

Effects of the Phosphodiesterase Type 4 Inhibitor Roflumilast on Early and Late Allergic Response and Airway Hyperresponsiveness in *Aspergillus-fumigatus*-Sensitized Mice

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Key Words

Airway hyperresponsiveness • Allergic response •
Asthma, mice • Chronic obstructive pulmonary disease •
Phosphodiesterase type 4 inhibitor • Pulmonary function •
Roflumilast, anti-inflammatory effect

Abstract

Background: Inhibitory effects of roflumilast on responses characteristic of allergic asthma were investigated in a fungal asthma model in BALB/c mice. **Methods:** Mice were sensitized with *Aspergillus* antigen (*Afu*) and exposed to *Afu* or vehicle, and given roflumilast 1 or 5 mg/kg. Early airway response (EAR) and late airway hyperresponsiveness (AHR) to methacholine were measured via plethysmography. Bronchoalveolar lavage (BAL) was used to assess inflammatory cell count. **Results:** In *Afu*-exposed mice, roflumilast dose-dependently reduced the EAR [26% at 1 mg/kg (NS) and 94% at 5 mg/kg ($p < 0.01$)] and AHR [46% at 1 mg/kg (NS) and 128% at 5 mg/kg ($p < 0.05$)]. Roflumilast 5 mg/kg reduced neutrophil, eosinophil and lymphocyte counts [87% ($p < 0.01$), 40% (NS) and 67% ($p < 0.01$), respectively] in BAL fluid versus controls. **Conclusions:** In this model, roflumilast inhibited the EAR, suppressed AHR and reduced inflammatory cell infiltration.

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Introduction

Allergic asthma involves a complex series of reactions within the airways that lead to the early airway response (EAR), characterized mainly by bronchoconstriction, as well as to airway hyperresponsiveness (AHR) and pulmonary inflammation in the late allergic reaction phase. Roflumilast is an oral, once-daily, phosphodiesterase type 4 (PDE4) inhibitor under investigation for the treatment of chronic obstructive pulmonary disease and asthma [1]. Roflumilast has demonstrated anti-inflammatory effects in vitro and in vivo [2–6]. Inhibition of PDE4 increases intracellular cyclic adenosine monophosphate concentrations, which, in turn, activates protein kinase A. Subsequently, phosphorylation and inactivation of target transcription factors lead to attenuation of cellular inflammatory activity [7]. Roflumilast inhibits chemotaxis and cytokine secretion in inflammatory cells, including neutrophils, eosinophils, monocytes and CD4+ T lymphocytes (type 2 T-helper lymphocytes), and reduces the release of tumor necrosis factor α , an important mediator in the pathophysiology of asthma [3]. In preclinical studies, roflumilast attenuated ovalbumin (OVA)-induced bronchoconstriction, EAR and AHR, and inhibited OVA-induced eosinophilia, levels of total protein and tumor

necrosis factor α in bronchoalveolar lavage (BAL) fluid [2, 5, 6], indicating a potential therapeutic role for roflumilast in the treatment of allergic asthma.

A well-established mouse model of allergic hypersensitivity utilizes sensitization and challenge with the antigen of *Aspergillus fumigatus* (*Afu*), an ubiquitous fungus of clinical importance [8, 9]. Significant pulmonary pathology in humans is associated with *Aspergillus*-induced bronchopulmonary aspergillosis. The related asthmatic lung disease is characterized by increased type 2 T helper cytokine generation and immunoglobulin (Ig) E and G levels, eosinophilia, airway obstruction, AHR and airway remodeling [10, 11]. Apart from airway inflammation with eosinophils and bronchial AHR, asthma also typically involves episodic bronchial obstruction. In previous studies, the presented mouse model of asthma has been proven successfully to display the EAR, AHR and inflammation, as well as the pharmacologic modulation of these endpoints [12, 13]. To date, investigations of the effect of roflumilast on pulmonary function have been reported in animal models of OVA-induced early allergic bronchoconstriction in guinea pigs [5] and OVA-induced early AHR in rats [6]. Data in animal models of asthma induced by sensitization with a human-relevant stimulus, such as the *Afu* antigen used in the present study, and data for the late allergic AHR are not available.

