Bioorganic & Medicinal Chemistry 21 (2013) 6359–6365

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

A synthetic compound, 4-acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl)pyrano[3,4-*c*]pyran-1,8-dione, ameliorates ovalbumin-induced asthma

Hwan-Suck Chung [a], Youngeun Kim [a], Sei Joong Oh [a], Hankyum Kim [a,b], Seung Ill Choi [c], Yujuan Zhang [c], Jin-Hyun Jeong [c], Hyunsu Bae [a,*]

[a] Department of Physiology, College of Korean Medicine, Kyung Hee University, #1 Hoeki-Dong, Dongdaemoon-gu, Seoul 130-701, Republic of Korea
[b] Department of Oriental Rehabilitation Medicine, Jaseng Hospital of Oriental Medicine, Seoul 135-896, Republic of Korea
[c] College of Pharmacy, Yonsei University, 162-1 Songdo-dong Yonsooku, Inchon 406-840, Republic of Korea

ARTICLE INFO

Article history:
Received 2 July 2013
Revised 19 August 2013
Accepted 24 August 2013
Available online 3 September 2013

Keywords:
4-Acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl)pyrano[3,4-*c*]pyran-1,8-dione
Asthma
CCL11
Ovalbumin
Eosinophil

ABSTRACT

Eosinophilia is one of the characteristic signs of allergic inflammation. Massive migration of eosinophils to the airways can cause epithelial tissue injury, contraction of airway smooth muscle and increased bronchial responsiveness. Previously, we discovered a new compound, 1*H*,8*H*-pyrano[3,4-*c*]pyran-1,8-dione (PPY), derived from the fruit of *Vitex rotundifolia* L. and evaluated its anti-inflammatory and anti-asthmatic properties. In this study, we synthesized a new modified compound, 4-acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl) pyrano[3,4-*c*]pyran-1,8-dione (PPY-345), which was based on the PPY skeleton, and we evaluated its anti-asthmatic effects. To evaluate the anti-asthmatic effect of PPY-345 in vitro, A549 lung epithelial cells were stimulated with TNF-alpha, IL-4 and IL-1-beta to induce the expression of CCL11 (Eotaxin), a chemokine involved in eosinophil chemotaxis. To characterize the anti-asthmatic properties of PPY-345 in vivo, we examined the influence of PPY-345 in an ovalbumin (OVA)-induced asthma model. PPY-345 treatments significantly reduced CCL11 secretion. PPY-345 treatment did not inhibit the translocation of NF-κB into the nucleus but suppressed the phosphorylation of signal transducers and activators of transcription 6 (STAT6). PPY-345 treatment significantly reduced airway hyperreactivity as measured by whole-body plethysmography. PPY-345 further reduced total cells, including eosinophil, macrophage and lymphocytes, in the BAL fluid, goblet cell hyperplasia and myosin light chain 2 positive smooth muscle cell area in the lung tissue. Additionally, PPY-345 significantly suppressed the levels of OVA–IgE present in the serum. These results suggested that PPY-345 could improve asthma symptoms in OVA-sensitized mice.

1. Introduction

Asthma is a chronic inflammatory disease characterized by airway inflammation, increased mucus production, intermittent airway obstruction and hyperresponsiveness.[1,2] Airway remodeling is specifically characterized by structural and morphometric changes to the airway, including subepithelial fibrosis, epithelial hypertrophy, goblet cell hyperplasia and smooth muscle hypertrophy.[3,4] The initial symptoms of asthma include airway inflammation, in which eosinophils play a crucial role.[5] Eosinophils are always present in excess in the airways of asthma patients, but their accumulation in the lungs decreases as asthmatic symptoms decrease. CCL11 (Eotaxin) is a small protein that is produced in the lungs of asthmatic patients and is a potent eosinophil chemoattractant. CCL11, a CC chemokine, stimulates the migration of eosinophils from small blood vessels to lung tissue by acting on the CC chemokine receptor CCR3, located on the leukocyte cell surface.[6] Asthmatic patients commonly take two types of medication: preventative and relief agents.[7] Oral or inhaled steroids are common drugs for the treatment or prevention of asthma, but steroids have many unpleasant side effects. Thus, research is currently underway to find asthma treatment alternatives.[8]

We previously isolated a novel natural compound, 1*H*,8*H*-Pyrano[3,4-*c*]pyran-1,8-dione (PPY), from the fruit of *Vitex rotundifolia* L. and evaluated its anti-inflammatory and anti-asthmatic effects. We reported that PPY treatment significantly reduced the expression of eotaxin, IL-8, IL-16, and vascular cell adhesion molecule-1 (VCAM-1) mRNA in A549 lung epithelial cells

Abbreviations: PPY-345, 4-acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl)pyrano[3,4-*c*]pyran-1,8-dione; BALF, bronchoalveolar lavage fluid; VCAM-1, vascular cell adhesion molecule-1; STAT6, signal transducers and activators of transcription 6; Dexa, dexamethasone; OVA, ovalbumin; AHR, airway hyperresponsiveness; H&E, hematoxylin and eosin; PAS, periodic acid Schiff; MLC2, myosin light chain 2; HRP, streptavidin-horseradish peroxidase; Penh, enhanced pause.

* Corresponding author. Tel./fax: +82 2 962 9316.
E-mail address: hbae@khu.ac.kr (H. Bae).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.08.045

that had been stimulated with cytokines. Additionally, in an ovalbumin-challenged mouse model, PPY profoundly inhibited eosinophil accumulation in the airways and reduced the levels of IL-4, IL-5, and IL-13 in the bronchoalveolar lavage fluid (BALF).[9] As a follow-up study, we have synthesized a new compound, 4-acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl)pyrano[3,4-*c*]pyran-1,8-dione (PPY-345) based on the PPY skeleton and evaluated its anti-asthmatic effects in vivo and in vitro.

