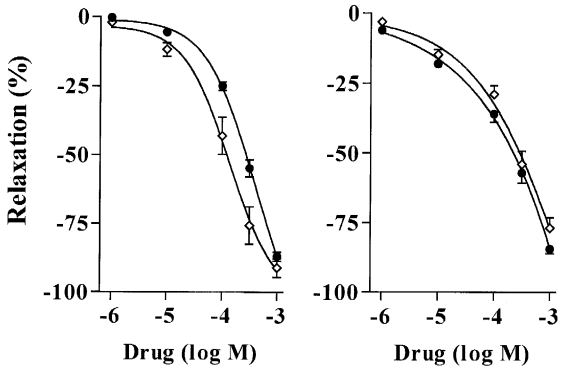

Fig. 4. Relaxation by PF-904 (●) and theophylline (◇) of the spontaneous (left panel) and histamine (0.1 mM)-induced (right panel) tone in human isolated bronchus. Ordinate scales: relaxation as % of inhibition produced by theophylline 3 mM. Abscissa scales: drug concentration as log *M*. Points are means ± S.E.M. of 6 experiments.

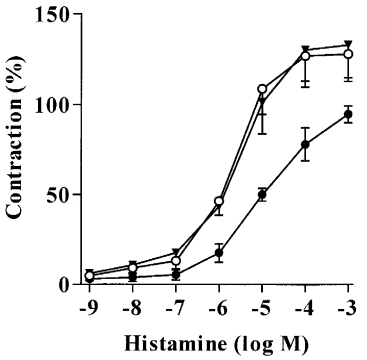

Fig. 5. Concentration–response curves for histamine in human isolated bronchus. Two consecutive curves were obtained. The initial curve served as control (not shown); the second curve was obtained in the absence (time-matched controls; ○) or presence of PF-904 (0.1 mM; ●) or theophylline (0.1 mM; ▼). Ordinates, contraction as % of maximal contraction obtained in the initial curve; abscissae, concentration of histamine as log *M*. Points are means ± S.E.M. of 4–6 experiments. The concentration–response curve for histamine was shifted right and downward in PF-904-treated tissues. The pEC_{50} values for histamine were 5.69 ± 0.06 ($n = 4$) in the absence vs. 4.86 ± 0.23 ($n = 5$; $P < 0.05$) in the presence of PF-904.

Glibenclamide (10 μM) antagonized the relaxant effect of cromakalim (pEC_{50} values for cromakalim were 6.12 ± 0.19 ($n = 6$) in the absence and 4.83 ± 0.05 ($n = 4$) in the presence of glibenclamide; $P < 0.05$) but had no effect against PF-904-induced relaxation (pEC_{50} values for PF-904 were 3.60 ± 0.11 in the absence and 3.40 ± 0.09 in the presence of glibenclamide; $n = 6$; $P > 0.05$). When added at the peak relaxation produced by PF-904 (1 mM; 0.86 ± 0.16 g, $n = 5$) or cromakalim (10 μM; 0.55 ± 0.08 g, $n = 6$; $P > 0.05$ vs. PF-904), glibenclamide (10 μM) immediately reversed the cromakalim- but not the PF-904-induced relaxation (data not shown). PF-904 produced complete relaxation in tissues precontracted with low (10 mM) or high (80 mM) concentrations of KCl (pEC_{50} values were 3.52 ± 0.08 and 3.45 ± 0.05, respectively, $n = 8$, $P > 0.05$) while cromakalim was only effective against the tone induced by low concentrations of KCl (data not shown).

Isoprenaline (10 nM–3 μM) and sodium nitroprusside (0.1 μM–1 mM) produced concentration-dependent relaxation of human isolated bronchus precontracted with acetylcholine (10 μM) with respective pEC_{50} values of 7.21 ± 0.11 ($n = 6$) and 5.23 ± 0.41 ($n = 5$). Preincubation with PF-904 or theophylline (each at 0.1 mM) resulted in small leftward shifts of the response curves for isoprenaline and sodium nitroprusside that failed to reach statistical significance, with as the only exception, the relaxation in response to isoprenaline in theophylline-treated tissues (leftward shift of 0.4 log unit).

