

ORIGINAL ARTICLE

Roflumilast Inhibition of Pulmonary Leukotriene Production and Bronchoconstriction in Ovalbumin-Sensitized and -Challenged Guinea Pigs

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This study investigated the effects of roflumilast, a PDE4 inhibitor, on slow-reacting substance of anaphylaxis (SRS-A)-mediated bronchoconstriction and pulmonary leukotriene (LT) release in ovalbumin (OVA)-sensitized and -challenged guinea pigs. Animals were treated with roflumilast orally (0.04, 0.12, 0.4, or 4 mg/kg) or placebo 1 hour before OVA challenge. Bronchoconstriction was quantified by measuring airway conductance (Gaw) and dynamic lung compliance (Cdyn). Roflumilast significantly attenuated the decrease in Gaw (50% inhibitory dose [ID₅₀] = 0.33 mg/kg) and Cdyn (ID₅₀ = 0.25 mg/kg) in a dose-dependent manner and significantly inhibited Cys-LT (ID₅₀ = 0.06 mg/kg) and LTB₄ (ID₅₀ = 0.05 mg/kg) release versus placebo-treated animals. Roflumilast did not affect LTD₄-induced bronchoconstriction. These findings support the role of roflumilast as an anti-inflammatory treatment for asthma.

Keywords phosphodiesterase 4 inhibitor, slow-reacting substance of anaphylaxis (SRS-A), leukotriene, asthma, guinea pig

INTRODUCTION

Asthma is a chronic disease affecting an estimated 300 million people worldwide (1). It is characterized by inflammation and airway hyperresponsiveness, leading to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing (2). Eosinophil and T lymphocyte (CD4⁺) infiltration and increased secretion of inflammatory mediators in asthma adversely affect the large and small airways (3). Currently, novel therapeutics that treat chronic inflammatory pulmonary diseases, such as asthma, are being sought. Proinflammatory cells (e.g., mast cells, eosinophils, and neutrophils), cytokines, and mediators found in the airways are key targets for drug development.

Leukotrienes (LTs) released from inflammatory cells are lipid-based signaling molecules in allergic and inflammatory responses in the respiratory system. Cysteinyl LTs (Cys-LTs) are synthesized by eosinophils, basophils, and mast cells, whereas leukotriene B₄ (LTB₄) is produced by neutrophils and macrophages (4). The Cys-LTs (LTC₄, LTD₄, and LTE₄) are potent bronchoconstrictors, controlling the inherent tone of human bronchi and increasing vascular permeability in the airways. These signaling molecules are chemoattractants for eosinophils and contribute to antigen-induced hyperresponsiveness. LTC₄ and LTD₄ are the major components in slow-reacting substance of anaphylaxis (SRS-A) and are important mediators of early allergic reaction in asthmatics (5, 6). Airway inflammation is also mediated by LTB₄, a chemoattractant for eosinophils, monocytes, and neutrophils. Leukotriene B₄ also regulates levels of proinflammatory cytokines, acts as a transcriptional activator of cytokines (e.g., interleukin

[IL]-2 and IL-6) (7, 8) and mediates histamine induction of nuclear factor kappaB (NF- κ B) and IL-8 (9). Agents that inhibit these proinflammatory mediators may play an important role in the treatment of inflammatory airway diseases.

Roflumilast, a phosphodiesterase type 4 (PDE4) inhibitor with anti-inflammatory activities, is in development for the treatment of patients with asthma. Roflumilast acts by inhibiting the breakdown of cyclic adenosine monophosphate (cAMP) through PDE4 inhibition (10, 11). Reinforcement of the cAMP signal in inflammatory cells may inhibit proliferation and chemotaxis of these cells and may decrease the release of inflammatory and cytotoxic mediators in the airways. In *in vitro* and *in vivo* preclinical studies, roflumilast inhibited inflammatory cell function—including chemotaxis, cytokine secretion, degranulation, respiratory burst, and proliferation in several inflammatory cell types—thus inhibiting inflammatory mediators that are important in the pathophysiology of asthma (12–14). Further, clinical studies have demonstrated that roflumilast inhibits late asthmatic reaction, attenuates airway hyperresponsiveness, and improves lung function in patients with asthma (15–17).

