Original Research

Co-inhalation of roflumilast, rather than formoterol, with fluticasone more effectively improves asthma in asthmatic mice

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Impact statement

Roflumilast, a selective phosphodiesterase-4 inhibitor, was approved for the treatment of chronic obstructive pulmonary disease (COPD). This study showed that co-inhalation of roflumilast and fluticasone significantly decreased airway hyperresponsiveness in ovalumin-asthmatic mice. Also, it more significantly improved inflammation and histopathological changes than co-inhalation of formoterol and fluticasone. The current results showed that inhaled roflumilast reduced counts of eosinophils, neutrophils, and macrophages in bronchoalveolar lavage fluid. Consequently, inhaled roflumilast might be of potential off-label benefit in treatment of eosinophilic and neutrophilic asthma and asthma-COPD overlap syndrome (ACOS). These results could also support other experimental and clinical studies addressing the same issue.

Abstract

Roflumilast is approved as an add-on therapy for chronic obstructive pulmonary disease. The inflammation in chronic obstructive pulmonary disease is mainly neutrophilic, while in asthma it is mainly eosinophilic, studies addressing role of roflumilast in eosinophilic inflammation are recommended. Also in severe asthma, the dominant inflammatory cells are neutrophils. Thus, roflumilast has a potential off-label use in the treatment of asthma. This study was designed to evaluate the effects of co-inhalation of roflumilast and fluticasone compared to that of formoterol and fluticasone in ovalbumin-sensitized and-challenged BALB/c mice. Besides normal control group, the ovalbumin-asthmatic mice were randomly divided into seven groups ($n = 8$): positive control, vehicle-treated, and five drug-treated groups. Treatments (μg/kg) were given as 15 min-inhalation once/day for five days as follows: roflumilast (500), formoterol (50), fluticasone (1000), roflumilast + fluticasone (500 + 1000), and formoterol + fluticasone (50 + 1000). Penh values were measured in conscious unrestrained mice using the single-chamber whole-body plethysmography. Airway hyperreactivity to inhaled methacholine was evaluated. Bronchoalveolar lavage fluid was used for the measurements of levels of IL-4, IL-5, TNF-α, OVA-specific IgE, and total and differential white cells. Lung sections were stained with hematoxylin and eosin and periodic acid-Schiff. The asthmatic mice showed significant increases in airway hyperreactivity which were significantly reversed by the combination treatments. The asthmatic mice showed significant increases in levels of IL-4, IL-5, TNF-α, ovalbumin-specific IgE, and total and differential white cells in bronchoalveolar lavage fluid. All treatments (except formoterol) significantly reversed these changes mainly with roflumilast + fluticasone. The asthmatic mice showed severe inflammatory infiltration and goblet cell hyperplasia which were maximally reversed by roflumilast + fluticasone, while minimally reversed by formoterol. In conclusion, co-inhalation of roflumilast + fluticasone more significantly improved inflammation and histopathological changes than co-inhalation of formoterol + fluticasone in ovalumin-asthmatic mice. Further studies are needed to help confirm the potential off-label add-on use of roflumilast in typical and atypical asthma and asthma-chronic obstructive pulmonary disease overlap syndrome.

Keywords: Asthma, fluticasone, formoterol, roflumilast, mice, off-label, ovalbumin

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Introduction

Bronchial asthma is a chronic airway inflammatory disease characterized by allergen-induced airway obstruction. The early phase of obstruction is manifested by acute bronchospasm, while the late phase, arising 8–24 h after allergen exposure, is associated with airway inflammatory cells influx. The severity of inflammation is indicated by airway hyperresponsiveness (AHR) and remodeling

(structural changes including subepithelial fibrosis and increases in goblet cells numbers and smooth muscle thickness).[1,2] The release of proinflammatory mediators from eosinophils and T-helper 2 (Th2) lymphocytes contributes to airway inflammation. Normally there is a balance between mediators from the Th1 cells (interleukin (IL)-2, IL-12, interferon (IFN)-γ, tumor necrosis factor (TNF)-α), and Th2 cells (IL-4, IL-5, IL-6, IL-9, and IL-13). Shift of the balance to Th1 or Th2 mediators favors development of autoimmune or atopic diseases, respectively.[3] The Th2 cells, eosinophils, B cells, and mast cells have important roles in asthma. IL-4 promotes inflammatory processes, differentiation of B cells to plasma cells which produce allergen-specific immunoglobulin E (IgE), mast cell proliferation, AHR, and structural airway changes. IL-5 is essential for the survival of B cell and activation of eosinophils leading to production of cysteinyl leukotrienes and eosinophil peroxidase that participate in AHR.[4,5]

Corticosteroids are the most effective long-term anti-inflammatory controller therapy in asthma. They inhibit the phospholipase A2 enzyme leading to suppression of the arachidonic acid pathway. Because the early anti-inflammatory therapy is a mainstay in asthma and to avoid systemic adverse effects of corticosteroids, combinations of inhalational corticosteroids (ICS) plus other agents were used to optimize therapy.[6] Fluticasone is an inhaled corticosteroid with greater anti-inflammatory effects in asthmatics compared to its congeners.[7] It reversed ovalbumin (OVA)-induced AHR and pulmonary eosinophilia in guinea pigs.[8]

Long-acting β_2 agonists (LABAs) are anti-asthmatic agents through stimulation of β_2 receptors leading to activation of adenylyl cyclase enzyme and increasing formation of the intracellular cAMP. LABAs "don't appear to have any clinically important antiinflammatory or proinflammatory effect."[9] LABAs are always used in a combination with ICS because LABAs monotherapy was reported to cause more asthma deterioration.[10] Formoterol is an LABA having a rapid onset of bronchodilation which is more rapid than that of its congeners and even nearly similar to that of salbutamonl (a short-acting β_2 agonist).[11]

Xanthines are non-selective phosphodiesterase (PDE) inhibitors used in the treatment of asthma through increasing the intracellular levels of c-AMP. Recently, selective inhibition of PDE-3 in the airway smooth muscle and of PDE-4 and PDE-7 in the inflammatory cells resulted in bronchodilatation and anti-inflammatory effects, respectively. Moreover, PDE-3 and PDE-4 inhibitors were shown to activate the mucociliary clearance in human airway epithelial cells. Thus, combined use of inhaled PDE-3 and PDE-4 inhibitors may be valuable in treating inflammatory airway diseases and decreasing the systemic adverse effects.[12,13] In spite of presence of PDE4 in airway smooth muscle and ability of its inhibitors to relax the tone of isolated human bronchial muscle,[14] most studies report that PDE4 inhibitors are ineffective bronchodilators.[15] PDE-4 inhibitors are very effective at inhibiting production of inflammatory mediators from neutrophils and eosinophils.[16,17]

