

Effects of KF19514, a Phosphodiesterase 4 and 1 Inhibitor, on Bronchial Inflammation and Remodeling in a Murine Model of Chronic Asthma

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ABSTRACT

Background: Phosphodiesterase 4 selective inhibitor may prevent airway inflammation and remodeling.

Objective: The aim of this study was to investigate the effects of KF19514, a phosphodiesterase 4 and 1 dual inhibitor, on chronic airway inflammation and remodeling following chronic exposure to aerosolized antigen in mice.

Methods: Ovalbumin (OVA) was administered intraperitoneally to BALB/c mice on days 0 and 14, and the mice were then exposed to aerosolized OVA daily for 4 weeks. Twenty-four hours following the final inhalation, bronchial responsiveness to acetylcholine was measured, and histologic examination and hydroxyproline content of the lung were evaluated.

Results: Bronchial responsiveness to acetylcholine, number of inflammatory cells and eosinophils in the lamina propria, thickness of epithelial and subepithelial collagen layers, and hydroxyproline content of the lung increased following chronic exposure to OVA for 7 weeks. KF19514 significantly prevented all of these changes.

Conclusions: Phosphodiesterase 4 and 1 inhibitors such as KF19514 may help prevent bronchial hyperresponsiveness and chronic asthma-induced airway remodeling.

KEY WORDS

airway hyperresponsiveness, airway remodeling, asthma, murine model, PDE inhibitor

INTRODUCTION

A characteristic feature of airway pathology in patients with chronic asthma, in addition to chronic eosinophilic airway inflammation, is an increase in the extracellular matrix within the lamina propria layer beneath the true epithelial basement membrane, namely subepithelial fibrosis.¹ Remodeling of the airway structure is thought to be due to an increased deposition of collagen I, III, and V; fibronectin; and tenascin in this area¹⁻³ in association with subepithelial myofibroblast proliferation,⁴ which

reflects the consequences of recurrent injury and aberrant repair processes in the airway wall. Subepithelial fibrosis may partially contribute to the increase in airway wall stiffness, poor airway reversibility, and poor responsiveness to inhaled corticosteroids even after prolonged therapy.⁵⁻⁷ The remodeling changes correlate with the development of airway hyperreactivity (AHR)⁸ and with persistent airflow limitations in chronic asthma patients.⁹ Long-term titration of inhaled glucocorticoid therapy to reduce AHR, which substantially improves clinical outcome, is associated with a significant reduction in subepithelial fibrosis.¹⁰

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Received 14 October 2008. Accepted for publication 16 December 2008.

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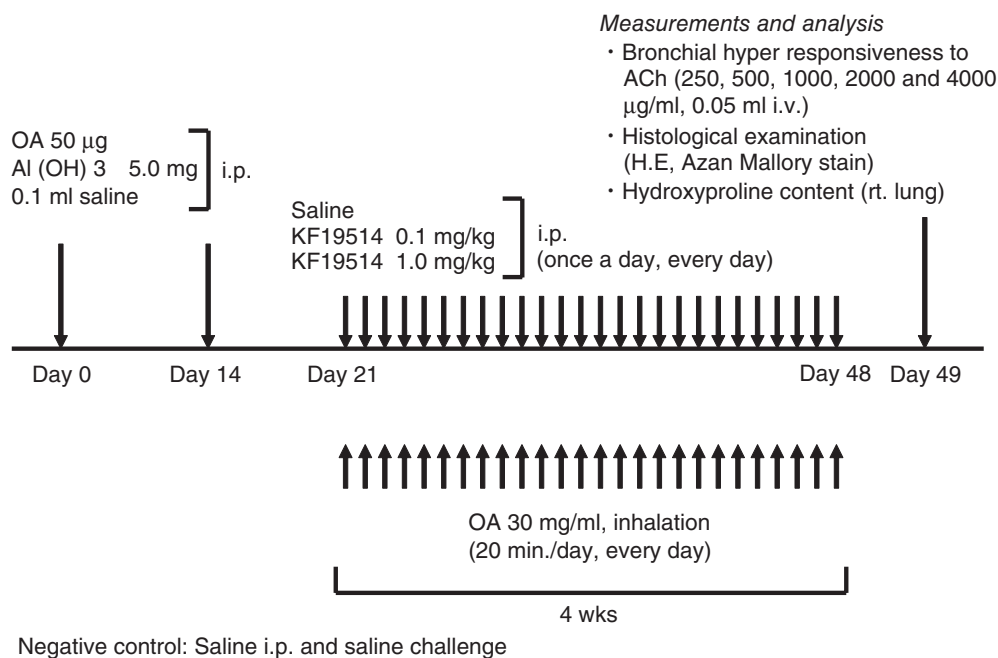