2. Materials and methods

2.1. Cells

A549 cells, a human type II-like epithelial lung cell line, were obtained from the Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). The cells were cultured in 100 mm tissue culture plates (Corning, Corning, NY, USA) in RPMI 1640 medium (Welgene, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and 100 U ml^{-1} penicillin–streptomycin (Invitrogen, Rockville, MD, USA). The plates were incubated at 37 °C at 100% humidity and 5% CO_2. The cells were sub-cultured every 3–4 days.

2.2. Synthesis of 4-acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl)pyrano[3,4-*c*]pyran-1,8-dione (PPY-345)

The synthetic pyranopyrandione was obtained[10] from the reaction of 3-methoxyphenol and diethyl ethoxymethylenemalonate, albeit in poor yield. The only structural commonality found in compounds is the benzopyran moiety, but they differ in their site of fusion with the other pyranone ring. The chemistry of pyrano[3,4-*c*]pyran-4,5-diones is largely unexplored. Except for MO calculations, that is, correlation of delocalization energy, p-bond order and p-charge density of 20 different theoretical pyranopyrandiones including pyrano[3,4-*c*]pyran-4,5-dione III, no other additional information is available in the literature. This has inspired us to develop an innovative route for the construction of this class of compounds in order to explore their therapeutic potential. PPY-345 was synthesized by the following procedure.[10] But PPY was not synthesized. At this point, PPY is available from extract. Furthermore, structure–activity relationship is under study.

2.2.1. Synthesis of 6-(3,4,5-trimethoxyphenyl)-4-(methylthio)-2-oxo-2*H*-pyran-3-carbonitrile (3)

A mixture of methyl 2-cyano-3,3-bis(methylthio)acrylate (**1**) (10 mmol), 1-(3,4,5-trimethoxyphenyl)ethanone (**2**) (11 mmol), and powdered KOH (12 mmol) in dry DMF(50 mL) was stirred at room temperature for 10–14 h. After completion of reaction, the mixture was poured into iced water with constant stirring. The precipitate thus obtained was filtered and purified on a silica-gel column with chloroform as eluent in 45% yield: ^{1}H NMR (400 MHz, $CDCl_3$, ppm) 7.05 (s, 2H), 6.59 (s, 1H), 3.94 (s, 6H), 2.73 (s, 3H).

2.2.2. Synthesis of PPY-345

A mixture of compound **3** (1 mmol), pentane-2,4-dione (1 mmol) and KOH (1.5 mmol) in dry DMF (10 ml) was stirred for 24 h at room temperature. The reaction mixture was poured onto crushed ice with vigorous stirring and then neutralized with 10% HCl. The precipitate obtained was filtered, washed with water and finally purified on a silica-gel column using 0.5% and 5% ethyl acetate in hexane as eluent for 1*H*,8*H*-pyrano[3,4-*c*]pyran-1,8-dione and 6-(2-bromophenyl)-4-(methylthio)-2-oxo-2*H*-pyran-3-carbonitrile, respectively, in 40% yield: ^{1}H NMR (400 MHz, $CDCl_3$, ppm) 7.55 (s, 1H), 7.28 (s, 2H), 3.88 (s, 9H), 2.17 (s, 6H). 4-Acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl)pyrano[3,4-*c*]pyran-1,8-dione (PPY-345) was dissolved in 50% ethanol. The chemical structures of PPY and PPY-345 and synthesis procedure of PPY-345 are shown in Figure 1.

2.3. Cell viability assay

The effect of PPY-345 on cell viability was examined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay. Briefly, A549 cells were seeded into 96-well plates at a density of 2×10^4 cells $well^{-1}$ and incubated at 37 °C for 24 h. The cells were then treated with 0, 5, 10, or 50 μM of PPY-345 for 24 h. After the addition of 20 μl $well^{-1}$ of CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI), the cells were cultured for an additional 4 h and the absorbance was recorded at 490 nm using a microplate reader (Sunrise, Tecan, Mannedorf, Switzerland). All experiments were performed in triplicate.

2.4. Eotaxin inhibition assay

Cells were plated in 24-well flat-bottomed plates (Corning, Corning, NY, USA). Upon reaching confluence, the medium was changed to serum free RPMI and the samples were then incubated for an additional 24 h. Concentrations of 0, 5, 10, or 50 μM of PPY-345 were added to the medium for 1 h, and the cells were stimulated with a combination of IL-1β (10 ng ml^{-1}) (Biosource,

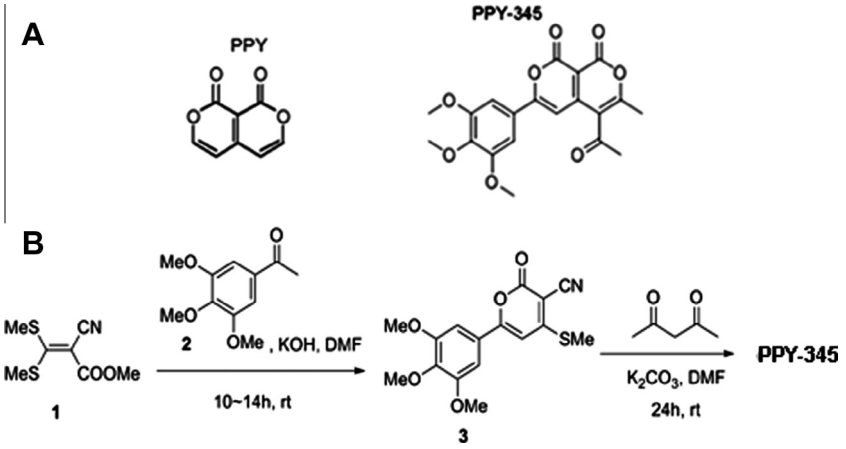

Figure 1. Chemical structures of (A) PPY and PPY-345; (B) and the synthesis procedure of PPY-345.