Roflumilast, a selective PDE-4 inhibitor, protected against inflammation and other mechanisms in chronic obstructive pulmonary disease (COPD) including its extrapulmonary effects.[18] Roflumilast elevates intracellular cAMP which mediates inhibition of nuclear factor-kappaB (NF-*κB)*. It inhibits the DNA binding activity of NF-*κB* by preventing inhibitor kappaB alpha phosphorylation and degradation. Also, it markedly inhibits phosphorylation of mitogen-activated protein (MAP) kinases that include protein kinase/c-Jun NH2-terminal kinase (JNK) and p38 MAP kinase. Thus, the anti-inflammatory activity of roflumilast seems to be mediated through suppression of NF-kappaB, p38 MAP kinase, and JNK activation in macrophages.[19] In a clinical trial, oral roflumilast for 12 weeks improved asthma symptoms because of its anti-inflammatory properties.[20] The PDE-4 selective inhibitors reduce production of several inflammatory mediators including histamine, leukotrienes, and cytokines such as TNF-α, IL-4, and IL-5[21] and thus roflumilast has a potential off-label use in treatment of asthma[22] Systemic roflumilast may cause nausea, vomiting, diarrhea, weight loss, and headache[23] and its use by inhalation might be a solution to avoid these systemic side effects.[24] Roflumilast is considered as an add-on therapy for COPD.[25] The inflammation in COPD is mainly neutrophilic, while in asthma it is mainly eosinophilic. Thus, studies addressing the role of roflumilast in eosinophilic inflammation are essential[26] and also in severe asthma, the dominant inflammatory cells are neutrophils.[27] Moreover, many patients with chronic airway obstruction may show manifestations of both asthma and COPD (asthma-COPD overlap syndrome: ACOS) based on genetic makeup and environmental conditions. The prevalence of ACOS is about 15–25% of the obstructive airway diseases in different communities and ACOS patients have worse outcomes compared with each disease alone.[28] Up till now, there is no sound evidence to support specific treatment for ACOS but addition of roflumilast to ICS/LABA treatment may be an option.[29]

Taken together, we hypothesize that inhaled roflumilast alone or combined with fluticasone improves the asthma-induced changes in mice more effectively than formoterol alone or combined with fluticasone, respectively. To investigate this hypothesis, the present study was designed to test effects of these drugs against airway response, hyperreactivity, inflammation, and remodeling in OVA-induced asthma in mice. This could help the potential off-label use of inhaled roflumilast as an alternative to formoterol in asthmatic and ACOS patients who are resistant or intolerant to formoterol/fluticasone therapy or as an add-on agent forming a triple combination.

Materials and methods

Animals and experimental design

The protocol of the study was approved by the Institutional Research Ethics Committee (the King Abdulaziz University Research Ethics Committee (KAU-REC)) and adhered to the international guidelines for the use of experimental animals. All drugs and chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless

mentioned otherwise. Female BALB/c mice (8–10 weeks old and about 30 g weight) were obtained from King Fahd Research Center, KAU and were housed in cages at 24°C in a 12-h light–dark cycle. All mice were acclimatized for at least one week before experimentation. Food and water were available ad libitum. Female mice were used because asthma is more frequent in women[30] and thus ''female sex is an independent risk factor for non-allergic asthma''.[31] In addition, female mice were reported to have more airway remodeling compared with male mice.[32] Another study reported that female mice developed more severe allergic inflammation than male mice but it was not associated with more marked airway remodeling.[33] Females are more likely to experience allergic asthma, to suffer from severe asthma symptoms, and to have medications' adverse effects. In males, testosterone seems to suppress asthma and there is an evidence for a steroid-sparing effect of dehydroepiandrosterone denoting its probable efficacy in treating asthma.[34] Thus, in the current study, female mice were used because female sex shows more severe asthmatic manifestations and to avoid the possible antiasthmatic effects of male sex hormones. Definitely differences between males and females should be kept in mind when ''designing, analyzing, and interpreting studies of smooth muscle responses in animal models and human subjects.''[35] The experimental design (Figure 1) included OVA sensitization and challenge to induce asthma in mice followed by administration of treatments and then measurement of the outcomes.

OVA sensitization and challenge

The mice were sensitized by intraperitoneal (i.p.) injection of 20 μg of OVA (grade III) in 0.1 ml of Alum (aluminium hydroxide powder, Al $(OH)_3$) on days 0 and 14. The phosphate-buffered saline (PBS) was used as a vehicle for OVA. On days 21–23 by using an ultrasonic nebulizer (Devilbiss, UK), mice were subjected to 20 min-aerosol of 1% OVA (10 mg/ml) to establish lung inflammation or PBS in the control group. On day 26, final challenges were given as 20-min aerosol of 5% OVA (50 mg/ml) or PBS in the control group.[36,37] The use of alum provokes ''T-helper 2 pattern and eosinophil-dominated bronchial inflammation which is the pattern encountered in most asthmatics.''[38]

Administration of drugs and treatment groups

The choice of the doses used was based on previous studies and pilot experiments. Regarding roflumilast doses of 250, 500, and 1000 μg/kg were tried in the pilot experiment. While the low dose showed non-significant effects, the medium and high doses showed dose-dependent effects. The medium dose was chosen to minimize adverse effects. Dimethyl sulfoxide (DMSO) was used as a vehicle for the drugs. In addition to the negative control (NC) group (sensitized and challenged with PBS), the OVA-sensitized and -challenged were randomly divided into seven groups (n = 8): positive control (PC) group (saline-treated), vehicle-treated group, and five drug-treated groups. Drugs were given by inhalation for 15 min once/day for five doses with the last dose given 5–6 h before the final OVA challenge. The saline and vehicle were given in the same manner.[8,36] The drugs given included roflumilast (R, 500 μg/kg),[26,39] formoterol (Fo, 50 μg/kg),[36] fluticasone (F, 1000 μg/kg),[40,41] roflumilast + fluticasone (R + F, 500 + 1000 μg/kg), and formoterol + fluticasone (Fo + F 50 + 1000 μg/kg).[42,40]

Measurement of airway hyperreactivity to methacholine (MCh)