The objective of this study was to investigate intragastric roflumilast for its potential to effectively reduce *Afu*-induced early-phase bronchoconstriction by measuring invasive lung function in BALB/c mice. The impact on AHR was also determined 24 h after the final allergen inhalation, and the pulmonary inflammatory response was subsequently characterized by analyzing the differential cell count in BAL fluid.

Methods

Animals

Female BALB/c mice (Charles River, Sulzfeld, Germany), 12 weeks old at the time of the first sensitization, were kept in a pathogen-free rodent facility (Fraunhofer ITEM, Hannover, Germany). The mice had free access to food and water. Care and use of experimental animals conformed to the 'Guide for the care and use of laboratory animals' published by the National Institutes of Health (NIH publication 85-23, revised 1985), and the study was approved by the appropriate governing authority (Bezirksregierung, Hannover, Germany).

Sensitization and Treatment

Mice were sensitized and boosted with *Afu* (Greer Laboratories Inc., Lenoir, N.C., USA) according to the protocol described

previously [13]. Specifically, on day 0, mice received intraperitoneally and subcutaneously injected *Afu* (5 μ g each), which was dissolved in saline (0.9% NaCl) and emulsified with incomplete Freund's adjuvant in a volume of 0.1 ml. On days 14 and 21, mice were boosted by inhalation in a closed Plexiglas® box for 12 min with *Afu* aerosol generated from a 1% solution in 0.9% NaCl (jet nebulizer, LC Star, 2.8 μ m mass median aerodynamic diameter; Pari GmbH, Starnberg, Germany). On day 23, mice were finally challenged with aerosolized *Afu*. A positive control group was *Afu* sensitized and *Afu* challenged like the drug-treated groups. A negative control group was *Afu* sensitized and exposed to the respective vehicle (NaCl on days 14 and 21, and gas-free water on day 23). Roflumilast (Nycomed GmbH, Konstanz, Germany) was suspended in an aqueous 4% methocel solution (Dow Chemicals, Midland, Md., USA) containing 1.3% polyethylene glycol (molecular weight 400; Serva, Heidelberg, Germany) and was administered intragastrically by gavage. In two groups, mice received either roflumilast 1 or 5 mg/kg 1 h before the first boosting with *Afu* aerosol, 1 day and 1 h before the second boosting with *Afu* aerosol, and 1 day and 1 h before the final challenge with *Afu* aerosol.

Allergen Challenge and Lung Function Measurements

Afu-Antigen-Induced EAR

On day 23, mice were anesthetized by intraperitoneal injections, with minimal supplementary injections where required, to a total dose of 23–32 mg/kg etomidate and 0.05–0.07 mg/kg fentanyl (both from Janssen-Cilag GmbH, Neuss, Germany). The technique for invasive, but repetitive, lung function measurements used in this study has been described recently [13, 14]. Briefly, mice were carefully intubated orotracheally. Two intubated, spontaneously breathing mice were then placed in the supine position in temperature-controlled body plethysmographs (type 871, HSE-Harvard Apparatus, March-Hugstetten, Germany). The orotracheal tube was directly attached to a pneumotachograph to determine tidal flow. To measure transpulmonary pressure, a water-filled PE-60 tube was inserted into the esophagus of the mice. Lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were calculated from transpulmonary pressure, tidal flow and volume signals over an entire breath cycle. Respiratory parameters were continuously recorded under baseline conditions as well as during and up to 15 min after challenge with *Afu* using HEM 3.5 software (Notocord, Croissy, France). Maximum R_L values were taken and expressed as percent changes from corresponding baseline values.