Table 4
Relaxant effects of PF-904 and theophylline on spontaneous and histamine (HA; 0.1 mM)-induced tone of human isolated bronchus

Drug	Tone	n/p [a]	pEC_{50} [b]	E_{max} (g)	RT [c]
PF-904	Spontaneous	10/5	3.57 ± 0.04	1.19 ± 0.16	1.35 ± 0.17
	HA-induced	10/4	3.66 ± 0.07	1.66 ± 0.27	1.93 ± 0.27
Theophylline	Spontaneous	9/5	3.91 ± 0.12	0.72 ± 0.10	—
	HA-induced	10/5	3.55 ± 0.08	1.85 ± 0.21	—

Data are means ± S.E.M. The plateau contraction produced by histamine (0.1 mM) was 1.30 ± 0.33 g and 0.92 ± 0.21 g for PF-904 and theophylline experiments, respectively.
[a] n/p = number of preparations/number of patients.
[b] $pEC_{50} = -\log EC_{50}$.
[c] RT = response to theophylline 3 mM added at the end of the experiment.

3.6. Inhibition of phosphodiesterase activity in human bronchus

PF-904 (10 μM–3 mM) and theophylline (30 μM–3 mM) produced a concentration-dependent inhibition of cyclic AMP and cyclic GMP phosphodiesterase activities (Fig. 6). The pIC_{50} values for PF-904 were 2.83 ± 0.25 ($n = 8$) and 2.90 ± 0.21 ($n = 8$) and those for theophylline were 3.78 ± 0.18 ($n = 8$) and 3.51 ± 0.12 ($n = 8$), respectively. PF-904 was significantly ($P < 0.05$) less potent than theophylline as inhibitor of cyclic AMP and cyclic GMP phosphodiesterase activities. When assayed against

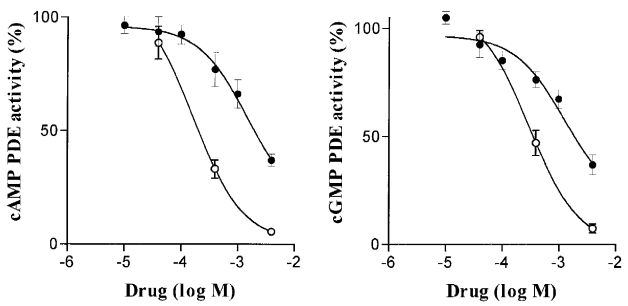

Fig. 6. Concentration–response curves for PF-904 (●) and theophylline (○) as inhibitors of cyclic AMP (left panel) and cyclic GMP (right panel) phosphodiesterase (PDE) activity in human bronchus. Ordinates: activity expressed as %; abscissae: drug concentration as log *M*. Points are means ± S.E.M. of 8 experiments.

phosphodiesterase IV activity, PF-904 and theophylline had pIC_{50} values of 3.43 ± 0.11 and 4.19 ± 0.12 ($n = 3$), respectively. PF-904 was significantly ($P < 0.05$) less potent than theophylline as inhibitor of phosphodiesterase IV activity. The potency of PF-904 and theophylline as phosphodiesterase IV inhibitors was greater than that against cyclic AMP phosphodiesterase activity but the differences failed to reach significance.

3.7. Inhibition of N-formylmethionyl–leucyl–phenylalanine-induced superoxide release by human polymorphonuclear leukocytes

Stimulation of human polymorphonuclear leukocytes with *N*-formylmethionyl–leucyl–phenylalanine (3 nM to 1 μM) elicited a concentration-related release of reactive oxygen species as detected by luminol-enhanced chemiluminescence with $pEC_{50} = 7.58 \pm 0.09$ ($n = 6$). A concentration of *N*-formylmethionyl–leucyl–phenylalanine close to its EC_{50} (30 nM) was selected for all subsequent experiments. Rolipram, a selective phosphodiesterase IV inhibitor (Raeburn and Advenier, 1995) reduced the chemiluminescence signal in a concentration-dependent manner with an IC_{50} value of 4.27 nM ($pIC_{50} = 8.37 \pm 0.47$; $n = 6$). Theophylline was also effective to inhibit superoxide release but its potency was lower than that of rolipram ($IC_{50} = 0.32$ mM; $pIC_{50} = 3.49 \pm 0.07$; $n = 6$). PF-904 (10–100 μM) inhibited superoxide release from *N*-formylmethionyl–leucyl–phenylalanine-stimulated human polymorphonuclear leukocytes, but failed to reach 50% inhibition at the highest concentration tested (Fig. 7). The IC_{25} value for PF-904 was 79.4 μM ($pIC_{25} = 4.10 \pm 0.04$; $n = 6$).