AHR was measured in the conscious unrestrained mice using the single chamber whole body plethysmography (Buxco Electronics, Wilmington, NC, USA) (WBP) and analyzed using Buxco software. Mice were acclimatized to the plethysmograph before experiments. AHR was expressed as Penh (enhanced pause) values which reflect ''effort of breathing'' and indicate the pause between inspiration and expiration in conscious animals. Increases in Penh values were used as an index of airway obstruction.[43] ''Penh may be more useful in models with predominant bronchial hyperresponsiveness due to peribronchial rather than parenchymal inflammation as has been postulated for the BALB/c mouse model of asthma.''[44] On day 26 prior to the last OVA challenge, baseline Penh measurements were continuously recorded then mice were challenged for 20 min with OVA except for the NC group which was challenged with PBS. To prevent hypoxia mice were provided with 30% oxygen. Recording of lung function continued for 15 min after the end of the OVA challenge. Twenty-four hours after the last OVA challenge, AHR to inhaled MCh (dissolved in 0.9% NaCl) was assessed. Mice were placed into the chamber of the WBP and the baseline Penh values were measured. Then mice were first nebulized with PBS and afterward with increasing doses of MCh (6.25–50 mg/ml) each for 3 min followed by readings of breathing parameters for 3 min after each nebulization to determine the Penh values.[45] MCh dose-response curves were generated

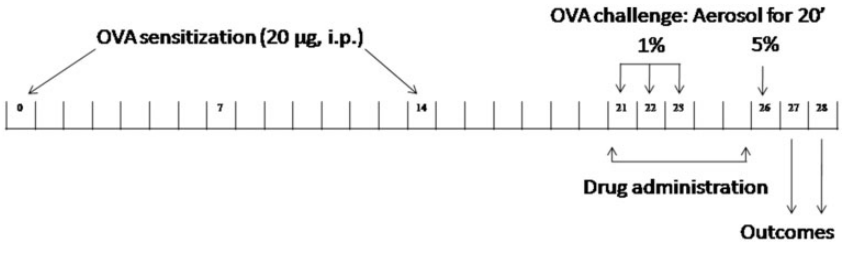

Figure 1 The experimental design in BALB/c mice. Time points for OVA sensitization and challenge, administration of drugs, and measurement of the outcomes

and ''the concentration of MCh that produced a 200% increase (PC_{200}) above baseline of the Penh value'' was recorded.[46]

Assay of levels of OVA-specific IgE and cytokines in the bronchoalveolar lavage fluid

Twenty-four hours after determination of AHR, the mice were anesthetized with pentobarbital (50 mg/kg, i.p.), and the bronchoalveolar lavage was done with PBS (at 37°C) using a tracheal tube. The recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 630 g for 7 min at 4°C and the supernatant was stored at −80°C. Measurements of the levels of IL-4, IL-5, TNF-α, and OVA-specific IgE were done using ELISA kits according to the manufacturer's instructions. The lower limits of detection of ELISA assay were 1.0, 4.0, 4.0, and 20.7 pg/ml, respectively (Biolegend San Diego, CA, US).[47,48]

Evaluation of cellular levels in the BALF

After centrifugation of the recovered BALF and collection of the supernatant, the cells in the BALF pellet were washed in saline, suspended in a lysing buffer to destroy the remaining erythrocytes. Then the total white cell counts were done using an automated cell counter (Cell Dyne 3500, Abbott Laboratories, NY, US). In order to perform the differential white cell counts, aliquots of the cells were placed on slides and then stained with Field's stain (TCS biosciences, Botolph Claydon, Buckingham, UK). After drying, 200 cells per slide were counted using a microscope (Optima X5Z-H) at ×400 magnification and cells were identified as eosinophils, neutrophils, lymphocytes, or monocytes.[45,46]

Histopathological examination

The lung was fixed in 10% neutral-buffered formalin, and paraffin sections (3 μm) were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The lung sections were taken along the primary bronchus. The whole section was examined blindly by two pathologists. The scoring has been based on the severity of the lung inflammation, thickness of the subepithelial smooth muscle layer, and hyperplasia/hypertrophy of the mucus-secreting cells as previously described.[48] The scoring ranged from 0 to 5 and the image-pro plus software was used. Score (0) means normal regular respiratory epithelium, thin subepithelial smooth muscle layer (up to 2 cell thickness), and no inflammatory response with lumen clear of mucoid secretions. Score (1) means abnormal respiratory epithelium (hyperplastic and hypertrophied), mild inflammatory response with mononuclear infiltrate and increased vascularity of mucosa, and mild increase in thickness of subepithelial smooth muscle layer (about 3–4 cell thickness). PAS stain shows mild increase in number of goblet cells (up to 20% increase compared to NC). Score (2) means mild to moderate inflammatory response with abnormal respiratory epithelium, inflammatory mononuclear infiltrate, increased vascularity of mucosa, and mild to moderate increase in thickness of subepithelial smooth muscle layer (about 4–6 cell thickness). PAS stain shows moderate increase in number of goblet cells (20–50% increase compared to NC). Score (3) means moderate inflammatory response with abnormal respiratory epithelium, inflammatory mononuclear infiltrate, increased vascularity of mucosa, and moderate increase in thickness of subepithelial smooth muscle layer (about 5–7 cell thickness). PAS stain shows moderate increase in goblet cells. Score (4) means moderate to severe inflammatory response with abnormal respiratory epithelium, inflammatory mononuclear infiltrate, increased vascularity of mucosa, moderate to severe increase in thickness of subepithelial smooth muscle layer (about 6–9 cell thickness). PAS stain shows severe increase in goblet cells (more than 50% increase compared to NC). Score (5) means severe inflammatory response with abnormal respiratory epithelium, markedly thickened subepithelial smooth muscle layer (about 8–12 cell thickness), accumulation of mucus in the airway lumen, and marked increase in vascular density with severe mononuclear inflammatory infiltrate in the peribronchial parenchymal areas. PAS stain shows severe increase in number of goblet cells.

Statistical analysis

All data were expressed as mean ± SEM and analyzed with SPSS 18. Comparisons for two groups were made using Student's *t*-test. The one-way analysis of variance (ANOVA) with Tukey's test was used for experiments in which more than two groups were compared. In order to compare the airway reactivity to MCh among groups, the dose-response curves were used to calculate the PC_{200} of MCh. A P value < 0.05 was considered statistically significant.

Results

Airway hyperresponsiveness to methacholine

The baseline Penh values did not show any significant variations among the different groups. The nebulization of MCh (6.25–50 mg/ml) dose-dependently enhanced the Penh values from 1-fold of saline aerosol to 2.99 ± 0.17-fold in the PC mice. The PC developed AHR shown by a shift of the Penh dose-response curve to the left and an increase of the maximal reactivity compared with the NC group. The monotherapies showed modest non-significant decreases in AHR compared with the PC group. The combination treatments (R + F and Fo + F) showed significant decreases of the Penh values induced by MCh concentrations of 25 and 50 (mg/ml) compared with the PC group and monotherapies with a non-significant difference in-between (Figure 2).