Camarillo, CA, USA), TNF-α (100 ng ml^{-1}) (Biosource), and IL-4 (100 ng ml^{-1}) (Biosource) and incubated for 24 h. The cell-free culture supernatants were then collected and assayed for eotaxin using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared to assess nuclear translocation of NF-κB and phosphorylation of signal transducers and activators of transcription 6 (STAT6). Briefly, dishes with cultured cells were washed with ice-cold PBS. The dishes were then scraped and the cells were transferred to microtubes. The cells were allowed to swell by adding 200 μl lysis buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM DTT, and 0.1 mM Na_3VO_4). The samples were incubated for 15 min on ice and gently mixed periodically. Next, 12.5 μl of 10% NP-40 was added and mixed carefully followed by a 5 min incubation. The tubes were vortexed twice for 10 s to disrupt the cell membranes and centrifuged for 30 s at 4 °C. Cytosol containing the supernatants was transfer to another tube. Pellets containing crude nuclei were resuspended in 10 μl extraction buffer (25 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM NaF, 1 mM DTT, 10% Glycerol, 0.2% NP-40 and 5 mM $MgCl_2$) and sonicated for 10 s three times. The samples were centrifuged at 15,000 rpm for 10 min to obtain the supernatant containing nuclear extracts. The extracts were stored at −70 °C until further usage.

2.6. Western blotting

The nuclear extracts were separated by 4-12% Tris-glycine (KOMA Biotech, Seoul, Korea) gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in TBS-Tween-20 for 1 h at room temperature, after which it was incubated with anti-p65 or p-STAT-6 or proliferating cell nuclear antigen (PCNA) antibody at 4 °C for overnight. After washing three times in TBS-Tween-20, the blot was incubated with a secondary antibody for 1 h. Antibody-specific proteins were then visualized with an enhanced chemiluminescence detection system as recommended (Amersham Corp., Newark, NJ). Band intensity was quantified by ImageJ analysis.

2.7. Animals

Balb/c male littermates (6 weeks of age, weighing 20–25 g) were purchased from Orient Bio (Seoungnam, South Korea). All mice were kept under specific pathogen-free conditions using air conditioners and a 12 h light·dark $cycle^{-1}$. In addition, all mice had free access to food and water during the experiments. The study was conducted according to the Rules for Animal Care and the Guiding Principles for Animal Experiments Using animals of the University of Kyung Hee Animal Care and Use Committee and in accordance with the recommendations of the Weatherall report 'The use of non-human primates in research.' The experimental procedure protocols were approved by the Animal Welfare and Animal Care Committee of the University of Kyung Hee Animal Care and Use Committee.

2.8. Induction of allergic asthma

Balb/c male mice 6 weeks of age were sensitized by intraperitoneal (ip) injection on days 0 and 14 with 100 μg of ovalbumin (OVA) (Sigma–Aldrich, St. Louis, MO, USA) precipitated with 20 mg of aluminum hydroxide in 200 μl of PBS. The mice were then challenged by administering 1% OVA in 50 μl PBS or PBS directly into the nostrils using a micropipette on days 15, 17, 19, 21, 23, and 25. Negative control mice were sensitized and challenged with PBS alone. On day 27, the mice were sacrificed and various tissues were collected for analyses.

2.9. Experimental protocol and design

All mice were randomly divided into four groups (n = 6 mice per group): (1) normal control mice (PBS group) were sensitized and challenged with normal saline; (2) OVA control mice (OVA group) were sensitized and challenged with OVA; (3) Positive control mice that were OVA-sensitized and orally injected 1 mg kg^{-1} dexamethasone (Sigma) (Dexa group); (4) OVA-sensitized mice that were intraperitoneally injected 10 mg kg^{-1} PPY-345 (PPY-345 group) (Fig. 2).

2.10. Measuring AHR to methacholine and analyzing BAL cells

Airway hyperresponsiveness (AHR) was measured 24 h after the final allergen challenge. Mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea), and baseline readings were taken for 3 min. The enhanced pause (Penh) was calculated according to the manufacturer's protocol [i.e., (expiratory time/relaxation time − 1) × (peak expiatory flow·peak inspiratory $flow^{-1}$)]. The results are expressed as the percentage increase in Penh following challenge with each concentration of methacholine (50 and 100 mg ml^{-1}).

2.11. Bronchoalveolar lavage (BAL)

Twenty-four hours after measuring the airway parameters, the mice were sacrificed with pentobarbital sodium (65 mg kg^{-1}, ip).

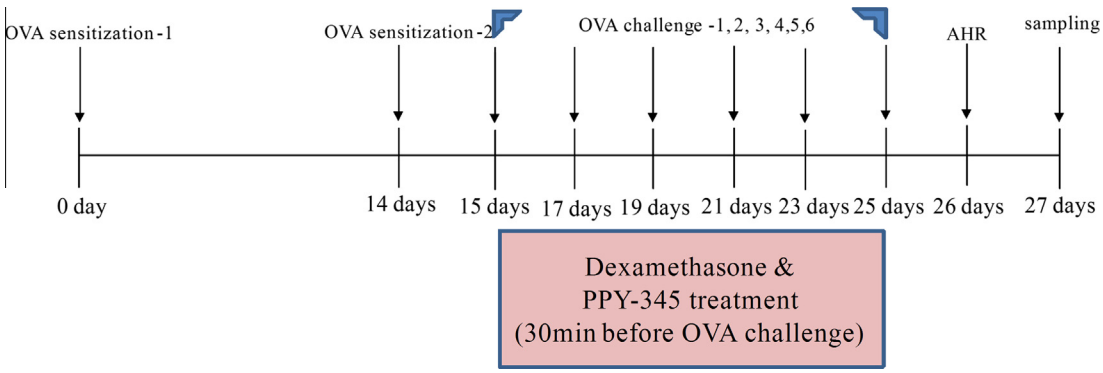

Figure 2. Schematic diagram of the experimental protocol.