Although the anti-inflammatory activity of roflumilast has been well documented *in vitro*, the underlying pharmacologic response in animal models has not been fully characterized. The objective of this study was to investigate the effects of roflumilast on acute bronchoconstriction and LT release in ovalbumin (OVA)-sensitized and -challenged guinea pigs.

METHODS

Animals

Male Dunkin Hartley guinea pigs (weight, 200 to 250 g at sensitization; 345 to 495 g during experiments) were obtained from Charles River (Sulzfeld, Germany). Guinea pigs were housed in groups of 3 to 4 in Macrolon cages (type IV) at 20° to 22°C with a 12-hour light-dark cycle and received food and

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water ad libitum (standard diet 3418 for guinea pigs; Provimi Kliba SA, Kaiseraugst, Switzerland). All animal experimentation was conducted in accordance with German national guidelines and legal regulations.

Study Design

Animals were sensitized on 2 consecutive days with 20 μ g OVA (Sigma, St. Louis, MO) and 20 mg $\text{Al}(\text{OH})_3$ in 0.5 mL saline, which was administered intraperitoneally. Experiments, which included OVA challenge, were conducted 2 to 3 weeks after sensitization.

Roflumilast (ALTANA Pharma, Konstanz, Germany) was administered 1 hour before OVA challenge. Roflumilast was dissolved in 4% methocel-suspension (methylhydroxypropyl cellulose; Dow Chemicals, Midland, MI) containing 20% polyethylene glycol 400 (PEG400; Merck-Schuchardt, Hohenheim, Germany). This solution was administered by gavage to conscious animals at doses of 0.04 ($n = 10$), 0.12 ($n = 10$), 0.4 ($n = 12$), and 4 mg/kg ($n = 12$). A 4%-methocel/PEG400 solution was used as a vehicle control (placebo).

Approximately 50 minutes before OVA challenge, the animals were anesthetized with 1.5 g/kg intraperitoneal urethane (12.5%) in a volume of 12 mL/kg. The right jugular vein was surgically exposed and cannulated with a small (outer diameter, 0.75 mm) polyethylene catheter for intravenous (IV) injection, as was the left carotid artery for blood pressure and heart rate measurements. The trachea was exposed in the neck region, and a y-shaped tracheal cannula (length, 2.8 cm; outer diameter, 3.1 or 2.5 mm) was inserted approximately 1.5 cm proximal of the carina. The tracheal cannula was connected to a heated Fleisch pneumotachograph (no. 0000; Hugo Sachs Elektronik [HSE], March-Hugstetten, Germany) and to a differential pressure transducer (Validyn DP45-14; HSE). To enhance the SRS-A response in this model, the animals were pretreated with 10 mg/kg IV indomethacin (in 0.5 M NaHCO_3 , 1.0 mL/kg; Sigma) 20 minutes before OVA challenge to block the cyclooxygenase pathway and to enhance the production of lipoxygenase mediators, and with 2.0 mg/kg pyrilamine (in saline; Sigma) 6 minutes before OVA challenge to inhibit bronchoconstriction resulting from endogenous histamine release. Animals were also pretreated with 0.1 mg/kg propranolol (in saline; Rhein-Pharma, Plankstadt, Germany) 5 minutes before OVA challenge to abolish endogenous adrenergic bronchodilation.

Approximately 15 minutes before OVA challenge, animals were mechanically ventilated. They received 1.5 mg/kg pancuronium bromide (Organon; Eppelheim, Germany) IV in a volume of 0.75 mL/kg to abolish spontaneous breathing. Ventilation was initiated with ambient room air using a small animal ventilator (type KTR4; HSE) at 60 breaths per minute, 7 mL/kg tidal volume (TV), and a 40:60 inspiration:expiration ratio. Blood pressure and heart rate were monitored to assess vital functions of the animals. Bronchoconstriction was induced by a 0.15-mg/kg OVA IV challenge (volume, 1 mL/kg). The negative control animals received a sham challenge.

Lung function parameters were measured for 12 minutes after OVA challenge. The primary lung function parameters were flow (F), integrated from the differential pressure transducer through the pneumotachograph, and pulmonary

inflation pressure (PIP), measured at the side port of the tracheal cannula against atmospheric pressure using a differential pressure transducer (MPX-11 DP; HSE). Conductance ($\text{Gaw} = \text{F}/\text{PIP}$ mL/s/cmH₂O) and dynamic lung compliance ($\text{Cdyn} = \text{TV}/\text{PIP}$ mL/cmH₂O) were calculated from the primary lung function parameters (18).