Levels of IL-4, IL-5, TNF-α, and OVA-specific IgE in the BALF

The PC mice showed significant increases of the BALF IL-4, IL-5, TNF-α, and OVA-specific IgE levels compared to the NC group. All treatments (except Fo) significantly reversed these OVA-induced changes mainly with the R + F group which showed a non-significant difference from the NC

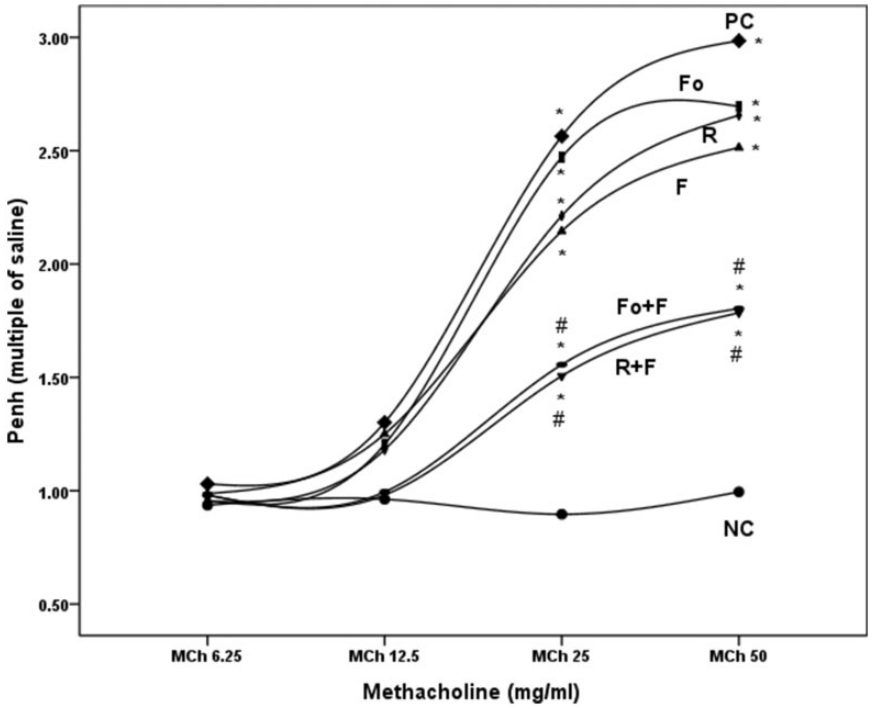

Figure 2 Effects of roflumilast, formoterol, fluticasone alone and in combinations on the airway responsiveness (AHR) in ovalbumin-sensitized and -challenged mice. AHR to increasing concentrations of methacholine (6.25–50 mg/ml) at 24 h post-final ovalbumin challenge was expressed as Penh values. Treatments (μg/kg/day by inhalation for 15 min for five days) included R (roflumilast, 500), Fo (formoterol, 50), F (fluticasone, 1000), R + F (roflumilast + fluticasone, 500 + 1000), and Fo + F (formoterol + fluticasone, 50 + 1000) (n = 8). Data are expressed as mean ± SEM. Comparisons were made using ANOVA with Tukey's post-hoc test. $^{*}P < 0.05$: the combination groups (R + F and Fo + F) vs. NC (normal control) group at MCh 25 ($P = 0.031$ and 0.015, respectively), R + F vs. NC at MCh 50 ($P = 0.011$), $^{**}P < 0.01$: Fo + F vs. NC ($P = 0.009$), $^{***}P < 0.001$: PC (positive control) and monotherapy groups vs. NC. $^{\#}P < 0.05$: Fo + F vs. R and F at MCh 25 ($P = 0.016$ and 0.041, respectively), R + F vs. F at MCh 25 ($P = 0.020$), the combination groups (R + F and Fo + F) vs. F at MCh 50 ($P = 0.024$ and 0.030, respectively), $^{\#\#}P < 0.01$: R + F vs. R at MCh 25 ($P = 0.007$), the combination groups (R + F and Fo + F) vs. R ($P = 0.004$ and 0.005, respectively) and vs. Fo ($P = 0.002$ and 0.003, respectively), $^{\#\#\#}P < 0.001$: the combination groups vs. Fo. $^{\dagger}P < 0.001$: the combination groups vs. PC

group. The combinations were significantly different from monotherapies and the R + F was significantly different from the Fo + F (Figure 3).

Cellular levels in the BALF

The PC mice showed significant increases of counts of the total white cells, eosinophils, neutrophils, lymphocytes, and monocytes in BALF compared to the NC group. All treatments (except Fo) significantly reversed these OVA-induced changes mainly with the R + F group. The combinations were significantly different from monotherapies and the R + F was significantly different from the Fo + F (Figure 4).

Histopathological findings

In the HE-stained sections (Figure 5 and Table 1), the PC group showed abnormal respiratory epithelium, severe inflammatory infiltrate in the peribronchial parenchymal areas, markedly increased mucosal vascular density, and markedly increased thickness of the subepithelial smooth muscle layer (score 5) compared with the NC group (score 0). Moreover, the PAS stain (Figure 6 and Table 1), the PC group showed severe increase in number of goblet cells. The combination of roflumilast and fluticasone nearly reversed and normalized these OVA-induced changes showing a score 1 picture. The roflumilast, fluticasone, and the combination of formoterol and fluticasone groups showed score 2 appearances, while the formoterol group showed a score 4 appearance.

Discussion

In the current study, the OVA-induced asthma in mice led to significant increases in AHR, levels of IL-4, IL-5, TNF-α, and OVA-specific IgE, and counts of total and differential (eosinophils, neutrophils, lymphocytes, and monocytes) white cells in BALF compared to the control mice. Moreover, the asthmatic mice showed severe peribronchial, perivascular, and alveolar septal inflammation and goblet cell hyperplasia. These results agree with previous studies.[41,49–52] In the current study, the monotherapies showed modest non-significant decreases in AHR, while the combination groups showed significant decreases in AHR at MCh concentrations of 25 and 50 (mg/ml). These two doses of MCh could be clinically relevant because maximum doses of 16, 25, and 32 mg/ml are commonly used in various pulmonary functions testing laboratories.[53] Also, doses ranging from 0.075 to 50 mg/ml were used to measure AHR in children with post-infectious bronchiolitis obliterans.[54] Regarding the increases in levels of cytokines (IL-4, IL-5, TNF-α), OVA-specific IgE, and total and differential white cells in BALF, all treatments (except formoterol) significantly reversed these OVA-induced changes mainly