BAL was collected by infusion and extraction of 1 ml of ice-cold PBS. This was repeated three times, and the lavages were pooled (mean volume, ±2.0 ml). Recovered BAL (70–80%) was centrifuged at 13,000 rpm for 10 min. The cell pallets were resuspended in 1 ml PBS, and the cells were adhered to glass slides using cytocentrifugation. Total viable cell counts were determined using a hemocytometer with trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes, and macrophages were determined on cytospin smears of BALF samples (5×10^5 cells·200 μl cells^{-1}) from individual mice stained with Diff-Quick staining (Life Technologies, Auckland, New Zealand) with 500 cells counted per sample. The BAL fluid was then centrifuged and the supernatants were kept at −70 °C. The results are expressed as total cell number $\times 10^4$.

2.12. Determination of IgE titers using ELISA

For serum analysis, 96-well immunomicroplates (Costar, NY, USA) were coated with monoclonal anti-mouse IgE antibodies. The serum was diluted 1:250 with 5% FBS in PBS (assay diluent), and IgE levels (BD Pharmingen) were measured using standardized sandwich ELISAs according to the manufacturer's protocol. Optical density was measured at 450 nm in a microplate reader (SOFT max PRO, version 3.1 software, CA, USA).

2.13. Histological examination

Trachea and lung tissues were removed from the mice, fixed in 4% paraformaldehyde and embedded in paraffin. After dehydration, the tissues were cut into 4 μm sections and stained with hematoxylin and eosin (H&E) or periodic acid Schiff reagent (PAS). For immunohistochemical detection of myosin light chain 2 (MLC2), 4 μm section of the lower trachea and lung tissue were treated with 0.3% H_2O_2–methanol for 20 min to block endogenous peroxidases, after which they were incubated at 4 °C overnight with anti-MLC2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in PBS, the slide was treated with a biotinylated secondary antibody for 20 min and then streptavidin-horseradish peroxidase (HRP) for 20 min. Immunoreactivity signals were visualized with DAB substrate (Zymed Laboratories, South San Francisco, CA, USA). Slides were counterstained with hematoxylin and finally mounted using Canada balsam (Showa Chemical Co. Ltd, Tokyo, Japan).

2.14. Statistical analysis

All results were expressed as the means ± SEM of at least three independent experiments performed in duplicate. One-way analysis of variance (ANOVA) and Tukey's test were used to compare the groups. Differences were considered to be significant at P <0.05.

3. Results

3.1. PPY-345 inhibits CCL11 secretion by cytokine-stimulated lung epithelial cells

To investigate the effects of PPY-345 on cultured cells, cell viability was monitored using the MTS assay in A549 lung epithelial cells. Concentrations of PPY-345 high as 50 μM did not affect cell viability (Fig. 3A). A solution of 0.5% ethanol was used as a vehicle control, which did not show any significant effects on A549 viability (data not shown). It is known that CCL11 (eotaxin-1) produced by lung epithelial cells is a potent chemoattractant for eosinophils. Therefore, inhibiting CCL11 secretion is potential one way to prevent asthma progression. In this study, we examined whether PPY-345 could inhibit CCL11 secretion in human lung epithelial cells stimulated with TNF-α, IL-4 and IL-1β. As shown in Figure 3B, CCL11 secretion was decreased by 75% in response to treatment with 5 μM PPY-345.

3.2. PPY-345 inhibits phosphorylation of STAT6 on cytokines stimulated lung epithelial cells

To further investigate the underlying mechanism behind the effects of PPY-345, we investigated its influence on NF-κB and STAT6, well-known transcription factors involved in allergic inflammation. When we analyzed p65 and p-STAT6 protein level in the nuclear fraction by Western blotting, we found that while translocation of p65 subunits to the nucleus was not inhibited, p-STAT6 protein levels were inhibited by PPY-345 treatment. These data indicated that PPY-345 treatment suppressed CCL11 production via inhibiting the STAT6 phosphorylation pathway (Fig. 4).

3.3. PPY-345 treatment reduced AHR

In an OVA-induced murine asthma model, AHR was significantly reduced by PPY-345 treatment as measured by whole-body plethysmography. AHR was evaluated by measuring enhanced pause (Penh) in response to nebulized methacholine. Bronchopulmonary hyperresponsiveness was significantly reduced