After recording baseline values, mice were challenged for 8 min with *Afu* aerosol (3% in gas-free water; Chromasolv®, Sigma-Aldrich, Deisenhofen, Germany), except for the negative control group, which was exposed to the vehicle (gas-free water). Dried aerosols were generated by a computer-controlled, jet-driven, aerosol generator system (Bronchy type III, particle size 2.8 μ m mass median aerodynamic diameter, Fraunhofer ITEM, licensed by Buxco, N.Y., USA), as previously described [14–16]. Aerosol concentrations were determined by 2 gravimetrically calibrated photometers for the 2 mice. The total inhaled dose of 35 μ g *Afu*, administered via the orotracheal tube, was calculated and controlled by a computerized feedback dose control system based on the continuously measured respiratory minute volume and aerosol concentration signals for each mouse. To prevent hypoxia, the mice were provided with 30% oxygen. Registration of lung

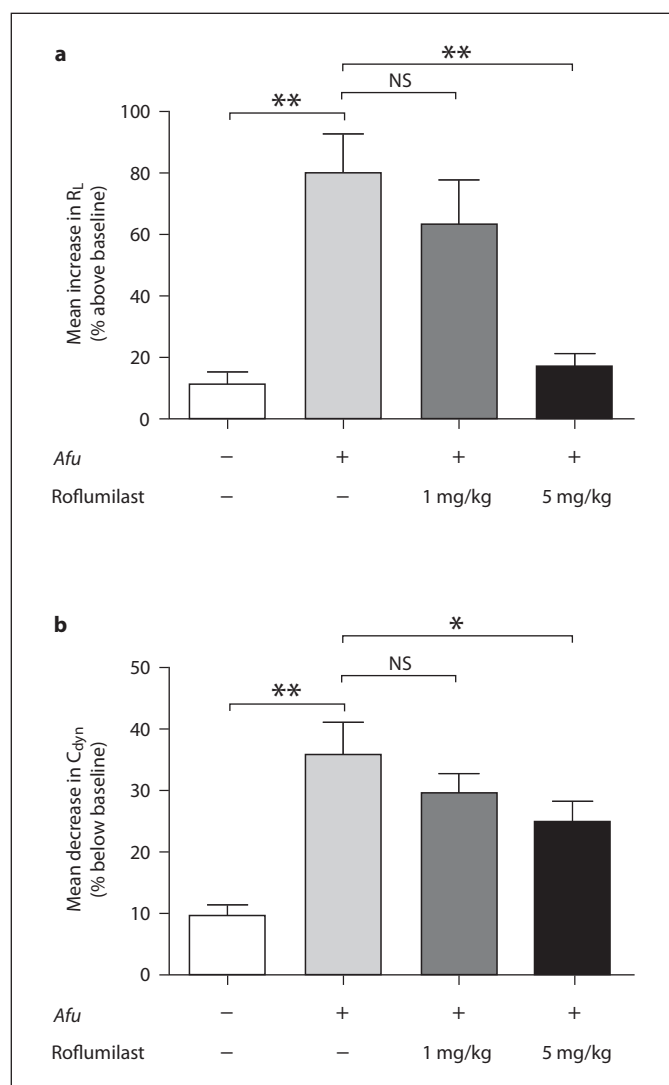


Fig. 1. Columns represent the maximum percent increase in R_L (a) or decrease in C_{dyn} (b) compared with baseline following inhibition of the *Afu*-antigen-induced EAR by roflumilast pretreatment. R_L and C_{dyn} were determined directly before, during and after challenge with aerosolized *Afu* in *Afu*-sensitized mice. Significant inhibition of EAR was observed in mice pretreated with roflumilast 5 mg/kg. Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$ compared with *Afu*-sensitized and *Afu*-challenged mice. Group size: $n = 11$ – 13 ; *Afu*-positive control (*Afu*+), $n = 18$. NS = Not significant.

function was continued for 15 min after the end of the exposure. After the measurements, mice were extubated as soon as they began recovering from anesthesia.

Afu-Antigen-Induced AHR

Twenty-four hours after the final allergen challenge with *Afu*, AHR to inhaled methacholine chloride (MCh) was assessed in the

same mice. Pulmonary function was measured noninvasively with a head-out body plethysmograph system for 4 mice, as previously described [13, 17–19]. Briefly, mice were placed in the plethysmographs, while their heads protruded through a neck collar into a ventilated exposure chamber. Monitoring of pulmonary function was started after a stable level had been reached. For air-flow measurements, a calibrated pneumotachograph with pressure transducer (HSE-Harvard Apparatus) was attached to each plethysmograph. Tidal mid-expiratory flow (EF_{50}) was calculated using HEM 3.5 software (Notocord) from the pneumotachograph tidal-flow signal for each breath. EF_{50} has been established as a measure of bronchoconstriction [13, 17–20]. A reduction in EF_{50} of more than 1.5 standard deviations of mean baseline values, which translates to a reduction of more than 20% versus baseline, is considered to indicate airway constriction.