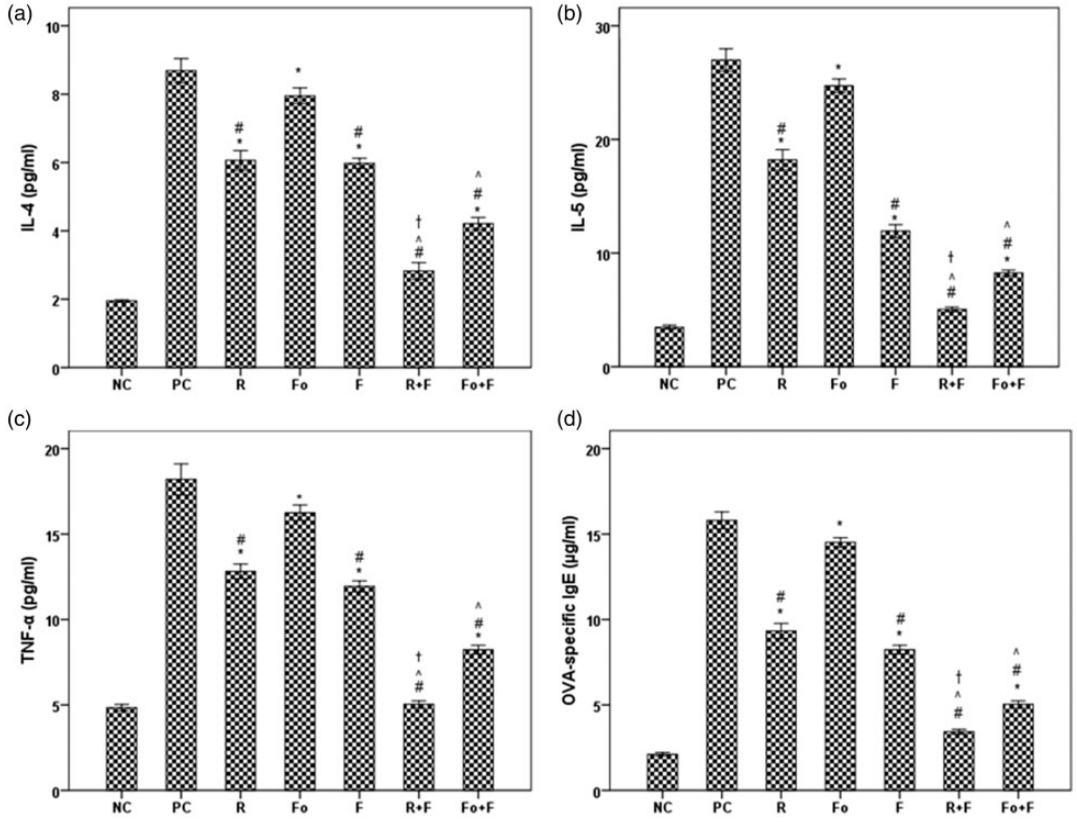

Figure 3 Effects of roflumilast, formoterol, fluticasone alone and in combinations at 24 h after the final ovalbumin challenge on: (a) IL-4, (b) IL-5, (c) TNF-α, and (d) OVA-specific IgE in the bronchoalveolar lavage fluid (BALF) in ovalbumin-sensitized and -challenged mice. Treatments (µg/kg/day by inhalation for 15 min for five days) included R (roflumilast, 500), Fo (formoterol, 50), F (fluticasone, 1000), R + F (roflumilast + fluticasone, 500 + 1000), and Fo + F (formoterol + fluticasone, 50 + 1000) (n = 8). Data are expressed as mean ± SEM. Comparisons were made using ANOVA with Tukey's post-hoc test. $^{*}P < 0.05$ vs. NC (normal control) group, $^{\#}P < 0.05$ vs. PC (positive control), $^{\wedge}P < 0.05$ the combination groups (R + F and Fo + F) vs. monotherapies, $^{\dagger}P < 0.05$ R + F vs. Fo + F (*P* values for IL-4, IL-5, TNF-α, and OVA-specific IgE = 0.002, 0.009, < 0.001, and 0.009, respectively)

with the roflumilast + fluticasone group. The combinations showed significant variations from monotherapies and the effects of the roflumilast + fluticasone combination were the most significant. The OVA-induced histopathological changes were greatly reversed by the roflumilast + fluticasone combination, moderately reversed by roflumilast, fluticasone, and the formoterol + fluticasone combination, and only mildly reversed by formoterol.

In asthmatic BALB/c mice, nine daily doses of nebulized fluticasone alone or in combination with salmeterol reduced AHR to MCh, airway inflammation, and cell counts to near-normal levels and also reduced mucin containing cells and remodeling, but salmeterol alone worsened AHR.[36] In OVA-asthmatic Brown Norway rats, inhaled fluticasone (1 and 10 mg for two weeks) inhibited the eosinophilic inflammation in BALF and airway mucosa. Only the higher dose decreased the elevated OVA-specific IgE serum level and inhibited airway thickening and goblet cell hyperplasia but unfortunately it was associated with systemic side effects.[41] In dogs, inhaled fluticasone significantly reduced BALF cells and blood eosinophils but did not affect airway function which may indicate steroid resistance.[55] In mice, inhalation of fluticasone (once daily for five days) suppressed concentration of Mycoplasma pneumoniae, pulmonary inflammation, and AHR.[56]

In OVA-asthmatic BALB/c mice, oral roflumilast decreased AHR and improved the peribronchial inflammatory cell infiltration, goblet cell hyperplasia, and subepithelial fibrosis. It significantly reduced the elevated counts of total cells, eosinophils, and lymphocytes and the elevated levels of IL-4, IL-5, and IL-13 in BALF indicating affection of the Th2 cytokine pathway and potential usefulness of roflumilast for treatment of chronic asthma.[57] In a mouse model of chronic asthma, oral roflumilast (5 mg/kg/day for 10 days) reduced expression of TNF-α, and IL-6.[58] Oral roflumilast (1000 µg) decreased AHR in asthmatic patients.[59] In a clinical trial, oral roflumilast decreased sputum eosinophils and neutrophils indicating an anti-inflammatory effect and a protective effect against allergen-induced airway inflammation, late phase bronchoconstriction, and AHR.[60] In OVA-sensitized guinea pigs, inhaled roflumilast (but not oral) for seven days alone or combined with inhaled salbutamol decreased specific airway resistance after histamine aerosol and decreased histamine-induced contractions of isolated tracheal preparation indicating that roflumilast has a direct bronchodilating effect. Moreover, it decreased the inflammatory markers and cells in BALF through enhancing apoptosis which removes the inflammatory cells without initiating an inflammatory response. Thus, roflumilast could be useful for asthmatic patients.[26] In