4. Discussion

The results obtained in the present study indicate that PF-904, when administered enterally, inhibits intravenous histamine-, arachidonic acid-, PAF-, and methacholine-induced bronchospasm in non-sensitized anaesthetized guinea pigs. Theophylline, a standard anti-asthma drug, was also effective in this in vivo model of bronchoconstriction. The potency (assessed as ID_{50} values) of PF-904 against these spasmogens was found to be between half (for histamine, arachidonic acid and PAF) and one fourth of that observed with theophylline. When the duration of action of equieffective doses of PF-904 and theophylline was compared, we found that significant effects of theophylline lasted for less than 60 min whereas PF-904 inhibited the bronchoconstrictor responses to histamine for 120 min. Additionally, pretreatment with PF-904 (100 mg/kg, intraduodenal) produced a marked inhibition of the bronchoconstriction elicited by inhaled antigen in sensitized guinea pigs. This inhibition was similar to that produced by theophylline (50 mg/kg) and rolipram (1 mg/kg) administered by the same route. Immediate bronchoconstriction produced by antigen in sensitized guinea pigs is related to mediator release, mainly histamine and lipoxygenase products (Evans et al., 1988). PF-904 inhibits the bronchoconstriction produced by histamine and other mediators in the guinea pig (this study), and this effect may account for the attenuation of antigen-induced airway responses. However, our study was not designed to show whether PF-904 is acting solely as an inhibitor of bronchospasm or whether it inhibits antigen-induced mediator release in the lung.

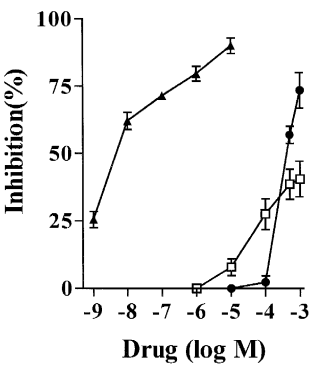

Fig. 7. Inhibitory effect of PF-904 (□), theophylline (●) and rolipram (▲) on superoxide release produced by *N*-formylmethionyl–leucyl–phenylalanine (30 nM, i.e., approx. EC_{50}) in human neutrophils. Abscissa: Drug concentrations as log *M*. Ordinate: inhibition (%). Data were derived from 6 different preparations and are given as means ± S.E.M.

In addition to the in vivo bronchoconstrictor studies, proper evaluation of anti-asthma drugs requires examination of drug effects on airways hyperreactivity, microvascular leakage and eosinophil infiltration. PAF is an inflammatory mediator which produces airways hyperreactivity in guinea pigs (Robertson and Page, 1987; Underwood et al., 1992). We have shown that intraduodenal PF-904 (50–100 mg/kg) significantly reduced the airways hyperreactivity evoked by intravenous PAF in non-sensitized anaesthetized guinea pigs. The ability of the compound to prevent hyperreactivity was similar to that observed for theophylline in doses found equieffective to inhibit histamine- and PAF-induced bronchoconstriction in the guinea pig in vivo (this study). In contrast, neither theophylline nor PF-904 prevented airway hyperreactivity 24 h after antigen challenge of sensitized guinea pigs, a model in which a wide array of endogenous mediators are involved (Coyle et al., 1988; Havill et al., 1990; Farmer et al., 1992; Ishida et al., 1993; Boichot et al., 1995). This finding is consistent with results of previous studies showing that non-selective phosphodiesterase inhibitors failed to prevent antigen-induced hyperreactivity (Sanjar et al., 1990; Gozzard et al., 1996) which contrasts with the established activity of selective phosphodiesterase IV inhibitors (Gozzard et al., 1996; Ortiz et al., 1996).

Airway microvascular leakage and eosinophil infiltration are regarded as important components of inflammatory reaction in asthma. PF-904 (100 mg/kg, intraduodenal) also attenuated antigen-induced airway (trachea and

main bronchi) microvascular leakage in guinea-pig as found with rolipram (Ortiz et al., 1996; this study). The anti-exudative effects of PF-904 against antigen-induced microvascular leakage may be explained by its antihistamine activity since histamine is an important mediator of leakage induced by antigen challenge in the guinea pig (Evans et al., 1988). The possible contribution of the inhibition of mediator release by PF-904 is uncertain at present. Antigen exposure produced an increase in the infiltration of eosinophils into the lungs of sensitized animals. The total cell count and macrophage numbers were also augmented. The accumulation of eosinophils is consistent with most findings (Coyle et al., 1988; Havill et al., 1990; Farmer et al., 1992). Oral PF-904 (200 mg/kg) effectively reduced eosinophil accumulation in airways of sensitized guinea pigs following antigen exposure. Theophylline and methylprednisolone also inhibited eosinophil recruitment in the guinea-pig airways after antigen exposure, which is consistent with previous reports showing that non-selective phosphodiesterase inhibitors and steroids as well as phosphodiesterase IV inhibitors reduce airway eosinophilia in sensitized guinea pigs challenged with antigen (Sanjar et al., 1990; Ortiz et al., 1996).