After recording baseline lung function for 2 min, the mice were exposed to aerosolized MCh at increasing concentrations (2.5, 5.0, 7.5, 10, 15, 20 and 30 mg/ml) using a Pari LC Star Nebulizer (Pari GmbH) until a decrease in EF_{50} of 50% was reached. Minimum values of EF_{50} (as percentage change from baseline) were plotted against inhalational MCh doses calculated for each mouse from measured minute volume and gravimetric MCh concentration. The effective inhalational dose (ED_{50}) was defined as the cumulative dose of MCh required to reduce EF_{50} to 50% of baseline.

Cell Counts in BAL Fluid

After the mice had been sacrificed using an overdose of pentobarbital sodium 25 h after allergen challenge, the lungs of each mouse were lavaged twice with 0.8 ml ice-cold 0.9% NaCl, as previously described [17]. BAL fluid was centrifuged, and the cell pellets were pooled and resuspended in phosphate buffer solution. Total cell counts were determined automatically using a Casy® cell counter (Schärfe System GmbH, Reutlingen, Germany). Cytospots were prepared and Pappenheim stained to evaluate the differential cell counts according to morphologic criteria.

Statistics

Values are reported as means $\pm$ standard error of the mean. To test for significant differences between groups, the Kruskal-Wallis and nonparametric Dunnett tests were performed. Differences were considered statistically significant when $p < 0.05$.

Results

Inhibition of Afu-Antigen-Induced EAR

No significant differences between groups were observed regarding the baseline R_L values (means ranged between 1.5 and 1.8 cm H_2O /ml/s). While challenge with the vehicle did not considerably affect R_L in the negative control group, inhalational *Afu* exposure in the positive control group led to a marked increase in R_L of $80.8 \pm 12.0\%$ ($p < 0.01$) above baseline. Treatment with roflumilast 1 or 5 mg/kg dose-dependently inhibited the EAR (fig. 1a). The increase in R_L was $63.0 \pm 14.9\%$ (NS) in the 1 mg/kg group and $16.0 \pm 5.3\%$ ($p < 0.01$) in the 5 mg/kg group, corre-

sponding to an inhibition of the EAR by 26 and 94%, respectively, compared with the positive control group.

In addition, a significant decrease in C_{dyn} of $36.8 \pm 5.4\%$ was observed in the positive control group compared with minor effects on C_{dyn} in the negative control group (fig. 1b). Mice treated with roflumilast 1 or 5 mg/kg showed dose-dependently lower responses of $30.5 \pm 3.3\%$ (NS) and $25.1 \pm 3.6\%$ ($p < 0.05$), corresponding to an inhibition of 24 and 44%, respectively, compared with the positive control group.

Inhibition of *Afu*-Antigen-Induced AHR

Afu-sensitized and *Afu*-challenged positive control mice were hyperresponsive to MCh (fig. 2). The ED_{50} of the positive controls was $12.8 \pm 1.8 \mu\text{g MCh}$ compared with $19.9 \pm 2.6 \mu\text{g MCh}$ for the *Afu*-sensitized, saline-challenged negative control mice ($p < 0.05$). Mice pretreated with roflumilast 1 or 5 mg/kg were dose-dependently less sensitive to MCh as they tolerated higher doses of MCh and showed ED_{50} values of $16.0 \pm 2.4 \mu\text{g MCh}$ (NS) and $21.9 \pm 3.0 \mu\text{g MCh}$ ($p < 0.05$), respectively, compared with the positive control group (fig. 2). Roflumilast at 1 mg/kg attenuated AHR by 46%. At 5 mg/kg, roflumilast completely prevented AHR, with ED_{50} MCh values exceeding baseline (128%).