Fig. 1 Experimental protocol

Phosphodiesterase (PDE) isoenzymes control the degradation of cyclic adenosine monophosphate and thus have key roles in regulating compartmentalized signaling responses to intracellular gradients of cyclic AMP.¹¹ The PDE4 family of enzymes, comprises four *PDE4* genes, which generate at least 16 different isoforms, exclusively hydrolyze cyclic AMP, and most of this activity occurs within cells.¹² KF19514, a novel potent PDE4 and PDE1 dual inhibitor, is an effective anti-inflammatory agent in animal models of acute allergen challenge, inhibiting antigen-induced bronchospasm.¹³⁻¹⁵ Roflumilast, a novel potent PDE4-selective inhibitor,¹⁶ inhibits airway inflammation and remodeling,¹⁷ however, whether PDE4 and PDE1 inhibitors prevent airway inflammation and remodeling in chronic asthma is unknown. We therefore examined the ability of KF19514 to inhibit airway inflammation, remodeling, and AHR in mice following chronic exposure to aerosolized antigen.

METHODS

ANIMALS

Specific pathogen-free female BALB/c mice (6 to 8 weeks of age) were obtained from Ninnox Laboratory Service (Ishikawa, Japan). After arrival at the Institute of Animal Experiments at our university, the mice were maintained in conventional animal housing facilities for 1 week before use and were allowed to drink and eat ad libitum. The experiment was performed according to the Principles of Laboratory Animal Care formulated by the Institute of Animal Experiments at Kanazawa University.

EXPERIMENTAL PROCEDURE

Sensitization of mice with OVA was performed according to the method by Temelkovski *et al.*¹⁸ with minor modifications. A mixed suspension of 50 g OVA and 5 mg aluminum hydroxide in 0.1 ml physiologic saline was administered intraperitoneally to mice once on Days 0 and 14. From Day 21, 30 minutes after intraperitoneal administration of 0.1 or 1.0 mg/kg of KF19514 or physiologic saline, mice were placed in a closed plastic inhalation chamber (length × width × height = 24 cm × 21 cm × 17 cm) and exposed to aerosol consisting of 3.0% w/v OVA suspended in physiologic saline for 20 minutes, every day for 4 weeks. Aerosol with particle diameters between 1 and 10 µm, was generated and administered at a flow of 0.3 ml/min using an ultrasonic nebulizer (Model C-13, Omron, Tokyo, Japan) in the exposure chamber. Control animals were treated in a similar way except that the physiologic saline did not contain OVA and aluminum hydroxide (Fig. 1).

MEASUREMENT OF AIRWAY RESPONSIVENESS

We anesthetized the mice with 2.5 mg, i.p. sodium pentobarbital 24 hours after the final inhalation of antigen then placed them in a supine position. The trachea was incised and cannulated with a 20-gauge nylon needle (inner diameter; 0.80 mm, length; 32 mm), and animals were artificially ventilated using a small-animal respirator (Model 1680, Harvard Apparatus, South Natick, MA, USA) adjusted to deliver a tidal volume of 0.5 ml at a rate of 60 strokes/minute. The change in lung resistance to inflation, and the lateral

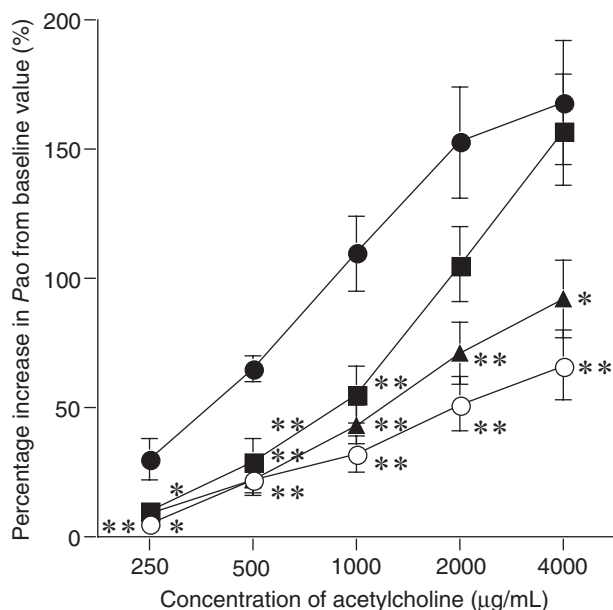


Fig. 2 Airway reactivity of unexposed and OVA-sensitized chronically exposed mice to saline alone or 0.1 mg/kg and 1.0 mg/kg KF19514, as assessed by the change in *Pao* in response to increasing concentrations of aerosolized acetylcholine. Circles indicate response in saline-exposed animals (naïve mice) ($n = 8$). Solid circles indicate response after administration of saline in OVA-sensitized mice ($n = 10$). Solid squares indicate response after administration of 0.1 mg/kg KF19514 in OVA-sensitized mice ($n = 10$). Solid triangles indicate response after administration of 1.0 mg/kg KF19514 in OVA-sensitized mice ($n = 10$). Each point indicates mean SEM. Significant differences compared with saline-treated OVA-sensitized mice are shown as *, $p < 0.05$; **, $p < 0.01$.