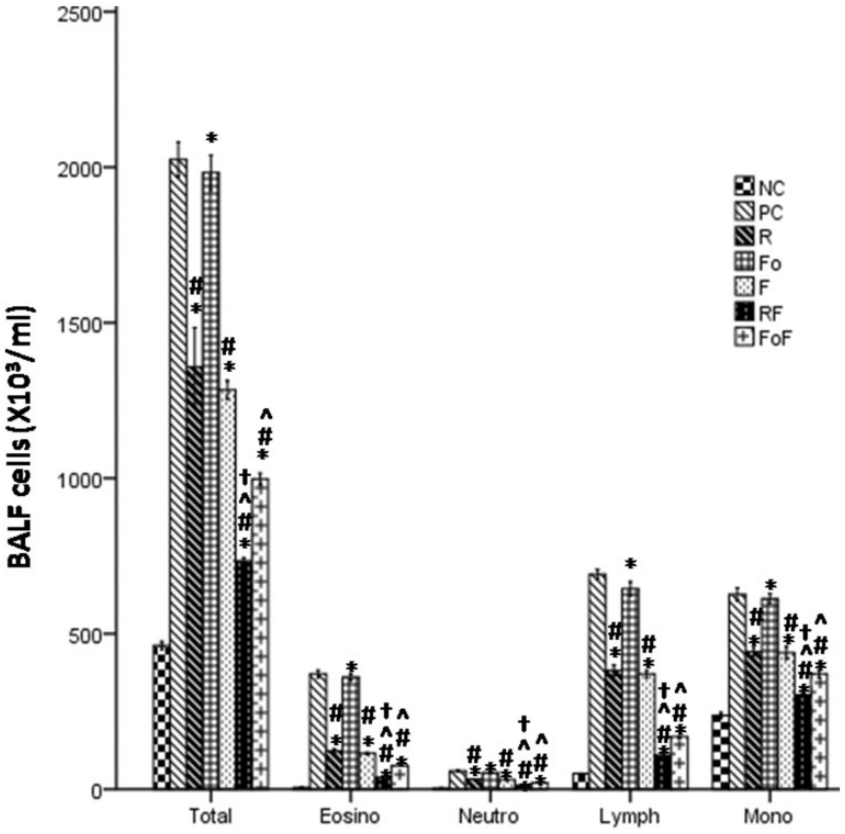

Figure 4 Effects of roflumilast, formoterol, fluticasone alone and in combinations at 24 h after the final ovalbumin challenge on total white cells, eosinophils, neutrophils, lymphocytes, and monocytes in the bronchoalveolar lavage fluid (BALF) in ovalbumin-sensitized and -challenged mice. Treatments (μg/kg/day by inhalation for 15 min for five days) included R (roflumilast, 500), Fo (formoterol, 50), F (fluticasone, 1000), RF (roflumilast + fluticasone, 500 + 1000), and FoF (formoterol + fluticasone, 50 + 1000) (n = 8). Data are expressed as mean ± SEM. Comparisons were made using ANOVA with Tukey's post-hoc test. $^{*}P < 0.05$ vs. NC (normal control) group, $^{\#}P < 0.05$ vs. PC (positive control), $^{\wedge}P < 0.05$ the combination groups (R + F and Fo + F) vs. monotherapies, $^{\dagger}P < 0.05$ RF vs. FoF (*P* values for total white cells, eosinophils, neutrophils, lymphocytes, and monocytes = 0.035, 0.008, 0.001, 0.038, and 0.038, respectively)

allergen-challenged Brown Norway rats at 24 h post-challenge, roflumilast given by nose-only inhalation decreased the inflammatory cell influx in BALF, reduced the antigen-induced interstitial airway edema, and improved lung functions.[61] In OVA-sensitized and -challenged BALB/c mice, the airway remodeling causes steroid resistance. Oral roflumilast significantly decreased all parameters of remodeling including airway inflammation, AHR, goblet cell hyperplasia, pulmonary fibrosis, and the levels of BALF IL-4, IL-5, and IL-13. These effects are due to significant inhibition of stem cell factor (SCF) level and hence SCF-induced proliferation of fibroblasts.[57]

Using both *in vitro* and *in vivo* experiments, roflumilast was suggested to be a potential new therapy for asthma and COPD. It relaxed OVA-induced contractions of isolated trachea from sensitized guinea pigs. Also, oral and intravenous roflumilast decreased allergen-induced bronchoconstriction, inflammatory infiltration, and elevated TNF-α level in BALF in rats and guinea pigs. In addition, roflumilast and its N-oxide metabolite were found to equally inhibit eosinophilia.[62] The bronchodilating effect of roflumilast is controversial. Previously no evidence was found for PDE4 inhibitors-induced bronchodilation in human COPD.[15] Also in animals, roflumilast did not protect against bronchospasm induced by leukotriene D_4 and serotonin.[63] Thus, the clinical effects of PDE4 inhibitors are due to anti-inflammatory rather than bronchodilating mechanisms.[64] Roflumilast is a modest bronchodilator which should never be used for relief of acute bronchospasm and it has not been studied as a corticosteroid-sparing agent in asthma or COPD.[23] On the other hand, in a multicentre clinical trial, roflumilast reduced allergen-induced bronchoconstriction and airway inflammation in asthmatic patients.[65]

Roflumilast N-oxide was found to activate the mucociliary clearance through regulation of the epithelial transmembrane conductance in human bronchi.[66] The results of six clinical trials reported useful effects of roflumilast in asthma through its anti-inflammatory mechanisms.[67] In 693 asthmatic patients, roflumilast (500 μg/day for 12 weeks) improved the mean forced expiratory volume in 1 s and the asthma manifestations without major adverse effects.[20] In a recent multicenter trial, roflumilast attenuated allergen-induced bronchoconstriction and airway inflammation in asthmatic patients.[65] Addition of roflumilast (500 mg) to high-dose fluticasone (500–1000 μg/day) for a month in patients with uncontrolled severe asthma better improved asthma than high-dose fluticasone alone possibly through affecting the neutrophilic component of the disease.[68] In OVA-sensitized and -challenged mice, roflumilast (5 mg/kg/day for 10 days orally) non-significantly decreased AHR but