Immediately after assessments of lung function during 12 minutes after OVA challenge, the lungs were gently lavaged 2 times with 6 mL/animal 4°C (~1.5 mL/100 g body weight) bronchoalveolar lavage (BAL)-buffer (phosphate-buffered saline containing 0.372% NaEDTA, pH 7.4, 10% Hanks' Balanced Salt Solution, buffered with 10% HEPES). Recovered BAL fluid (BALF) was centrifuged (5 minutes, 5°C, 21,000 $\times$ g) and supernatants were stored at -20°C. Supernatants were assayed for Cys-LTs and LTB₄ using enzyme immunoassay kits (EIA 520501 and 520111; Cayman Chemicals, Ann Arbor, MI). Assays were read at 405 nm in a Tecan Spectra reader (Tecan Deutschland GmbH, Crailsheim, Germany).

Mechanism of Action

To further explore the mechanism responsible for inhibition by roflumilast of OVA-induced bronchoconstriction and pulmonary LT release, the following experiments were undertaken. First, to demonstrate the Cys-LT mediation of the OVA-induced bronchoconstriction, animals were pretreated with the specific Cys-LT receptor antagonist, montelukast (Vorin Laboratories LTD, Secunderabad, India), 1 hour before OVA challenge at doses of 0.018 ($n = 10$), 0.06 ($n = 10$), 0.1 ($n = 10$), and 0.3 mg/kg ($n = 5$). Second, to demonstrate that roflumilast did not act via Cys-LT receptor antagonism, the effect of different doses of roflumilast (0.04, 0.4, 4, and 12 mg/kg, $n = 10$ animals per group) on LTD₄-induced (1 μ mol/animal) bronchoconstriction was investigated. Inhibition of LTD₄-induced (1 μ mol/kg) bronchoconstriction by oral montelukast (0.3 mg/kg, $n = 7$) was investigated as a control.

Data Analysis

A PC-based software program, model Ap1/8 (version V1.10f; PO-NE-MAH Inc., distributed by HSE) was used for data acquisition, analysis, and archiving. This program was used to calculate Gaw and Cdyn. All parameters were averaged at 1-minute intervals during the 30-minute prechallenge and at 10-second intervals from the 1-minute prechallenge to the 12-minute postchallenge. GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used for biostatistical analysis and curve-fit calculations to determine the 50% inhibitory dose (ID_{50}) values. The percentage decrease over time of Gaw and Cdyn and the area under the curve (AUC) were determined from 1- to 12-minute postchallenge (AUC_{1-12}) data for each animal. The percent inhibition of OVA-induced decrease of Gaw and Cdyn was calculated on the basis of AUC_{1-12} values in reference to placebo.

The percent inhibition of pulmonary Cys-LT and LTB₄ release was calculated on the basis of measured LT concentrations in the BAL in reference to placebo. Outlying data were eliminated using Grubb's test, and 1-way analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test was used to determine significance between treated animals and the corresponding placebo population. Statistical

significance was accepted at $p < 0.05$. All data are shown as mean or mean $\pm$ standard error of the mean (SEM). Dose-response curves and ID_{50} values were determined using log-linear regression analysis.

RESULTS

Inhibition of OVA-Induced Bronchoconstriction by Roflumilast

Ovalbumin-induced bronchoconstriction was characterized by a decrease in Gaw and Cdyn, beginning approximately 1 minute after OVA challenge and reaching a maximum decrease of Gaw (85%) at 7 minutes postchallenge (Figure 1A) and of Cdyn (92%) at 8 minutes postchallenge (Figure 1B).

Roflumilast inhibited the OVA-induced decrease in Gaw and Cdyn in a dose-dependent manner (Figure 1). The first significant inhibition was seen after pretreatment with 0.4