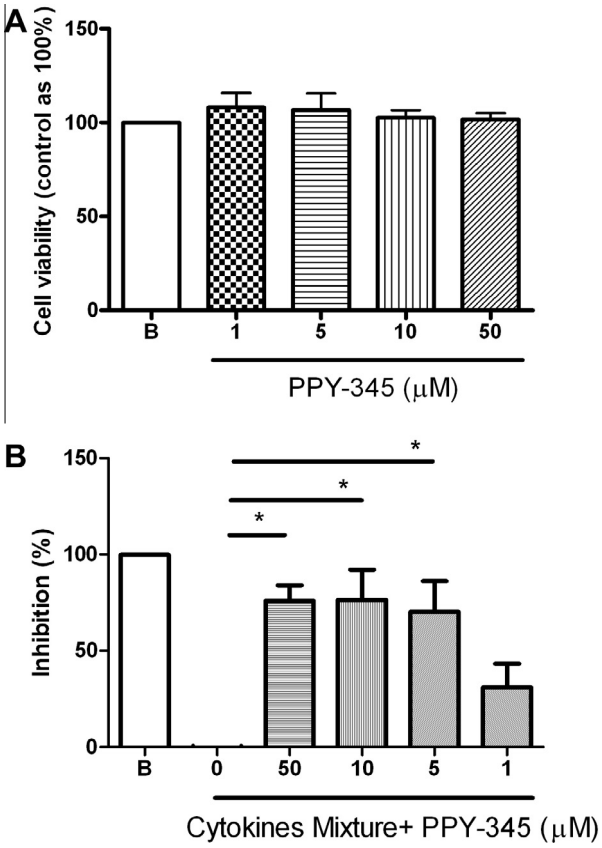

Figure 3. Effect of PPY-345 on (A) cell viability and (B) eotaxin secretion levels in A549 cells. (A) A549 cells were treated with various concentrations of PPY-345 and cultured for 24 h. Cell viability was measured by the MTS assay. (B) A549 cells were treated with different concentrations of PPY-345 for 1 h, after which TNF-α (100 ng ml^{-1}), IL-4 (100 ng ml^{-1}), and IL-1β (10 ng ml^{-1}) were added. The cells were then incubated for 24 h, and the supernatants were used assessed by ELISA. Inhibition was calculated as 'Inhibition of X (%) = (cytokines mixture treated sample value − X) × 100/(cytokines mixture treated sample value − blank value)'. The values shown are the mean ± SEM of three independent experiments. $^*P$ <0.01 compared with the PPY-345 non-treated samples value. 'B' indicates blank.

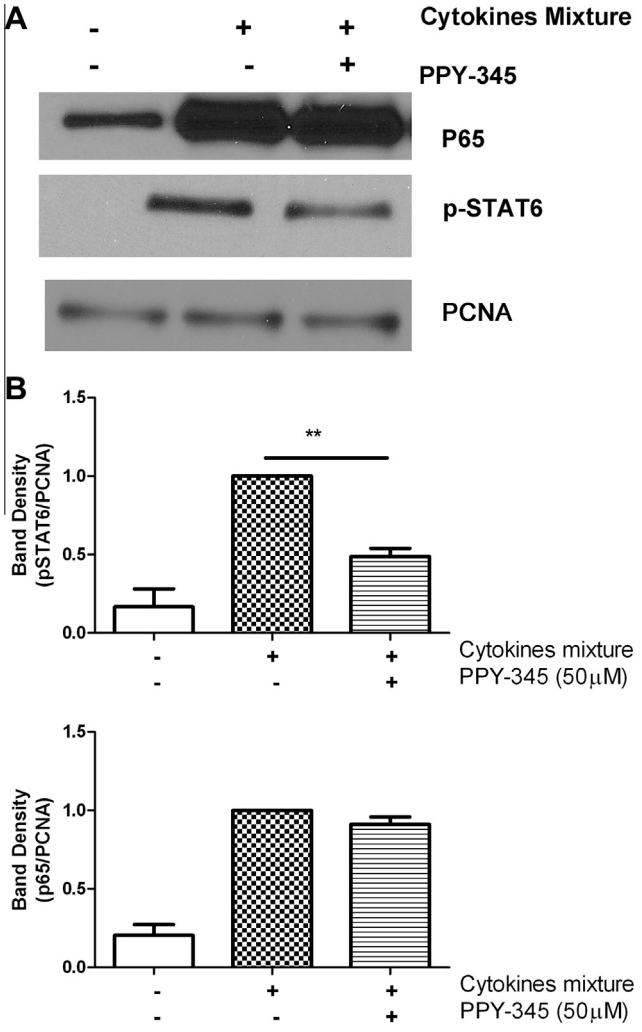

Figure 4. Influence of PPY-345 on the nuclear translocation of NF-κB and STAT6 phosphorylation in A549 cells stimulated with TNF-α (100 ng ml^{-1}), IL-4 (100 ng ml^{-1}), and IL-1β (10 ng ml^{-1}). (A) A549 cells treated with 50 μM PPY-345 were incubated for 1 h with TNF-α, IL-4, and IL-1β. Nuclear extracts were prepared and analyzed for the presence of NF-κB, phosphorylated STAT6 and PCNA by western blotting, as described in Section 2. (B) Band intensities of p-STAT6/PCNA or p65/PCNA were quantified by densitometric analysis of three experiments. $^{**}P$ <0.01.

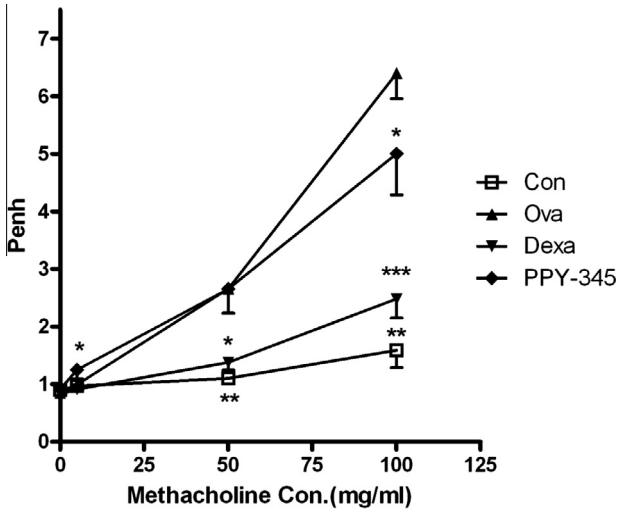

Figure 5. Effects of PPY-345 on airway hyperresponsiveness to methacholine in an OVA-induced murine asthma model. After the mice inhaled 50 or 100 mg ml^{-1} of methacholine, AHR was measured and is shown as Penh values. Data are represented as the mean ± SEM (n = 6 mice per group). $^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.001 versus OVA.