pressure in the tracheal tube (pressure at the airway opening; *Pao*, cmH₂O), was determined using the modified method by Konzett and Rossler described by Jones *et al.*¹⁹ using a pressure transducer (Model TP-603T, Nihon Koden Kogyo, Tokyo, Japan). Since the change in *Pao* following inhalation of leukotriene C₄ (LTC₄) represents the average of the changes in pulmonary resistance (*R_L*) and reciprocal dynamic lung compliance ($1/C_{dyn}$),²¹ we used *Pao* as an overall index of bronchial response to the bronchoactive agent. Airway reactivity to intravenous acetylcholine²⁰ was measured 24 hours after the final inhalation of antigen. Spontaneous breathing was blocked by injection of pancuronium bromide (0.1 mg/kg) into the jugular vein. Ten minutes after initiating artificial ventilation, when the *Pao* was stabilized, five successive intravenous administrations of acetylcholine hydrochloride (250, 500, 1000, 2000, and 4000 g/mL, 0.05 mL/animal) were performed at 5-minute intervals with constant monitoring of the bronchopulmonary response. *Pao* was continuously recorded as

described above and expressed as a percentage of the baseline value.

HISTOLOGIC STUDY

After the measurement of acetylcholine-induced bronchoconstriction as described above, the mice were killed with an overdose of pentobarbital. The left lung was removed and inflated with 1 mL of 10% neutral buffered formalin and sectioned. After fixation overnight in formalin, the tissue was embedded in paraffin. The sections were cut in the longitudinal plane of the bronchus and lung, stained in one batch with either haematoxylin and eosin (H&E) for measuring the thickness of the epithelial cell layer and the inflammatory cell (lymphocyte, neutrophil, eosinophil) numbers, or Azan-Mallory solution for determining subepithelial collagen deposition. The sections were then coded for morphometry. Histologic analysis was performed by an observer with no knowledge of the experimental procedures.

After general observation, the thickness of the epithelial cell layer was measured on H&E-stained sections, randomly selected from each animal. The analyses were made along either side of the longitudinally cut airway wall. The measurements were made only in bronchi greater than 150 μm in diameter and at locations where the epithelium appeared intact and well-orientated to minimize the influence of tangential sectioning, using an oil immersion objective ($\times 1000$) and an eyepiece graticule as previously described.^{18,21} The data were recorded to the nearest half division on the reticle (0.5 μm). After the measurements were completed in one field, the microscopic stage was advanced a linear distance of two microscopic fields and measurements were repeated. For each animal, the average thickness of the epithelial cell layer was determined for 20 fields and at least 75 points (mean 81 ± 3) at 20 μm intervals. The areas covered 1.6 mm of the basement membrane length. The mean thickness of the epithelial cell layer was then calculated.

The density of eosinophils in the lamina propria was also measured in H&E-stained sections. The lamina propria was defined as the zone between the epithelial basement membrane and the luminal border of the tracheal cartilage plates and smooth muscle layer in the bronchi. Eosinophils were readily identified based on specifically staining granules in cytoplasm. When eosinophils were counted in a field, the length of the epithelial cell layer was measured. For each animal, at least 15 fields, which covered 2.3 mm of epithelial basement membrane length were analyzed, and the number of cells per 1000 μm² basement membrane was determined. Measurement of the epithelial thickness was performed in the same field used for cell counting, with at least 23 (mean 28 ± 2) measurements for each animal.