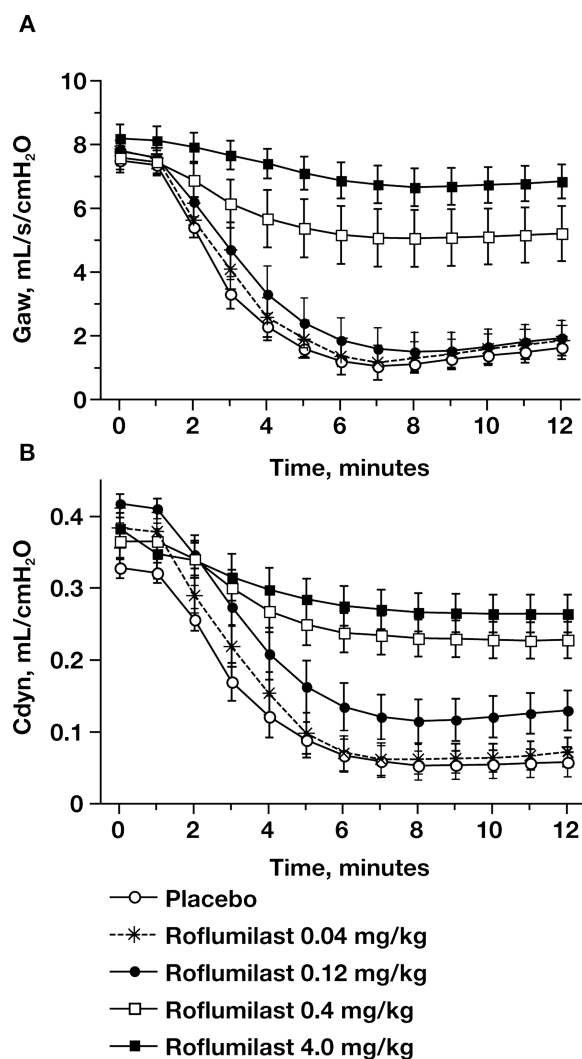


FIGURE 1.—Time course of ovalbumin (OVA)-induced bronchoconstriction in OVA-sensitized guinea pigs: inhibition by roflumilast. Roflumilast dose-dependently inhibited OVA-induced decrease in (A) airway conductance (Gaw) and (B) airway compliance (Cdyn) measured in anesthetized and mechanically ventilated guinea pigs. Data are shown as mean $\pm$ SEM.

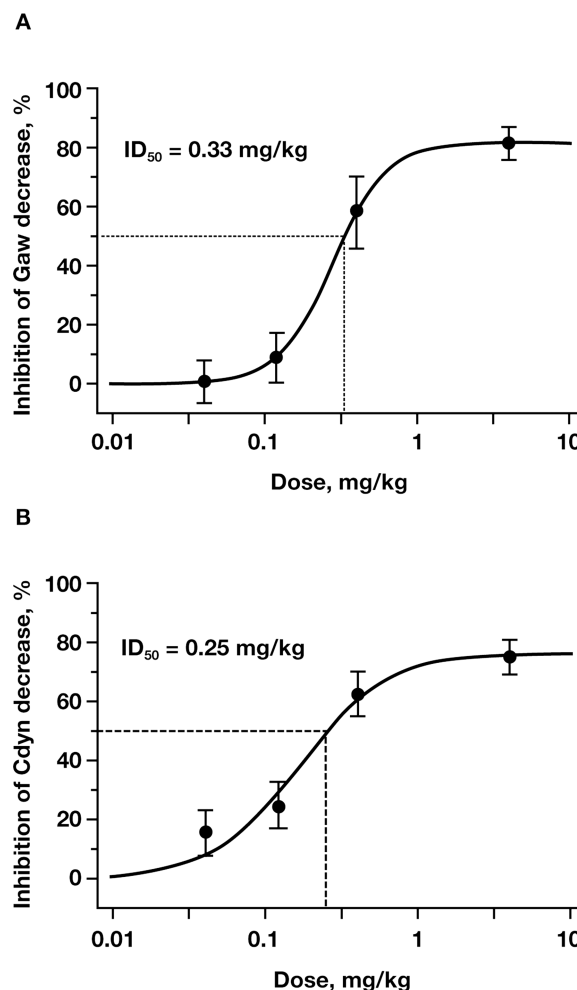


FIGURE 2.—Dose-response curve of ovalbumin (OVA)-induced bronchoconstriction in OVA-sensitized guinea pigs: inhibition by roflumilast. Roflumilast dose-dependently inhibited OVA-induced (A) airway conductance (Gaw) and (B) airway compliance (Cdyn) decrease measured in anesthetized and mechanically ventilated guinea pigs. Data are shown as mean $\pm$ SEM.

mg/kg roflumilast: the decrease in Gaw was reduced by 58% ($p < 0.05$) and the decrease in Cdyn was reduced by 64% ($p < 0.001$) compared with placebo. Maximum inhibition of the decrease in Gaw (81%; $p < 0.001$) and Cdyn (75%; $p < 0.001$) was seen at 4 mg/kg compared with placebo. Roflumilast attenuated the decrease in Gaw with an $ID_{50} = 0.33$ mg/kg (Figure 2A) and a similar decrease in Cdyn with an $ID_{50} = 0.25$ mg/kg (Figure 2B).