Inhibition of *Afu*-Antigen-Induced Airway Inflammation

A marked inflammatory reaction with high numbers of inflammatory cells was observed in the BAL fluid of *Afu*-challenged positive control mice compared with saline-challenged negative controls ($5.19 \pm 0.28 \times 10^5$ and $3.50 \pm 0.29 \times 10^5$ total cells/mouse, respectively; $p < 0.01$). This influx of inflammatory cells was due to changes in the mean number per mouse of: eosinophils 0.04×10^5 versus 0.98×10^5 cells (fig. 3a); neutrophils 0.26×10^5 versus 0.75×10^5 cells (fig. 3b), and lymphocytes 0.02×10^5 versus 0.28×10^5 cells (fig. 3c), in the negative and positive control groups, respectively. All changes in the negative versus positive control groups were statistically significant ($p < 0.01$). The number of macrophages was unchanged ($3.0\text{--}3.2 \times 10^5$ cells/mouse). While treatment with roflumilast 1 mg/kg had no significant effect on inflammatory cells, roflumilast 5 mg/kg inhibited the influx of eosinophils by 40% (NS; fig. 3a), neutrophils by 87% ($p < 0.01$; fig. 3b) and lymphocytes by 67% ($p < 0.01$; fig. 3c) compared with the positive control group. The mean total cell counts per mouse were 5.5×10^5 cells (NS) and 4.0×10^5 cells ($p < 0.05$) for the roflumilast 1 and 5 mg/kg groups, respectively.

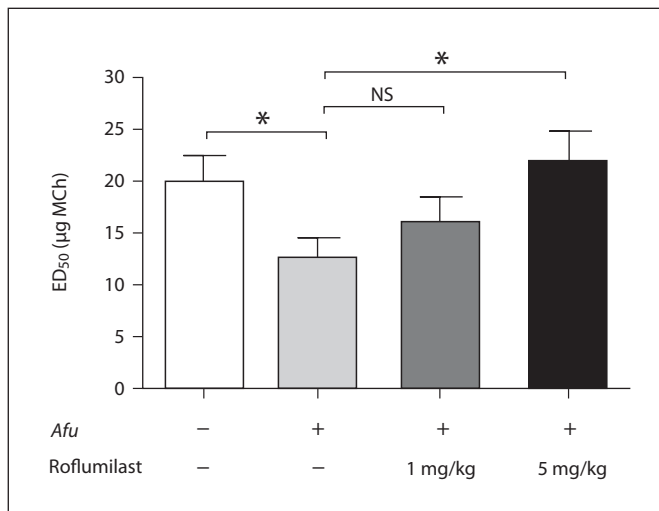
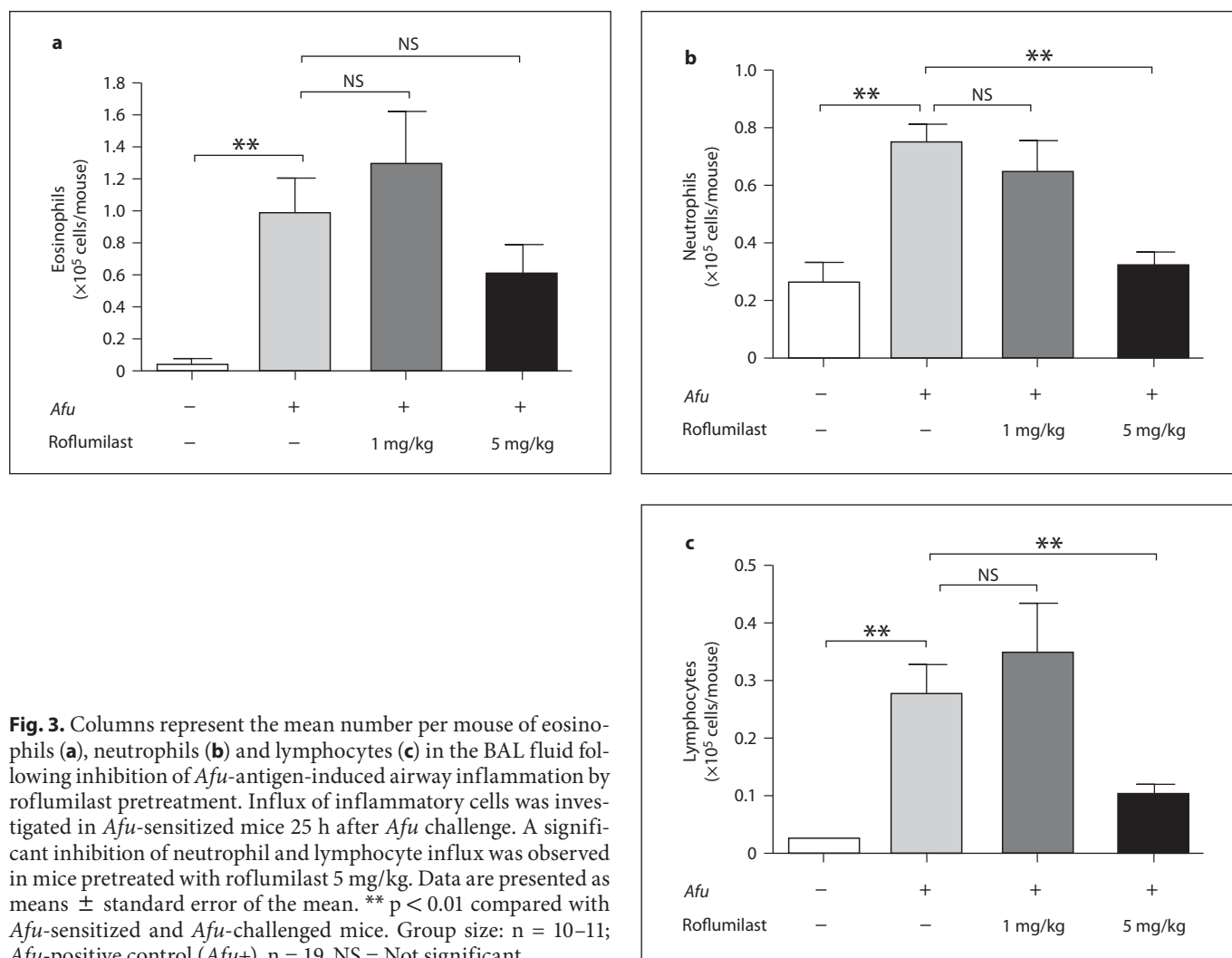