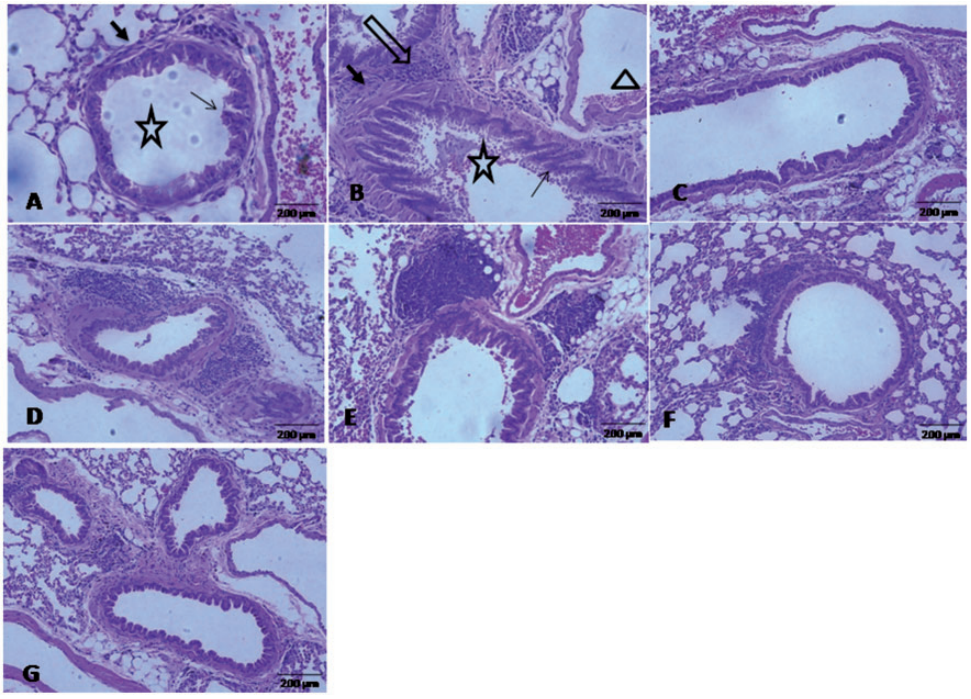

Figure 5 Sections in lung stained by HE (×20) showing effects of roflumilast, formoterol, fluticasone alone and in combinations in OVA-induced asthma in mice. (a) Normal control (NC) group showing regular respiratory epithelium (thin arrow), thin subepithelial smooth muscle layer (up to 2 cell thickness) (bold arrow), and the airway lumen is clear of mucoid secretions (star). No inflammatory response (score 0). (b) Ovalbumin-induced asthma (PC) group showing abnormal respiratory epithelium (hyperplastic and hypertrophied) (arrow), severe inflammatory infiltrate in peribronchial parenchymal areas (thick arrow), markedly increased vascular density (triangle), and marked increase in thickness of subepithelial smooth muscle layer (about 8–12 cell thickness) (bold arrow) and accumulation of mucus in the airway lumen (star) (score 5). (c) Roflumilast-treated group, (e) Fluticasone-treated group, and (g) Formoterol + Fluticasone-treated group showing mild to moderate inflammatory response with abnormal respiratory epithelium, inflammatory infiltrate, increased vascularity of mucosa, and mild to moderate increase in thickness of subepithelial smooth muscle layer (about 4–6 cell thickness) (score 2). (d) Formoterol-treated group showing moderate to severe inflammatory response with abnormal respiratory epithelium, inflammatory infiltrate, increased vascularity of mucosa, and moderate to severe increase in thickness of subepithelial smooth muscle layer (about 6–9 cell thickness) (score 4). (f) Roflumilast + Fluticasone-treated group showing mild inflammatory response with abnormal respiratory epithelium, mild inflammatory infiltrate, increased vascularity of mucosa, and mild increase in thickness of subepithelial smooth muscle layer (about 3–4 cell thickness) (score 1). (A color version of this figure is available in the online journal.)

Table 1 Effects of treatments on the airway histopathological parameters in ovalbumin-induced asthma in mice

Groups	Airway epithelial thickness (μm)	Airway inflammatory cell infiltrate (score)	Subepithelial smooth muscle thickness (μm)	Number of goblet cells (/100 μm)
NC	8.73 ± 0.50	0.22 ± 0.10	9.53 ± 0.23	0.36 ± 0.10
PC	24.76 ± 1.34	5.16 ± 0.35	21.05 ± 0.75	1.92 ± 0.15
R	16.08 ± 1.31*,**,***	2.16 ± 0.08$^{\#\#\#}$	14.71 ± 0.75$^{\#\#\#}$	0.89 ± 0.02$^{\wedge\wedge,\wedge\wedge\wedge}$
Fo	21.48 ± 1.55***	4.47 ± 0.23$^{\#\#\#}$	18.93 ± 0.36$^{\#\#\#}$	1.75 ± 0.10$^{\wedge\wedge\wedge}$
F	15.58 ± 1.06**,***	2.09 ± 0.05$^{\#\#\#}$	14.60 ± 0.84$^{\#\#\#}$	1.06 ± 0.08$^{\wedge\wedge\wedge}$
R + F	9.80 ± 0.29*,**,***	0.94 ± 0.08$^{\#\#,\#\#\#}$	10.25 ± 0.27$^{\#,\#\#\#}$	0.51 ± 0.03$^{\wedge,\wedge\wedge,\wedge\wedge\wedge}$
Fo + F	14.96 ± 1.22**,***	1.97 ± 0.11$^{\#\#\#}$	13.25 ± 0.73$^{\#\#\#}$	0.92 ± 0.02$^{\wedge\wedge,\ \wedge\wedge\wedge}$

Note: Treatments (μg/kg/day by inhalation for 15 min for five days) included R (roflumilast, 500), Fo (formoterol, 50), F (fluticasone, 1000), R + F (roflumilast + fluticasone, 500 + 1000), and Fo + F (formoterol + fluticasone, 50 + 1000) (n = 8). The image-pro plus software was used. Data are expressed as mean ± SEM. Comparisons were made using ANOVA with Tukey's post-hoc test. $^{*}P < 0.05$: R vs. Fo ($P = 0.022$), R + F vs. F and Fo + F ($P = 0.011$ and 0.032, respectively), $^{**}P < 0.01$: R, F, and Fo + F vs. NC (normal control) ($P = 0.001$, 0.001, and 0.005, respectively), R + F vs. R ($P = 0.004$), F and Fo + F vs. Fo ($P = 0.009$ and 0.003, respectively), $^{***}P < 0.001$: all treatments (except Fo) vs. PC (positive control), Fo vs. NC. $^{\#}P < 0.05$: R + F vs. Fo + F ($P = 0.018$), $^{\#\#}P < 0.01$: R + F vs. Fo + F ($P = 0.002$), $^{\#\#\#}P < 0.01$: all treatments (except R + F) vs. NC, all treatments vs. Fo and PC, R + F and Fo vs. R and F, R + F vs. monotherapy, Fo + F vs. Fo, $^{\wedge}P < 0.05$: R + F vs. R and Fo + F ($P = 0.047$ and 0.023, respectively), $^{\wedge\wedge}P < 0.01$: R and Fo + F vs. NC ($P = 0.001$ and 0.001), R + F vs. F ($P = 0.001$), $^{\wedge\wedge\wedge}$<0.001: all treatments vs. Fo and PC, Fo and F vs. NC.