Inhibition of OVA-Induced Pulmonary LT Release by Roflumilast

Release of Cys-LT and LTB₄ in the lungs after OVA challenge was measured in BALF, and the effect of pretreatment with roflumilast on LT concentration was determined (Table 1). Roflumilast reduced Cys-LT and LTB₄ in BALF in a dose-dependent manner (Figure 3). First significant inhibition of Cys-LT (74%; $p < 0.05$) and LTB₄ (66%; $p < 0.05$) was seen after pretreatment with 0.12 mg/kg roflumilast. Maximum inhibition was achieved at 4 mg/kg, yielding 98%

TABLE 1.—Roflumilast inhibition of OVA-induced pulmonary leukotriene release.

Dose (mg/kg)	n	Cys-LT (pg/mL±SEM)	Inhibition (%±SEM)	n	LTB4 (pg/mL±SEM)	Inhibition (%±SEM)
Placebo	9	54 ± 17	—	9	43 ± 13	—
0.04	10	35 ± 13	35 ± 25	10	26 ± 9	39 ± 21
0.12	10	14 ± 4*	74 ± 8	11	15 ± 3*	66 ± 8
0.4	12	8 ± 3†	85 ± 6	13	14 ± 5*	67 ± 10
4.0	12	1 ± 1†	98 ± 1	10	13 ± 5*	69 ± 11

OVA = ovalbumin; n = number of animals; Cys-LT = cysteinyl leukotrienes; SEM = standard error of the mean; LTB4 = leukotriene B4.

* $p < 0.05$ vs placebo.

† $p < 0.01$ vs placebo.

inhibition of Cys-LT ($p < 0.01$) and 69% inhibition of LTB4 ($p < 0.05$). The ID_{50} values for inhibition of Cys-LT and LTB4 release by roflumilast were 0.06 mg/kg (Figure 3A) and 0.05 mg/kg (Figure 3B), respectively.

OVA-Induced Bronchoconstriction Is Mediated via Cys-LTs

The specific Cys-LT receptor antagonist montelukast inhibited the OVA-induced decrease in Gaw and Cdyn in a

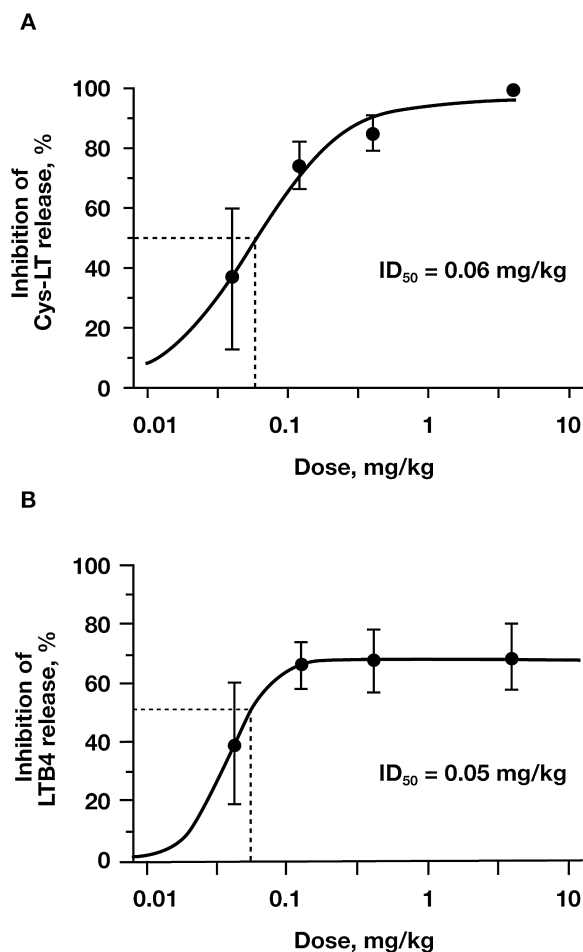


FIGURE 3.—Ovalbumin (OVA)-induced pulmonary leukotriene release in OVA-sensitized guinea pigs: inhibition by roflumilast. Roflumilast dose-dependently inhibited OVA-induced pulmonary (A) cysteinyl leukotriene (Cys-LT) and (B) leukotriene B4 (LTB4) release measured in bronchoalveolar lavage fluid. Data are shown as mean % inhibition ± SEM.