The thickness of the subepithelial collagen deposi-

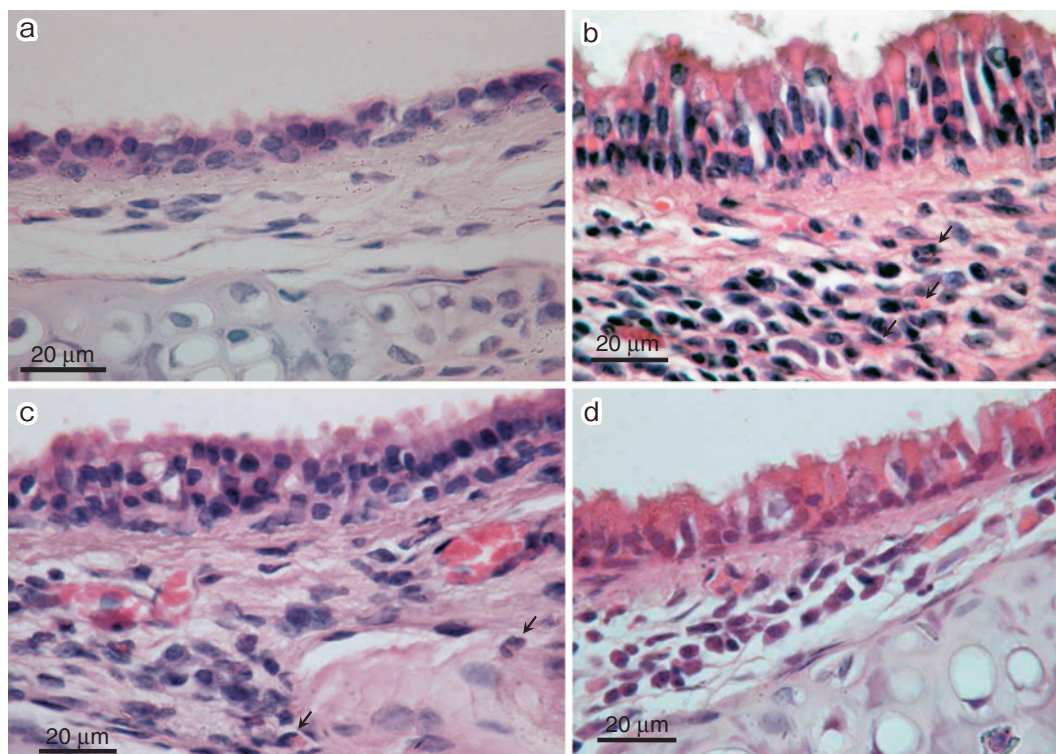


Fig. 3 Photomicrographs showing inflammatory cells and eosinophils (arrows) in the lamina propria and thickening of the epithelial and subepithelial collagen layers. Lung tissues were obtained from unexposed mice (a) OVA-sensitized chronically exposed mice treated with saline; (b) 0.1 mg/kg KF19514; (c) 1.0 mg/kg KF-19514 and (d) (H&E stain). Original magnification, $\times 1000$.

tion areas was analyzed on Azan-Mallory-stained sections in the same way as described above for determining the thickness of the epithelium basement membrane. On average, the positive zones beneath the epithelium were measured in 20 fields and at 80 points on each section. The data were taken along 1.5 to 1.8 mm of the basement membrane from which the mean thickness of the subepithelial collagen layer was calculated for each mouse.

MEASUREMENT OF AMOUNT OF HYDROXYPROLINE IN THE LUNG

The right lung of each mouse was removed and stored at -80°C for the measurement of hydroxyproline immediately after the measurement of acetylcholine-induced bronchoconstriction. The extent of pulmonary fibrosis was evaluated by estimating total lung collagen deposition as reflected by hydroxyproline content measured in control and antigen-exposed mice. Measurement of the amount of hydroxyproline was performed according to the method described by Huszar *et al.*²³ with minor modifications.

CHEMICALS

The following chemicals were used: OVA (Sigma Chemicals, St. Louis, MO, USA), sodium pentobarbi-

tal (Abbott Laboratories, North Chicago, IL, USA), aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan), pancuronium bromide (Tocris Cookson, Bristol, UK), acetylcholine hydrochloride (Wako Pure Chemical Industries, Osaka, Japan), and KF19514 (5-phenyl-3-(3-pyridil) methyl-3*H*-imidazo [4,5-*c*] [1,8]naphthyridin-4 (5*H*)-one (Chemical Synthesis Division of Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo, Shizuoka, Japan).

STATISTICAL ANALYSIS

The mean and standard error (SEM) were calculated for all parameters in this study. Statistical analyses were performed by analysis of variance (ANOVA) for four groups and unpaired *t*-test for two groups. Dose-response curves for acetylcholine-induced bronchoconstriction were also analyzed by two-factor repeated measures ANOVA. A *P* value of 0.05 or less was considered to indicate a statistically significant difference.