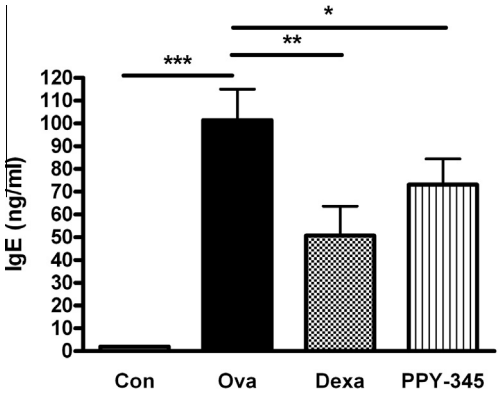

Figure 6. Effects of PPY-345 treatment on systemic IgE production in the serum of OVA-sensitized asthmatic mice. Total IgE levels were measured by ELISA. The data shown represent the means ± SEM. $^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.001.

in PPY-345-treated mice when compared with the OVA group at 100 mg ml^{-1} of methacholine (Fig. 5). The dexamethasone-treated group, used as a positive control, also exhibited significantly reduced Penh values (P <0.001). These data indicate that PPY-345 suppressed AHR in asthmatic mice.

3.4. PPY-345 decreased plasma IgE concentration

Cross-linking of allergen specific IgE antibodies on the surface of mast cells causes them to be degranulated and subsequently leads to AHR.[11] To investigate the systemic effects of PPY-345 treatment, IgE levels were measured in blood after 48 h of the last challenge, as elevated IgE production is a key characteristic of systemic asthmatic responses. Plasma levels of IgE were significantly decreased in PPY-345-treated mice (Fig. 6).

3.5. PPY-345-inhibited inflammatory cell influx into the BAL fluid

One hallmark of allergic airway disease is the accumulation of eosinophils, neutrophils, lymphocytes, and macrophages in the lungs.[12] To evaluate the effects of PPY-345 treatment on the recruitment of immune cells to the airways, we measured total cell numbers as well as the numbers of eosinophils, lymphocytes and macrophages in BAL fluid. In the OVA-immunized animals, the total number of infiltrating cells was approximately 2.5-fold higher in the BAL fluid than in control animals. However, PPY-345 treatment decreased the total number of infiltrating cells up to similar level as that observed in the dexamethasone treatment group (Fig. 7). Eosinophils were counted based on their morphological characteristics and were visualized by Diff-Quick staining. Eosinophils numbers in the PPY-345-treated group were significantly decreased compared to the OVA group. The numbers of lymphocytes and macrophages in the BAL fluid were also significantly lowered by PPY-345 treatment (Fig. 7).

3.6. Effect of PPY-345 on eosinophil infiltration and goblet cell hyperplasia in lung

H&E staining of lung sections revealed that more eosinophils had infiltrated between the trachea and blood vessels in OVA-sensitized mice than in normal control mice (Fig. 8A). In asthmatic mice treated with PPY-345, eosinophil infiltration was significantly

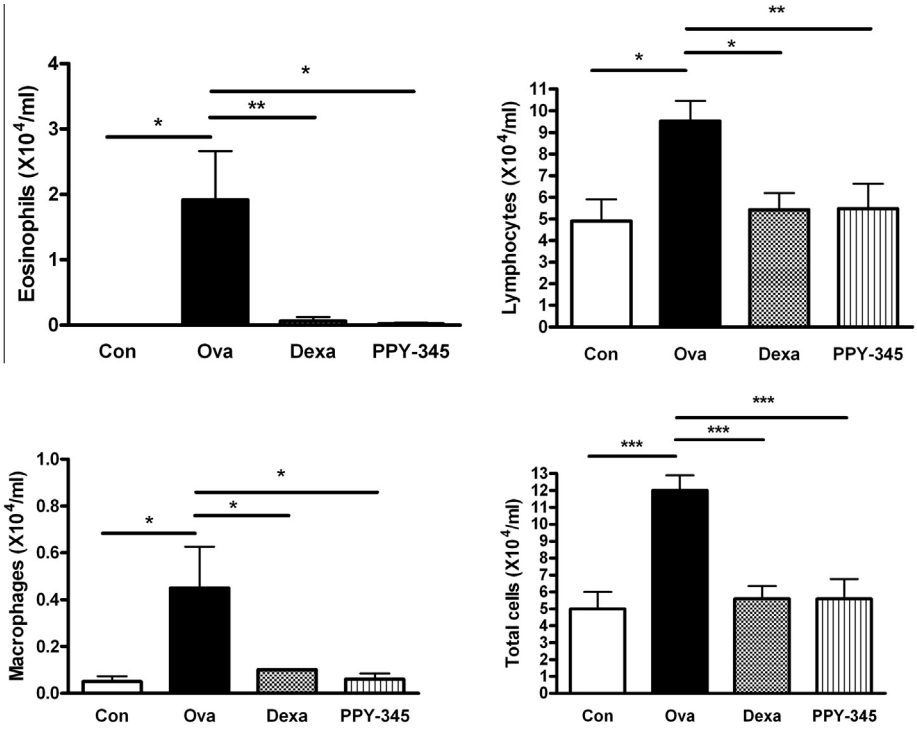

Figure 7. Effects of PPY-345 on inflammatory cells accumulation in the BAL fluid. BAL cells were separated using a cytospin, after which they were stained with Diff-Quick. Differential cell counting was performed using standard morphological criteria. The data shown represent the means ± SEM. $^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.001.

decreased in the lung. PAS staining further revealed goblet cell hyperplasia in the airways of the asthmatic mice. PPY-345 or dexamethasone treatment suppressed this goblet cell hyperplasia (Fig. 8B). In addition, OVA-challenged mice expressed myosin light chain 2 (MLC2) in the peribronchial muscle layer of the lung, and PPY-345 treatment decreased the thickness of this smooth muscle layer (Fig. 8C and D). These data demonstrated that PPY-345 had significant potential to effectively inhibit airway remodeling.