Fig. 2. Columns represent the effective inhalational dose of MCh in micrograms required to decrease EF_{50} by 50% following inhibition of *Afu*-antigen-induced AHR by roflumilast pretreatment. EF_{50} was determined noninvasively directly before, during and after provocation with aerosolized MCh in *Afu*-sensitized mice 24 h after *Afu* challenge. Significant, complete inhibition of AHR was observed in mice pretreated with roflumilast 5 mg/kg. Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$ compared with *Afu*-sensitized and *Afu*-challenged mice. Group size: $n = 10\text{--}12$; *Afu*-positive control (*Afu*+), $n = 16$. NS = Not significant; ED_{50} = the effective inhalational dose of MCh required to reduce mid-expiratory flow (EF_{50}) to 50% of baseline.

Discussion

There are several publications available on the anti-inflammatory efficacy of roflumilast. Considering the studies in animal models of asthma, roflumilast has been reported to reduce OVA-induced bronchoconstriction in vitro [2] and in vivo in guinea pigs [5], to inhibit early adenosine-mediated AHR and the inflammatory response in OVA-sensitized rats [6], and to decrease inflammation and remodeling of the lungs in a chronic mouse model of OVA-induced asthma [4]. The present study is the first to report inhibitory effects of roflumilast on the EAR, late AHR against MCh and inflammatory reactions in mice sensitized and challenged with *Afu* extract containing the human pathogenic *Afu*.