RESULTS

ACETYLCHOLINE-INDUCED BRONCHOCONSTRICTION

We first evaluated the bronchial responsiveness to acetylcholine. Figure 2 shows dose-response curves for the effect of intraperitoneal administration of

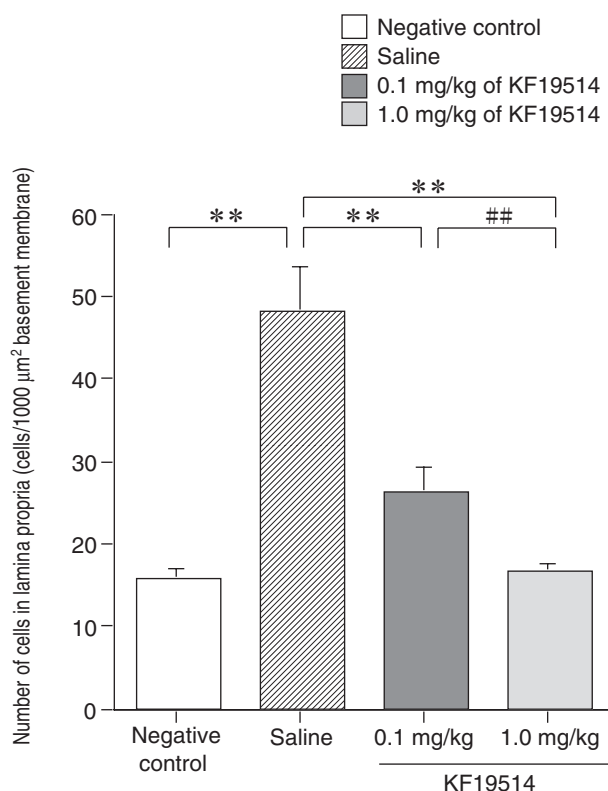


Fig. 4 Inflammatory cells in the lamina propria of the trachea of OVA-sensitized, chronically exposed mice treated with saline ($n = 10$) compared to unexposed control mice ($n = 8$) or to mice treated with 0.1 mg/kg KF19514 ($n = 10$) or 1.0 mg/kg KF19514 ($n = 10$). Each bar indicates mean SEM. Significant differences compared with saline-treated OVA-sensitized mice are shown as **, $p < 0.01$. Significant differences compared with 0.1 mg/kg KF19514-treated OVA-sensitized mice are shown as ##, $p < 0.01$.

KF19514 on acetylcholine-induced bronchoconstriction. Chronic exposure to OVA induced significant bronchial-hyperresponsiveness in OVA-sensitized mice ($n = 10$), as compared with saline-exposed animals (naïve mice, $n = 8$; $p < 0.01$). The dose-response curves of the acetylcholine-induced increase in *Pao* was significantly and dose-dependently inhibited by KF19514 (Fig. 2).

ANALYSIS OF MORPHOLOGIC CHANGES AFTER CHRONIC ANTIGEN EXPOSURE

Thereafter we evaluated the morphologic changes after chronic OVA exposure in mice. Consistent with the morphologic analyses of H&E-stained tissue samples (Fig. 3), chronic OVA exposure resulted in significant increases in the amount of peribronchial and perivascular infiltration of inflammatory cells, predominantly consisting of eosinophils infiltrating the lamina propria in the airway of OVA-sensitized mice ($n = 10$), as compared with saline-exposed mice ($n =$

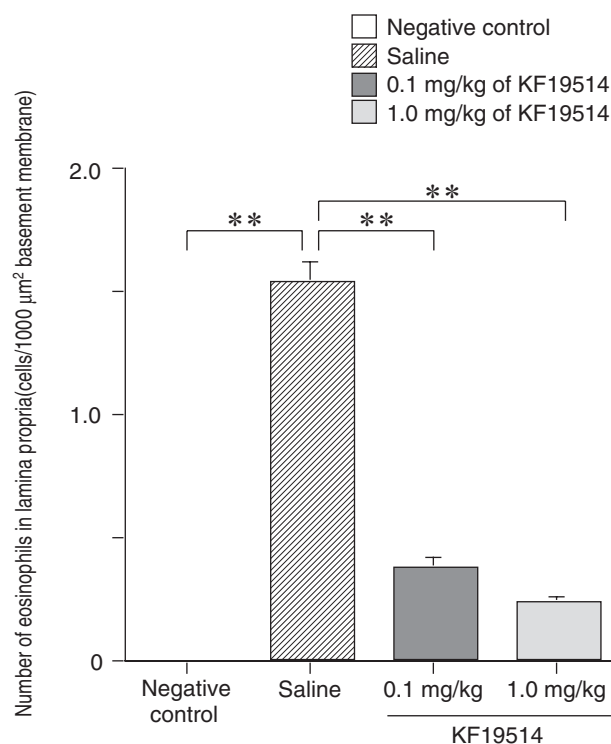


Fig. 5 Eosinophils in the lamina propria of the trachea of OVA-sensitized, chronically exposed mice treated with saline ($n = 10$) compared to unexposed control mice ($n = 8$) or to mice treated with 0.1 mg/kg KF19514 ($n = 10$) or 1.0 mg/kg KF19514 ($n = 10$). Each bar indicates mean SEM. Significant differences compared with saline-treated in OVA-sensitized mice are shown as **, $p < 0.01$.