TABLE 2.—Montelukast inhibition of OVA-induced bronchoconstriction.

Dose (mg/kg)	Decrease in Gaw			Decrease in Cdyn		
	Inhibition (%)	SEM	n	Inhibition (%)	SEM	n
0.018	35†	10	10	34†	9	10
0.06	36†	8	10	41†	10	10
0.1	71†	8	10	72†	7	10
0.3	88†	5	5	84†	4	5

OVA = ovalbumin; Gaw = airway conductance; Cdyn = airway compliance; SEM = standard error of the mean; n = number of animals.

† $p < 0.01$ vs placebo.

dose-dependent manner, proving the Cys-LT mediation of bronchoconstriction (Table 2). Maximum inhibition of the decrease in Gaw (88%; $p < 0.01$) and Cdyn (84%; $p < 0.01$) was seen at 0.3 mg/kg compared with placebo.

Roflumilast Is Not Acting Via Cys-LT Receptor Antagonism

Challenge with 1 μ mol LTD4 per animal resulted in approximately 80% maximal bronchoconstriction (i.e., decrease in Gaw; Figure 4). Roflumilast, up to the excessive dose of 12 mg/kg, exerted no significant inhibitory effect on LTD4-induced bronchoconstriction (Figure 4), providing evidence that inhibitory activity of roflumilast is not mediated via Cys-LT receptor antagonism and not a result of direct bronchospasmolytic activity. As expected, the known selective Cys-LT receptor antagonist montelukast at a dose of 0.3 mg/kg significantly inhibited (83% ± 32%; $p < 0.01$) LTD4-induced bronchoconstriction (Figure 4).

DISCUSSION

The pathophysiology of asthma involves complex interactions between multiple inflammatory mediators and the influx of numerous inflammatory cell types. Therapeutic agents under development to treat patients with asthma are frequently designed to target the inflammatory components underlying this disease. Roflumilast inhibits inflammatory cell influx and cytokine release in the airways (12, 13). The objective of this study was to determine the effect of roflumilast, a PDE4 inhibitor with anti-inflammatory activity, on pulmonary inflammation and on an associated class of mediators—the leukotrienes.

The PDE4 inhibitor roflumilast is in clinical development for the treatment of chronic obstructive pulmonary disease (COPD) and asthma. Inhibition of PDE4 results in an inhibition of intracellular cAMP metabolism in key inflammatory cells in the airways. Reinforcement of the cAMP signal in inflammatory cells prevents proliferation and infiltration of these cells into the airways, decreases the release of inflammatory and cytotoxic mediators, and prevents airway smooth muscle constriction (10, 11). In inflammatory cells, roflumilast acts as an intracellular inhibitor to reduce proinflammatory activities (12, 13). In this study, roflumilast attenuated OVA-induced LT release into the airway lumen in a dose-dependent manner, leading to attenuation of bronchoconstriction. Because of inhibition of cyclooxygenase pathway by indomethacin, histamine-induced bronchoconstriction by pyrilamine, and endogenous catechol production by propranolol, this animal model is designed to represent mainly Cys-LT-dependent bronchoconstriction.