4. Discussion

In our previous study, we screened the anti-asthmatic effects of 270 medicinal plants and we found that the fruit of *Vitex rotundifolia* L. had the highest effects in our in vitro experiments. After several rounds of fractionation and activity testing, a novel compound named PPY was isolated.[9] As a follow-up study, we synthesized a PPY derivative based on the PPY skeleton and evaluated its activity. We demonstrated that PPY-345 inhibited the production of CCL11 in proinflammatory cytokine-activated A549 cells. PPY-345 did not inhibit the proinflammatory cytokine-induced nuclear translocation of NF-κB but did inhibit the phosphorylation of STAT6.

We previously showed that PPY treatment suppresses the MAPK/NF-κB pathway.[9] Therefore, we hypothesized that the PPY derivative, PPY-345, would also inhibit the MAPK/NF-κB pathway. However, PPY-345 did not inhibit the translocation of NF-κB into nucleus (Fig. 4). According to Fulkerson et al., three chemokines, CCL11, CCL17, and CCL22, are known to be STAT6 dependent.[13] Murine models of allergic airway inflammation have demonstrated that overexpression of the cytokine products of Th2 cells, specifically IL-4 and IL-13, is sufficient for the induction of numerous lung chemokines and the development of pulmonary eosinophilia.[14,15]

IL-4 and IL-13 share the IL-4 receptor α chain (IL-4Rα), which mediates the phosphorylation of JAK1 and JAK3 and, subsequently, phosphorylation of IL-4Rα. STAT6 monomers are then recruited to the phosphorylated docking tyrosine residues on IL-4Rα and phosphorylated by JAKs, resulting in STAT6 dimerization and translocation to the nucleus. STAT6 is required for many IL-4- and IL-13-mediated responses, including CCL11 expression.[13,16,17]

Bronchial asthma is characterized by reversible airway obstruction in response to allergens, chronic eosinophilic airway inflammation, and non-specific airway hyper-responsiveness (AHR).[18]

In this study, we used an OVA-induced murine model of allergic airway disease. In the OVA-immunized asthma model, AHR is closely associated with eosinophilia, as evidenced by increased numbers of eosinophils in the BAL fluid.[19] The results of the present study indicated that PPY-345 treatment improved methacholine-induced AHR in OVA-immunized asthmatic mice (Fig. 5). Specific IgE production is a hallmark of allergic diseases.[20] In the present study, PPY-345 treatment suppressed the increased antigen-specific IgE production characteristic of OVA-induced allergic asthma in mice (Fig. 6).

The infiltration of inflammatory cells, including eosinophils, into the BAL fluid was lowered to the levels observed in the dexamethasone or control groups following PPY-345 treatment (Fig. 7). The decreased accumulation of eosinophils was also confirmed in the PPY-345-treated lung samples (Fig. 8A). During an asthma attack, in addition to tracheal contraction, goblet cells secrete more mucus, which causes obstruction and difficulty breathing through narrowed airways. In our study, PPY-345 treatment reduced the MLC2 positive muscle area and the numbers of PAS-positive goblet cells, suggesting that PPY-345 effectively inhibited airway remodeling in asthma (Fig. 8).

In conclusion, we demonstrated that PPY-345 treatment profoundly inhibited CCL11 production by cytokine-activated lung epithelial cells in addition to impairing the recruitment of inflammatory cells, including eosinophils, in a murine asthma model. These results indicated that PPY-345 reduced airway hyperresponsiveness, OVA-specific IgE production, and airway