10). Intraperitoneal administration of KF19514 inhibited an increase in inflammatory cells, consisting of eosinophils, in the lamina propria in the airway of mice after chronic OVA exposure in a dose-dependent manner (Fig. 4, 5). Moreover, chronic OVA exposure induced significant increases in the thickness of the epithelial and subepithelial collagen layers in the airway of OVA-sensitized mice, compared with saline-exposed mice. Intraperitoneal administration of KF19514 decreased the thickness in epithelial and subepithelial collagen layers after chronic OVA exposure in a dose-dependent manner (Fig. 6, 7).

HYDROXYPROLINE OF THE LUNG

We evaluated the amount of hydroxyproline in the lung. Chronic exposure to OVA resulted in a significant increase in the hydroxyproline content of the right total lung, compared with saline-exposed mice. Intraperitoneal administration of KF19514 inhibited the increase in hydroxyproline content after chronic OVA exposure in a dose-dependent manner (Fig. 8).

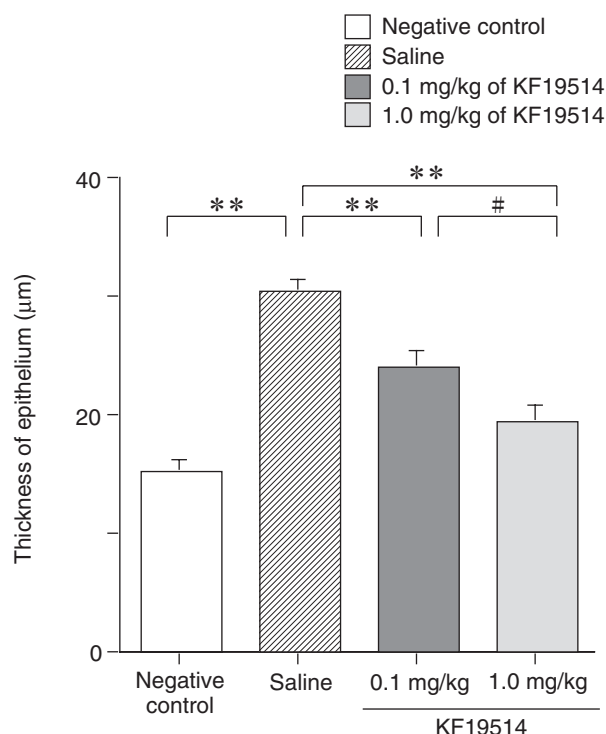


Fig. 6 Thickness of epithelium in the trachea of OVA-sensitized, chronically exposed mice treated with saline ($n = 10$) compared to unexposed control mice ($n = 8$) or to mice treated with 0.1 mg/kg KF19514 ($n = 10$) or 1.0 mg/kg KF19514 ($n = 10$). Each bar indicates mean SEM. Significant differences compared with saline-treated OVA-sensitized mice are shown as **, $p < 0.01$. Significant differences compared with 0.1 mg/kg KF19514-treatment in OVA-sensitized mice are shown as #, $p < 0.05$.

DISCUSSION

We set out to determine whether selective PDE 4 and 1 dual inhibitor KF19514 might be useful for preventing bronchial hyperresponsiveness and airway remodeling in asthma. Airway remodeling is now considered to be an important feature of chronic asthma and is postulated to promote the progression of the disease to a chronic irreversible stage.²⁴ The pathophysiology of remodeling and its effects on airway physiology remain to be fully established. Airway inflammation and remodeling in chronic asthma are characterized by airway eosinophilia, hyperplasia of goblet cells and smooth muscle, and subepithelial fibrosis. Chronic airway inflammation is an important aspect of asthma pathogenesis.¹ Eosinophil infiltration into the airway and airway hyperreactivity are now thought to be regulated by Th2 cytokines and chemokines.²⁵ Interleukin-4 (IL-4) and IL-13 are essential for immunoglobulin E production, while IL-5, eotaxin, and RANTES, a member of the 8-kDa cytokine family possesses chemotactic activity for