Afu is ubiquitous in nature and is typically found in moist areas of decaying organic matter, such as hay and compost. The tiny spores of *Afu* ($2\text{--}3 \mu\text{m}$) allow for deposition deep into the lungs, which can lead to fungal infections (allergic bronchopulmonary aspergillosis) or



pneumonitis (farmer's lung) [21], and can induce sensitization together with other fungi or common allergens in atopic patients. Furthermore, skin reactivity to fungal allergens has been reported in patients with life-threatening asthma, and patients with severe asthma requiring multiple hospital admissions are commonly sensitized to mold [22]. In addition, asthma severity has been shown to correlate with the degree of dampness and mold growth in the homes of asthma patients [23].

In this study, allergen challenges were performed in the intubated mouse to administer the antigen directly into the lungs and to exclude nasal effects, as well as to obtain high inspiratory-antigen concentrations and high deposition of the dose. The EAR, starting almost immediately after antigen exposure, is an important patho-

physiologic feature of allergic asthma that can be effectively treated by fast-acting substances, such as β_2 -agonists. A therapeutic effect of roflumilast on the EAR has only been investigated in OVA-sensitized guinea pigs to date [5]. This is the first study that has demonstrated pronounced effects of roflumilast on antigen-induced bronchoconstriction during the EAR in the lungs of BALB/c mice sensitized with *Afu*.

After *Afu* challenge, a marked increase in R_L , as well as a significant decrease in C_{dyn} , was observed. R_L reflects airway resistance predominantly in the larger, central airways, whereas C_{dyn} is thought to reflect obstruction in the smaller, more peripheral airways [24–26]. In the present study, the positive control group showed a marked increase in R_L and a decrease in C_{dyn} , which typically rep-

resents EAR with central and peripheral bronchoconstriction, as shown previously using the same mouse model and techniques [12, 13]. Pretreatment with roflumilast 5 mg/kg inhibited the EAR in both the central and peripheral airways.

In a different EAR model, OVA-sensitized guinea pigs were treated with oral roflumilast and challenged with OVA (0.15 mg/kg intravenously) after 1 h following premedication with indomethacin, pyrilamine and propranolol to assess the predominantly leukotriene-mediated bronchoconstriction. Oral pretreatment with roflumilast at 0.4 or 4 mg/kg significantly inhibited the EAR by 58 and 81%, pulmonary cysteinyl leukotriene release by 85 and 98%, and leukotriene B₄ release by 67 and 69%, respectively [5]. Because guinea pigs develop a more IgG- than IgE-mediated asthmatic response, and the EAR had to be abated by pretreatment with antihistamines, it was of interest to see whether these findings could be validated in another species using a more IgE-mediated asthma model. Despite the differences in the two animal models, the data on roflumilast efficacy for resistance (large airway response) presented here were comparable to data obtained in the guinea pig model. Furthermore, in patients with allergic asthma, oral roflumilast administered once daily modestly attenuated allergen-induced EAR by 28% [27].

In the present study, 1 day after antigen challenge, AHR was assessed by noninvasive head-out plethysmography and by evaluation of the EF₅₀. This method has been proven to be valid in assessing bronchoconstriction, especially in AHR asthma models [13, 17, 18, 20, 28]. AHR in the late allergic phase is a central pathophysiologic alteration in bronchial asthma in humans and is a significant marker of the severity and progression of this disease. In comparison with the negative controls, mice in the positive control group required a significantly lower MCh dose to produce a 50% decrease in EF₅₀ and, therefore, had developed a marked AHR that underlines the validity of the *Afu*-asthma model.

In roflumilast-treated mice, AHR was significantly attenuated in a dose-dependent manner. While roflumilast 1 mg/kg led to a 46% inhibition of AHR, which was not statistically significant, the 5 mg/kg dose significantly inhibited AHR, and ED₅₀ MCh values exceeded baseline (128% inhibition). These data are comparable to those obtained in OVA-sensitized Brown Norway rats pretreated with intragastric roflumilast 3 mg/kg, which showed a statistically significant inhibition of AHR by 68% 48 h after OVA challenge [29]. The findings are also in good accordance with those observed in another OVA chal-

lenge model in Brown Norway rats [6]. Here, oral pretreatment with roflumilast (1 or 3 mg/kg) led to a marked inhibition of adenosine-mediated AHR in OVA-sensitized rats 200 min after OVA challenge. In addition, in a chronic asthma model in OVA-sensitized mice, roflumilast slightly decreased AHR; however, this result was not statistically significant [4].