PF-904 relaxed spontaneous and histamine elevated tone in human respiratory muscle in vitro with a potency and efficacy similar to that of theophylline (Cortijo et al., 1993; this study). In addition, histamine-induced contraction was impaired after pretreatment with PF-904. Therefore, PF-904 had both spasmolytic and anti-spasmogenic activity, in particular against the tone raised by histamine. These results extend to the human airways earlier findings for guinea-pig tissues. The relaxation produced by PF-904 was not antagonized by glibenclamide (10 μM), an established K_{ATP} channel blocker (Small et al., 1993) nor was it impaired in tissues precontracted with high concentrations of KCl. These data suggest that relaxation in response to PF-904 is not mediated through activation of cromakalim-sensitive K^+-channels (Small et al., 1993).

PF-904 and theophylline (each at 0.1 mM) showed a tendency to enhance the relaxation produced by isoprenaline and sodium nitroprusside but the leftward shifts in the concentration–response curves for these relaxants failed to reach significance except for theophylline potentiation of the relaxation to isoprenaline. The lack of consistency of some phosphodiesterase inhibitors to produce a substantial increase of isoprenaline- and sodium nitroprusside-induced relaxation in human bronchus was reported earlier (Torphy et al., 1993). Therefore, we studied the inhibitory effects of PF-904 on cyclic AMP and cyclic GMP phosphodiesterase activities of human isolated bronchus. PF-904 inhibited in a concentration-dependent fashion, and with similar potency and efficacy, cyclic AMP and cyclic GMP phosphodiesterase activities as well as cyclic AMP phosphodiesterase IV activity (this study). These results indicate that PF-904 is a non-selective phosphodiesterase inhibitor. This compound was less potent than theophylline, a known non-selective phosphodiesterase inhibitor (Raeburn and Advenier, 1995). The potency of theophylline as inhibitor of human bronchial phosphodiesterase activity (approx. 0.2–0.3 mM) and its potency as relaxant of human bronchial tone (approx. 0.1–0.3 mM) were remarkably similar, suggesting that non-selective inhibition of phosphodiesterase contributes to human bronchodilatation by theophylline. In contrast, the potency of PF-904 as inhibitor of phosphodiesterase (approx. 1.3–1.5 mM; approx. 0.4 mM against phosphodiesterase IV) is lower than its potency as relaxant of human airways (approx. 0.2–0.3 mM). This suggests that mechanisms other than phosphodiesterase inhibition contribute to airway smooth muscle relaxation by PF-904.

Oxygen radicals may be released by various inflammatory leukocytes accumulated in the airways, thus contributing to lung damage. The present results confirmed the activity of rolipram, a selective phosphodiesterase IV inhibitor (Raeburn and Advenier, 1995), as inhibitor of *N*-formylmethionyl–leucyl–phenylalanine-induced superoxide release by human neutrophils (Schudt et al., 1991; Cortijo et al., 1996). Unselective phosphodiesterase inhibitors also cause a marked decrease of the oxidative burst after human polymorphonuclear leukocytes activation by agonists like *N*-formylmethionyl–leucyl–phenylalanine as previously shown (Schudt et al., 1991). PF-904 was more effective than theophylline at low concentrations (10–100 μM) but its efficacy remained below 50% at the highest concentration tested (1 mM). These concentrations of PF-904 (10–100 μM) also produce significant relaxation of human bronchus in vitro. The potential importance of these findings will depend on the plasma concentrations achievable after oral administration of PF-904 in humans.

In conclusion, this study showed that oral PF-904 inhibits bronchoconstriction caused by a variety of spasmogens in the guinea pig in vivo; prevents airways hyperreactivity to histamine after PAF exposure, and reduces antigen-induced bronchospasm, eosinophil lung infiltration, and airway microvascular leakage in sensitized guinea pigs. In vitro studies showed that PF-904 is a relaxant of human bronchus and inhibits human bronchial cyclic AMP and cyclic GMP phosphodiesterase activity. In addition, relaxant concentrations of PF-904 reduce superoxide generation by human neutrophils. Although no animal model is truly representative of human bronchial asthma, these in vivo findings, together with the in vitro results with human tissues, suggest that PF-904 is a candidate for further investigation as a possible anti-asthma agent.

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