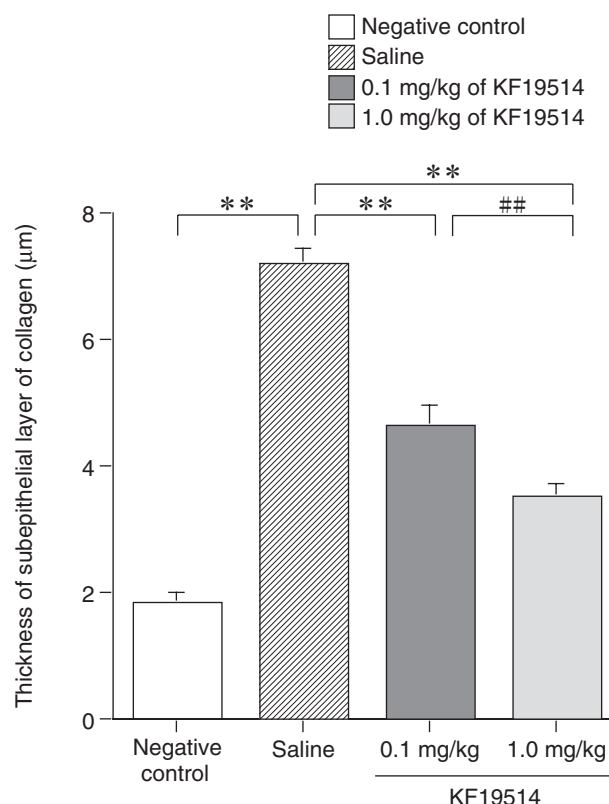


Fig. 7 Thickness of subepithelial collagen layer of the trachea of OVA-sensitized, chronically exposed mice treated with saline ($n = 10$) compared to unexposed control mice ($n = 8$) or to mice treated with 0.1 mg/kg KF19514 ($n = 10$) or 1.0 mg/kg KF19514 ($n = 10$). Each bar indicates mean SEM. Significant differences compared with saline-treated OVA-sensitized mice are shown as **, $p < 0.01$. Significant differences compared with 0.1 mg/kg KF19514-treated OVA-sensitized mice are shown as ##, $p < 0.01$.

monocytes and CD4 T cells, are important for eosinophil recruitment.²⁶ Monocyte chemoattractant protein-1 is involved in airway hyperreactivity via its capability to degranulate mast cells.²⁷

In this study, we used a mouse model of chronic asthma established by inducing airway inflammation and airway wall remodeling over a 4-week period and promoted through continued antigen exposure.¹⁸ We investigated the effect of PDE 4 and 1 dual inhibitor KF19514 in this mouse model of antigen-induced chronic airway inflammation and fibrosis. Chronic exposure of OVA-sensitized mice to aerosolized OVA-induced bronchial hyperresponsiveness and airway eosinophilic inflammation and fibrosis. Intraperitoneal administration of KF19514 inhibited the chronic OVA exposure-induced bronchial hyperresponsiveness, airway inflammation, and fibrosis in a dose-dependent manner.

Recently, at least 11 different cyclic nucleotide PDE isozymes were identified based on functional

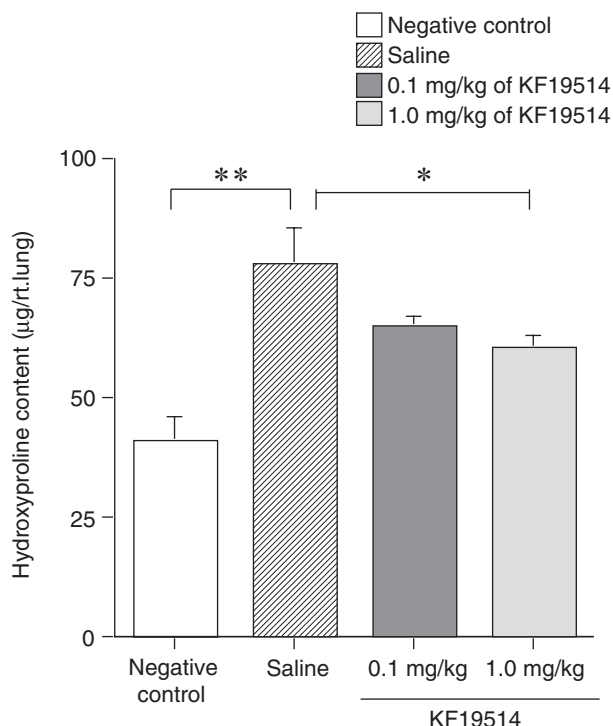


Fig. 8 Hydroxyproline content in OVA-sensitized, chronically exposed mice treated with saline ($n = 10$) compared to unexposed control mice ($n = 8$) or to mice treated with 0.1 mg/kg KF19514 ($n = 10$) or 1.0 mg/kg KF19514 ($n = 10$). Each bar indicates mean SEM. Significant differences compared with saline-treated in OVA-sensitized mice are shown as *, $p < 0.05$; **, $p < 0.01$.