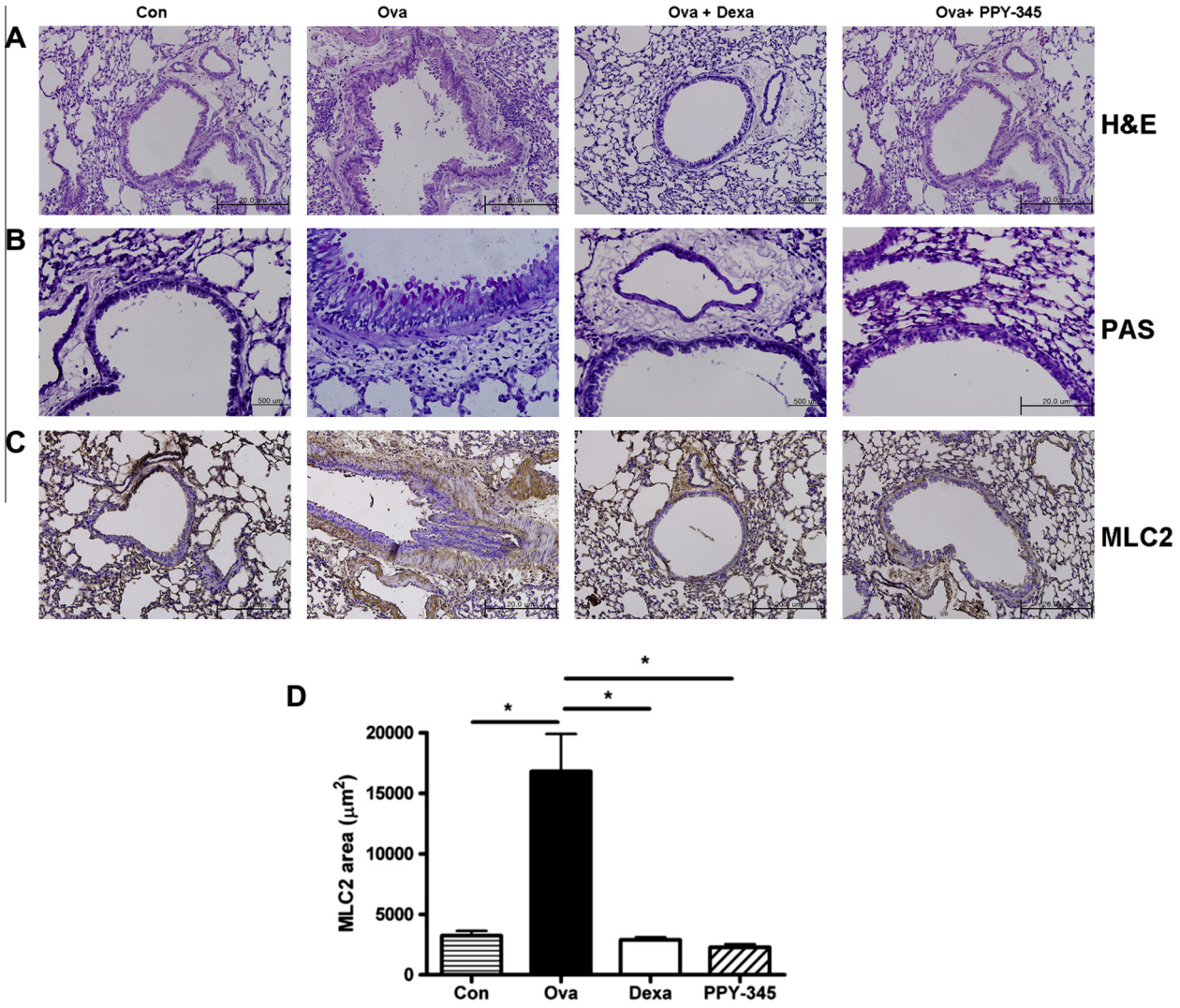

Figure 8. Effect of PPY-345 on eosinophil infiltration and goblet cell hyperplasia in the lung. (A) H&E staining of lung tissue to detect eosinophil infiltration. (B) Lung sections were stained with PAS stain to analyze goblet cell hyperplasia. Goblet cells are indicated by the arrows. (C) MLC2 expression was analyzed by IHC staining to evaluate airway smooth muscle thickness. (D) Graphs represent the area of MLC2-positive smooth muscle in 5 bronchi. The data shown represent the means ± SEM. $^{**}P$ <0.01.

remodeling in a murine asthma model at least in part via inhibiting eosinophil infiltration to the lungs.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government [MEST] (No. 2012-0005755).

References and notes

1. Bousquet, J.; Jeffery, P. K.; Busse, W. W.; Johnson, M.; Vignola, A. M. *Am. J. Respir. Crit. Care Med.* **2000**, *161*, 1720.
2. Roche, W. R.; Beasley, R.; Williams, J. H.; Holgate, S. T. *Lancet* **1989**, *1*, 520.
3. Aikawa, T.; Shimura, S.; Sasaki, H.; Ebina, M.; Takishima, T. *Chest* **1992**, *101*, 916.
4. Jeffery, P. K. *Am. Rev. Respir. Dis.* **1991**, *143*, 1152.
5. Kay, A. B. *J. Allergy Clin. Immunol.* **1991**, *87*, 893.
6. Pease, J. E.; Williams, T. J. *Curr. Opin. Pharmacol.* **2001**, *1*, 248.
7. Thomson, N. C.; Bicknell, S.; Chaudhuri, R. *Curr. Opin. Allergy Clin. Immunol.* **2012**, *12*, 241.
8. Mark, J. D. *Pediatr. Clin. North Am.* **2007**, *54*, 1007.
9. Lee, H.; Han, A. R.; Kim, Y.; Choi, S. H.; Ko, E.; Lee, N. Y.; Jeong, J. H.; Kim, S. H.; Bae, H. *Int. J. Immunopathol. Pharmacol.* **2009**, *22*, 591.
10. Singh, F. V.; Dixit, M.; Chaurasia, S.; Raghunandan, R.; Maulik, P. R.; Goel, A. *Tetrahedron Lett.* **2007**, *48*, 8998.
11. Bradding, P.; Walls, A. F.; Holgate, S. T. *J. Allergy Clin. Immunol.* **2006**, *117*, 1277.
12. Busse, W. W.; Lemanske, R. F., Jr. *N. Engl. J. Med.* **2001**, *344*, 350.
13. Fulkerson, P. C.; Zimmermann, N.; Hassman, L. M.; Finkelman, F. D.; Rothenberg, M. E. *J. Immunol.* **2004**, *173*, 7565.
14. Li, L.; Xia, Y.; Nguyen, A.; Feng, L.; Lo, D. *J. Immunol.* **1998**, *161*, 3128.
15. Wills-Karp, M.; Luyimbazi, J.; Xu, X.; Schofield, B.; Neben, T. Y.; Karp, C. L.; Donaldson, D. D. *Science* **1998**, *282*, 2258.
16. Kaplan, M. H.; Schindler, U.; Smiley, S. T.; Grusby, M. J. *Immunity* **1996**, *4*, 313.
17. Leonard, W. J.; O'Shea, J. J. *Annu. Rev. Immunol.* **1998**, *16*, 293.
18. Gonzalo, J. A.; Lloyd, C. M.; Kremer, L.; Finger, E.; Martinez, A. C.; Siegelman, M. H.; Cybulsky, M.; Gutierrez-Ramos, J. C. *J. Clin. Invest.* **1996**, *98*, 2332.
19. Shi, H. Z.; Qin, X. J. *Allergy* **2005**, *60*, 986.
20. Sutton, B. J.; Gould, H. J. *Nature* **1993**, *366*, 421.