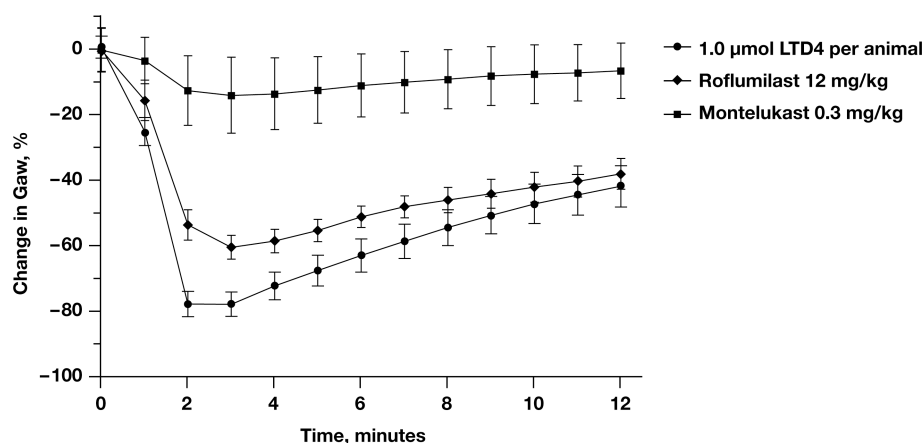


FIGURE 4.—Leukotriene D₄-induced bronchoconstriction in ovalbumin (OVA)-sensitized guinea pigs: inhibition by cysteinyl leukotriene receptor antagonist. Leukotriene D₄ induced a dose-dependent decrease of airway conductance (Gaw). Montelukast (0.3 mg/kg) abolished LTD₄-induced bronchoconstriction, but roflumilast 12 mg/kg did not reduce the decrease in Gaw. Data are shown as mean % decrease $\pm$ SEM.

As expected, Cys-LT antagonism by montelukast abolished OVA-induced bronchoconstriction, confirming the Cys-LT dependence of the OVA-induced bronchoconstriction model. To elucidate the mechanism of action of roflumilast, LTD₄ was used to elicit bronchoconstriction similar to that observed in OVA-challenged guinea pigs. In this model, roflumilast administered up to 12 mg/kg failed to inhibit LTD₄-induced bronchoconstriction. Therefore, roflumilast appears to suppress bronchoconstriction in OVA-challenged animals because of an inhibition of Cys-LT release, as it does not attenuate LTD₄-induced bronchoconstriction. This indicates that the inhibition of bronchoconstriction by roflumilast cannot be attributed to antagonism of the Cys-LT receptor and is not a result of direct bronchospasmolytic action. Rather, roflumilast appears to inhibit Cys-LT production and bronchoconstriction by other means, such as inhibition of mast cell degranulation and subsequent LT release, down-regulating levels of proinflammatory mediators leading to Cys-LT production, or limiting the influx of inflammatory cells capable of producing Cys-LTs into the airways. Similar inhibitory activity of other investigational PDE4 inhibitors (e.g., rolipram, cilomilast, YM976) has been demonstrated in guinea pig models of allergen- and LTD₄-induced bronchoconstriction (19–22). All PDE4 inhibitors inhibit OVA-induced bronchoconstriction; however, some (e.g., cilomilast) partially mediate the effect through direct bronchospasmolytic activity (19, 20, 22). Because roflumilast prevents bronchoconstriction through anti-inflammatory properties rather than bronchospasmolytic activity, it may provide complementary therapy to conventional bronchodilators.

This study also demonstrated that roflumilast inhibits Cys-LT and LTB₄ release into the lungs. Reduction of Cys-LT levels may lead to inhibition of eosinophil chemotaxis and antigen-induced airway hyperresponsiveness, indicating that roflumilast may provide inhibitory action concerning Cys-LT-mediated effects similar to those of montelukast, a selective Cys-LT receptor antagonist. However, pretreatment with roflumilast also decreased levels of LTB₄, suggesting a greater range of anti-inflammatory activity than montelukast,

which specifically interrupts Cys-LT signaling. Thus, roflumilast inhibits LTs and numerous other proinflammatory mediators, indicating multilevel anti-inflammatory effects (13). Several PDE4 inhibitors, including roflumilast, inhibit receptor agonist fMLP-stimulated LTB₄ release by neutrophils; however, like other PDE4 inhibitors tested, roflumilast did not inhibit Ca²⁺-stimulated LTB₄ release, suggesting that roflumilast may affect receptor-mediated release of LTB₄ (13).

This study further supports the anti-inflammatory activities of the PDE4 inhibitor, roflumilast, which include inhibition of LT production and the previously demonstrated inhibition of cytokine and chemokine release and inflammatory cell influx (12, 13). This is the first description of the mechanistic action of a PDE4 inhibitor on the inhibition of Cys-LT and LTB₄ release into the lungs in vivo, as well as the anti-inflammatory, not bronchospasmolytic, inhibition of allergen-induced, LT-mediated bronchoconstriction by roflumilast. Taken together, these findings provide further support for the role of roflumilast as an anti-inflammatory treatment for asthma.

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