significantly reduced eosinophils, chronic inflammatory cells, subepithelial fibrosis, and thickening of the airway epithelium. Thus, roflumilast is an effective inhibitor of airway inflammation and remodeling.[51] The ability of roflumilast to reduce sputum eosinophils, neutrophils, and macrophages in COPD patients makes it potentially useful in typical and atypical asthma and in ACOS to reduce COPD-related exacerbations; however, this is still under investigation. A clinical trial has been designed to evaluate effects of roflumilast 500 g on

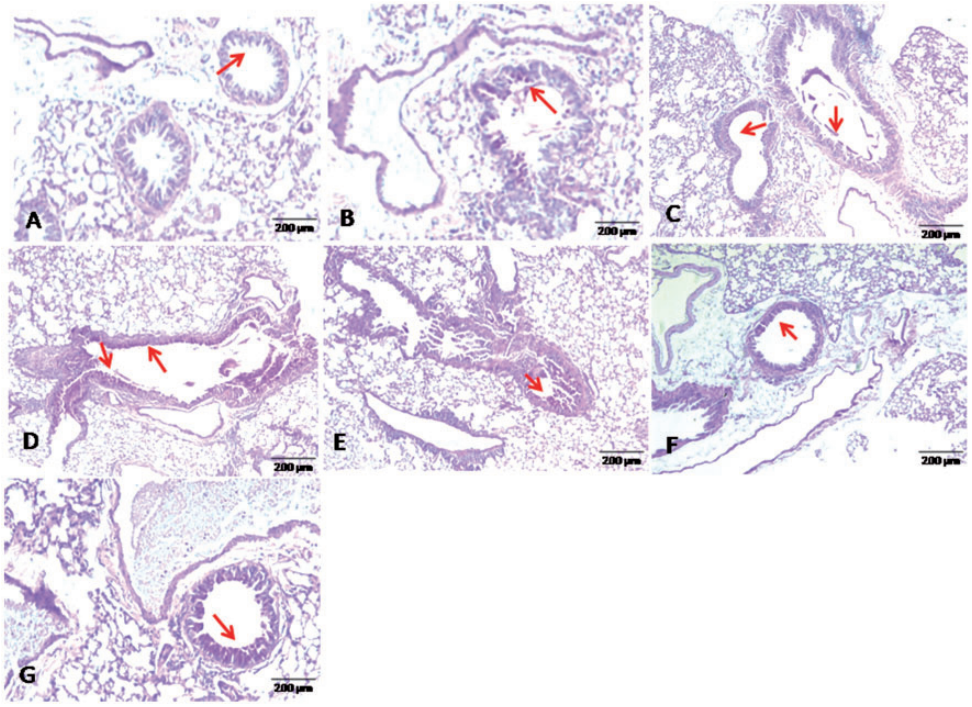

Figure 6 Sections in lung stained by PAS (×20) showing effects of roflumilast, formoterol, fluticasone alone and in combinations on the number of goblet cells (red arrows) in OVA-induced asthma in mice. (a) Normal control (NC) group showing normal goblet cell number. (b) OVA-induced asthma (PC) group showing severe increase in number of goblet cells. (c) Roflumilast-treated group, (e) Fluticasone-treated group and (g) Formoterol + Fluticasone showing mild to moderate increase in number of goblet cells. (d) Formoterol-treated group showing moderate to severe increase in number of goblet cells. (f) Roflumilast + Fluticasone-treated group showing mild increase in number of goblet cells. (A color version of this figure is available in the online journal.)

exacerbation rate in patients with COPD treated with a fixed dose combination of LABA and ICS.[69]

In OVA-sensitized and -challenged mice, formoterol (1.5–150 μg/kg) given intranasally at 1 and 3 h after the final OVA challenge dose-dependently reversed the increased Penh but neither inhibit AHR to aerosolized MCh nor affect the inflammatory cell influx in BALF.[37] Formoterol (given i.p.) reduced airway goblet cell hyperplasia in asthmatic female BABL/c mice.[70] However, in human, LABAs monotherapy was associated with increased asthma mortality possibly because LABAs have a bronchodilating effect while lack an anti-inflammatory effect, and then they control the asthmatic manifestations while mask inflammation. Also, certain patients may have a rare vulnerability to an adverse effect of LABAs. Thus, LABAs should only be used in combination with ICS and it was found that adding LABAs to ICS in uncontrolled asthmatic patients improved asthma manifestations and decreased exacerbations.[71] The fixed-dose fluticasone/formoterol inhaler (available in low, medium and high doses) used for treatment of persistent asthma shows an efficacy which is greater than that of either agent alone in addition to good safety.[42] In human, addition of low doses of inhaled β_2 agonists to low doses of ICS produced more inhibition of cellular proliferation and cytokines production in lung because β_2 agonists causes more activation of the corticosteroid receptors[72] and corticosteroids increase the β_2 receptor transcription.[73] Investigating the effects of a triple therapy of roflumilast, formoterol, and fluticasone is an interesting point for a further research to detect whether superior to the two-drug regimens. Limitations of the current study included use of only female mice and lack of measurement of the TGF-beta 1 level and MUC5B expression.

In conclusion, combined inhalation of roflumilast and fluticasone significantly decreased airway hyperresponsiveness in ovalumin-asthmatic mice. Also, it more significantly improved inflammation and histopathological changes than combined inhalation of formoterol and fluticasone. Further studies are needed to bring such bench results to clinical settings. Based on its ability to reduce counts of eosinophils, neutrophils, and macrophages in BALF shown in the current study, inhaled roflumilast might be of potential off-label usefulness in treatment of typical and atypical asthma and ACOS. It could be valuable add-on therapy to formoterol/fluticasone combination in these disorders.

Authors' contributions: HM designed the study and wrote the paper; HM, MR, and MS conducted the pharmacological experiments; AA and NK conducted the histopathological work; HM, HH, MR, and MS analyzed the statistical data, interpreted the results, and corrected the paper.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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