In an allergen challenge study in patients with mild asthma, a single oral dose of roflumilast significantly decreased the development of late AHR against histamine by approximately 1.1 doubling dilutions [30]. In a further clinical study of patients with allergic asthma, treatment with roflumilast for 4 weeks significantly reduced AHR against adenosine monophosphate [31].

In the present study, we also investigated the late inflammatory response and observed pronounced elevation of total cell numbers and the number and percentage of eosinophils, neutrophils and lymphocytes in the BAL fluid of the positive control mice compared with the negative control group. Treatment with the high dose of roflumilast (5 mg/kg) resulted in a decrease in the total cell count. This was mainly due to a suppressed influx of eosinophils, neutrophils and lymphocytes.

Our findings of the inhibitory effects of roflumilast on pulmonary inflammatory cell accumulation are in good accordance with previous studies in other animal models and with recently reported clinical data. In OVA-sensitized Brown Norway rats, for example, roflumilast led to a marked decrease in eosinophilia 48 h after allergen challenge [2] and inhibited pulmonary neutrophilia 4 h after allergen challenge [6]. In addition, roflumilast inhibited accumulation of eosinophils and CD3+ T cells in a chronic asthma model in OVA-sensitized mice [4].

In a 4-week study in patients with stable asthma, roflumilast significantly reduced sputum eosinophilia by 57% [32]. In a separate allergen challenge study in patients with stable asthma, roflumilast demonstrated its anti-inflammatory properties by attenuating the allergen-induced influx of inflammatory cells, mainly eosinophils and neutrophils, into the airways [33]. There is also evidence from preclinical studies that the suppression of inflammatory cell recruitment by roflumilast is due to inhibition of the release of chemotactic mediators (e.g. leukotriene B₄), inhibition of the expression of adhesion molecules (e.g. E- and P-selectin) and by the reduction of leukocyte-endothelial interaction regarding leukocyte rolling, adhesion and emigration [34].

In the present study, we investigated the effect of roflumilast at doses of 1 and 5 mg/kg. The inhibition of the *Afu*-antigen-challenge-mediated changes in the lung of mice was modest at 1 mg/kg, but pronounced and statistically significant at 5 mg/kg. These therapeutic effects, and the dose-dependency observed, can be explained by the estimated mean free-plasma levels and by the potency of roflumilast and its active metabolite roflumilast N-oxide in inhibiting PDE4 enzymes. The inhibition of the catalytic domain of the PDE4 enzymes, PDE4A, B, C and D, has previously been reported, with IC₅₀ values of 0.11–0.61 nmol/l for roflumilast and 0.31–3.2 nmol/l for roflumilast-N-oxide [35]. Based on these IC₅₀ values, a dose of roflumilast 1 mg/kg is expected to result in mean free plasma concentrations of roflumilast and its N-oxide metabolite that are below or in the range of their IC₅₀ values. A dose of roflumilast 5 mg/kg results in higher mean free-plasma concentrations that exceed IC₅₀ values 2- to 20-fold [unpubl. data]. Despite blood samples not being analyzed in the present study, this estimation offers a rationale as to why the use of roflumilast at 1 mg/kg in this study and in previous studies in mice represents a marginally effective dose, while significant therapeutic effects occur with roflumilast 5 mg/kg.

Conclusion

Roflumilast has shown efficacy in key markers of asthma in a mouse model of fungal allergic airway reactions. Roflumilast inhibited the EAR, suppressed AHR and reduced inflammatory cell infiltration. These anti-inflammatory effects of roflumilast provide further support for its use in the treatment of patients with asthma.

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Conflicts of Interest

This study was conducted as contract research by Fraunhofer ITEM. This study was sponsored by Nycomed GmbH (formerly Altana Pharma AG), Konstanz, Germany. The authors H.-G.H., M.M., R.K., N.K. and A.B. are members of Fraunhofer ITEM. L.W. is a former employee of Altana Pharma AG (now Nycomed GmbH), and R.B. is an employee of Nycomed GmbH; both were representatives of the sponsor for this study.

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