characteristics, such as substrate specificity and susceptibility to selective inhibitors.²⁸ KF19514 is a new potent and highly-selective inhibitor of PDE4 and PDE1 isoenzymes.²⁹ The IC_{50} values of KF19514 for PDE1, PDE2, PDE3, PDE4, and PDE5, derived from canine tracheal smooth muscle, are 0.27, > 10 , > 10 , 0.40, and > 10 $\mu\text{mol/L}$, respectively.¹³ Both oral and intravenous administration of PDE4 inhibitors such as rolipram and Ro-20-1724 have antiallergic effects³⁰⁻³³ as well as bronchodilator effects.^{34,35} Howell and coworkers³⁰ reported that rolipram has pulmonary antiallergic effects, including the inhibition of antigen-induced full and leukotriene-dependent bronchoconstriction and prevention of antigen-induced airway hyperreactivity, with minimal relaxant effects on airway smooth muscle. Teixeira *et al.*³⁶ reported that rolipram effectively inhibits allergic- and mediator-induced eosinophil accumulation. On the other hand, PDE1 inhibition by KF19514 does not seem to contribute to the inhibition of inflammatory cell accumulation, because vinpocetine, a selective PDE1 inhibitor, does not inhibit eosinophil accumulation.^{37,38} These findings suggest that the inhibitory effects of KF19514 on bronchial hyperresponsiveness, airway inflammation, that develop following chronic

antigen exposure result from KF19514 antagonism of PDE4 and not PDE1.

Selective PDE4 inhibitors, which act against the catalytically active site, inhibit the function of a variety of inflammatory and immunomodulatory cells.³⁹ These compounds decrease the production of numerous relevant inflammatory mediators, including histamine and leukotrienes,⁴⁰ proinflammatory cytokines such as tumor necrosis factor- α and IL-1,⁴¹ and T-lymphocyte-derived cytokines such as IL-4 and IL-5.⁴² Corbel *et al.* reported that selective PDE4 inhibitors such as RP 73-401 might modulate the expression of airway remodeling-associated mediators such as matrix metalloproteinase-9 and transforming growth factor- α and that this effect may be at least partially mediated by the balance between tumor necrosis factor- and IL-10.⁴³

Here, we did not evaluate inflammatory mediators such as histamine and leukotrienes, proinflammatory cytokines such as tumor necrosis factor- α , IL-1, IL-4, and IL-5, and airway remodeling-associated mediators such as matrix metalloproteinase-9 and transforming growth factor- α . Airway inflammation and remodeling are thought to have a profound effect on airway response. Inflammatory cells give rise to airway responsiveness by releasing chemical mediators, cytokines, and chemokines in the local microenvironment, resulting in physiologic changes observed in asthma and sustained airway inflammation. Increased airway wall thickness caused by the remodeling process strengthens the effect of smooth-muscle contractions as explained by Poiseuille's law of laminar flow, and contributes to airway hyperresponsiveness.⁴⁴ Roflumilast, a novel potent PDE4-selective inhibitor, is a potent inhibitor of airway inflammation and remodeling.¹⁷

PDE1 was implicated in human vascular smooth muscle proliferation^{45,46} and it is tempting to speculate that one or more PDE1 isoenzymes could serve the same function in airway myocytes. In humans, PDE1s are encoded by three genes (PDE1A, PDE1B and PDE1C), Rybalkin *et al.*⁴⁵ found that PDE1C was markedly induced in proliferating, but not in quiescent, smooth muscle cells that are derived from human aorta. Furthermore, as in human airway myocytes, this enzyme was the predominant PDE in these cells. The induction of PDE1C lowers cAMP in vascular myocytes and therefore relieves an endogenous 'break', which allows mitogenesis to proceed unhindered. Given that airway remodeling is a characteristic of COPD and other airway inflammatory diseases, such as chronic severe asthma, dual-specificity inhibitors of PDE1C and PDE4 might selectively target proliferating airway smooth muscle cells and, by doing so, retard the remodeling process (PDE1C-mediated) and arrest inflammation (by way of PDE4 inhibition).⁴⁷

KF19514, a PDE4 and PDE1 dual inhibitor, showed

an antiallergic effect, probably via its inhibitory activity of antigen-induced mediator release, in addition to a functional bronchoprotective effect via PDE4 inhibition and inhibition through the remodeling of airway smooth muscle via PDE1 and PDE4 inhibition. This dual inhibitor of PDE4 and PDE1 is more clinically useful in preventing airway remodeling in asthma than the inhibitor of only PDE4. In conclusion, our results indicate that selective PDE 4 and 1 dual inhibitor KF19514 might be useful for preventing bronchial hyperresponsiveness and airway remodeling in asthma.

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